SCIEX

Answers for Science.
Knowledge for Life.™

Metabolite Identification Using TripleTOF® Technology & MetabolitePilot™

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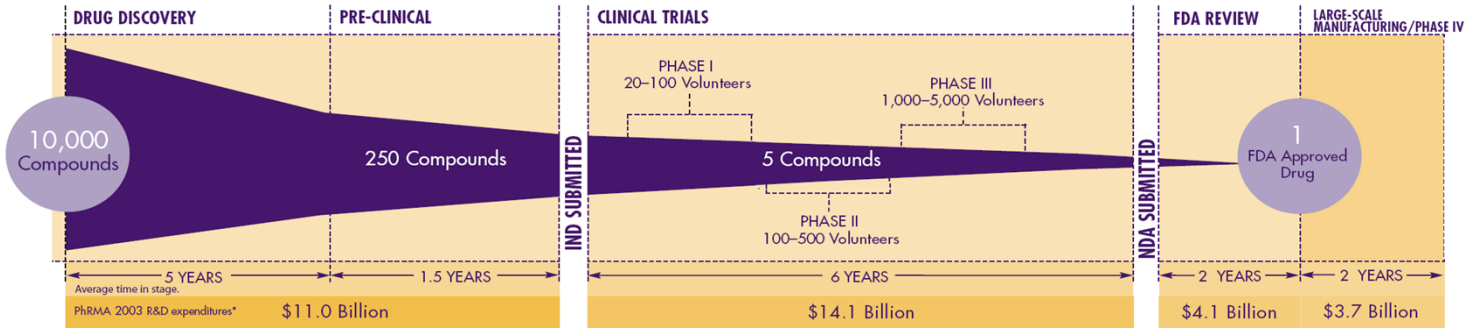
Pharmaceutical & Metabolomics

Fast Compound Method Development

Fast Screening for Reactive Metabolites

Definitive Metabolite Identification



Complementary Platforms

Metabolite Identification Workflows with Real-Time Multiple Mass Defect Filtering and SWATH™ Acquisition

Key Challenges of Met ID in Complex Biological Matrices

- **Missing, low-level drug metabolites** in complex biological matrices such as bile, plasma, and tissue extracts
- **Incomplete metabolite information** leading to repeated sample analysis and decreased productivity
- **Non-definitive metabolite identification and characterization** due to inadequate MS/MS information
- **Multiple, non-integrated software platforms** complicate data processing, slowing metabolite ID and structure elucidation

AB SCIEX for Drug Metabolism

1. TripleTOF™ Platform Capabilities
2. Acquisition Strategies
   - Real-Time Filtering
     - Multiple Mass Defect Filtering (MMDF)
     - Dynamic Background Subtraction
   - Quant/Qual Acquisition
     - Data Dependent Acquisition (IDA)
     - SWATH™
3. Digital record of information (SWATH)
4. Software (MetabolitePilot™, MultiQuant™)
5. Selectivity (SelexION™)

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The TripleTOF™ 5600+ System

- Speed – Up to 100MS/MS per cycle in IDA
- Resolution Up to 35K
- Mass accuracy ~ sub 2 ppm MS and MS/MS
- Dynamic Range for both quant and qual
  - QqQ like performance
- Workflow specific solutions
  - Real-time IDA algorithms (MMDF, DBS)
- SWATH™ Acquisition

The TripleTOF™ 6600 System

- Powerful Performance for Qualitative and Quantitative Analysis
- Linear Dynamic Range
  - Greater than 5 orders
- Extended Q1 mass range
  - Up to 2250 m/z
- Fast Acquisition Rates
  - Up to 100 MS/MS per cycle in IDA
  - Up to 200 MS/MS per cycle in SWATH
- High Mass Accuracy
  - Improved mass stability resulting in easier opera frequency
  - < 0.5 ppm w/ internal reference
  - < 2 ppm RMS external
- Higher Resolution
  - >35,000 in TOF MS
  - >20,000 or >30,000 in TOF MS/MS

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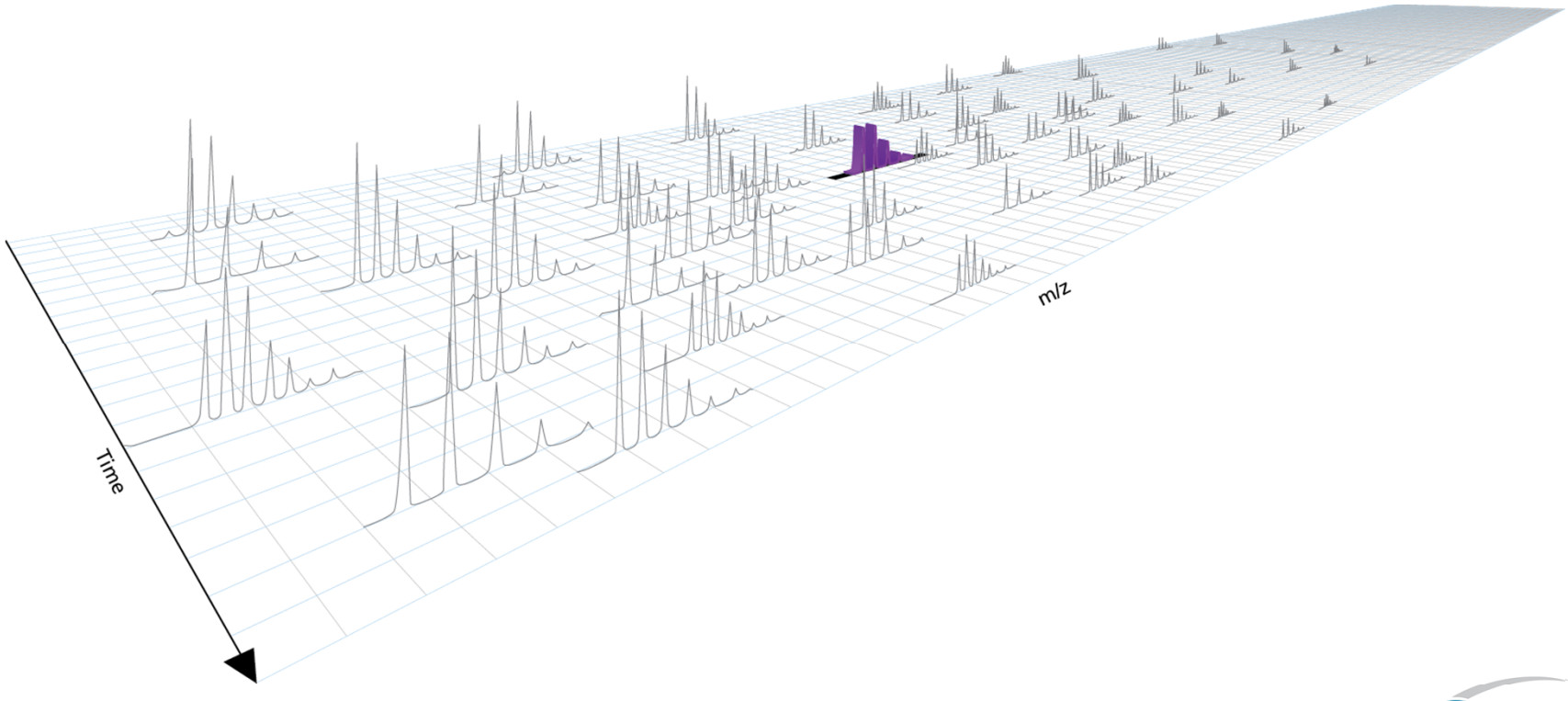
Data Acquisition Strategies

