



Answers for Science.
Knowledge for Life.™



Advancing your Forensic Toxicology Analyses; Adopting the Latest in Mass Spectrometry Innovations

**Goal of analytical
chemistry**

=

**Separate / differentiate analytes
from other components**



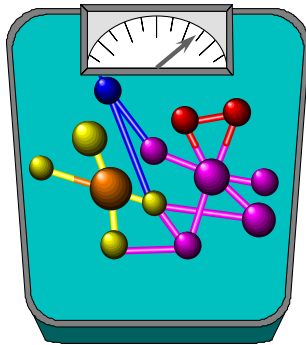
Analyte

Other components

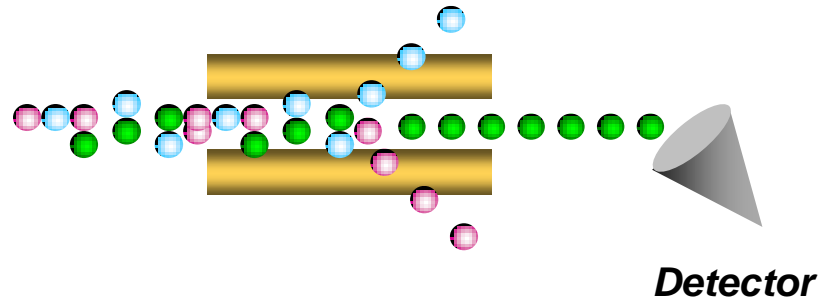
- Most of the sample
- Masks the analyte
- Highly selective tools are required to filter out the other components

Mass Spectrometry

- By measuring the mass of the analytes, mass spectrometers provide enhanced selectivity over other types of detectors.



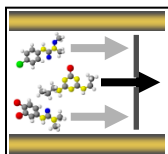
A mass spectrometer is essentially a “molecular scale”



Quadrupole MS systems are mass filters. Only the ion of interest is allowed to reach the detector

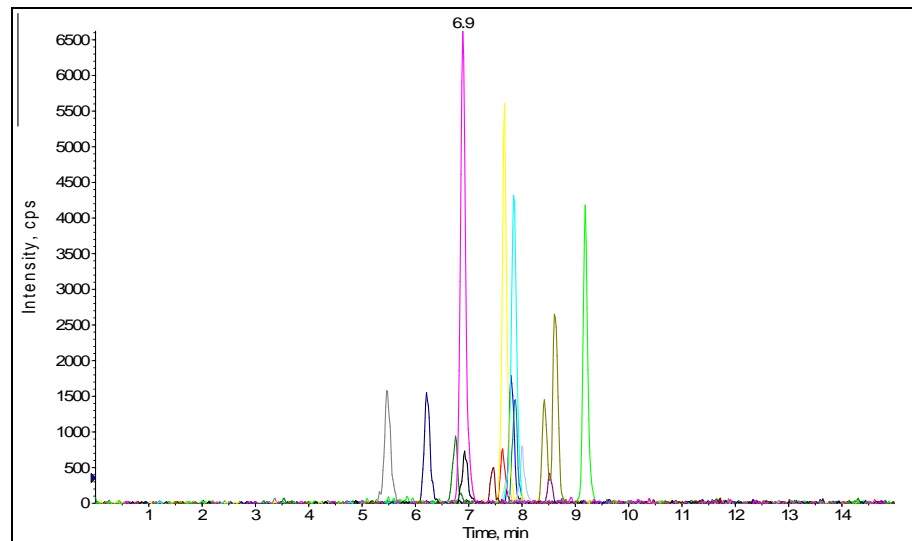
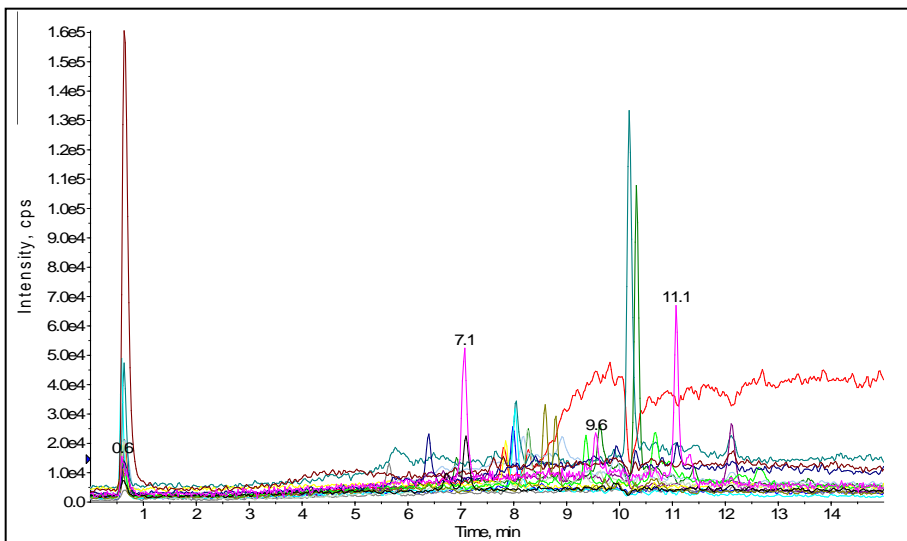
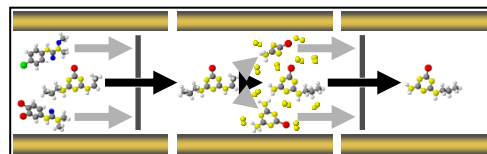
Single Quadrupole (MS)

- Lower selectivity
- Many interferences



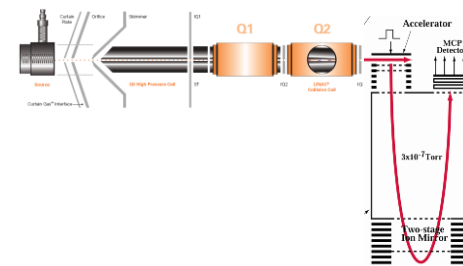
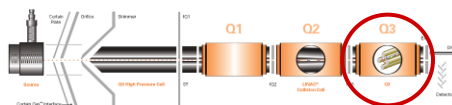
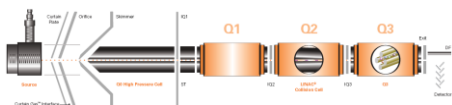
Triple Quadrupole (MS/MS)

- Excellent selectivity
- Few interferences



MS/MS provides far superior selectivity, therefore fewer interferences, because fragmentation patterns are so specific to each parent ion.

Key Features of AB SCIEX LC-MS/MS Systems



AB SCIEX Triple Quad™
System

QTRAP® system

TripleTOF® system

- **Quantitation**
- **ID with MRM ratio**

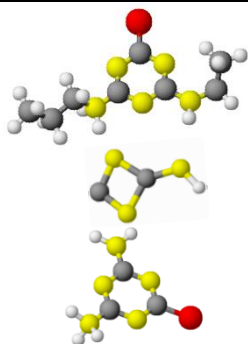
- **Quantitation**
- **ID with MRM ratio**
- **ID with MS/MS library searching**

- **Quantitation**
- **ID with accurate mass**
- **ID with MS/MS library searching**
- **ID true unknowns**
- **Retrospective data processing**

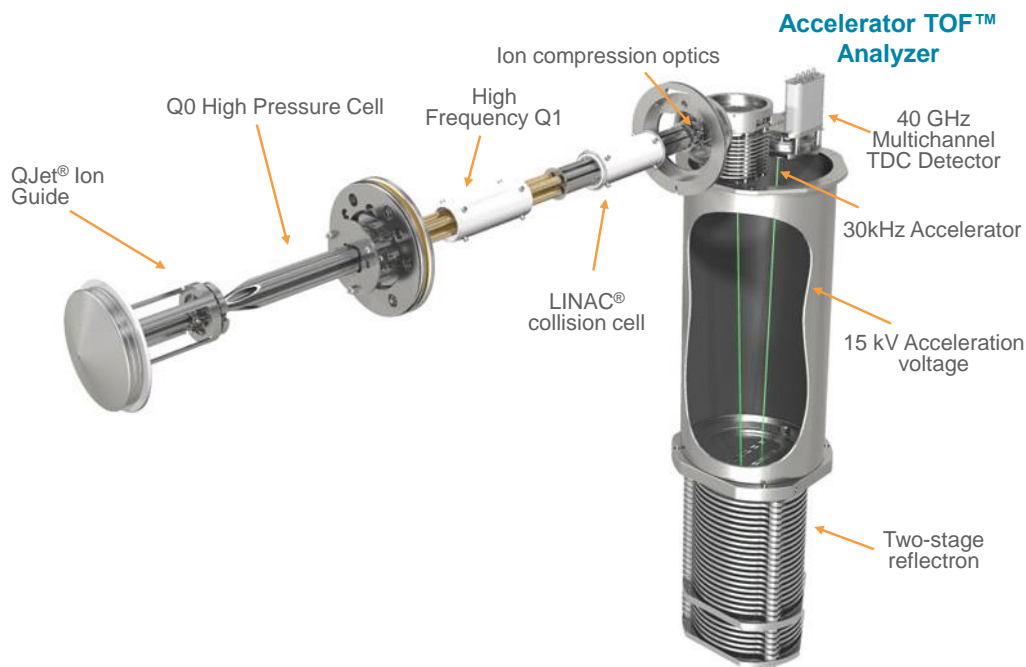
increasing confidence in compound ID

Principle of Time-of-Flight (TOF) Mass Spectrometry

- Ion packets are pulsed and accelerated into a TOF analyzer.
- Separation of ions is based on the time to traverse the flight tube, and arrive at the detector, on a nanosecond time scale.
 - Smaller m/z ions move faster than heavier m/z ions
- Higher resolution is achieved with longer flight path (longer TOF tubes, reflectors, faster acceleration).



