# Extraction of a Drugs of Abuse Panel from Oral Fluid Using ISOLUTE® SLE+ After Collection with the Oral-Eze Collection Device Prior to UPLC-MS/MS Analysis

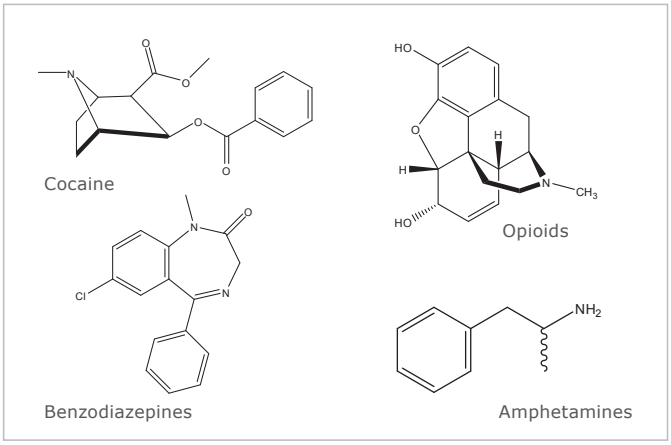


**Figure 1.** Example structures by class

## Introduction

This application note describes the extraction of 47 drugs of abuse from oral fluid matrix after sampling via Oral-Eze collection devices, prior to UPLC-MS/MS analysis. **Figure 1** shows examples of these structures by class.

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

This application note describes an effective and efficient ISOLUTE® SLE+ protocol optimized for both 400 µL and 1 mL sample capacity column formats.

## Analytes

**Table 1.** Analytes

| | | | | |
|---|---|---|---|---|
| Amphetamine | Methamphetamine | MDA | MDMA | MDEA |
| Mephedrone | Morphine | Hydromorphone | Oxymorphone | Dihydrocodeine |
| Oxycodone | Hydrocodone | Codeine | 6-MAM | Methadone |
| EDDP | Cocaine | Benzoylecgonine | 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 | Bromazepam | Zaleplone | Zopiclone | Zolpidem |
| Fentanyl | Norfentanyl | Ketamine | Norketamine | Buprenorphine |
| Norbuprenorphine | PCP | | | |

## Sample Preparation Procedure

**Sample Pre-treatment:** Following oral fluid collection (as per manufacturer instructions), remove paddle, add internal standard as required, and 4% aqueous ammonium hydroxide (10 µL) to each collection device. Vortex mix.

**Format:** **ISOLUTE® SLE+ 400 µL Sample Volume Columns, part number 820-0055-B**

**Sample Loading:** Load 300 µL of the pre-treated oral fluid onto the column and apply a pulse of vacuum or positive pressure (3–5 seconds) to initiate flow. Allow the sample to absorb for 5 minutes.

**Analyte Extraction** Apply dichloromethane (1 mL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of DCM (1 mL) and allow to flow for another 5 minutes under gravity. Apply vacuum or positive pressure (5–10 seconds) to pull through any remaining extraction solvent.

**Format:** **ISOLUTE® SLE+ 1 mL Sample Volume Columns, part number 820-0140-C**

**Sample Loading:** Load 600 µL of the pre-treated oral fluid onto the column and apply a pulse of vacuum or positive pressure (3–5 seconds) to initiate flow. Allow the sample to absorb for 5 minutes.

**Analyte Extraction** Apply dichloromethane (2.5 mL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of DCM (2.5 mL) and allow to flow for another 5 minutes under gravity. Apply vacuum or positive pressure (5–10 seconds) to pull through any remaining extraction solvent.

**Post Elution and Reconstitution:** Before evaporation, add 50 mM HCl in methanol (100 µL) to each collection tube. This will stabilize amphetamines, bath salts and ketamine, and minimize analyte losses during evaporation.

Dry the extract in a stream of air or nitrogen using a SPE Dry (40 °C, 20 to 40 L/min) or TurboVap (1.0 bar at 40 °C for 40 mins).

