Quantitative forensic toxicology

Consideration, experimentation and implementation

TREND SETTERS

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Utility of Standard Addition for Quantitative Forensic Toxicology: Consideration, Experimentation, and Implementation

Alex J Krotulski, PhD – Associate Director (CFSRE) & Program Manager (NPS Discovery)

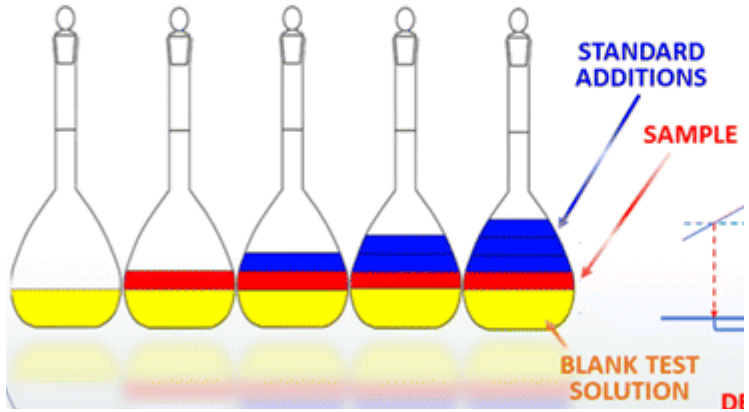
Trendsetters – SCIEX – April 27, 2021

Standard Addition

- **Definition:** A type of quantitative analysis approach whereby the standard is added directly to the aliquots of analyzed sample
  - Internal calibration model (as opposed to an external calibration model)

- Various scientific areas use standard addition

- Mechanism to provide accurate and reliable quantitative results in the absence of a traditionally validated assay
  - Rarely encountered substances
  - Novel Psychoactive Substances (NPS)



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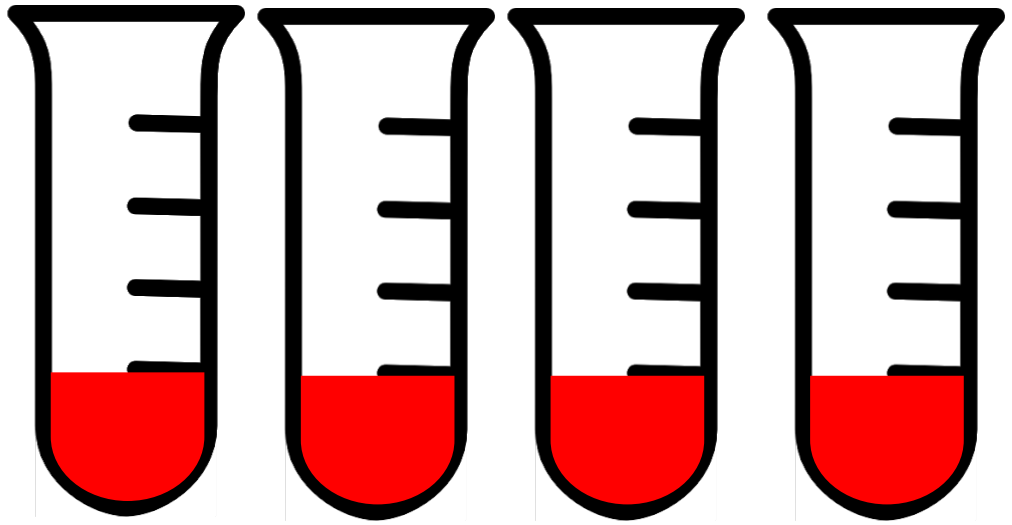
Standard Addition

- Briefly… how standard addition works:



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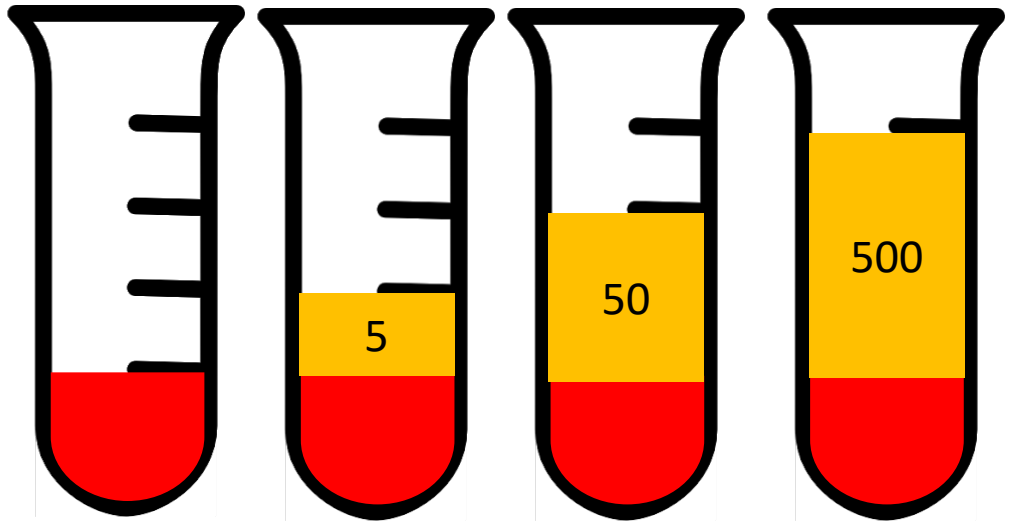

