Screening of Fentanyl Analogs in Whole Blood using LC-QTOF Analysis

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Sam Houston State University

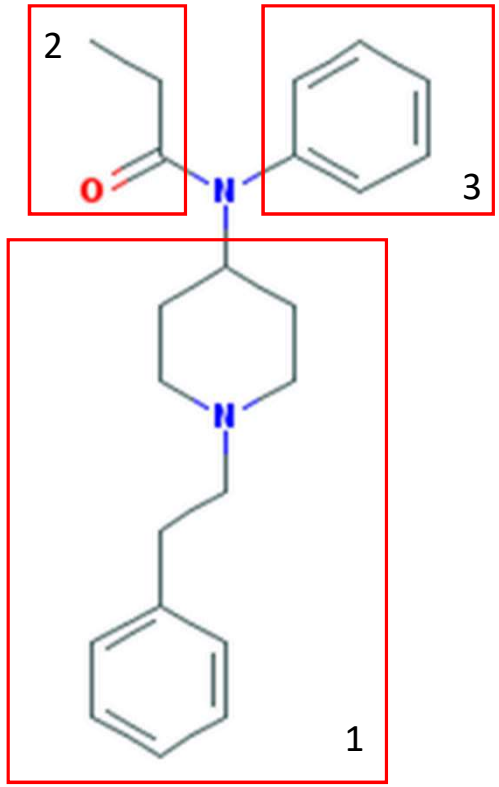
Disclosure

FSF Lucas Research Grant Recipient

Fentanyl Analogs

Fentanyl Analogs

- Introduction into illicit drug market as cutting agents for heroin
- Up to 10,000x potency of morphine
  1. phenylethyl piperidine
  2. propanamide
  3. phenyl groups
- Temporarily placed under Schedule I
- Increase in opioid related deaths



Previous Research

- Gergov et al. (2009)
  - Simultaneous detection and quantification of 25 opioids (9 fentalogs) using LC-MS/MS
- Verplaetse et al. (2010)
  - Detection of fentanyl and metabolite using UHPLC-MS/MS
  - Use of SPE
- Viaene et al. (2016)
  - Quantification of 16 opioids in plasma using LC-QQQ & LC-QTOF
- Shoff et al. (2017)
  - Screening method for 44 opioids (13 fentalogs) using LC- Ion trap MS

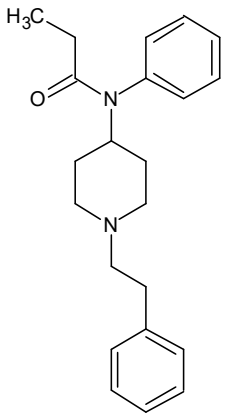
Need for…

(1) advanced screening methods to distinguish similar structure fentanyl analogs using high resolution mass spectrometry

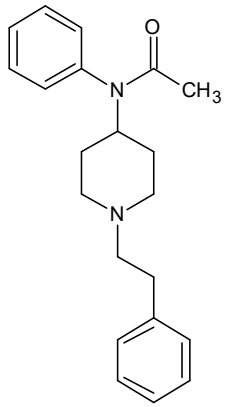
(2) an optimized solid phase extraction protocol

Objectives

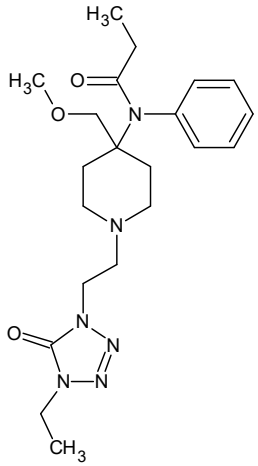
- Develop a method for the comprehensive screening of 14 fentalogs using LC-QTOF
- Optimize an SPE extraction for fentalogs from whole blood
- Validate the method according to SWGTOX guidelines for screening methods
- Apply validated method to authentic case samples previously testing positive for fentanyl or analogs



