LC-MS/MS Approaches for Identifying Emerging NPS

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Disclaimer

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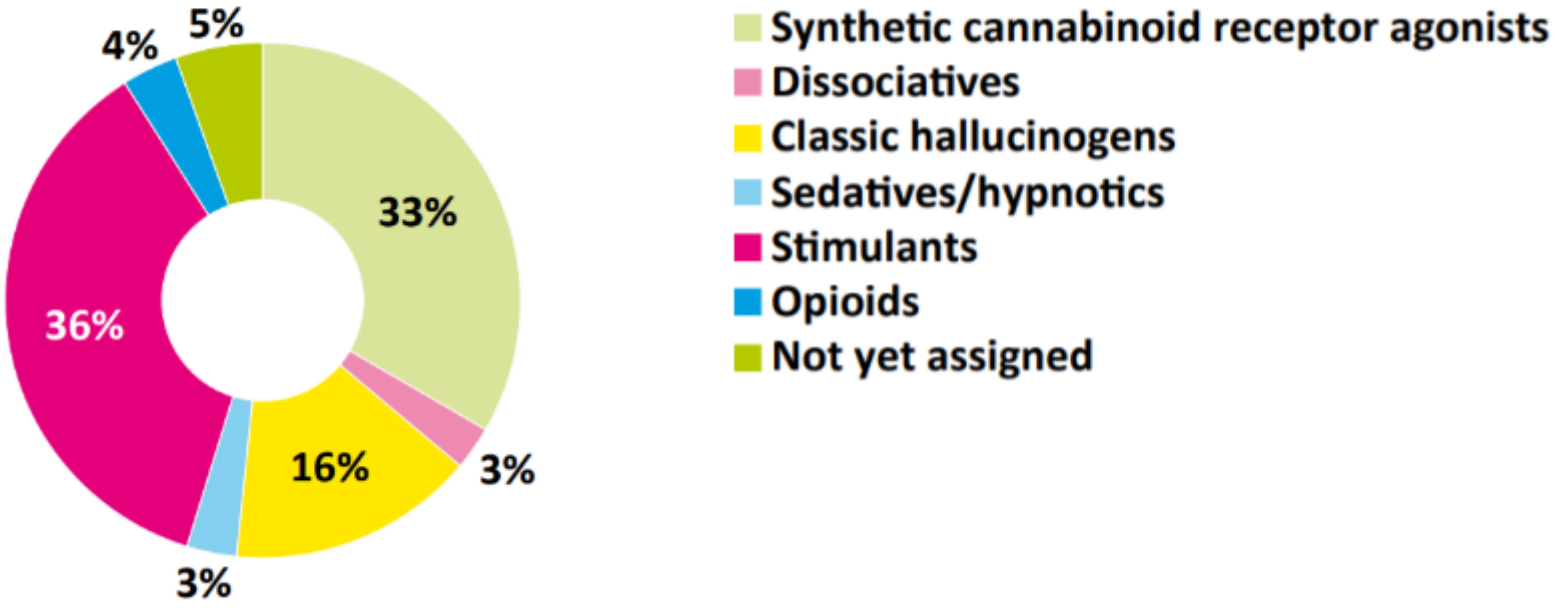
Emerging NPS

- Over 23 tons of NPS were seized in 2015
- From 2009-2016, **739** different NPS reported
  - Only 60 seemed to “disappear”

FIG. 10 | Proportion of new psychoactive substances, by pharmacological effect, December 2016

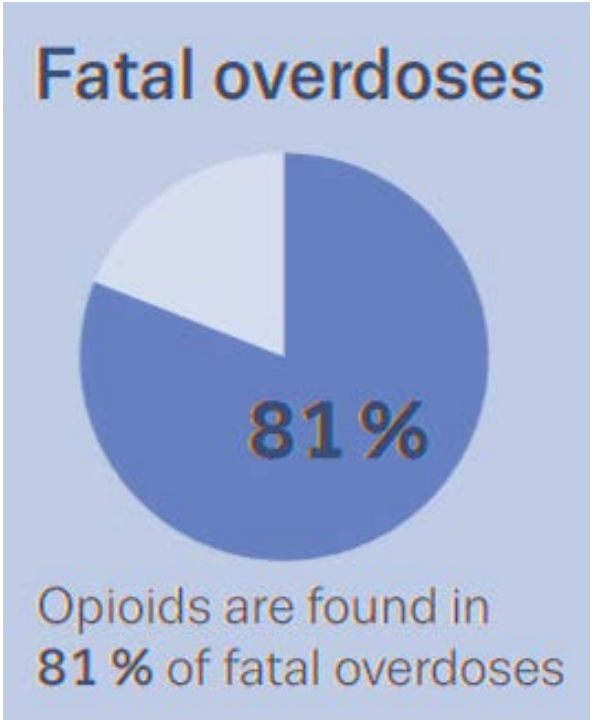


UNODC World Drug Report 2017

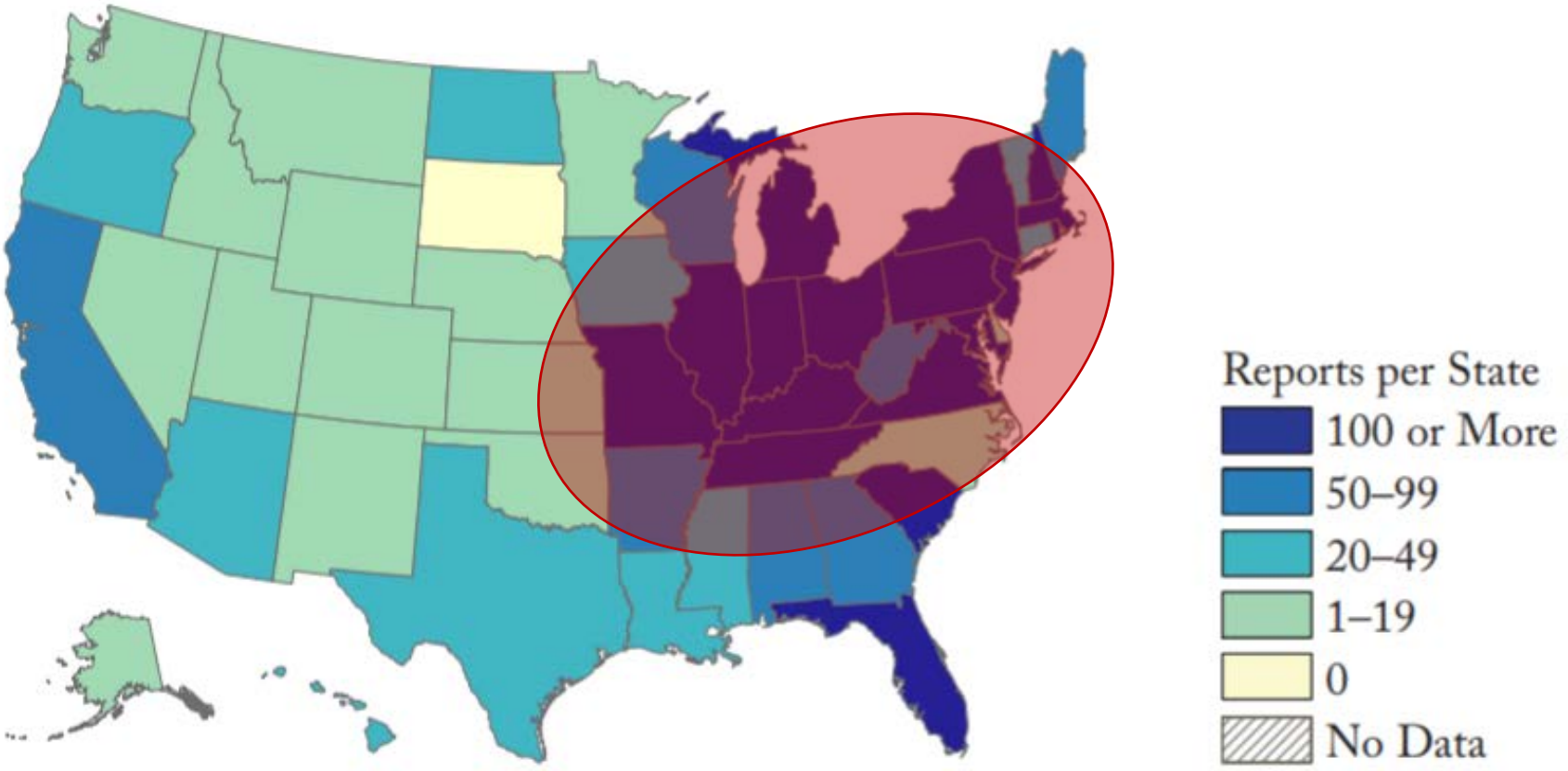
For Forensic Use

Novel Opioids in Europe

- 25 new opioids since 2009
  - 18 fentanyl-related compounds
- Over 60% of opioid seizures in 2015 were fentalogs
- 2 L of synthetic opioids seized in 2015
- New nasal sprays
- Small role in European drug market



