New Applications:

Environmental

- Update EPA Method 8321B-Determination of Phenoxyacid Herbicides in Water by Solid Phase Extraction and LC-MS/MS Detection
- Determination of Pesticide Residues in Blueberries by AOAC QuEChERS Approach and Dispersive SPE Cleanup with a Novel Sorbent ChloroFiltr®
- A Determination of Veterinary Drug Residues in Milk Using Polymeric SPE and UHPLC-MS/MS Analysis
- EPA Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Aqueous Direct Injection and LC/MS/MS
- Detection of Aflatoxins in Milk at Picogram Levels Using SPE and LC-MS/MS

Forensic

- Simultaneous Determination of Prescription and Designer Benzodiazepines in Urine by SPE and LC-MS/MS
- SOFT-Determination of 35 Pesticides and 3 Cannabinoids in Marijuana Edibles
- Extraction of Synthetic and Naturally Occurring Cannabinoids in Urine Using SPE and LC-MS/MS
- LC-MS/MS Method for 89 Banned or Controlled Drugs in the Horse Racing Industry
- Updated- Quantitative Analysis of EtG and EtS in Urine Using Clean Screen® ETG and LC-MS/MS

Clinical

- Thyroid Hormones in Serum and Plasma Using SPE Extraction and UHPLC-MS/MS Analysis

Determination of Phenoxyacid Herbicides in Water by Solid Phase Extraction and LC-MS/MS Detection

UCT Part Numbers:
ECHLD156-P (Enviro Clean® HL DVB 500mg/6mL, PE Frits)
VMFSTFR12 (Sample Transfer Tubes)

EPA Method 8321B*

Procedure:

1. **Sample Pretreatment**
   a) Adjust sample pH to <1 with 1:1 sulfuric acid in water, low pH is critical to obtain high recoveries.

2. **Cartridge Conditioning**
   a) Attach sample transfer tubes (**VMFSTFR12**) to the top of the SPE cartridges (**ECHLD156-P**), and attach the SPE cartridges to an SPE manifold.
   b) Wash the SPE cartridges (with transfer tubes connected) using 10 mL methylene chloride, let solvent soak sorbent for 2 min before drawing to waste, leave full vacuum on for 1 min.
   c) Condition the SPE cartridges with 10 mL methanol, leave a thin layer above the frit.
   d) Equilibrate the SPE cartridges with 15 mL DI water, leave a thin layer above the frit.

3. **Sample Loading**
   a) Insert the stainless steel ends of the sample transfer tubes into sample bottles, adjust vacuum for a fast dropwise sample flow (about 20-25 mL/min).
   b) After all sample is passed through, dry the SPE cartridges under full vacuum for 10 min.

4. Analyte Elution

a) Insert the collection vials to the manifold.

b) Rinse the sample bottles with 5 mL acetonitrile, apply the rinse to the SPE cartridges. Let the elution solvent soak the sorbent for 1-2 min before drawing through slowly.

c) Repeat the elution (step 4b) with 2 additional aliquots of 5 mL acetonitrile.

Instrumental Analysis

Analyze the eluate directly by LC-MS/MS, or concentrate to 1 mL and analyze by HPLC.

Note: Use acid washed sodium sulfate and glassware if SPE eluates need be dried and concentrated.

Results

Compound	LCS1 Recovery%	LCS2 Recovery%	RPD%
2,4-D	96.6	96.2	0.4
MCPA	95.0	94.4	0.6
Dichlorprop	93.6	92.7	1.0
Mecoprop	94.4	93.4	1.1
2,4,5-T	94.2	93.0	1.3
Dichlorobenzoic acid	89.5	87.6	2.1
2,4-DB	85.1	83.7	1.7
Acifluorfen	108.6	88.4	20.5
Silvex	103.1	86.0	18.1
Bentazone	89.8	89.7	0.1

*EPA Method 8321B SOLVENT-EXTRACTABLE NONVOLATILE COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/THERMOSPRAY/MASS SPECTROMETRY (HPLC/TS/MS) OR ULTRAVIOLET (UV) DETECTION

UCT, LLC • 2731 Bartram Road • Bristol, PA 19007 • 800.385.3153 • 215.781.9255 • www.unitedchem.com • Email: methods@unitedchem.com

Determination of Pesticide Residues in Blueberries by AOAC QuEChERS Approach and Dispersive SPE Cleanup with a Novel Sorbent ChloroFiltr®

UCT Part Numbers:
ECMSSA50CT-MP - Mylar pouch containing 6 g $MgSO_4$ and 1.5 g NaOAc with 50-mL centrifuge tubes included
CUMPSGGC182CT - 2 mL centrifuge tube containing 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18, and 50 mg ChloroFiltr®
SLAQ100ID21-3UM - Selectra® Aqueous C18, 100 x 2.1 mm, 3 µm
SLAQGDC20-3UM - Selectra® Aqueous C18, Guard column, 10 x 2.1 mm, 3 µm
SLGRDHLDR - Guard Cartridge Holder

Summary:

Blueberry has been ranked as one of the healthiest fruits for its high antioxidant content that helps combating free radicals, which could damage DNA and cellular structures [1]. Application of pesticides during plant cultivation is common to increase product yield, therefore it is valuable developing effective analytical methods for the determination of pesticide residues in blueberries, which however is challenging due to matrix complexity as blueberries are rich in anthocyanins, sugars, polyphenols, vitamins, minerals, and other interfering components.

This application outlines a simple, fast, and cost-effective method for the determination of multi-class pesticides, including one of the most problematic pesticides, pymetrozine in blueberries. The acetate buffered AOAC QuEChERS protocol demonstrated higher extraction efficiency for pymetrozine than the other 2 QuEChERS protocols (the EN citrate buffered or the original unbuffered), thus was selected for the extraction of pesticide residues in blueberries. 15 g of homogenized blueberries were extracted with 15 mL of acetonitrile (MeCN) containing 1% acetic acid (HAc). 6 g magnesium sulfate ($MgSO_4$) and 1.5 g sodium acetate (NaOAc) were employed to enhance phase separation and partition of pesticides into the MeCN layer. After shaking and centrifugation, 1 mL of the supernatant was transferred to a 2-mL dSPE tube containing the optimized cleanup sorbents of 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18, and 50 mg

ChloroFiltr®. Residual water was absorbed by $MgSO_4$, anthocynins, polyphenols, sugars and organic acids were removed by PSA, lipids and other non-polar interferences were retained by C18, while chlorophylls were removed by ChloroFiltr®, resulting in clean extract for LC/MS/MS analysis. UCT's aqueous C18 HPLC column was used for analyte retention and separation, which showed superior retention especially for several very polar pesticides, such as methamidophos and acephate.

Procedure:

1. **QuEChERS extraction**
    a) Weigh 15 ± 0.3 g of homogenized blueberry sample into 50-mL centrifuge tubes.
    b) Add triphenyl phosphate (TPP) as internal standard (IS) (optional), and appropriate amounts of spiking solution to fortified samples.
    c) Add 15 mL of MeCN with 1% HAc. Cap and shake for 1 min at 1000 strokes/min using a Spex 2010 Geno-Grinder.
    d) Add salts (6 g $MgSO_4$ and 1.5 g NaOAc) from pouch (**ECMSSA50CT-MP**) to the 50-mL tube, and vortex for 10 sec to break up salt agglomerates.
    e) Shake for 1 min at 1000 strokes/min using the Geno-Grinder.
    f) Centrifuge at 3000 rcf for 5 min.

2. **dSPE cleanup**
    a) Transfer 1 mL of the supernatant to a 2-mL dSPE tube (**CUMPSGGC182CT**).
    b) Shake for 1 min at 1000 strokes/min using the Spex 2010 Geno-Grinder.
    c) Centrifuge at 3000 rcf for 5 min.
    d) Transfer 0.2 mL of the cleaned extract into a 2-mL auto-sampler vial, add 0.2 mL of reagent water, and vortex for 30 sec.
    e) The samples are ready for LC-MS/MS analysis.

LC-MS/MS Method:

HPLC: Thermo Scientific Dionex UltiMate 3000® LC System		
Column: UCT, Selectra®, aQ C18, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, aQ C18, 10 x 2.0 mm, 3 µm		
Column temperature: 40 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
Injection volume: 2 µL		
Gradient program:		
Time (min)	**A%** (10 mM ammonium acetate in DI water)	**B%** (0.1% formic acid in MeOH)
0	100	0
1	50	50
3.5	50	50
6	5	95
9	5	95
9.1	100	0
14	100	0

Divert mobile phase to waste from 0 – 1.5 and 11.5 – 14 min to prevent ion source contamination.

MS parameters	
Instrumentation	Thermo Scientific TSQ Vantage tandem MS
Polarity	ESI +
Spray voltage	3500 V
Vaporizer temperature	450 °C
Ion transfer capillary	350 °C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	40 arbitrary units
Q1 and Q3 peak width	0.4 and 0.7 Da
Collision gas and pressure	Ar at 1.5 mTorr
Cycle time	0.5 sec
Acquisition method	EZ Method (scheduled SRM)

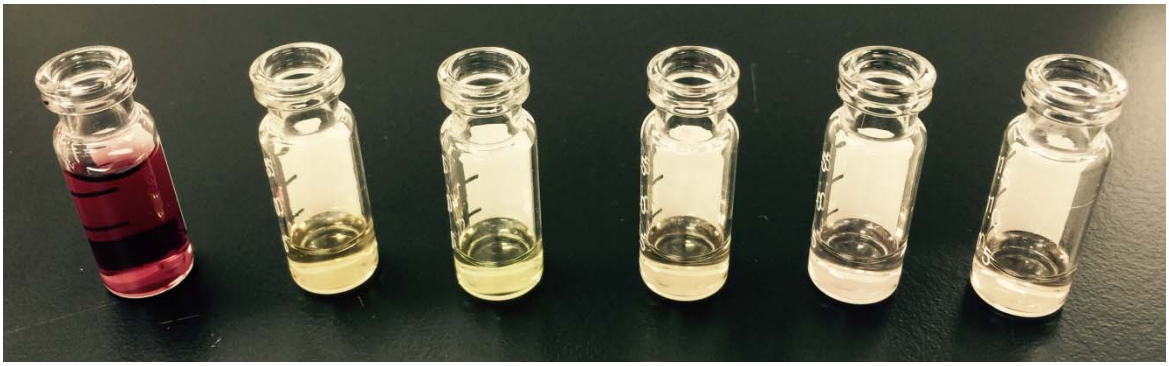
SRM Table						
Compound	**Precursor**	**Product 1**	**CE1**	**Product 2**	**CE2**	**S-lens RF**
Metamidophos	142.0	94.1	14	125.0	13	50
Acephate	184.0	143.0	6	95.0	25	33
Aldicarb sulfoxide	207.1	89.1	13	69.1	16	32
Oxydemeton methyl	247.0	169.0	13	109.0	27	57
Pymetrozine	218.1	105.1	20	176.1	17	63
Dichrotophos	238.1	112.1	12	127.0	18	52
Triethylphosphorothioate	199.0	125.0	16	143.0	14	55
Dimethoate	230.0	125.0	22	171.0	15	50
Carbendazim	192.1	160.1	18	132.1	29	60
Dichlorvos	220.9	109.0	17	127.0	13	62
Thiabendazole	202.0	175.1	25	131.1	31	70
Fenamiphos sulfone	336.1	266.0	19	188.0	26	75
Fenamiphos sulfoxide	320.1	233.0	24	108.1	40	60
Simazine	202.1	132.0	19	124.1	16	66
Tebuthiuron	229.1	172.1	16	116.0	26	55
Carbaryl	202.1	145.1	11	127.1	30	38
Flutriafol	302.1	70.1	17	123.0	28	69
Famphur	326.0	217.0	20	93.0	30	68
Thionazin	249.0	113.0	23	97.0	28	58
DEET	192.1	119.1	17	91.1	29	64
Atrazine	216.1	174.1	16	68.1	34	66
Malathion	331.0	127.0	12	99.0	25	55
Triadimefon	294.1	197.1	14	69.1	20	65
Pyrimethanil	200.1	107.1	24	183.1	23	68
Acetochlor	270.1	224.1	10	148.1	18	58
Sulfotep	323.0	97.0	37	115.0	30	60
Tebuconazole	308.1	70.1	21	125.0	33	66
Zoxamide	336.0	187.0	21	159.0	38	74
Diazinon	305.1	169.1	20	153.1	20	68
TPP (IS)	327.1	152.1	35	77.1	38	95
Cyprodinil	226.1	93.1	33	77.1	43	70
Pyrazophos	374.1	222.1	20	194.1	31	100
Profenofos	372.9	302.9	17	128.0	42	73
Ethion	385.0	142.9	26	199.0	6	56
Chlorpyrifos	349.9	97.0	32	197.9	19	67

Results:

Selection of dSPE cleanup sorbents:

Different sorbent mixtures (A - E) were packed in 2-mL dSPE centrifuge tubes for blueberry extract cleanup:

A. 150 mg $MgSO_4$ and 50 mg PSA
B. 150 mg $MgSO_4$ and 150 mg PSA
C. 150 mg $MgSO_4$, 50 mg PSA, and 50 mg C18
D. 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18, and 7.5 mg GCB
E. 150 mg $MgSO_4$, 50 mg PSA, 50 mg C18, and 50 mg ChloroFiltr®



Photographs, from left to right: crude blueberry extract, and extracts cleaned with sorbent mixture A, B, C, D, and E, respectively.

Illustrated in the above picture, cleanup of blueberry extracts with PSA only (A and B) or PSA and C18 (C) is inefficient for complete pigment removal. With the addition of either GCB (D) or ChloroFiltr® (E), colorless extracts were obtained; therefore sorbent mixtures D and E were selected for the recovery study.

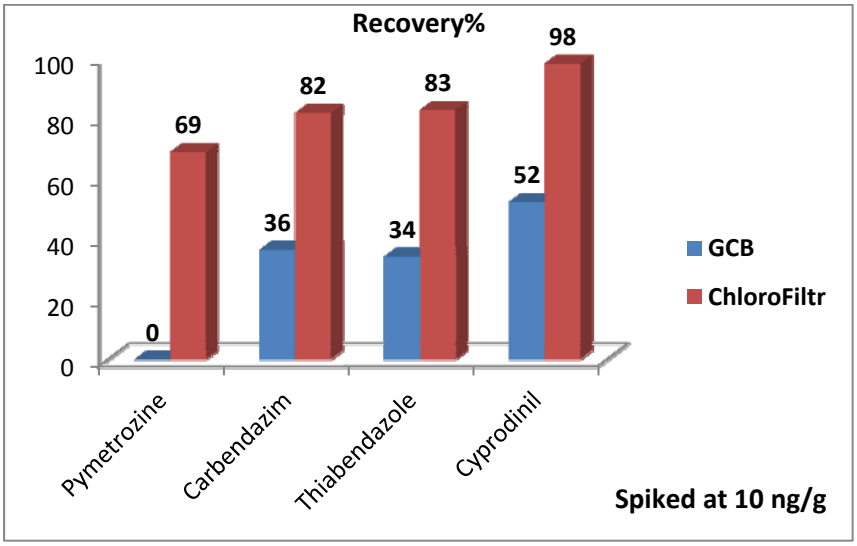
Recovery study:

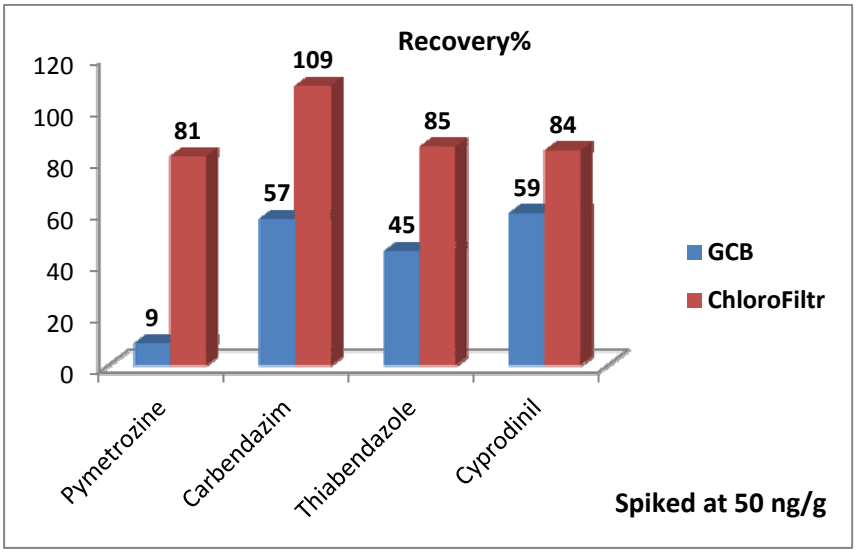
Blueberry samples were fortified with 10 ng/g and 50 ng/g of pesticides, and underwent the AOAC QuEChERS extraction and dSPE cleanup with 150 mg MgSO4, 50 mg PSA, 50 mg C18, and 7.5 mg GCB (D) or 50 mg ChloroFiltr® (E) as described above. The mean recoveries and RSD% of 6 replicated samples are listed in the table below.

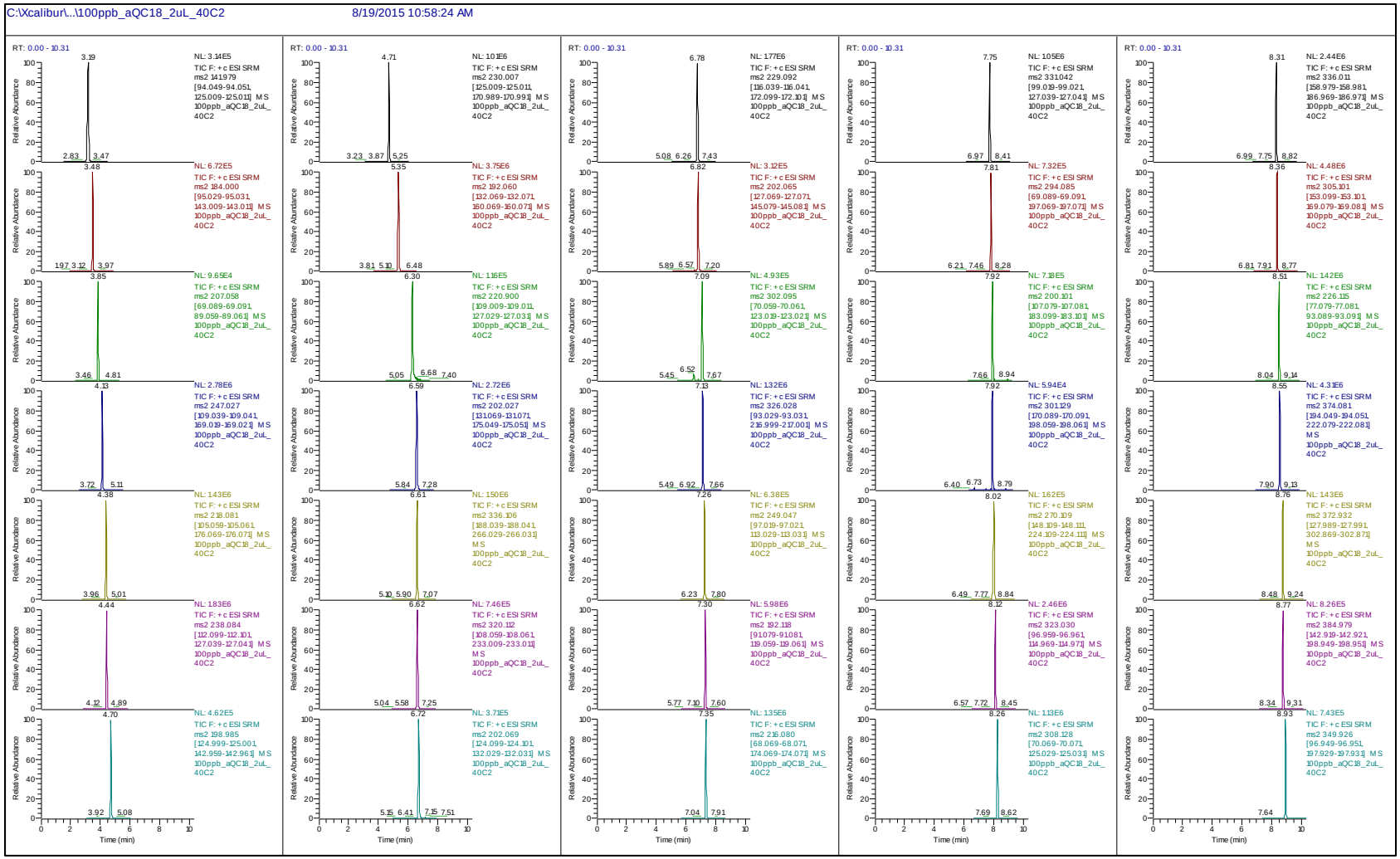
Accuracy and Precision of Pesticides in Spiked Blueberries