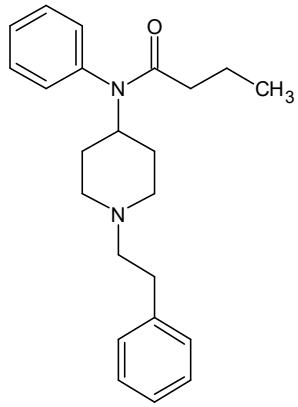
Fentanyl
336.22016



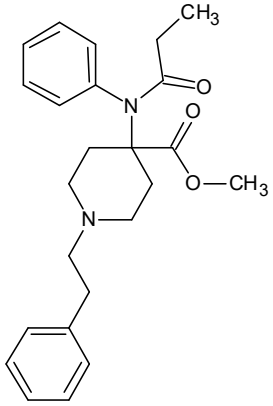
Acetyl Fentanyl
322.20451



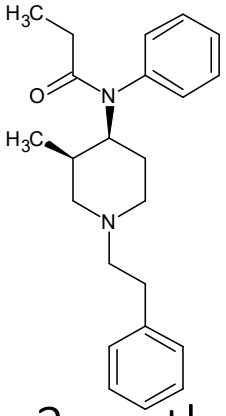
Alfentanil
416.25359



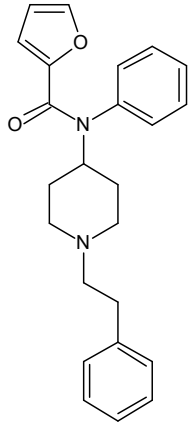
Butyryl fentanyl
350.23581



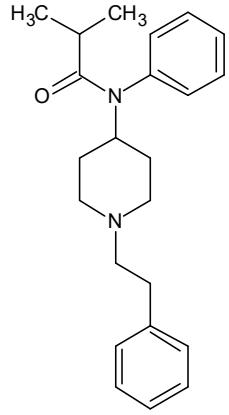
Carfentanil
394.22564



Cis-3-methyl fentanyl
350.23581

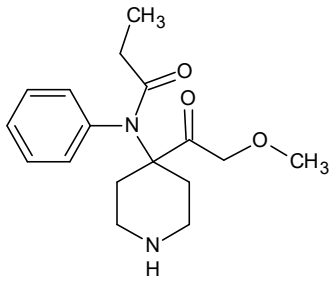


Furanyl fentanyl
374.19943



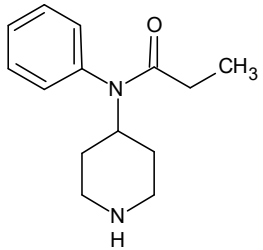
Isobutyryl fentanyl
350.23581

*Metabolite

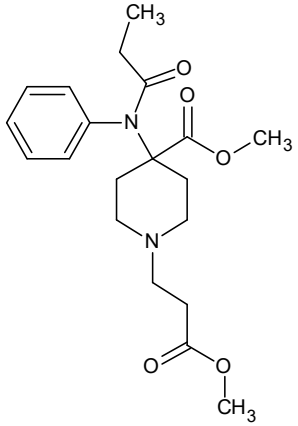


Norcarfentanil
290.16304

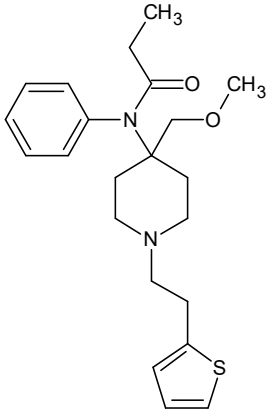
*Metabolite



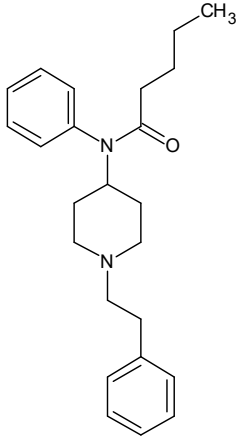