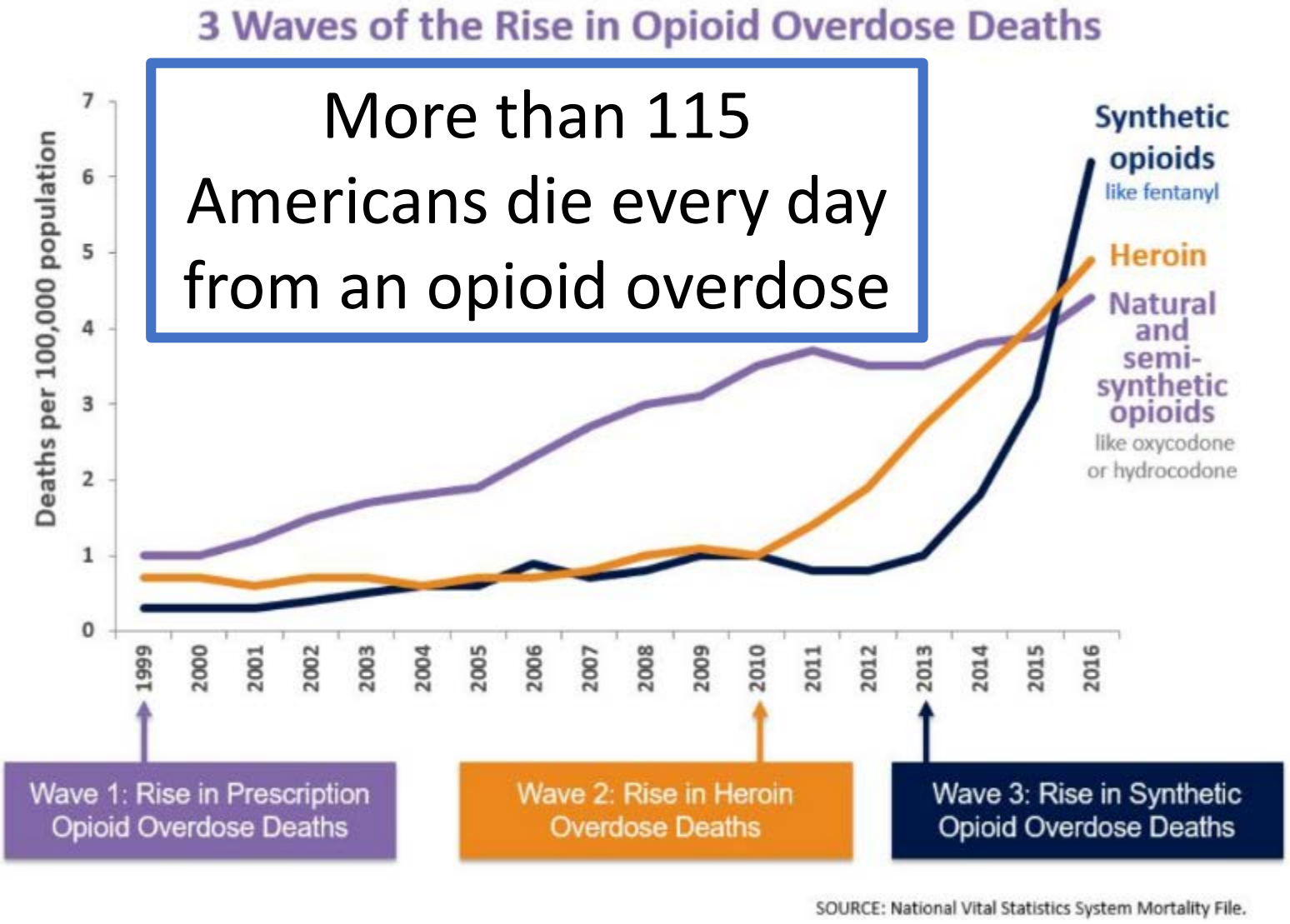
Fentanyls in the US



NFLIS Brief: Fentanyl, 2001-2015

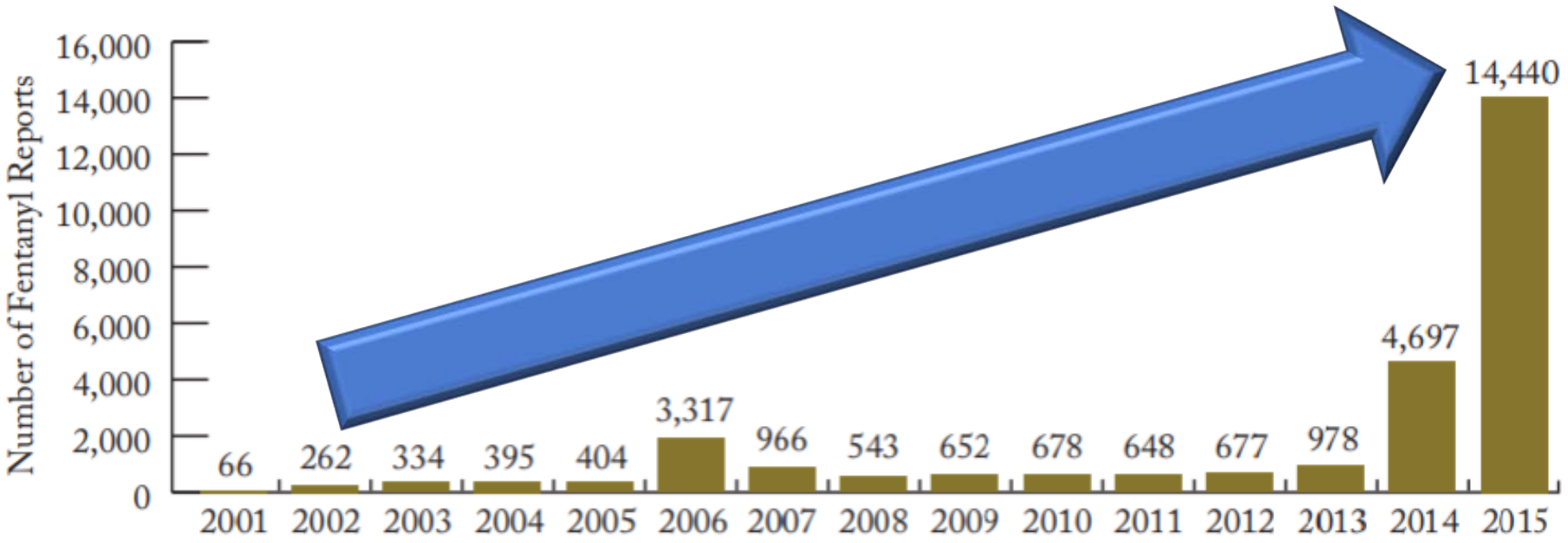
For Forensic Use

Opioids in the US



Fentanyl in the US

- Fentanyl-related seizures from 2001 to 2015



Fentanyls in the US

- Fentanyl-related seizures soared from 2015 to 2016

Table 1 National Annual Estimates of Fentanyl and Fentanyl-Related Substances Reported in NFLIS, 2015–2016[1]

Fentanyl and Fentanyl-Related Substances	2015		2016		Total	
	Number	Percent	Number	Percent	Number	Percent
Fentanyl	14,440	84.59%	34,199	84.99%	48,639	84.87%
Acetyl fentanyl	2,412	14.13%	1,669	4.15%	4,080	7.12%
Furanyl fentanyl	0	0.00%	2,273	5.65%	2,273	3.97%
Carfentanil	0	0.00%	1,251	3.11%	1,251	2.18%
3-Methylfentanyl	1	0.01%	427	1.06%	428	0.75%
Butyryl fentanyl	205	1.20%	93	0.23%	298	0.52%
Fluoroisobutyryl fentanyl	0	0.00%	82	0.20%	82	0.14%
p-Fluoroisobutyryl fentanyl	0	0.00%	76	0.19%	76	0.13%
p-Fluorobutyryl fentanyl	2	0.01%	72	0.18%	74	0.13%
Valeryl fentanyl	0	0.00%	52	0.13%	52	0.09%
Acryl fentanyl	0	0.00%	26	0.06%	26	0.04%
p-Fluorofentanyl	8	0.05%	5	0.01%	13	0.02%
ANPP	0	0.00%	8	0.02%	8	0.01%
o-Fluorofentanyl	0	0.00%	3	0.01%	3	0.01%
Beta-hydroxythiofentanyl	3	0.02%	0	0.00%	3	0.01%
Acetyl-alpha-methylfentanyl	1	0.01%	0	0.00%	1	0.00%
Alpha-methylfentanyl	0	0.00%	1	0.00%	1	0.00%
4-Methoxy-butyryl fentanyl	0	0.00%	*	*	*	*
Total[2]	17,071	100.00%	40,236	100.00%	57,308	100.00%

NFLIS Brief: Fentanyl, 2015-2016

For Forensic Use

Opioids have reached national crisis level…

But what about their impact on forensic toxicologists?

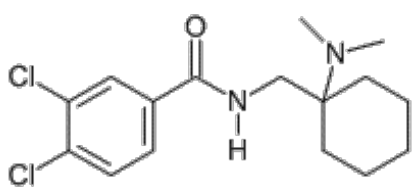
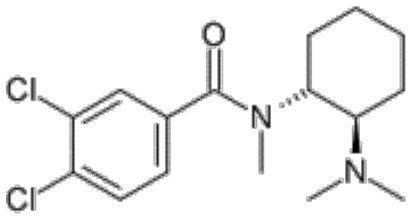
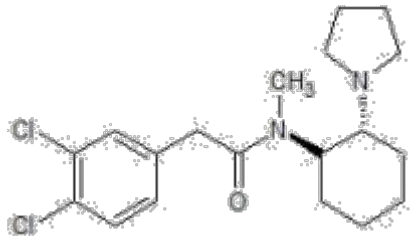
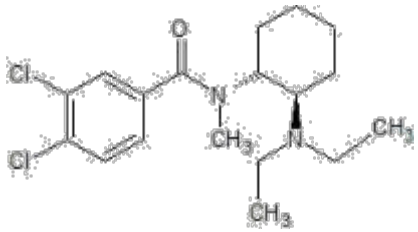
Goals of Forensic Toxicologists

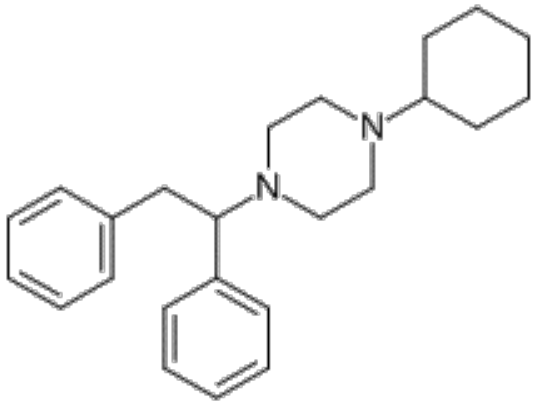
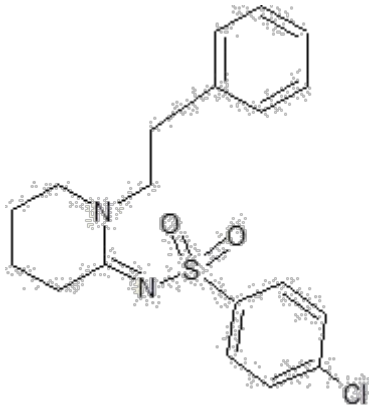
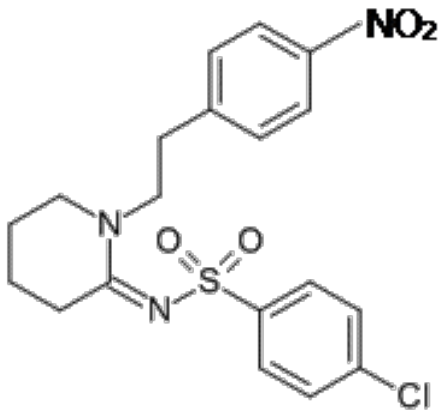
- Keep methods up to date, without sacrificing:
  - Run time
  - Sample volumes
  - Ease of sample preparation
  - Analytical sensitivity
  - Analytical scope

LC-MS/MS Approaches in Our Lab…

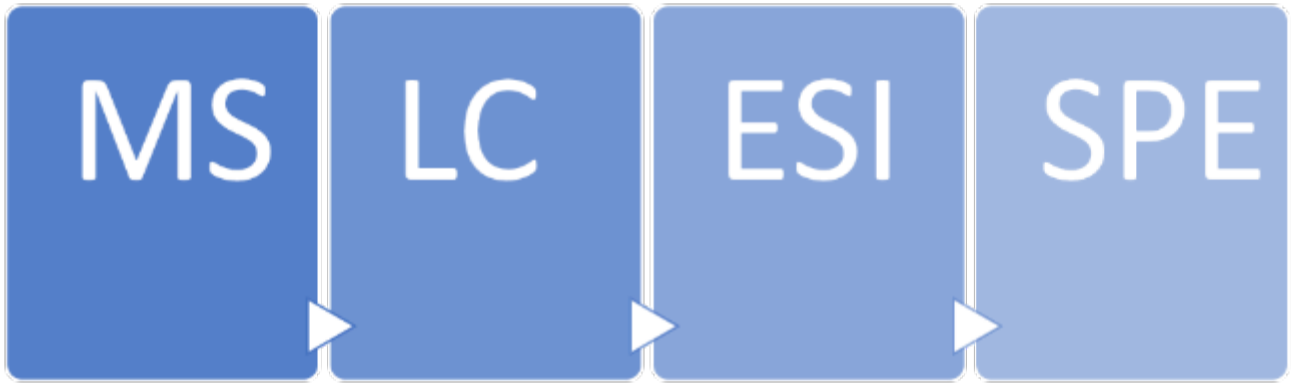
- Novel synthetic opioids (NSO) by QQQ
  - U-series compounds
  - W-series compounds
  - MT-45
  - Metabolites

