

HRMS in Regulated Bioanalysis?

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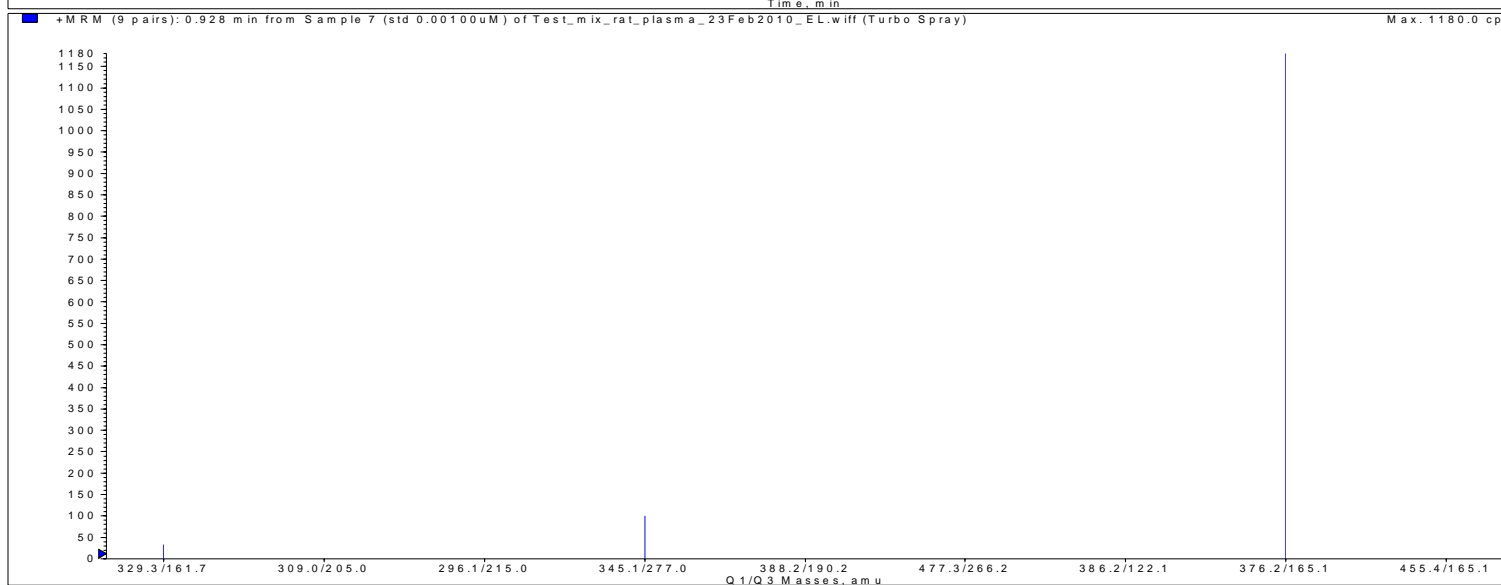
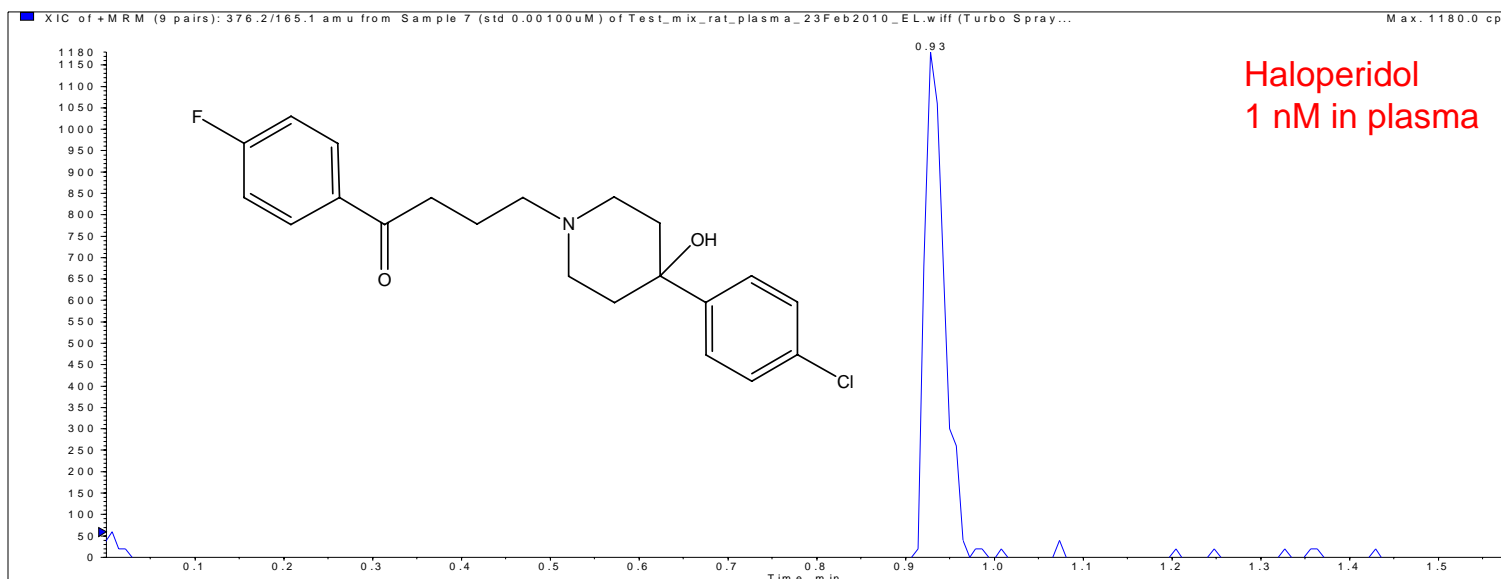
Triple Quadrupole Mass Spectrometry

- Selective
 - Based on two stages of mass selection, parent m/z and fragment ion m/z
- Sensitive
 - Beam instrument with high duty cycle
- Robust
 - Calibration stable over long period
- Compact Data Sets
 - MRM data files are very small and easy to process
- 25+ years of constant development for quantitative applications

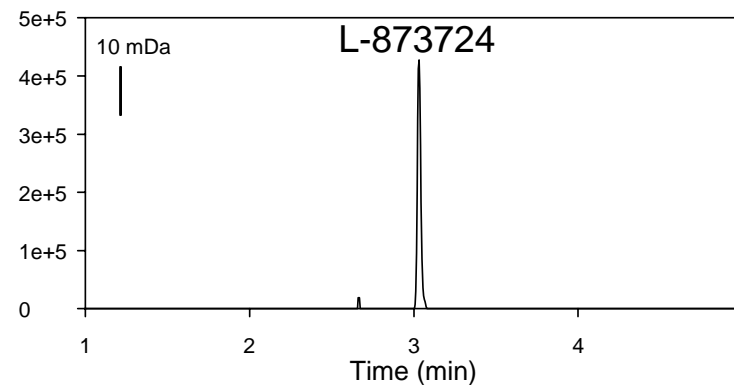
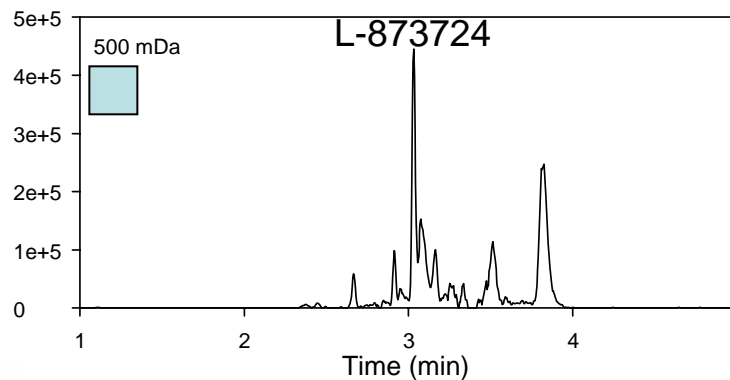
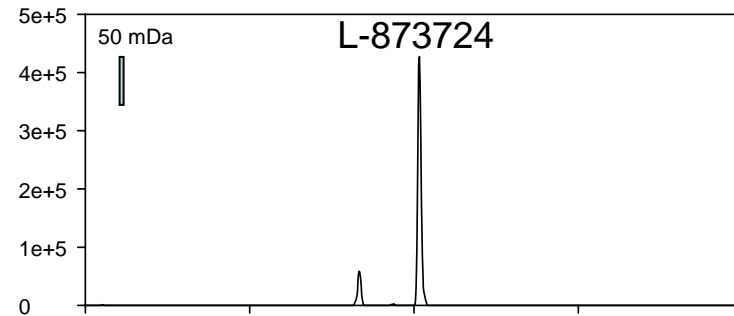
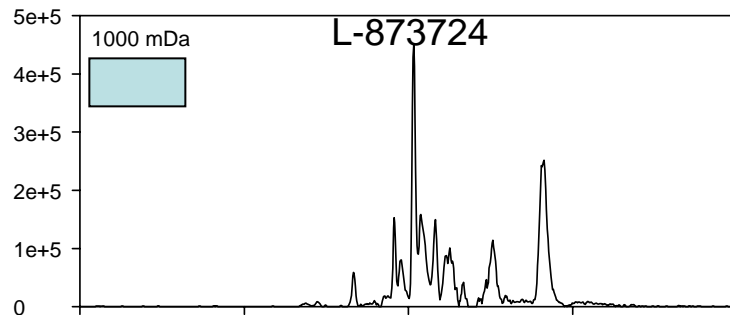
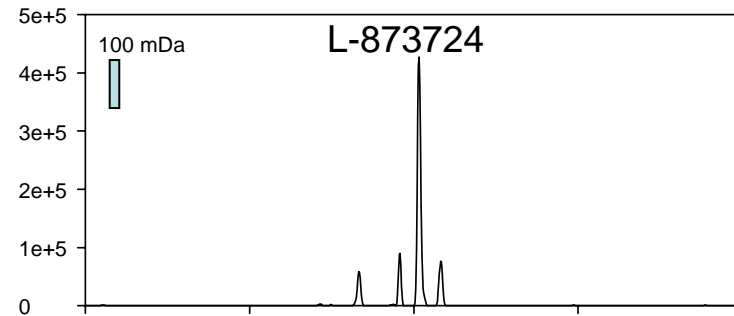
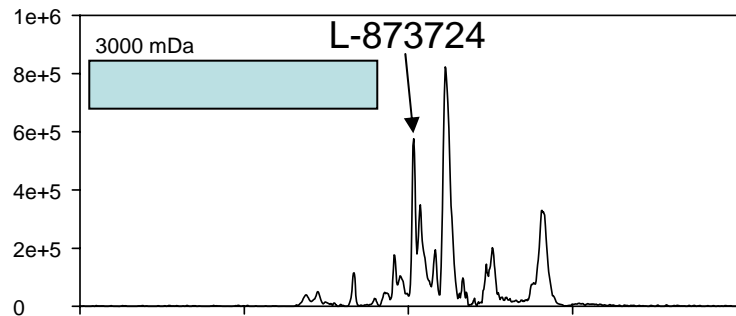
High Resolution Mass Spectrometry