Compound	dSPE cleanup with PSA/C18/GCB				dSPE cleanup with PSA/C18/ChloroFiltr®			
	Spiked at 10 ng/g		Spiked at 50 ng/g		Spiked at 10 ng/g		Spiked at 50 ng/g	
	Recovery%	RSD%	Recovery%	RSD%	Recovery%	RSD%	Recovery%	RSD%
Methamidophos	**82.8**	2.5	**92.8**	1.0	**85.0**	3.7	**89.3**	1.2
Acephate	**82.8**	11.0	**83.1**	12.8	**86.1**	11.3	**89.5**	13.3
Aldicarb_sulfoxide	**87.0**	6.5	**95.6**	12.7	**103.4**	11.5	**82.1**	12.9
Oxydemeton_methyl	**96.6**	5.9	**94.0**	7.4	**96.2**	5.9	**84.2**	8.2
Dichrotophos	**65.7**	20.1	**103.6**	10.7	**91.6**	13.3	**102.3**	15.6
Pymetrozine	**0.0**	na	**8.7**	9.4	**68.8**	11.6	**81.3**	13.4
Dimethoate	**62.8**	16.1	**103.0**	6.2	**101.1**	10.9	**94.9**	10.6
Triethylphosphorothioate	**65.0**	11.9	**99.9**	6.7	**96.4**	12.5	**95.8**	10.4
Carbendazim	**36.3**	9.9	**57.0**	11.1	**81.7**	5.4	**108.7**	9.5
Dichlorvos	**91.0**	6.1	**103.3**	1.8	**95.4**	4.9	**91.5**	3.8
Fenamiphos_sulfone	**96.1**	1.9	**104.1**	1.6	**97.2**	5.5	**97.9**	9.4
Fenamiphos_sulfoxide	**94.5**	2.3	**99.2**	1.4	**101.0**	4.2	**94.9**	10.5
Simazine	**94.5**	7.3	**103.7**	3.1	**99.4**	8.0	**94.5**	6.0
Carbaryl	**103.5**	3.3	**104.2**	3.1	**95.4**	3.7	**98.7**	6.0
Tebuthiuron	**95.6**	2.0	**101.1**	1.9	**97.9**	1.9	**97.7**	6.7
Thiabendazole	**34.1**	7.0	**44.5**	7.7	**82.7**	2.9	**85.1**	9.8
Famphur	**103.3**	3.1	**109.4**	1.4	**98.1**	10.0	**102.7**	1.7
Flutriafol	**96.4**	2.3	**105.9**	0.9	**92.1**	3.5	**97.4**	1.4
Thionazin	**104.3**	2.8	**105.6**	1.8	**90.8**	14.9	**97.6**	4.3
Atrazine	**116.9**	2.7	**116.1**	1.8	**84.6**	12.3	**86.6**	11.5
DEET	**127.6**	3.8	**112.9**	2.5	**85.1**	24.9	**84.7**	18.8
Malathion	**94.5**	7.5	**108.2**	2.0	**95.4**	0.9	**104.2**	3.7
Triadimefon	**89.9**	3.3	**104.3**	2.0	**91.4**	5.5	**99.0**	1.6
Pyrimethanil	**62.6**	9.3	**72.8**	3.8	**81.9**	5.9	**89.3**	2.3
Acetochlor	**95.7**	3.7	**105.2**	2.8	**102.1**	5.7	**97.8**	2.4
Sulfotep	**94.2**	3.3	**107.7**	1.7	**96.3**	3.0	**106.3**	1.2
Tebuconazole	**93.0**	3.5	**102.6**	1.7	**87.3**	2.1	**93.5**	1.8
Zoxamide	**99.0**	2.5	**109.1**	1.1	**89.1**	2.1	**96.3**	2.2
Diazinon	**93.8**	2.4	**103.1**	0.9	**93.4**	3.5	**96.4**	1.1
Cyprodinil	**52.2**	5.9	**59.2**	6.6	**98.1**	3.4	**83.6**	1.7
Pyrazophos	**70.2**	5.5	**76.1**	7.5	**94.5**	2.0	**99.3**	1.3
Ethion	**96.8**	4.0	**100.9**	6.1	**95.9**	3.3	**93.6**	2.0
Profenofos	**91.0**	3.6	**98.9**	3.9	**88.1**	2.5	**87.3**	1.6
Chlorpyrifos	**88.7**	2.1	**98.5**	3.4	**94.6**	0.9	**89.1**	2.2

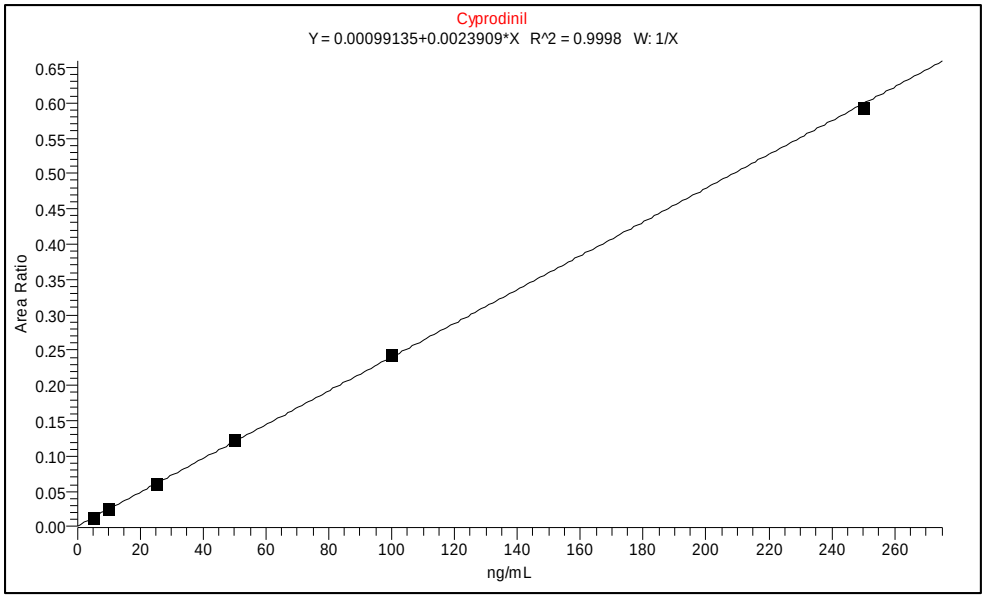
The recoveries of several pesticides such as pymetrozine, carbendazim, thiabendazole, and cyprodinil, were found be adversely affected by GCB, but unaffected when ChloroFiltr® was used; therefore, this sorbent combination was selected for blueberry extract cleanup in the final optimized procedure. The graphs below demonstrate the recovery comparison using GCB versus ChloroFiltr® at 2 contrasting levels (10 and 50 ng/g).







Chromatograms of 34 Pesticides and TPP (IS) in 1:1 MeCN:H_2O (100 ppb) using UCT Aqueous C18 HPLC Column
(Compound order can be found in the SRM table.)



Matrix-matched Calibration Curve of Cyprodinil (R^2 = 0.9998)

References:

[1] http://www.whfoods.com/genpage.php?tname=foodspice&dbid=8

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A Determination of Veterinary Drug Residues in Milk Using Polymeric SPE and UHPLC-MS/MS Analysis

UCT Part Numbers:
ECHLD126-P – EnviroClean® HLDVB , 200 mg/6mL SPE cartridge, PE Frit
SLDA100ID21-18UM – Selectra® DA, 100 × 2.1 mm, 1.8 µm HPLC column
SLDAGDC20-18UM – Selectra® DA, 10 × 2.0 mm, 1.8 µm guard cartridge
SLGRDHLDR – Guard cartridge holder

Summary:

This application note outlines a multi-class, multi-reside method for the determination of 49 representative veterinary drugs in milk using a simple, solid-phase extraction (SPE) procedure and analysis by UHPLC-MS/MS. To achieve fast and simultaneous extraction of the various drug residues, a generic liquid extraction procedure using EDTA/acetic acid buffer is conducted prior to extraction on a polymeric SPE cartridge. UHPLC separation is carried out with a Selectra® DA column, which exhibits alternative selectivity to a C18 phase and is capable of enhanced retention for the more polar drugs. The method was evaluated for each compound at three varying concentrations (1, 10 and 100 µg/kg). For most compounds, recoveries were between 70% and 120% and reproducibility was <20%. In addition, the majority of compounds could be accurately detected at a concentration of 1 µg/kg, demonstrating that the presented method is sufficient to monitor a wide range of veterinary drugs in milk. The drugs investigated belonged to several different classes, including β-agonists, macrolides, amphenicols, sulfonamides, tetracyclines and quinolones.

Introduction:

Veterinary drugs are frequently administered to food-producing animals, including dairy cows, to treat and prevent disease and/or increase growth rates. The inappropriate or illegal use of these drugs can result in the presence of their residues in food of animal origin which could pose a potential threat to human health. Milk is an important food commodity that is consumed by a large portion of the population, including infants. To ensure food safety and prevent the unnecessary exposure of consumers to veterinary drugs, it is vital to test milk for drug residues. The United

States, European Union (EU), CODEX and other international organizations have established maximum residue limits (MRLs) for veterinary drugs in a variety of biological matrices, including milk [1-3]. The MRLs for milk are typically lower than those set for other biological matrices (muscle, liver and kidney) and span a wide concentration range (low µg/kg to >1000 µg/kg). In addition, a number of drugs are prohibited for use in food producing animals or are unauthorized for use in lactating animals and require very low detection limits (≤2 µg/kg).

Milk is a complex matrix containing dissolved fats, carbohydrates, proteins and minerals (including calcium), which can complicate the development of a fast, easy and reliable analytical method for the identification and quantification of veterinary drug residues. Development of a multi-class, multi-residue (MMR) method can be challenging not only due to the inclusion of a large number of drugs with diverse physicochemical properties, but also on account of the complex sample matrix and the instability of certain drug classes (e.g. β-lactams, tetracyclines and macrolides). A MMR method should ideally be capable of extracting a wide range of drugs, reduce major matrix interferences, obtain good analyte recovery, be reproducible and achieve adequate limits of detection (LOD's). The use of a generic sample preparation procedure, such as SPE using a polymeric sorbent, is a suitable approach for achieving these goals. Ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) is the detection system of choice for veterinary drugs as it allows rapid detection of trace-level residues in complex matrices. However, the diverse physicochemical properties of the veterinary drugs still pose challenges and analytical conditions must be optimized to obtain adequate sensitivity of all the compounds as well as good retention and peak shape of problematic compounds.

Sample Preparation Procedure:

A. Sample extraction

1. Weigh 5 g of milk into a 15 mL polypropylene centrifuge tube.
2. Add 5 mL of 0.1M EDTA-Na_2 + 2% acetic acid.
3. Vortex for 5 minutes to de-proteinize the milk.
4. Centrifuge for 5 minutes at ≥ 3500 *g*.

Note: A larger volume of extraction solvent or a second extraction of the milk sample (5mL buffer) can be carried out if deemed necessary.

B. SPE extraction

1. Condition SPE cartridge with:
   a) 1 × 3 mL methanol
   b) 1 × 3 mL ultrapure water
2. Apply the supernatant to the SPE cartridge, taking care to avoid any transfer of the lipid layer. If required, use a low vacuum to draw the sample through (≤5 mL/min).

C. Wash cartridge

1. 1 × 3 mL ultrapure water.
2. 1 × 3 mL 10% methanol.
3. Dry cartridge under vacuum (≥10 inHg) for 5-10 minutes to remove residual water.
4. 1 × 3 mL hexane.
5. Dry cartridge under vacuum (≥10 inHg) for 1 minute to remove residual hexane.

D. Elution

1. Elute with 3 mL acetone.
2. Evaporate the sample to dryness at 35-40°C under a gentle stream of nitrogen.
3. Reconstitute in 1 mL of methanol:water (50:50, v/v).
4. Filter extract with a 0.22 µm nylon (or other suitable membrane) syringe filter into an autosampler vial.

LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 1.8 µm (p/n: SLDA100ID21-18UM)
Guard column	UCT Selectra® DA, 10 × 2.0 mm, 1.8 µm (p/n: SLDAGDC20-18UM)
Guard column	p/n: SLGRDHLDR
Column temp.	60°C
Flow rate	400 µL/min
Injection volume	5 µL
Autosampler temp.	10°C
Wash solvent	Methanol
Divert valve	Divert to waste at 0-1.5 and 12-16.5 min to reduce ion source

Time (min)	Mobile phase A Water + 0.1% formic acid	Mobile phase B Methanol + 0.1% formic acid
0.0	95%	5%
0.5	70%	30%
4.0	70%	30%
5.0	40%	60%
8.0	40%	60%
8.5	0%	100%
12.0	0%	100%
12.1	95%	5%
16.5	95%	5%

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ
Ionization mode	ESI^+ & ESI^-
Spray voltage	4000 V
Vaporizer	450°C
Capillary	350°C
Sheath gas	55 arbitrary units
Auxiliary gas	45 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering	0 V
Q1 and Q3 peak	0.7 Da
Collision gas	Argon
Collision gas	1.7 mTorr
Acquisition	EZ method (scheduled SRM)
Cycle time	0.5 sec
Software	Xcalibur™ version 2.2

SRM Transitions							
Analyte	t_R (min)	Precursor ion	Product ion 1	CE 1 (V)	Product ion 2	CE 2 (V)	S-lens (V)
Sulfanilamide	2.14	156.00	92.11	12	108.10	10	45
Albuterol	2.55	240.12	121.07	28	148.07	18	51
Albuterol-D_3 (IS)	2.55	243.13	124.15	28	151.15	17	50
Lincomycin	3.13	407.16	126.11	29	359.23	17	93
Ampicillin	3.82	350.08	106.07	17	192.03	14	70
Trimethoprim	3.90	291.10	123.06	24	230.11	22	88
Trimethoprim-$^{13}C_3$ (IS)	3.90	294.09	233.19	22	264.16	25	70
Thiamphenicol(ESI^-)	4.10	353.92	121.08	54	184.99	21	70
Sulfadiazine	4.15	251.02	92.10	25	156.02	15	66
Sulfathiazole	4.30	255.98	92.08	27	156.01	13	66
Norfloxacin	4.35	320.09	233.08	24	276.11	16	82
Ormetoprim	4.53	275.11	123.08	26	259.12	26	90
Thiabendazole	4.57	202.01	131.07	32	175.05	25	75
Thiabendazole-D_6 (IS)	4.57	208.02	137.14	33	181.10	25	70
Oxytetracycline	4.60	461.08	337.09	28	426.14	18	82
Cefalexin	4.85	348.04	157.98	6	174.00	14	55
Ofloxacin	5.06	362.10	261.09	26	318.15	17	92
Ciprofloxacin	5.25	332.09	231.05	34	288.15	17	82
Ciprofloxacin-^{15}N-$^{13}C_3$ (IS)	5.25	336.09	235.10	36	291.21	16	80
Tetracycline	5.30	445.08	154.04	15	410.18	17	76
Sulfamethoxazole	5.38	254.09	92.07	26	148.08	17	55
Sulfamethoxazole-$^{13}C_6$ (IS)	5.38	260.03	98.15	26	162.07	15	61
Sulfamerazine	5.41	265.03	92.09	28	155.96	16	72
Lomefloxacin	6.04	352.09	265.08	22	308.16	16	78
Sulfamethizole	6.12	270.99	92.08	26	156.01	13	62
Chloramphenicol (ESI^-)	6.43	320.93	121.04	35	152.01	19	70
Cefotaxime	6.50	455.99	124.97	43	166.96	19	74

Enrofloxacin	6.51	360.12	245.12	24	316.19	17	86
Demeclocycline	6.55	465.03	430.12	19	448.13	14	92
Sulfachloropyridazine	6.76	284.98	92.10	27	156.01	14	70
Sulfamethazine	6.80	279.05	124.09	25	186.03	16	68
Sulfamethazine-$^{13}C_6$ (IS)	6.80	285.06	124.17	24	186.07	16	76
Azithromycin	6.85	749.11	116.00	38	591.45	24	128
Sarafloxacin	6.88	386.07	299.06	27	342.17	17	90
Clindamycin	6.96	425.10	126.10	29	377.19	17	95
Chlortetracycline	7.05	479.04	153.99	27	444.13	19	95
Cefazolin	7.15	454.97	111.92	31	155.97	14	68
Doxycycline	7.24	445.08	321.08	28	428.18	16	79
Diphenhydramine	7.34	256.12	115.07	65	165.05	62	45
Carbadox	7.40	263.04	129.06	30	231.06	12	69
Sulfadimethoxine	7.60	311.03	108.05	29	156.04	19	87
Erythromycin	7.91	734.37	157.99	28	576.41	16	107
Erythromycin-$^{13}C_2$ (IS)	7.91	736.36	160.07	28	578.41	13	108
Cephalothin	8.10	418.95	204.01	16	359.05	10	55
Penicillin G	8.15	367.09	114.04	31	160.02	14	71
Anhydroeythromycin	8.23	716.36	158.01	27	558.36	15	112
Clarithromycin	8.53	748.38	157.99	27	590.41	15	120
Ceftiofur	8.73	523.95	124.92	54	240.96	14	100
Penicillin V	8.83	383.09	114.04	32	160.03	15	70
Tylosin	8.97	916.42	173.92	34	772.47	23	173
Roxithromycin	9.30	837.43	157.95	31	679.47	16	120
Oxolinic acid	9.39	262.03	160.05	36	216.04	29	66
Oxacillin	9.52	434.08	144.01	31	160.00	15	75
Cloxacillin	10.10	468.04	160.00	16	177.96	31	85
Flumequine	10.40	262.04	126.05	48	202.03	32	70
Virginiamycin	10.80	526.19	337.08	19	355.08	16	83

CE = collision energy

Results and Discussion:

Summary of the recovery, reproducibility and method performance data generated (n=12 each).

	1 µg/kg		10 µg/kg		100 µg/kg			
Analyte	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**	**LCL (µg/kg)**	**Linearity (R^2)**
Sulfanilamide	87.1[a]	1.6	82.6	12.2	75.2	8.2	0.5	0.9993
Albuterol	100.1	6.5	106.6	4.4	105.9	1.7	0.5	0.9996
Lincomycin	109.3	5.8	87.4	9.1	94.0	4.6	0.5	0.9974
Ampicillin	109.8	10.4	92.3	7.8	96.3	3.6	0.5	0.9983
Trimethoprim	94.8	5.6	105.6	3.6	103.1	1.8	0.5	0.9992
Thiamphenicol	81.3	23.3	103.3	7.7	80.2	17.2	0.5	0.9962
Sulfadiazine	88.7	10.1	89.7	7.8	86.7	6.1	0.5	0.9985
Sulfathiazole	90.0	8.9	92.4	12.8	98.4	4.4	0.5	0.9990
Norfloxacin	96.4	4.7	98.0	5.5	98.1	3.3	0.5	0.9992
Ormetoprim	95.3	6.1	101.6	10.1	98.0	4.2	0.5	0.9974
Thiabendazole	96.5	5.7	102.3	4.5	101.6	1.5	0.5	0.9997
Oxytetracycline	98.8	11.6	90.9	13.8	96.2	4.2	0.5	0.9984
Cefalexin	69.3[a]	7.8	78.4	25.9	82.1	21.6	1.0	0.9981
Ofloxacin	101.2[a]	4.9	83.7	9.1	100.1	7.3	0.5	0.9988
Ciprofloxacin	95.0	3.5	99.4	4.2	100.9	1.8	0.5	0.9988
Tetracycline	108.8	10.0	99.0	5.2	99.4	7.7	0.5	0.9993
Sulfamethoxazole	89.0	9.2	101.6	3.6	101.7	2.5	0.5	0.9994
Sulfamerazine	87.7	10.7	88.9	10.8	100.1	12.4	0.5	0.9975
Lomefloxacin	107.1	4.4	111.0	4.6	108.5	1.9	0.5	0.9989
Sulfamethizole	91.0	13.5	95.2	12.2	98.8	5.0	0.5	0.9990
Enrofloxacin	96.4	4.2	84.4	5.6	99.5	4.8	0.5	0.9985
Chloramphenicol	98.0	12.2	110.3	7.4	101.5	4.1	0.5	0.9959
Cefotaxime	96.6	11.3	90.7	6.2	92.1	3.2	0.5	0.9979
Demeclocycline	100.3	11.1	95.2	5.8	95.9	7.3	0.5	0.9991
Sulfachloropyridazine	85.6	9.0	82.7	15.1	88.7	14.3	0.5	0.9994
Sulfamethazine	97.7	9.1	99.4	4.3	99.7	2.4	0.5	0.9996

Azithromycin	71.0	7.7	83.4	8.0	82.8	6.2	0.5	0.9981
Sarafloxacin	79.0	16.2	102.4	11.5	98.5	6.1	0.5	0.9994
Clindamycin	82.4	19.2	81.0	12.3	86.2	19.0	0.5	0.9978
Chlortetracycline	108.1	4.7	89.7	8.6	92.2	5.4	0.5	0.9999
Cefazolin	96.1	1.9	97.3	7.2	101.7	3.8	1.0	0.9980
Doxycycline	102.9	4.3	87.2	6.1	92.7	5.3	0.5	0.9988
Diphenhydramine	92.3	8.2	96.6	6.0	97.8	8.0	0.5	0.9999
Carbadox	82.3	11.4	95.3	12.2	95.9	6.5	0.5	0.9962
Sulfadimethoxine	74.4	14.0	74.6	7.4	81.4	5.5	0.5	0.9991
Erythromycin	97.1	8.0	102.6	3.8	100.1	1.4	0.5	0.9970
Cephalothin	105.4	5.4	96.5	11.1	99.8	6.0	0.5	0.9997
Penicillin G	103.2	5.4	98.0	7.1	99.9	2.5	0.5	0.9995
Anhydroeythromycin	112.7	9.8	107.8	6.7	100.2	4.4	0.5	0.9971
Clarithromycin	104.5	7.1	99.3	7.3	100.8	4.9	0.5	0.9986
Ceftiofur	57.1	7.7	67.8	20.3	66.2	25.9	0.5	0.9982
Penicillin V	101.7	10.1	88.9	6.2	96.7	5.9	0.5	0.9990
Tylosin	82.5	7.4	71.4	5.2	79.4	6.1	0.5	0.9995
Roxithromycin	97.2	9.1	92.4	10.5	95.6	4.2	0.5	0.9986
Oxolinic acid	101.0	5.4	97.5	7.9	99.5	3.9	0.5	0.9986
Oxacillin	92.8	10.5	83.4	5.8	88.6	6.6	0.5	0.9990
Cloxacillin	87.6	11.5	79.0	6.7	84.2	3.5	0.5	0.9982
Flumequine	80.8	13.9	93.0	6.8	91.8	10.5	0.5	0.9992
Virginiamycin	89.3	16.1	91.4	9.3	92.7	10.0	0.5	0.9979

[a]n=6.