- Traditional Strategies - IDA, MRM & MRM^{HR}
- MRM^{HR} – High resolution MRM quantitation
- Standard 0.7 Da Q1 Window
- A single analyte is selected, fragmented and a MS/MS spectrum is acquired. Further experiments are acquired in a looped fashion across the LC gradient



Data Acquisition Strategies

- Real Time Algorithms on TripleTOF™ Series
- **Separate from data processing algorithms**
- **Eliminates MS/MS triggering on background noise**
- **Determine which ion(s) are significantly changing with time**
- **Select the best ion(s) to target for MS/MS**
- **Applied during UPLC/MS acquisition**
- **Part of information dependent data acquisition (IDA) logic**

Data Acquisition Strategies

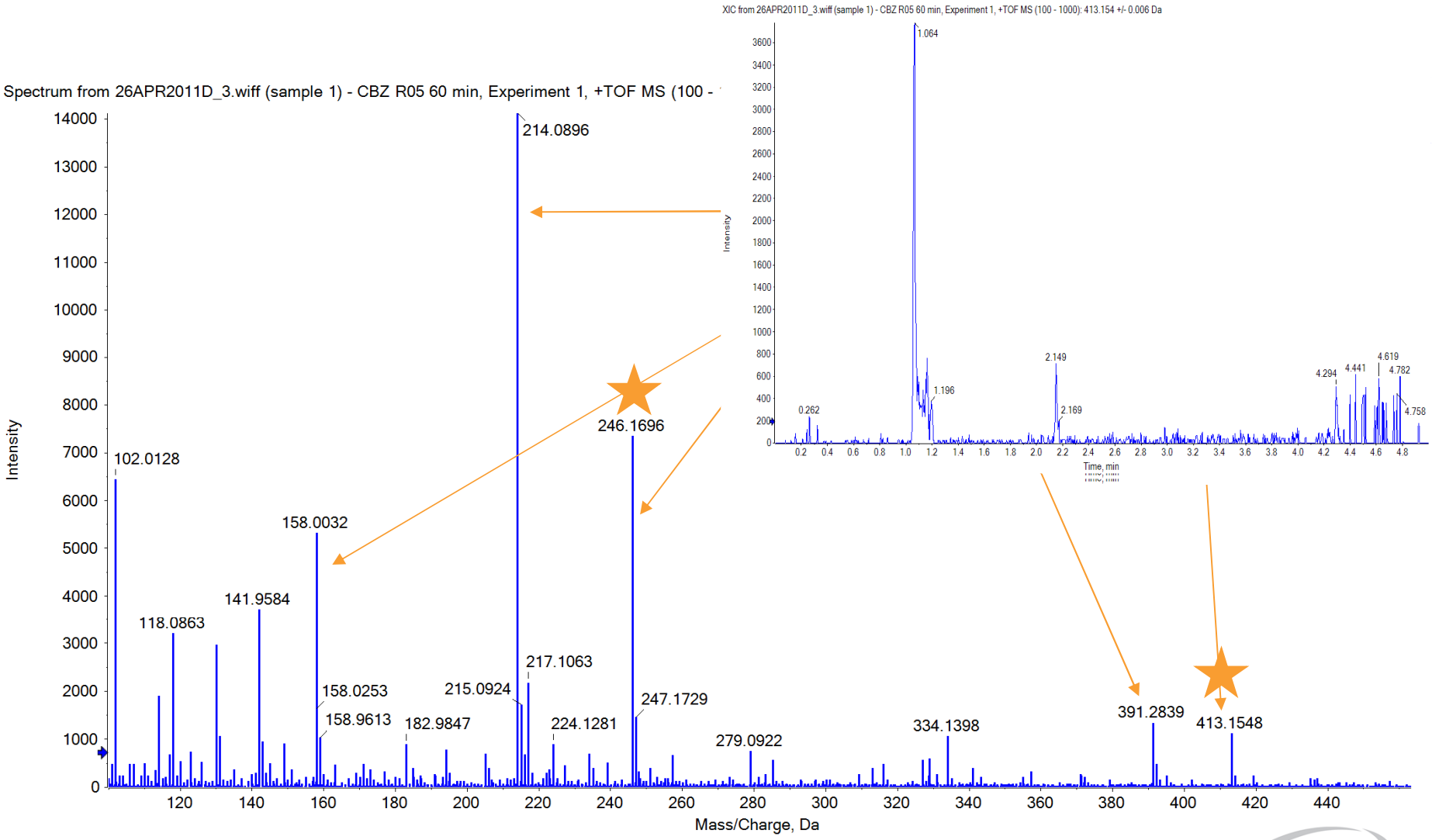
- Benefits of Real Time Algorithms on TripleTOF™ Series