- Selective?
 - Based on resolving power
- Sensitive?
 - Pulsed (TOF) or Indirect Detection (FT traps) based instruments
- Robust?
 - Calibration stable over long period (using internal calibration protocols)
- Large Data Sets
 - Full scan data files are large and time consuming to process
- ~3-5 years of recent development for quantitative applications

Selectivity: SRM Based Quantitation



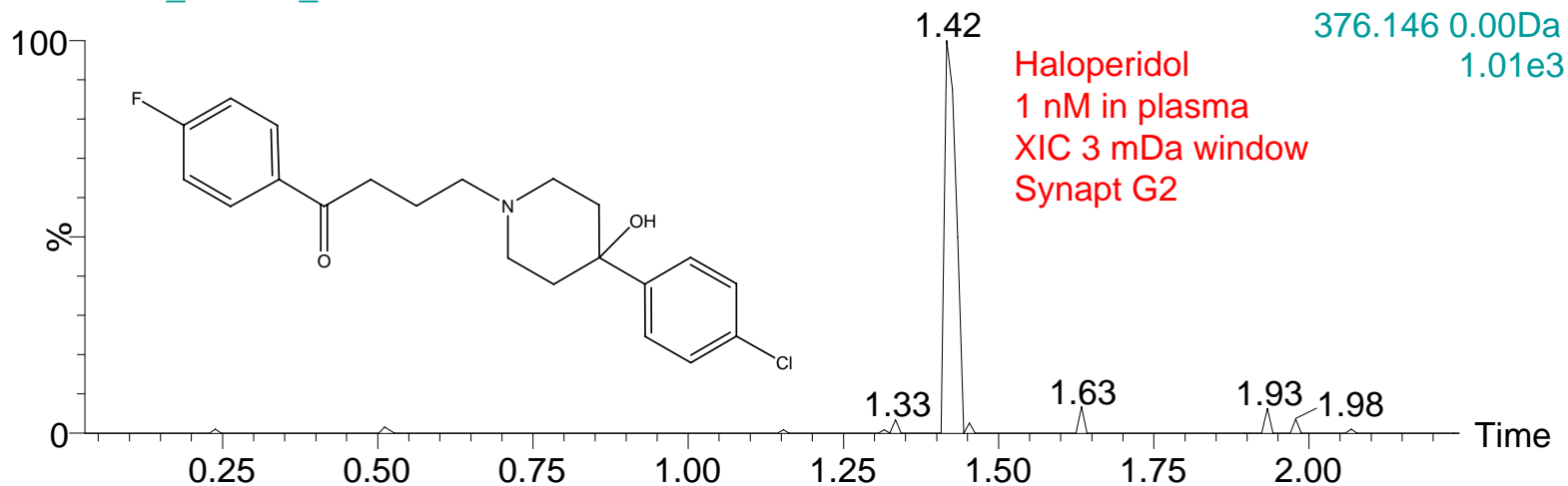
Why Mass Accuracy and Stability Matters: Narrow Window XIC from Rat Plasma t=15 min



Full Scan Based Quantitation

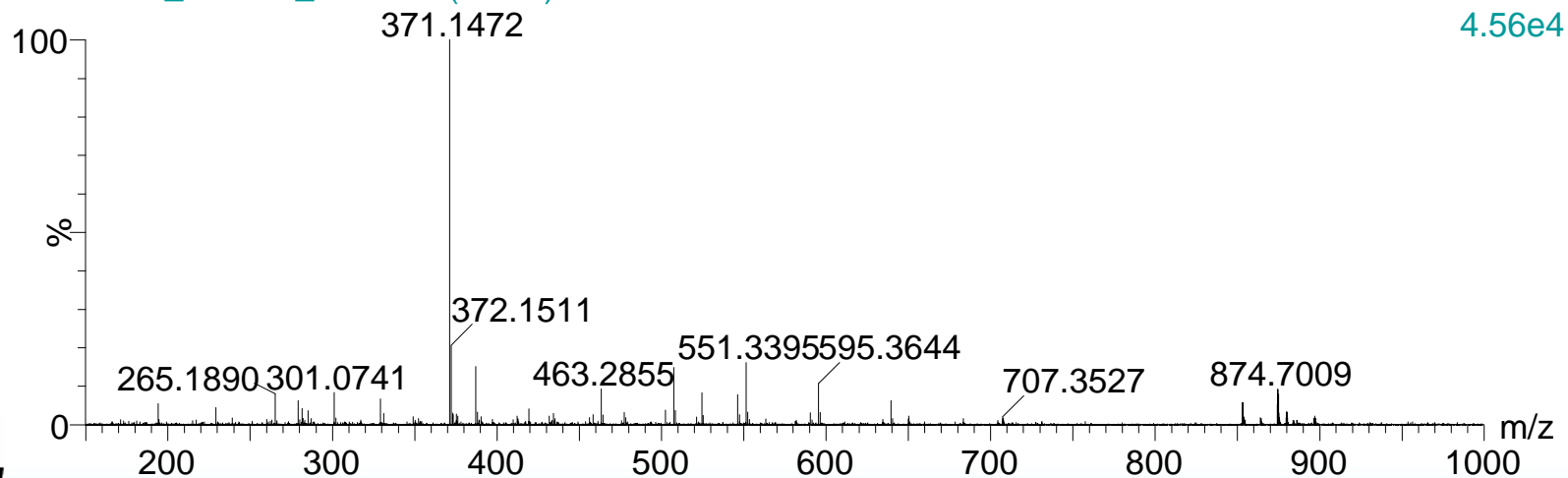
Standard, 6 compound Mix, 0.001

24feb2010_PKTest_010



Standard, 6 compound Mix, 0.001

24feb2010_PKTest_010 149 (1.416)