All data has been acquired using the AB SCIEX TripleTOF® 5600+ System

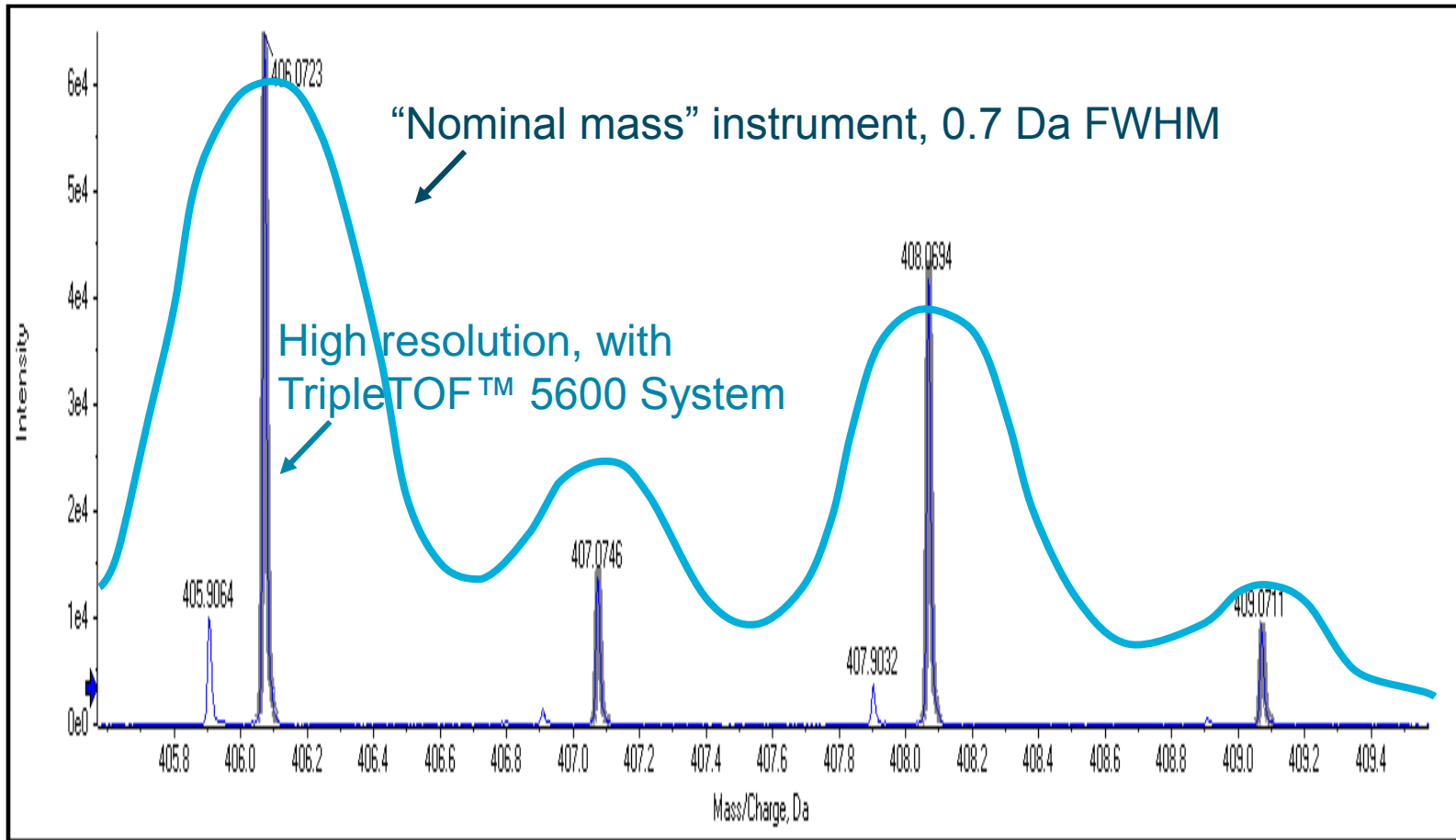


- Stable mass accuracy of ~1ppm RMS
- Fully automated calibration of MS and MS/MS
- Resolution ~30,000
- 100 Hz acquisition rate
- Linear dynamic range ~ 4 orders

Hybrid quadrupole / time-of-flight (TOF)
mass spectrometer

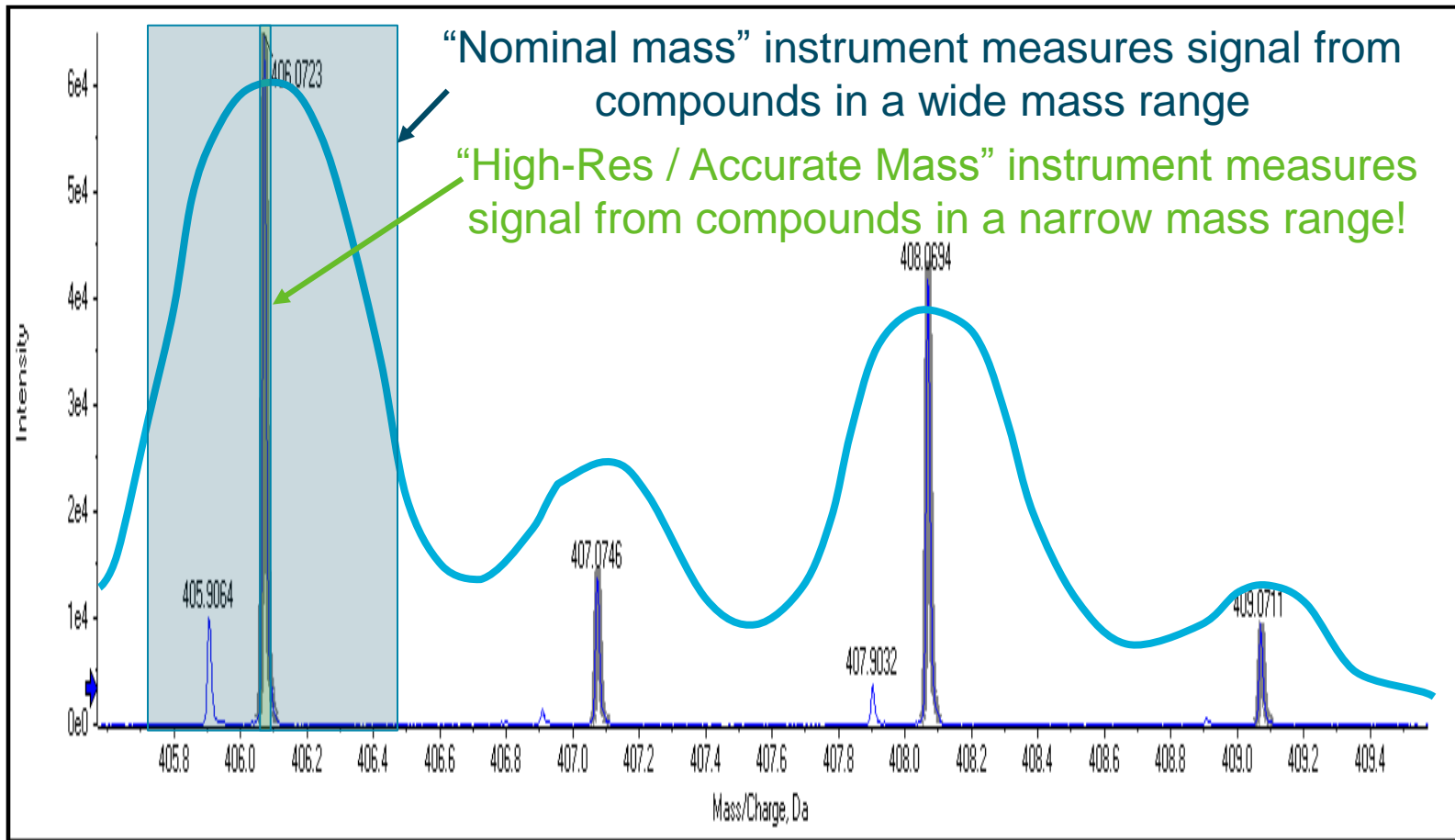
Why consider TOF technology for screening?

- Greater specificity, with high-resolution measurements



Why consider TOF technology for screening?

- Greater specificity, with high-resolution measurements



Why consider TOF technology for screening?

- Greater specificity compared to Triple Quadrupole Mass Spectrometers, with high-resolution measurements

A simple example:

4 different molecular formulae with nominal mass = 28 Da

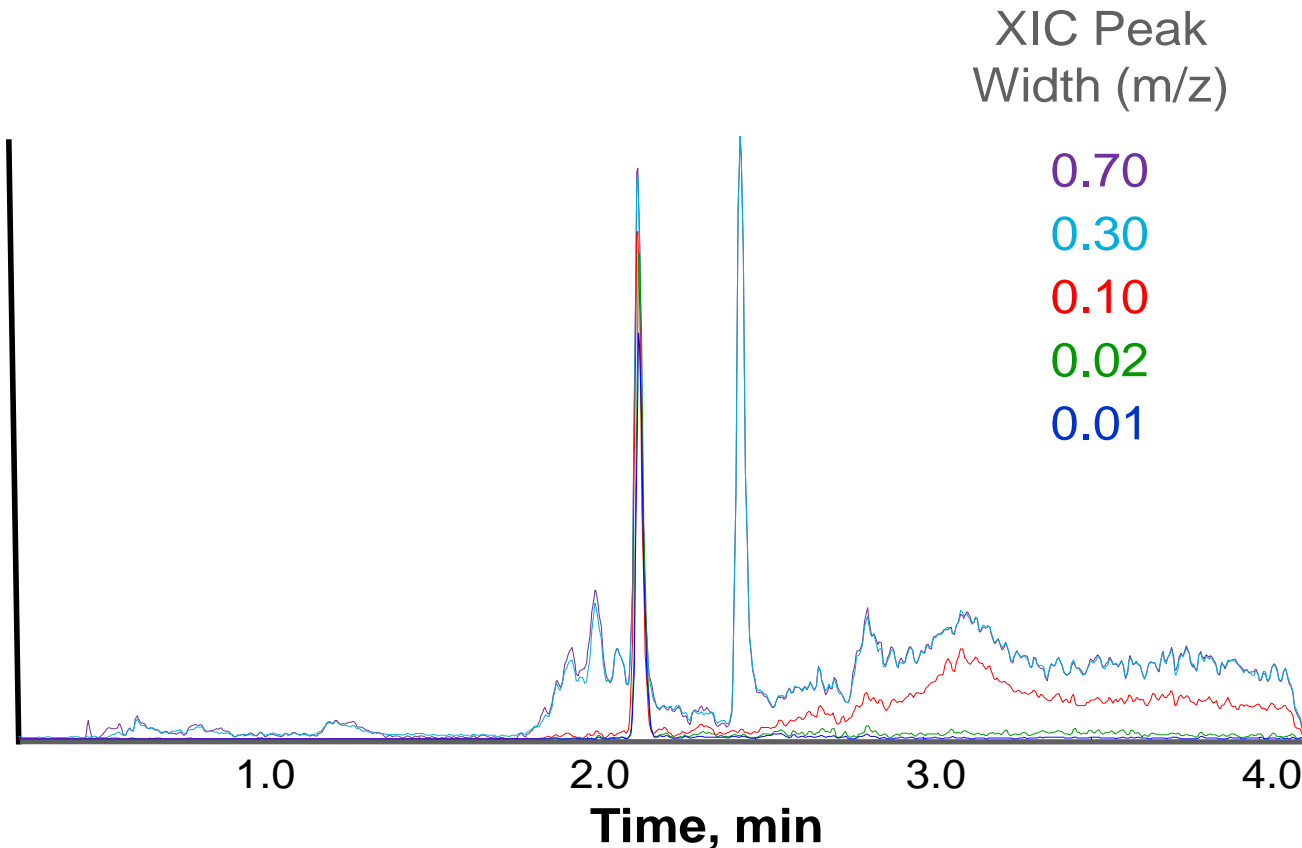
N₂	=	2 X 14.0031	= 28.0061
CO	=	12.0000 + 15.9949	= 27.9949
C₂H₄	=	(2 X 12.0000) + (4 X 1.0078)	= 28.0312
CH₂N	=	12.0000 + (2 X 1.0078) + 14.0031	= 28.0187