Increased productivity through:

- **Single injection workflow for both TOF MS and TOF MS/MS**
- **Obtain more relevant data (increased MS/MS triggering efficiency)**
- **UPLC time scale (2-3 sec peak width)**
- **Complex In-vivo samples, plasma with PEG's, bile samples, tissue samples**

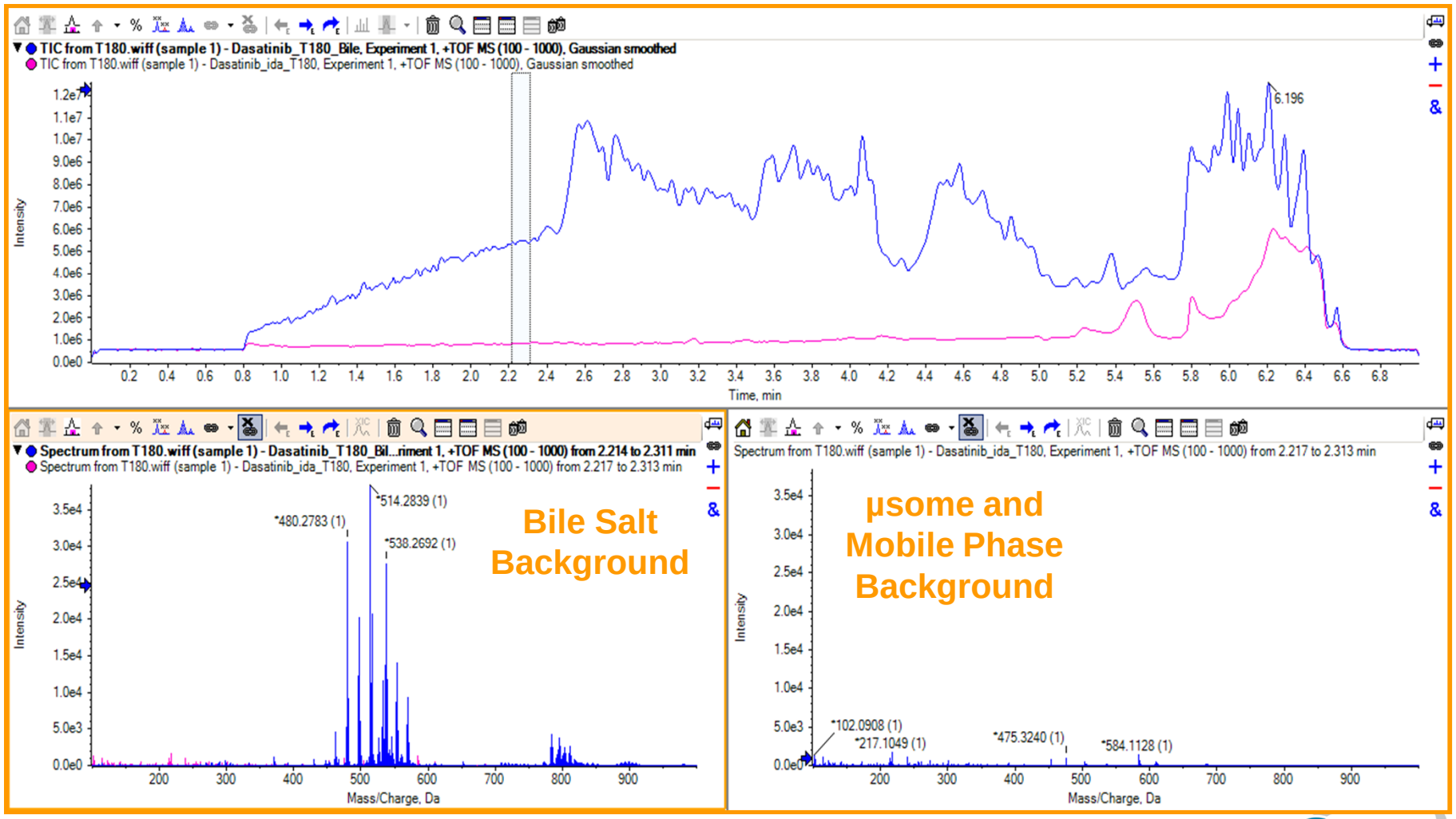
Dynamic Background Subtraction

- Increasing IDA Efficiency



Dynamic Background Subtraction – Bile Example

- Profound impact on IDA efficiency when dealing with high background as with bile samples

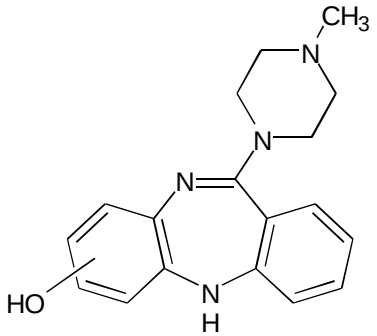


Real-Time Multiple Mass Defect Filter (MMDF)

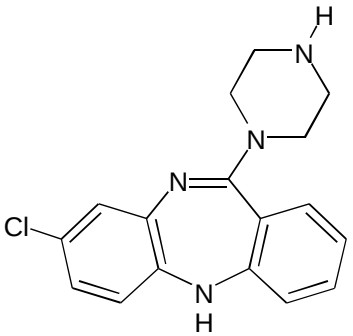
- MMDF in non exclusive mode
  - Using the mass defects based on formula
    - Parent
    - Major phase II
    - Predicted cleavages (optional)
    - Easy to implement
  - Useful as a broad general Qual/Quant screen
- Non exclusive mode also allows for simultaneous unpredicted approach
- This is a real-time algorithm for IDA target selection
  - Unique to our software
- In combination with Dynamic Background Subtraction

- The difference between the exact mas and the nominal mass of a compound is known as the mass defect
- In impurity profiling or metabolism studies closely related molecules like a parent and its impurity should have similar mass defects
- We take advantage of this fact during data acquisition to perform MS/MS only on ions that fall within a small window. In the case of Dextromethorphan that is less than a 60mDa window