NSO by QQQ

AH-7921	U-47700	U-50488	U-49900
			

MT-45	W-15	W-18
		

NSO Method Development

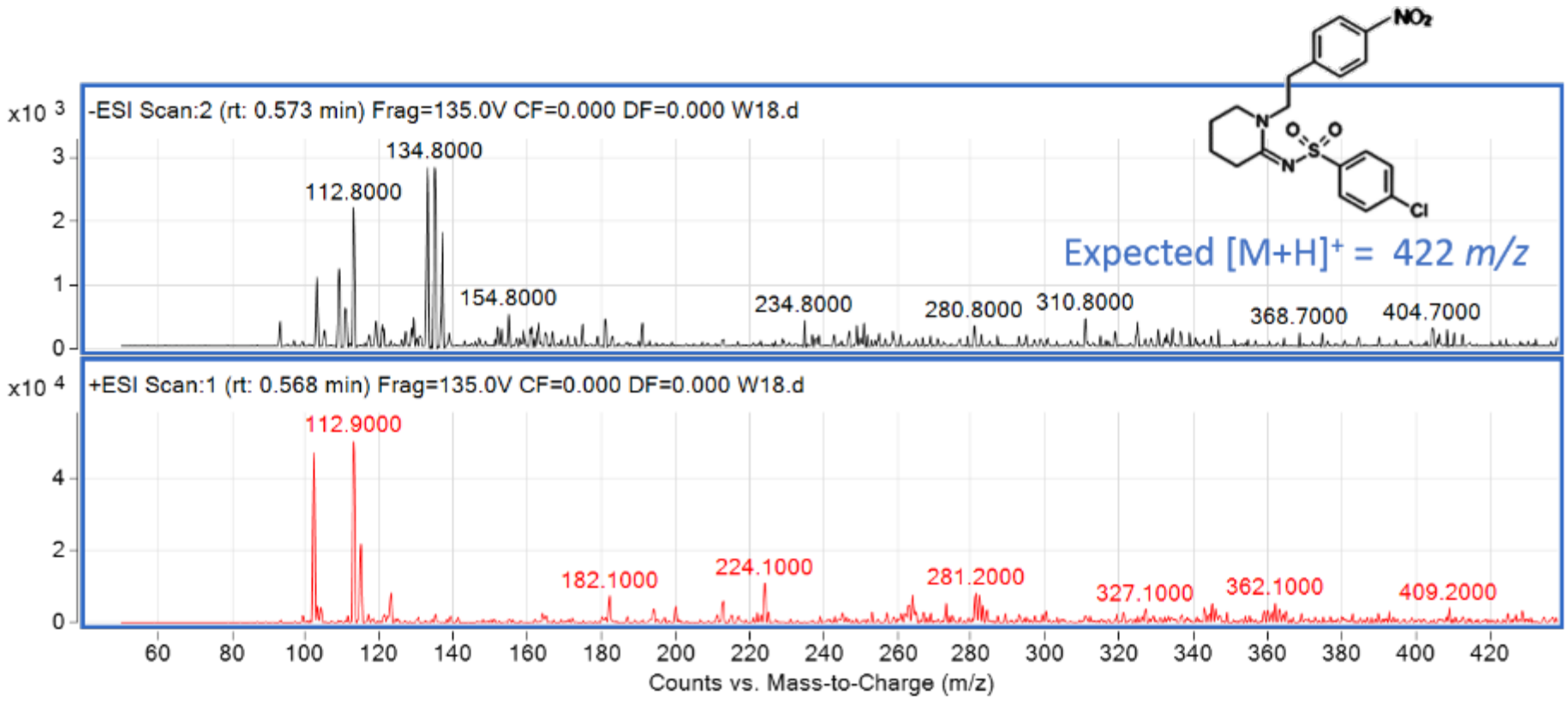


NSO Method Development: MS

- Typical Workflow:
  - Obtain reference standards
  - Dilute in 50:50 Mobile Phase
    - 0.1% Formic Acid in H_2O/Acetonitrile
  - Inject without a column into MS using MS2 Scan
    - Default source settings
    - Positive ESI (or both if not sure)
  - Confirm precursor ion
  - Proceed to Optimizer for MRM
    - Collect 4-6 transitions and narrow down later!

But what happens when you cannot see your reference standard??

NSO Optimization: MS



Expected $[M+H]^+$ = 422 *m/z*

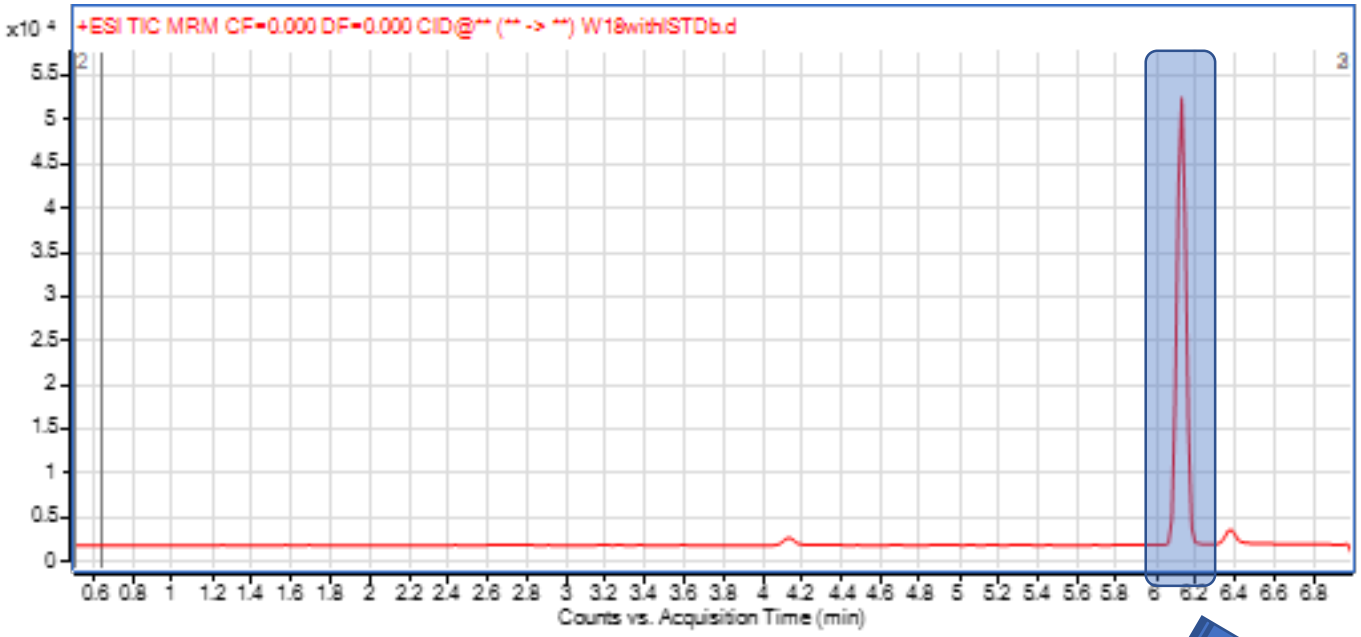
MS | LC | ESI | SPE

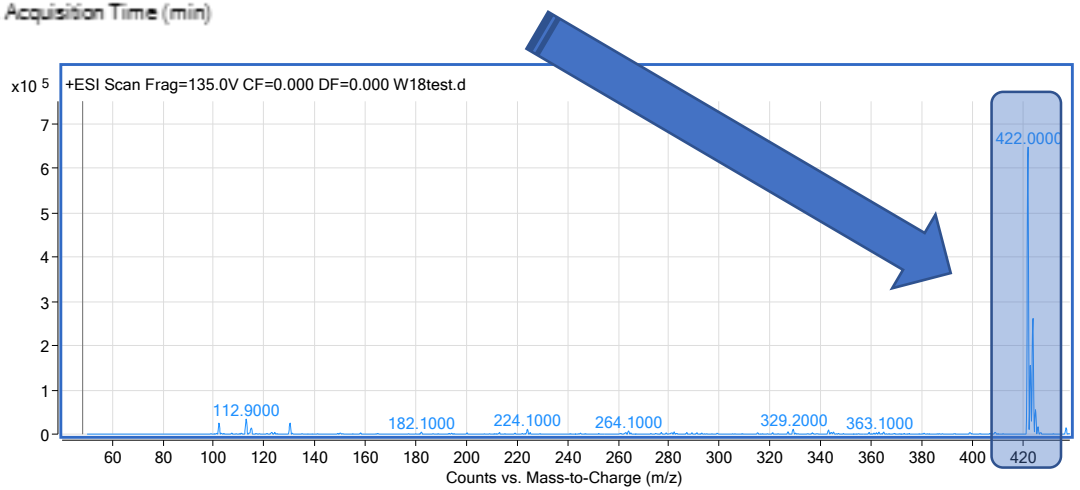
For Forensic Use

NSO Optimization: MS

- Mobile phase choice
  - Necessary for ionization
  - Can reduce or produce adducts
- May be subject to in-source fragmentation
  - Reduce Fragmentor Voltage

NSO Optimization: MS





MS LC ESI SPE

For Forensic Use

NSO Optimization: MS

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	Fragmentor (V)	RT (min)	Δ RT (min)	Internal Standard
U-47700	329.2	172.9	37	117			U-47700-d_6
	329.2	144.9	61	117			
AH-7921	329.2	144.9	57	107			700-d_6
	329.2	46.2	21	107			
U-49900	357.1	172.9	37	120			700-d_6
	357						
U-50488	369.1	158.9	49	115	3.665	1	U-47700-d_6
	369.1	112.1	37	115			
MT-45	349.5	181.0	20	112	4.511	1	MT-45-d_{11}
	349.5	77.1	93	112			
W-18	422.1	111.0	50	153	6.392	1	W-18-d_4
	422.1	75.1	50	153			
W-15	377.1	111.0	41	145	6.640	1	W-18-d_4
	377.1	75.1	97	145			
U-47700-d_6	335.2	172.9	37	112	2.733	1	-
	335.2	144.9	57	112			
MT-45-d_{11}	360.2	181.0	20	132	4.491	1	-
	360.2	77.1	93	132			
W-18-d_4	426.1	115.0	53	143	6.391	1	-
	426.1	78.1	105	143			