Upon dryness, reconstitute with 200 µL of mobile phase A : mobile phase B (80:20, v:v)

## UPLC Conditions

**Instrument:** Waters ACQUITY UPLC

**Column:** ACE EXCEL 1.7 µm C18 prototype column (100 x 2.1 mm id)

**Mobile Phase:** **A:** 5 mM ammonium acetate (aq)

**B:** 5 mM ammonium acetate in methanol

**Flow Rate:** 0.3 mL/min

**Table 2.** Gradient conditions

| Time | % A | % B | Curve |
|---|---|---|---|
| 0 | 90 | 10 | 1 |
| 10 | 10 | 90 | 6 |
| 11.9 | 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 11.9 minutes.
**Curve 6:** Linear Gradient

## Mass Spectrometry Conditions

**Instrument:** 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 3.** 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 |

| Compound | MRM Transition | Cone Voltage (V) | Collision Energy (eV) |
|---|---|---|---|
| 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 |
| Bromazepam | 316.1 > 182.1 | 40 | 30 |
| 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 |
| PCP | 244.2 > 159.9 | 20 | 15 |

## Results

Oral fluid mixed with collection device buffer was spiked with 1 ng of analytes per loaded sample (n=7), equating to 10 ng/mL when extracting 300 µL (or 100 µL of actual oral fluid).

The percentage analyte recoveries for the various drug classes can be seen in **Figures 2–4**. RSD's ranged from 1.2%–9.1%.

## Oral-Eze Method Scale up for Amphetamines, Bath Salts and Opiates

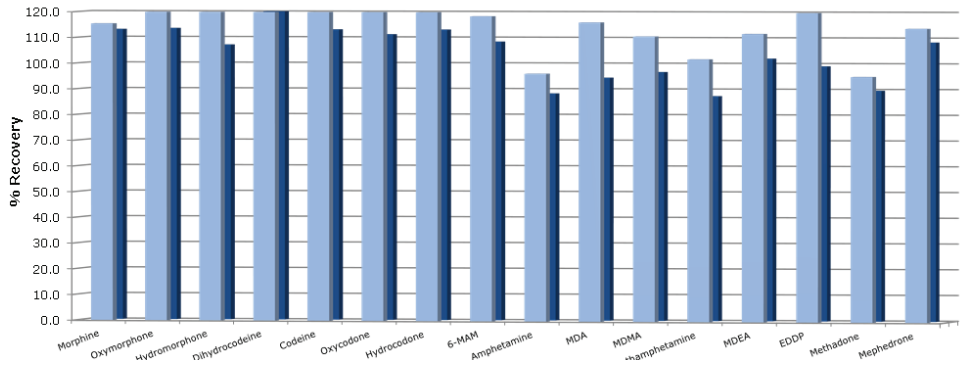


**Figure 2.** Recovery profile for amphetamines, bath salt and opiates from Oral-Eze collected oral fluid using ISOLUTE® SLE+ 400 µL and 1 mL columns.