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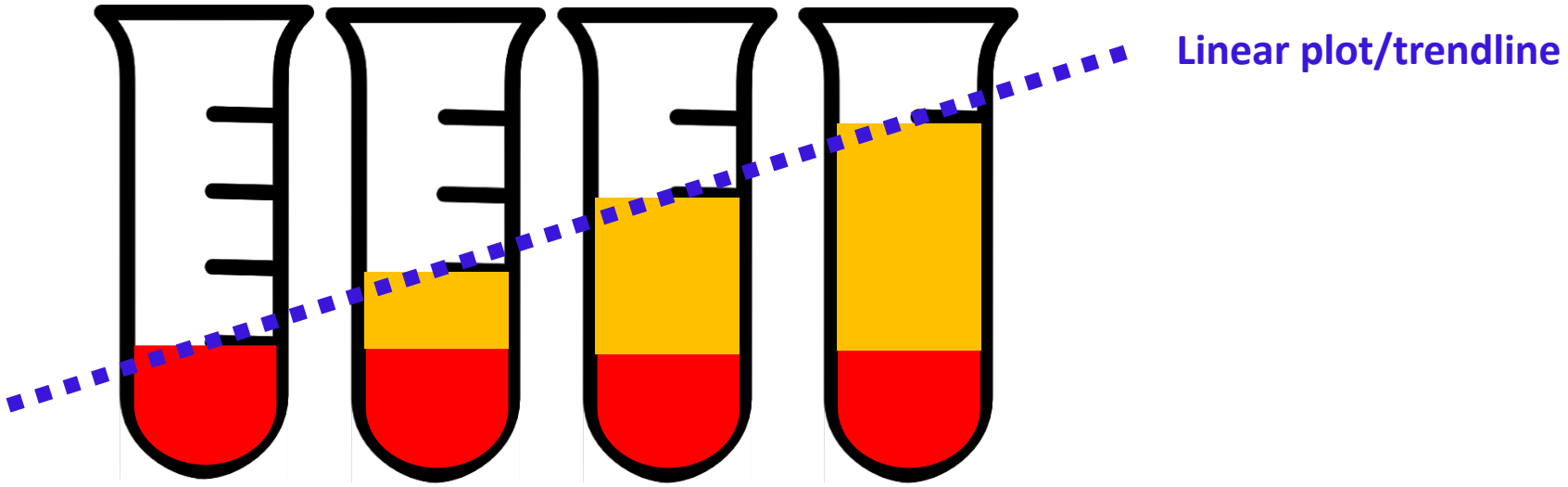

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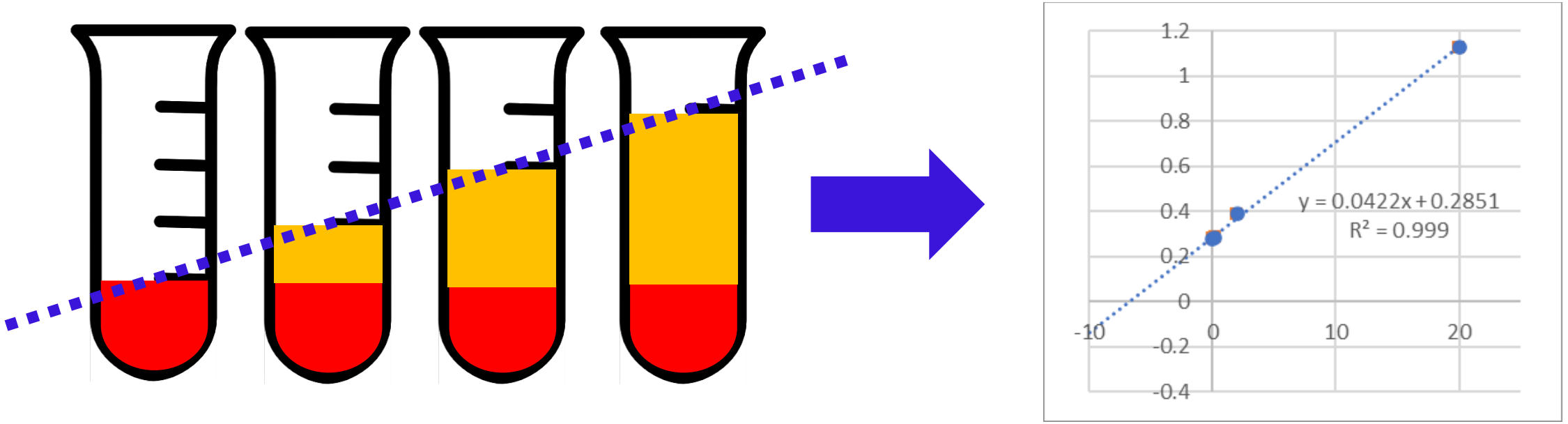

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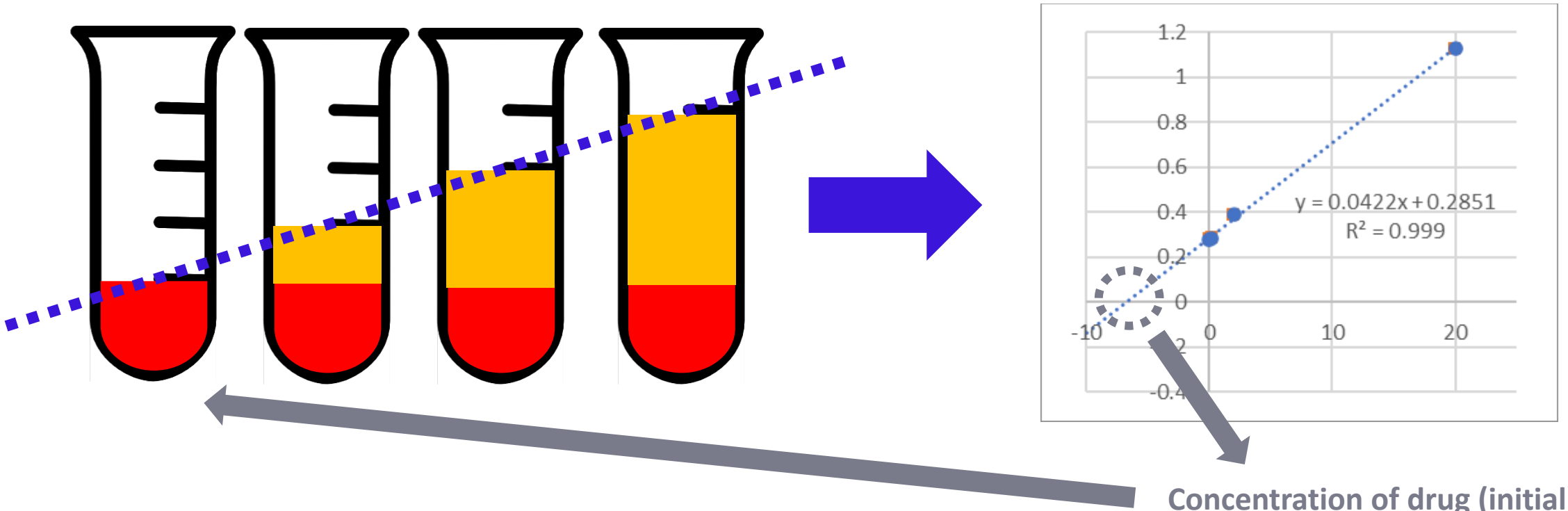