Choice of Acquisition: dMRM

Differentiate Structural Isomers

CHOICE OF ISTD

NSO Optimization: LC

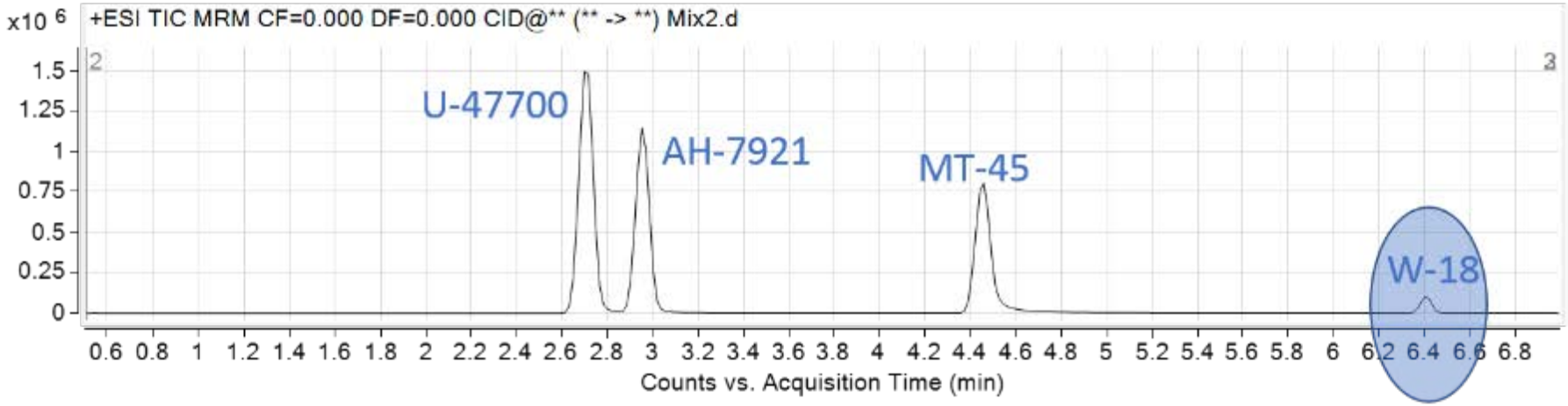
- Typical Workflow:
  - Choose generic mobile phase
    - 0.1% Formic Acid in Water
    - 0.1% Formic Acid in Acetonitrile
  - Start with Poroshell column
  - Test out a generic gradient
    - 90:10 to 10:90
  - Inject standards to acquire all optimized MRMs

But what happens when you cannot see your reference standard??

NSO Optimization: LC

- Mobile phase choice
  - Necessary for ionization
  - Can reduce or produce adducts
  - Can greatly influence peak shape & response
- Switch from MRM to dynamic MRM after establishing chromatography

NSO Optimization: LC



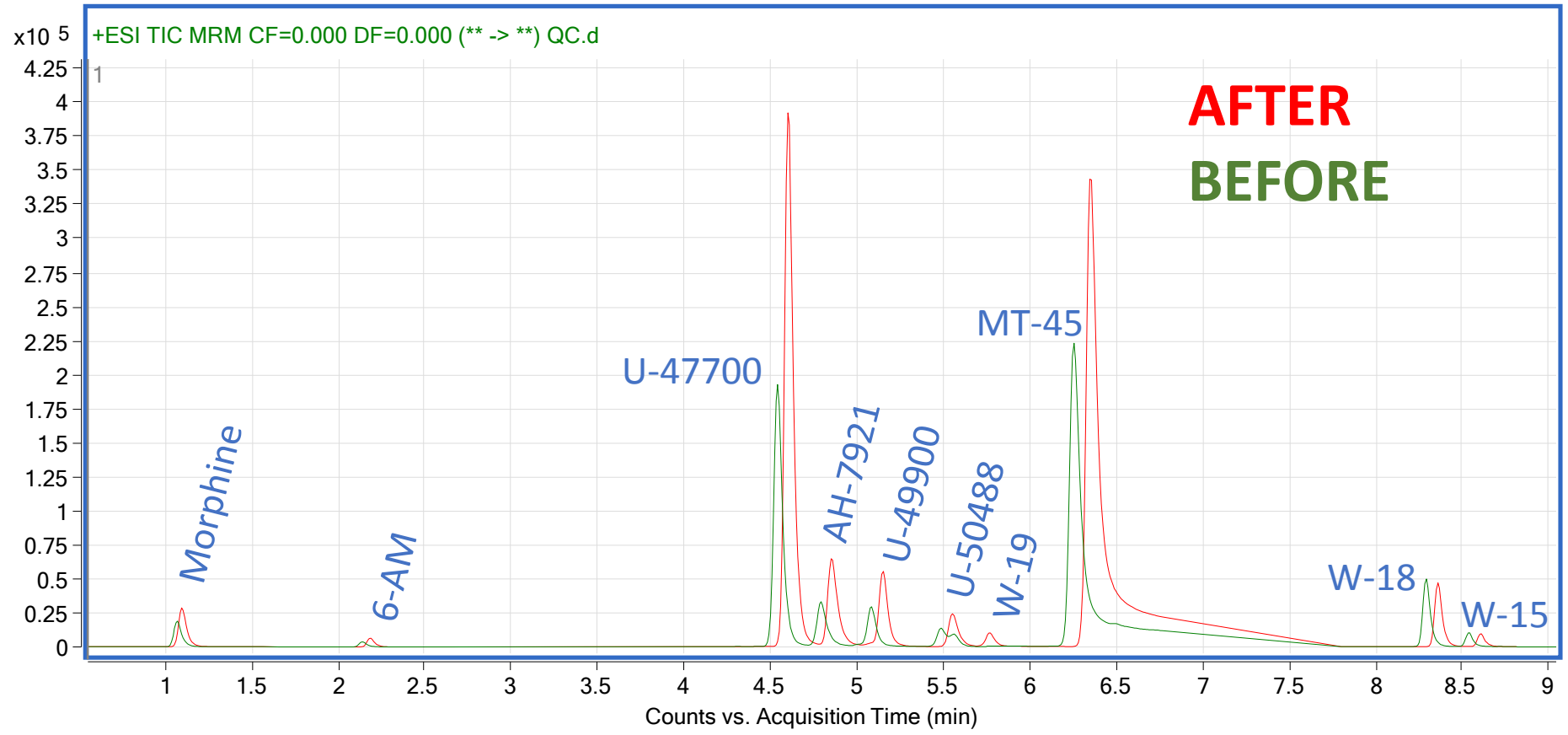
MS | LC | ESI | SPE

For Forensic Use

NSO Optimization: ESI

- Typical Workflow:
  - Examine peak responses and estimate LOD
  - Using optimized chromatography, inject mixed standards and evaluate source settings
  - Source Optimizer software