## Oral-Eze Method Scale up for Benzodiazepines

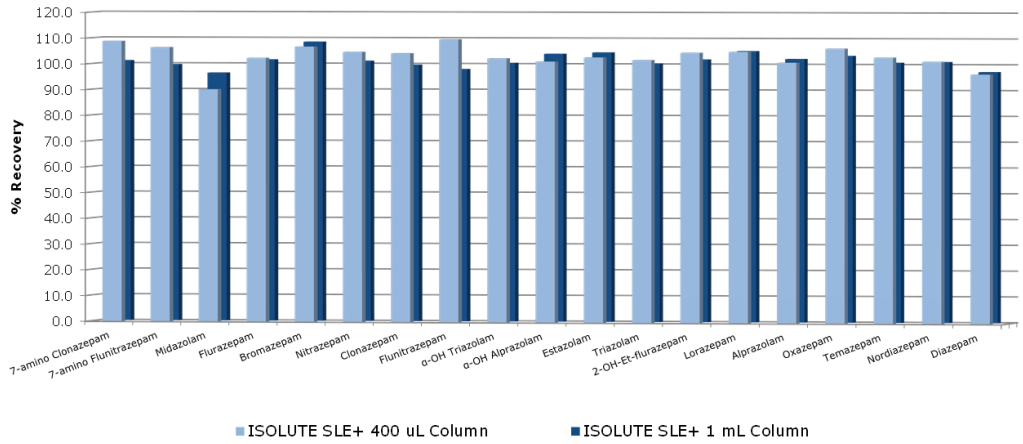


**Figure 3.** Recovery profile for benzodiazepines from Oral-Eze collected oral fluid using ISOLUTE® SLE+ 400 µL and 1 mL columns.

## Oral-Eze Method Scale up for Other Drug Classes

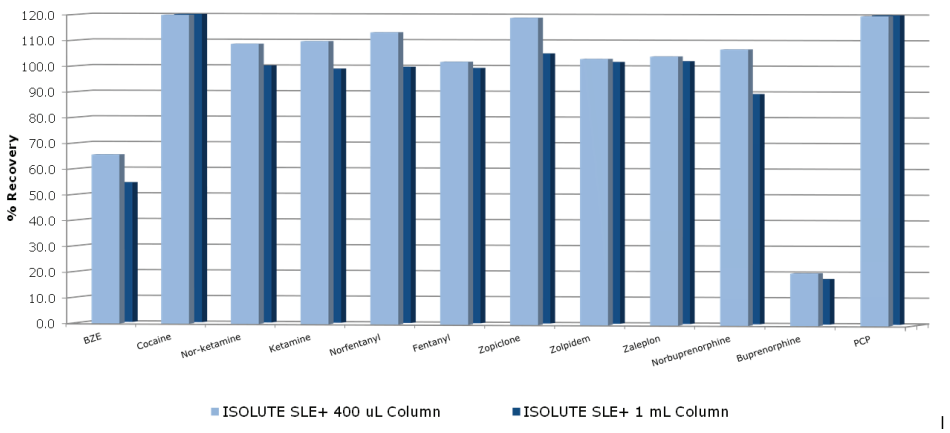


**Figure 4.** Recovery profile for multi-class analytes from Oral-Eze collected oral fluid using ISOLUTE® SLE+ 400 µL and 1 mL columns.

## Calibration Curves

Calibration curves were generated using oral fluid spiked at concentrations of 1–500 ng/mL, with internal standards spiked at 10 ng/mL for deuterated drug-metabolites and 100 ng/mL for deuterated drug-parents, prior to extraction on 1 mL columns. Figures 5–8. demonstrate good coefficients for all analytes ($r^2 > 0.99$). Quadratic function was observed at the top end of the calibration curve for many analytes (the excluded points seen in the figures below). Dilution of these samples was performed to improve linearity, using a reconstitution volume of 1 mL instead of 200 µL.