Cannot be distinguished by a “nominal mass” instrument

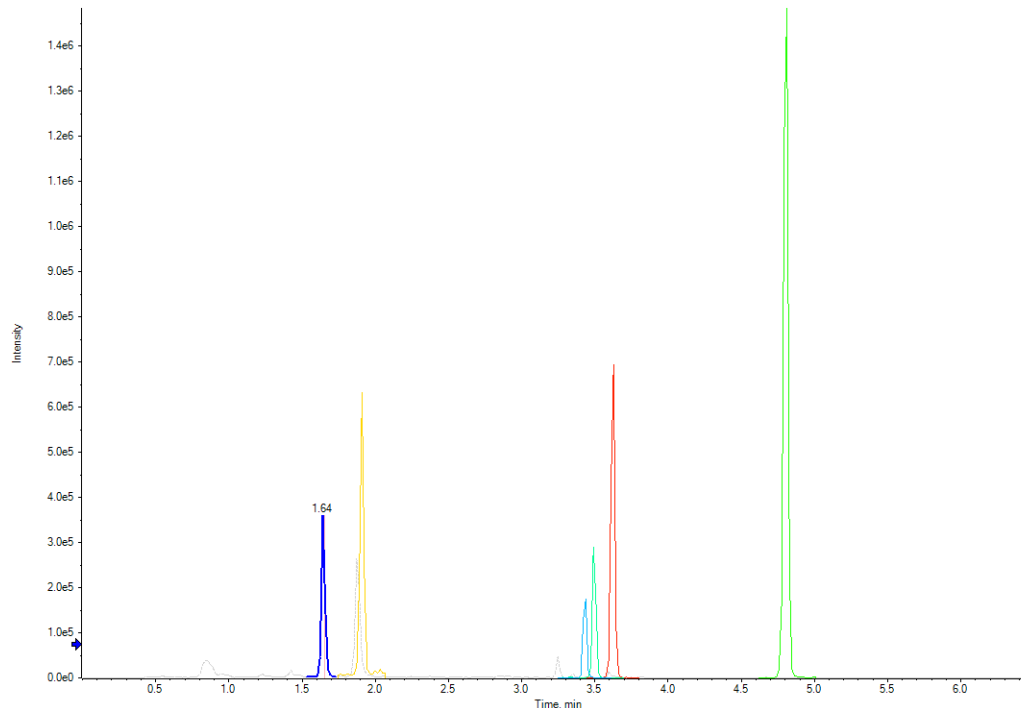
Why consider TOF technology for screening?

- Greater specificity compared to Triple Quadrupole Mass Spectrometers, with high-resolution measurements
- Improved S/N with narrower XIC extraction windows



Why consider TOF technology for screening?

- Greater specificity compared to Triple Quadrupole Mass Spectrometers, with high-resolution measurements
- Improved S/N with narrower XIC extraction windows
- Possible to re-interrogate data for unanticipated compounds



XIC (± 5 mDa) for:

Alprazolam

Benzoyllecgonine

Buprenorphine

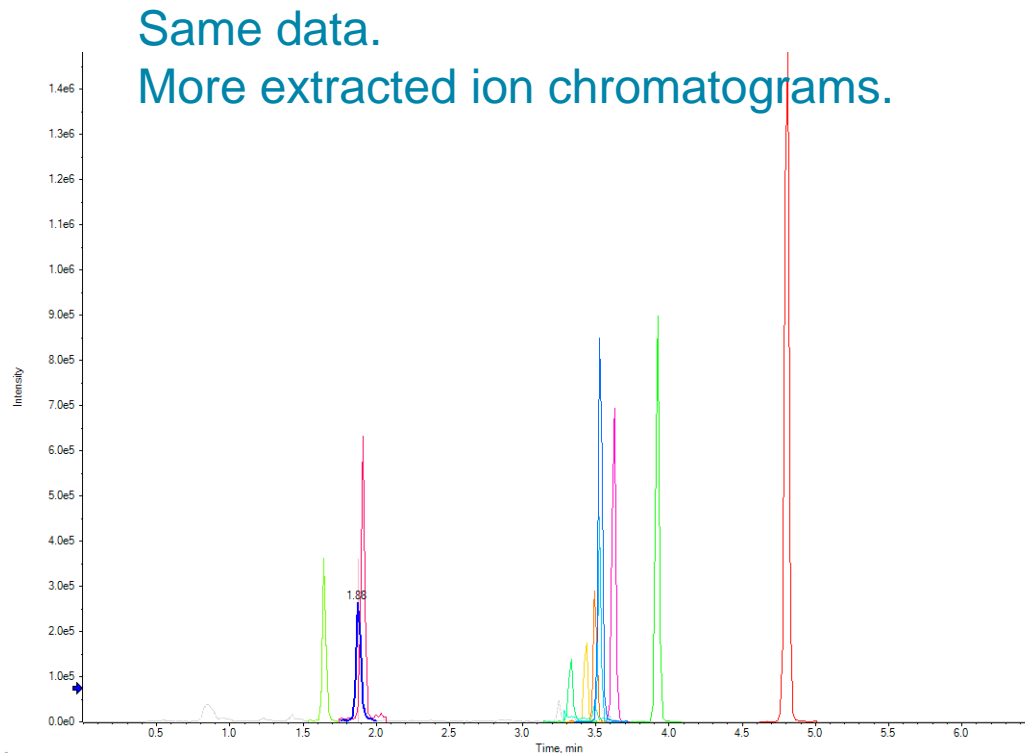
Carisoprodol

Clonazepam

Codeine

Why consider TOF technology for screening?

- Greater specificity compared to Triple Quadrupole Mass Spectrometers, with high-resolution measurements
- Improved S/N with narrower XIC extraction windows
- Possible to re-interrogate data for unanticipated compounds



XIC (± 5 mDa) for:

Alprazolam
Benzoyllecgonine
Buprenorphine
Carisoprodol
Clonazepam
Codeine
Diazepam
EDDP
Fentanyl
Flunitrazepam
Flurazepam
Hydrocodone

The Million \$\$\$ Question(s):

Is TOF-MS sufficiently selective? Can we get away from MS/MS if we use a high-resolution accurate mass system?

Why ask this question?

- TOF-MS methods are extremely convenient, because they are generic, and non-targeted
 - No method development is required for new compounds
 - It is possible to re-interrogate the data later, to look for other compounds
- We *know* that high-resolution TOF-MS provides far better selectivity than nominal mass (quadrupole) MS instrumentation
 - It is reasonable to wonder if the selectivity of TOF-MS is comparable to MS/MS on a triple quadrupole

Methods:

- 50+ compounds were spiked into authentic urine matrix
- Analysis was performed using both TOF-MS and TOF-MS/MS
- Extracted ion chromatograms (XICs) were generated using an extraction window of $\pm 5\text{mDa}$

Sample Prep:

- Spiked urine was diluted 10x with mobile phase A, vortexed, centrifuged, and injected directly onto the MS system

HPLC Conditions

- Flow rate = 500uL/minute
- Run-time = 6.5 minutes
- Column = Phenomenex Kinetex C18, 50x3.0mm, 2.6um
- Mobile phase A = 10mM ammonium formate
- Mobile phase B = 1:1 methanol:acetonitrile