Chromatographic separation

The unique chemistry of the Selectra® DA column, which contains a polyaromatic stationary phase, provides orthogonal selectivity to a traditional C18 column and offers a high degree of retention and selectivity for aromatic compounds. The stationary phase is capable of retaining analytes through hydrophobic (dispersive) interactions as well as through pi-pi (π- π) interactions which exhibit a substantial increase in retention for dipolar, unsaturated or conjugated analytes. The Selectra® DA column is ideally suited for the analysis of veterinary drug residues, as most compounds (and metabolites) possess aromatic functionality.

In the final UHPLC-MS/MS method, methanol was chosen as the organic mobile phase solvent, as it was found to give better overall peak shape than acetonitrile, particularly for the tetracycline and fluoroquinolone antibiotics. A hold was included in the gradient to improve chromatographic separation and all compounds were successfully eluted in <12 min. The enhanced retention of the Selectra® DA column ensured that the most polar compound included in the method, sulfanilamide, didn't elute until >2 minutes (30% methanol). Although it was possible to start the gradient at a higher percentage of organic solvent (20%) and reduce the overall run time, this required the use of a smaller injection volume (2 µL) which negatively affected the method sensitivity. Ultimately, the best sensitivity was obtained by starting the gradient at 5% methanol and using a 5 µL injection volume.

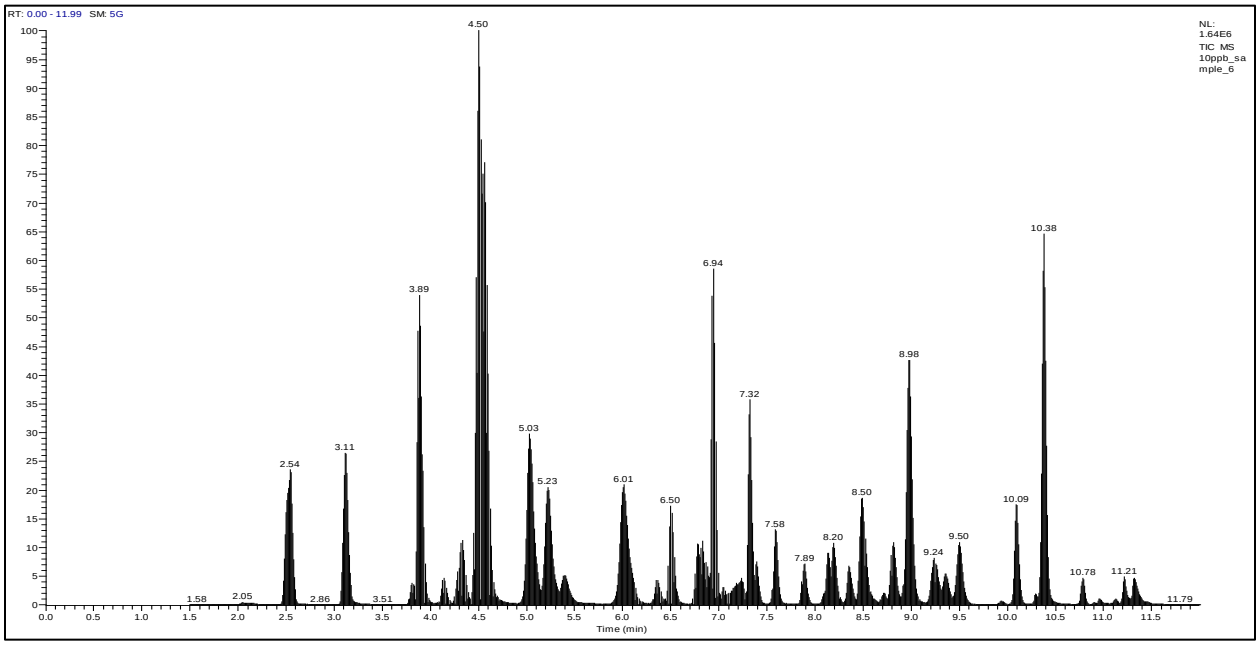

Figure 1. TIC chromatogram of an extracted milk sample (10 µg/kg) containing the 49 veterinary drugs and 7 internal standards.

Sample preparation procedure:

Prior to instrumental analysis, a sample pre-treatment step is required to concentrate the analytes of interest and eliminate non-desirable matrix components. This is particularly important for the analysis of veterinary drugs in milk because of their low regulatory limits and the larger sample size required to obtain necessary method sensitivity. One of the biggest difficulties in milk analysis is the high fat, protein, and calcium content that can often interfere with instrumental analysis. The instability of certain drug classes, namely β-lactams, tetracyclines and macrolides, causes additional complications by limiting the conditions that can be used for sample extraction and cleanup. Therefore, the sample preparation procedure was optimized to remove as much co-extracted matrix components as possible while minimizing any loss of the veterinary drug residues.

A simple deproteinization procedure using an EDTA/acetic acid buffer (sample pH should be 4-4.5) followed by centrifugation to separate the proteins and lipids was carried out prior to SPE extraction and cleanup. The inclusion of EDTA in the extraction buffer prevents the complexation of drugs with metal ions (e.g. calcium), particularly the tetracyclines and fluoroquinolones. After application of the sample supernatant to the SPE cartridge, the sorbent was washed with 10% methanol to remove polar matrix components and hexane to remove lipophilic compounds. Acetone was used as the SPE elution solvent as it was found to be more effective than methanol, particularly for hydrophobic compounds that contain multiple aromatic functional groups and are strongly retained on the DVB sorbent. Furthermore, acetone is a volatile organic solvent that is readily removed by evaporation under mild conditions (35-40°C). Filtration of the sample extract prior to LC-MS/MS analysis and the use of isotopically labeled internal standards and matrix-matched calibration curves are recommended in order to obtain the best possible results.

For most compounds, the recovery was between 70% and 120% and the reproducibility <20%. Only a a small number of compounds gave results outside of the acceptable limits, which was due to analyte instability (cefalexin and ceftiofur) or inadequate sensitivity at the lowest concentration level (sulfanilamide and thiamphenicol). In addition, all compounds could be accurately detected at a concentration of 10 µg/kg and the vast majority of compounds at 1 µg/kg, demonstrating that the presented method is suitable for monitoring a wide range of veterinary drug residues in milk.

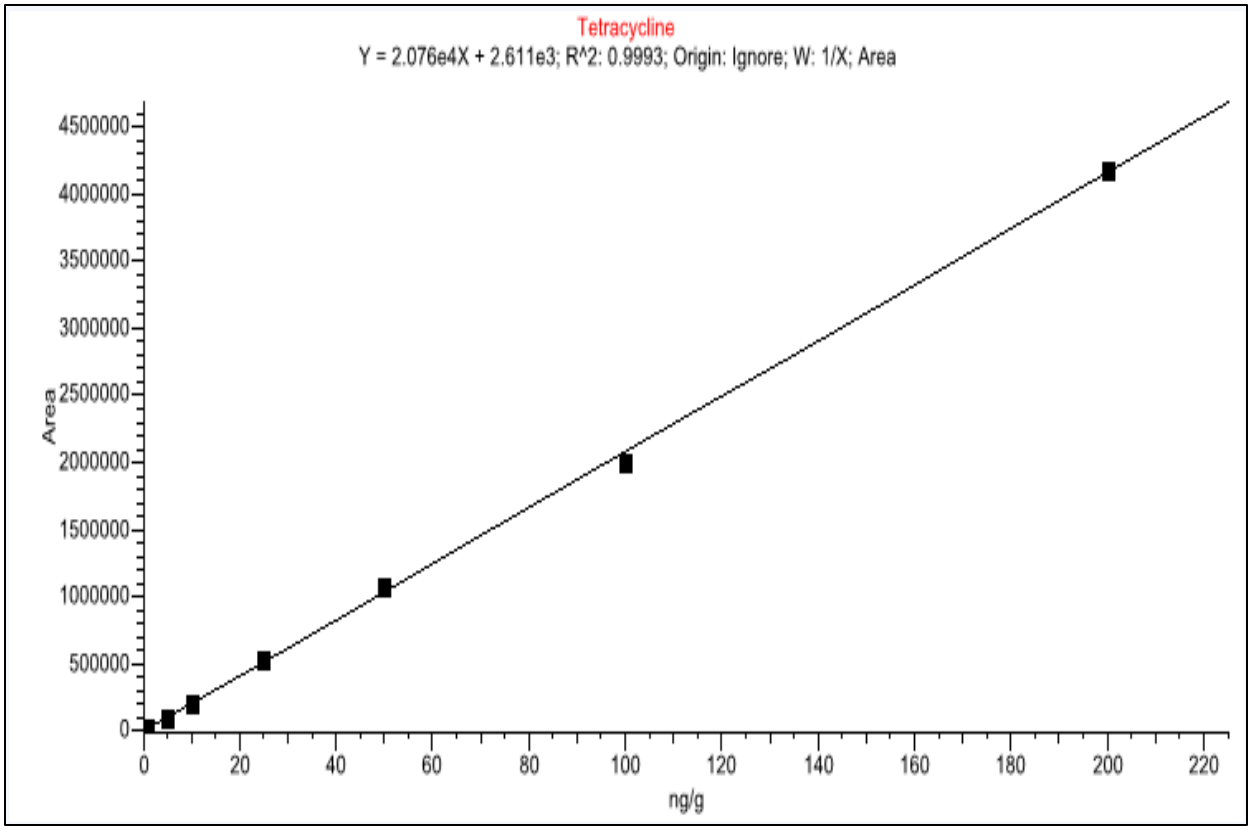

Figure 2. Example of an eight-point matrix-matched calibration curve (0.5-200 µg/kg, equivalent to 2.5-1000 ng/mL in final extract).

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EPA Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Aqueous Direct Injection and LC/MS/MS

UCT Part Numbers:
SLAQ100ID21-3UM - Selectra® Aqueous C18, 100 x 2.1mm, 3µm
SLAQGDC20-3UM - Selectra® Aqueous C18, Guard column, 10 x 2.0mm, 3µm
SLGRDHLDR - Guard Cartridge Holder

Summary:

Cyanotoxins are toxins that are produced by some species of photosynthetic cyanobacteria (also known as blue-green algae) blooming under certain conditions [1], such as stagnant or slow moving warm water with high-level nutrients like phosphates and nitrogen. Cyanotoxins are dangerous to humans and wildlife, affecting their livers (hepatotoxic), nervous systems (neurotoxic), and skin (acutely dermatotoxic). Human exposure to cyanotoxins can occur through ingestion of either the contaminated drinking water or exposed fish and shellfish, in addition to inhalation or dermal contact with the contaminated recreational water [2].

This application note describes a direct aqueous injection and liquid chromatography tandem mass spectrometry (DAI-LC/MS/MS) method for the determination of cylindrospermopsin (hepatotoxin) and anatoxin-a (neurotoxin) in drinking water under EPA Method 545 [3]. These two target analytes are also included in the UCMR4 screening compound list that will be monitored by public drinking water systems soon.

UCT's Aqueous C18 HPLC column was utilized for analyte retention and separation, which had demonstrated excellent consistency in peak area and retention times. 7-point calibration curves were constructed for analyte quantification. The responses were linear ($R^2 \geq 0.9982$) over the analytical range from 0.1 to 10 µg/L. Excellent accuracy (93.6 – 110.3%) and precision (RSD% < 10%, n=7) were achieved in fortified reagent water and tap water samples.

Procedure:

1. Preserve water samples with 1 g/L of sodium bisulfate (antimicrobial) and 0.1 g/L of ascorbic acid (dechlorination).
2. Add 10 µL of 0.5-2 ng/µL internal standard mixture to 2-mL vials, and appropriate amounts of spiking solutions for fortified samples, and bring the final volume to 1 mL with the preserved water samples.
3. Vortex the samples for 30 sec and analyze by LC-MS/MS equipped with an Aqueous C18 HPLC column.

LC-MS/MS method:

HPLC: Thermo Scientific Dionex UltiMate 3000® LC System		
Column: UCT, Selectra®, aQ C18, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, aQ C18, 10 x 2.0 mm, 3 µm		
Column temperature: 30 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
Injection volume: 50 µL		
Gradient program:		
Time (min)	**A%** (50 mM acetic acid in DI water)	**B%** (MeOH)
0	100	0
1.5	100	0
4.5	70	30
6	70	30
6.1	10	90
7.5	10	90
7.6	100	0
13.5	100	0

Divert mobile phase to waste from 0 – 1.8 and 7 – 13.5 min to prevent ion source contamination.

MS parameters	
Instrumentation	Thermo Scientific TSQ Vantage tandem MS
Polarity	ESI +
Spray voltage	4000 V
Vaporizer temperature	400 °C
Ion transfer capillary temperature	350 °C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	25 arbitrary units
Q1 and Q3 peak width (FWHM)	0.2 and 0.7 Da
Collision gas and pressure	Ar at 1.5 mTorr
Cycle time	0.6 sec
Acquisition method	EZ Method (scheduled SRM)

Retention Times and SRM Transitions							
Compound	**Rt (min)**	**Precursor**	**Product 1**	**CE 1**	**Product 2**	**CE 2**	**S-Lens RF**
Uracil-d4	2.05	115.1	98.1	16	72.1	14	45
L-phenylalanine-d5	4.58	171.1	125.2	14	106.1	28	47
Cylindrospermopsin	5.40	416.1	194.1	31	176.1	31	106
Anatoxin-a	5.74	166.1	149.1	12	131.0	15	62

Results:

Relative Standard Deviation (RSD) of Peak Area and Retention Times by aQ C18 HPLC Column

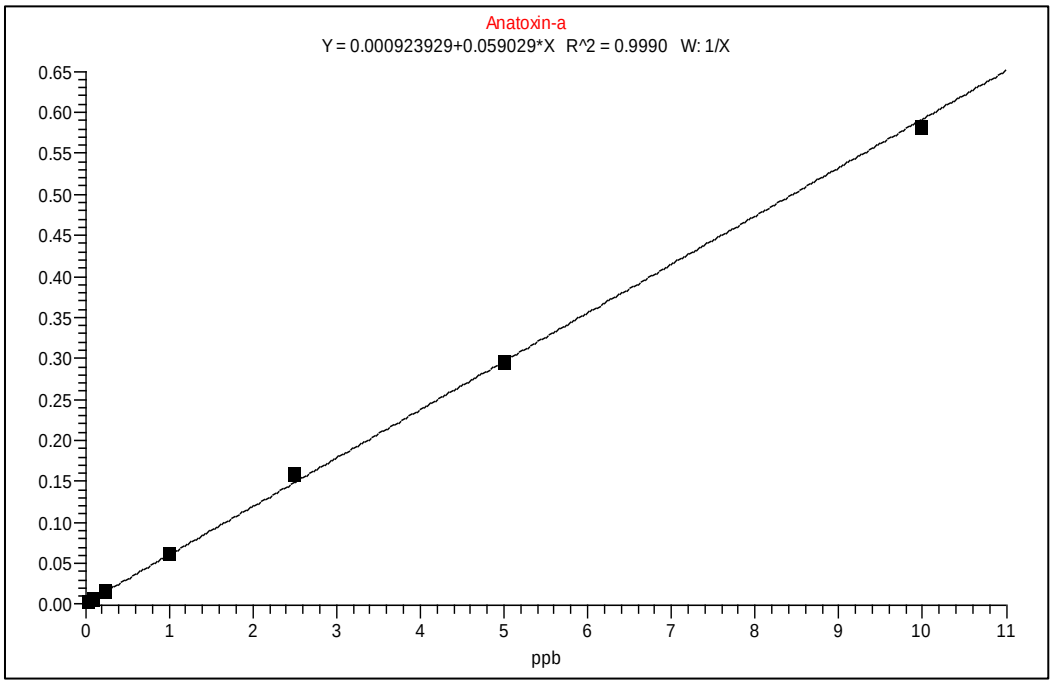
Compound	**RSD% (n=44)**	
	Peak Area	**Retention Time**
Uracil-d4	3.7	0.1
L-phenylalanine-d5	2.5	0.4
Cylindrospermopsin	ND	0.1
Anatoxin-a	ND	0.3

ND: not determined.

Analytical Range and Linearity Data

Compound	Analytical range (µg/L)	Linearity (R^2)	
		Reagent water	Tap water
Cylindrospermopsin	0.1 - 10	0.9989	0.9992
Anatoxin-a	0.1 - 10	0.9990	0.9982

Calibration Curve of Anatoxin-a in Reagent Water



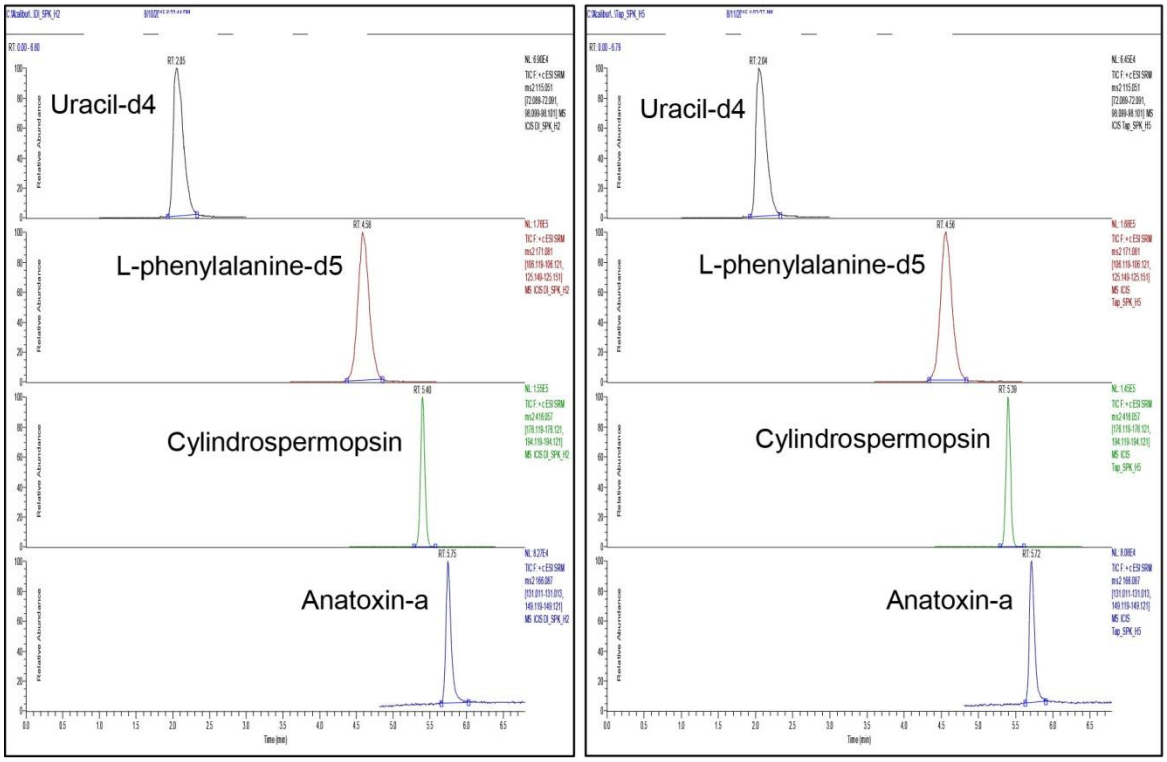
Accuracy and Precision in Fortified Reagent Water (n=7)

Compound	Spiked at 0.1 µg/L		Spiked at 2.5 µg/L	
	Recovery%	RSD%	Recovery%	RSD%
Cylindrospermopsin	**99.8**	4.5	**105.8**	2.3
Anatoxin-a	**101.5**	8.6	**103.1**	2.0

Accuracy and Precision in Fortified Tap Water (n=7)

Compound	Spiked at 0.1 µg/L		Spiked at 2.5 µg/L	
	Recovery%	RSD%	Recovery%	RSD%
Cylindrospermopsin	**100.4**	6.4	**110.3**	0.7
Anatoxin-a	**93.6**	5.2	**106.8**	0.6

Chromatograms of Fortified Water Samples



Reagent Water at 2.5 µg/L

Tap Water at 2.5 µg/L

References:

[1] https://en.wikipedia.org/wiki/Cyanotoxin

[2] http://www.waterrf.org/PublicReportLibrary/4548a.pdf

[3] http://water.epa.gov/scitech/drinkingwater/labcert/upload/epa815r15009.pdf

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Detection of Aflatoxins in Milk at Picogram Levels Using SPE and LC-MS/MS

UCT Part Numbers:
ECHLD126-P – EnviroClean® HL DVB , 200 mg/6 mL SPE cartridge
SLC-18100ID21-3UM – Selectra® C18, 100 × 2.1 mm, 3 µm HPLC column
SLC-18GDC20-3UM – Selectra® C18, 10 × 2.0 mm, 3 µm guard cartridge
SLGRDHLDR – Guard cartridge holder

August 2015

Summary:

Aflatoxins are naturally occurring mycotoxins that are produced by several species of fungi (*Aspergillus flavus* and *Aspergillus parasiticus*). They are classified by the International Agency for Research on Cancer as group 1 carcinogens (compounds known to be carcinogenic in humans) [1]. Aflatoxins can occur in food products as a result of fungal contamination of crops (prior to harvest or during storage). There are approximately 20 related aflatoxin metabolites, although only B1, B2, G1 and G2 are normally found in food [2]. Of these, aflatoxin B1 is the most biologically active and most commonly encountered [3].