Norfentanyl
232.15756



Remifentanil
376.19982

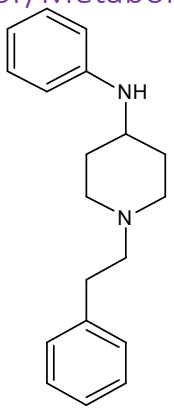


Sufentanil
386.20280



Valeryl fentanyl
364.25146

*Precursor/Metabolite



4-ANPP
280.19395

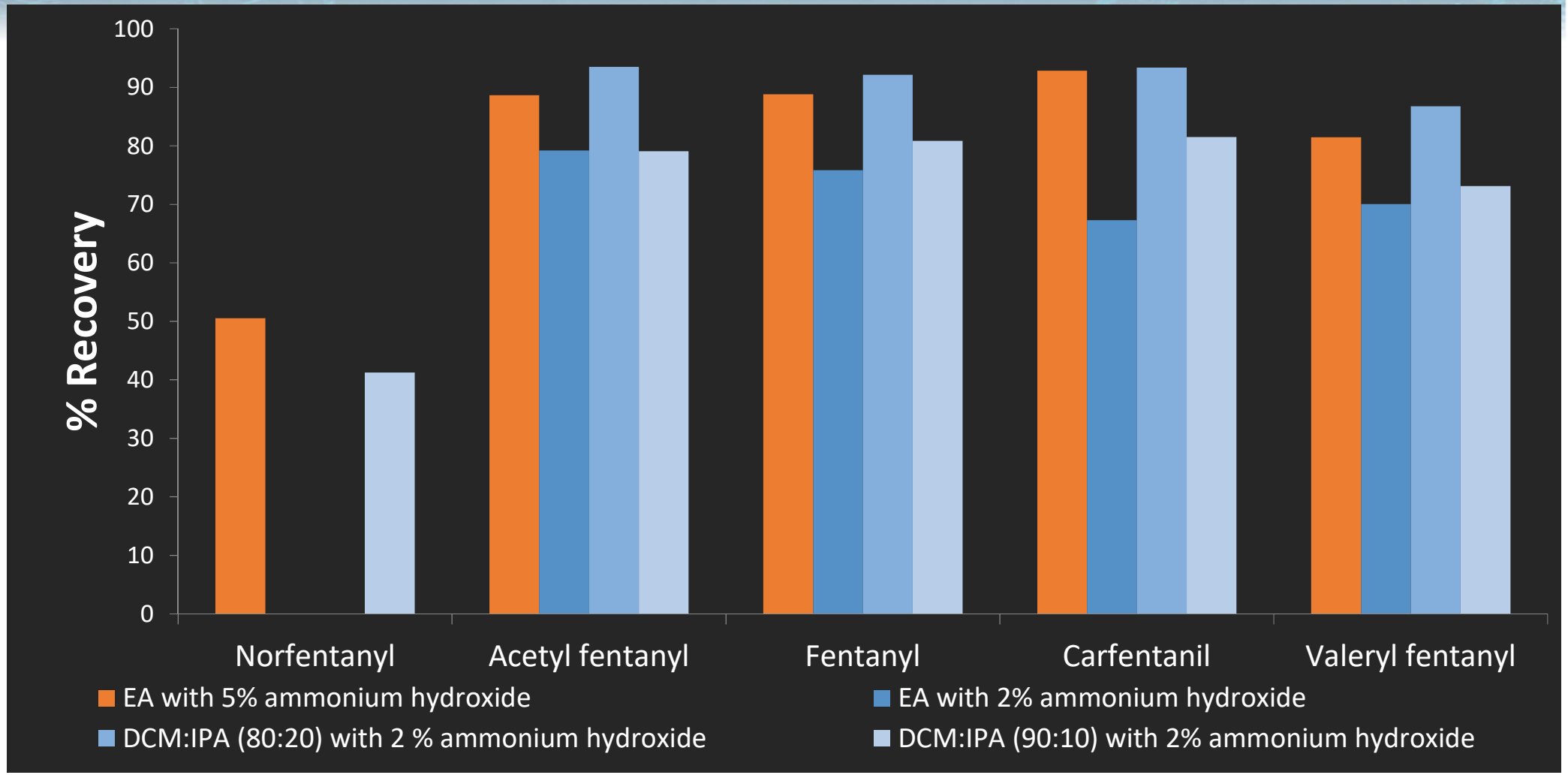
LC-QTOF Conditions

Agilent Technologies 1290 Infinity LC	
Column	Poroshell 120 EC-C18 (2.1 x 100mm x 2.7 µm)
Mobile Phase	A: 0.1% Formic Acid in water B: 0.1% Formic Acid in ACN
Flow Rate	0.400 mL/min
Agilent Technologies 6530 Accurate Mass time-of-flight MS	
Ionization Type	Electrospray ionization (ESI)-positive
Collision Energies	TOF Mode: 0 eV All Ions: 0, 10, 20 eV
Injection Volume	5 µL
Mass Range	100-1000 m/z

Method Development: Extraction

- Blood volume:
