Automated Extraction of Synthetic Cannabinoids (SPICE) from Urine using ISOLUTE® SLE+ Prior to LC-MS/MS

This application note describes the extraction of a range of SPICE drugs and metabolites in urine which are typically screened in forensic toxicology panels using ISOLUTE® SLE+ in a 96-well plate format. Both manual (Biotage Pressure+ 96) and automated (TECAN Freedom EVO® 100) processing conditions are described.

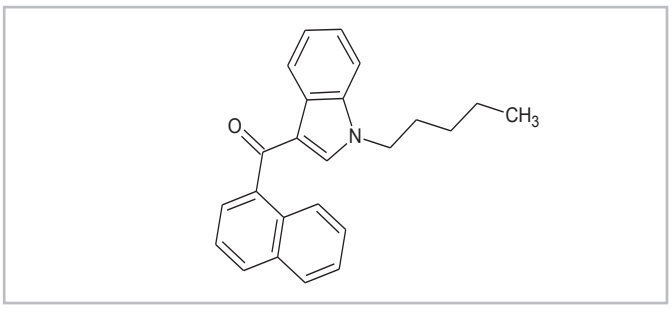

Figure 1. Structureof JWH 018

Introduction

Synthetic cannabinoids have become an increasing problem as an ever changing target for detection during drug screening. A clean and fast extraction method is needed for accurate detection and quantitation of illicit drugs in biological matrices. Supported liquid extraction addresses this need.

ISOLUTE SLE+ Supported Liquid Extraction plates and 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 time.

Analyte

JWH-018, JWH-073, JWH-200, JWH-250, JWH-250-N-(5-hydroxypentyl), JWH-018-N-5-(pentanoic acid), JWH-073 N-(3-hydroxybutyl), JWH 018 N-(4-hydroxypentyl), XLR-11, UR-144, UR-144 (5-Chloropentyl), UR-144-(pentanoic acid), UR-144-(5-hydroxypentyl)

Fully Automated Sample Preparation Workflow using Tecan Freedom EVO® 100 Work Station

Format:	ISOLUTE SLE+ 400 µL Supported Liquid Extraction Plate, part number 820-0400-P01
TECAN Pre-planning:	The pipetting strategy of buffers, samples and solvents was planned to minimize pipette tip usage and add solutions as needed with no risk of sample cross contamination. The sample, buffer and solvent volumes are limited to the 96-well volumes and should be predetermined based on empirical knowledge. Calibration, QC and fortified standards were prepared offline.
Urine Hydrolysis Station: (96-well collection plate)	Pipette in this exact order: ammonium acetate (100 mM, pH 5, 380 µL), β-glucuronidase (5000 units/mL, 10 µL), urine (400 µL, calibration standard, QC standard, blank or patient sample) and internal standard (20 µL) into designated wells. Agitate sample plate and heat to recommended enzyme conditions for 60 minutes. This step can also be conducted offline.
Pre-treatment Station: (96-well collection plate)	Pipette hydrolyzed urine samples (400 µL) and ammonium hydroxide (2%, v/v, 400 µL) into designated wells. Mix the solution by aspirating and dispensing several times into well.
Extract Station: (ISOLUTE SLE+ plate)	Pipette 400 µL of pre-treated samples into designated wells of ISOLUTE SLE+ extraction plate. Apply a short pulse of vacuum to initiate flow and then allow sample to absorb for 5 minutes. Apply ethyl acetate (2 x 700 µL) into each well and allow solvent to flow under gravity. Apply brief vacuum pulse.
Post extraction:	Evaporate sample to dryness and reconstitute in mobile phase

Biotage®

Manual Sample Preparation Workflow for Biotage® Pressure+ 96 Manifold

Format: ISOLUTE SLE+ 400 µL Supported Liquid Extraction Plate, part number 820-0400-P01

Urine Hydrolysis:: Add β-glucuronidase at a concentration of 5000 units/mL to 1 mL of patient urine, fortified calibration standards and QC standards in appropriate container. Add ammonium acetate (pH 5, 100 mM, 1 mL). Spike the solution with internal standard. Incubate sample as per instructions with enzyme.