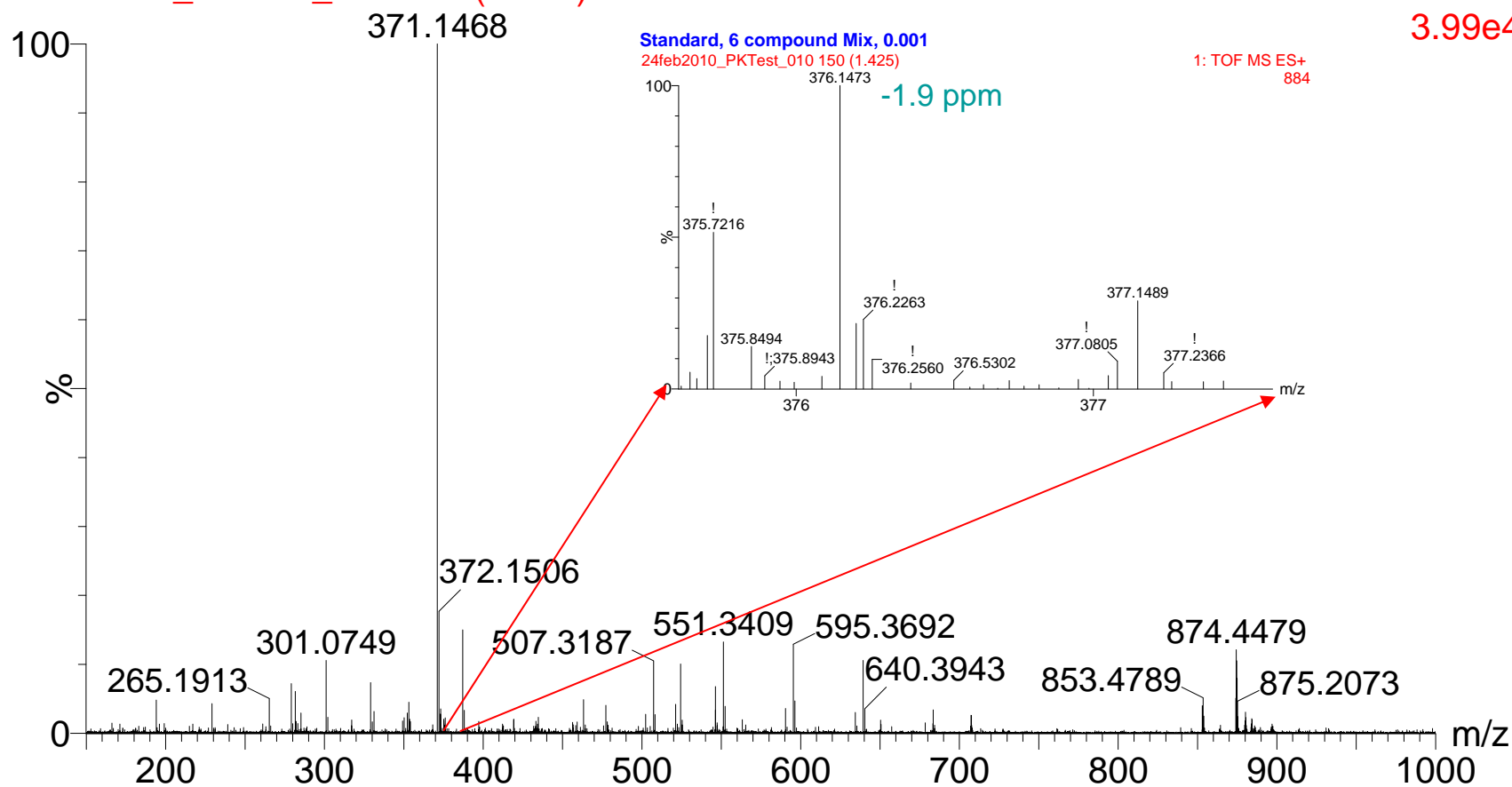


Plasma is a Dirty Matrix

Standard, 6 compound Mix, 0.001

24feb2010_PKTest_010 150 (1.425)

1: TOF MS ES+
3.99e4



Why Resolution Matters: Rat Plasma t=15 min

Experimental
Data

482.1712
R=22000
-1.53 ppm

481.5 482.0 482.5 483.0 483.5

$M/\Delta M = 20000$

481.5 482.0 482.5 483.0 483.5

$M/\Delta M = 10000$

481.5 482.0 482.5 483.0 483.5

$M/\Delta M = 5000$

481.5 482.0 482.5 483.0 483.5

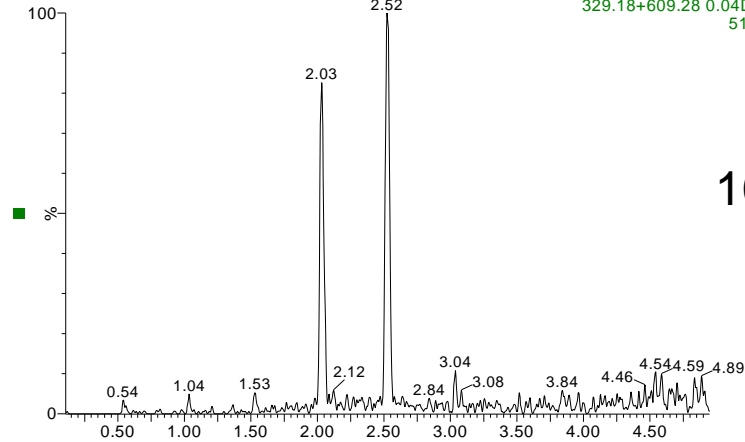
m/z

At a resolution <20,000 FWHM the compound will be over quantified due to un-resolved contaminants.

Both improved mass resolution and chromatographic resolution are needed for accurate quantification.

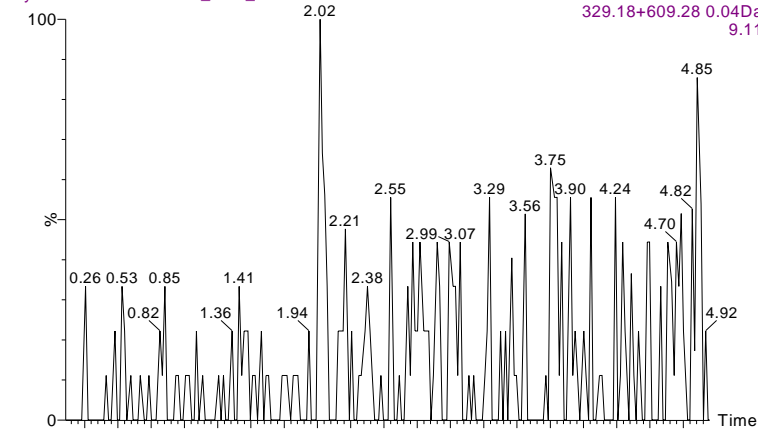
Sensitivity Comparison Between Q-TOF Synapt G1 QToF and Xevo G1 QToF

S3, MAX-RP UAA056 04-Feb-2009 17:18:41
 0.0
 SystemCheck20090202_10nM_3 Sm (SG, 2x1); Sm (SG, 2x1) 1: TOF MS ES+
 329.18+609.28 0.04Da 51.1



10nM

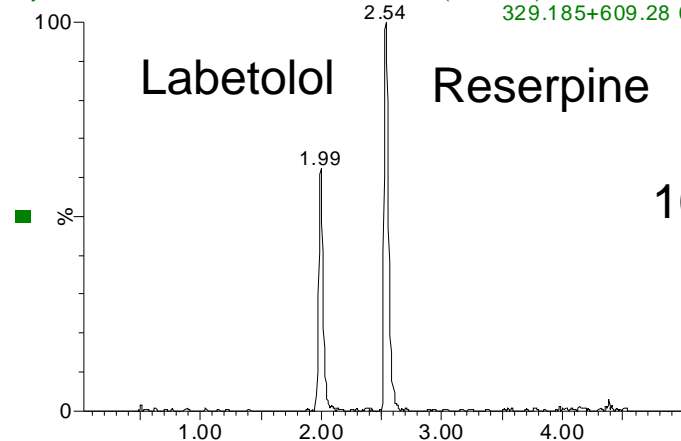
SystemCheck20090202_1nM_1 1: TOF MS ES+
 329.18+609.28 0.04Da 9.11



1nM

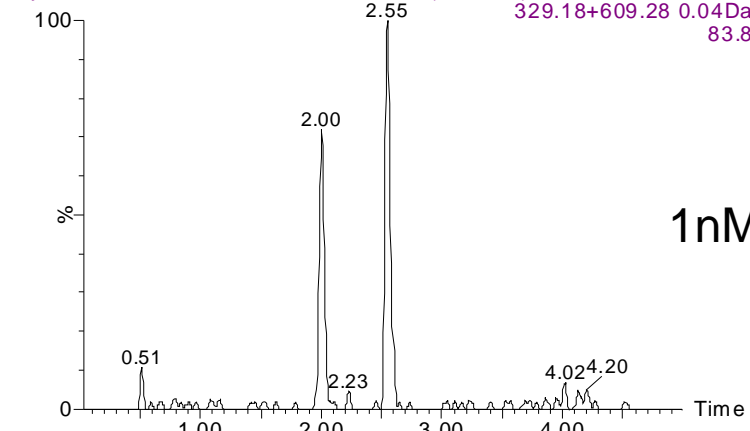
Conditions: full scan at 10,000 resolution; scan time: 0.2s; source temp. 550°C; flow rate 0.5 mL/min; 5 uL injection volume;

Xevo QToF MS 04-Feb-2009
 YAA019 16:55:29
 Systemcheck_2009_02_05_10nM_2 Sm (SG, 2x3) 1: TOF MS ES+
 329.185+609.28 0.04Da 1.07e3