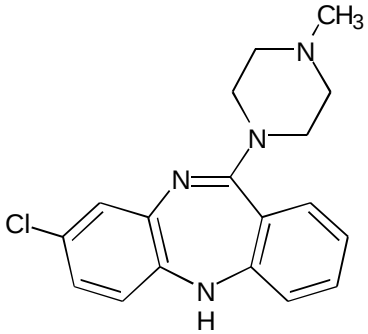
Mass Defect in Metabolism



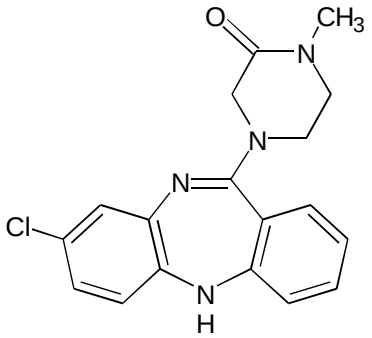
308.1637 Da



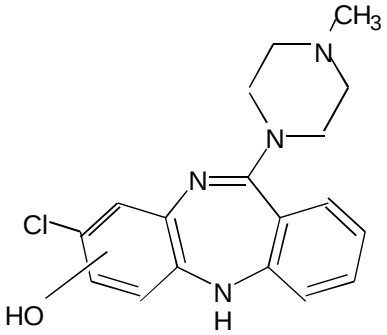
312.1141 Da



326.1298 Da

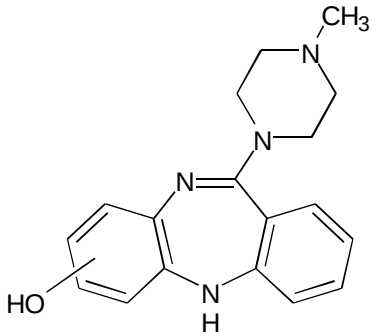


340.1091 Da

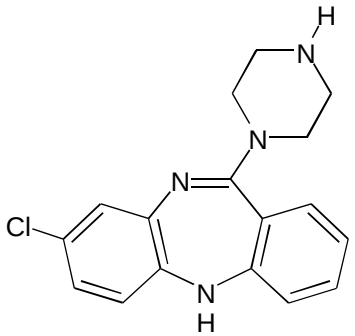


342.1247 Da

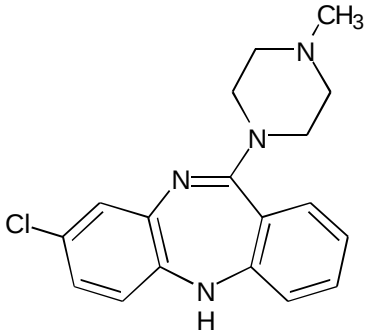
Mass Defect in Metabolism



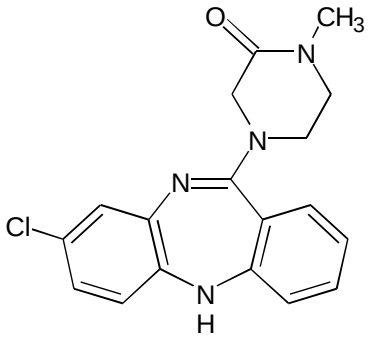
.1637 Da



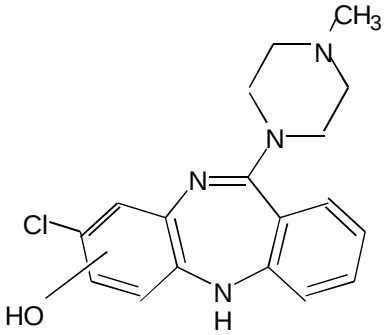
.1141 Da



.1298 Da

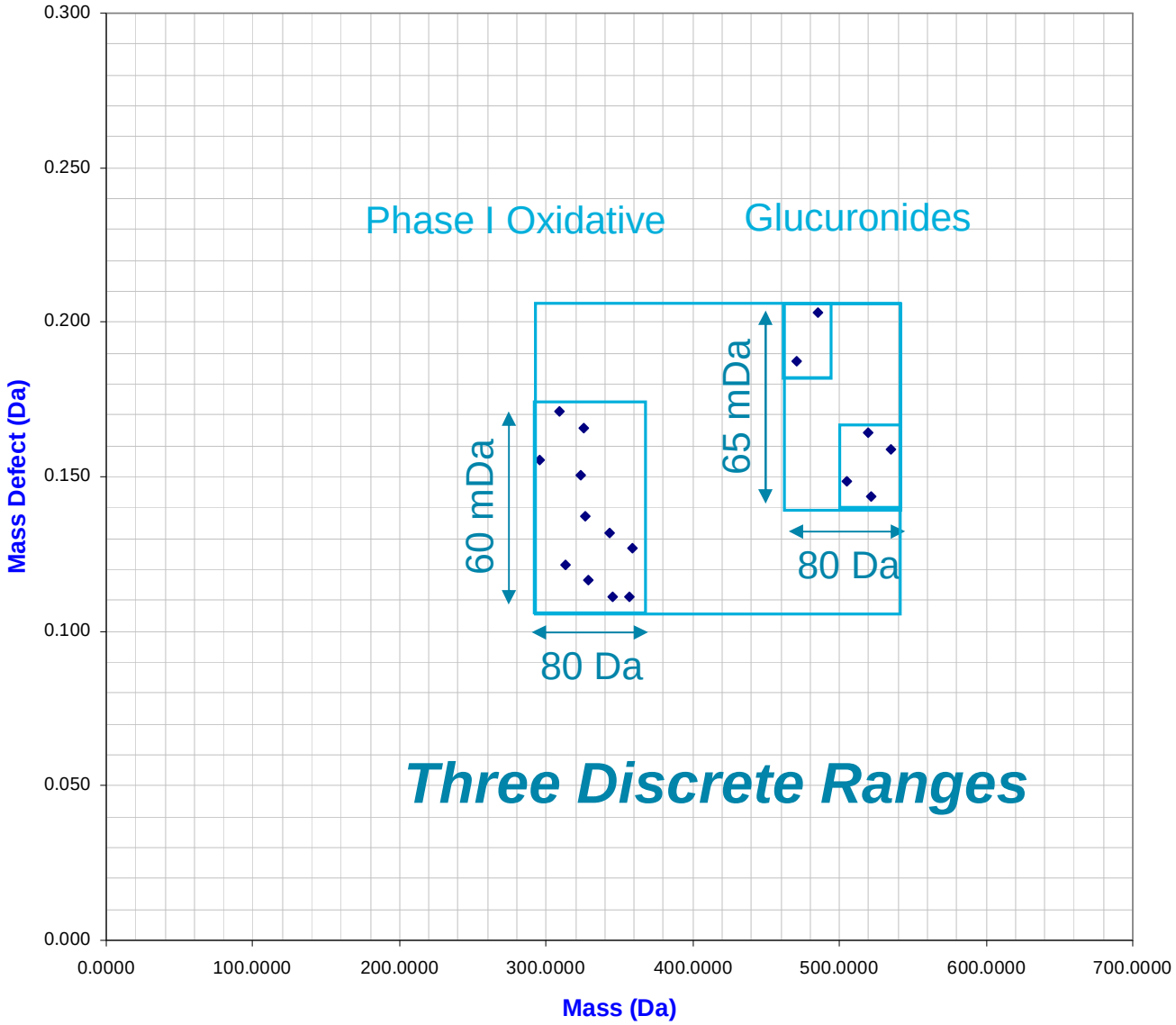


.1091 Da



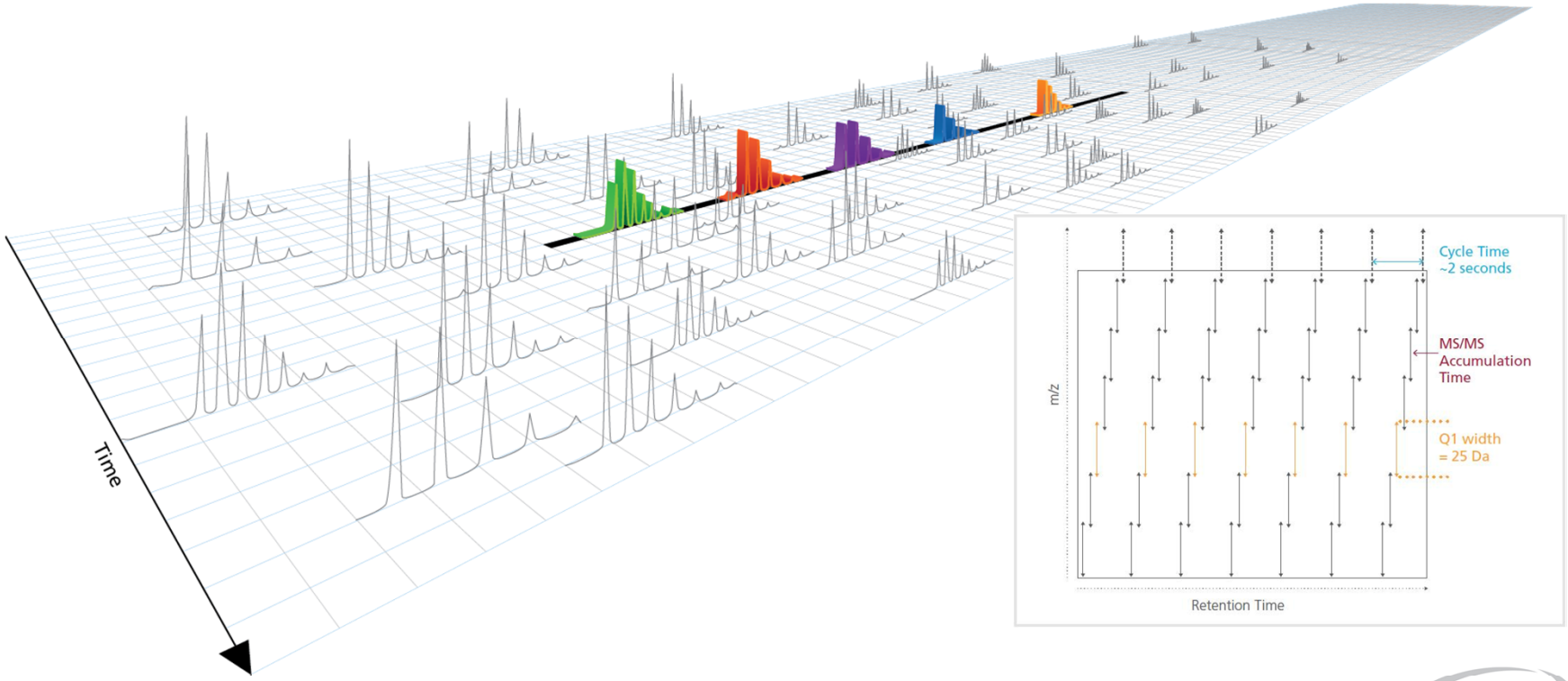
.1247 Da

Mass Defect Distribution – Clozapine Metabolites



Data Acquisition Strategies

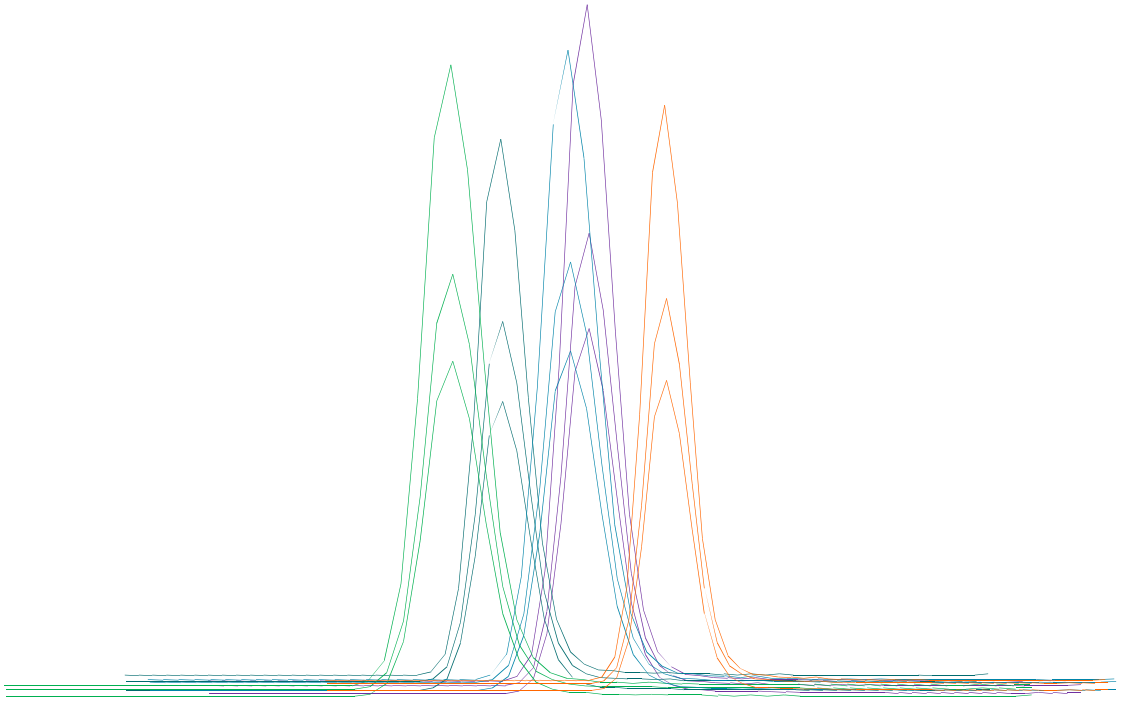
- MS/MS^{ALL} with SWATH™ Acquisition
- SWATH is a data independent workflow
- Acquire all data with a single acquisition method