Sample Pretreatment: Load 400 µL of hydrolyzed urine samples onto the ISOLUTE® SLE+ 96-well plate. Apply a short pulse of positive pressure and allow samples to absorb for 5 minutes.

Analyte elution: Apply ethyl acetate (2 x 700 µL). Apply short pulses of pressure and collect eluent.

Post extraction: Evaporate sample to dryness and reconstitute in mobile phase

HPLC Conditions

Instrument: Agilent 1200 Liquid Handling System (Agilent Technologies, Berkshire, UK)

Column: Mac-MOD ACE Excel 2 C18-AR, 2.1 x 100 mm i.d. (Mac-MOD Analytcal, Chadds Ford, PA.)

Mobile Phase: A: 0.1% formic acid in water
B: 0.1% formic acid in methanol

Isocratic: 15% A/ 85% B at 300 µL/min; 10 minute run time

Injection Volume: 10 µL

Temperature: Ambient

Mass Spectrometry Conditions

Applied Biosystems/MDS Sciex 4000 Q-Trap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA.) equipped with a Turbo Ionspray® interface for mass analysis.

Ion Source Temperature: 500 °C

Scan Function	Analyte	MRM Transition	Declustering Potential (DP)	Collision Energy (CE)	Cell Exit Potential (CXP)
1	JWH-073	328>155	40	30	16
2	JWH-018	342>155	40	30	16
3	JWH-018 N-(4-hydroxypentyl)	358>155	40	30	16
4	JWH-018 5-pentanoic acid	372>155	40	30	16
5	JWH-073 N-(3-hydroxybutyl)	344>155	40	30	16
6	JWH-250 N-(5-hydroxypentyl)	352>120.9	40	30	16
7	JWH-200	385>155	40	30	16
8	JWH-250	336>121	40	30	16
9	d5-JWH-018 N- (4-hydroxypentyl	363.5> 155	40	35	16
10	XLR-11	330>125	30	35	16
11	UR-144	312.5>125	30	35	16
12	UR-144 5-Chloro-pentyl	346.9>125	30	35	16
13	UR-144 Pentanoic Acid	342.5>125	30	35	16
14	UR-144 5-Hydroxy-pentyl	328.5>125	30	35	16

Table 1. MRM transitions for SPICE drugs in positive mode Turbo Ionspray

Results

The TECAN Freedom EVO® 100 liquid handling system was set up to fully automate the process of extracting drugs from biological matrix using ISOLUTE® SLE+ in a 96-well plate format. The overall process requires a series of workstations dedicated to an enzymatic hydrolysis, sample pre-treatment and sample extraction sub-process. **Figure 3** shows an illustration of the work platform set-up to facilitate processing of patient samples, calibration standards and QC standards through each sub-process station. Each sub-process is conducted in a 96-well plate. **Figure 4** shows a photograph of the workstation illustrated in **Figure 3.**

Blank human urine was fortified with a suite of SPICE standards listed in **Table 1**. The extraction of SPICE analytes from multiple biological matrices was recently reported in Application note 774. This same methodology was adapted for the programming of the TECAN Freedom EVO 100 to facilitate automation. Urine calibrators and QC standards were prepared offline and loaded onto TECAN liquid handler. The manual extraction of the SPICE suite was conducted on a Biotage PRESSURE+ 96 manifold. Initial recoveries of the analytes from the ISOLUTE SLE+ 400 µL 96-well plate were determined using the semi-automated process (**Figure 2**) The number of averaged samples used to determine the recoveries was seven with percent relative standard deviations less than 10.

