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

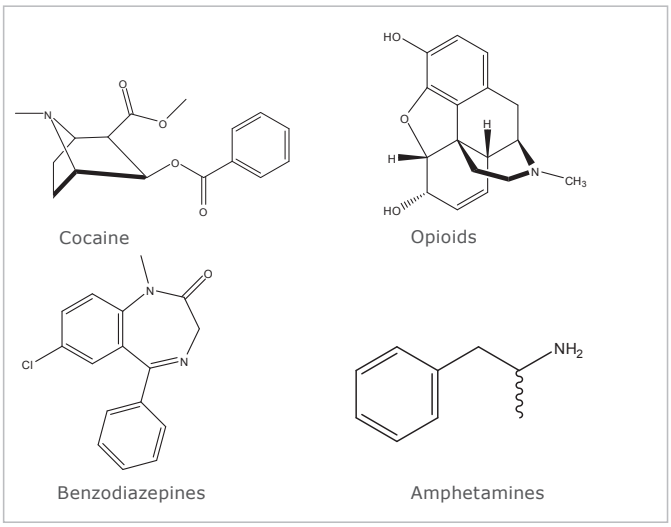

Figure 1. Example structures by class

Introduction

This application note describes the extraction of 47 drugs of abuse from oral fluid matrix after sampling via Quantisal collection devices (Immunalysis), 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 200 µL and 500 µL sample volumes.

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, add 15 µL of concentrated ammonium hydroxide to each collection device (see notes for source of ammonium hydroxide). Vortex mix.

Format: **ISOLUTE® SLE+ 400 µL Sample Volume Columns, part number 820-0055-B (also available in tabless format, part number 820-0055-BG)**

Sample Loading: Load 200 µL of the pre-treated oral fluid device buffer (equivalent to 50 µL of neat 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 dichloromethane (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 (also available in tabless format, part number 820-0140-CG)**

Sample Loading: Load 500 µL of the pre-treated oral fluid device buffer (equivalent to 125 µL of neat 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 (3 mL) and allow to flow under gravity for 5 minutes. Apply a further aliquot of dichloromethane (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: (used for both column formats) Before evaporation, add 100 µL of 50 mM HCl in methanol 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): 20 µL loop

Sample Temperature: 20 °C

Column Temperature: 40 °C

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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 buffer was spiked with 1 ng of analytes (n=7), equating to:

- 8 ng/mL when extracting 500 µL (125 µL neat oral fluid)
- Or 20 ng/mL when extracting 200 µL (50 µL neat oral fluid)

The % analyte recoveries for the various drug classes can be seen in **Figures 2–4**. RSD's ranged from 1.3–9.3%.

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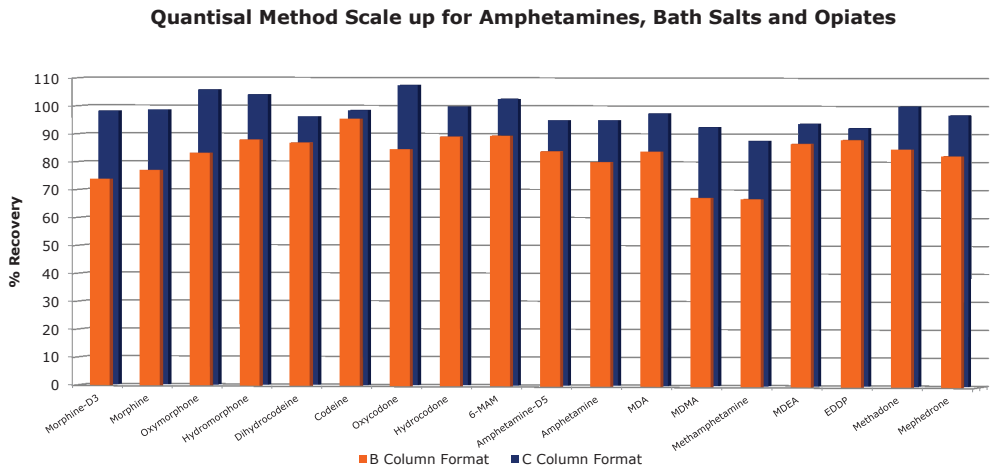

Figure 2. Recovery profile for amphetamines, bath salt and opiates from Quantisal-collected oral fluid using ISOLUTE® SLE+ 400 µL (B) columns and 1 mL (C) columns.