  - 0.25 mL vs. 0.50 mL
- Reconstitution volume:
  - 50, 100, 200 µL
- Injection volume:
  - 2 µL vs. 5 µL
- Assessment: analyte response & peak shape

Method Development: Extraction



Extraction procedure

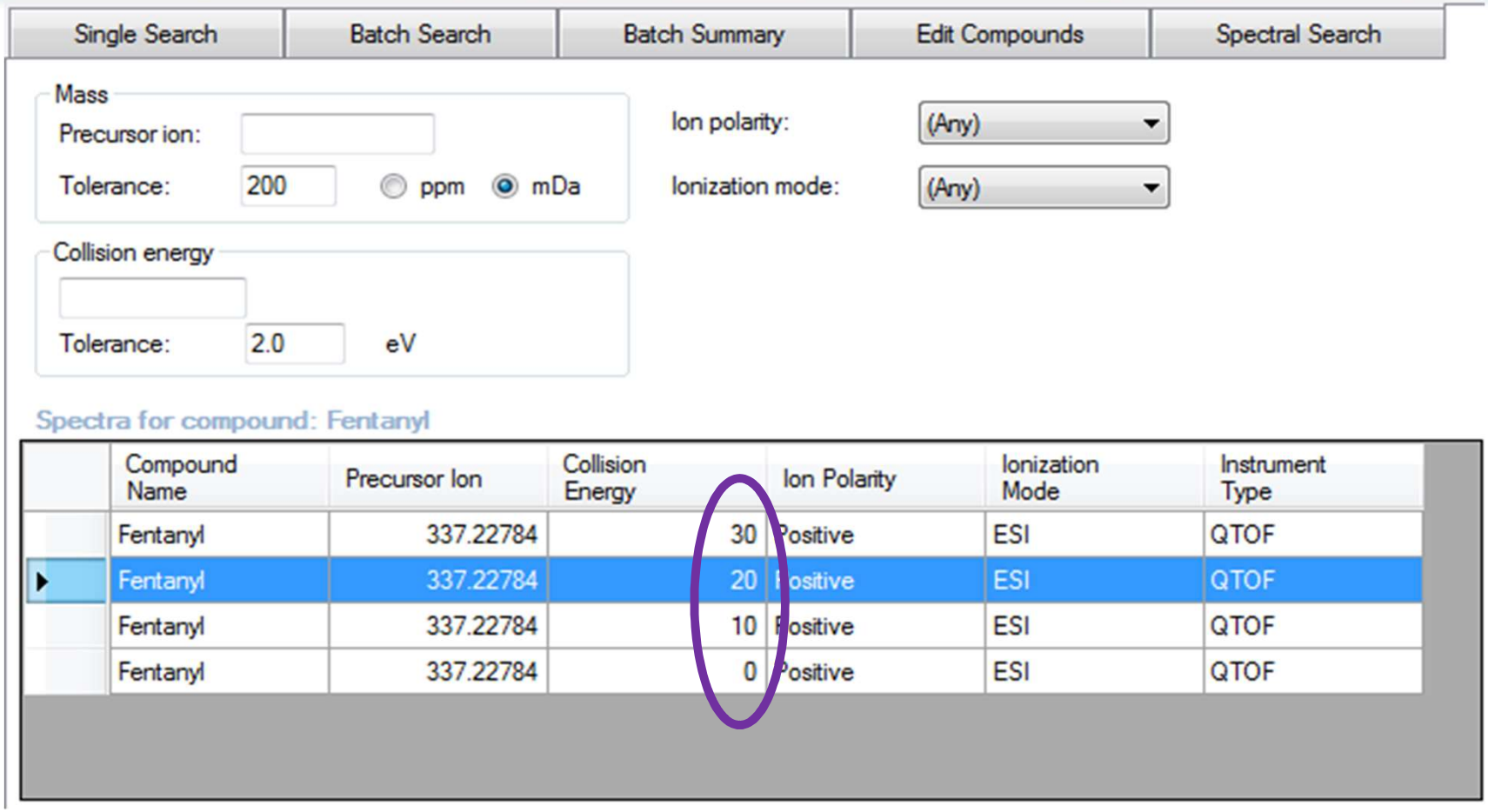
0.25 mL	• **250 µL blood** + ISTD **(12.5 µL)** & standard mixes + 2.5 mL phosphate buffer (pH 6)
	• **Centrifuge at 2500 rpm for 5 minutes**
Add	• Sample to column (CEREX® Clin II)
1 mL	• DI water wash
1 mL	• 1M acetic acid wash
Dry	• Under vacuum for 5 mins
1 mL	• Hexane wash
1 mL	• Ethyl Acetate wash
1 mL	• Methanol wash
1 mL	• DCM wash
1 mL	• Elute basic drugs **5% ammonium hydroxide in Ethyl Acetate**
Dry	• Dry down; **Recon 50 µL of 50:50 MP**