10nM

Systemcheck_2009_02_05_1nM_1 Sm (Mn, 2x3) 1: TOF MS ES+
 329.18+609.28 0.04Da 83.8



1nM

Xevo is about 10-20x more sensitive than the Synapt

Future HRMS Workflows

- Quantitative analysis:
 - Able to reach level of sensitivity given by the SRM
 - Good Linearity
- Method selectivity:
 - Can resolve chromatographic interference
 - Decrease baseline noise (signal to noise)
- Method development:
 - Reduce method development working days
 - Methods (extraction and chromatographic) simplified

Small Molecule - MK-A

- Clinical method- monoisotope 821, Human plasma, Isocratic, 2x50 mm UPLC, L/L cleanup

Expected Conc. (ng/mL)	No. Of Values Used	API 4000 %		API 5000 %		AB 5500 %		AB 5600 ^a %		AB 5600 ^b %	
		% C.V.	Accuracy	% C.V.	Accuracy	% C.V.	Accuracy	% C.V.	Accuracy	% C.V.	Accuracy
1	6 of 6	6.0	102	8.7	100	4.2	101	11.7	102	n/a	n/a
2.5	6 of 6	2.1	96	3.0	99	2.1	99	11.0	97	15.8	99
5	6 of 6	2.1	97	2.8	102	1.1	99	6.4	96	8.2	100
10	6 of 6	1.9	99	1.6	101	2.2	101	5.0	97	4.3	102
100	6 of 6	1.7	100	0.8	103	1.2	102	2.9	101	6.5	106
500	6 of 6	1.3	103	0.9	102	0.6	104	5.0	103	3.8	105
1000	6 of 6	1.4	101	0.8	99	0.7	100	2.7	101	5.3	99
1750	6 of 6	1.4	100	1.2	98	1.9	97	4.1	102	2.7	94
2000	6 of 6	1.7	100	0.7	97	1.4	97	1.6	101	2.7	93
Range of Values		1.3 - 6.0	96 - 103	0.7 - 8.7	97 - 102	0.6 - 4.2	97 - 102	1.6 - 11.7	96 - 103	2.7 - 15.8	93 - 106

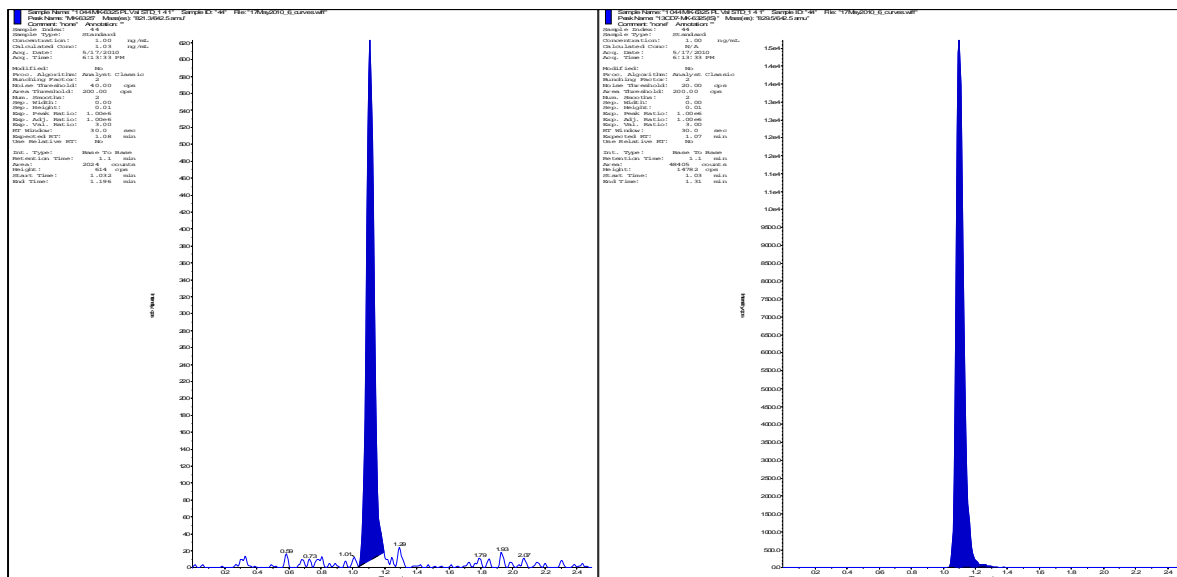
^a - AB 5600 TOF quant with 40 mDa width

^b - AB 5600 MRM quant with 40 mDa width

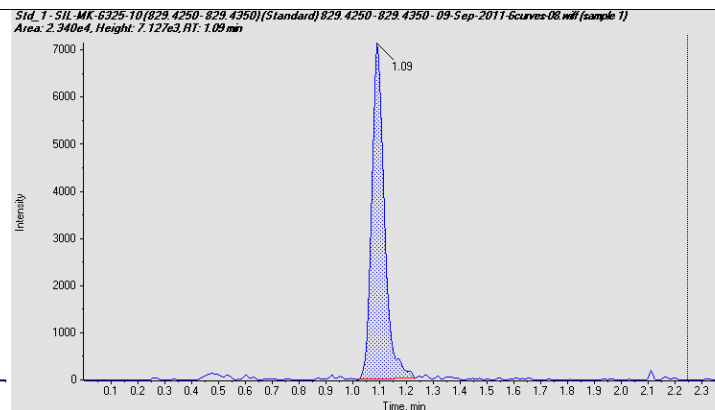
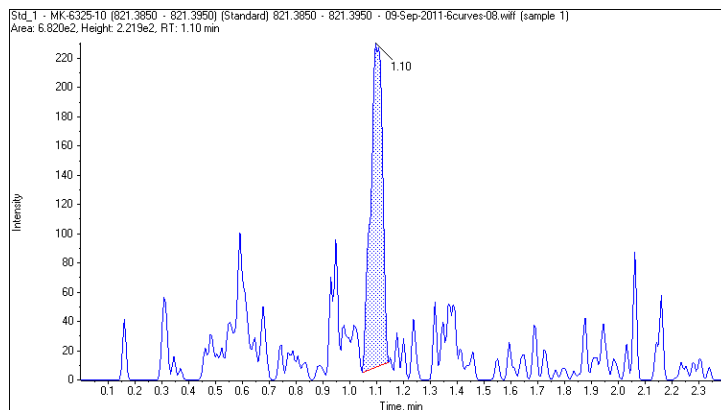
LLOQ Chromatograms

Representative Chromatogram at LLOQ (1 ng/mL)

API-4000



API-5600



Small Molecule - MK-A

Sensitivity of Detection:

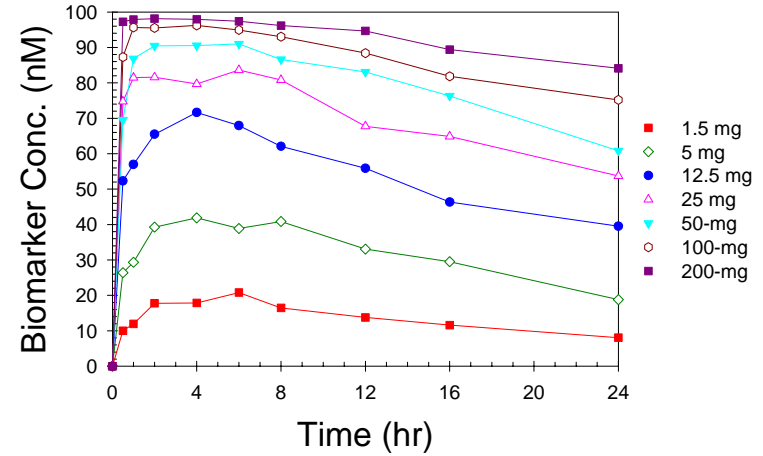
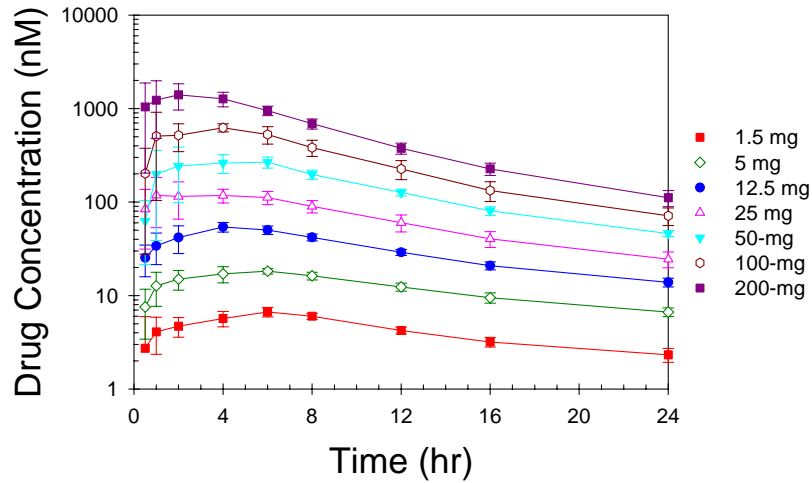
MS	Vol (uL) Injection	Intensity at LLOQ	Average Noise	S/N	Intensity at ULOQ	Range of IS Response
API 4000	8	600	20	30	8.0×10^5	$3.8 \times 10^4 - 4.8 \times 10^4$
API 5000	2	850	30	28	7.5×10^5	$7.5 \times 10^4 - 1.2 \times 10^5$
AB 5500	2	1250	50	25	2.3×10^5	$1.3 \times 10^5 - 1.6 \times 10^5$
AB 5600 ^a	8	450	100	5	8.0×10^5	$5.0 \times 10^4 - 7.0 \times 10^4$
AB 5600 ^b	8	42	4	11	7.0×10^3	$2.0 \times 10^4 - 3.0 \times 10^4$

