

Extraction of a Drugs of Abuse Panel from Oral Fluid Using ISOLUTE® SLE+ After Collection with the Intercept Oral Fluid Drug Test Kit Prior to UPLC-MS/MS Analysis

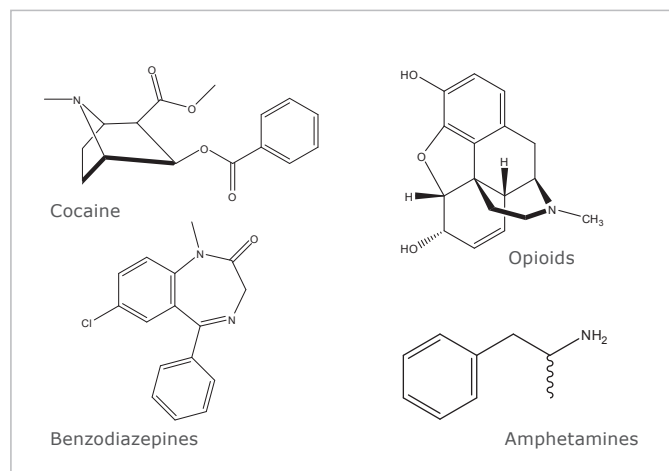


Figure 1. Example structures by class

Introduction

This application note describes the extraction of 47 drugs of abuse from oral fluid matrix collected using the Intercept Oral Fluid Drug Test Kit (Orasure Technologies), 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 the 1 mL capacity column format.

Analytes

Table 1. Analytes

Amphetamine	Methamphetamine	MDA	MDMA	MDEA
Mephedrone	Morphine	Hydromorphone	Oxymorphone	Dihydrocodeine
Oxycodone	Hydrocodone	Codeine	6-MAM	Methadone
EDDP	Cocaine	Benzoyllecgonine	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, add 0.5% aqueous ammonium hydroxide (10 µL) to each collection device. Vortex mix. Dilute 250 µL of the contents of the device with 250 µL HPLC grade water Vortex mix.
Format:	ISOLUTE® SLE+ 1 mL sample volume columns, part number 820-0140-C
Sample Loading:	Load 480 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 ethyl acetate (3 mL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of ethyl acetate (3 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 minimise 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 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

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

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)	Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
Amphetamine	136.0 > 118.9	16	9	Zopiclone	389.2 > 245.1	20	17
Amphetamine-D5	141.0 > 123.9	16	9	Norbuprenorphine	414.3 > 101.0	55	42
Methamphetamine	150.0 > 90.9	22	17	Ketamine	238.1 > 124.9	25	27
MDA	180.1 > 105.0	16	23	Nitrazepam	282.2 > 236.1	40	25
MDMA	194.1 > 163.0	20	13	Flunitrazepam	314.2 > 268.2	40	25
MDEA	208.2 > 163.0	22	13	Clonazepam	316.1 > 270.1	40	25
Hydromorphone	286.2 > 185.1	44	29	α -OH-triazolam	359.1 > 331.1	45	26
Morphine	286.2 > 201.0	42	25	Oxazepam	287.2 > 241.0	30	21
Morphine-D3	289.2 > 201.0	42	25	Estazolam	295.2 > 267.2	40	24
BZE	290.1 > 168.0	30	18	Temazepam	301.1 > 255.1	30	22
BZE-D3	293.1 > 171.0	30	18	Zolpidem	308.2 > 235.1	45	35
Oxymorphone	302.2 > 198.1	34	37	Alprazolam	309.2 > 281.2	40	26
Dihydrocodeine	302.2 > 199.1	42	33	Methadone	310.2 > 265.2	26	15
Oxycodone	316.2 > 241.2	34	27	Lorazepam	321.1 > 275.1	30	22
Mephedrone	178.1 > 160.0	35	12	Bromazepam	316.1 > 182.1	40	30
Norfentanyl	233.1 > 84.0	25	19	α -OH-alprazolam	325.2 > 297.1	40	25
7-amino-flunitrazepam	284.2 > 135.0	40	27	2-OH-ethyl-flurazepam	333.2 > 109.0	40	27
7-amino-clonazepam	286.2 > 121.0	40	30	Triazolam	343.0 > 308.1	45	27
Hydrocodone	300.2 > 199.1	46	33	Nordiazepam	271.1 > 139.9	40	28
Codeine	300.3 > 215.1	42	25	Diazepam	285.2 > 154.0	40	27
6-MAM	328.2 > 165.1	44	33	Diazepam-D5	290.2 > 154.0	40	27
6-MAM-D3	331.2 > 165.1	44	33	Midazolam	326.2 > 291.2	45	29
Cocaine	304.2 > 182.0	30	20	Fentanyl	337.3 > 105.0	35	40
Norketamine	224.1 > 124.9	20	23	Flurazepam	388.2 > 315.1	35	23
EDDP	278.2 > 234.2	26	30	Buprenorphine	468.3 > 101.0	55	42
Zaleplone	306.2 > 264.2	40	22	PCP	244.2 > 159.9	20	15