- Generate high resolution quantitative XICs on all analytes



Data Acquisition Strategies

- MS/MS^{ALL} with SWATH™ Acquisition

- Q1 is a variable or fixed window from 1-25Da to allow a number of precursors through
- All ions fragmented in the collision cell and a high resolution composite MS/MS spectrum acquired
- Stepping across the mass range in a loped fashion each cycle to produce composite MS/MS spectra of all precursors eluting off the column



Benefits of SWATH™ for Met ID

1. **Comprehensive quantitative and qualitative analysis** of all the sample components **in a single injection**
2. **Informative SWATH™ MSMS for better metabolites** structure prediction and site modification including (Less complex spectrum than traditional DIA techniques)
   - **MS/MS for Low level** metabolite ID
   - SWATH™ MS/MS **retains isotope pattern for each fragment**
3. **High resolution quantification** reduces potential for interferences, yet maintains the sensitivity and dynamic range of leading triple quads (Selective quant using product ion mass and sum product ions- MRM style Quant)
4. **Ultimate safety net** for capturing both predicted and unpredicted metabolites
5. **Easy and Retrospective**
   - Requires **no sample-specific method development**
   - Creates a **digital archive** of all analytes, enabling retrospective investigations without re-acquisition

What Makes SWATH Unique for Metabolite ID?

Unique Qualitative Features

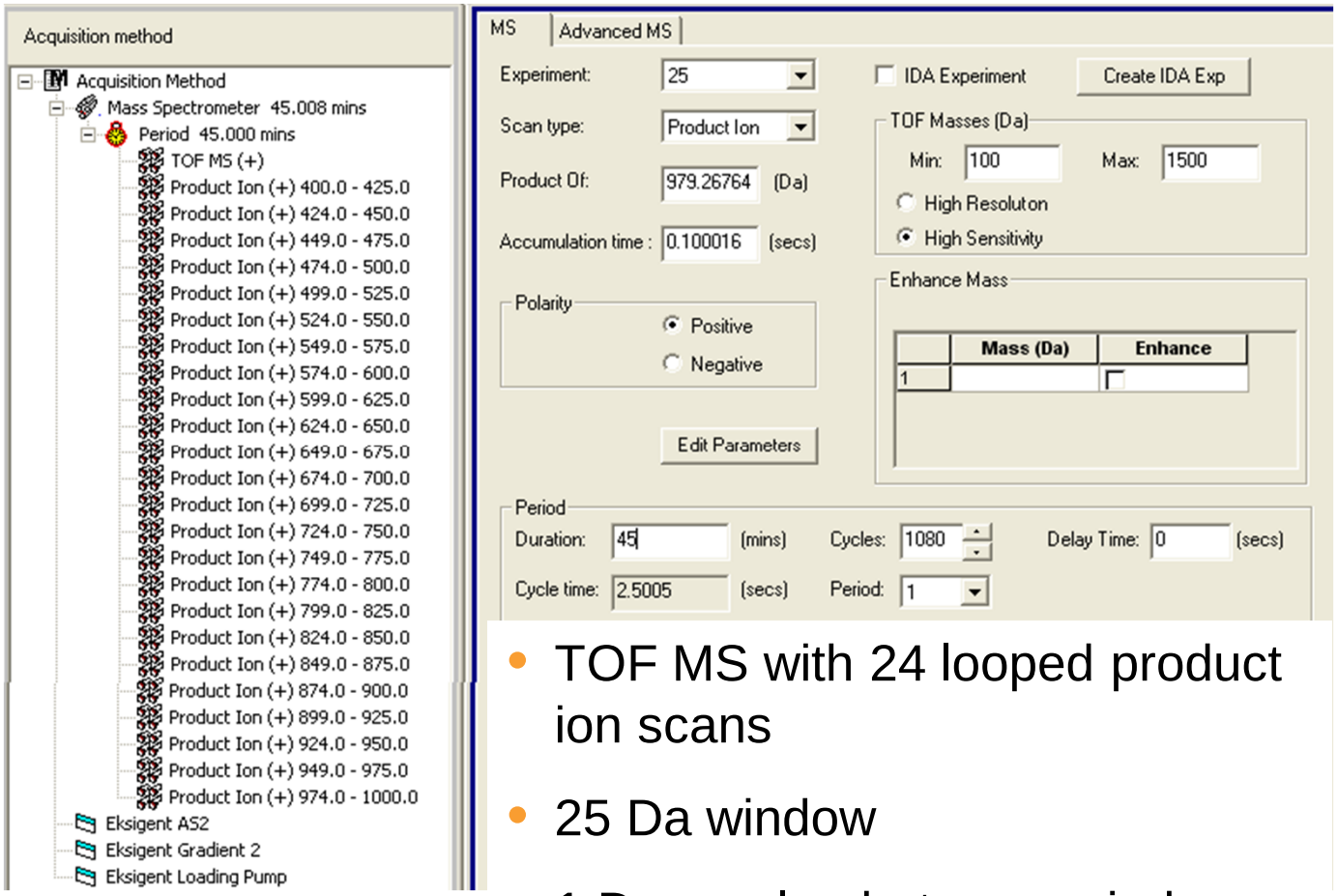
- **Less complex MS/MS spectrum** than traditional DIA techniques
- Wider Q1 selection **retains isotope pattern for each fragment**
  - Good for C14/SIL metabolism studies
- **100% MS/MS for Low level** metabolite