Personal Compound Database and Library (PCDL)

Single Search Results: 17 hits

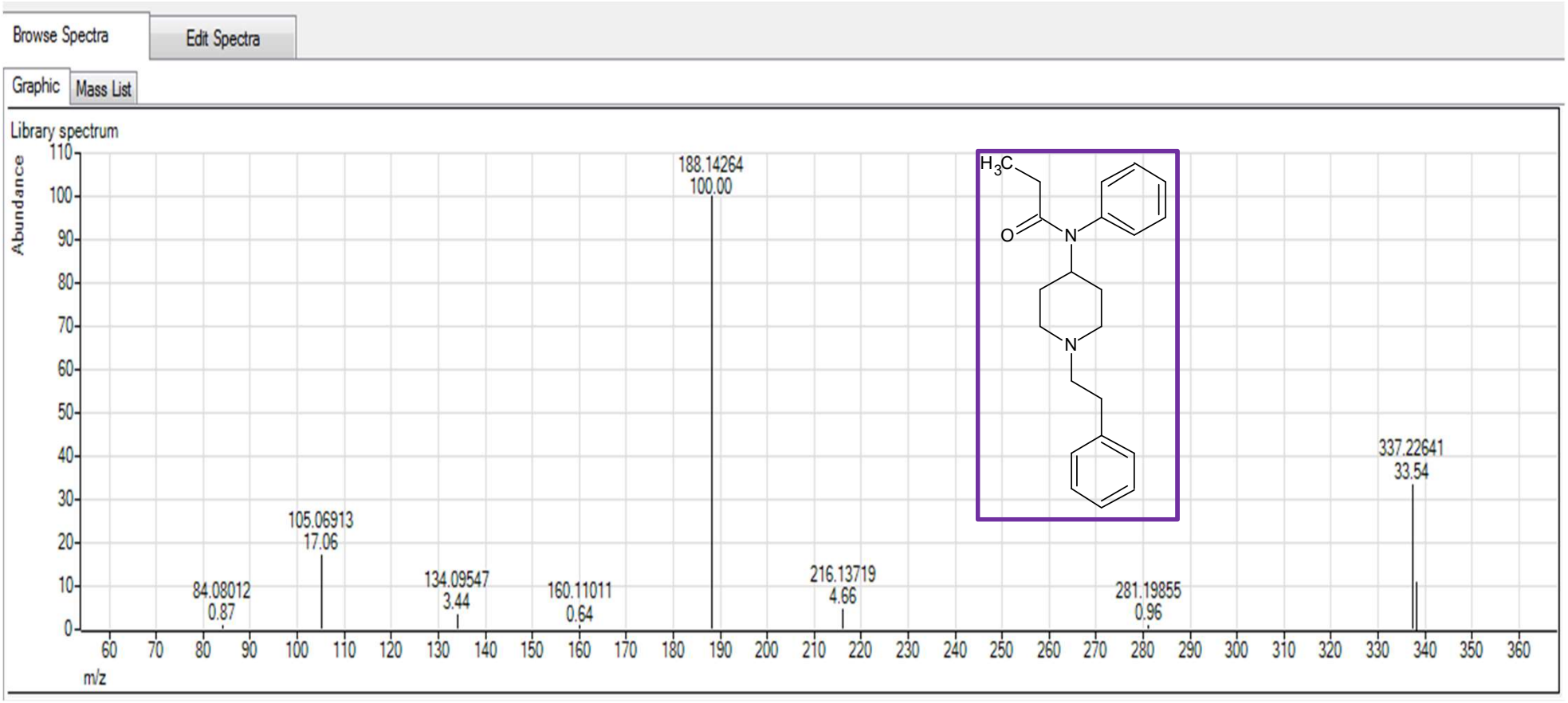
Compound Name	Formula	Mass	Anion	Cation	RT (min)	CAS	ChemSpider	IUPAC Name	Num Spectra
D5- Norfentanyl	C14H15D...	237.18895			2.385				4
Norfentanyl	C14H20N...	232.15756			2.402				4
Norcarfentanil	C16H22N...	290.16304			2.728				4
Remifentanil	C20H28N...	376.19982			3.138				4
Acetyl fentanyl	C21H26N...	322.20451			3.515				4
Alfentanil	C21H32N...	416.25359			4.063				4
4-ANPP	C19H24N2	280.19395			4.080				4
D5-Fentanyl	C22H23D...	341.25155			4.165				4
Fentanyl	C22H28...	336.22016			4.182				4
Furanyl fentanyl	C24H26N...	374.19943			4.405				4
D5-Carfentanil	C24H25D...	399.25703			4.730				4
cis-methyl fentanyl	C23H30N...	350.23581			4.747				4
Carfentanil	C24H30N...	394.22564			4.747				4
Isobutyryl fentanyl	C23H30N...	350.23581			4.850				4
Butyrl fentanyl	C23H30N...	350.23581			4.850				4
Sufentanil	C22H30N...	386.20280			5.227				4
Valeryl fentanyl	C24H32N...	364.25146			5.620				4