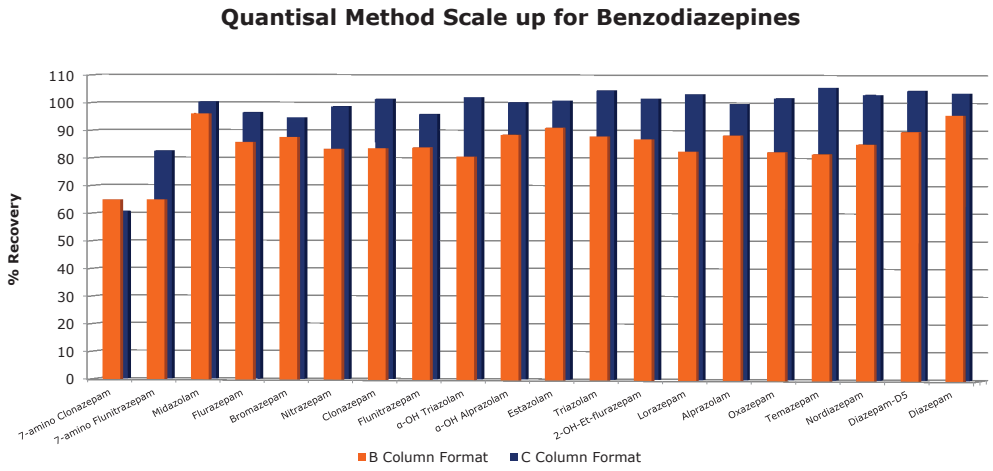

Figure 3. Recovery profile for benzodiazepines from Quantisal-collected oral fluid using ISOLUTE® SLE+ 400 µL (B) columns and 1 mL (C) columns.

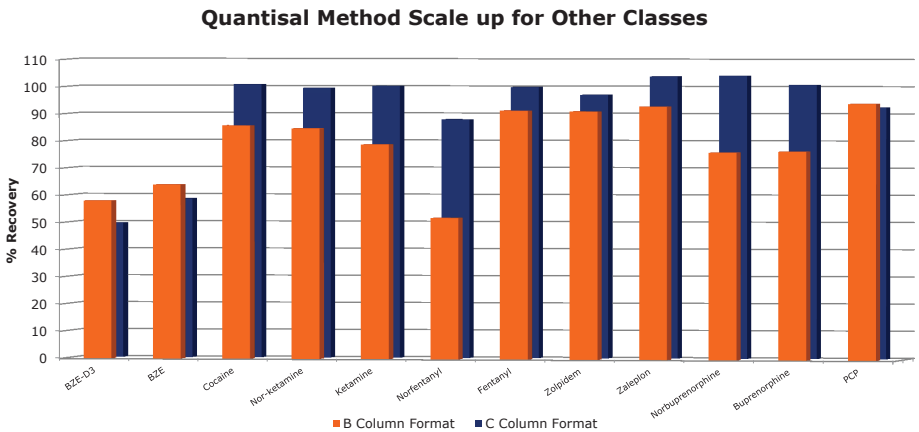

Figure 4. Recovery profile for multi-class analytes from Quantisal-collected oral fluid using ISOLUTE® SLE+ 400 µL (B) columns and 1 mL (C) columns.

Calibration Curves

400 µL capacity column and 1 mL capacity column processing: Calibration curves were generated using OF 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, shown in **Figures 5–8**. Good coefficients of determination were obtained 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 1500 µL as opposed to 200 µL.