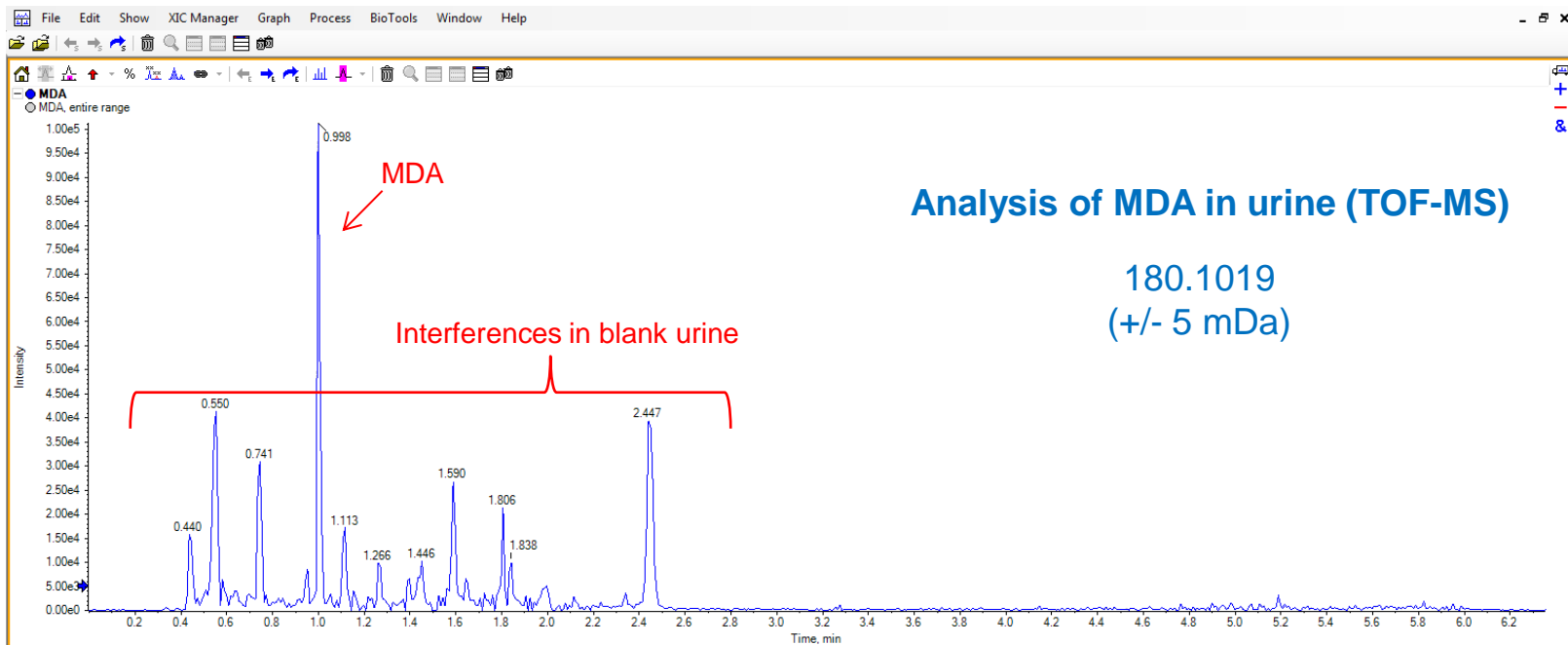


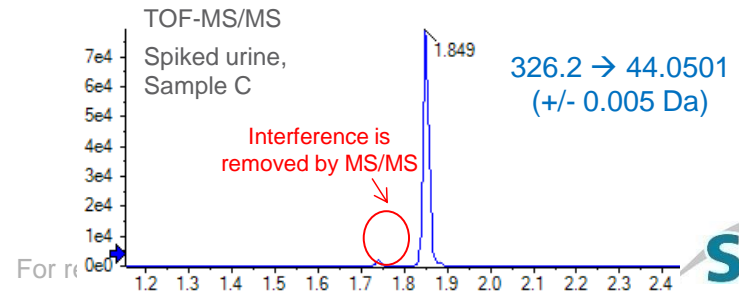
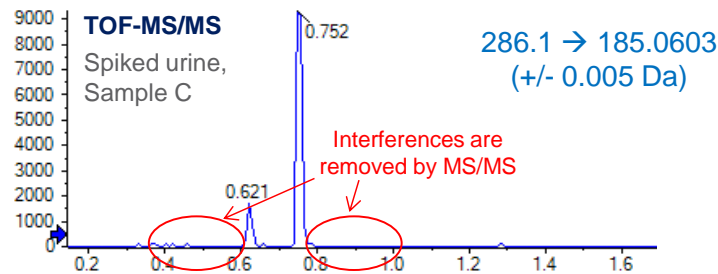
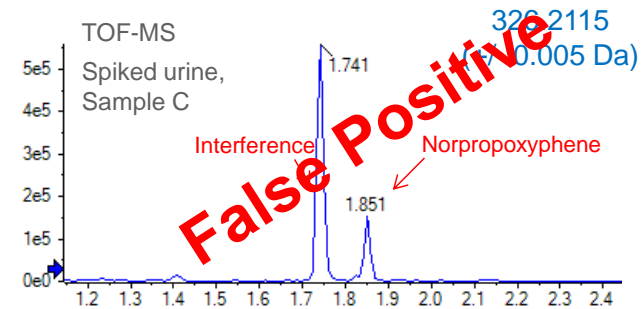
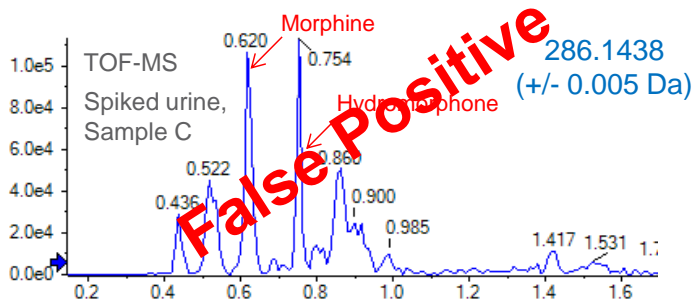
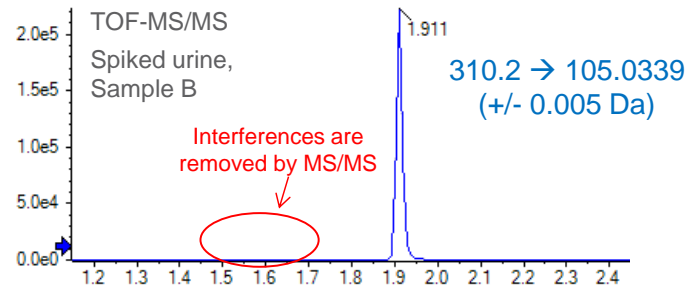
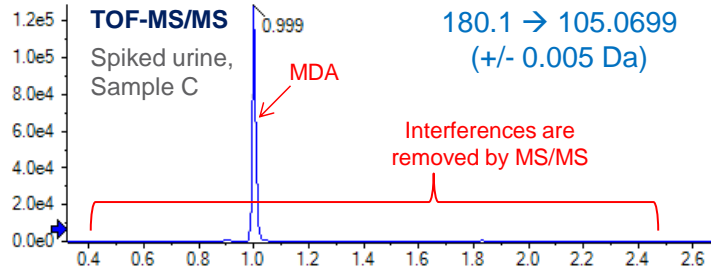
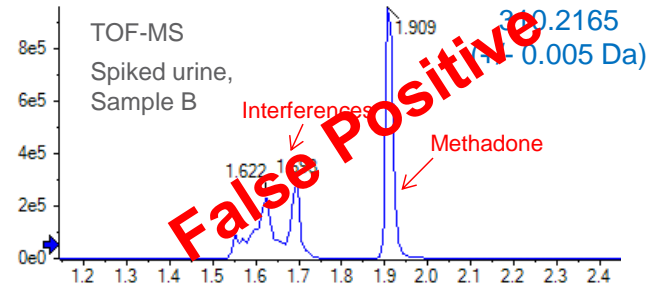
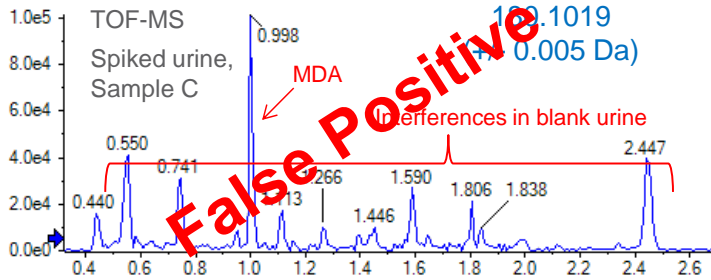
TripleTOF® 5600+
System

- Using only TOF-MS, even with a small extraction mass range (+/- 5mDa), there is still a possibility of observing interferences.
- In spiked urine, many compounds display interferences within the RT window.
- **MS/MS** is required for unambiguous ID (either XIC from MS/MS, or library search)

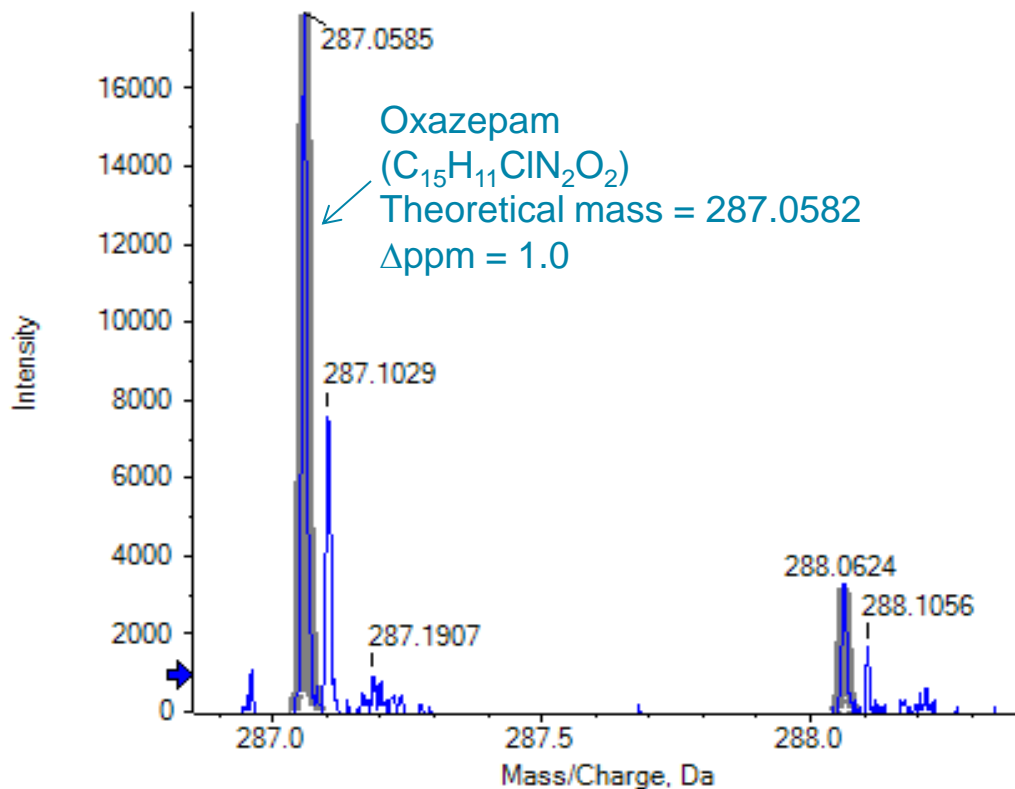
OR...

- **Chromatographic separation** is absolutely essential.





- With TOF-MS, identification of compounds relies upon:
 - (i) accurate mass measurements (within several ppm of theoretical value)
 - (ii) isotope patterns (matches the 'expected' pattern)

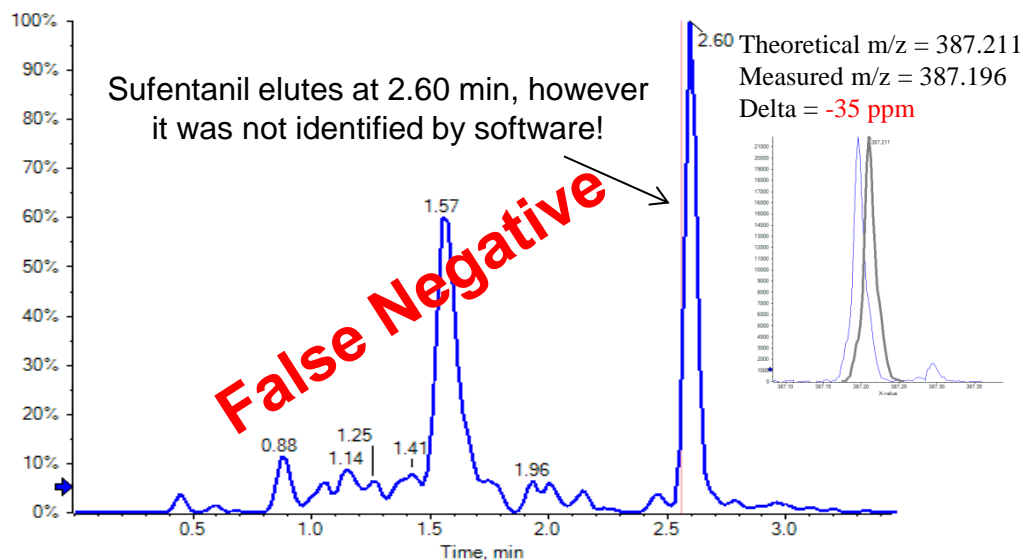


Example: TOF-MS enables the separation of Oxazepam from an interference having the same nominal mass.