Personal Compound Database and Library (PCDL)

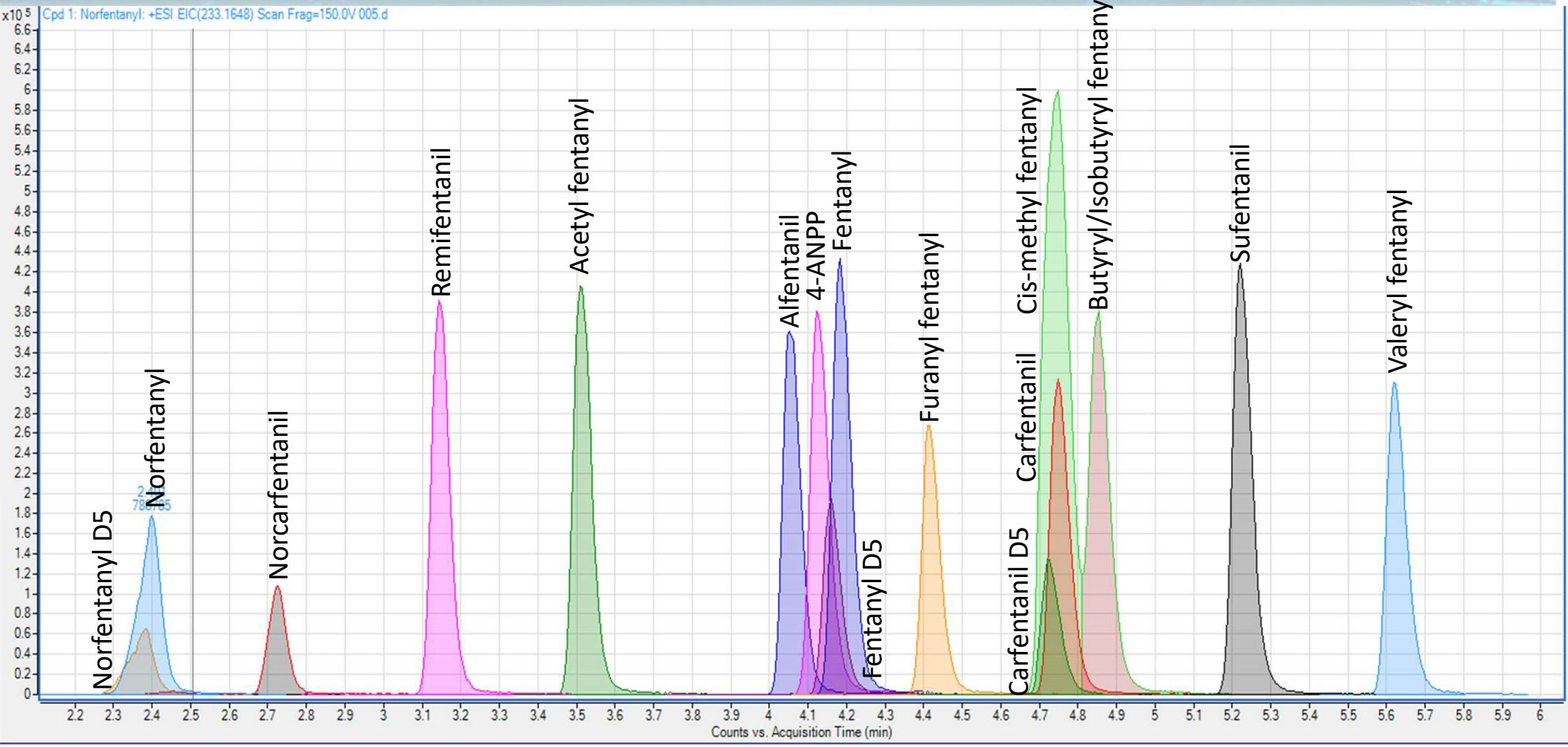


Personal Compound Database and Library (PCDL)

- Fentanyl mass spectrum produced from 20 eV



Chromatography



Data Acquisition Modes

Time of Flight

- Presumptive
- Allows all ions to pass to the detector- no collision energies applied
- Precursor Ion determination
- **"Qualify" based on:**
  - **Formula matching parameters:**
    - ± 0.35 min from RT
    - ± 15 ppm Mass
    - Abs peak area >10,000 counts

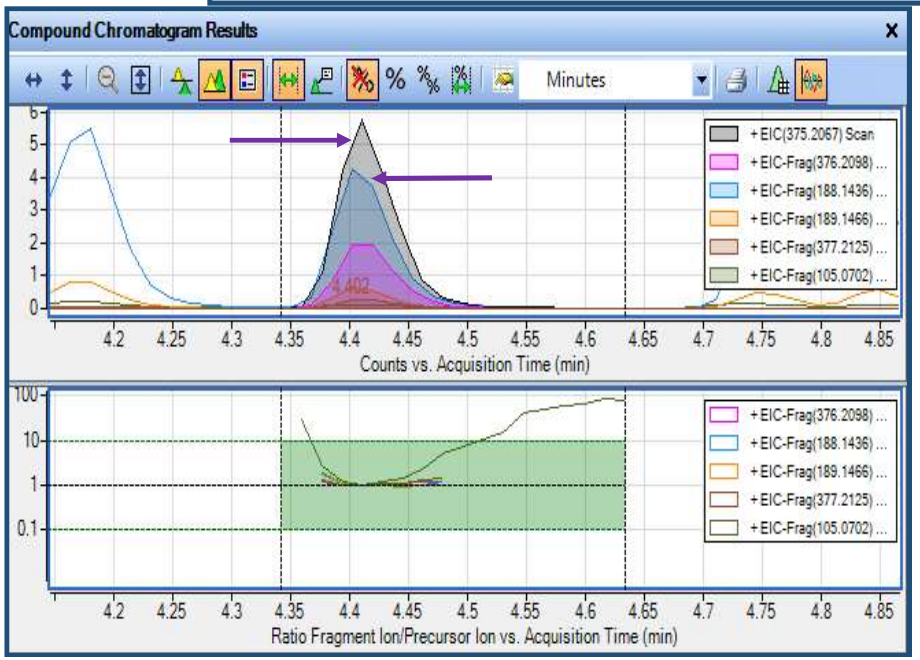
All Ions Fragmentation

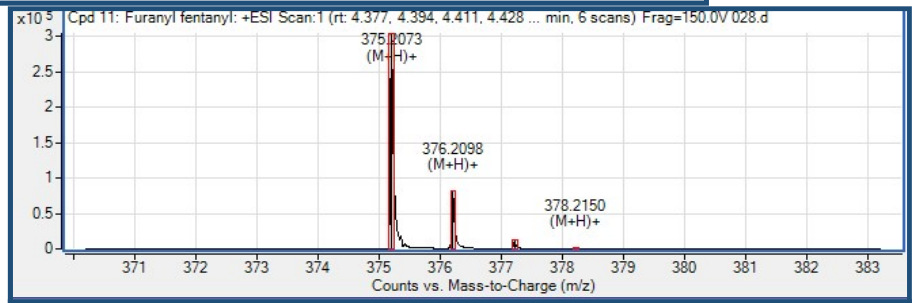
- Confirmatory
- Three collision energies applied
- Software pairs fragment ions to precursor ions
- **"Qualify" based on:**
  - **Formula matching parameters:**
    - Abs peak area >5,000
  - **Ion confirmation parameters:**
    - Co-elution Score >60
    - S/N > 3
    - Minimum number of ions: 2
    - ± 0.15 min from RT