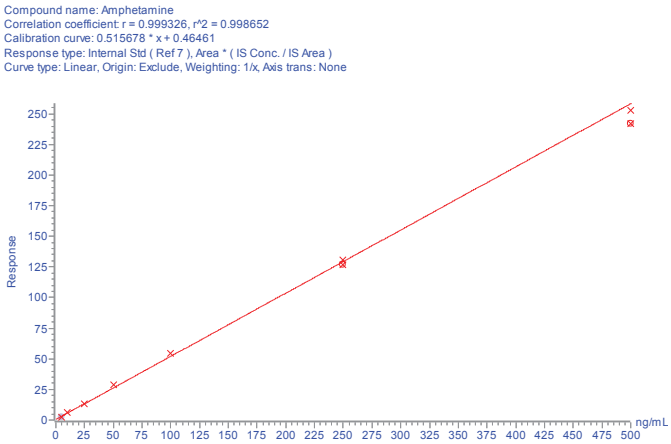

Figure 5. Calibration Curve for amphetamine using ISOLUTE® SLE+ 400 µL columns

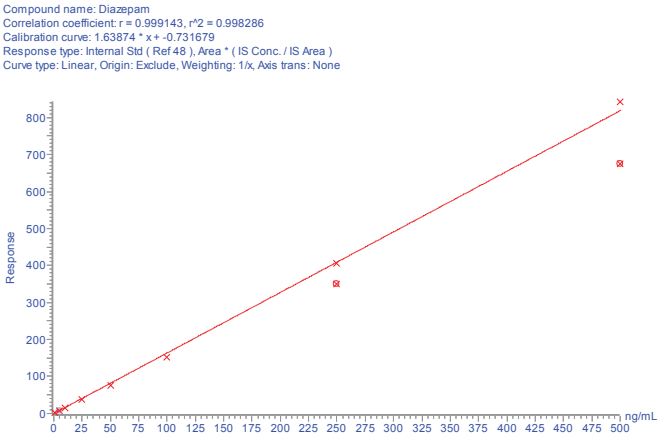

Figure 6. Calibration Curve for diazepam using ISOLUTE® SLE+ 400 µL columns

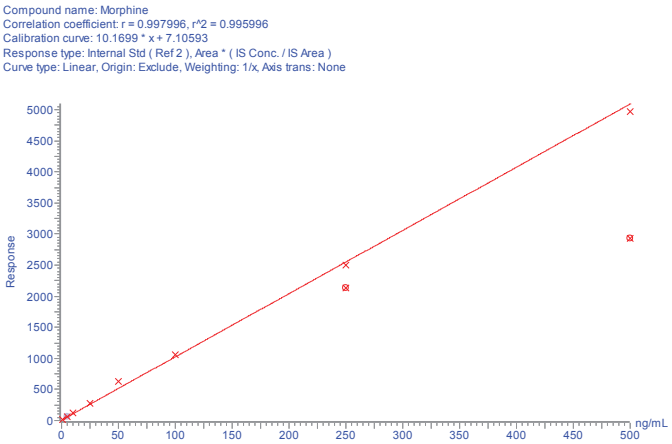

Figure 7. Calibration Curve for morphine using ISOLUTE® SLE+ 400 µL columns

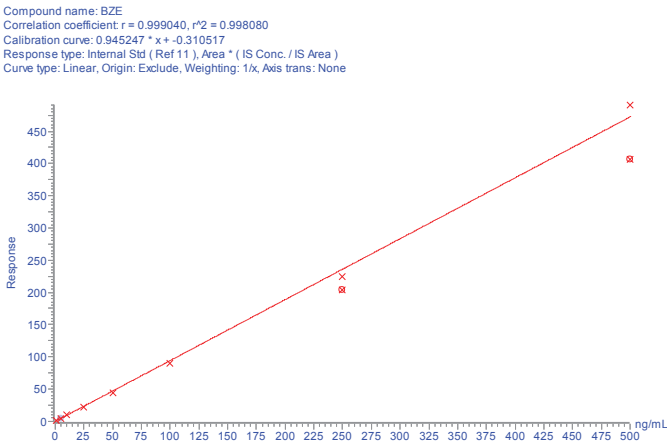

Figure 8. Calibration Curve for Benzoylecgonine (BZE) using ISOLUTE® SLE+ 400 µL columns