Selecting “Up-Spike” Concentrations

- Can be difficult to impossible (at times)
  - Use screening data for estimation
- **General approaches:**
  - If you know estimated concentration:
    - I.e., approximately 10 ng/mL
    - Up-spike at 50%, 100%, and 200%
    - More specific, but less comprehensive and more time consuming
  - If you know ball-park concentration:
    - I.e., somewhere around 1-10 ng/mL
    - Up-spike at 0.2, 2, and 20 ng/mL for all
    - Span two orders of magnitude or more, can be comprehensive, can drop data point*

- **Good:**
  - Estimated concentration: 7 ng/mL
  - Up-spike at 4, 10, and 20 ng/mL
  - Actual concentration: 5.8 ng/mL
- **Bad:**
  - Estimated concentration: 500 ng/mL
  - Up-spike at 2, 5, and 10 ng/mL
  - Actual concentration: ??? ng/mL
  - *OR*
  - Estimated concentration: ??? ng/mL
  - Up-spike at 5, 10, and 50 ng/mL
  - Actual concentration: 0.2 ng/mL
- **Up-spikes *should* bracket your final conc.**

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CONSIDERATIONS

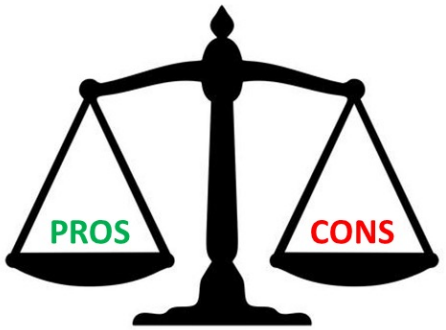
Considerations

- **The Good and The Bad:**

- Pros:
  - "Self-validating" approach
  - Different requirements for *validation*
  - Resource savings on small scale
  - When executed properly, more accurate

- Cons:
  - Consumed sample volume (2+ mL per assay)
  - Need to know a ballpark quantitative value
  - For 10+ samples, can become time consuming and resource consuming

- **Additional Considerations:**
  - Desired quantitative range
  - Instrumentation
    - GC-MS vs. LC-MS/MS vs. LC-HRMS
  - Matrix type
    - Tissue, vitreous, bile, etc.
  - Matrix effects
    - Can you replicate postmortem blood?
  - Internal standard
  - Etc.



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Considerations

- Standard addition **can not** be applied without assessment of the analytical technique

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Standard additions: myth and reality†

Stephen L. R. Ellison[a] and Michael Thompson[b]

DOI: 10.1039/b717660k

Standard additions is a calibration technique devised to eliminate rotational matrix effects in analytical measurement. Although the technique is presented in almost every textbook of analytical chemistry, its behaviour in practice is not well documented and is prone to attract misleading accounts. The most important limitation is that the method cannot deal with translational matrix effects, which need to be handled separately. In addition, because the method involves extrapolation from known data, the method is often regarded as less precise than external calibration (interpolation) techniques. Here, using a generalised model of an analytical system, we look at the behaviour of the method of standard additions under a range of conditions, and find that, if executed optimally, there is no noteworthy loss of precision.

Recommendations for standard additions

1. Make sure that the analytical method is effectively linear over the whole of the required working range.
2. Make sure that any translational interference is eliminated separately.
3. Only one level of added analyte is necessary, with repeated measurements if better precision is required.
4. Let the concentration of the added analyte be as high as is consistent with linearity, and ideally at least five times the original concentration of analyte.

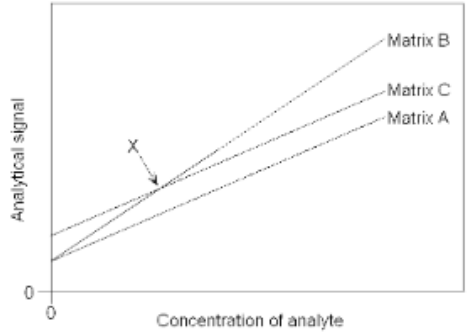

Figure 1. Different types of matrix effect on the analytical signal. Matrix A is the calibration matrix. With Matrix B a rotational effect changes the size of the signal derived from the analyte, but not the intercept. With Matrix C the intercept has been shifted by a translational effect, but the slope is unaffected. At point X the two matrix effects fortuitously have the same outcome.

https://doi.org/10.1039/b717660k

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EXPERIMENTATION

Experimentation

- **Method development and workflow** *(same a traditionally validated assay)*
  - Analytical method
  - Spiking mixes / internal standard
  - Sample preparation protocol / extraction

- **Example:**
  - SCIEX TripleTOF® 5600+ LC-MS/MS System
  - Column, mobile phase, and gradient
  - MRM transitions / MS parameters
  - Isotonitazene and fentanyl-D5 / 0.1 and 1 ng/µL
  - Liquid-liquid extraction

Table I. LC Gradient Conditions

Time (min)	%A	%B	Flow (mL/min)
Initial	50	50	0.4
1.0	50	50	0.4
4.0	5	95	0.4
5.0	5	95	0.4
5.1	50	50	0.4
6.0	50	50	0.4

Table II. MRM Parameters

Analyte	Cone (V)	Precursor (m/z)	Collision (V)	Product (m/z)	Dwell (s)
Isotonitazene	50	411.2	46	106.9	0.053
			22	100.0	0.053
			44	72.0	0.053
Fentanyl-d_5	56	342.2	24	188.0	0.053
			40	105.0	0.053

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Experimentation

- **Method verification** (or "validation")
  - Modeled after ASB Standard 036: *Standard Practices for Method Validation in Forensic Toxicology*
  - These are our proposed experiments to assess the use of standard addition

Required:
- Linearity (target range)
- Limit of detection
- Carryover
- Interferences

May be required:
- "Controls" (accuracy and precision)
- Stability studies
- Recovery

Not required:
- Ion suppression / enhancement
- Dilution integrity

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IMPLEMENTATION

Implementation

- [Screening] – Analysis of sample(s) by LC-QTOF-MS
- Method development and verification
- [Confirmation] – Application to authentic sample(s)
- Calculation of concentration (x-intercept)