^a - AB 5600 TOF quant with 40 mdalton width

^b - AB 5600 MRM quant with 40 mdalton width

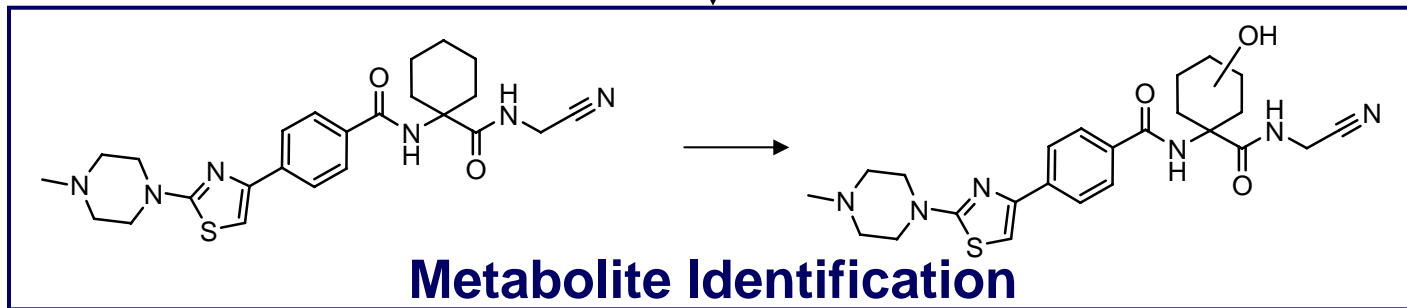
API 5600 is equivalent to the API 4000 in terms of LLOQ. The API 5600 can meet all assay analytical requirements.

