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APPLICATIONS

Acids, Neutrals and Bases Using Novum™ Simplified Liquid Extraction (SLE)

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Introduction

A common practice when performing a liquid-liquid extraction (LLE) or SLE method is to adjust pH to neutralize analytes of interest in order to maximize their LogD value (getting them to be as hydrophobic as possible). This facilitates their partition from an aqueous sample into an extracting organic solvent. Due to the differences in pKa values between acids and bases, there is no pH adjustment that could neutralize both acids and bases effectively. Because of this, it is not usually recommended to combine both acids and bases in a single liquid extraction.

In this technical note, we will break with tradition and show how a specific pH manipulation can lead to a successful extraction of a relatively hydrophobic acid (THC-COOH), polar bases (buprenorphine and norbuprenorphine) and neutrals (barbiturates).

We developed a SLE application for acids (THC-COOH), neutrals (barbiturates), and bases (Norbuprenorphine and Buprenorphine) from a urine matrix containing β-glucuronidase followed by two LC-MS/MS methods using a Kinetex® EVO C18 LC column and SCIEX API 4000™ mass spec in negative mode electrospray ionization (ESI-) and a Kinetex Biphenyl LC column and SCIEX API 4000 mass spec in positive mode electrospray ionization (ESI+).

Materials and Methods

Reagents and Chemicals

Secobarbital, Amobarbital, Phenobarbital, Butalbital, Pentobarbital, Pentobarbital-D5, 11-nor-9-Carboxy-Δ9-THC (COOH-THC) and 11-nor-9-Carboxy-Δ9-THC-D3 (COOH-THC-D3) standards were purchased from Cerilliant® (Round Rock, TX). Campbell β-Glucuronidase Enzyme was purchased through Campbell Science Products, 100,000 units/mL (Rockford, IL). Formic acid was purchased from Sigma-Aldrich® (St. Louis, MO). HPLC-grade acetonitrile, methanol, methyl tert-butyl ether, ethyl acetate and hexane were purchased from Honeywell™ (Morris Plains, NJ).

Experimental Conditions

Sample Pre-treatment

Each sample was comprised of 200 µL urine, 25 µL β-glucuronidase, 25 µL Ammonium acetate buffer (100 mM, pH 4.0), 180 µL Ammonium bicarbonate buffer (100 mM, pH 9.0), and 20 µL of internal standards (concentration of analytes range from 0.5 µg/mL-25 µg/mL depending on analyte).

Supported Liquid Extraction (SLE)

96-Well Plate:	Novum SLE MAX 96-Well Plate
Part No.:	8E-S138-5GA
Condition:	Load 450 µL pretreated sample and pulse vacuum (~5" Hg) for 2-3 seconds or until sample has completely entered the sorbent. Wait 6 minutes.
Elute:	2x 900 µL aliquots of Ethyl acetate. Allow to flow via gravity. At the completion of the second aliquot, apply vacuum at 5" Hg for 15 seconds to complete the extraction and remove residual solvent from tips.
Dry down:	Evaporate eluate to dryness at room temperature under a gentle stream of Nitrogen
Reconstitute:	Reconstitute in 100 µL of Methanol/Water (20:80) with 100 ng/mL of COOH-THC-D3, 250 ng/mL of Ammobarbital-D5 and 100 ng/mL of Morphine-D6

LC Conditions (ESI+)

Analytical Column: Kinetex 2.6 µm Biphenyl 100 Å
Dimension: 50 x 2.1 mm
Part No.: 00B-4622-AN
Recommended Guard:: SecurityGuard™ ULTRA Biphenyl
Guard Part No.: AJ0-9209
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Acetonitrile
Gradient:

Time (min)	%B
0	5
5	100
5.1	5
7	5

Injection Volume: 4 µL
Flow Rate: 0.5 µL/min
Temperature: Ambient
Instrument: Agilent® 1260 LC
Detector: MS/MS (SCIEX API 4000™) ESI+

LC Conditions (ESI-)

Analytical Column: Kinetex 2.6 µm EVO C18 100Å
Dimension: 50 x 2.1 mm
Part No.: 00B-4725-AN
Recommended Guard:: SecurityGuard ULTRA EVO C18
Guard Part No.: AJ0-9298
Mobile Phase: A: 10 mM Ammonium bicarbonate,, pH 9
B: Acetonitrile
Gradient:

Time (min)	%B
0	5
2	15
5	20
5.01	60
6	60
6.1	5
7.5	5

Injection Volume: 3 µL
Flow Rate: 0.5 µL/min
Temperature: Ambient
Instrument: Agilent 1260 LC
Detector: MS/MS (SCIEX API 4000) ESI-

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Figure 1.
Chromatogram in ESI+ mode (Buprenophrine/Norbuprenorphine/THC-COOH)

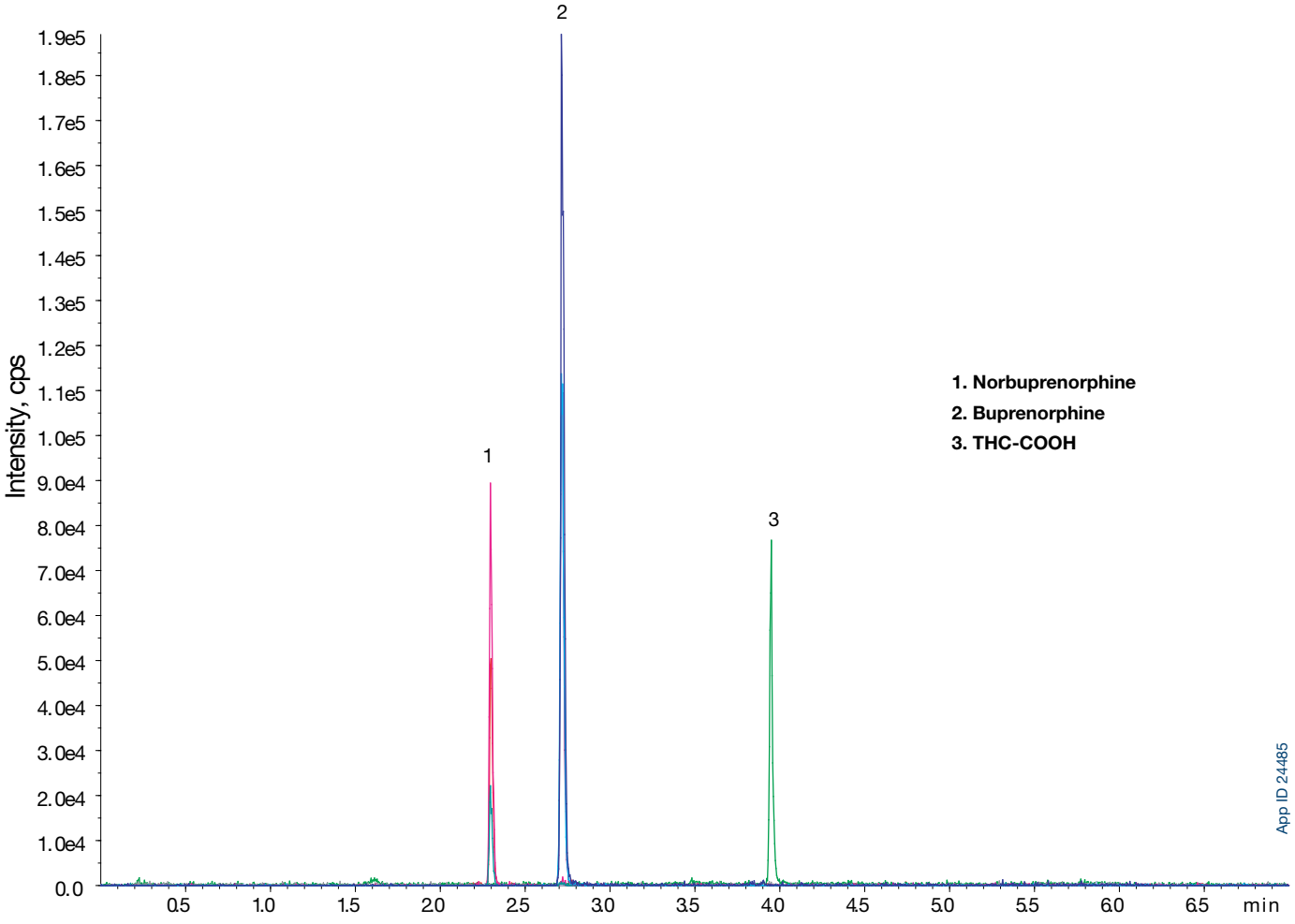

Figure 2.
Chromatogram in ESI- Mode (Barbiturates Mix)