Aflatoxins can occur in milk as a result of dairy animals consuming contaminated feed. The main residue of concern in milk is aflatoxin M1, the major metabolite of B1. The intake of contaminated milk, even at low concentrations, is a significant threat to human health, especially to children who are a major consumer of dairy products. Therefore, the US Food and Drug Administration has established a tolerance of 0.50 µg/kg for aflatoxin M1 in milk [4], while the European Union has imposed more stringent limits - 0.050 µg/kg in raw milk and 0.025 µg/kg in infant formula [5]. No limits have been established for aflatoxin B1, B2, G1 and G2 in milk.

This application note outlines a method for the low level determination of aflatoxins in milk using a polymeric solid-phase extraction (SPE) cartridge. Analysis is performed by LC-MS/MS using a Selectra® DA HPLC column. The method was optimized to allow the detection of aflatoxins at the low regulatory concentrations required. Recovery studies were carried out by spiking whole milk at two concentration levels (0.025 and 0.5 µg/kg). Matrix-matched calibration curves, ranging from 0.01-2 µg/kg, were used for quantitation. The mean recovery was found to be in the range of 84 to 100%, and repeatability was ≤7%.

Procedure:

Aflatoxins are relatively unstable in light and air, particularly in polar solvents or when exposed to oxidizing agents, ultraviolet light, or solutions with a pH below 3 or above 10. They should be protected from ultraviolet light as much as possible.

1. **Sample extraction 1 (aqueous extraction)**
   a) Weigh 20 g of milk into a 50 mL polypropylene centrifuge tube.
   b) Add 200 µL of glacial acetic acid.
   c) Vortex for 5 minutes to deproteinize the milk.
   d) Centrifuge for 5 minutes at ≥ 4000 *g*.

2. **SPE extraction**
   a) Condition SPE cartridge with:
      1. 1 × 3 mL methanol
      2. 1 × 3 mL ultrapure water
   b) Apply the supernatant to the SPE cartridge, taking care to avoid any transfer of the lipid layer. If required, use a low vacuum to draw the sample through (≤5 mL/min).

3. **Sample extraction 2 (solvent extraction)**
   a) Add 10 mL acetone to any residual milk solids from the aqueous extraction.
   b) Vortex for 2 minutes to extract the aflatoxins.
   c) Centrifuge for 5 minutes at ≥ 4000 *g*.
   d) Transfer the supernatant to a clean polypropylene tube and evaporate to ≤0.5 mL at 50°C under a gentle stream of nitrogen.
   e) Add 10 mL ultrapure water and vortex briefly.
   f) Apply sample to the SPE cartridge (same cartridge as step B).

4. **Wash cartridge**
   a) 1 × 3 mL ultrapure water.
   b) 1 × 3 mL 50% methanol.
   c) Dry cartridge under vacuum (≥10 inHg) for 5-10 minutes to remove residual water.
   d) 1 × 3 mL hexane.
   e) Dry cartridge under vacuum (≥10 inHg) for 1 minute to remove residual hexane.

5. **Elution**
   a) Elute the aflatoxins with 4 mL acetone.
   b) Evaporate the sample to dryness at 50°C under a gentle stream of nitrogen.
   c) Reconstitute in 1 mL of methanol:water (50:50, v/v).
   d) Filter extract with a 0.22 µm nylon (or other suitable membrane) syringe filter into an autosampler vial.

LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000
HPLC column	UCT Selectra® C18, 100 × 2.1 mm, 3 µm **(p/n: SLC-18100ID21-3UM)**
Guard column	UCT Selectra® C18, 10 × 2.0 mm, 3 µm **(p/n: SLC-18GDC20-3UM)**
Guard column holder	**p/n: SLGRDHLDR**
Column temp.	40°C
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Acetonitrile + 0.1% formic acid
Flow rate	300 µL/min
Gradient	0 min (5% B), 5-6 min (hold 100% B), 6.1-11 min (equilibrate 5% B)
Injection volume	20 µL
Autosampler temp.	10°C
Wash solvent	Methanol
Divert valve	Divert to waste at 0-4 and 6-11 min to reduce ion source contamination

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ
Ionization mode	ESI^+
Spray voltage	3500 V
Vaporizer	400°C
Capillary	350°C
Sheath gas pressure	55 arbitrary units
Auxiliary gas	45 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering	0 V
Q1 and Q3 peak	0.2 and 0.7 Da
Collision gas	Argon
Collision gas	2.2 mTorr
Acquisition method	EZ method (scheduled SRM)
Cycle time	0.6 sec
Software	Xcalibur™ version 2.2

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**S-lens (V)**
Aflatoxin M1	4.7	329.0	273.0	21	259.0	23	120
Aflatoxin G2	4.9	331.0	245.0	27	189.0	38	115
Aflatoxin G1	5.0	329.0	243.0	25	199.0	46	117
Aflatoxin B2	5.0	315.0	287.0	24	259.0	27	111
Aflatoxin B1	5.2	313.0	241.0	35	285.0	21	111

Results and Discussion:

Accuracy & Precision Data for Whole Milk				
	0.025 µg/kg (n=5)		0.5 µg/kg (n=5)	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
Aflatoxin M1	94.43	3.54	90.91	4.31
Aflatoxin B1	89.82	4.19	84.34	4.31
Aflatoxin B2	93.27	3.76	88.27	7.51
Aflatoxin G1	92.51	5.48	89.28	7.06
Aflatoxin G2	100.05	1.71	93.51	6.83

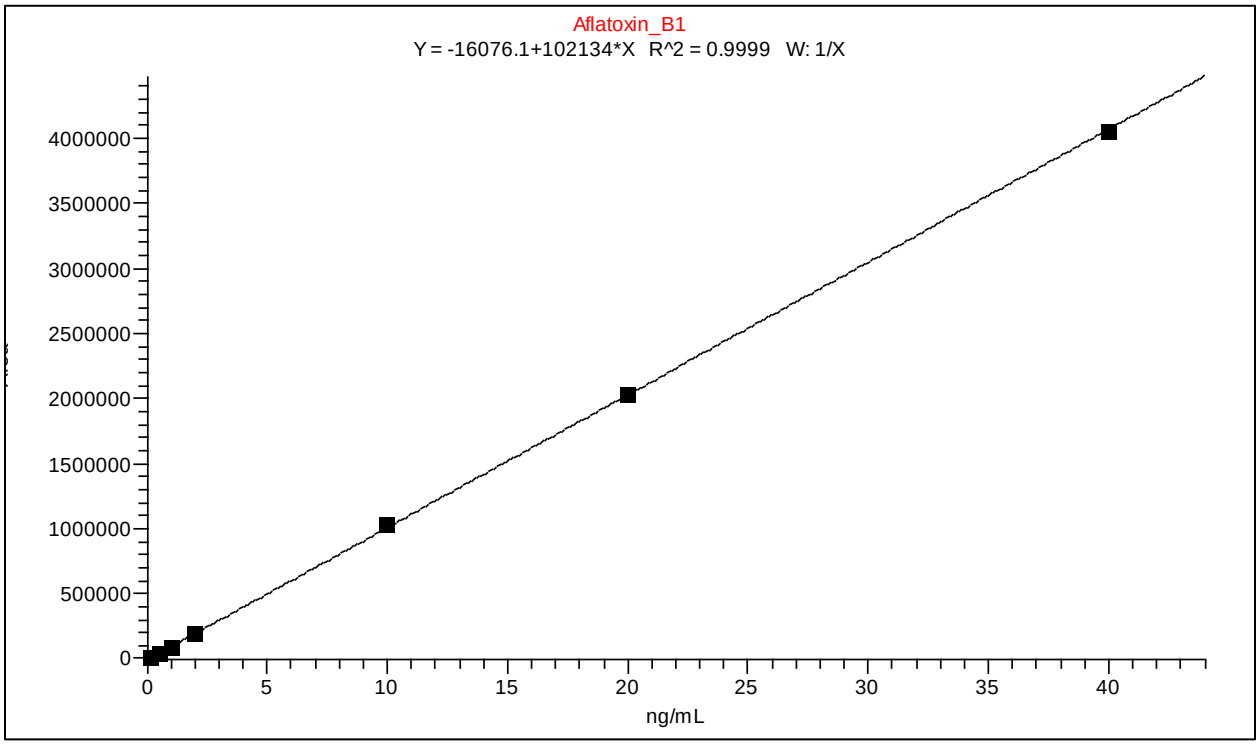

Figure 1. Example of a seven point matrix-matched calibration curve (0.2, 0.5, 1, 2, 10, 20 and 40 ng/mL; equivalent to 0.01, 0.025, 0.05, 0.1, 0.5, 1 and 2 µg/kg in milk).

Chromatograms

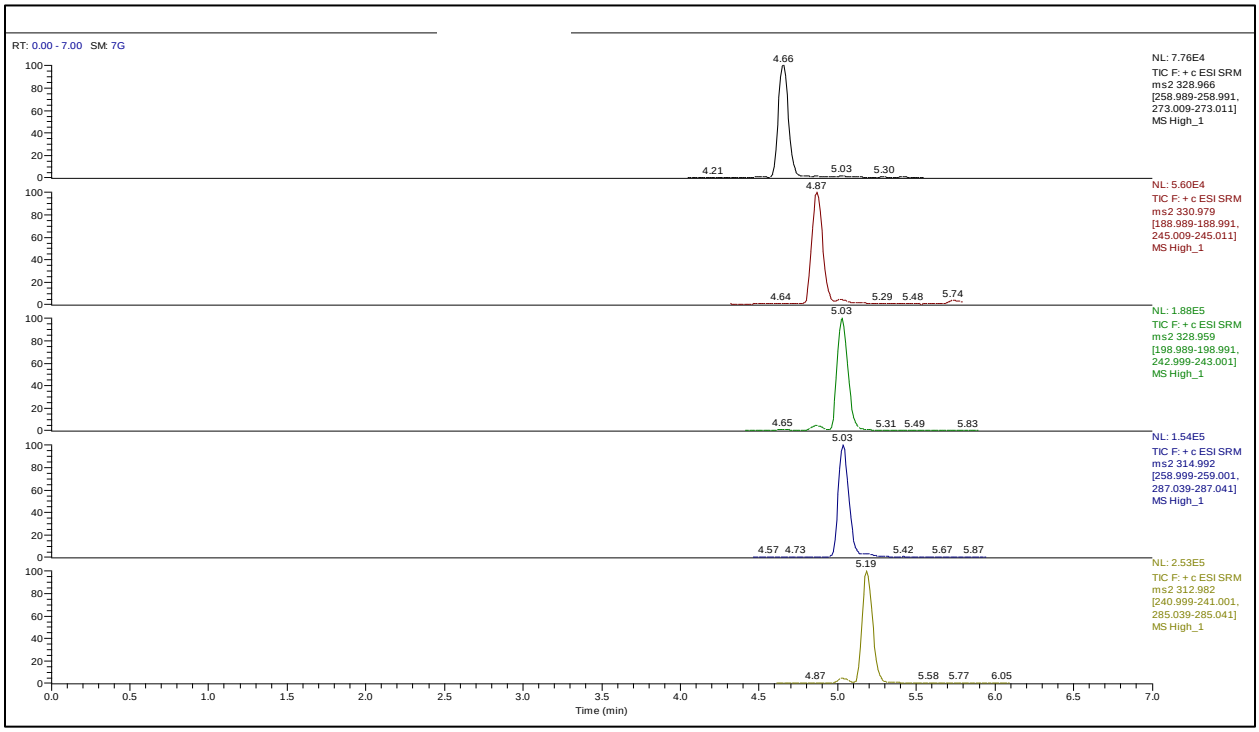

Figure 2. Chromatogram of an extracted milk sample fortified at 0.5 µg/kg.

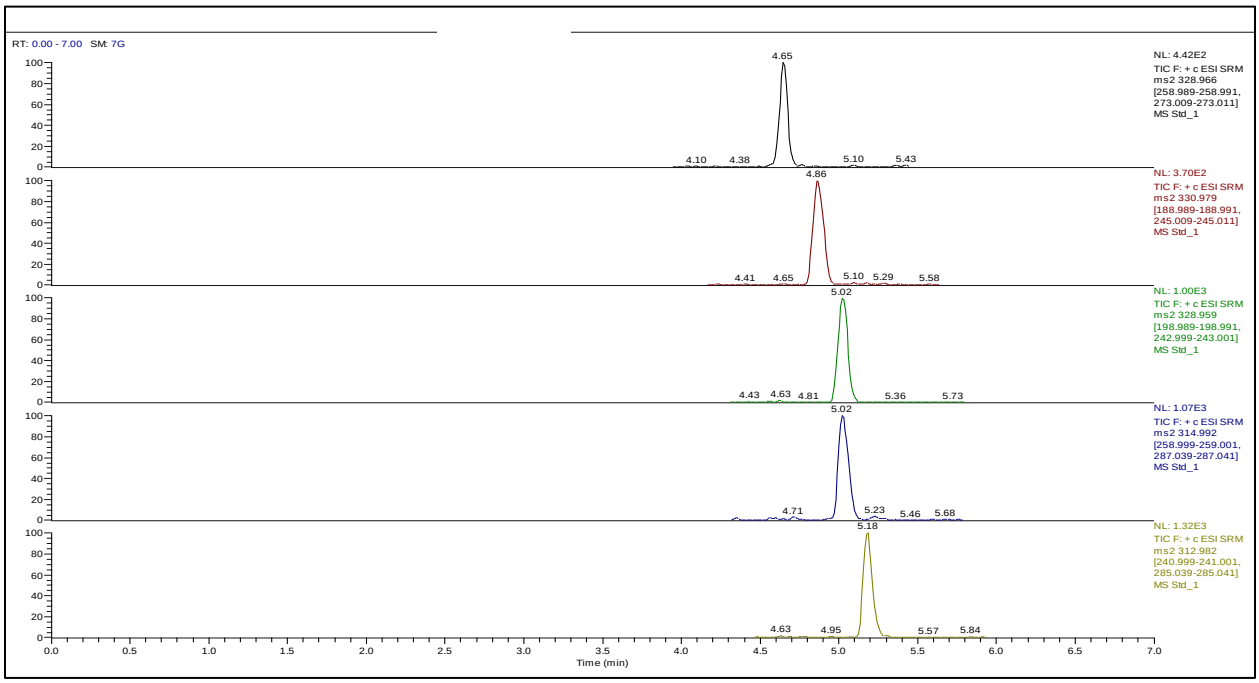

Figure 3. Chromatogram of the lowest matrix-matched calibration point (0.01 µg/kg).

Prior to instrumental analysis, a sample pre-treatment step is required to concentrate the analyte(s) of interest and eliminate non-desirable matrix components. This is particularly important for the analysis of aflatoxins in milk because of the low regulatory limits established for aflatoxin M1 and the larger sample size required to obtain the necessary method sensitivity. SPE is ideally suited to achieving these objectives. One of the biggest difficulties in milk analysis is the high fat and protein content that can often interfere with instrumental analysis. The sample preparation procedure was therefore optimized to remove as much co-extracted matrix components as possible.

Initially, a simple deproteinization step using acetic acid followed by centrifugation was executed to separate the proteins and lipids prior to SPE extraction. However, the recoveries were found to be low (<40%) using this approach, which is most likely caused by the adsorption of the aflatoxins onto proteins or lipids in the milk. It was determined that a solvent extraction step was necessary to adequately extract the aflatoxin residues from milk prior to SPE cleanup. Due to the high water content of milk, direct extraction with an organic solvent would result in a large volume of supernatant that could not be directly applied to the SPE cartridge (organic content too high) or require a time consuming evaporation step to remove the solvent. As a result, a two-step extraction procedure incorporating an initial aqueous extraction step was included in the final method. This simple step removes most of the water from the sample prior to a second extraction with acetone, a volatile organic solvent that is readily removed by evaporation. This extract is then reconstituted in water prior to application to the SPE cartridge. The SPE sorbent was washed with 50% methanol to remove medium to highly polar matrix components and hexane to remove lipophilic compounds. Acetone was used as the SPE elution solvent as it was found to be more effective than methanol and very easy to remove by evaporation. Filtration of the final sample extract prior to LC-MS/MS analysis and the use of matrix-matched calibration curves and/or isotopically labeled internal standards are recommended to obtain optimal results.

References:

1. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, International Agency for Research on Cancer (IARC) website, http://monographs.iarc.fr/ENG/Classification/index.php (accessed June 2015).
2. European Mycotoxins Awareness Network website, http://services.leatherheadfood.com/eman/FactSheet.aspx?ID=6 (accessed June 2015).
3. European Food Safety Authority website, http://www.efsa.europa.eu/en/topics/topic/aflatoxins.htm (accessed June 2015)
4. FDA Compliance Policy Guide, Sec. 527.400 Whole Milk, Lowfat Milk, Skim Milk - Aflatoxin M1.
5. Commission Regulation (EU) No 165/2010. Amending Regulation (EC) No 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. *Official Journal of the European Union*, Feb 26, 2010, pp L 50/8 – L 50/12.

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Simultaneous Determination of Prescription and Designer Benzodiazepines in Urine by SPE and LC-MS/MS

UCT Part Numbers:
CSXCE106 – Clean Screen® XCEL, 130mg/6mL
SPHACE5001-5- Select pH Buffer, 100 mM Acetate pH 5.0
BETA-GLUC-50 - 50mL β- glucuronidase enzyme - liquid form
SLDA100ID21-3UM- Selectra® DA Column, 100 x 2.1mm, 3µm
SLDAGDC21-3UM- Selectra® DA Guard Column, 10 x 2.1mm, 3µm
SLGRDHLDR - Guard Cartridge Holder

SUMMARY:

Benzodiazepines, frequently referred to as "Benzos", are prescribed for the treatment of anxiety, insomnia, muscle spasms, alcohol withdrawal and seizure-prevention on account of their ability to depress the central nervous system. Generally, these drugs are deemed safe and highly effective when used properly and for short durations of time. However, long term use can lead to both physical and psychological dependence consequentially triggering abuse[1].

Benzodiazepines are also recurrently utilized as illegal recreational drugs. In this case, they may be ground to a powder, mixed with water and injected, as well as being swallowed as pills. Their administration is often accompanied by the use of other drugs, such as alcohol and opioids for an enhanced overall effect. Similar to other commonly abused compounds, such as cannabinoids or amphetamines, "legal" alternatives have been developed for Benzos as well in an attempt to bypass the controlled substances act. These new designer drugs are structural or functional analogs of the controlled substance designed to not only mimic the pharmacological effects of the original drug, but also avoid illegal classification and/or detection in a standard drug test.[2]

Keeping up with the ever changing designer drug market has proven to be a real challenge for laboratories across the country. Given that these compounds are derived from "template structures", it will prove valuable for labs to have a method that can not only target current metabolites of interest, but also the latest ones being formulated.

PROCEDURE:

Sample Pretreatment

To 1 mL of urine sample, add 1 mL of 100 mM Acetate Buffer (pH= 5) and 25-50 µL of concentrated Selectrazyme™ β-glucuronidase (**BETA-GLUC-50**).
Vortex and heat for 1-2 hours at 65° C; Allow sample to cool
Do not adjust pH~ sample is ready to be added to the extraction column.