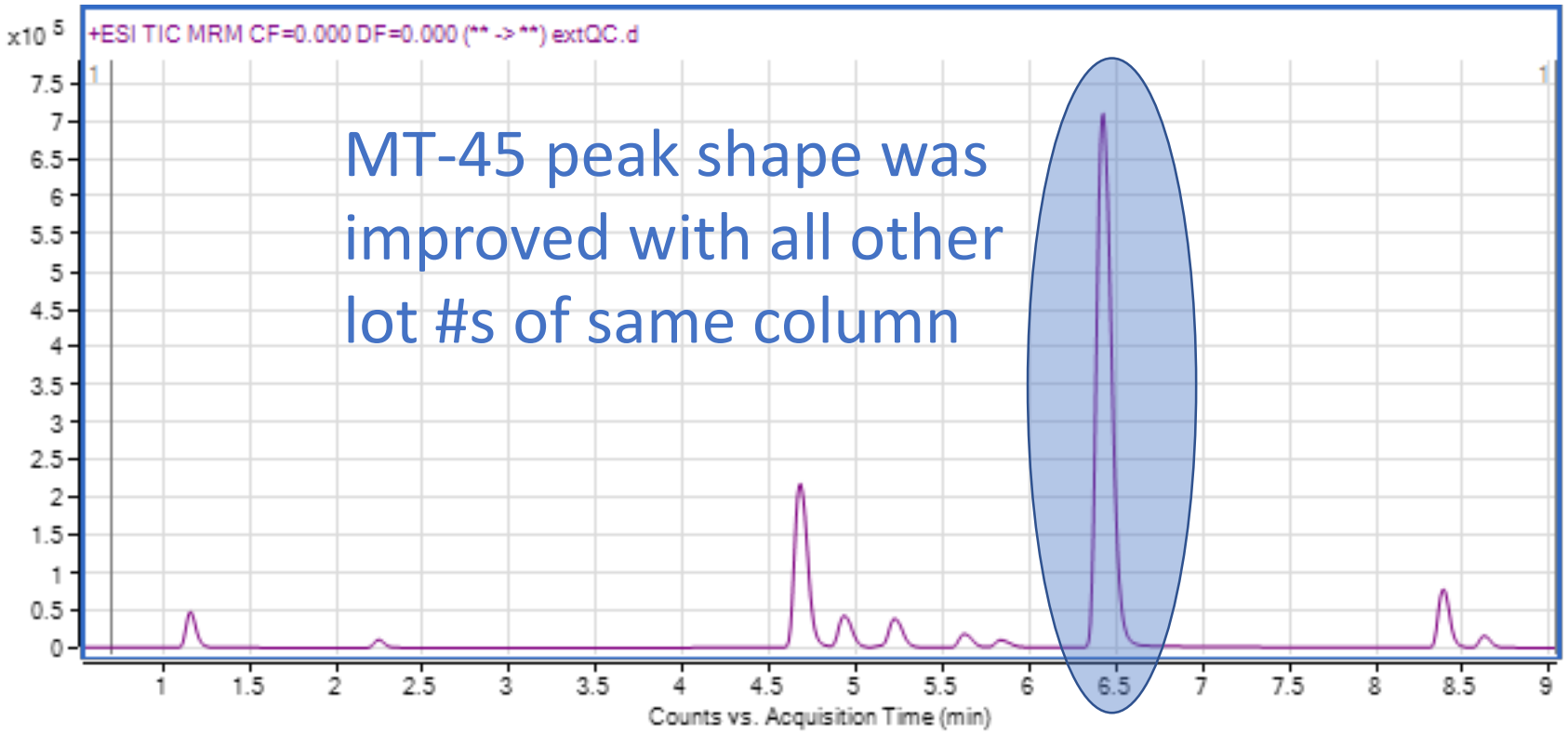
NSO Optimization: ESI



MS | LC | ESI | SPE

For Forensic Use

NSO Optimization: ESI



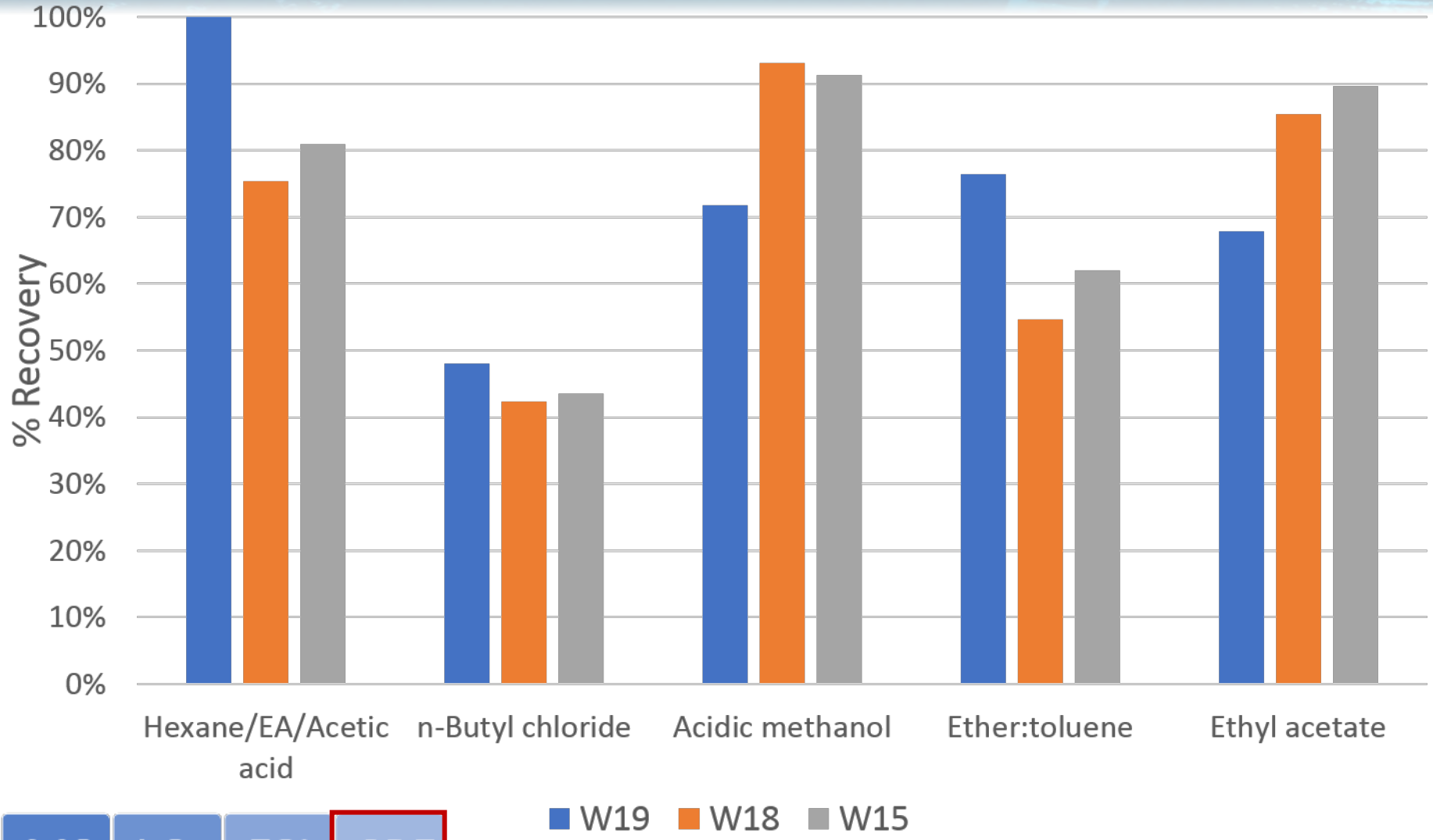
MS | LC | ESI | SPE

For Forensic Use

NSO Optimization: SPE

- Typical Workflow:
  - Polymeric, mixed-mode SPE cartridge
  - Start with basic steps:
    - Buffer sample (phosphate buffer)
    - Load sample
    - Wash (water, acid)
    - Dry
    - Wash (hexane)
    - Elute acidic/neutral (ethyl acetate)
    - Wash (methanol)
    - Elute basic (DCM:IPA with ammonia)
  - Always examine Matrix Effects & Recovery as you go!

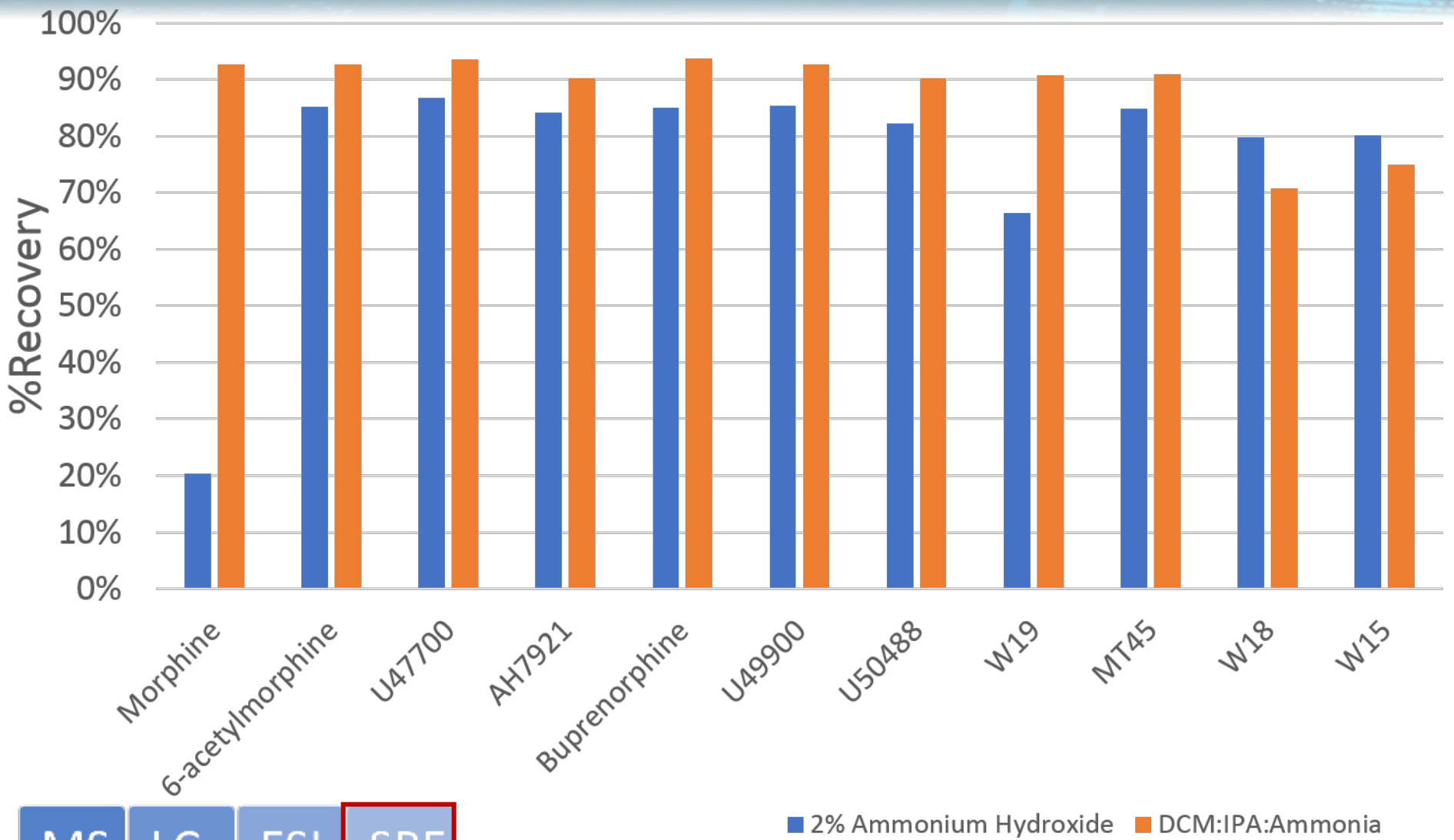
NSO Optimization: SPE



MS LC ESI SPE

For Forensic Use

NSO Optimization: SPE



MS LC ESI SPE

For Forensic Use

NSO Optimization: SPE

- Adjust volumes
  - Sample volume
  - Buffer volume
  - Wash volume
  - Elution volume
  - Reconstitution volume
- Adjust solvent choices
  - Wash solvents
  - Acidic/neutral elution solvent
  - Basic elution solvent
- Examine challenging concentrations
- Examine multiple sources of matrix
- Eliminate unnecessary transitions after development
- Readjust LC parameters if cannot eliminate interferences

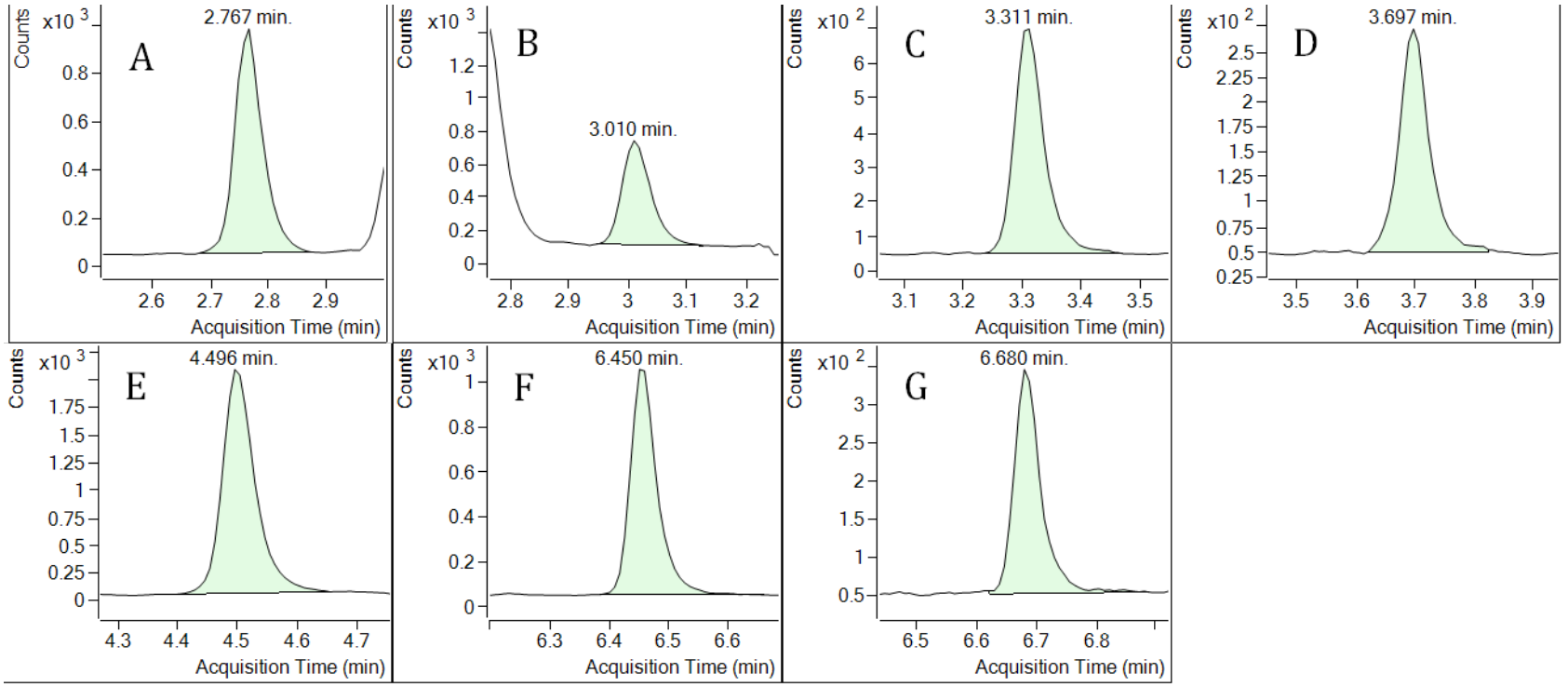
NSO Final Method

- 0.5 mL blood
- SPE
  - Elute acidic/neutral with ethyl acetate
  - Elute basic with 80/20 DCM/IPA with 5% ammonia
  - Reconstitute in 1 mL (!)
- Mobile phase: 0.05% formic acid with 5mM ammonium formate
- Gradient elution, 0.5 mL/min, 11 min run time
- Poroshell column (120 EC-C18)