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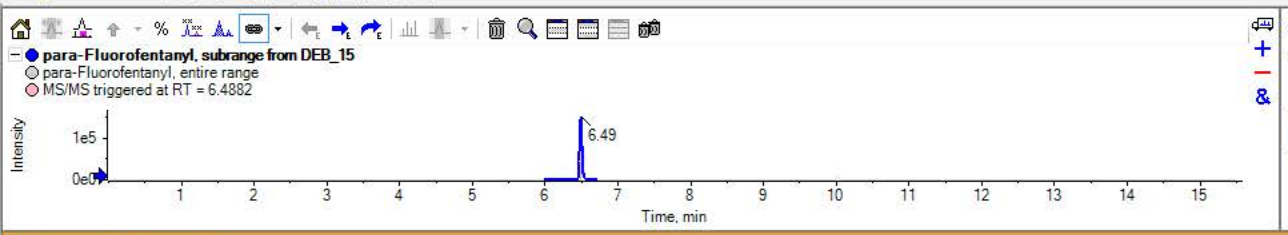
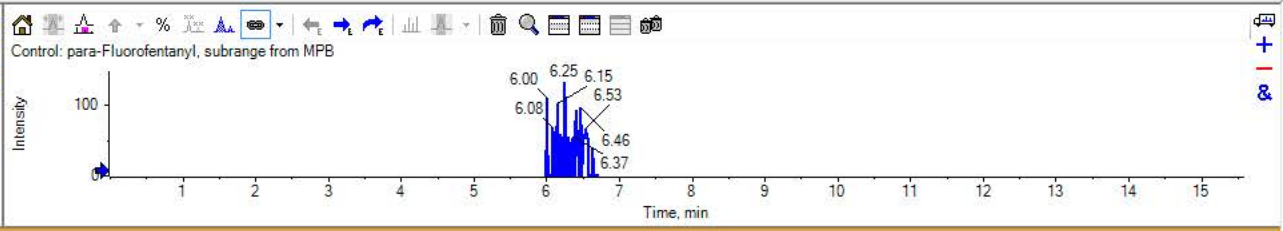
PeakView - [MasterView - New Session]

File Edit Show MasterView Graph Process Quantitation BioTools Bio Tool Kit Window Help

Default





MasterView

New Session

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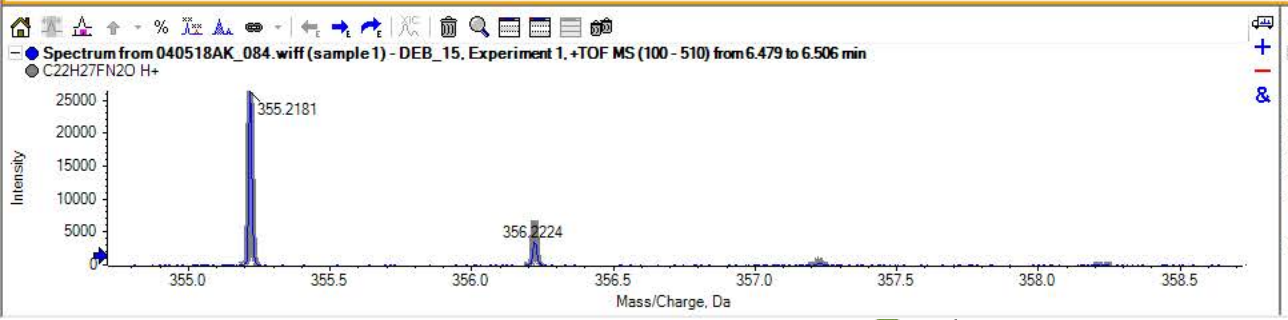
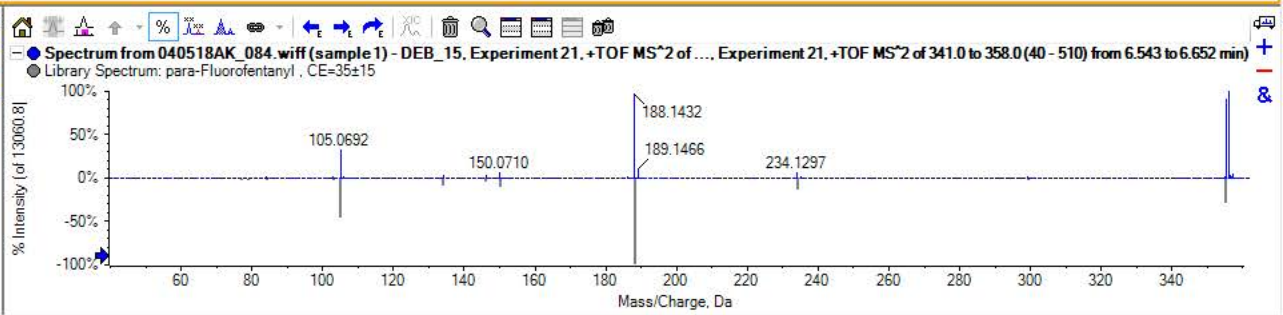
✓	040518AK_084	DEB_15	44
✓	040518AK_085	DEB_16_0	41
✓	040518AK_086	DEB_12_0	70
✓	040518AK_087	DEB_13	59
✓	040518AK_088	DEB_15_0	91
✓	040518AK_089	DEB_11_0	62
✓	040518AK_090	MPB	10
✓	040518AK_091	SRM	77
✓	040518AK_092	DOP_13_0	41
✓	040518AK_093	DOP_14	25
✓	040518AK_094	DOP_15	81
✓	040518AK_095	DOP_16	65
✓	040518AK_096	DOP_17	40
✓	040518AK_097	DOP_18	65
✓	040518AK_098	DOP_19	53