SPE Method:

1. Attach SPE cartridges (**CSXCE106**) to a glass block manifold or positive pressure manifold.
2. Load the pretreated sample, adjust vacuum or pressure for a slow dropwise sample flow (1-2mL/min).
3. Wash the SPE cartridges with 3 mL of Acetate Buffer pH 5 (**SPHACE5001-5)**. Dry the SPE cartridges under full vacuum or pressure for 5 minutes.
4. Repeat the wash with 3 mL Methylene Chloride. Dry the SPE cartridges under full vacuum or pressure for 10 min.
5. Insert collection rack with test tubes to the manifold, and elute the retained analytes with 3 mL of Ethyl Acetate:NH_4OH (98:2).
6. Evaporate the eluate to dryness at 45 ° C under a gentle stream of nitrogen, and reconstitute with 100 µL of 50% MeOH in DI water.
7. Vortex the extract for 30 sec and transfer to 200-µL inserts held in 2-mL vials.

HPLC Conditions	
Instrumentation	Agilent 1200 Binary Pump SL
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 3 µm (p/n: SLDA100ID21-3UM)
Guard column	UCT Selectra® DA, 10 × 2.0 mm, 3 µm (p/n: SLDAGDC21-3UM)
Guard column holder	p/n: SLGRDHLDR
Column temp.	40° C
Mobile phase A	Water + 0.1% formic acid
Mobile phase B	Methanol + 0.1% formic acid
Flow rate	300 µL/min
Gradient	0 min,70%B; 1-6 min,100%B; 6-9 min,100%B; 9.01-13 min, 70%B
Injection volume	10 µL
Autosampler temp.	10° C
Wash solvent	Methanol

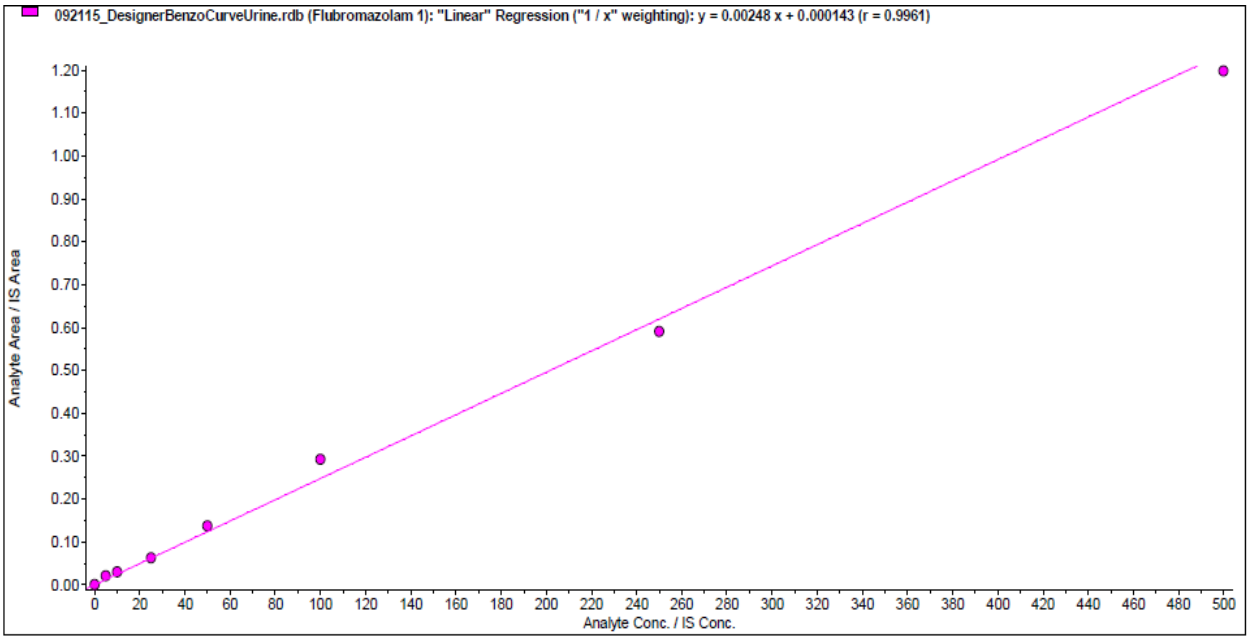
MS Conditions	
Instrumentation	API 4000 QTRAP MS/MS
Ionization mode	ESI^+
Spray voltage	4200 V
Vaporizer temperature	650° C
Capillary temperature	350° C
Sheath gas pressure	40 arbitrary units
Auxiliary gas pressure	5 arbitrary units
Ion sweep gas	0 arbitrary units

MRM Transitions (ESI positive, dwell time: 50 ms)					
Compound		Retention Time (min.)	MRM Transitions		
			Q1	Q3 ion 1	Q3 ion 2
1	7-amino Clonazepam	1.56	286.1	222.3	250.2
2	Midazolam	1.79	326.0	291.0	222.0
3	Lorazepam	2.38	321.1	303.3	275.0
4	Oxazepam	2.54	287.1	241.3	104.2
5	Clonazepam	2.66	316.1	270.2	241.2
6	Flubromazepam	3.02	335.0	226.1	186.0
7	Alpha-Hydroxy-Alprazolam	3.10	325.2	297.1	216.3
8	Nordiazepam	3.19	271.1	104.1	165.2
9	Phenazepam	3.26	352.0	185.9	206.0
10	Pyrazolam	3.45	356.0	206.1	167.2
11	Temazepam	3.57	301.1	255.2	177.2
12	Flubromazolam	4.11	372.9	345.0	292.2
13	Alprazolam	4.22	309.2	205.3	281.2
14	Diclazepam	4.49	321.0	229.1	154.1
15	Diazepam	4.80	285.1	193.2	154.1
16	Etizolam	5.46	345.06	316.1	291.1

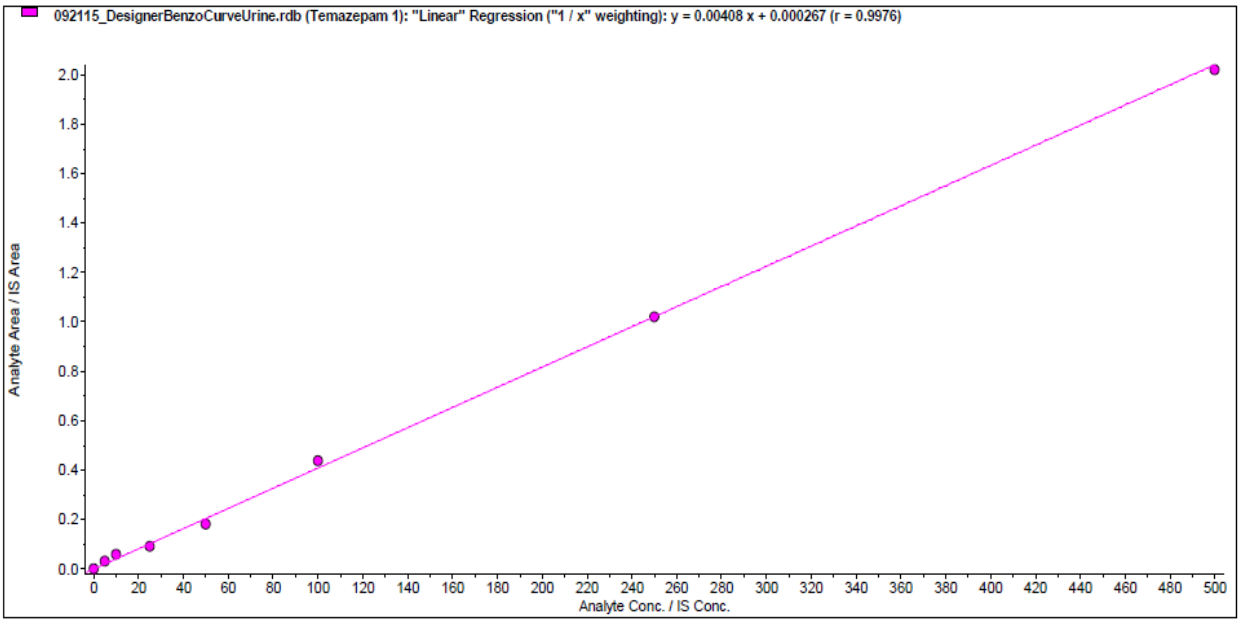
RESULTS:

Analyte	Absolute Extraction Recovery (%, n= 3)			Matrix Effect (%, n= 3)			Overall Extraction Efficiency (%, n= 3)		
	15 ng/mL	75 ng/mL	200 ng/mL	15 ng/mL	75 ng/mL	200 ng/mL	15 ng/mL	75 ng/mL	200 ng/mL
Diclazepam	78	66	71	28	19	19	56	54	57
Etizolam	89	79	91	5	2	7	84	77	85
Flubromazepam	92	86	79	0	-3	-9	93	88	86
Flubromazolam	90	76	87	-6	-8	-2	95	83	89
Phenazepam	98	77	85	24	17	24	74	64	64
Pyrazolam	81	72	86	21	18	18	64	59	71
7-Amino Clonazepam	90	67	85	36	19	11	58	54	76
Alpha-Hydroxy-Alprazolam	98	92	82	-43	-30	-29	140	119	107
Alprazolam	86	79	80	-58	-47	-42	137	116	114
Clonazepam	84	77	82	20	11	16	67	69	68
Diazepam	95	84	88	14	7	4	82	78	85
Lorazepam	106	78	92	24	15	23	81	66	71
Nordiazepam	114	91	103	41	29	27	68	64	75
Oxazepam	107	88	97	15	7	15	91	82	83
Temazepam	74	67	73	5	2	8	71	66	68
Midazolam	64	59	75	50	41	25	32	34	56

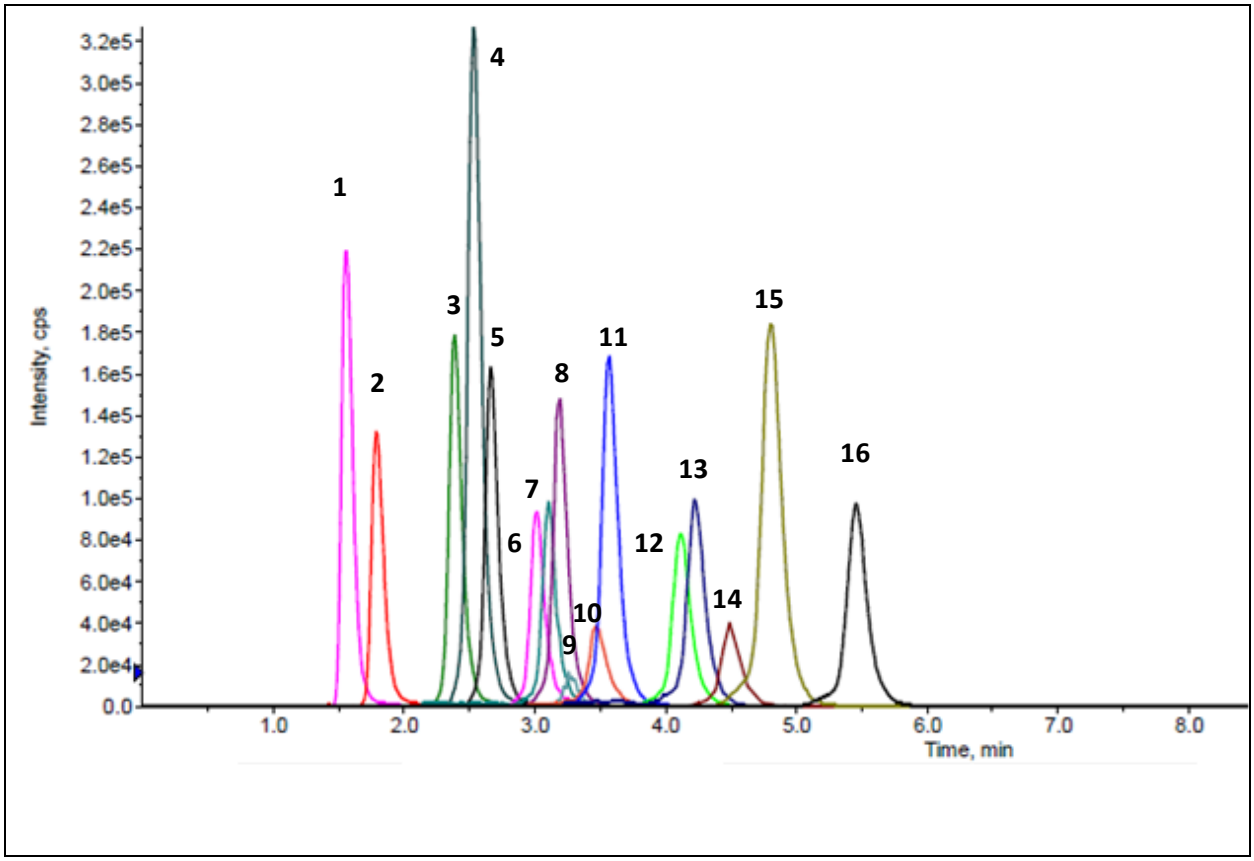
Matrix Matched Calibration Curve: Designer Benzodiazepine (Flubromazolam)



Matrix Matched Calibration Curve: Prescription Benzodiazepine (Temazepam)



Chromatogram of 100 ng/mL solvent standard



Discussion:

Traditional benzodiazepines primarily function as neutral analytes, however, there are some that have weakly basic functional groups allowing for ionization at a fixed pH. The Clean Screen® XCEL I column was chosen for this application due to its capability of simultaneously extracting neutral and basic compounds, while eliminating the need for time-consuming column conditioning and extensive solvent usage for sample cleanup.

The urine samples were adjusted to a pH of 5 for optimal enzyme hydrolysis. Following incubation, they were loaded directly onto the Clean Screen® XCEL I extraction columns. Although the majority of the analytes are primarily retained via hydrophobic interactions, use of this pH allows for any potential ionizable groups to be fully charged and retained via ion exchange. The percentage of organic in the wash was optimized in order to provide sufficient cleanup for the benzodiazepines without compromising on overall analyte recovery. After trying several various solvent combinations of Hexane, Methylene Chloride and buffer containing a percentage of Acetonitrile, it was determined that a buffer wash sequentially followed by a Methylene Chloride wash gave the cleanest final extract with the best overall recovery. An elution solvent of Ethyl Acetate with 2% Ammonium Hydroxide was chosen on account of its ability to not only displace any hydrophobic interactions, but also simultaneously disrupt ionic retention factors.

Conclusion:

1. By utilizing UCT's Clean Screen® XCEL I extraction columns in conjunction with the Selectra®DA HPLC column, prescription and synthetic Benzodiazepine levels can be monitored simultaneously reducing both sample preparation and instrumental analysis time.
2. The universal nature of this extraction method makes it applicable to existing Benzodiazepines along with other newly emerging analogs.
3. It is strongly recommended to use matrix-matched calibration curves, which include isotopically labeled internal standards to compensate for any remaining matrix that is not removed via the extraction procedure.

References:

1. "Benzodiazepines Drug Class Information on RxList.com" RxList. N.p., 2 Apr. 2015. Web 28 Sept. 2015.
2. http://www.ncbi.nlm.nih.gov/pubmed/24259203

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Determination of 35 Pesticides and 3 Cannabinoids in Marijuana Edibles

UCT Part Numbers:
ECQUUS950CT-MP – QuEChERS salts for THC Potency and Pesticide Testing- 50 mL Centrifuge Tubes included
ECQUUS142CT- Dispersive SPE sorbent blend for Pesticide Testing in Edibles- 2 mL Centrifuge Tubes included
SLAQ100ID21-3UM- Selectra® Aqueous C18 HPLC Column 100 x 2.1 mm, 3µm
SLAQGDC20-3UM- Selectra® Aqueous C18 Guard Column, 10 x 2.1mm, 3µm
SLGRDHLDR - Guard Cartridge Holder

Introduction:

As of July 2015, 23 states and Washington D.C. in USA have legalized the medical use of marijuana, while 4 states and Washington D.C. have legalized the recreational use of marijuana. As a result, many forensic toxicology labs are looking for fast, reliable, and cost-effective methods to determine cannabis potency and pesticides in edibles. This application utilizes the advantages of QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) to extract 35 pesticides and 3 cannabinoids including tetrahydrocannabinol (THC), cannabidiol (CBD), and cannabinol (CBN) in edibles, followed by either serial dilutions for cannabis potency analysis, or a dispersive solid phase extraction (dSPE) cleanup for pesticide residue analysis. This hybrid method allows the QuEChERs technique, which is extensively used in the food testing industry, to be utilized in a forensic setting.

Procedure:

(a) Sample pre-treatment

1. For hard candies and chocolate, grind to a fine powder using a SPEX 6770 freezer mill.

Figure 1: Hard candy before (left) and after (right) freezer mill grinding

2. For gummy samples, cut into slim pieces. Although freezer mill can grind gummies to powder at low temperature with the use of liquid nitrogen, it returns to gel state when temperature goes up to room temperature, thus gummy samples should be cut instead of ground.
3. For sodas, degas for 30 min by sonication.

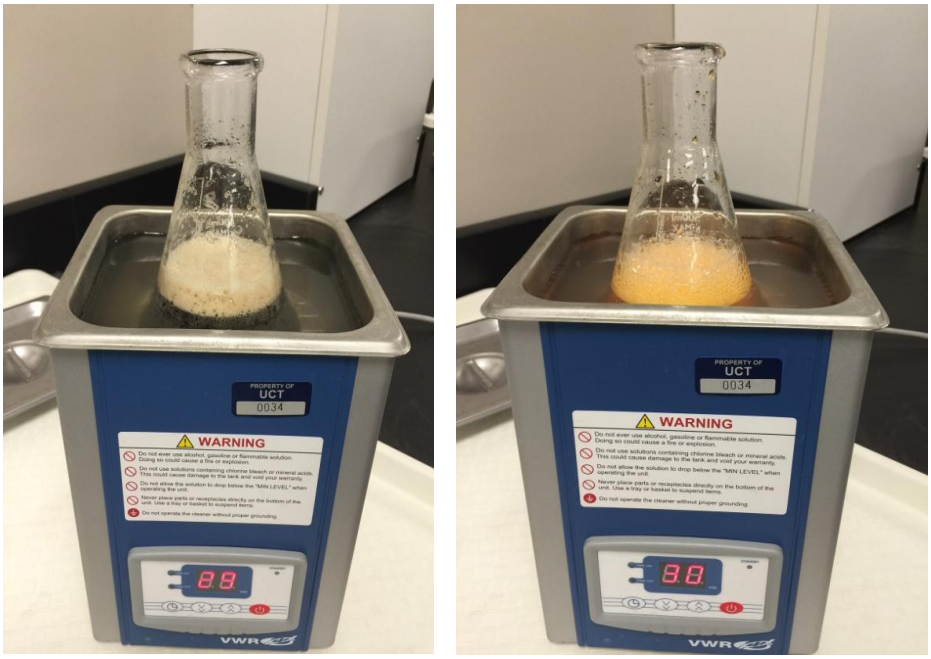

Figure 2: Degassing of Reef cola (left) and Orange kush (right)

(b) QuEChERS extraction

1. Weigh 1 g of the pre-treated samples (hard candies, gummies, brownies, chocolate, and oil) into 50-mL centrifuge tubes, add internal standard (optional) and 10 mL of reagent water, and hydrate for 1 hr using a horizontal shaker. For sodas, add 10 mL of the degassed sample and internal standard (optional) to 50-mL centrifuges.
2. Add 10 mL of acetonitrile (MeCN) with 1% acetic acid.
3. Add QuEChERS extraction salts from pouches (**ECQUUS950CT-MP**), and vortex for 10 sec to break up salt agglomerates.

4. Shake for 1 min at 1000 stroke/min using a SPEX Geno/Grinder. For gummy samples, add 2 metal balls and shake for 10 min at 1000 stroke/min.
5. Centrifuge at 3000 rcf for 5 min.

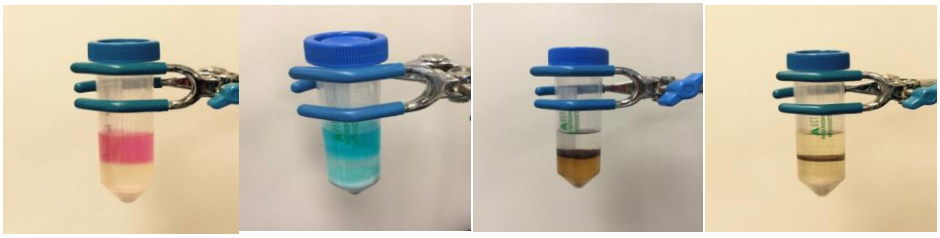

Figure 3: Samples after QuEChERS extraction (from left to right: hard candies, gummies, soda, and chocolate)

(c) dSPE cleanup for pesticide residue analysis

1. Transfer 1 mL of the supernatants to 2-mL dSPE tube (**ECQUUS142CT**).
2. Shake for 1 min at 1000 stroke/min using the SPEX Geno/Grinder.
3. Centrifuge at 3000 rcf for 5 min.
4. Transfer 200 µL extract to the 2-mL auto-sampler vials, add 200 µL of DI water, and vortex for 30 sec.

Figure 4: Comparison of QuEChERS extracts before and after dSPE cleanup (from left to right: hard candies and gummies)

(d) Make serial dilutions for cannabinoid analysis

1. Perform serial dilutions (200 to 20,000 times depending on the cannabinoid concentration in different samples) of the QuEChERS extracts to 100 to 200 ng/mL.
2. Spike the diluted samples with 50 and 150% of the target cannabinoids, which are used to quantify the cannabinoid concentration according to the standard addition method.