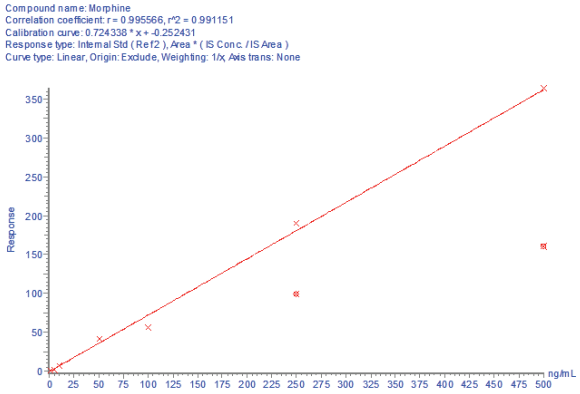


**Figure 5.** Calibration Curve for morphine using ISOLUTE® SLE+ 400 µL columns.

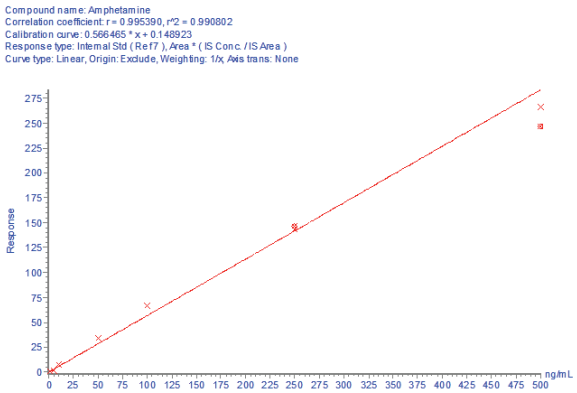


**Figure 6.** Calibration Curve for amphetamine using ISOLUTE® SLE+ 400 µL columns.

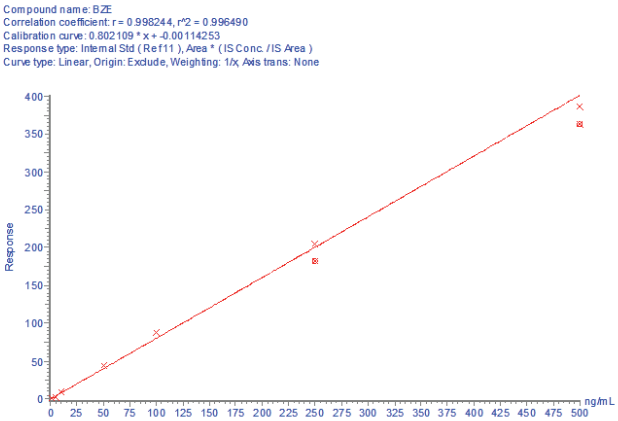


**Figure 7.** Calibration Curve for benzoylecgonine (BZE) using ISOLUTE® SLE+ 400 µL columns.

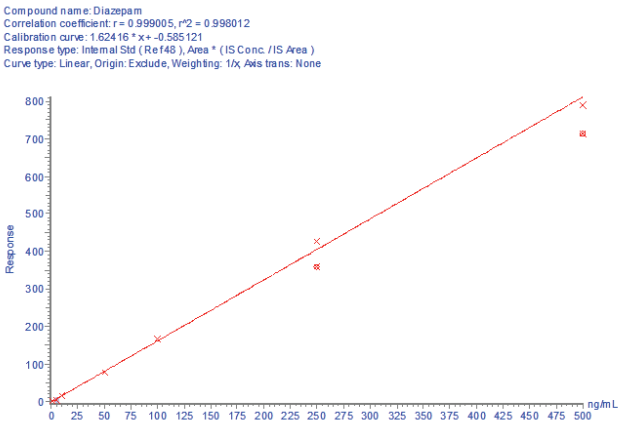


**Figure 8.** Calibration Curve for diazepam using ISOLUTE® SLE+ 400 µL columns.

**Table 4.** Estimated LOQ's based on S/N ratios from 1 ng/mL and 10 ng/mL extracted samples for the 400 µL and 1mL capacity formats respectively are:

| Analyte | Estimated LOQ (ng/mL) | Estimated LOQ (ng/mL) |
|---|---|---|
| Amphetamine | 0.08 | 0.04 |
| Methamphetamine | 0.05 | 0.02 |
| MDA | 0.025 | 0.01 |
| MDMA | 0.05 | 0.02 |
| MDEA | 0.035 | 0.02 |
| Hydromorphone | 0.2 | 0.1 |
| Morphine | 0.01 | 0.005 |
| BZE | 0.2 | 0.1 |
| Oxymorphone | 0.05 | 0.02 |
| Dihydrocodeine | 0.05 | 0.02 |
| Oxycodone | 0.2 | 0.1 |
| Mephedrone | 1 | 0.75 |
| Norfentanyl | 0.02 | 0.01 |
| 7-amino-flunitrazepam | 0.2 | 0.15 |
| 7-amino-clonazepam | 0.2 | 0.15 |
| Hydrocodone | 0.2 | 0.15 |
| Codeine | 0.035 | 0.01 |
| 6-MAM | 0.02 | 0.01 |
| Cocaine | 0.02 | 0.01 |
| Norketamine | 0.02 | 0.01 |
| EDDP | 0.02 | 0.01 |
| Zaleplone | 0.02 | 0.01 |
| Zopiclone | 1 | 0.35 |
| Norbuprenorphine | 0.02 | 0.01 |