Multiplexed Analysis



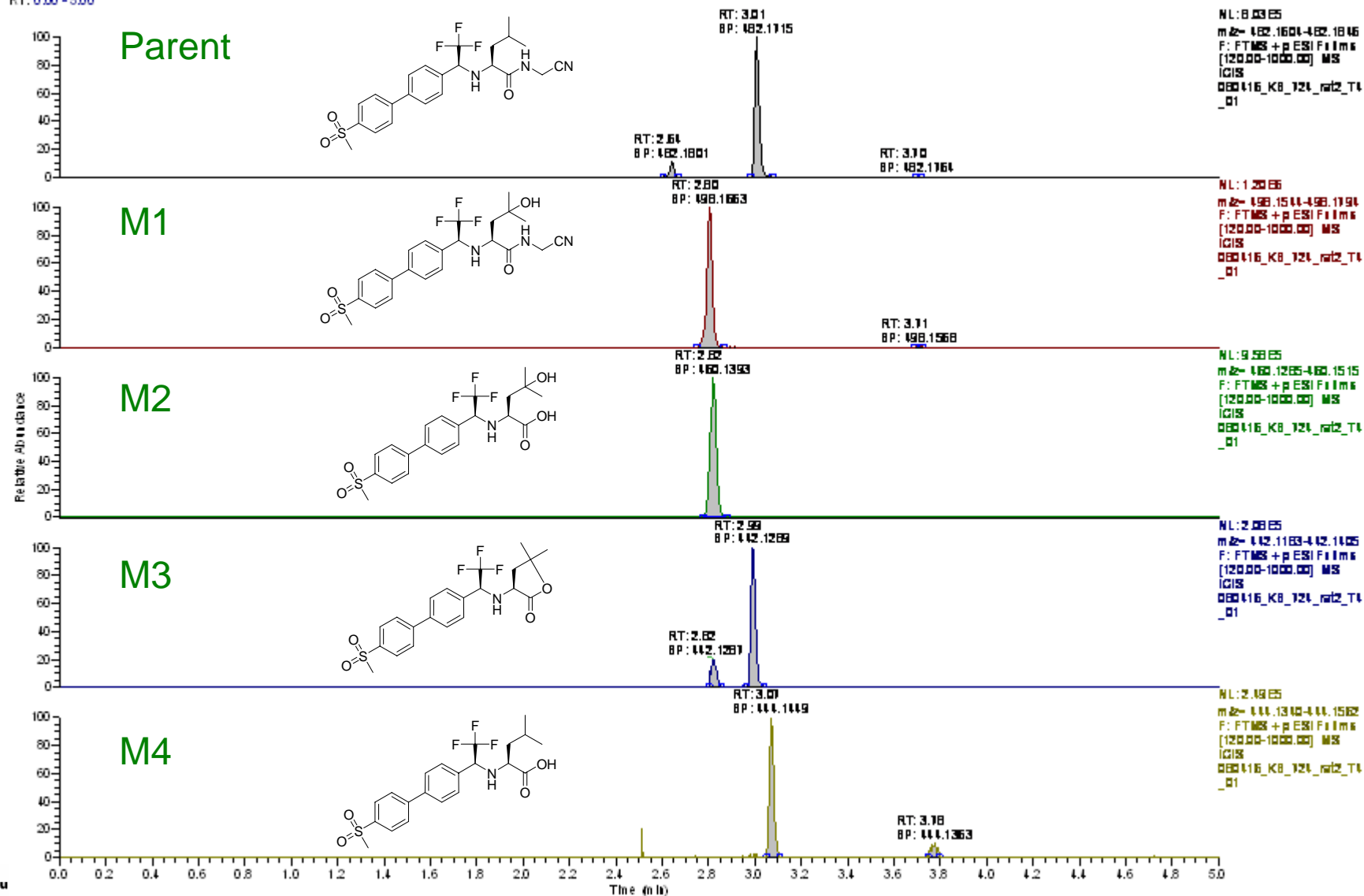
Drug Measurements

Biomarker Measurements

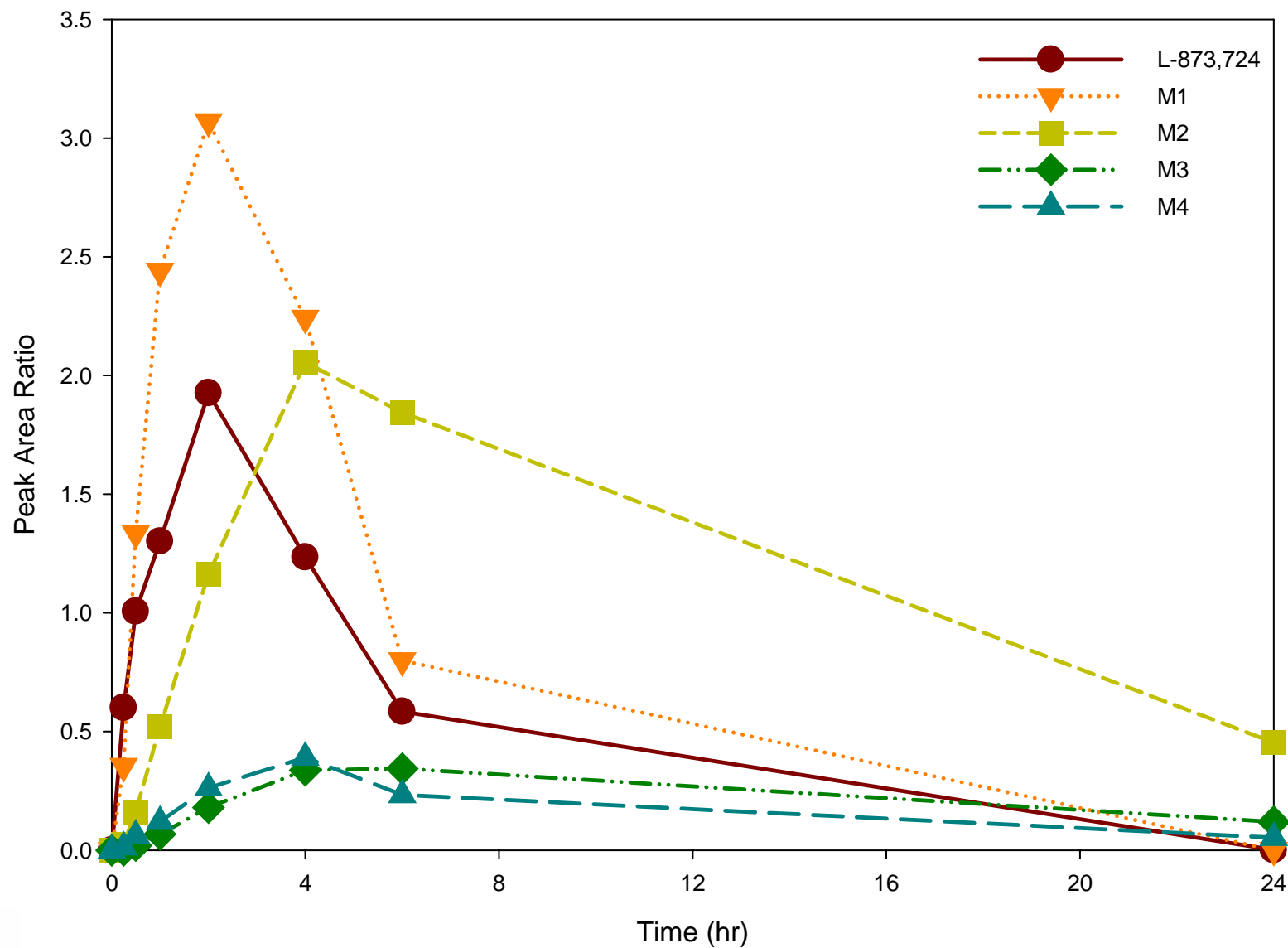


In vivo Metabolites (4 hr rat plasma time point)

RT: 0.00 - 5.00



Metabolite Profiles in Plasma



Leveraging HRMS Data

- The ability to detect both the target compound and a variety of other endogenous and exogenous species can be very useful during method development.
- Interferences from co-eluting metabolites can be understood and resolved to improve the overall method.
- Chromatographic optimization can be undertaken without the need for reference standards.

HRMS and DBS

- Clinical DBS analysis of MK1775 and MK0518 indicated low recovery from stored QC DBS relative to fresh QC DBS.
- The objective of this study was to analyze the DBS extracts from stored and fresh QC cards to see if there any degradation occurred.
- If no degradation products are observed, it may indicate that extraction efficiency of stored DBS is not the same as fresh DBS.

Sample Preparation and Analysis

- DBS High QC's extracted using validated protocol
- 5 uL of extract was injected onto UPLC-HRMS
 - Waters Xevo Qtof
- Data processed to look for parent and potential degradation products

MK-1775

Expected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
Parent	C27H32N8O2	-0.0015	501.2711	-1.5	8.13	227.60

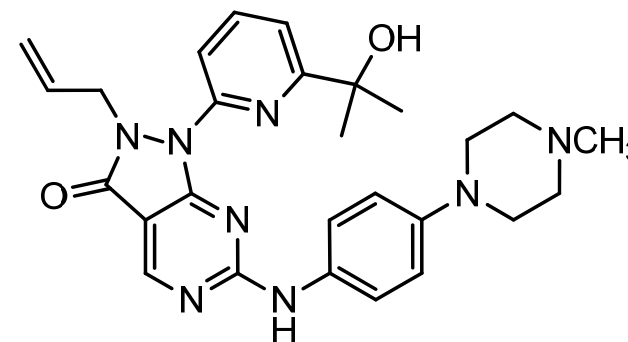
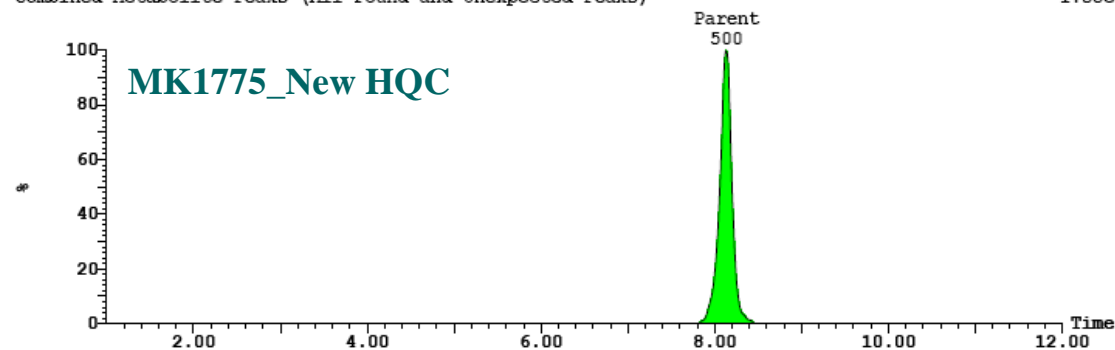
Unexpected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
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Total metabolites: 1

Combined Metabolite Peaks (All Found and Unexpected Peaks)

1.35e3



Expected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
Parent	C27H32N8O2	0.0008	501.2734	0.8	8.08	93.60
+O2-H2	C27H30N8O4	29.9767	531.2493	2.5	9.35	6.80
+O2-H2	C27H30N8O4	29.9793	531.2520	5.2	9.49	6.50

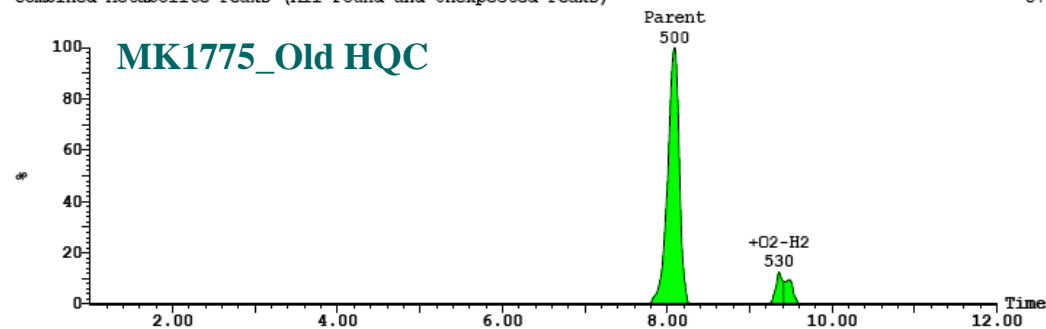
Unexpected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
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Total metabolites: 3

Combined Metabolite Peaks (All Found and Unexpected Peaks)

576



Analysis of fresh versus stored QC's by HRMS indicated degradation of the compound. Stability is only established for a shorter time.

MK-0518

Expected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
Parent	C ₂₀ H ₂₁ FN ₆ O ₅	-0.0005	445.1630	-0.5	7.42	97.00

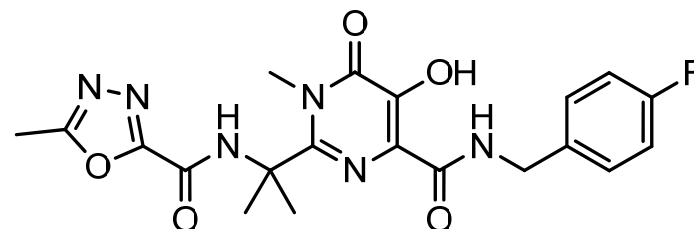
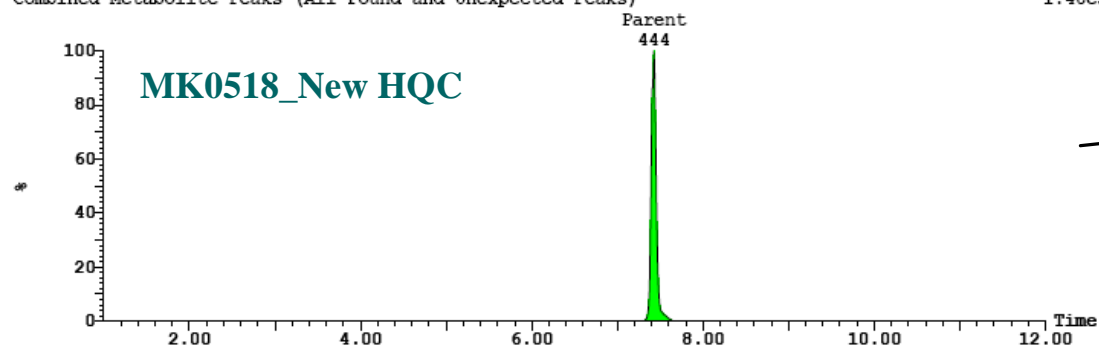
Unexpected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
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Total metabolites: 1

Combined Metabolite Peaks (All Found and Unexpected Peaks)

1.48e3



Expected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
Parent	C ₂₀ H ₂₁ FN ₆ O ₅	-0.0026	445.1609	-2.6	7.43	55.00