(e) Analyze by LC/MS/MS

1. Analyze samples by LC/MS/MS (Thermo Scientific UltiMate 3000 LC system coupled to TSQ Vantage tandem MS) equipped with the Selectra®Aqueous C18 HPLC column (**SLAQ100ID21-3UM**).

Instrument Parameters (Pesticides):

HPLC: Thermo Scientific Dionex UltiMate 3000® LC System		
Column: UCT, Selectra®, aQ C18, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, aQ C18, 10 x 2.0 mm, 3 µm		
Column temperature: 40 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
Injection volume: 2 µL		
Gradient program:		
Time (min)	**A%** (10 mM ammonium acetate in DI water)	**B%** (0.1% formic acid in MeOH)
0	100	0
1	50	50
3.5	50	50
6	5	95
9	5	95
9.1	100	0
14	100	0

MS parameters	
Instrumentation	Thermo Scientific TSQ Vantage tandem MS
Polarity	ESI +
Spray voltage	3500 V
Vaporizer temperature	450 °C
Ion transfer capillary	350 °C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	40 arbitrary units
Q1 and Q3 peak width (FWHM)	0.4 and 0.7 Da
Collision gas and pressure	Ar at 1.5 mTorr
Cycle time	0.5 sec
Acquisition method	EZ Method (scheduled SRM)

SRM Table						
Compound	**Precursor**	**Product 1**	**CE1**	**Product 2**	**CE2**	**S-lens RF**
Metamidophos	142.0	94.1	14	125.0	13	50
Acephate	184.0	143.0	6	95.0	25	33
Aldicarb sulfoxide	207.1	89.1	13	69.1	16	32
Oxydemeton methyl	247.0	169.0	13	109.0	27	57
Pymetrozine	218.1	105.1	20	176.1	17	63
Dichrotophos	238.1	112.1	12	127.0	18	52
Triethylphosphorothioate	199.0	125.0	16	143.0	14	55
Dimethoate	230.0	125.0	22	171.0	15	50
Carbendazim	192.1	160.1	18	132.1	29	60
Dichlorvos	220.9	109.0	17	127.0	13	62
Thiabendazole	202.0	175.1	25	131.1	31	70
Fenamiphos sulfone	336.1	266.0	19	188.0	26	75
Fenamiphos sulfoxide	320.1	233.0	24	108.1	40	60
Simazine	202.1	132.0	19	124.1	16	66
Tebuthiuron	229.1	172.1	16	116.0	26	55
Carbaryl	202.1	145.1	11	127.1	30	38
Flutriafol	302.1	70.1	17	123.0	28	69
Famphur	326.0	217.0	20	93.0	30	68
Thionazin	249.0	113.0	23	97.0	28	58
DEET	192.1	119.1	17	91.1	29	64
Atrazine	216.1	174.1	16	68.1	34	66
Malathion	331.0	127.0	12	99.0	25	55
Triadimefon	294.1	197.1	14	69.1	20	65
Pyrimethanil	200.1	107.1	24	183.1	23	68
Bifenazate	301.1	170.1	18	198.1	6	48
Acetochlor	270.1	224.1	10	148.1	18	58
Sulfotep	323.0	97.0	37	115.0	30	60
Tebuconazole	308.1	70.1	21	125.0	33	66
Zoxamide	336.0	187.0	21	159.0	38	74
Diazinon	305.1	169.1	20	153.1	20	68
TPP (IS)	327.1	152.1	35	77.1	38	95
Cyprodinil	226.1	93.1	33	77.1	43	70
Pyrazophos	374.1	222.1	20	194.1	31	100
Profenofos	372.9	302.9	17	128.0	42	73
Ethion	385.0	142.9	26	199.0	6	56
Chlorpyrifos	349.9	97.0	32	197.9	19	67

Instrument Parameters (Cannabanoids):

HPLC: Thermo Scientific Dionex UltiMate 3000® LC System		
Column: UCT, Selectra®, aQ C18, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, aQ C18, 10 x 2.0 mm, 3 µm		
Column temperature: 40 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
Injection volume: 5 µL		
Gradient program:		
Time (min)	**A%** (10 mM ammonium acetate in DI water)	**B%** (0.1% formic acid in MeOH)
0	40	60
0.5	40	60
3	5	95
7	5	95
7.1	40	60
10	40	60

MS parameters	
Instrumentation	Thermo Scientific TSQ Vantage tandem MS
Polarity	ESI +
Spray voltage	3500 V
Vaporizer temperature	450 ° C
Ion transfer capillary temperature	350 ° C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	40 arbitrary units
Q1 and Q3 peak width (FWHM)	0.4 and 0.7 Da
Collision gas and pressure	Ar at 1.5 mTorr
Cycle time	0.5 sec
Acquisition method	EZ Method (scheduled SRM)

SRM Table						
Compound	**Precursor**	**Product 1**	**CE1**	**Product 2**	**CE2**	**S-lens RF**
CBD	315.0	193.1	20	123.0	30	77
CBN	311.1	223.1	19	293.2	14	73
THC	315.2	193.1	19	123.1	31	73

Results:

A. Pesticide residue analysis

6-point matrix-matched calibration curves with concentrations at 5, 10, 25, 50, 100, and 250 ng/mL were generated. The responses were found to be linear ($R^2 >$ 0.99) over the concentration range. The limit of quantitation (LOQ) of this method was found to be 50 ng/g in the edibles, and 5 ng/mL in the soda samples.

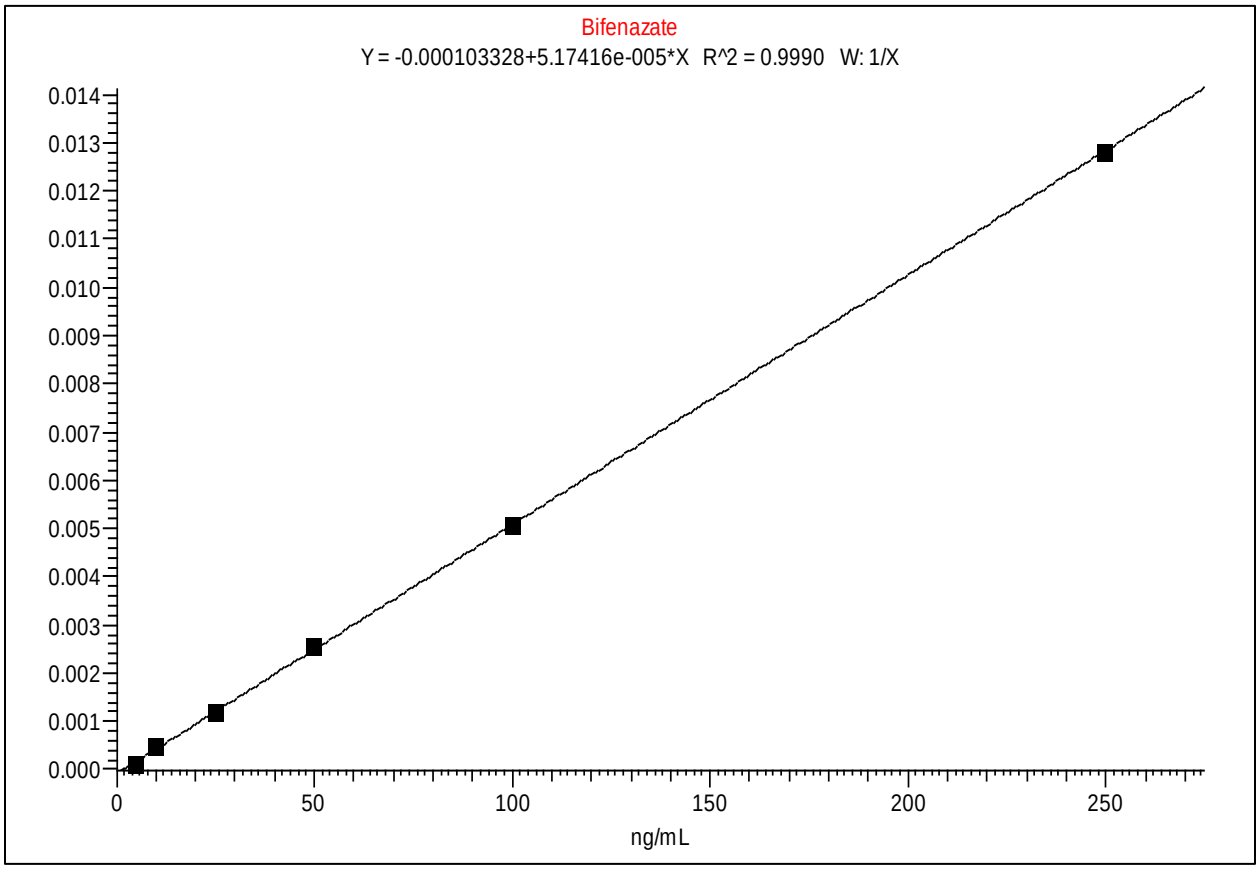

Figure 5: Matrix-matched calibration curve of Bifenazate (R^2 = 0.9990)

Table 1: Accuracy and Precision of Pesticides in Spiked Samples

Compound	Spiked at 10 ng/mL		Spiked at 50 ng/mL	
	Recovery%	RSD% (n= 6)	Recovery%	RSD% (n= 6)
Methamidophos	**80**	11	**83**	12
Acephate	**81**	14	**93**	12
Aldicarb_sulfoxide	**93**	13	**95**	23
Oxydemeton_methyl	**74**	16	**80**	23
Dichrotophos	**90**	15	**75**	14
Pymetrozine	**57**	20	**59**	10
Dimethoate	**105**	16	**87**	12
Triethylphosphorothioate	**97**	14	**82**	14
Carbendazim	**98**	15	**74**	12
Dichlorvos	**97**	12	**97**	11
Fenamiphos_sulfone	**121**	11	**108**	15
Fenamiphos_sulfoxide	**99**	14	**96**	16
Simazine	**121**	14	**107**	14
Carbaryl	**93**	10	**103**	14
Tebuthiuron	**105**	9	**105**	17
Thiabendazole	**70**	7	**78**	8
Famphur	**101**	13	**101**	13
Flutriafol	**92**	14	**96**	10
Thionazin	**103**	11	**99**	12
Atrazine	**99**	24	**95**	13
DEET	**105**	30	**97**	12
Malathion	**102**	23	**115**	14
Triadimefon	**97**	21	**101**	18
Bifenazate	**154**	23	**98**	21
Pyrimethanil	**83**	14	**84**	16
Acetochlor	**96**	16	**101**	12
Sulfotep	**100**	15	**99**	13
Tebuconazole	**85**	2	**87**	5
Zoxamide	**86**	3	**91**	5
Diazinon	**92**	4	**92**	3
Cyprodinil	**77**	5	**77**	3
Pyrazophos	**94**	4	**97**	3
Ethion	**92**	3	**92**	5
Profenofos	**87**	8	**88**	6
Chlorpyrifos	**90**	9	**93**	9

Table 2: Pesticide residues detected in edibles

Brand	Product	Detected pesticides
Keef Cola	Keef Cola	Not detected
Keef Cola	Orange Kush	10 ng/mL Bifenazate
Dixie Brands	Elixir	14 ng/mL Bifenazate
Nectar Bee	Cherry Lime Hard Candy	Not detected
Nectar Bee	Sour Fruit Ring Strawberry	Not detected
Wana	Sour Gummies	Not detected
EdiPure	Sweet 'n Sours	Not detected
EdiPure	Mixed Drops	Not detected
Growing Kitchen	Fantastic brownie	97 ng/g Bifenazate
Incredibles	Mile High Mint	Not detected
Incredibles	Cookie and Cream	Not detected
Incredibles	Monkey Bar	Not detected
Elite Botanicals	CBD Oil	1221 ng/g Bifenazate

B. Cannabis potency determination by standard addition method

Example: Cookie and cream bar, labeled with 30 mg of THC in 45 grams (equals 667 µg/g)

After QuEChERS extraction of 1 g of the ground cookie and cream sample into 10 mL MeCN, the concentration of THC in the supernatant will be 66.7 µg/mL. Serial dilutions (x10x50= 500) were made to dilute the extract to about 133 ng/mL, then the diluted samples were spiked with 70 (about 50%) and 210 ng/mL (about 150%) cannabinoids. The peak areas were plotted against the diluted sample (0), 50% spiked (70 ng/mL) and 150% spiked (210 ng/mL) samples, a 3-point linear curve (Figure 6) was generated. The concentration in the diluted sample was calculated by dividing the intercept by the slope. With the calculated concentration, the peak areas were re-plotted (Figure 7) and a linear curve with R^2 of 0.9999 was obtained, indicating that the standard addition method is effective for accurate analyte quantitation.

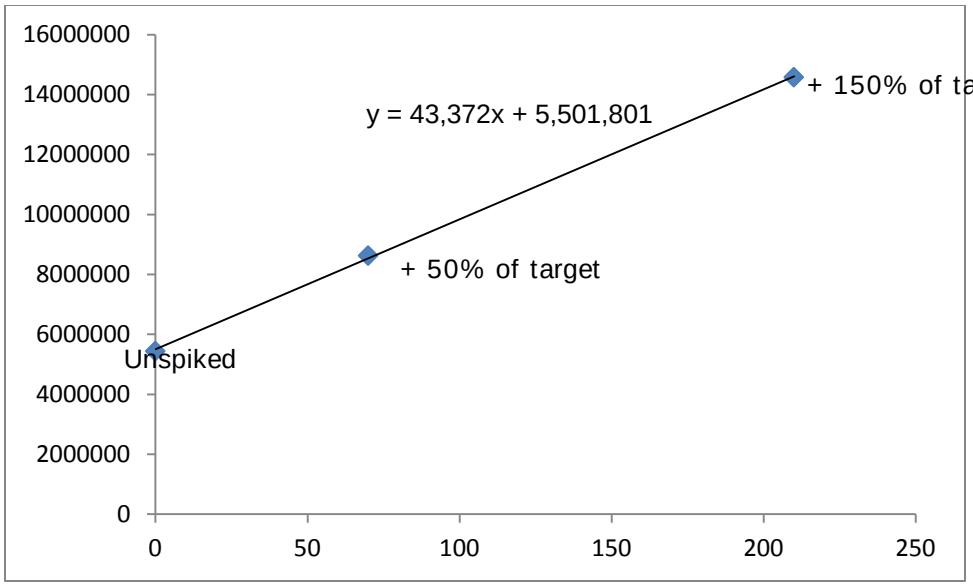

Figure 6: Plot of peak area against the unspiked sample (0), and samples spiked at 50% (70 ng/mL) and 150% (210 ng/mL) of cannabinoids

Calculations:

THC conc. in the diluted sample = 5501801/43372 = 127 ng/mL

THC in the cookie and cream bar = 127 ng/mL x 500 x 10 mL/g x 45 g x 10^{-6} mg/ng = 29 mg (very close to the labeled 30 mg THC)

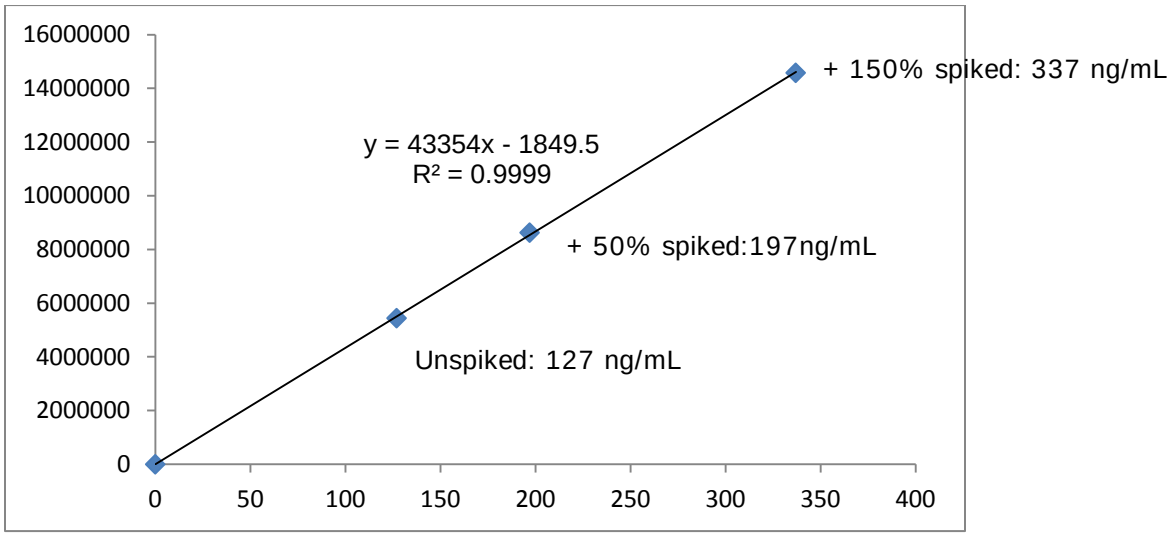

Figure 7: Re-plot of peak area against the actual concentrations: unspiked sample (127 ng/mL), and samples spiked at 50% (197 ng/mL) and 150% (337 ng/mL) of cannabinoids

Table 3: Comparison of labeled and detected cannabinoids in edibles (unit:mg)

Brand	Edibles	CBD		CBN		THC	
		Labeled	Detected	Labeled	Detected	Labeled	Detected
Keef Cola	Keef Cola	NA	ND	NA	ND	10	7
Keef Cola	Orange Kush	NA	ND	NA	ND	10	6
Dixie Brands	Elixir	NA	ND	NA	ND	90	60
Nectar Bee	Cherry Lime Hard Candy	NA	ND	NA	ND	10	6
Nectar Bee	Sour Fruit Ring Strawberry	NA	ND	NA	ND	10	8
Wana	Sour Gummies	NA	ND	NA	ND	100	95
EdiPure	Sweet 'n Sours	NA	**28**	NA	ND	100	31
EdiPure	Mixed Drops	NA	ND	NA	ND	100	49
Growing Kitchen	Fantastic brownie	NA	ND	NA	ND	10	14
Incredibles	Mile High Mint	NA	ND	NA	ND	100	74
Incredibles	Cookie and Cream	NA	ND	NA	ND	100	29
Incredibles	Monkey Bar	NA	ND	NA	ND	100	69
Elite Botanicals	CBD Oil	**500**	**493**	< 5	ND	5	12

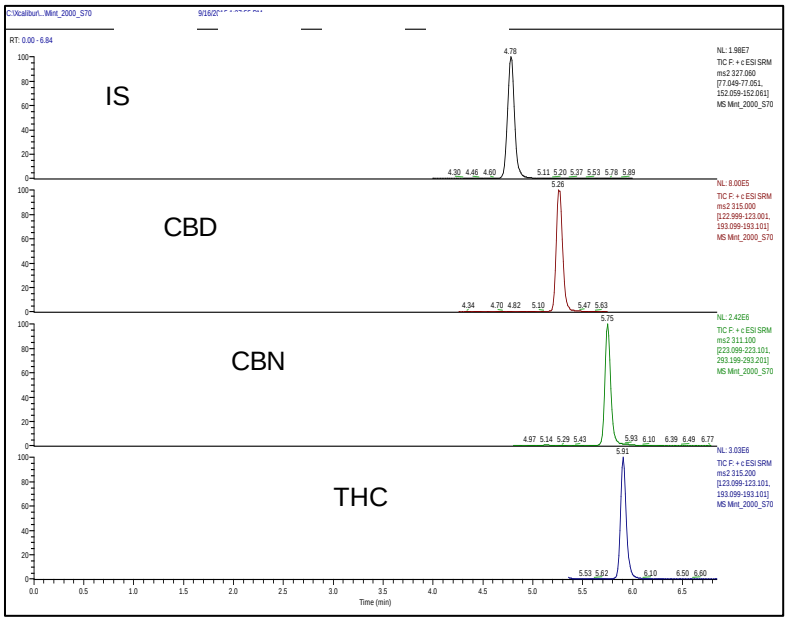

Figure 8: Chromatogram of the diluted mint milk chocolate sample (2000 times dilution of the QuEChERS extract) spiked with 70 ng/mL of cannabinoids

CONCLUSIONS:

A fast and effective method was developed for the determination of pesticide residues and cannabis potency in edibles. Pesticide residues and cannabinoids were extracted using the QuEChERS approach, followed by either a proprietary blend of dSPE sorbents for pesticide analysis, or serial dilutions for cannabinoid potency test. Bifenazate, commonly used to control mites on agricultural products, was found to be present in two soda products as well as oil and brownie edibles. The detected amounts of cannabinoids were compared to those listed on the labels of the cannabis infused food products. Of the tested products, 23% were accurately labeled within (+ /-) 10% of expected concentrations, while others were either higher or lower than claimed amounts.

ACKNOWLEDGEMENT

Keith Tucker (Vice President of Marketing at *SPEX* SamplePrep, LLC) is acknowledged for kindly providing the 6770 Freezer mill and 2010 Geno/grinder. Erik Swiatkowski (UCT) is thanked for his help in grinding samples using the SPEX 6770 freezer mill.