Results

Oral fluid mixed with buffer was spiked with 10 ng of analytes per loaded sample (n=7), equating to 125 ng/mL when extracting 480 μ L of loaded sample (or 80 μ L of raw oral fluid).

The % analyte recoveries for the various drug classes can be seen in **Figures 2–4**. RSD's ranged from 1.8–9.8%.

Amphetamines, Bath Salts and Opiates Recoveries

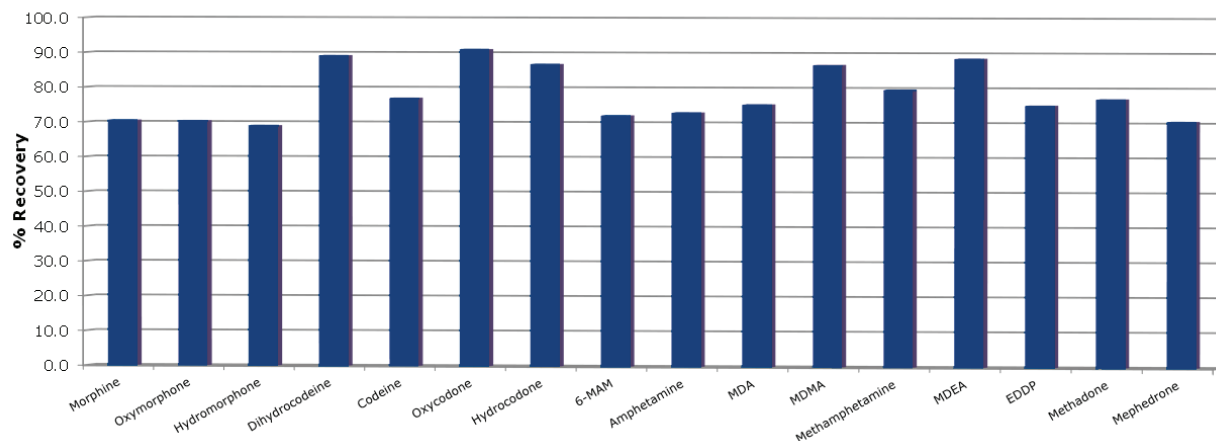


Figure 2. Recovery profile for amphetamines, bath salt and opiates from Intercept-collected oral fluid using ISOLUTE® SLE+ 1 mL columns.