NSO Method Validation

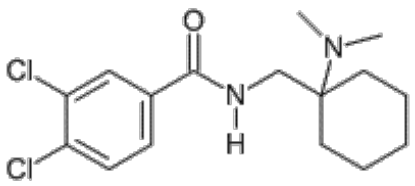
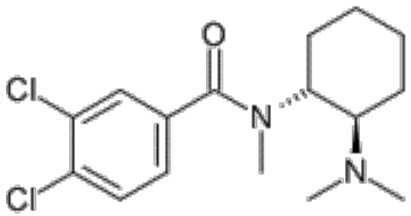
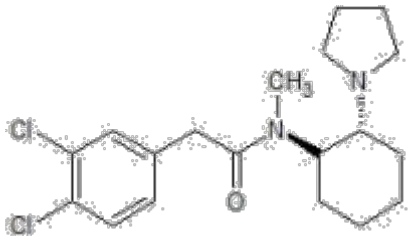
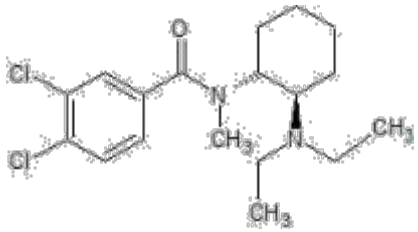
- SWGTOX Guidelines
- LOD: 0.125 ng/mL
- Linearity: 0.25 – 100 ng/mL (1 – 100 for W-18)
- Matrix effects <±19%
- Recovery 62.3 – 92.0%
- R^2 > 0.99 (n=5)
- Bias & precision <±17%
- Stable: 22°C (24 h), 4°C (72 h), autosampler (4°C, 72 h)

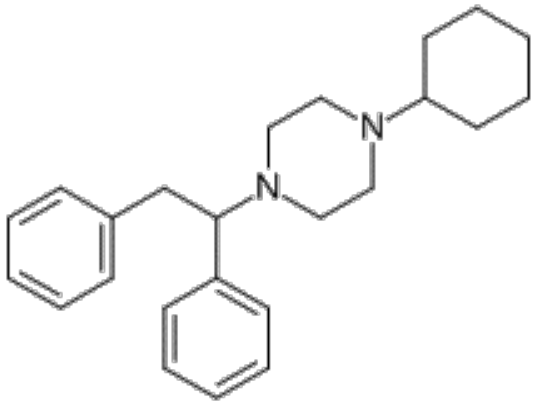
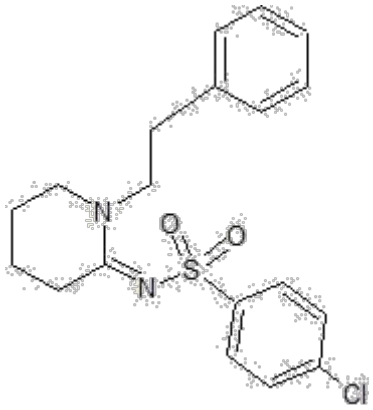
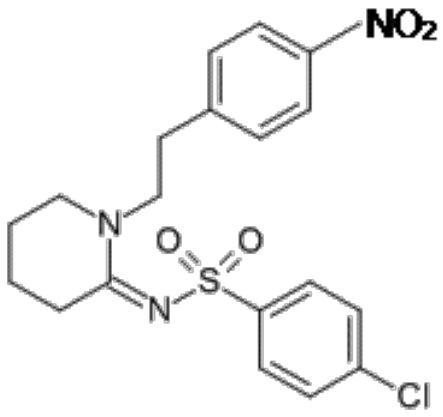
NSO at LOQ



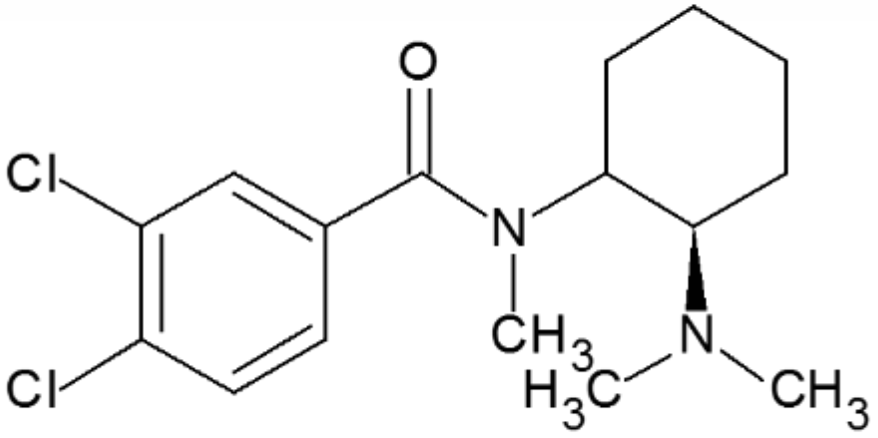
Extracted ion chromatograms for quantifying transitions at their LLOQs for (A) U-47700, (B) AH-7921, (C) U-49900, (D) U-50488, (E) MT-45, (F) W-18, and (G) W-15

U-47700 by QQQ

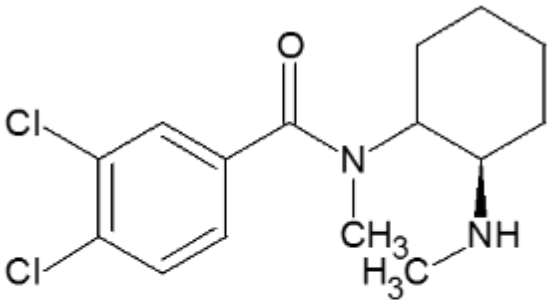
AH-7921	U-47700	U-50488	U-49900
			

MT-45	W-15	W-18
		

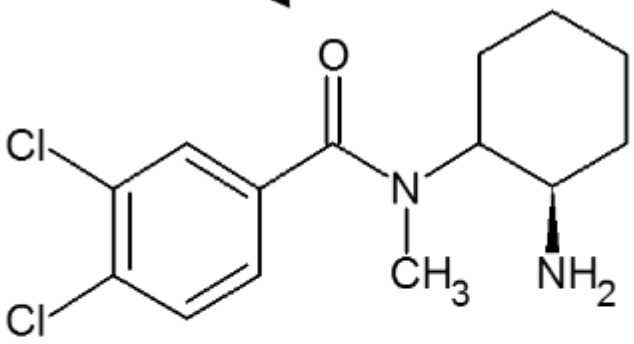
U-47700 by QQQ



U-47700

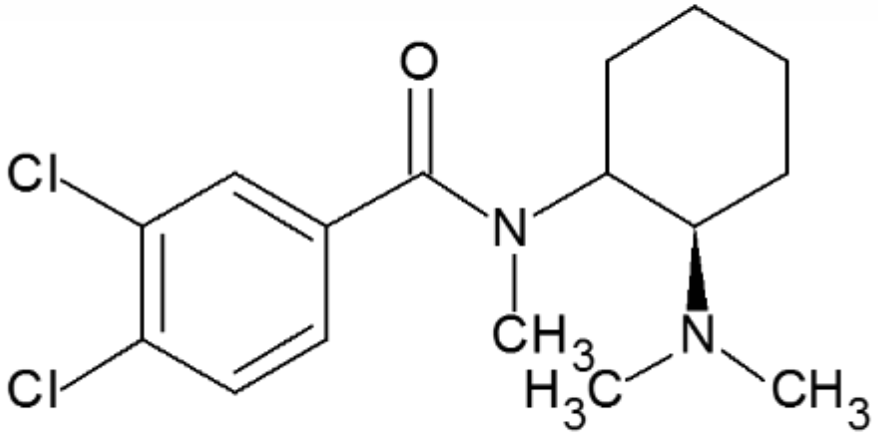


N-desmethyl-U-47700

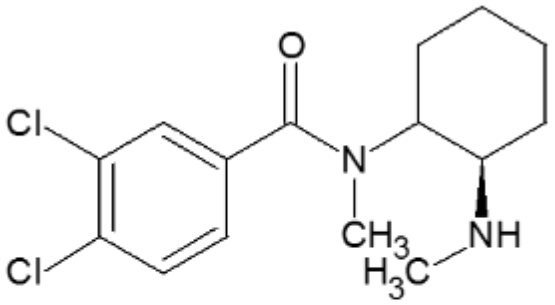


N,N-didesmethyl-U-47700

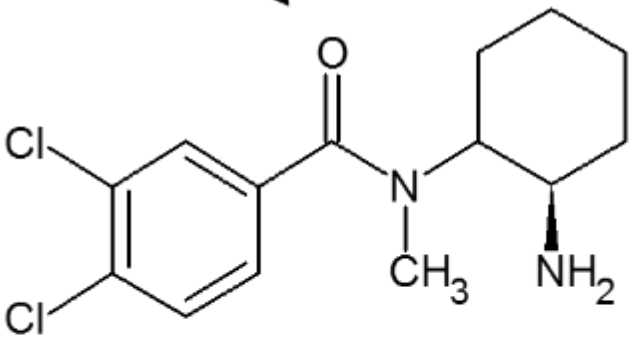
U-47700 by QQQ



U-47700



dm-U4

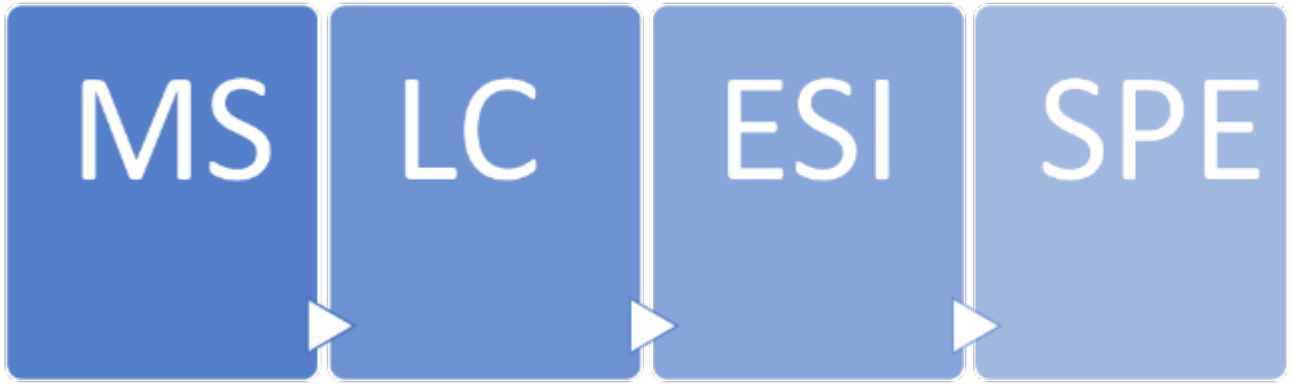


ddm-U4

For Forensic Use

U-47700 by QQQ

- Start with established method previously validated for all the synthetic opioids