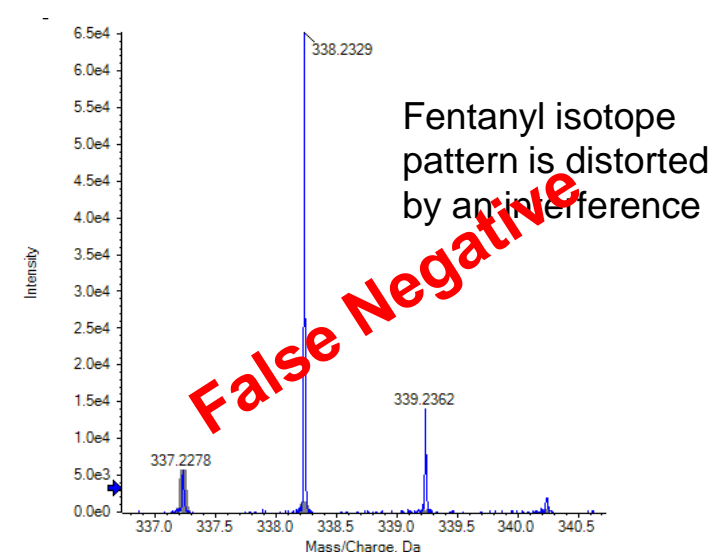
- With TOF-MS, identification of compounds relies upon
 - (i) accurate mass measurements (within several ppm of theoretical value)
 - (ii) isotope patterns (matches the 'expected' pattern)

Problem #1: Accurate mass measurements can be distorted by co-eluting, isobaric compounds

Problem #2: Isotope patterns can be distorted by co-eluting (non-isobaric!) compounds



Example 1. The measured mass for Sufentanil (RT=2.60 min), spiked into a urine sample, differed from the theoretical accurate mass by -35 ppm, due to the presence of a co-eluting interference.



Example 2. The Fentanyl isotope pattern is distorted due to the presence of a co-eluting compound, at M+1.

We conclude that...

- 1. TOF-MS** alone is not sufficiently selective for definitive compound ID in complex sample matrices.
 - Interferences are frequently observed in the high-resolution XICs
 - Distorted accurate mass measurements are observed, due to co-eluting interferences
 - Distorted isotope patterns are common, due to co-eluting interferences
- 2. TOF-MS/MS** provides ultra-selective XICs (MRM^{HR}), that are free from interferences.
- 3. TOF-MS/MS** enables library searching of compound-specific fragmentation patterns.
 - Does not rely solely on accurate mass measurements and isotope patterns

TOF-MS/MS acquisition, with *Scheduled MRM^{HR}*

1. TOF-MS is acquired continuously throughout the run
 2. TOF-MS/MS scans are triggered at the appropriate RT window for each compound.
- In this experiment, there are **14 TOF-MS/MS scans** (accumulation = 50 msec), to account for the maximum # of overlapping RT windows for **52 target compounds**.

Experimental Method

TOF-MS (+)

Product ion (+) 328.2

Product ion (+) 300.2

...

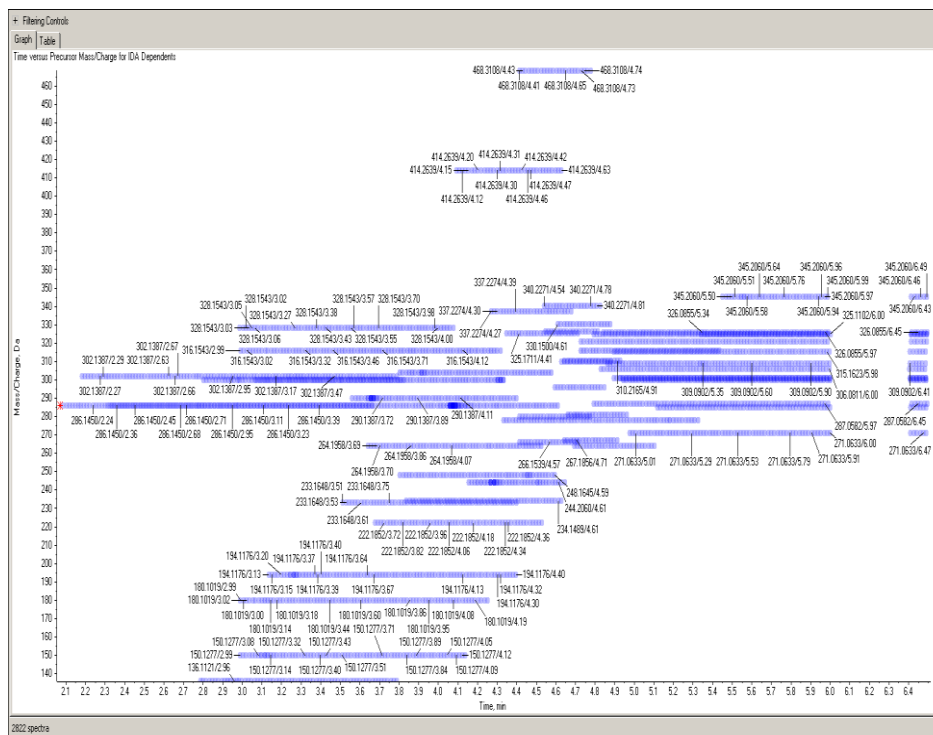
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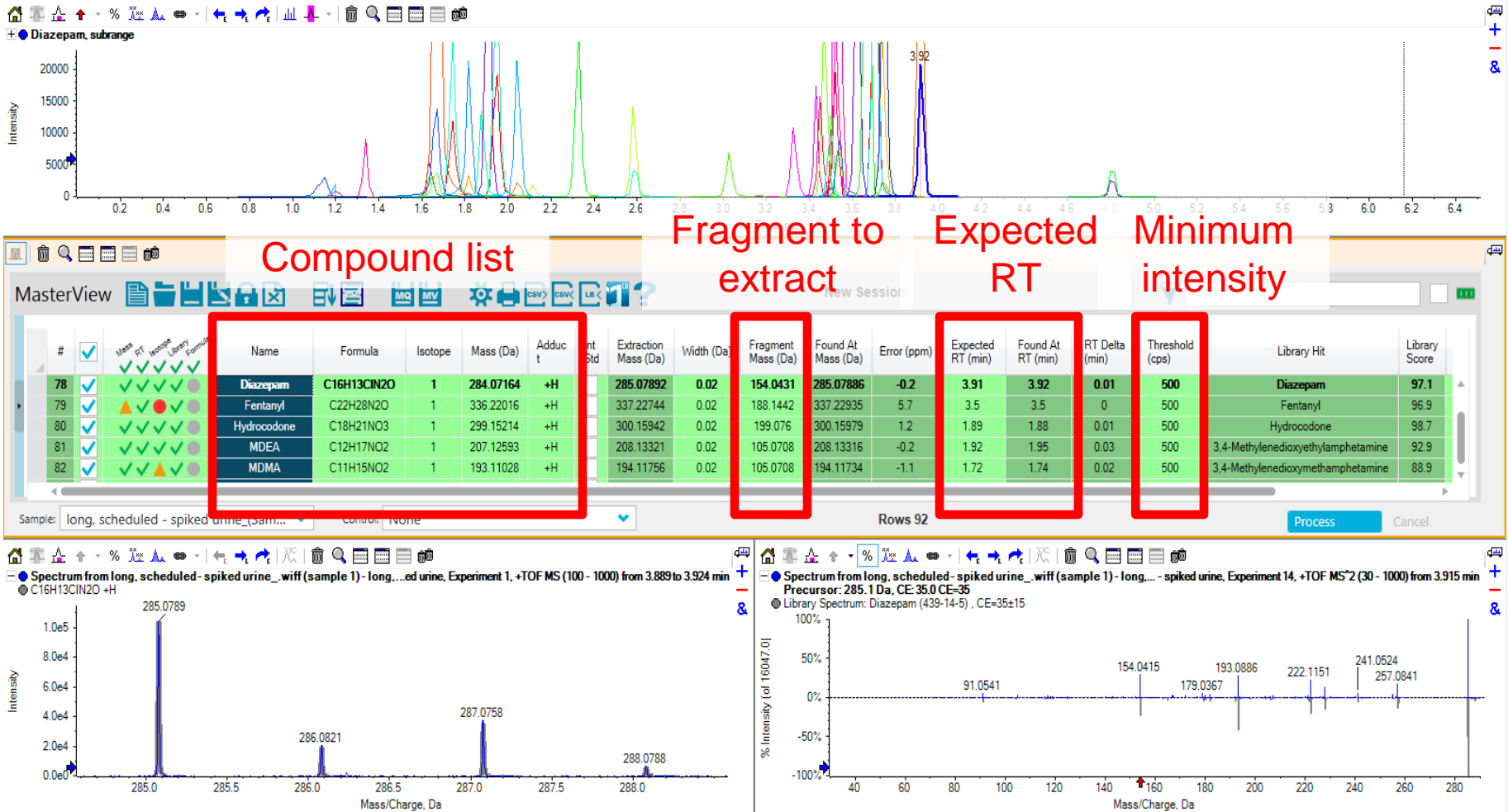
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Product ion (+) 286.1