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Extraction of Synthetic and Naturally Occurring Cannabinoids in Urine Using SPE and LC-MS/MS

UCT Part Numbers:
SSHLD063– Styre Screen® HLD, 60 mg/ 3 mL cartridge
BETA-GLUC-50 - 50mL β- glucuronidase enzyme - liquid form
SPHACE5001-5 - Select pH Buffer Pouch, 100mM Acetate buffer, pH 5.0
SLDA100ID21-3UM - Selectra® DA, 100 x 2.1mm, 3µm
SLDAGDC21-3UM - Selectra® DA, Guard, 10 x 2.1mm, 3µm
SLGRDHLDR - Guard Cartridge Holder

September 2015

Summary:

Synthetic cannabinoids (Spice) are a family of compounds that when consumed mimic the effects of marijuana. These products are often marketed as "legal alternatives to cannabis" or "legal highs" and have dramatically increased in popularity among different drug user populations. The biggest hurdle for testing facilities is keeping up with the ever-changing synthetic analogs being produced by illicit drug makers in an attempt to avoid detection.

Designing methods to detect synthetic cannabinoids is a rapidly moving target for laboratories. Currently, the best methods for detection are liquid chromatography/ tandem mass spectrometry (LC-MS/MS) and gas chromatography/mass spectrometry (GC/MS). Historically, typical protocols target JWH-018 and JWH-073 and their metabolites. Such targeted protocols are generally limited by the availability of reference standards and lack of standardized testing criteria.

While much work still needs to be done to develop standardized methods for synthetic cannabinoids, one approach some laboratories have taken is to set the limit of detection as low as analytically possible. By paring UCT's Styre Screen® HLD polymeric solid phase extraction column with the Selectra®DA HPLC column, one can ultimately produce a cleaner more concentrated sample leading to enhanced LOD's/LOQ's. Although these compounds are ever changing they are derived from "template structures", which allows them to produce the same desired effect while still being legal. Having a method that can not only target

current metabolites of interest, but also the new ones being created is vital for laboratories to keep up with the constantly changing market.

Procedure:

Sample Pretreatment

1. To 1.0 mL of urine, add 2 mL of 100Mm Acetate buffer (pH 5.0) and 50 µL of beta-glucuronidase, vortex for 30 sec and heat at 65° C for 1-2 hours.
2. Allow sample to cool

SPE Method:

1. Attach SPE cartridges (UCT part#:**SSHLD063**) to a glass block manifold or positive pressure manifold.
2. Load the pretreated sample.
3. Apply vacuum or pressure for a slow drop-wise sample flow.
4. Wash the sample test tubes with 3 mL 100Mm Acetate buffer (pH 5.0) followed by 3 mL of 25% MeOH in 100Mm Acetate buffer (pH 5.0).

   Note: The MeOH:H_2O ratio has been optimized to produce a clean extract without any analyte loss.
5. Dry the SPE cartridges under full vacuum or pressure for 10 min.
6. Insert collection rack with test tubes to the manifold, and elute the retained compounds with 1 x 3 mL of Ethyl Acetate.
7. Evaporate the SPE eluate to dryness at 45 ° C under a gentle stream of nitrogen, and reconstitute with 100 µL of 50% MeOH in D.I. H_2O.
8. Vortex the extract for 30 sec and transfer to 200-µL inserts held in 2-mL autosampler vials for LC-MS/MS analysis.

LC-MS/MS method:

Instrumentation:	AB Sciex API 4000 QTrap MS/MS with Agilent 1200 Binary Pump
Column:	UCT, Selectra®, DA, 100 x 2.1 mm, 3 µm
Guard column:	UCT, Selectra®, DA, 10 x 2.1 mm, 3 µm
Column temperature:	50 °C
Column flow rate:	0.300 mL/min
Auto-sampler temperature:	10 °C
Injection volume:	10 µL

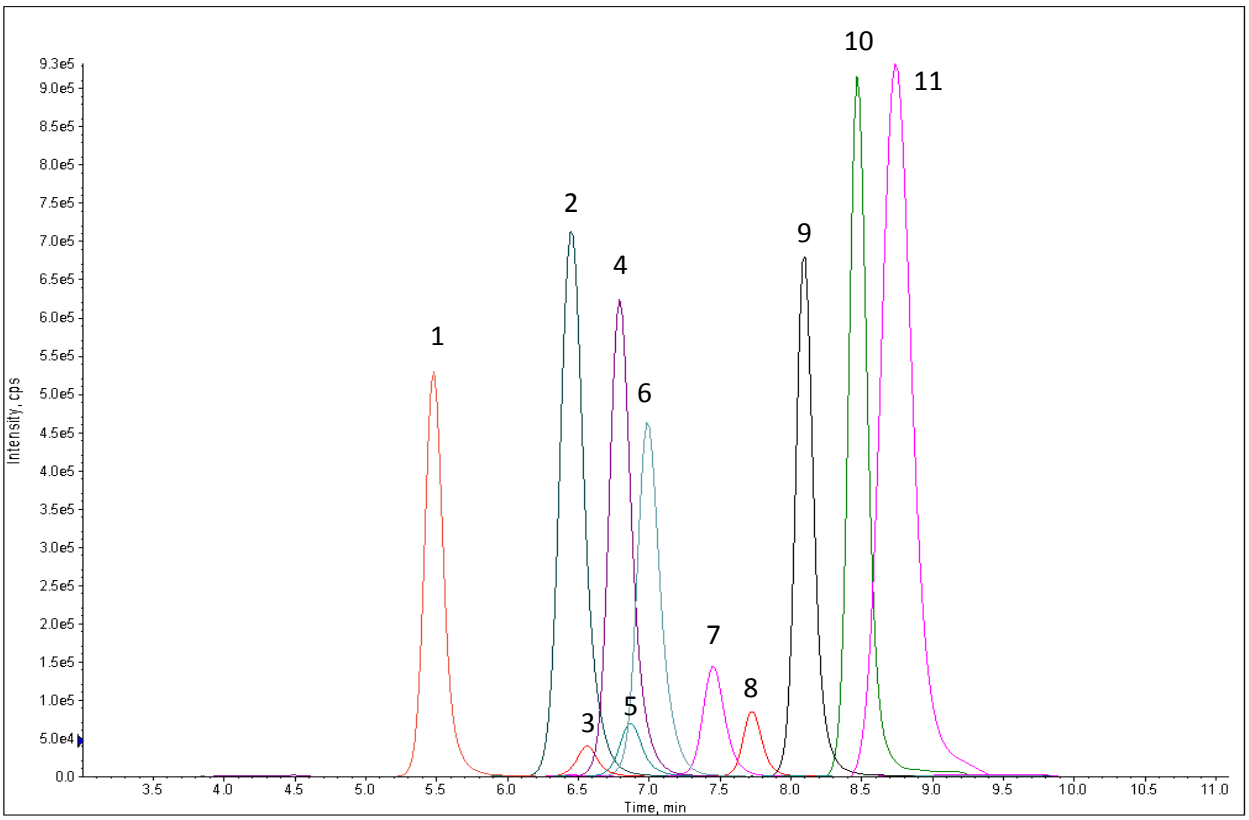
Gradient program:		
Time (min)	**A%** (0.1% formic acid in D.I. H_2O)	**B%** (0.1% formic acid in MeOH)
0	50	50
1	20	80
4	20	80
5	0	100
9.5	0	100
10	50	50
14	50	50

	Analyte	**MRM Transitions**		**Rt (min)**
		Q1	**Q3**	
1	JWH-200	385.097	154.900	5.48
2	THC-OH	331.135	313.300	6.45
3	Cannabidiol	315.142	192.900	6.56
4	JWH-073 N Butanoic Acid	358.118	155.000	6.79
5	THC-COOH	345.101	327.100	6.87
6	JWH-018 N Pentanoic Acid	372.108	154.900	6.99
7	Cannabinol	311.051	223.000	7.46
8	THC	315.200	193.000	7.73
9	JWH-250	336.113	120.800	8.09
10	JWH-073	328.082	155.000	8.47
11	JWH-018	342.113	154.900	8.73

Results:

Accuracy and Precision Data of Spiked Urine Samples

Compound Name	2.5 ng/mL		7.5 ng/mL		75 ng/mL		300 ng/mL	
	Avg. Recovery %	RSD% (n= 3)	Avg. Recovery %	RSD% (n= 3)	Avg. Recovery %	RSD% (n= 3)	Avg. Recovery %	RSD% (n= 3)
THC	81.0	4.7	82.0	5.8	74.0	6.2	70.0	5.4
JWH200	94.0	5.2	102.0	7.7	94.0	6.5	95.0	5.5
JWH073	81.0	5.5	93.0	6.3	89.0	7.5	89.0	6.4
JWH250	98.0	6.7	103.0	5.2	93.0	5.2	94.0	4.1
JWH018	77.0	4.3	93.0	4.5	83.0	4.1	81.0	3.8
CBN	81.0	6.8	81.0	6.7	69.0	4.8	66.0	6.8
CBD	86.0	5.5	91.0	6.9	78.0	5.4	76.0	3.4
THC-COOH	97.0	6.2	114.0	4.9	115.0	6.3	109.0	6.9
THC-OH	97.0	7.8	103.0	5.8	91.0	7.6	95.0	7.4
JWH073 Butanoic Acid	89.0	6.1	96.0	5.5	91.0	6.5	93.0	5.2
JWH018 Pentanoic Acid	98.0	4.8	99.0	3.2	92.0	8.1	91.0	5.1
Overall Mean	**89.0**	**5.7**	**96.0**	**5.6**	**88.0**	**6.2**	**87.0**	**5.4**



Chromatogram of a 100 ng/mL solvent standard

Discussion:

The effects produced by synthetic cannabinoids are very similar to those induced from natural cannabinoid use. Currently, the most common way for screening individuals for recent cannabinoid usage is by immunoassay. Commercially available THC immunoassays do not cross react with synthetic cannabinoids which means labs have to develop mass-spectrometry based screening tests for these designer drugs. This simple extraction not only produces clean, concentrated extracts for spice drugs, but also THC and its metabolites.

The structures and pKa values of synthetic cannabinoids and their metabolites make them ideal candidates for clean-up via solid phase extraction (SPE). Opting to go with a polymeric resin allowed for the elimination of a conditioning step which saved on time and solvent usage. Several combinations of buffer/methanol washes were evaluated for optimal cleanliness and recovery

ranging from 75% buffer/25% methanol to 50%buffer/50%methanol. Although good recovery was achieved for most analytes under all conditions it was noted that going above 25% methanol caused the metabolites of JWH compounds to be lost in the wash. 100% Ethyl Acetate was determined to be the best elution solvent after also evaluating 50%Ethyl Acetate/50%Hexane and 85%Ethyl Acetate/15% IPA solvent combinations.

Conclusion:

a) By utilizing UCT's SSHLD extraction columns and corresponding methodology, both THC and synthetic cannabinoid levels can be monitored simultaneously reducing both analyst time and instrument time.

b) The universal nature of this extraction method makes it amenable to other various synthetic cannabinoids and metabolites, which is important due to the continuous evolution of newly synthesized chemical analogs.

c) It is strongly recommended to use matrix-matched calibration curves, which include isotopically labeled internal standards to compensate for any remaining matrix that is not removed via the extraction procedure.

References

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LC-MS/MS Method for 89 Banned or Controlled Drugs in the Horse Racing Industry

UCT Part Numbers:
SLDA100ID21-3UM – Selectra® DA HPLC Column 100 x 2.1 mm, 3µm
SLDAGDC21-3UM – Selectra® DA Guard Cartridge Column 10 x 2.0 mm, 3µm
SLGRDHLDR - Guard Column Holder

The world of equine drug testing is constantly evolving. New regulations regarding banned and controlled substances can be found throughout North America, Europe and Australia [1,2]. The need for a reliable LC-MS/MS method for the determination and separation of a wide variety of drug classes is critical. In addition to traditional drugs of abuse, this equine testing panel also encompasses drug classes such as bronchodilators, diuretics, antihistamines and non-steroidal anti-inflammatory medications. The unique polyaromatic phase of the Selectra® DA HPLC column allows for efficient analysis and baseline separation of multiple drug classes at once, including several complex isobaric compounds.

Instrumentation Parameters:

HPLC Conditions		
HPLC: Agilent 1200 Series		
Column: UCT, Selectra®, DA, 100 x 2.1 mm, 3 µm		
Guard column: UCT, Selectra®, DA, 10 x 2.0 mm, 3 µm		
Column temperature: 40 °C		
Column flow rate: 0.300 mL/min		
Auto-sampler temperature: 10 °C		
Injection volume: 10 µL		
Gradient program:		
Time (min)	**A%** (0.1% formic acid in H_2O)	**B%** (0.1% formic acid in MeOH)
0	95	5
5	40	60
10	40	60
10.1	0	100
16.5	0	100
17	95	5
22	95	5

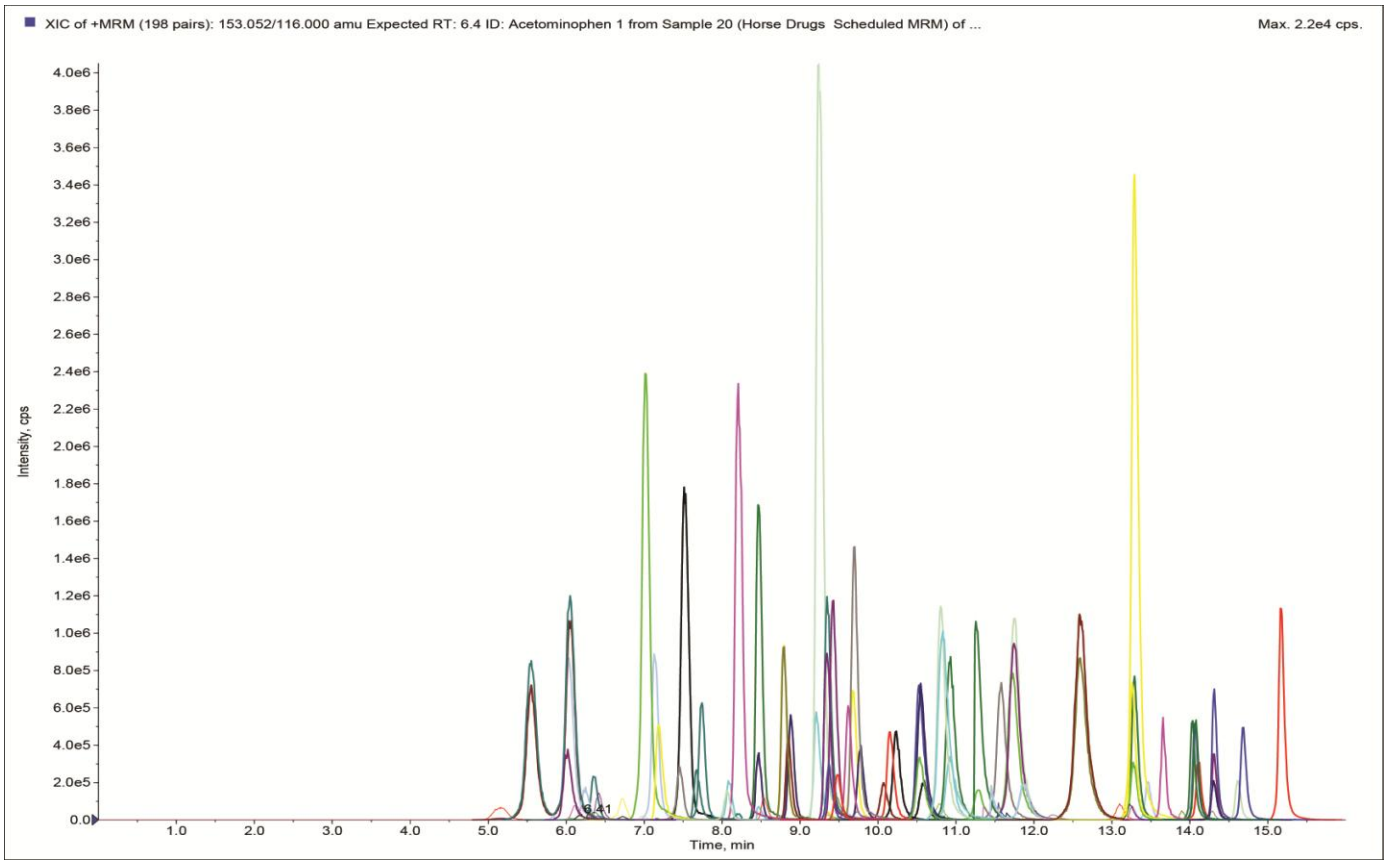
MS parameters	
Instrumentation	AB Sciex 4000 Q Trap
Polarity	ESI +
Spray voltage	5000 V
Vaporizer temperature	650 °C
Collision gas	Medium
Cycle time	6.2 sec
Acquisition method	Scheduled MRM

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1**	**Product ion 2**	**CE 2**	**DP (V)**
Acetaminophen	6.4	153.0	116.0	29	115.1	43	96
Albuterol	5.3	241.0	149.1	25	223.1	15	41
Alprazolam	13.0	308.9	205.3	17	281.2	17	31
Amitriptyline	11.9	278.2	91.1	17	105.1	17	46
Amphetamine	6.0	136.1	91.2	23	119.1	13	51
Aripiprazole	13.0	448.1	277.9	17	278.1	17	31
Atenolol	6.3	267.1	145.0	35	190.0	25	61
Baclofen	6.4	214.1	150.9	23	196.9	17	36
Benzoylecognine	9.2	209.1	168.0	25	105.1	39	76
Benzocaine	9.6	166.2	138.0	15	120.0	23	31
Bupivacaine	9.1	289.0	140.3	35	84.1	59	51
Buprenorphine	10.6	468.4	55.1	87	83.2	87	81
Butorphanol	9.7	329.2	311.2	33	158.2	61	71
Caffeine	10.0	195.0	138.0	27	110.0	33	36
Carprofen	13.8	274.2	228.0	15	193.2	43	36
Chlordiazepoxide	10.2	300.0	227.1	21	283.0	35	56
Chlorothiazide	14.2	295.9	215.2	29	250.0	19	31
Citalopram	10.0	325.0	109.1	39	262.0	27	46
Clenbuterol	8.3	277.1	202.9	25	259.0	15	66
Clidinium	9.7	352.2	142.2	45	96.0	75	66
Clonazepam	13.4	316.1	270.2	39	241.2	37	56
Clonzapine	9.5	327.0	270.0	35	192.0	63	56
Cocaine	9.2	304.1	182.0	30	95.1	73	46
Cocaine D3	9.2	307.1	185.1	23	85.0	51	91
Cyclothiazide	10.7	390.1	317.2	33	100.0	49	41
Dextrorphan	8.6	258.1	157.1	53	199.1	37	91
Diazepam	14.5	285.1	193.2	43	154.1	37	56
Dothiepin	11.1	296.1	251.1	25	225.1	25	61
Edrophonium	8.3	203.0	168.0	25	132.0	25	96
Ephedrine	5.5	166.2	148.0	11	117.0	25	36
Estazolam	12.5	295.3	100.2	31	208.1	31	61
Fentanyl	10.5	338.3	189.1	29	105.0	57	71
Fentanyl D5	10.5	342.2	188.3	39	105.0	53	51
Flunitrazepam	14.1	313.9	268.2	33	239.2	45	36
Flurazepam	10.9	388.1	315.1	37	134.1	67	46

Furosemide	14.2	332.1	91.1	57	119.0	33	66
Gabapentin	6.2	172.0	154.0	15	137.0	25	61
Gabapentin D10	6.1	182.1	164.1	15	147.3	29	51
Hexobarbital	10.7	273.0	157.0	17	81.0	29	41
Hydrochlorothiazide	14.3	297.9	252.2	19	217.1	29	36
Hydrocodone	8.0	300.0	199.0	39	128.0	50	46
Hydromorphone	6.7	286.0	185.0	41	157.0	50	46
Imipramine	11.7	280.9	85.9	25	58.1	63	61
Imipramine D3	11.7	284.0	61.2	49	208.2	35	41
Ketamine	8.8	238.1	125.0	30	220.0	21	36
Ketamine D4	8.8	242.2	129.1	41	224.3	27	66
Levorphanol	8.7	258.1	157.2	59	199.1	35	61
Lidocaine	7.4	235.1	86.0	27	58.1	55	36
Loratadine	14.9	383.1	337.2	31	267.2	45	76
Lorazepam	13.1	321.0	303.5	21	275.0	29	46
Lormetazepam	14.0	334.9	289.1	31	317.2	17	76
Maprotiline	11.8	278.1	250.1	27	117.0	33	41
MDA	7.1	180.2	163.1	15	105.0	31	46
MDEA	8.3	208.1	163.0	19	105.1	37	41
MDMA	7.7	194.2	163.1	19	105.1	33	76
Mepenzolate	9.5	340.2	130.0	41	77.0	101	46
Meprobamate	11.3	219.0	191.0	31	178.0	31	126
Methadone	13.2	310.1	265.0	19	105.0	35	56
Methamphetamine	7.0	150.0	91.1	27	119.1	15	46
Methamphetamine D5	7.0	155.2	92.1	25	121.2	15	26
Mianserin	10.3	265.0	208.0	31	91.0	61	66
Midazolam	10.8	326.0	291.0	50	222.0	50	60
Mirtazepine	9.4	266.1	72.0	27	195.1	61	61
Morphine	6.2	286.3	152.0	79	165.0	50	46
Nalbuphine	8.4	359.2	341.1	31	255.0	43	71
Nalorphine	13.0	311.3	266.1	25	105.1	41	71
Naloxone	7.5	328.0	310.0	27	211.9	55	46
Naltrexone	14.3	343.0	308.0	35	315.3	35	46
Naproxen	13.7	231.1	185.0	21	170.2	37	36
n-Butylscopolammonium	9.2	360.1	130.1	59	194.1	31	51
Neostigmine	7.5	224.1	72.0	49	209.3	29	66
Nitrazepam	11.7	281.1	86.0	31	208.1	31	36
Nortriptyline	11.7	264.1	190.9	33	105.1	33	51
Olonzapine	7.1	313.0	256.0	33	84.0	35	66
Orphenadrine	10.5	270.1	166.0	37	165.1	63	26
Oxazepam	13.2	287.0	241.3	31	104.2	21	46
Oxazeam D5	13.2	292.1	274.0	19	246.1	33	66
Oxycodone	7.9	316.0	140.0	39	256.0	50	31
Oxymorphone	6.3	302.0	227.0	37	198.0	50	36
Paroxetine D6	12.3	336.1	76.2	53	198.1	25	81
Paroxetine	12.4	330.0	192.0	27	123.0	37	61
Prazepam	15.1	325.1	271.0	29	140.0	57	56
Promethazine	11.2	285.1	86.0	29	71.1	61	41
Propanolol	9.6	261.1	116.1	27	184.1	25	66
Protriptyline D5	11.5	268.1	192.1	35	156.1	35	16