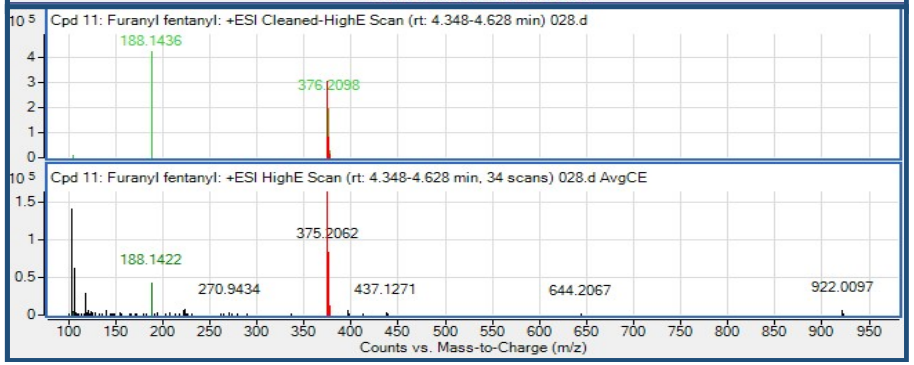
All Ions Fragmentation

Best	Name	Formula	m/z	Mass	Mass (Tgt)	Diff (ppm)	Score (Tgt)	RT	RT
◉	Furanyl fentanyl	24 H26 N2 O2	375.2073	374.1998	374.1994	-1.11	98.98	4.411	

m/z	CE	FV	Coelution Score	Flags(FIs)	Height	SNR	RT	RT Diff	Compound Name
376.2098	10		99.4	Qualified	194067.4	∞	4.402	0.009	Furanyl fentanyl
188.1436	20		98.5	Qualified	24505.7	672.5	4.402	0.009	Furanyl fentanyl
189.1466	20		98.6	Qualified	65535.2	205.5	4.402	0.009	Furanyl fentanyl
377.2125	10		99.3	Qualified	28385.7	60.1	4.402	0.009	Furanyl fentanyl
105.0702	20		94.3	Qualified	11170.6	6.9	4.402	0.009	Furanyl fentanyl







Method Validation: Limit of Detection

- LOD determined based on qualification parameters in MassHunter
  - Includes S/N >3
- TOF LODs: 0.1-0.5 ng/mL
- AIF LODs: 0.1-1.0ng/mL
- Overall, TOF mode has lower LOD than AIF mode

Analyte	TOF Mode	AIF Mode
Norfentanyl	0.5	>1.0
Norcarfentanil	1.0	1.0
Remifentanil	0.25	1.0
Acetyl Fentanyl	0.25	1.0
Alfentanil	0.1	0.5
4-ANPP	0.1	>1.0
Fentanyl	0.1	1.0
Furanyl fentanyl	0.25	0.5
Carfentanil	0.1	0.25
Cis-methyl fentanyl	0.25	0.1
Butyryl/Isobutyryl fentanyl	0.25	0.1
Sufentanil	0.1	0.25
Valeryl fentanyl	0.1	0.1

Method Validation

- Method validated according to SWGTOX guidelines for a qualitative analysis

Recovery (%)	Matrix Effects (%)	Interferences/ Carryover	24 hr Autosampler Stability (% Bias)	24 hr Refrigeration Stability (% Bias)
57.2-103.0	-35.2-14.4 Without nor-analytes: 1.0-14.4	None	-1.16-3.69	-10.46-18.10