Benzodiazepines Recoveries

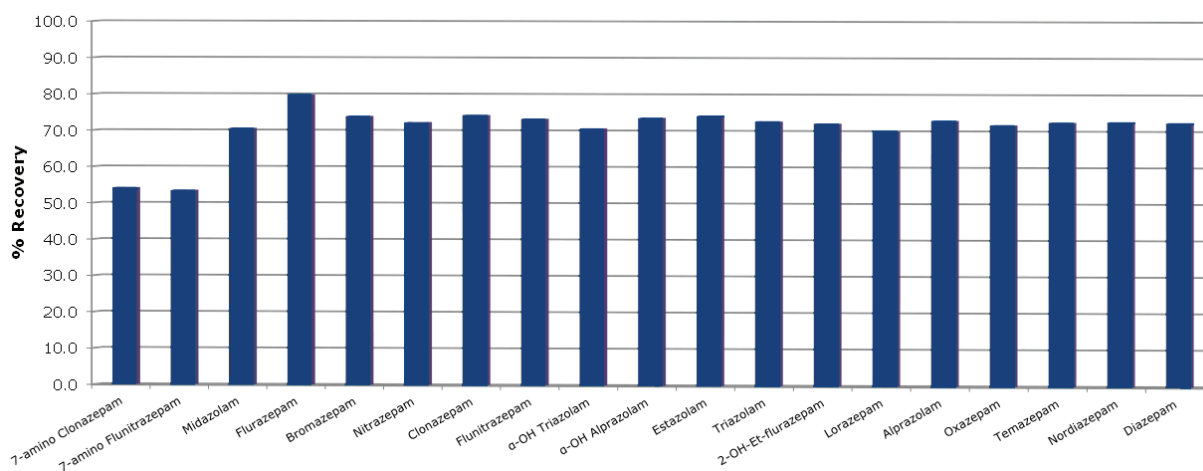


Figure 3. Recovery profile for benzodiazepines from Intercept-collected oral fluid using ISOLUTE® SLE+ 1 mL columns.

Other Drug Recoveries

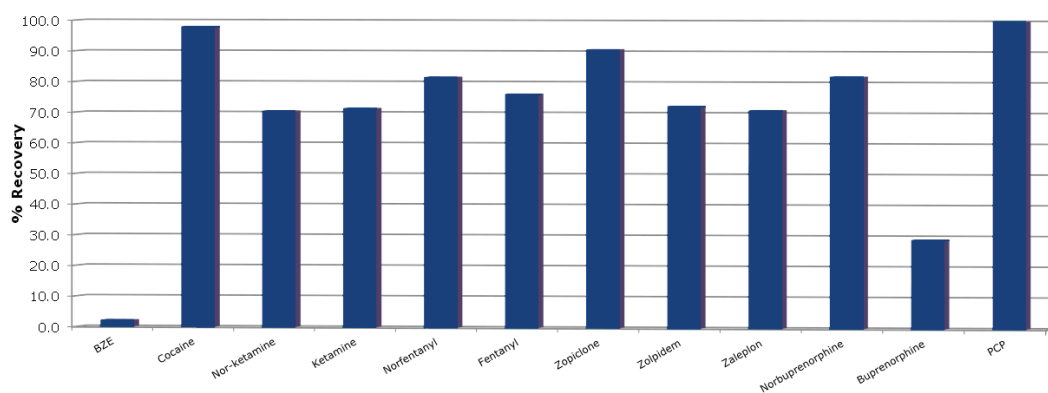


Figure 4. Recovery profile for multi-class analytes from Intercept-collected oral fluid using ISOLUTE® SLE+ 1 mL columns.

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 capacity ISOLUTE® SLE+ 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 μ L.

Compound name: Morphine
Correlation coefficient $r = 0.995544$, $r^2 = 0.991107$
Calibration curve: $0.699405 \cdot x + 0.555574$
Response type: Internal Std (Ref 2), Area* (IS Conc./IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

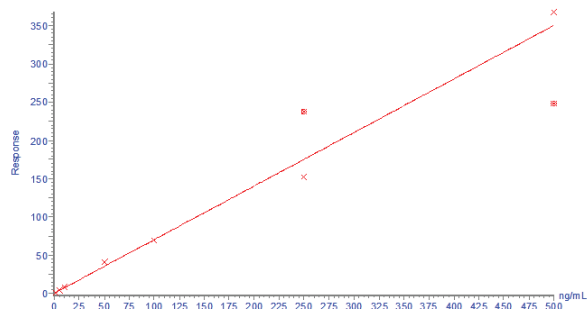


Figure 5. Calibration Curve for morphine using ISOLUTE® SLE+ 1 mL columns.