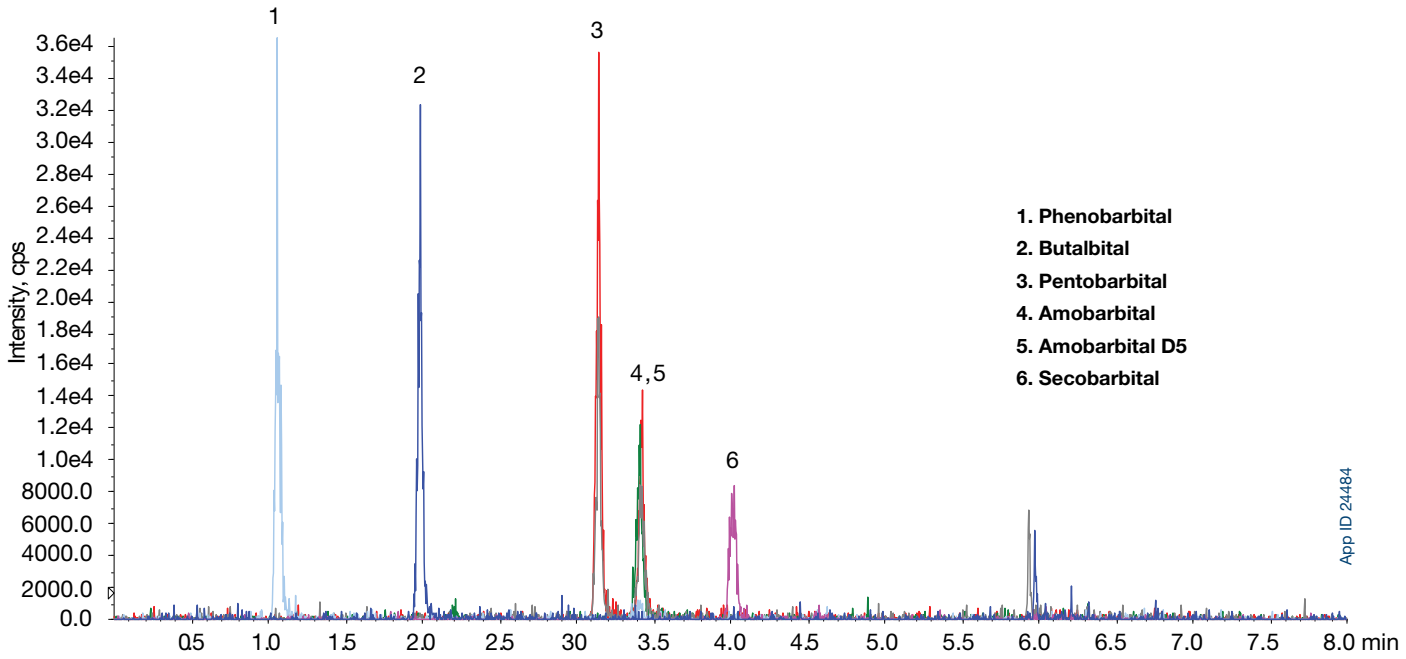

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Table 1.
Percent Recovery for All Analytes Under Optimized Conditions (pH 9, Ethyl acetete extraction)

Analyte	Average % Recovery	%CV
Buprenorphine	103	9
Norbuprenoprhine	97	11
THC-COOH	95	9
Pentobarbital	85	10
Butalbital	99	1
Phenobarbital	85	10
Amobarbital	96	6
Secobarbital	84	5

Discussion

Since the working concentrations for norbuprenorphine, buprenorphine and THC-COOH are much lower than barbiturates, the recovery data for these compounds was more critical, and thus the positive mode panel was screened and optimized first. Only the samples that showed acceptable recovery were subsequently rerun in negative mode to determine if this SLE method is also suitable for the barbiturates. This process can be significantly improved through use of polarity switching on the LC-MS/MS. However, this process forced a better understanding of the chemistry in play, while hopefully leading to a better explanation than what would be ascertained through a more brute force approach.