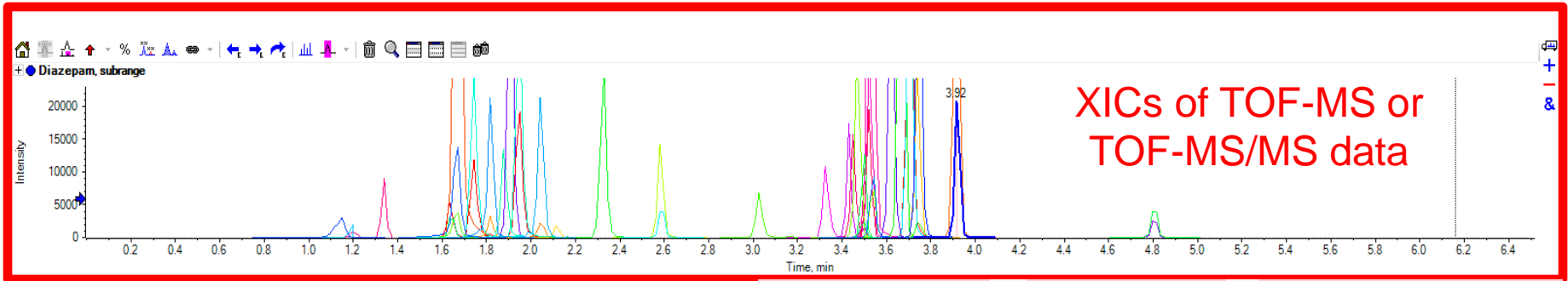
Software makes data interpretation easy

1. You tell the software what compounds to look for.



Software makes data interpretation easy

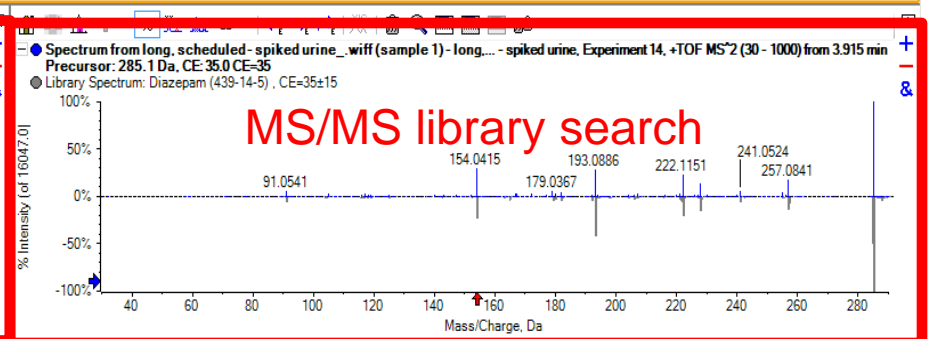
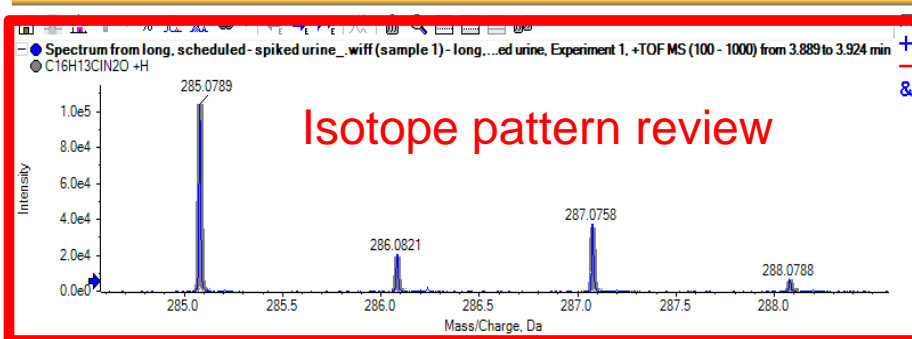
2. The software automatically assesses / displays all the relevant criteria for compound ID



MasterView

#	Mass	RT	Isotope	Library	Name	Formula	Isotope	Mass (Da)	Adduct	Int Std	Extraction Mass (Da)	Width (Da)	Fragment Mass (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	Found RT (min)	RT Delta (min)	Threshold (cps)	Library Hit	Library Score
78	✓	✓	✓	✓	Diazepam	C16H13ClN2O	1	284.07164	+H		285.07892	0.02	154.0431	285.07886	-0.2	3.91	3.92	0.01	500	Diazepam	97.1
79	✓	✓	✓	✓	Fentanyl	C22H28N2O	1	336.22016	+H		337.22744	0.02	188.1442	337.22935	5.7	3.5	3.5	0	500	Fentanyl	96.9
80	✓	✓	✓	✓	Hydrocodone	C18H21NO3	1	299.15214	+H		300.15942	0.02	199.076	300.15979	1.2	1.89	1.88	0.01	500	Hydrocodone	98.7
81	✓	✓	✓	✓	MDEA	C12H17NO2	1	207.12593	+H		208.13321	0.02	105.0708	208.13316	-0.2	1.92	1.95	0.03	500	3,4-Methylenedioxyethylamphetamine	92.9
82	✓	✓	✓	✓	MDMA	C11H15NO2	1	193.11028	+H		194.11756	0.02	105.0708	194.11734	-1.1	1.72	1.74	0.02	500	3,4-Methylenedioxymethamphetamine	88.9

Sample: long_scheduled_spiked_urine_(Sam... Control: None







“All truths are easy to understand once they are discovered; the point is to discover them” – Galileo Galilei

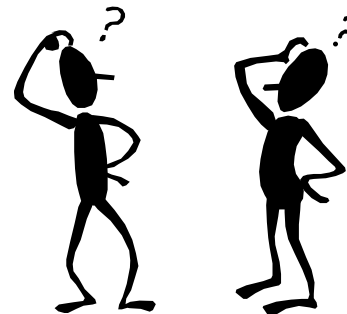
MS/MS^{ALL}

- Until we have all the information, we don't even know what questions we should be asking!
 - We have seen that *targeted* TOF-MS/MS provides the highest selectivity... but one needs to know *what* to target.
- Ultimate Goal: Collect high-resolution MS/MS information on *every analyte* in your sample (MS/MS^{ALL}).

Toxicologist's Wishlist:

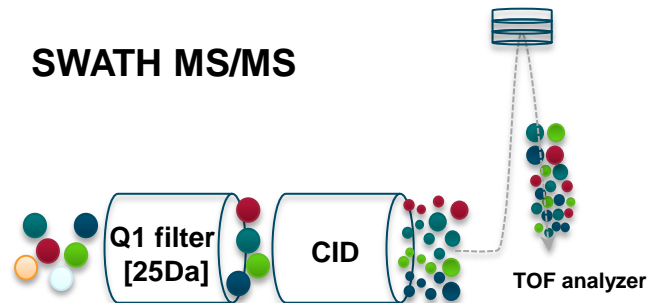
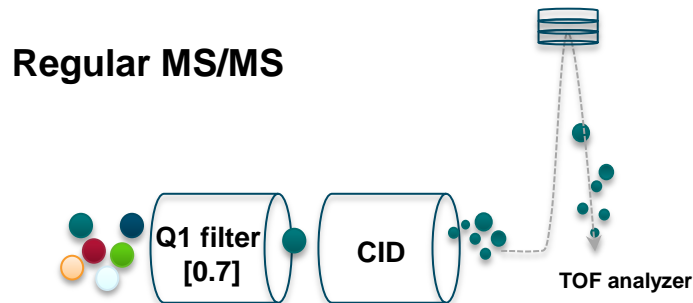
-  Generic method, non-targeted
-  Maximum selectivity
-  Digital record of everything in your sample
-  Possibility to re-interrogate the data later

How to accomplish this???



What is SWATH® acquisition?

- It is the collection of MS/MS in a generic and comprehensive fashion
- Relies on Q1 to provide an additional level of selectivity
 - Wide Q1 isolation window is stepped across the mass range of interest
 - Assists in simplifying MS/MS spectra, and hence the interpretation
 - Filters endogenous precursors that could generate interfering fragment ions



Comparing MRM^{HR} and SWATH[®] acquisition

1. Both techniques employ TOF-MS full-scan
 - Provides accurate mass measurement, and isotope pattern, for parent ions
2. Both techniques employ looped product ion scans
 - MRM^{HR} collects dedicated MS/MS for targeted compounds
 - SWATH[®] collects MS/MS for all compounds by using wide Q1 isolation windows, stepped across a mass range

MRM^{HR}

Experimental Method

TOF-MS

Product ion, 328.2

Product ion, 300.2

...

...

...

...

Product ion, 286.1

SWATH[®]

Experimental Method

TOF-MS

Product ion, 150-170

Product ion, 170-190

...

...

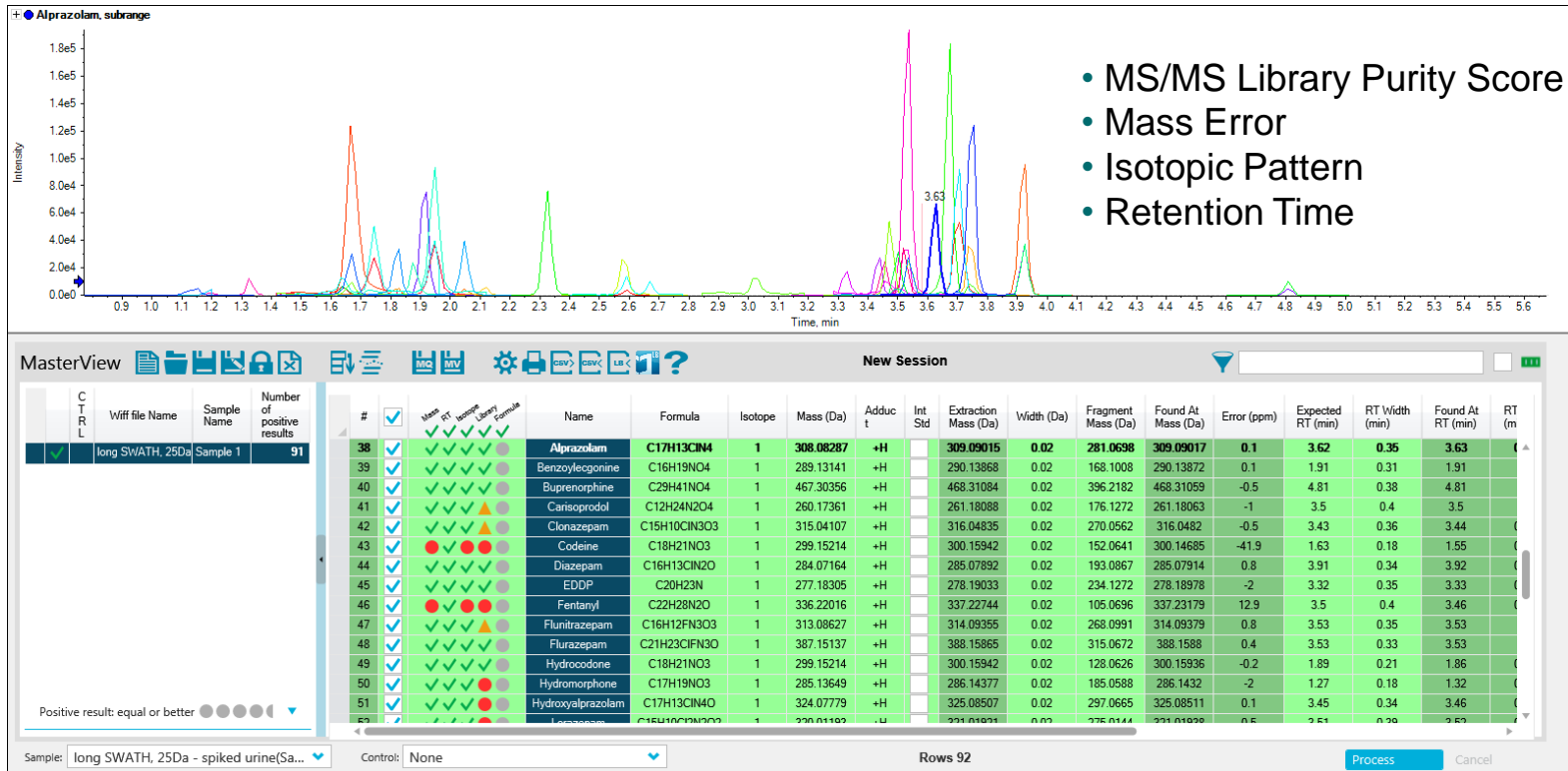
...