- Estimated optimization:
  - MS: Optimize metabolites & add MRMs
  - LC: Shorten run time
  - SPE: Decrease sample volume (due to application)

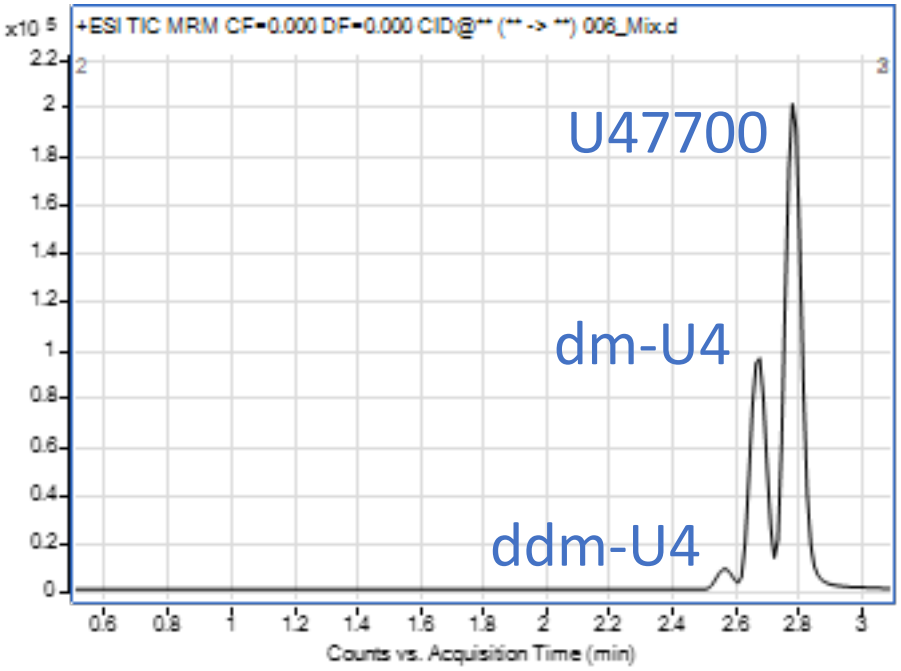
U-47700 Optimization: MS

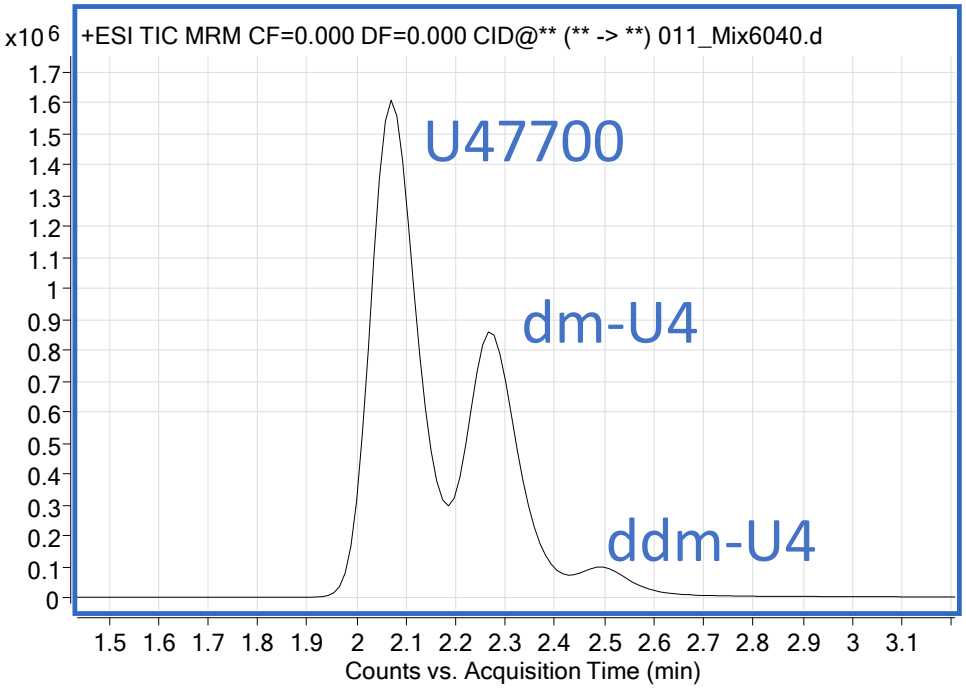
- Optimization:
  - ✓MS: Optimize metabolites & add MRMs

Analyte	Precursor Ion (m/z)	Product Ion (m/z)	Collision Energy (V)	Fragmentor (V)	Dwell (ms)	Internal Standard
U-47700	329.2	172.9	32	117	20	U-47700-d_6
	329.2	144.9	56	117	20	
dm-U4	315.2	172.9	32	107	20	U-47700-d_6
	315.2	144.9	52	107	20	
ddm-U4	301.1	189.9	20	120	20	U-47700-d_6
	301.1	144.9	56	120	20	
U-47700-d_6	335.2	172.9	36	112	20	-
	335.2	144.9	60	112	20	