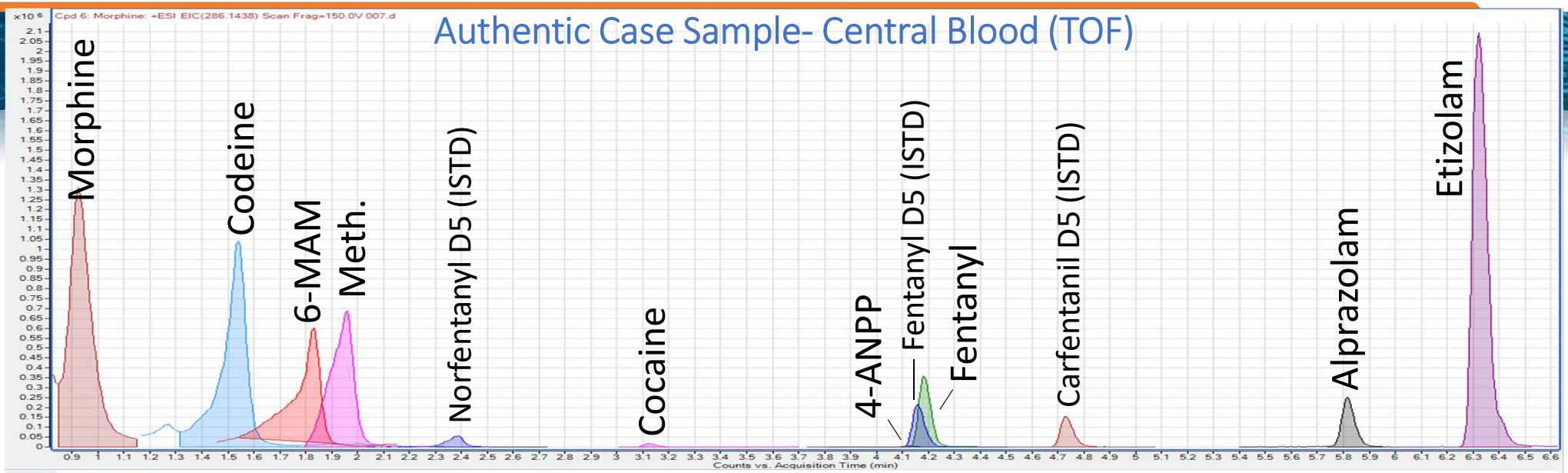
Expansion of PCDL

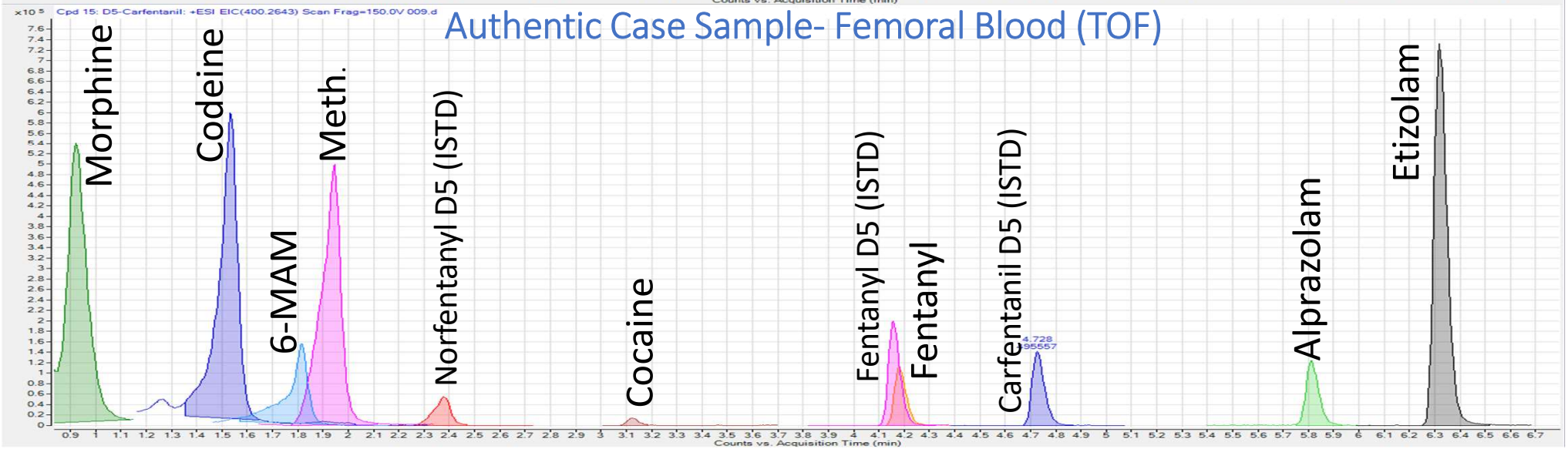
- Additional fentanyl analogs
  - 138 commercially available compounds
  - Unknown estimation based on mass

- Additional drugs of abuse
  - 93 compounds
  - Unknown determination based on mass, RT, and spectral information

Postmortem Blood Samples (n=32)

Fentanyl Analog	Number of Samples Detected		Other Drugs of Abuse	Number of Samples Detected
Furanyl fentanyl	16		U-47700	15
4-ANPP	16		Morphine	10
Cis-methyl fentanyl	4		Alprazolam	8
Fentanyl	4		Cocaine	7
Norfentanyl	2		Etizolam	5
Valeryl fentanyl	1		Oxycodone	5
			Mitragynine	4
			Cyclobenzaprine	4
			6-MAM	4
			Hydrocodone	2
			Codeine	2
			Methamphetamine	2
			Buprenorphine	1


Authentic Case Sample- Central Blood (TOF)
Cpd 6: Morphine: +ESI EIC(286.1438) Scan Frag=150.0V 007.d
Morphine
Codeine
6-MAM
Meth.
Norfentanyl D5 (ISTD)
Cocaine
4-ANPP
Fentanyl D5 (ISTD)
Fentanyl
Carfentanil D5 (ISTD)
Alprazolam
Etizolam
Counts vs. Acquisition Time (min)


Authentic Case Sample- Femoral Blood (TOF)
Cpd 15: D5-Carfentanil: +ESI EIC(400.2643) Scan Frag=150.0V 009.d
Morphine
Codeine
6-MAM
Meth.
Norfentanyl D5 (ISTD)
Cocaine
Fentanyl D5 (ISTD)
Fentanyl
Carfentanil D5 (ISTD)
4.728
Alprazolam
Etizolam
Counts vs. Acquisition Time (min)

Results

- Fentanyl analogs not detected by the submitting laboratory:
  - Valeryl fentanyl
  - Norfentanyl (presumptive)
  - 4-ANPP

- Some analytes not detected by present method
  - Presumptive detection based on mass in Expanded Fentalog PCDL
  - Addition of emerging analogs to PCDL

Conclusions

- This research represents a valid method for:
  - Extracting fentanyl analogs from whole blood using SPE
  - Screening for fentanyl analogs using LC-QTOF
  - Application to authentic postmortem case samples

- PCDL is beneficial for screening toxicological samples

Acknowledgments

- Sam Houston State University Department of Forensic Science
- Research Advisor: Dr. Madeleine Swortwood
- Research Team: Christina & Michael
- Forensic Science Foundation- Lucas Grant Award
- Colleagues assisting with safety, supervision, and instrumentation

Thank you!

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