Product ion, 510-530

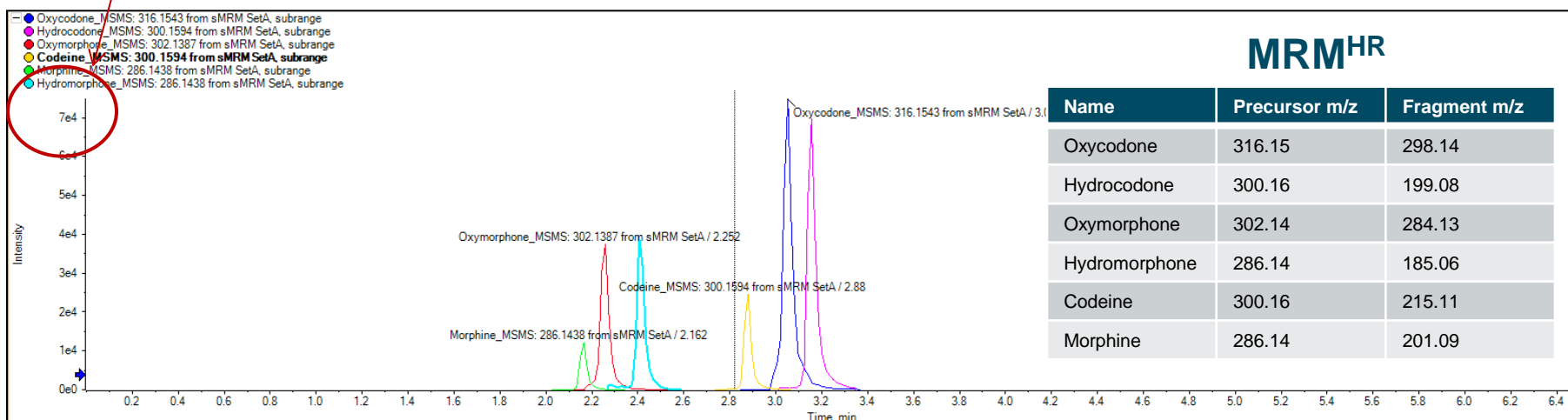
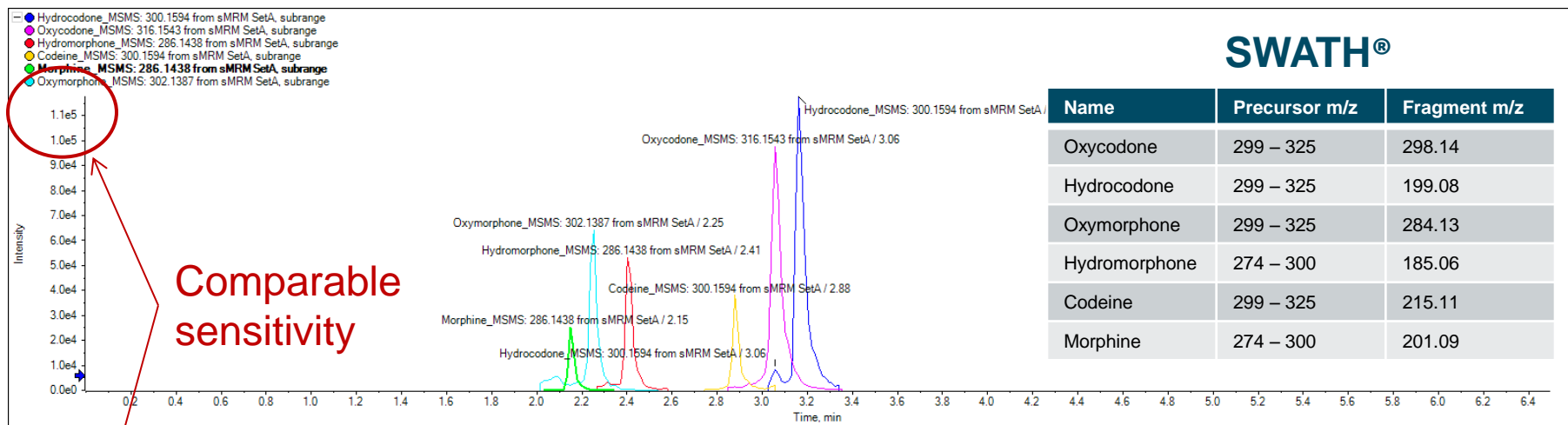
Product ion, 530-550

Processing SWATH®-MS/MS Data

- Same as MRM^{HR}
 - User tells software the mass of the parent ion, and the fragment ion
 - Software automatically extracts XIC from the appropriate SWATH® scan (i.e. the mass range containing the parent ion)

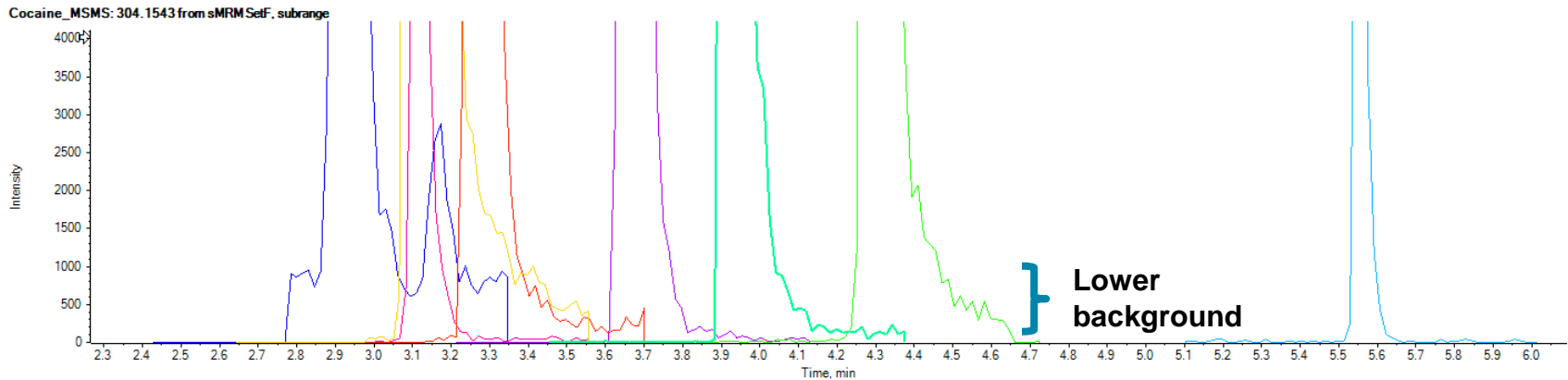


Comparing MRM^{HR} and SWATH[®] acquisition

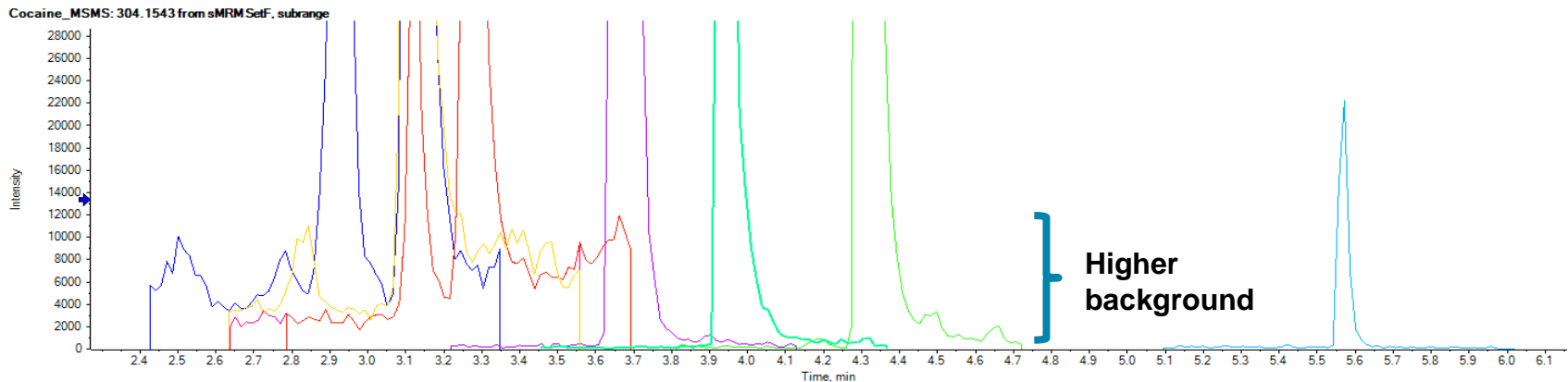


What about background?