U-47700 Optimization: LC

- Acetonitrile vs Methanol

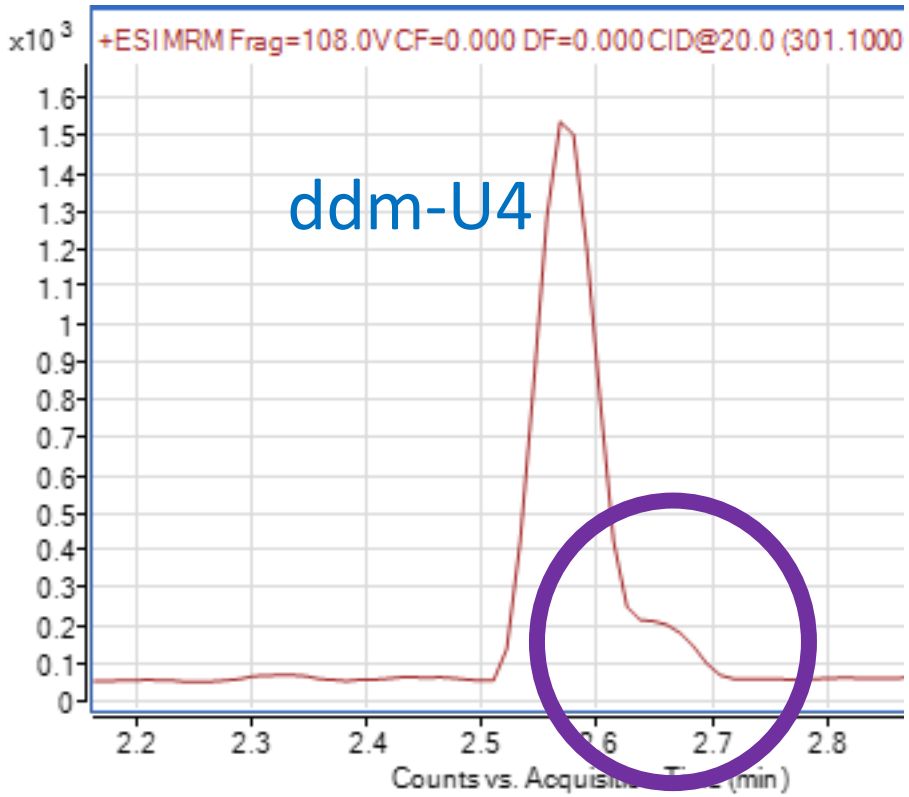


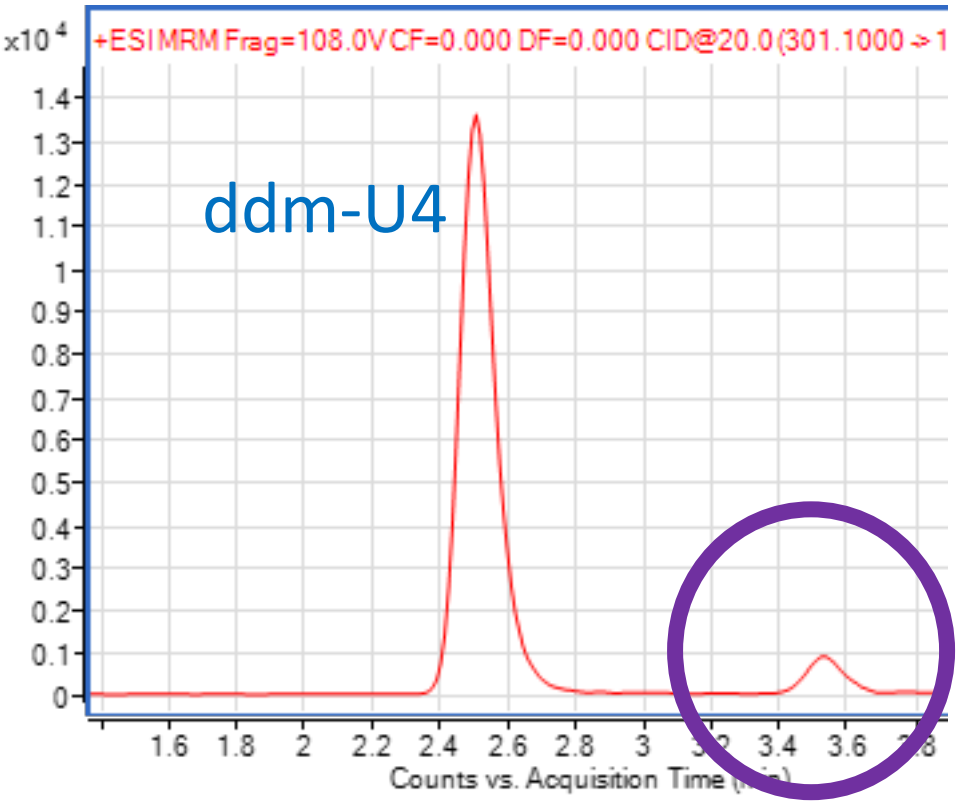


For Forensic Use

U-47700 Optimization: LC

- Acetonitrile vs Methanol





MS | LC | ESI | SPE

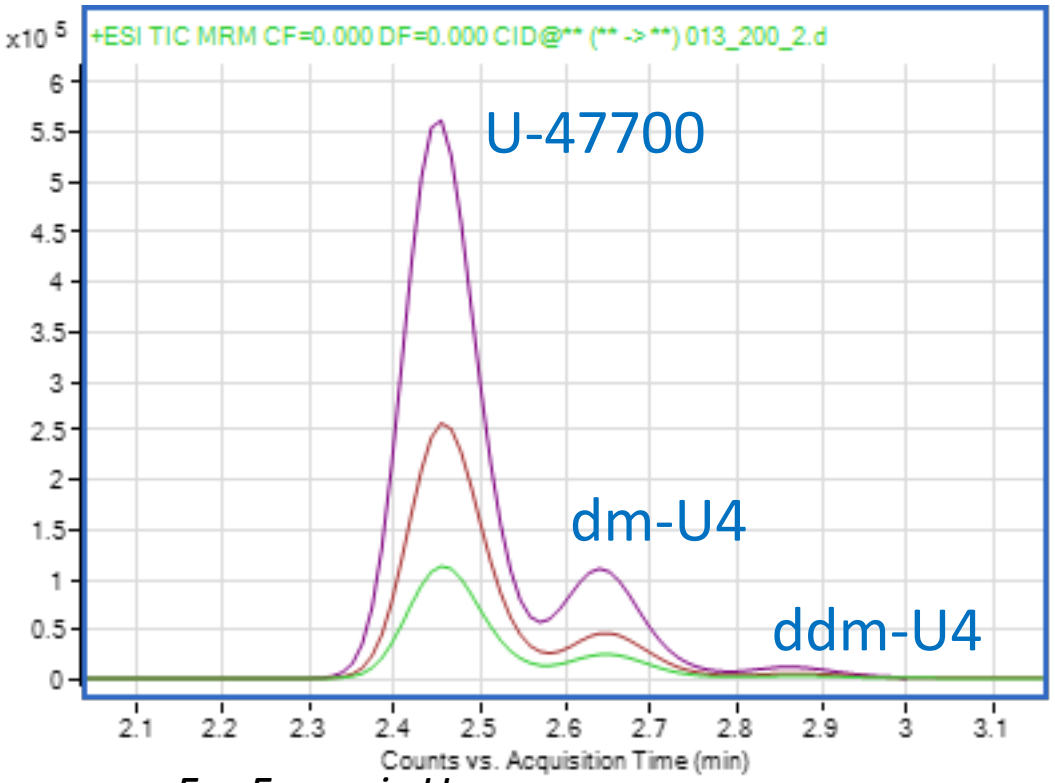
For Forensic Use

U-47700 Optimization: SPE

✓SPE: Decrease sample volume (due to application)

- To maximize analytical sensitivity for least sensitive analyte (ddm-U4), optimized:
  - Reconstitution volume

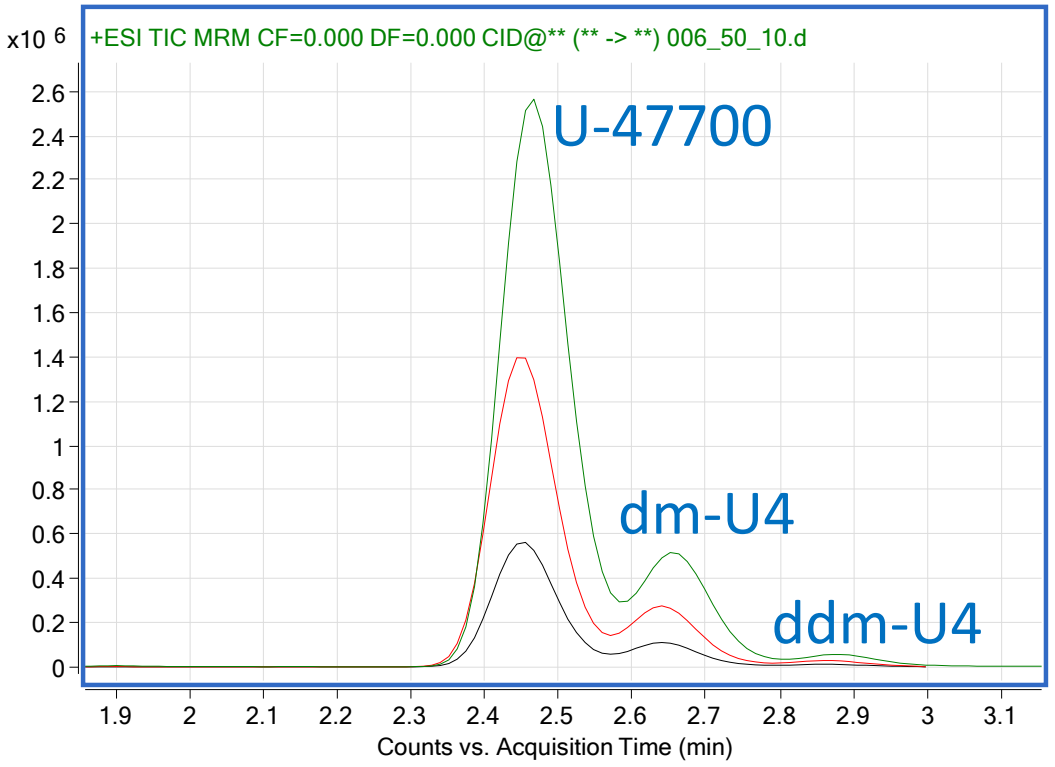
- 50 µL
- 100 µL
- 200 µL



U-47700 Optimization: SPE

✓SPE: Decrease sample volume (due to application)

- To maximize analytical sensitivity for least sensitive analyte (ddm-U4), optimized:
  - Injection volume
    - 10 µL
    - 5 µL
    - 2 µL



U-47700 Final Method

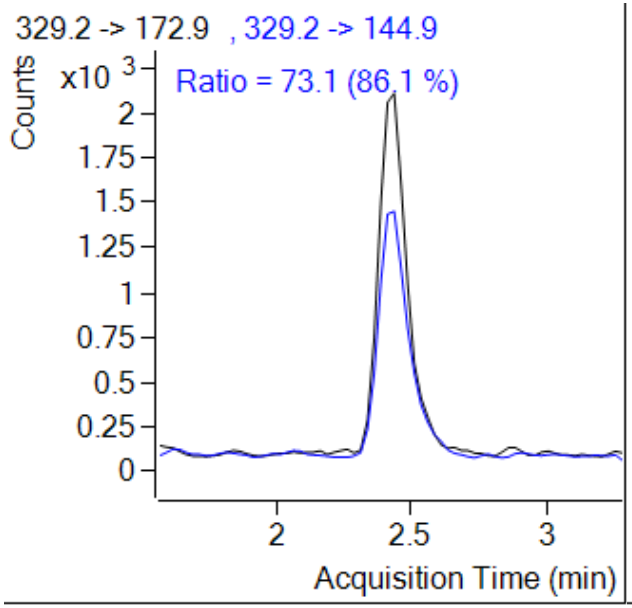
- 0.1 mL plasma
- SPE
  - No acidic/neutral elution
  - Elute basic with 80/20 DCM/IPA with 5% ammonia
  - Reconstitute in 50 µL
- Mobile phase A: 0.05% formic acid with 5mM ammonium formate
- Mobile phase B: 0.1% formic acid in Methanol
- Isocratic elution, 0.4 mL/min, 6 min run time
- Zorbax column (Eclipse Plus C18)

U-47700 Method Validation

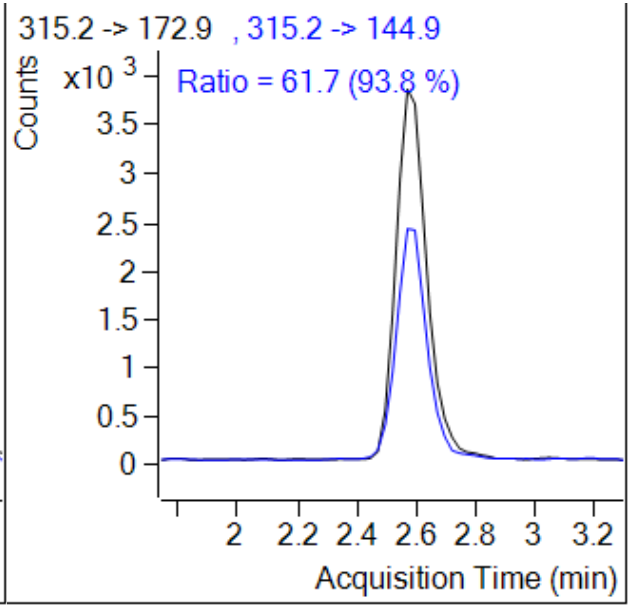
- SWGTOX Guidelines
- LOD: 0.05 ng/mL
- Linearity: 0.1 – 100 ng/mL (0.5 – 100 for ddm-U4)
- Matrix effects <±5%
- Recovery 79.4 – 88.0%
- $R^2 > 0.99$ (n=5)
- Bias & precision <±13.6%
- Stable: 22°C (24 h), 4°C (72 h), 3 freeze/thaw cycles, autosampler (4°C, 72 h)

U-47700 & Metabolites at LOQ

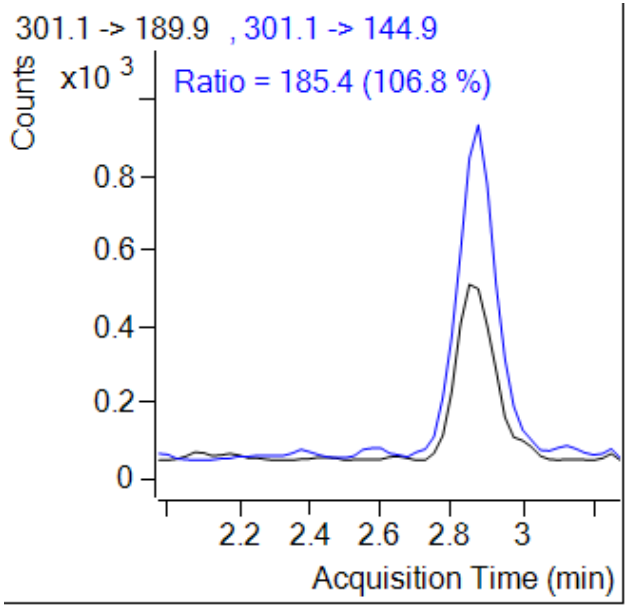
U-47700



dm-U4



ddm-U4



Emerging NPS by LC-MS/MS

- Pay attention to structure & pKa as it will influence:
  - ESI mode
  - Mobile phase choice
  - Extraction performance
- Spend the time optimizing chromatography
  - Consider different columns, modifiers, & solvents
- Don’t skimp on extraction development
  - May need to consider multiple elution steps
  - Optimize volumes as necessary
- Always consider the analytical sensitivity for what you are trying to achieve

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- National Institute of Justice, Award No. 2017-R2-CX-0019
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Swortwood Research Group 2017-2018

Questions?

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