Protriptyline	11.5	264.2	191.1	29	91.2	29	51
Pseduoephedrine D3	6.0	169.1	151.1	15	117.0	29	56
Pseudoephedrine	6.0	166.2	148.0	11	117.0	25	36
Risperidone	10.6	411.1	191.1	39	69.0	81	61
Sertraline	12.9	305.9	274.9	17	159.1	33	31
Theophylline	8.2	180.9	123.9	27	95.9	33	31
Trazodone	10.3	372.1	148.1	47	95.9	85	56
Triazolam	14.3	343.0	308.1	39	315.0	37	81
Trimipramine	12.4	295.1	100.2	51	58.1	51	51
Vecuronium	9.2	557.4	100.0	71	356.4	55	121
Venlafaxine	11.9	278.2	233.1	25	105.2	29	31
Warfarin	14.0	308.9	163.0	19	251.1	27	51
Zolpidem	14.0	309.0	163.0	23	251.0	49	61
Zopiclone	9.3	388.9	244.9	19	217.1	27	46

Chromatogram:



REFERENCES:

1. http://www.fei.org/sites/default/files/FEI_Prohibited_Substances_List_and_DB.pdf
2. http://arcicom.businesscatalyst.com/model-rules---standards.html

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Quantitative Analysis of EtG and EtS in Urine Using Clean Screen®ETG and LC-MS/MS

UCT Part Numbers:
CSETG203 – Clean Screen® ETG 200 mg in a 3 mL SPE cartridge
SLETG100ID21-3UM - Selectra® ETG HPLC column, 100 x 2.1 mm, 3 µm
SLETGGDC20-3UM - Selectra® ETG guard column, 10 x 2.0 mm, 3 µm
SLGRDHLDR - guard cartridge holder

August 2015

1. **Prepare Sample**
   To 200 µL of urine sample with 5% formic acid add appropriate deuterated analogues of EtG/EtS.
   Vortex for 30 seconds.

2. **Condition Clean Screen®ETG Extraction Column**
   1 x 2 mL MEOH containing 1% formic acid.
   1 x 2 mL D.I. H_2O containing 1% formic acid.

   Note: Aspirate at < 3 inches Hg to prevent sorbent from drying out.

3. **APPLY SAMPLE:**
   Load sample at 1-2 mL / minute.

4. **Dry Column**
   10 minutes at full vacuum or pressure.

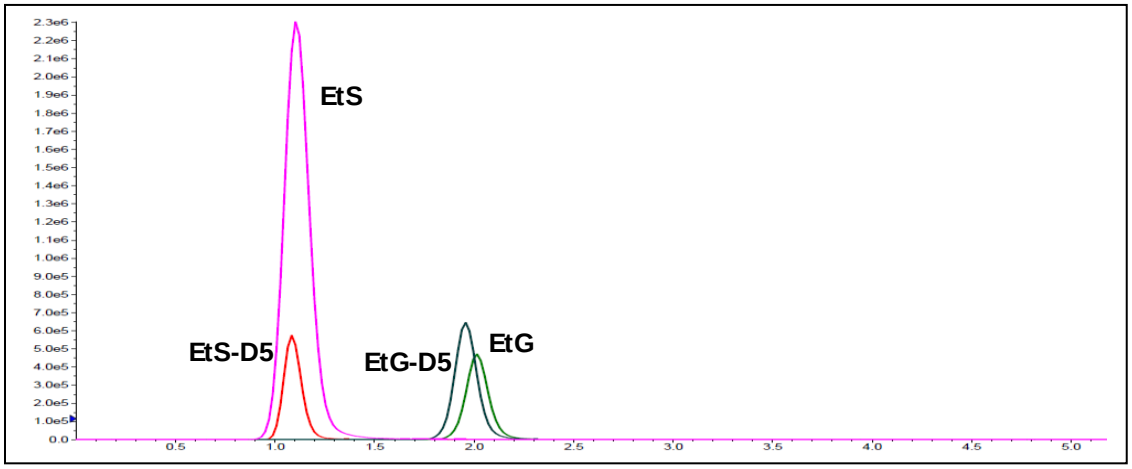
5. **Elute EtG/EtS:**
   1 x 2 mL MEOH containing 1% formic acid.
   Collect eluate at 1-2 mL /minute.

6. **Evaporate/Reconstitute:**
   Evaporate eluate under a gentle stream of nitrogen < 40ºC.
   Dissolve the residue in 200 µL of D.I. H_2O.

LC-MS/MS method:

Instrument: Agilent 1200 Binary Pump SL
Detector: AB Sciex API 4000 Q Trap MS/MS
Column: UCT Selectra® ETG HPLC column, 100 x 2.1 mm, 3 µm
Guard Column: UCT Selectra® ETG, 10 x 2.0 mm, 3 µm
Column Temperature: 30 ° C
Column Flow Rate: 0.3 mL/min
Injection Volume: 10 µL
Gradient Program:

Time (min)	% Mobile Phase A (0.1% Formic Acid in water)	% Mobile Phase B (0.1% Formic Acid in ACN)
0	100	0
1.5	100	0
1.7	0	100
2.7	0	100
3.0	100	0
6.0	100	0



MRM transitions (ESI⁻, 50 ms dwell time)				
Compound	**Rt (min)**	**Q1 ion**	**Q3 ion 1**	**Q3 ion 2**
EtS-D5	1.28	130.1	97.8	79.7
EtS	1.31	125.1	95.8	96.9
EtG-D5	1.66	226.1	85.1	74.9
EtG	1.69	220.9	85.1	75.1

Results:

Excellent recoveries were achieved with EtG at 96% and EtS at 98.3%.The extraction efficiency was evaluated by fortifying samples at two concentrations (250 ng/mL and 2500 ng/mL). RSD values were less than 5.3% (n= 4 at each concentration).

Recovery and RSD% from Urine Spiked at 2 Levels

Compound	Spiked at 250 ng/mL		Spiked at 2500 ng/mL	
	Recovery%	RSD% (n= 4)	Recovery%	RSD% (n= 4)
EtG	96.0	4.8	102.9	4.4
EtS	98.3	6.5	109.6	3.9
Overall mean	97.15	5.65	106.25	4.15

Discussion:

Upon re-evaluation of UCT's original EtG extraction method utilizing Clean Screen® ETG columns, it was noted that the previously employed aqueous wash step resulted in significant loss of both EtG and EtS. Also, it was discovered that there was significant sample breakthrough on the carbon-based extraction column using 0.5 mL of sample or higher due to a lack of sufficient capacity. As a result, the method was modified using decreased sample volume as to not overload the column and without the use of the aqueous wash step. Surprisingly, the cleanliness of the extract was not compromised and excellent recoveries were achieved.

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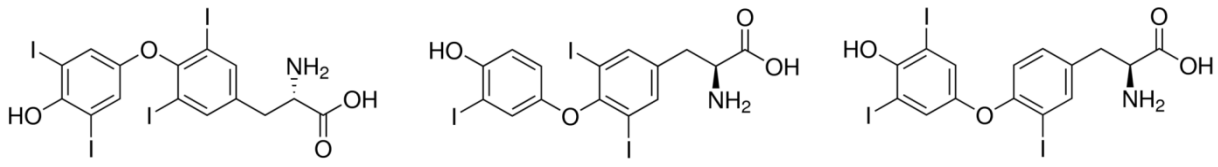
Thyroid Hormones in Serum and Plasma Using SPE Extraction and UHPLC-MS/MS Analysis

UCT Part Numbers:

CSDAU203 - CLEAN SCREEN® DAU, 200mg / 3mL tube
SLDA100ID21-18UM-Selectra® DA HPLC column, 100 x 2.1 mm, 1.8µm
SLDAGDC20-18UM - Selectra®DA guard column, 10 x 2.1 mm, 1.8 µm
SLGRDHLDR - guard cartridge holder

Introduction:

Thyroid hormones are endogenous hormones that play an important role in many biological processes, including growth, development and metabolism. Most of the thyroid hormones circulating in the blood are bound to proteins and only a small fraction of circulating hormones are in their biologically active free form (unbound). Together with the thyroid stimulating hormone (thyrotropin), the concentrations of circulating thyroid hormones are used to assess thyroid function and diagnose thyroid disease, such as hyperthyroidism and hypothyroidism, or to monitor treatment status. It is therefore essential to have accurate and sensitive analytical methods that are capable of measuring low levels of thyroid hormones in serum or plasma. The thyroid hormones can be measured as free hormones, which are indicators of hormone activity in the body, or as total hormones (free hormones plus protein bound hormones). The structures of the three primary thyroid hormones of interest, thyroxine (T_4), triiodothyronine (T_3) and reverse triiodothyronine (rT_3), are shown in Figure 1.



Thyroxine (T_4) Triiodothyronine (T_3) Reverse Triiodothyronine (rT_3)

Figure 1. Structures of the thyroid hormones.

This application note describes the development of an analytical method for the sensitive and accurate determination of free and total thyroid hormones in serum and plasma using a mixed-mode SPE extraction procedure and UHPLC-MS/MS analysis. Chromatographic separation, including baseline resolution of triiodothyronine and reverse triiodothyronine, is conducted in 3 minutes using a Selectra® DA column. Ultra-low hormones and steroids serum (Golden West Biologicals, Inc.®) was used to generate accuracy and precision data. The method was evaluated at two concentrations (0.2 and 1 ng/mL) using procedures for free and total hormones. Recoveries of the three hormones ranged from 87 to 100% and reproducibility was ≤3.5%.

Procedure:

1. Sample preparation for free hormones:

a) To 1 mL of plasma add 1 mL of 0.1M HCl.
b) Vortex for 30 seconds.

Sample preparation for total hormones:

a) To 1 mL of plasma add 2 mL acetonitrile.
b) Vortex briefly and let stand for 5 min.
c) Centrifuge at ≥1500 rcf for 5 minute.
d) Transfer supernatant to a clean glass tube.
e) Add 2 mL of 0.1M HCl and vortex for 30 seconds.

2. SPE extraction

a) Condition SPE cartridge with:
   1. 1× 3 mL methanol.
   2. 1× 3 mL 0.1M HCl.
b) Apply the sample to the SPE cartridge (if required, use a low vacuum to draw the sample through at ≤3 mL/min).

3. Wash cartridge

a) 1× 3 mL 0.1M HCl.
b) 1× 3 mL methanol.
c) Dry cartridges for ~ 30 seconds under a high vacuum to remove excess methanol.

4. Elution

a) Elute with 1× 3 mL methanol containing 2% ammonium hydroxide ($MeOH:NH_4OH$, 98:2 v/v).
b) Evaporate the sample to dryness under a gentle stream of nitrogen.
c) Reconstitute in 100 µL of methanol and vortex for 1 minute.
d) Transfer sample to an autosampler vial containing a low volume insert.

LC-MS/MS Conditions:

HPLC Conditions	
Instrumentation	Thermo Scientific™ Dionex™ Ultimate™ 3000
HPLC column	UCT Selectra® DA, 100 × 2.1 mm, 1.8 µm (p/n: SLDA100ID21-18UM)
Guard column	UCT Selectra® DA, 10 × 2.0 mm, 1.8 µm (p/n: SLDAGDC20-18UM)
Guard column holder	p/n: SLGRDHLDR
Column temp.	50° C
Mobile phase A	water + 0.1% formic acid
Mobile phase B	acetonitrile + 0.1% formic acid
Flow rate	400 µL/min
Gradient	0 min, 30% B; 2.5-3.5 min, 100% B; 3.6-6 min, 30%B
Injection volume	10 µL
Autosampler temp.	10° C
Wash solvent	Methanol
Divert valve	Divert to waste at 0-2.2 and 3.5-6 min to reduce ion source contamination

MS Conditions	
Instrumentation	Thermo Scientific™ TSQ Vantage™ (QqQ)
Ionization mode	ESI^+
Spray voltage	4000 V
Vaporizer temperature	450° C
Capillary temperature	350° C
Sheath gas pressure	50 arbitrary units
Auxiliary gas pressure	50 arbitrary units
Ion sweep gas	0 arbitrary units
Declustering potential	0 V
Q1 and Q3 peak width	0.7 Da
Collision gas	Argon
Collision gas pressure	2.3 mTorr
Acquisition method	EZ method (scheduled SRM)
Cycle time	0.4 sec
Software	Xcalibur™ version 2.2

SRM Transitions							
Analyte	**t_R (min)**	**Precursor ion**	**Product ion 1**	**CE 1 (V)**	**Product ion 2**	**CE 2 (V)**	**S-lens (V)**
Triiodothyronine (T3)	2.61	651.7	605.7	15	197.0	61	115
Reverse triiodothyronine (rT3)	2.77	651.7	605.8	15	507.8	15	112
Thyroxine (T4)	2.94	777.6	731.7	17	323.8	39	130
Thyronine-13C6	2.94	783.6	737.7	17	329.9	48	115

Results:

	0.2 ng/mL		1 ng/mL	
Analyte	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**
Thyroxine	94.4	2.5	95.2	1.4
Triiodothyronine	99.5	3.5	96.5	2.3
Reverse Triiodothyronine	99.8	2.3	91.5	1.8

Table 1. Accuracy and precision data in plasma using free hormone procedure (n= 6 each).

	0.2 ng/mL		1 ng/mL	
Analyte	**Recovery (%)**	**RSD (%)**	**Recovery (%)**	**RSD (%)**
Thyroxine	94.7	1.7	92.6	1.5
Triiodothyronine	96.5	0.8	91.1	3.5
Reverse Triiodothyronine	95.2	2.0	87.1	2.2

Table 2. Accuracy and precision data in plasma using total hormone procedure (n= 6 each).

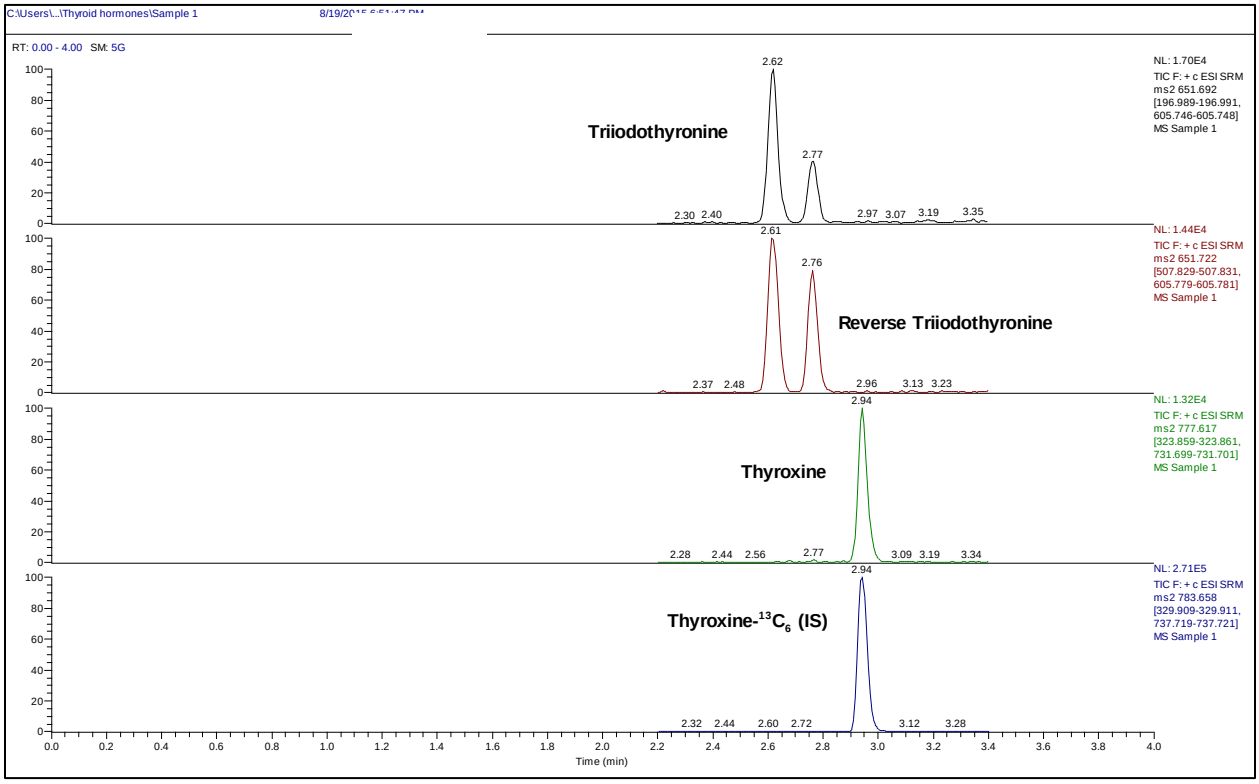


Figure 1. Chromatogram of an extracted plasma sample (0.2 ng/mL).

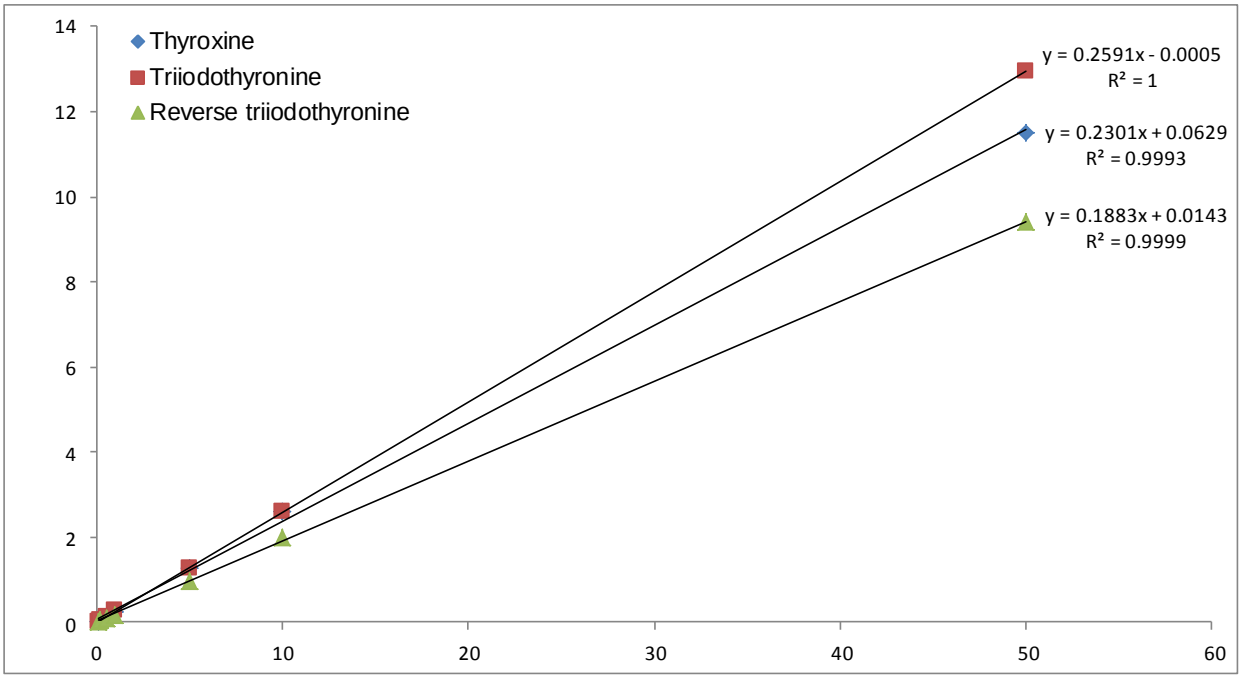


Figure 2. Example of extracted matrix-matched calibration curves (0.1, 0.2, 0.5, 1, 5, 10, and 50 ng/mL).

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