Compound name: Amphetamine
Correlation coefficient $r = 0.998889$, $r^2 = 0.997778$
Calibration curve: $0.688492 \cdot x + 0.230558$
Response type: Internal Std (Ref 7), Area* (IS Conc./IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

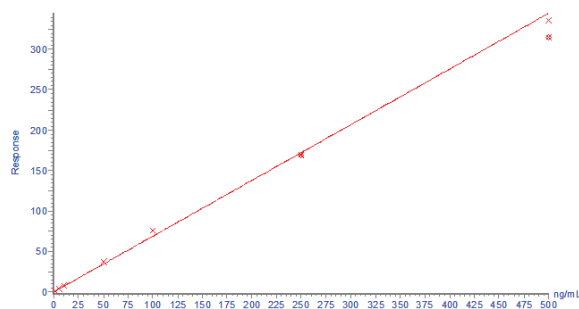


Figure 6. Calibration Curve for amphetamine using ISOLUTE® SLE+ 1 mL columns.

Compound name: BZE
Correlation coefficient $r = 0.999243$, $r^2 = 0.998487$
Calibration curve: $0.833417 \cdot x + 1.11491$
Response type: Internal Std (Ref 11), Area* (IS Conc./IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

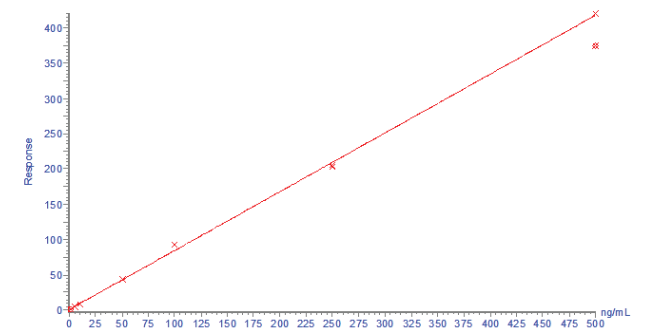


Figure 7. Calibration Curve for benzoylcegonine (BZE) using ISOLUTE® SLE+ 1 mL columns.

Compound name: Diazepam
Correlation coefficient $r = 0.999735$, $r^2 = 0.999471$
Calibration curve: $1.43422 \cdot x + 0.00187844$
Response type: Internal Std (Ref 46), Area* (IS Conc./IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None

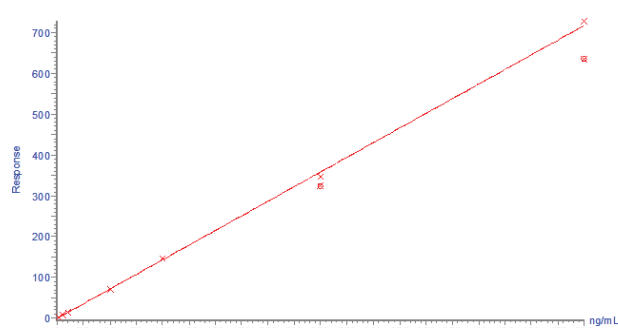


Figure 8. Calibration Curve for diazepam using ISOLUTE® SLE+ 1 mL columns

Table 4. Estimated LOQ's based on S/N ratios from 1 ng/mL extracted samples are:

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

Additional Notes

*Benzoylcegonine 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 BZE recovery is required, dichloromethane 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. 480 μ L sample loaded on a 1 mL capacity column. We were unable to use 95/5 DCM/IPA as elution solvent (as per the equivalent urine assay) due to high levels of co-extracted interferences originating from the collection device buffer.

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. 0.5% aqueous ammonium hydroxide, used to modify pH prior to extraction, was prepared by the addition of 50 μ L of commercially available 28–32% grade to 9.95 mL UHPLC grade water.

Blowdown Stability

Amphetamines, bath salts and ketamines can suffer losses on evaporation when drying in the more volatile free base form. To overcome this effect we added 100 μ L 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 μ L concentrated hydrochloric acid to 11.95 mL HPLC grade methanol. The hydrochloric acid stock is commercially available ~12M.

Ordering Information

Part Number	Description	Quantity
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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