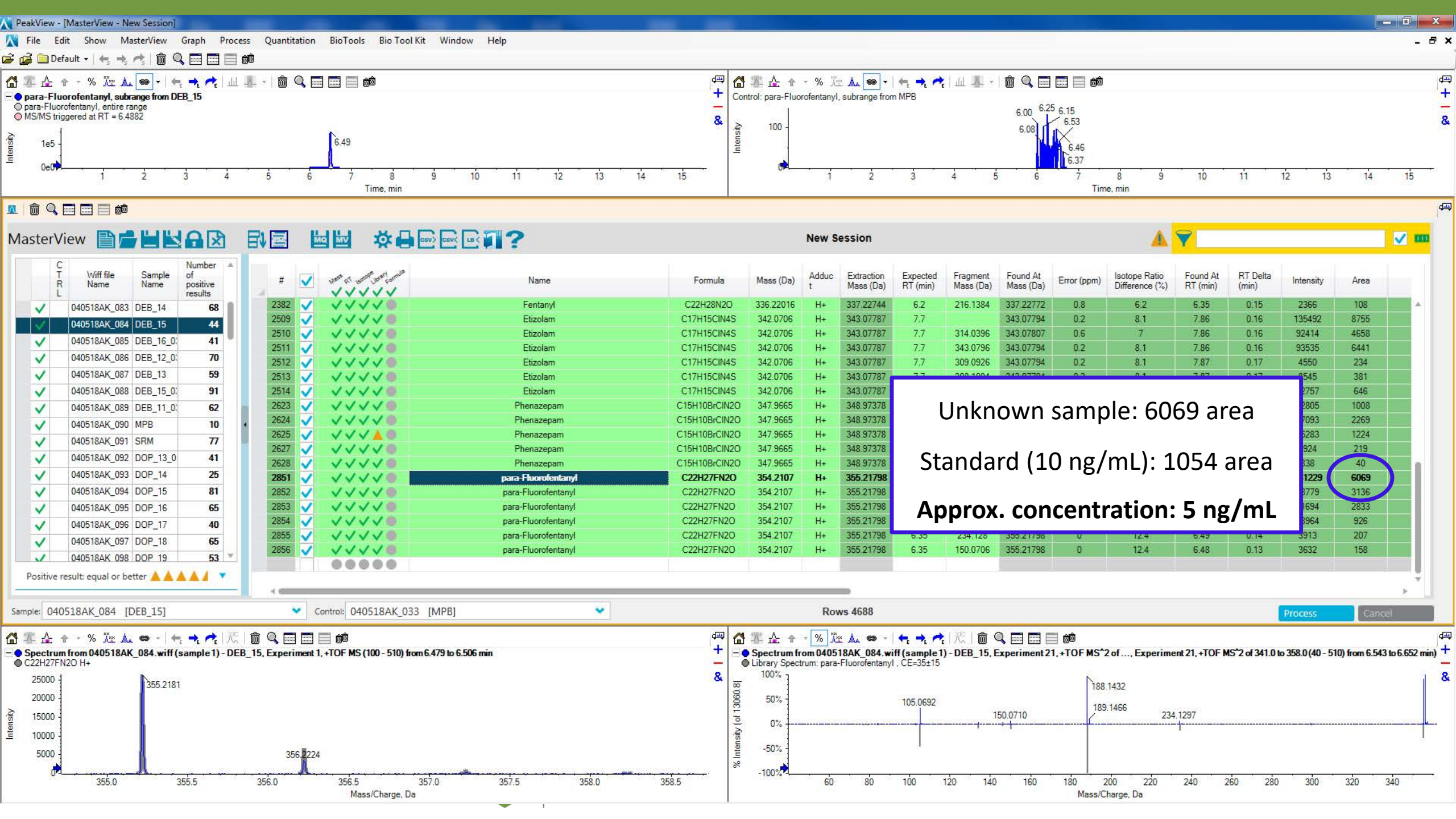
Positive result: equal or better

#	Name	Formula	Mass (Da)	Adduct	Extraction Mass (Da)	Expected RT (min)	Fragment Mass (Da)	Found At Mass (Da)	Error (ppm)	Isotope Ratio Difference (%)	Found At RT (min)	RT Delta (min)	Intensity	Area
2382	Fentanyl	C22H28N2O	336.22016	H+	337.22744	6.2	216.1384	337.22772	0.8	6.2	6.35	0.15	2366	108
2509	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7		343.07794	0.2	8.1	7.86	0.16	135492	8755
2510	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7	314.0396	343.07807	0.6	7	7.86	0.16	92414	4658
2511	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7	343.0796	343.07794	0.2	8.1	7.86	0.16	93535	6441
2512	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7	309.0926	343.07794	0.2	8.1	7.87	0.17	4550	234
2513	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7	308.1094	343.07794	0.2	8.1	7.87	0.17	8545	381
2514	Etizolam	C17H15ClN4S	342.0706	H+	343.07787	7.7	310.1005	343.07794	0.2	8.1	7.86	0.16	12757	646
2623	Phenazepam	C15H10BrClN2O	347.9665	H+	348.97378	7.86		348.97459	2.3	7.1	8.03	0.17	22805	1008
2624	Phenazepam	C15H10BrClN2O	347.9665	H+	348.97378	7.86	348.9733	348.97362	-0.5	2.6	8.03	0.17	47093	2269
2625	Phenazepam	C15H10BrClN2O	347.9665	H+	348.97378	7.86	183.9757	348.97362	-0.5	2.6	8.01	0.15	25283	1224
2627	Phenazepam	C15H10BrClN2O	347.9665	H+	348.97378	7.86	242.0609	348.97362	-0.5	2.6	8.03	0.17	4924	219
2628	Phenazepam	C15H10BrClN2O	347.9665	H+	348.97378	7.86	320.9792	348.97362	-0.5	2.6	8.03	0.17	838	40
2851	**para-Fluorofentanyl**	**C22H27FN2O**	**354.2107**	**H+**	**355.21798**	**6.35**		**355.21806**	**0.2**	**12.5**	**6.49**	**0.14**	**151229**	**6069**
2852	para-Fluorofentanyl	C22H27FN2O	354.2107	H+	355.21798	6.35	355.2168	355.21798	0	12.4	6.49	0.14	58779	3136
2853	para-Fluorofentanyl	C22H27FN2O	354.2107	H+	355.21798	6.35	188.1432	355.21798	0	12.4	6.49	0.14	61694	2833
2854	para-Fluorofentanyl	C22H27FN2O	354.2107	H+	355.21798	6.35	105.0701	355.21798	0	12.4	6.49	0.14	18964	926
2855	para-Fluorofentanyl	C22H27FN2O	354.2107	H+	355.21798	6.35	234.128	355.21798	0	12.4	6.49	0.14	3913	207
2856	para-Fluorofentanyl	C22H27FN2O	354.2107	H+	355.21798	6.35	150.0706	355.21798	0	12.4	6.48	0.13	3632	158

Sample: 040518AK_084 [DEB_15] Control: 040518AK_033 [MPB] Rows 4688 Process Cancel