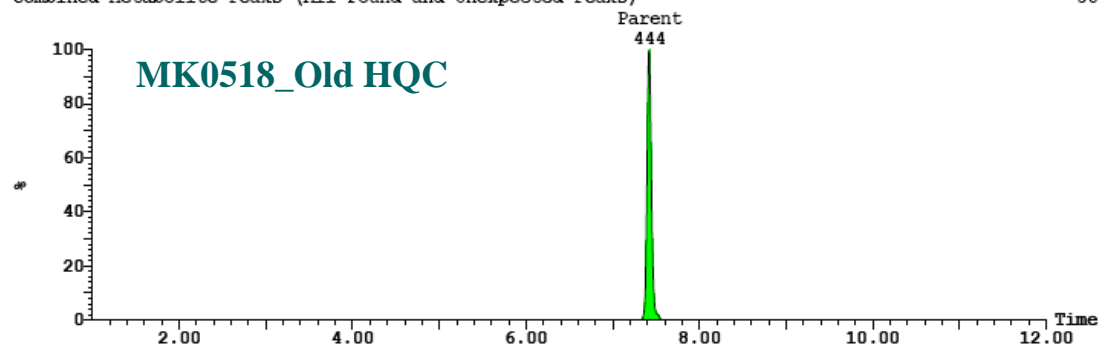
Unexpected Metabolites:

Metabolite Name	Formula	Mass Difference	m/z Found	mDa	Time	Area Abs
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Total metabolites: 1

Combined Metabolite Peaks (All Found and Unexpected Peaks)

867



Analysis of fresh versus stored QC's by HRMS indicated the compound was stable.

Stability is established for a longer time and new extraction method developed.

Future Drug Development Workflows

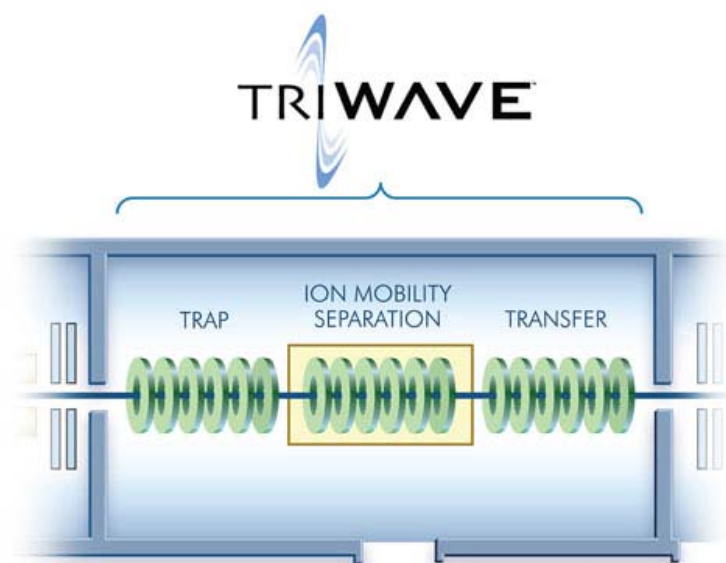
- When compared to a SRM, will HRMS instruments:
 - Resolve chromatographic problems?
 - Can have improved selectivity with HRMS, but will not fix “ionization” issues
 - Perform bioanalytical assays?
 - Yes
 - Simplify method development time?
 - Yes
 - Be robust and reliable?
 - Yes
 - Generate manageable data files?
 - ?, yet to be done – significant hurdle
 - Enable data processing throughput?
 - ?, need suitable software

Future Challenges

- HRMS hardware is ready for routine assay support
 - Sensitivity, Linearity, Selectivity are acceptable and improving
- Software continues to lag hardware
 - How to handle large datasets?
 - Validate data processing steps?
 - Centroid vs. Profile data?
 - Data reduction acceptable or not?
- Cost vs. Benefit of ownership
 - QQQ's are a commodity with large user base
 - HRMS are more complex with fewer users
 - Future developments in hardware and software will narrow the gap between QQQ and HRMS

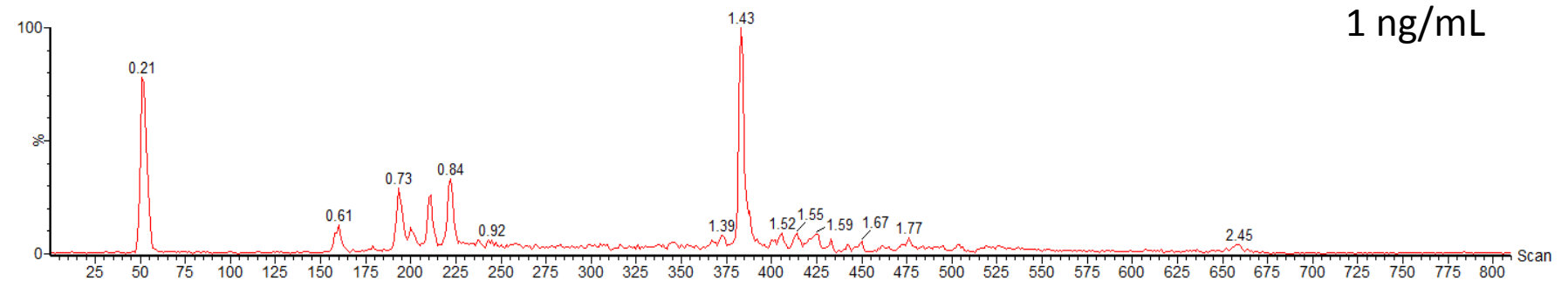
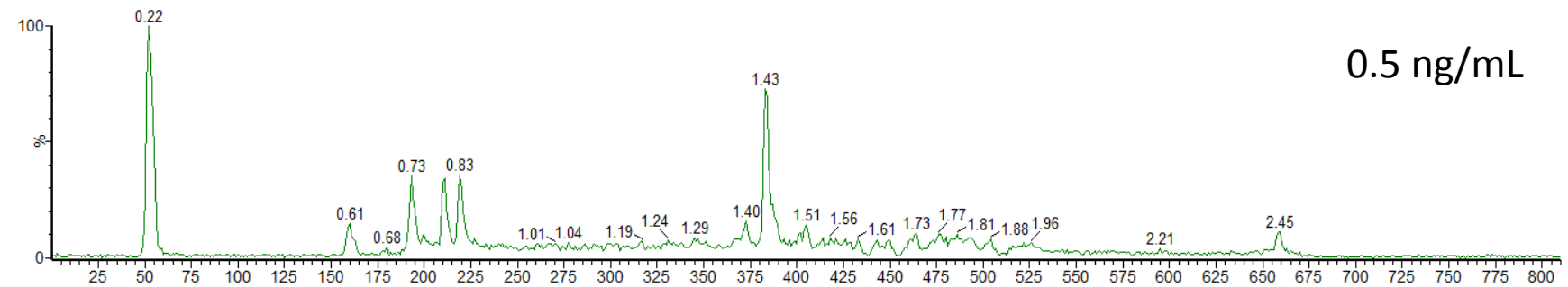
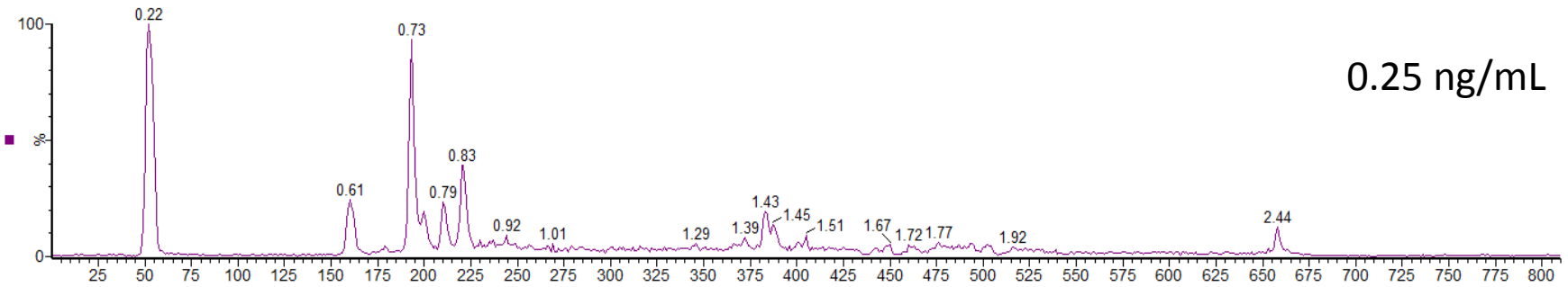
Ion Mobility with HRMS

- Waters G2-S using Ion Mobility
 - Additional level of selectivity beyond mass resolving power by using ion mobility
 - Early data looks promising, but much more work is needed
 - Data file size and data processing software will need to be addressed