Table 4. MRM Conditions

Analyte	Estimated LOQ 400 µL Columns (ng/mL)	Estimated LOQ 1 mL Columns (ng/mL)
Amphetamine	< 0.3	< 0.1
Methamphetamine	< 0.2	< 0.075
MDA	< 0.75	< 0.2
MDMA	< 0.15	< 0.1
MDEA	< 0.1	< 0.025
Hydromorphone	< 0.05	< 0.01
Morphine	< 0.15	< 0.04
BZE	< 0.01	< 0.075
Oxymorphone	< 0.2	< 0.05
Dihydrocodeine	< 0.05	< 0.02
Oxycodone	< 0.05	< 0.04
Mephedrone	< 1	< 0.5
Norfentanyl	< 0.05	< 0.04
7-amino-flunitrazepam	< 0.05	< 0.01
7-amino-clonazepam	< 0.05	< 0.02
Hydrocodone	< 0.3	< 0.2
Codeine	< 0.3	< 0.2
6-MAM	< 0.05	< 0.01
Cocaine	< 0.05	< 0.04
Norketamine	< 0.1	< 0.02
EDDP	< 0.15	< 0.1
Zaleplone	< 0.02	< 0.01
Zopiclone	< 0.1	< 0.025
Norbuprenorphine	< 0.1	< 0.015

Analyte	Estimated LOQ 400 µL Columns (ng/mL)	Estimated LOQ 1 mL Columns (ng/mL)
Ketamine	< 0.025	< 0.01
Nitrazepam	< 0.25	< 0.01
Flunitrazepam	< 0.2	< 0.01
Clonazepam	< 0.02	< 0.01
α-OH-triazolam	< 0.025	< 0.02
Oxazepam	< 0.075	< 0.025
Estazolam	< 0.01	< 0.01
Temazepam	< 0.025	< 0.01
Zolpidem	< 0.025	< 0.02
Alprazolam	< 0.02	< 0.01
Methadone	< 0.1	< 0.02
Lorazepam	< 0.1	< 0.05
Bromazepam	< 0.3	< 0.2
α-OH-alprazolam	< 0.02	< 0.015
2-OH-ethyl-flurazepam	< 0.025	< 0.01
Triazolam	< 0.01	< 0.01
Nordiazepam	< 0.01	< 0.01
Diazepam	< 0.05	< 0.01
Midazolam	< 0.05	< 0.02
Fentanyl	< 0.025	< 0.02
Flurazepam	< 0.025	< 0.015
Buprenorphine	< 0.2	< 0.075
PCP	< 0.025	< 0.02

Additional Notes

Blowdown Stability

Amphetamines, bath salts and ketamine suffer blow down issues when drying in the 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. This does not adversely affect recovery of the other analyte classes.

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. 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.
4. Concentrated ammonium hydroxide stock used to modify pH prior to extraction, is commercially available (28–32%).

Extract Cleanliness

To avoid co-extraction of the additives used in the oral fluid device an underload strategy was used, i.e. 200 µL of sample loaded on a 400 µL capacity column, and 500 µL loaded on a 1 mL capacity column. We were unable to use DCM/IPA (95/5, v/v) as per the equivalent urine assay due to high levels of co-extracted interferences. The use of ethyl acetate or MTBE as elution solvents provided high, reproducible analyte recoveries but suffered from lack of extraction of the cocaine metabolite BZE, hence DCM was selected as the final elution solvent. The use of DCM followed by MTBE is also a possibility if other drugs that are not extracted with DCM as single solvent are present.

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Ordering Information

Part Number	Description	Quantity
820-0055-B	ISOLUTE® SLE+ 400 µL Supported Liquid Extraction Column	50
820-0055-BG	ISOLUTE ®SLE+ 400 µL Supported Liquid Extraction Column (tabless)	50
820-0140-C	ISOLUTE® SLE+ 1 mL Supported Liquid Extraction Column	30
820-0140-CG	ISOLUTE® SLE+ 1 mL Supported Liquid Extraction Column (tabless)	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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