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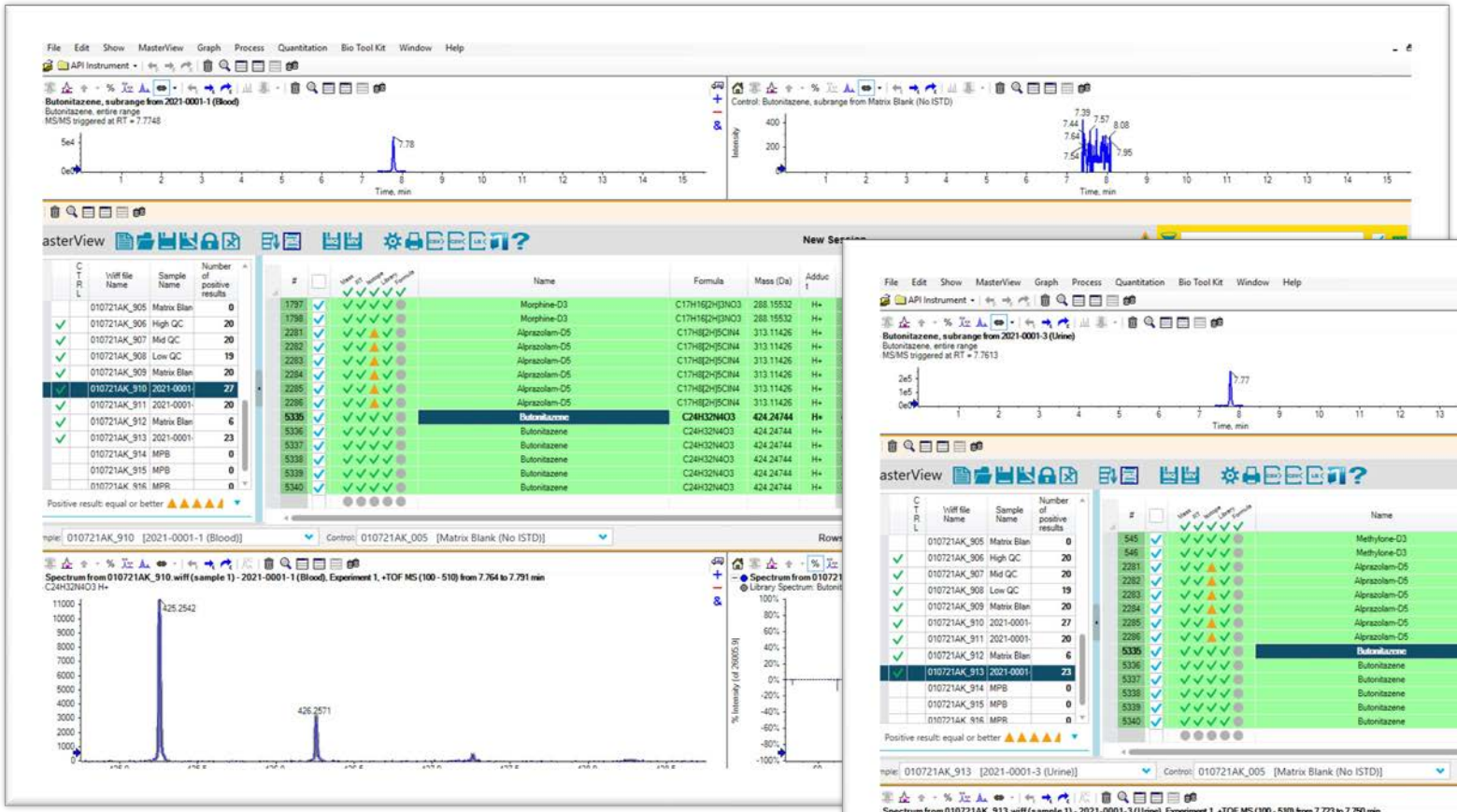
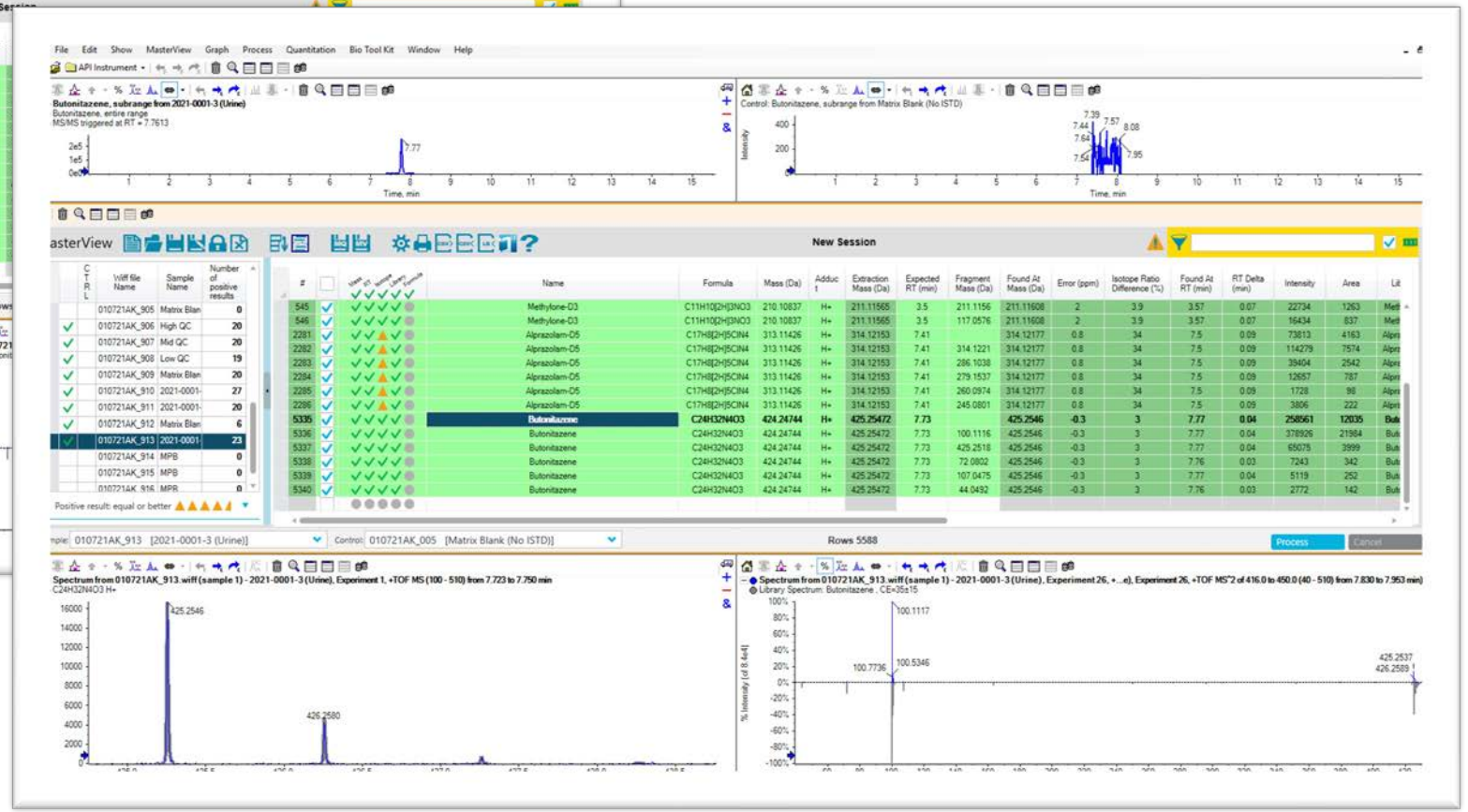
para-Fluorofentanyl, subrange from DEB_15
para-Fluorofentanyl, entire range
MS/MS triggered at RT = 6.4882
6.49
Intensity
Time, min
Control: para-Fluorofentanyl, subrange from MPB
6.00 6.25 6.15 6.53 6.08 6.46 6.37
MasterView
New Session
Wiff file Name
Sample Name
Number of positive results
040518AK_083 DEB_14 68
040518AK_084 DEB_15 44
040518AK_085 DEB_16_0 41
040518AK_086 DEB_12_0 70
040518AK_087 DEB_13 59
040518AK_088 DEB_15_0 91
040518AK_089 DEB_11_0 62
040518AK_090 MPB 10
040518AK_091 SRM 77
040518AK_092 DOP_13_0 41
040518AK_093 DOP_14 25
040518AK_094 DOP_15 81
040518AK_095 DOP_16 65
040518AK_096 DOP_17 40
040518AK_097 DOP_18 65
040518AK_098 DOP_19 53
Positive result: equal or better
Name
Formula
Mass (Da)
Adduct
Extraction Mass (Da)
Expected RT (min)
Fragment Mass (Da)
Found At Mass (Da)
Error (ppm)
Isotope Ratio Difference (%)
Found At RT (min)
RT Delta (min)
Intensity
Area
2382 Fentanyl C22H28N2O 336.22016 H+ 337.22744 6.2 216.1384 337.22772 0.8 6.2 6.35 0.15 2366 108
2509 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 7.7 343.07794 0.2 8.1 7.86 0.16 135492 8755
2510 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 7.7 314.0396 343.07807 0.6 7 7.86 0.16 92414 4658
2511 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 7.7 343.0796 343.07794 0.2 8.1 7.86 0.16 93535 6441
2512 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 7.7 309.0926 343.07794 0.2 8.1 7.87 0.17 4550 234
2513 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 7.7 343.07787 381
2514 Etizolam C17H15ClN4S 342.0706 H+ 343.07787 646
2623 Phenazepam C15H10BrClN2O 347.9665 H+ 348.97378 1008
2624 Phenazepam C15H10BrClN2O 347.9665 H+ 348.97378 2269
2625 Phenazepam C15H10BrClN2O 347.9665 H+ 348.97378 1224
2627 Phenazepam C15H10BrClN2O 347.9665 H+ 348.97378 219
2628 Phenazepam C15H10BrClN2O 347.9665 H+ 348.97378 40
2851 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 6069
2852 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 3136
2853 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 2833
2854 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 926
2855 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 207
2856 para-Fluorofentanyl C22H27FN2O 354.2107 H+ 355.21798 6.35 150.0706 355.21798 0 12.4 6.48 0.13 3632 158
Unknown sample: 6069 area
Standard (10 ng/mL): 1054 area
Approx. concentration: 5 ng/mL
Sample: 040518AK_084 [DEB_15]
Control: 040518AK_033 [MPB]
Rows 4688
Process
Cancel
Spectrum from 040518AK_084.wiff (sample 1) - DEB_15, Experiment 1, +TOF MS (100 - 510) from 6.479 to 6.506 min
C22H27FN2O H+
355.2181
356.2224
Mass/Charge, Da
Spectrum from 040518AK_084.wiff (sample 1) - DEB_15, Experiment 21, +TOF MS^2 of ..., Experiment 21, +TOF MS^2 of 341.0 to 358.0 (40 - 510) from 6.543 to 6.652 min)
Library Spectrum: para-Fluorofentanyl , CE=35±15
105.0692
150.0710
188.1432
189.1466
234.1297
% Intensity (of 13060.8)
Mass/Charge, Da