A series of urine matrix calibration standards at 3, 5, 10, 25, 50 and 100 ng/mL were prepared offline. In-house QC check standards were prepared using a different urine matrix from the one used to prepare calibration standards. The samples were loaded onto the TECAN Freedom EVO 100 and extracted on the ISOLUTE SLE+ 400 µL 96-well plate without performing the hydrolysis step. Calibration curves were generated for each analyte. **Figure 5** shows a typical calibration curve generated for each analyte. The mass spec response for each extracted QC standard was measured against the appropriate calibration curve to determine the concentration for each analyte. The measured concentrations and the percent accuracy (in parentheses) for each QC analyte are listed in **Table 2**.

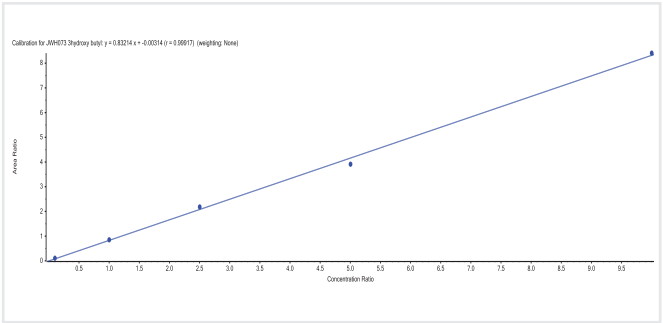

Figure 5. Calibration curve for JWH073 3-hydroxybutyl (r^2>0.99) generated from calibration standards extracted using TECAN Freedom EVO 100 liquid handling system. This curve is typical for 5-level standard curve determination for all of the SPICE drug analytes.

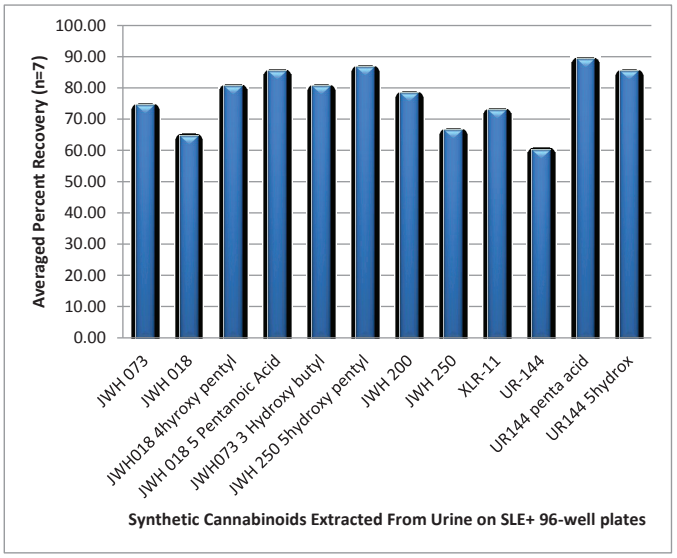

Figure 2. Plot of averaged recoveries (n=7) for SPICE drugs spiked into urine at 10ng/mL and extracted using the Biotage Positive PRESSURE+ 96 manifold.

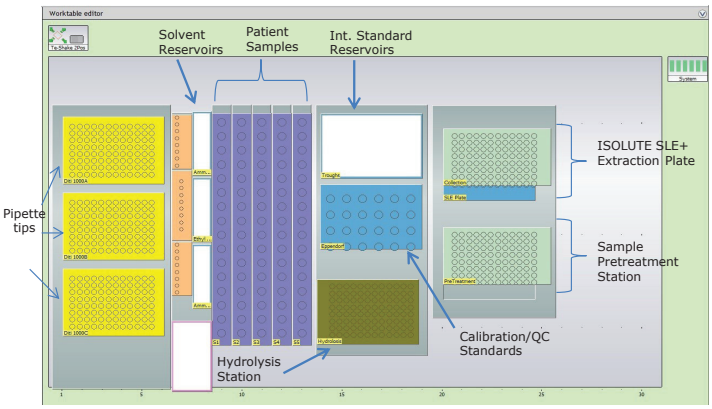

Figure 3. Illustration of Tecan Freedom Evo® 100 workstation layout for fully automated SPICE drug extraction from urine. The process set-up includes a 96-well plate station for hydrolysis of urine using enzyme, acid or base. Sample pre-treatment is facilitated in a separate 96-well plate prior to loading onto ISOLUTE® SLE+ plate for sample extraction.