Below are the various conditions that were screened for this method.

pH of Sample Diluent:

Our method development began with optimizing the sample diluent (see sample pre-treatment) by testing 3 different pHs: 8, 9, and 9.5. These 3 pH levels were selected based on the individual analyte's LogD calculations provided by the computation website, www.chemicalize.com. In each scenario, the goal was to target a LogD >1 for each analyte. Ultimately, pH 9 provided the best results.

pH 8	• Poor recovery of Norbuprenorphine, which is important because of the low cutoffs for Norbuprenorphine
pH 9	• Best recovery • Recovery values presented in **Table 1**
pH 9.5	• Lower and more inconsistent %CV for THC-COOH • Low recovery of the most polar barbiturates (Secobarbital and Butalbital)

Elution Solvent

In addition to the discussed deviations in pH, various solvent mixtures were screened in order to identify which produced the highest recoveries and lowest %CVs for all analytes of interest. While some showed promise, ultimately it was difficult to find a solvent scheme that showed consistent recovery for Norbuprenorphine, Buprenorphine and THC-COOH at pH 9.

For example:

MTBE	Provided low recovery of buprenorphine and nor buprenorphine as well as THC-COOH in comparison to Ethyl Acetate
MTBE/ETAC (1:3)	%CVs > 15 % for all compounds in positive mode
MTBE/IPA (95:5)	%CVs > 20 % for all compounds in positive mode
Ethyl Acetate	Recovery values presented in **Table 1**
ETAC/Hexane (1:3) and ETAC/ Hexane (3:1)	Any mixture tried with hexane result in recoveries that were not acceptable for the opioid based compounds
Chloroform/ IPA (95:5) and DCM/ IPA (95:5)	Both of these chlorinated solvent schemes provided no recovery for THC-COOH, while also yielding a very dirty baseline in comparison to other solvents tested

Conclusion

By implementing an ammonium bicarbonate buffer, that is neither too basic to diminish recovery of THC-COOH and barbiturates, nor one that is too acidic to reduce recovery of norbuprenorphine, the pH 9 dilution was shown to provide the best middle ground for maximizing Log D value for each of the compounds tested without significantly compromising a single analyte or class within the suite.

While other solvents are similar in effective polarity, ethyl acetate as an extraction solvent provided the highest recoveries and lowest % RSDs, while showing a background acceptable for quantitation.

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Ordering Information

Novum™ SLE 96-Well Plates

Novum Simplified Liquid Extraction (SLE) Well Plates		
Part No.	**Description**	**Unit**
8E-S138-FGA	Novum SLE MINI 96-Well Plate	1/pk
8E-S138-5GA	Novum SLE MAX 96-Well Plate	1/pk

Novum SLE Tubes

Novum Simplified Liquid Extraction (SLE) Tubes		
Part No.	**Description**	**Unit**
8B-S138-FAK	Novum SLE 1 cc tubes	100/pk
8B-S138-5BJ	Novum SLE 3 cc tubes	50/pk
8B-S138-JCH	Novum SLE 6 cc tubes	30/pk
8B-S138-KDG	Novum SLE 12 cc tubes	20/pk

Kinetex® Core Shell LC Columns

2.6 µm Minibore Columns (mm)				SecurityGuard™ ULTRA Cartridges‡
Phases	**30 x 2.1**	**50 x 2.1**	**100 x 2.1**	**3/pk**
EVO C18	00A-4725-AN	00B-4725-AN	00D-4725-AN	AJ0-9298
Biphenyl	00A-4622-AN	00B-4622-AN	00F-4622-AN	AJ0-9209

‡ SecurityGuard ULTRA Cartridge requires holder, Part No.: AJ-9000.

Presston™ 100 Positive Pressure Manifold

Part No.	Description
AH0-9334	Presston 100 Positive Pressure Manifold, 96-Well Plate
AH0-9342	Presston 100 Positive Pressure Manifold, 1 mL Tube Complete Assembly
AH0-9347	Presston 100 Positive Pressure Manifold, 3 mL Tube Complete Assembly
AH0-9343	Presston 100 Positive Pressure Manifold, 6 mL Tube Complete Assembly

Presston 100 Tube Adapter Kits (for AH0-9334)

Part No.	Description
AH0-9344	1 mL Tube Adapter Kit
AH0-9345	3 mL Tube Adapter Kit
AH0-9346	6 mL Tube Adapter Kit

WARRANTY Phenomenex warrants that for a period of 12 months following delivery, the Presston 100 Positive Pressure Manifold you have purchased will perform in accordance with the published specifications and will be free from defects in materials or workmanship. In the event that the Presston 100 Positive Pressure Manifold does not meet this warranty, Phenomenex will repair or replace defective parts. Please visit www.phenomenex.com/Presston for complete warranty information.

guarantee

If Phenomenex products in this technical note do not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

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