Sample #	Notes	Sample ID and Comments
1	Calibration Model	Reagent blank (DI water)
2		Standard 1: 100 ng/mL
3		Blank Blood -- No ISTD added
4		Standard 2: 50 ng/mL
5		Standard 3: 20 ng/mL
6		Standard 4: 10 ng/mL
7		Standard 5: 5 ng/mL
8		Standard 6: 1 ng/mL
9		Standard 7: 0.5 ng/mL
10		Standard 8: 0.25 ng/mL
11		Standard 9: 0.1 ng/mL
12		Blank Blood – with ISTD added
13	Standard Addition Assessment (5 ng/mL)	Std Add-1 (No up-spike)
14		Std Add-2 (0.2 ng/mL up-spike)
15		Std Add-3 (2 ng/mL up-spike)
16		Std Add-4 (20 ng/mL up-spike)
17	Standard Addition Assessment (10 ng/mL)	Std Add-1 (No up-spike)
18		Std Add-2 (0.2 ng/mL up-spike)
19		Std Add-3 (2 ng/mL up-spike)
20		Std Add-4 (20 ng/mL up-spike)
21	Recovery (20 ng/mL)	Pre-spike-1
22		Pre-spike-2
23		Pre-spike-3
24		Post-spike-1
25		Post-spike-2
26		Post-spike-3
27	Matrix Interferences (10 different sources)	Matrix Blood 1
28		Matrix Blood 2
29		Matrix Blood 3
30		Matrix Blood 4
31		Matrix Blood 5
32		Matrix Blood 6
33		Matrix Blood 7
34		Matrix Blood 8
35		Matrix Blood 9
36		Matrix Blood 10
37	Analyte/Internal Standard Interferences (Highest Calibrator and Normal ISTD Conc.)	Highest Cal. 1 – 100 ng/mL
38		Highest Cal. 2 – 100 ng/mL
39		Highest Cal. 3 – 100 ng/mL
40		Highest Cal. 4 – 100 ng/mL
41		Highest Cal. 5 – 100 ng/mL
42		Internal Standard 1 – 20 ng/mL
43		Internal Standard 2 – 20 ng/mL
44		Internal Standard 3 – 20 ng/mL
45		Internal Standard 4 – 20 ng/mL
46		Internal Standard 5 – 20 ng/mL