Unique Quantitative Features

- **Selective MS/MS Quantification-** MRM style using single product ion or sum multiple product ions
- Possibility o**f Multicomponent Quantification** in single acquisition method
  - (Total mAb, Conjugated & Free SM)

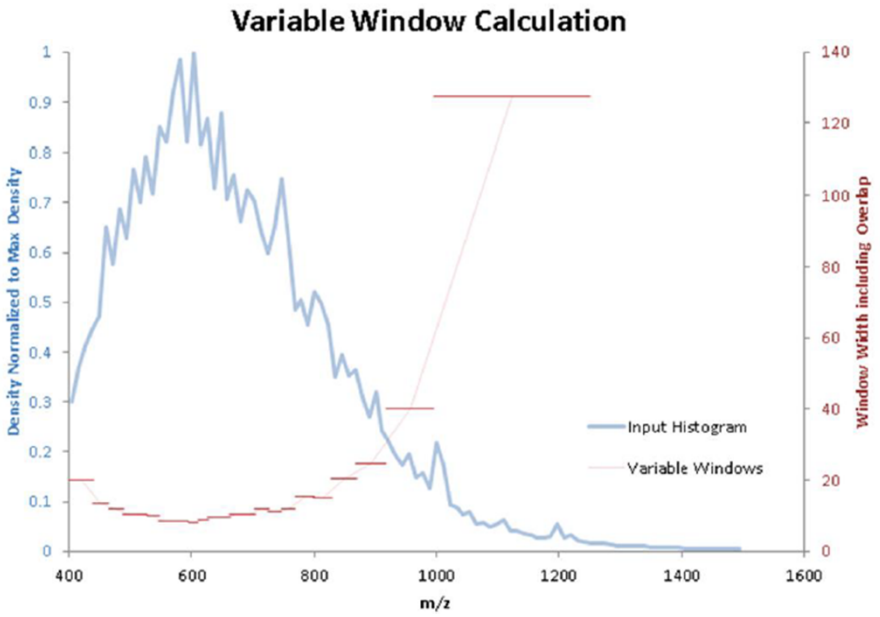
Creating a SWATH™ method in Analyst

- MS/MS^{ALL} with SWATH™ Acquisition - Method Builder



- TOF MS with 24 looped product ion scans
- 25 Da window
- 1 Da overlap between windows for complete coverage