| Analyte | Estimated LOQ (ng/mL) | Estimated LOQ (ng/mL) |
|---|---|---|
| Ketamine | 0.01 | 0.005 |
| Nitrazepam | 0.01 | 0.005 |
| Flunitrazepam | 0.01 | 0.005 |
| Clonazepam | 0.02 | 0.01 |
| α-OH-triazolam | 0.02 | 0.01 |
| Oxazepam | 0.035 | 0.015 |
| Estazolam | 0.02 | 0.01 |
| Temazepam | 0.01 | 0.005 |
| Zolpidem | 0.02 | 0.01 |
| Alprazolam | 0.035 | 0.015 |
| Methadone | 0.02 | 0.01 |
| Lorazepam | 0.035 | 0.02 |
| Bromazepam | 0.035 | 0.02 |
| α-OH-alprazolam | 0.02 | 0.01 |
| 2-OH-ethyl-flurazepam | 0.035 | 0.02 |
| Triazolam | 0.02 | 0.01 |
| Nordiazepam | 0.035 | 0.015 |
| Diazepam | 0.02 | 0.01 |
| Midazolam | 0.01 | 0.005 |
| Fentanyl | 0.01 | 0.005 |
| Flurazepam | 0.02 | 0.01 |
| Buprenorphine | 0.25 | 0.1 |
| PCP | 0.05 | 0.035 |

## Additional Notes

*Buprenorphine extraction recovery is low compared to samples fortified with the analyte after supported liquid extraction, however the LLOQ values in the table illustrate that this is not an obstacle to effective quantitation. If increased Buprenorphine recovery is required, or if a non-chlorinated solvent is required, ethyl acetate may be used as an alternative extraction solvent.

### Extract Cleanliness

Due to the nature of the buffers used in the oral fluid device and to avoid their co-extraction, an underload strategy was used i.e. 300 µL of sample loaded on a 400 µL capacity column, and 600 µL of sample loaded on a 1 mL capacity column.

### Solution Preparation

1. 5 mM ammonium acetate aq: Weigh 0.1927 g and dissolve in 500 mL UHPLC grade water.
2. 5 mM ammonium acetate in methanol: Weigh 0.1927 g and dissolve in 500 mL UHPLC grade methanol.
3. 4% aqueous ammonium hydroxide, used to modify pH prior to extraction, was prepared by the addition of 200 µL of commercially available 28–32% grade to 4.8 mL UHPLC grade water.

### Blowdown Stability

Amphetamines, bath salts and ketamines can suffer losss on evaporation when drying in the more volatile free base form. To overcome 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.

50 mM HCl in methanol is prepared by adding 50 µL concentrated hydrochloric acid to 11.95 mL HPLC grade methanol. The hydrochloric acid stock is commercially available ~12M.



Biotage®

## Ordering Information

| Part Number | Description | Quantity |
|---|---|---|
| 820-0055-B | ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Column | 50 |
| 820-0140-C | ISOLUTE® SLE+ 1 mL Supported Liquid Extraction Column | 30 |
| PPM-48 | Biotage® PRESSURE+ 48 Positive Pressure Manifold for Columns | 1 |
| SD-9600-DHS-EU | Biotage® SPE Dry Sample Concentrator System 220/240 V | 1 |
| SD-9600-DHS-NA | Biotage® SPE Dry Sample Concentrator System 100/120 V | 1 |
| C103198 | TurboVap® LV, Evaporator 100/120V | 1 |
| C103199 | TurboVap® LV, Evaporator 220/240V | 1 |

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