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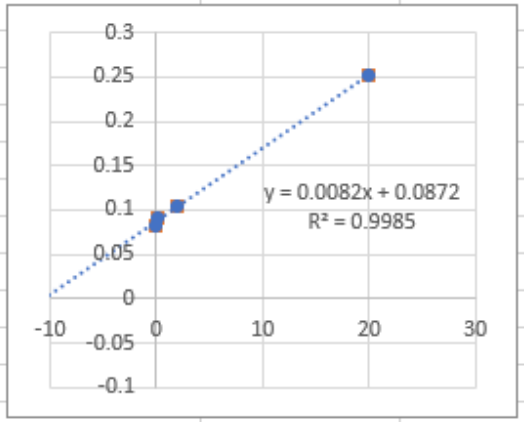
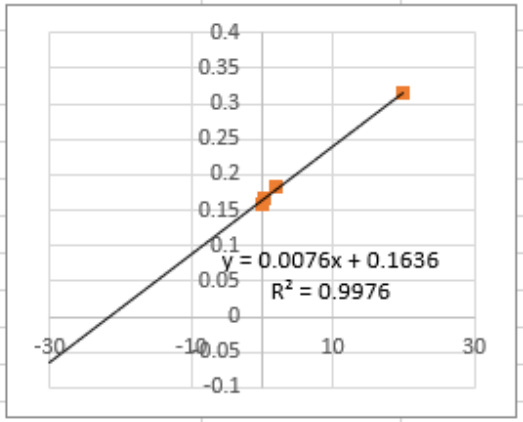
NPS DISCOVERY

Concentration: 10 ng/mL		
Sample Name	Conc. (ng/mL)	PAR
Target Sample	0	0.083
Up-spike #1	0.2	0.092
Up-spike #2	2	0.105
Up-spike #3	20	0.252
Y-Intercept	-	**0.0872**
X-Intercept	**10.6**	-



Concentration: 20 ng/mL		
Sample Name	Conc. (ng/mL)	PAR
Target Sample	0	0.159
Up-spike #1	0.2	0.166
Up-spike #2	2	0.183
Up-spike #3	20	0.316
Y-Intercept	-	**0.1636**
X-Intercept	**21.4**	-



Recovery	
Pre-1	0.157
Pre-2	0.158
Pre-3	0.169
Pre-Avg	0.161
Post-1	0.175
Post-2	0.149
Post-3	0.176
Post-Avg	0.167
Recovery	**96.8**

Matrix	No issues
Standard	No issues
ISTD	No issues
Carryover	Mininal (<1000 area)

Verification Data ↑

"Authentic" Sample →

WO# XXXXXXXX CFSRE# XXXX-XXXX-X		
Sample Name	**Conc. (ng/mL)**	**PAR**
Target Sample	0	0.103
Up-spike #1	0.2	0.1
Up-spike #2	2	0.112
Up-spike #3	20	0.285
Y-Intercept	-	**0.0983**
R2 Correl	-	**0.998**
X-Intercept	**10.6**	-
Cal. Curve	**11.5**	**-9%**

cfsre NPS DISCOVERY

Butonitazene Quantitation

Sample ID	Concentration (ng/mL)	Peak Area Ratio (Response)	[Butonitazene] (ng/mL
Standard Addition 1.1 (Blood)	0	0.197	
Standard Addition 1.2	0.5	0.228	
Standard Addition 1.3	5	0.554	**Blood**
Standard Addition 1.4	50	3.402	3.3
Standard Addition 2.1 (Serum)	0	0.196	
Standard Addition 2.2	0.5	0.234	
Standard Addition 2.3	5	0.687	**Serum**
Standard Addition 2.4	50	4.58	2.4
Standard Addition 3.1 (Urine)	0	1.134	
Standard Addition 3.2	0.5	1.186	
Standard Addition 3.3	5	1.713	**Urine**
Standard Addition 3.4	50	6.641	10

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Conclusions / Take Aways

- Standard addition is a scientifically valid approach for quantitation in forensic toxicology
  - Toxicologists should consider its utility in their labs
- Sample volume consumed may be an issue
- Method assessment is still **required**
- Allows for quantitation when other methods or approaches are not available
  - Particularly useful for new and emerging NPS
- *There are other ways to perform standard addition*

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PAPER
Toxicology

Brorphine—Investigation and quantitation of a new potent synthetic opioid in forensic toxicology casework using liquid chromatography-mass spectrometry

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Abstract

New synthetic opioids continue to appear as novel psychoactive substances (NPS) on illicit drug markets. Isotonitazene emerged in mid-2019, becoming the most prevalent NPS opioid in the United States within a few months. Notification by the Drug Enforcement Administration of its intent to schedule isotonitazene in mid-2020 led to its decline in popularity and replacement with a new NPS opioid: brorphine. Brorphine is a potent synthetic opioid, but little information was previously available regarding its toxicity or involvement in impairment and death. Our laboratory developed an assay for the identification and quantitative confirmation of brorphine using standard addition. Quantitative analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). *In vitro* and *in vivo* metabolism

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NPS DISCOVERY

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  - Joseph Homan
  - Donna Papsun
- **Forensic partners**

- **Provide Feedback or Insights**
  - We would love to hear from you
  - Fellow colleagues are looking for different perspectives and innovative approached
  - Email: alex.krotulski@cfsre.org
  - *Does your lab use standard addition?*
  - *Do you have an assessment process?*
  - *Do you use standard addition in a high-volume, high-throughput environment?*
  - *Do you have other ideas about implementation?*

Thank You!

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Quantitative forensic toxicology

Consideration, experimentation and implementation

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