- MRM^{HR} (“unit” Q1 isolation window)



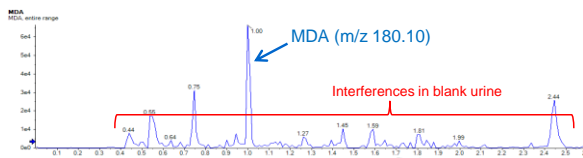
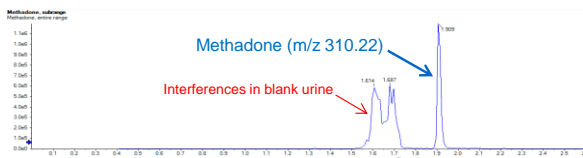
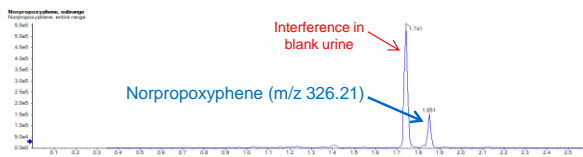
- SWATH[®] acquisition (wide Q1 isolation window)



Comparison of TOF, TOF-MS/MS, and SWATH[®] acquisition for targeted drug screening

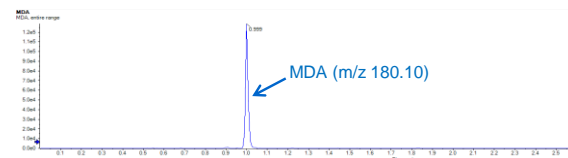
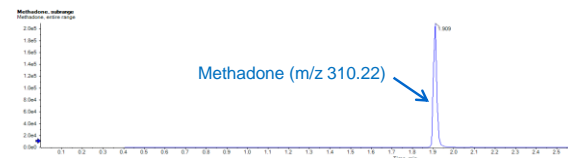
TOF-MS

- Generic, non-targeted method
- Low selectivity (MS only)
- Retrospective data mining



TOF-MS/MS (a.k.a. MRM^{HR})

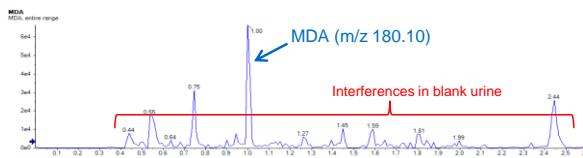
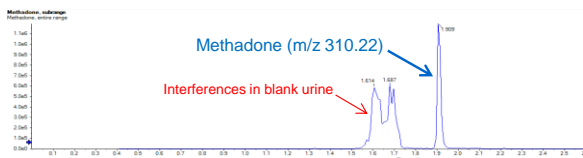
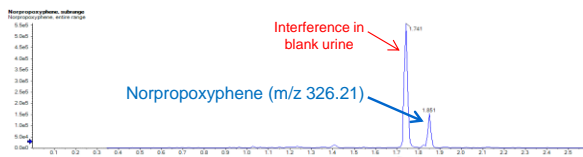
- Targeted acquisition method
- Highest selectivity (MS/MS)
- Retrospective data mining



Comparison of TOF, TOF-MS/MS, and SWATH[®] acquisition for targeted drug screening

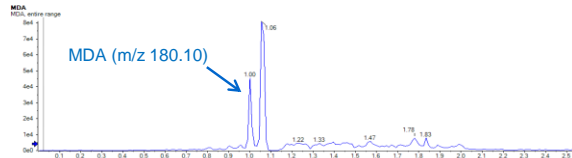
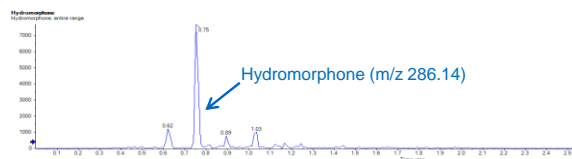
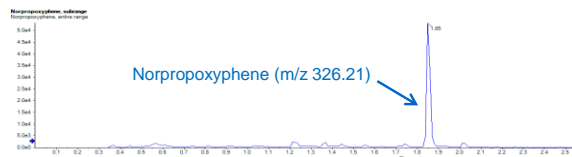
TOF-MS

- Generic, non-targeted method
- Low selectivity (MS only)
- Retrospective data mining



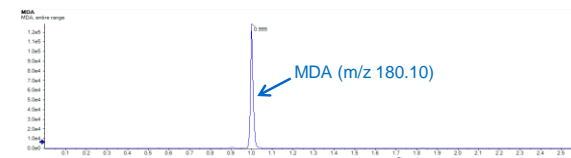
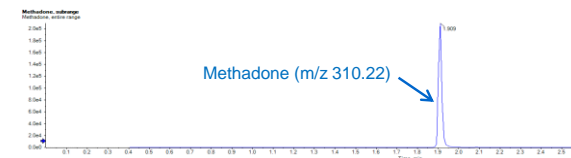
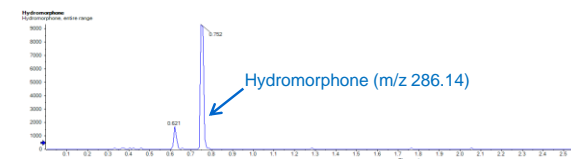
SWATH TOF-MS/MS

- Generic, non-targeted method
- High selectivity (MS/MS)
- Retrospective data mining



TOF-MS/MS (a.k.a. MRM^{HR})

- Targeted acquisition method
- Highest selectivity (MS/MS)
- Retrospective data mining

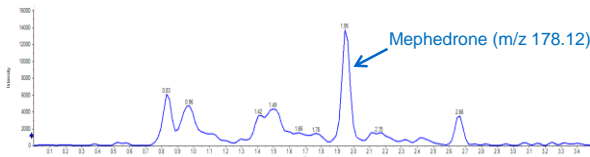
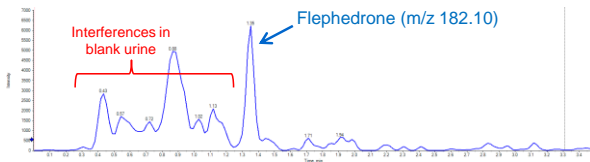


Comparison of TOF, TOF-MS/MS, and SWATH® acquisition for retrospective data analysis

- MRM^{HR} is a targeted technique – no retrospective data mining
- TOF-MS enables data mining, however lacks selectivity
- SWATH-MS/MS is generic, selective, and allows retrospective data analysis

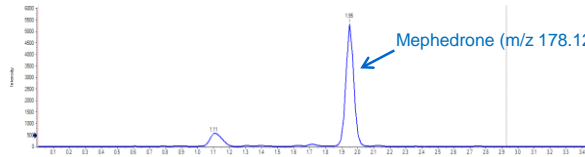
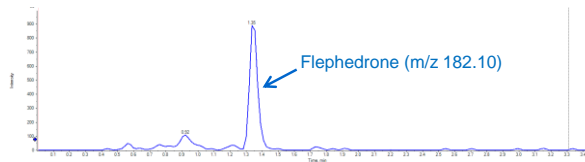
TOF-MS

- Generic, non-targeted method ✔
- Low selectivity (MS only) ✘
- Retrospective data mining ✔



SWATH TOF-MS/MS

- Generic, non-targeted method ✔
- High selectivity (MS/MS) ✔
- Retrospective data mining ✔



TOF-MS/MS (a.k.a. MRM^{HR})

- Targeted acquisition method ✘
- Highest selectivity (MS/MS) ✔
- Retrospective data mining ✘

N/A

N/A

Advantages of SWATH® acquisition

1. SWATH® acquisition method is **generic**, and **non-targeted**
2. SWATH data can be **re-interrogated** for both MS and MS/MS information (and XICs)
3. SWATH cycle time **does not increase** as the number of target compounds increases
4. Sensitivity of SWATH is **comparable to MRM^{HR}**, however the background is higher with SWATH®

Disadvantages of SWATH® acquisition

1. SWATH® is slightly less selective than MRM^{HR}
2. Background is slightly higher than MRM^{HR}, but better than TOF-MS.
3. SWATH® cycle times may be longer
 - Dependent on the mass range covered
 - Dependent on the size of the Q1 isolation windows

Does SWATH® acquisition satisfy the wishlist?

Toxicologist's Wishlist:

SWATH® acquisition

 Generic method, non-targeted



 Maximum selectivity



 Digital record of everything in your sample

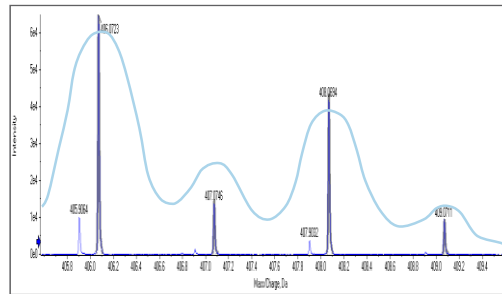


 Possibility to re-interrogate the data later

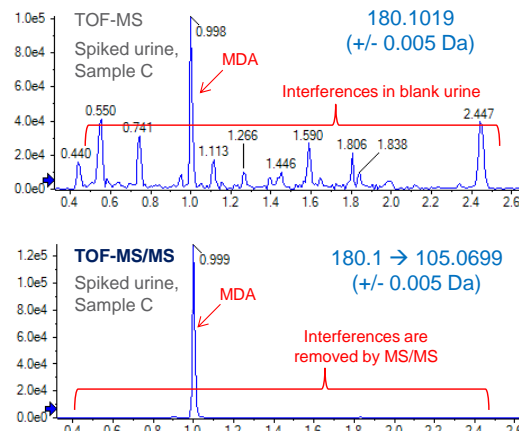


Conclusions

- High-resolution accurate mass MS systems (e.g. Time-of-Flight) provide incredible selectivity VS ‘nominal mass’ instruments.

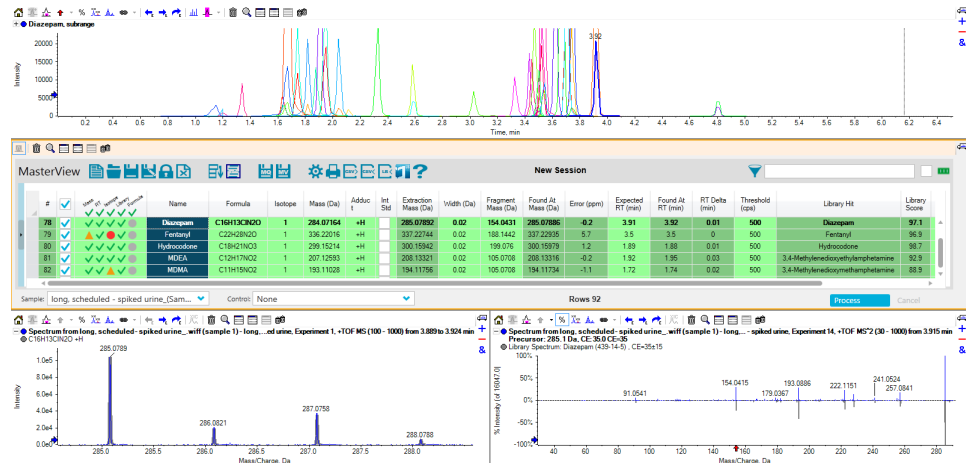


- MS/MS is required, even on a high-res MS system, to remove chemical interferences and to avoid false positives/negatives.







Conclusions

- MRM^{HR} acquisition employs looped TOF-MS and TOF-MS/MS scans, to provide:
 - Maximum specificity for all targeted compounds (via MS/MS)
 - Ability to re-interrogate the data, for unanticipated compounds (via MS)
- Definitive compound ID is achieved by assessing:
 - High-resolution XICs of MS/MS data
 - MS/MS fragmentation pattern
 - Accurate mass
 - Isotope pattern
 - Retention time



Conclusions

- SWATH[®] acquisition is a mode of Data-Independent Acquisition that...
 - provides TOF-MS *and* TOF-MS/MS information for all compounds in a sample (even unanticipated compounds)
 - enables monitoring of any number of analytes, with no increase in experimental cycle time
 - captures a complete digital record of everything in your sample
 - enables retrospective data analysis

	Generic method, non-targeted	✓
	Maximum selectivity	✓
	Digital record of everything in your sample	✓
	Possibility to re-interrogate the data later	✓

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Questions?