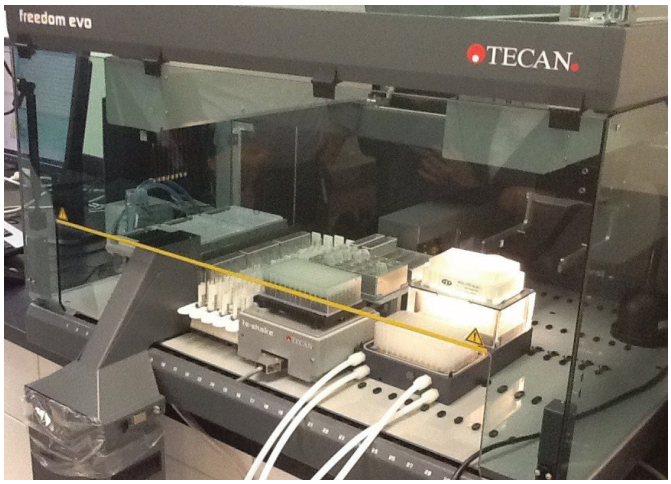

Figure 4. Picture of actual TECAN Freedom EVO® 100 liquid handling workstation used for SPICE drug extraction processing.

Calculated Concentration of Fortified QC standards (% Accuracy)		
Drug Analyte	**QC 75 ng/mL**	**QC 15 ng/mL**
JWH-073	52.76 (70)	17.95 (119)
JWH-018	55.41 (74)	16.40 (109)
JWH-018 N- (4-hydroxypentyl)	62.57 (83)	13.74 (92)
JWH-018 5-pentanoic acid	70.22 (94)	15.35 (102)
JWH-073 N-(3-hydroxybutyl)	66.17 (88)	14.92 (99)
JWH-250 N-(5-hydroxypentyl)	74.43 (99)	15.13 (101)
JWH-200	81.04 (108)	17.64 (118)
JWH-250	57.43 (77)	13.93 (93)
XLR-11	65.24 (87)	15.29 (102)
UR-144	65.17 (87)	16.81 (112)
UR-144 5-Chloro-pentyl	83.21 (110)	15.99 (106)
UR-144 Pentanoic Acid	73.65 (98)	15.48 (103)
UR-144 5-Hydroxy-pentyl	59.29 (80)	12.95 (86)

Table 2. Results of fortified urine spiked with known amounts of SPICE drugs to create in-house QC standards to test against calibration curve generated from calibration standards. All of the samples were extracted using the TECAN Freedom EVO® 100 workstation.

Acknowledgements

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References

Extraction of Synthetic Cannabinoids (SPICE) and Metabolites from Urine, Plasma, and Whole Blood using ISOLUTE SLE+ Prior to LC-MS-MS Analysis. Biotage Application Note 774, December 2012.

Victor E. Vandell, Bradford Elmore, Simon Fogarty, Frank Kero and Elena Gairloch, “A Method for Automated Extraction of Pain Medication from Urine for Analysis by LC-MS/MS” MSACL 2013, San Diego, California.

Ordering Information

Part Number	Description	Quantity
820-0400-P01	ISOLUTE® SLE+ + 400 µL Supported Liquid Extraction Plate	1
PPM-96	Biotage® Pressure+ 96 Positive Pressure Manifold	1
SD-9600-DHS-NA	Biotage® SPE Dry 96	1

For the latest application notes and more information about ISOLUTE® SLE+ visit www.biotage.com

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