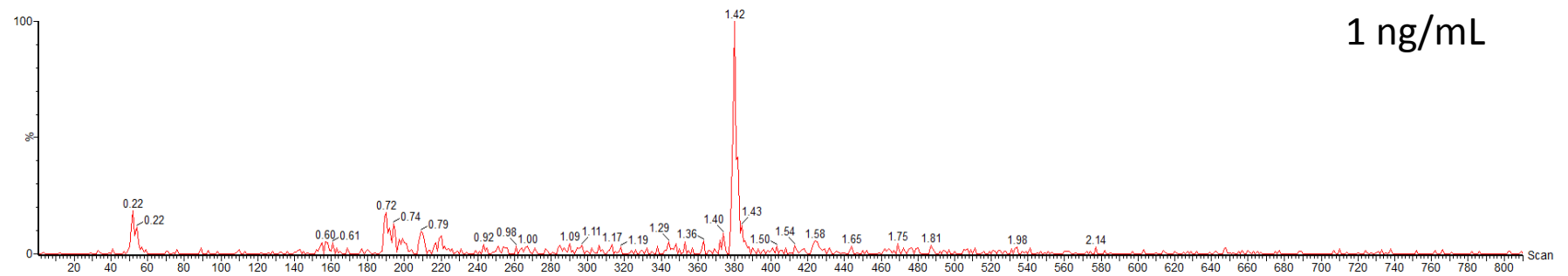
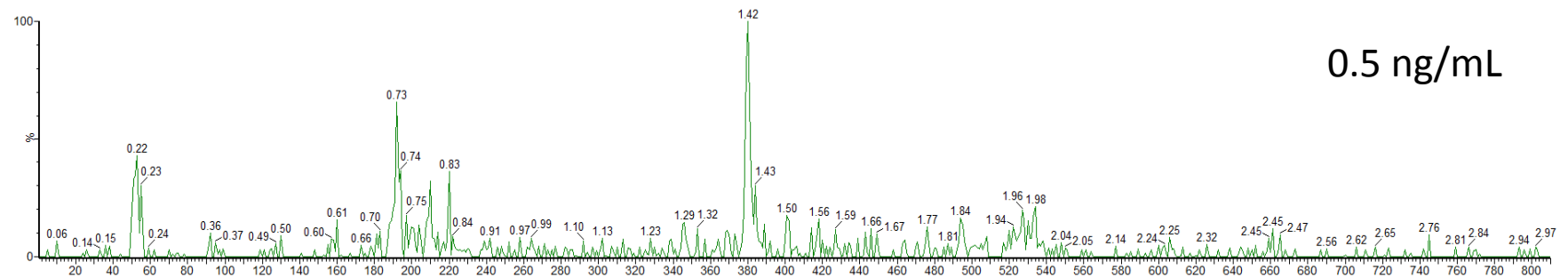
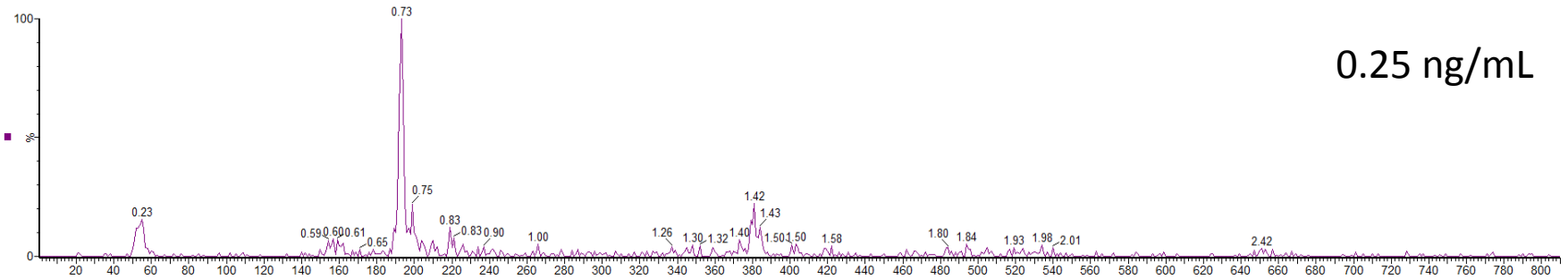


Tamoxifen

20mDa XIC, MS Only, IMS OFF

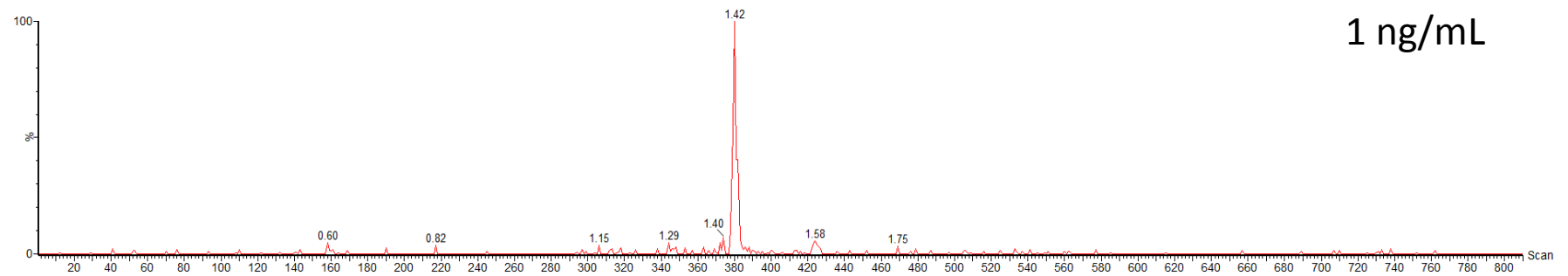
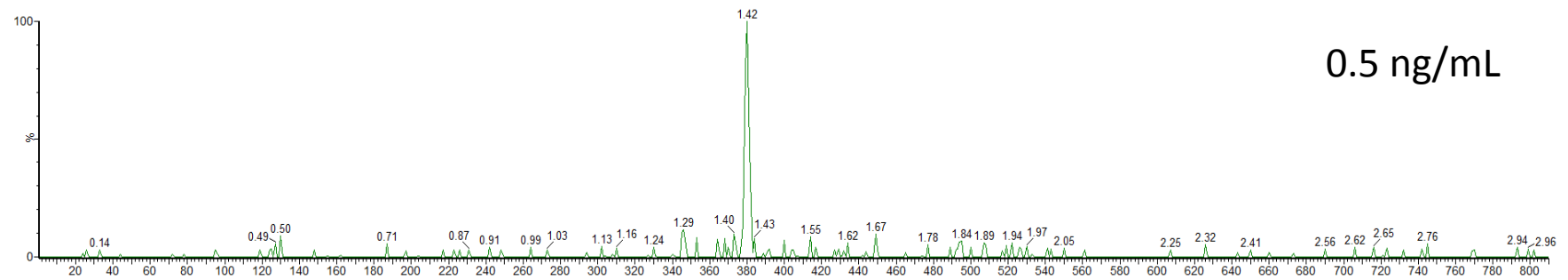
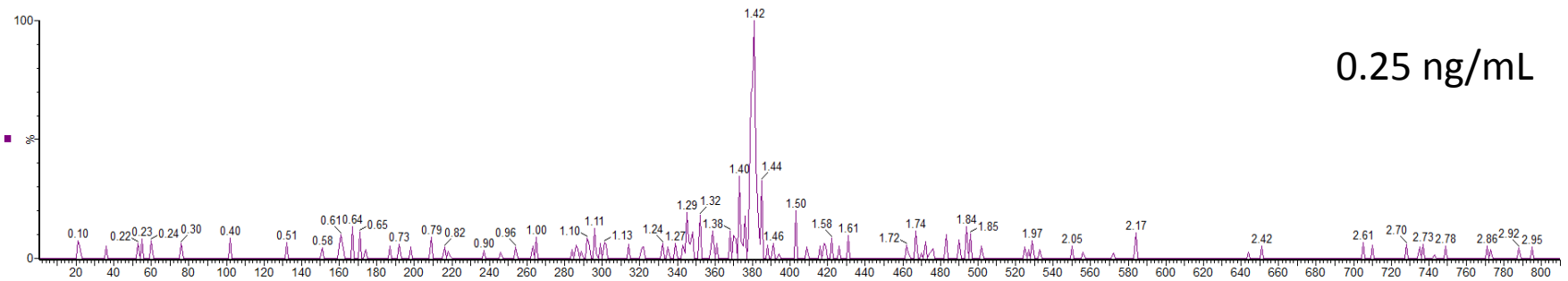


Tamoxifen 20mDa XIC, IMS full scan DT 1-200 (no filter)



Tamoxifen

20mDa XIC + 92-102 DT filter



What if...

- HRMS was developed instead of QQQ technology?
 - Full scan based data acquisition with sensitivity and selectivity equal to today's modern QQQ with fast data processing used routinely in today's bioanalytical labs.
- What would the discussion be like if QQQ's just came along now?
 - “I have to figure out how the compound fragments to analyze it?”
 - “I need a different MS method for every compound?”
 - “I have to know what I am looking for before I run my samples?”
 - “Nominal mass? Really? You must be joking?”
 - “What about all the other things in my samples?”

Are Triple Quadrupoles Dead?