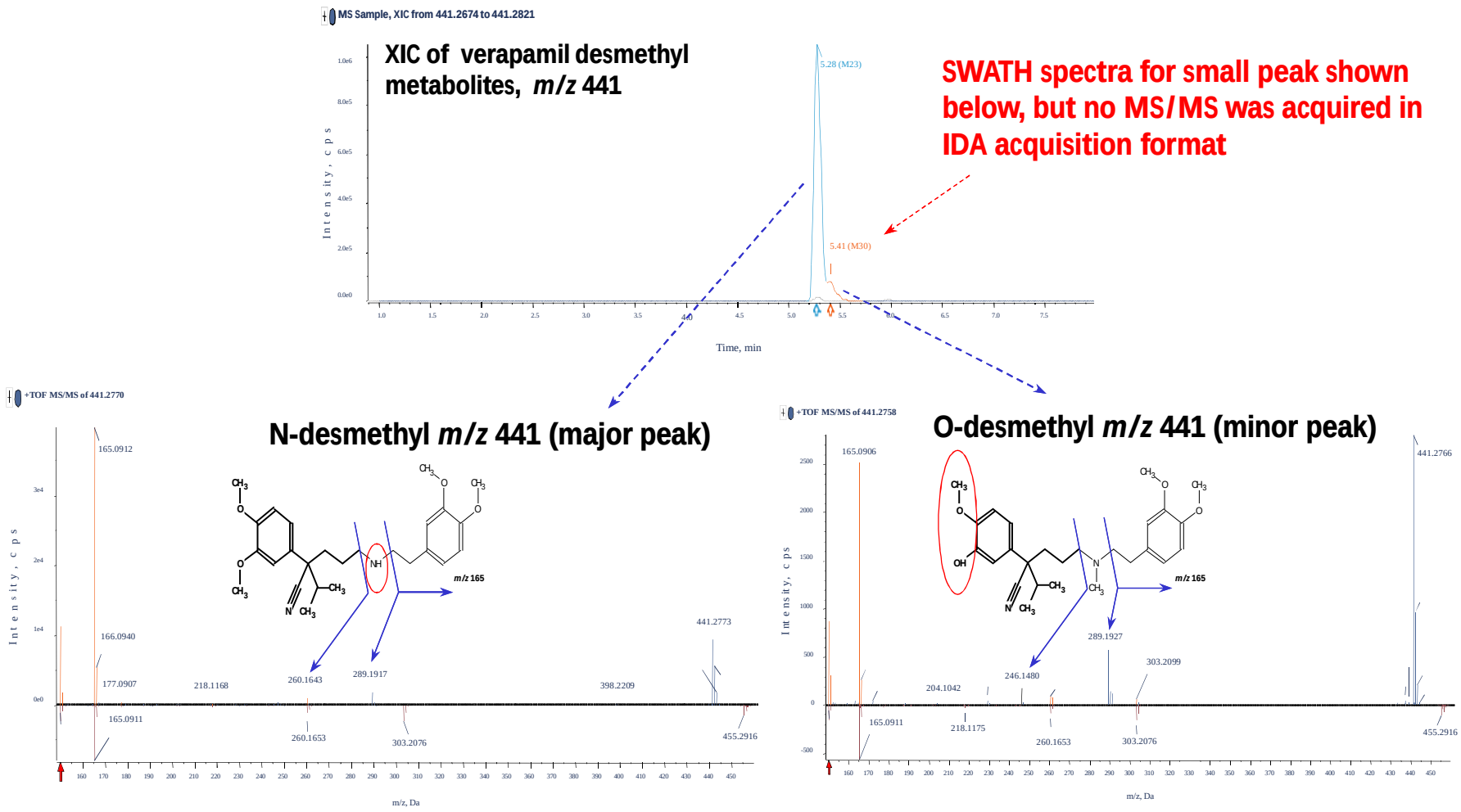
Variable Window SWATH™ Acquisition



- Adjust Q1 selection window to facilitate detailed coverage of target mass range
- Reduce number of precursors for increased qualitative specificity and quantitative accuracy
- Simple interface for acquisition method building
- Text file import capability for full control over acquisition windows

High quality MS/MS Spectra for low level metabolites

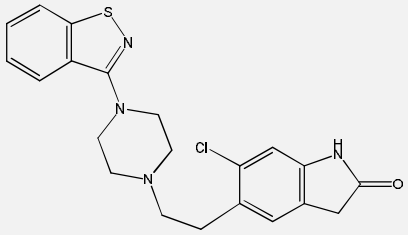
MS/MS Spectra Acquired for Minor Metabolites

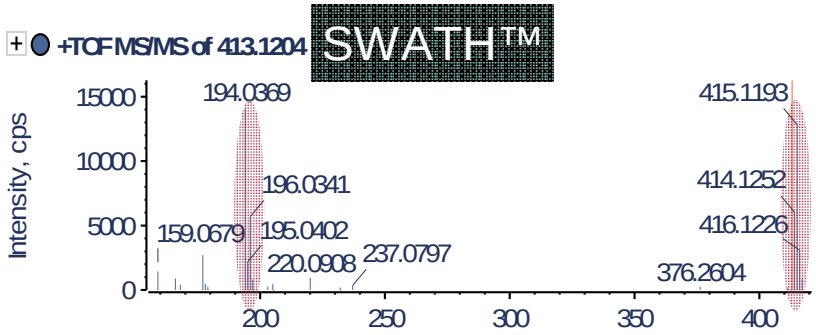


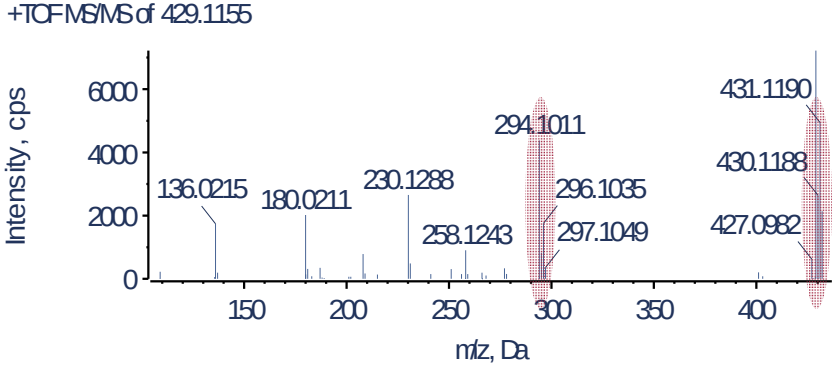
R. Schneider et al.: Exploiting Variable SWATH Techniques to Maximize the Quality of MS/MS Spectra for Metabolite Identification Studies, ASMS Conference 2014

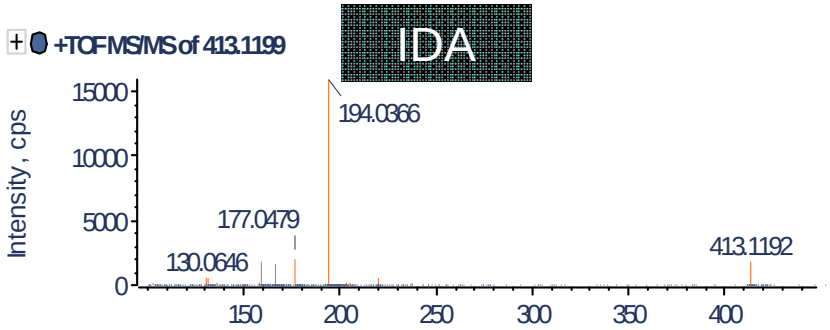
SWATH™ Acquisition vs. IDA

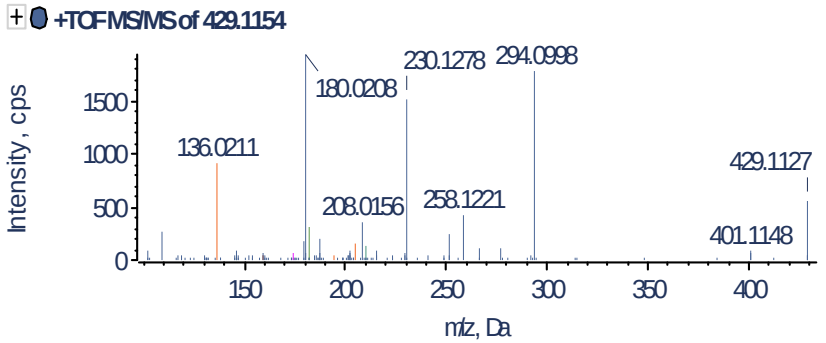
Ziprasidone











- **All the major product ions** present in SWATH™ as compared to 1 Da isolation IDA
- **Get more confidence** in compound ID with low level MS/MS
- **Enables MS/MS quantitation** on all discovered metabolites
- **Retain data, not samples, for years to come with SWATH™ Acquisition**

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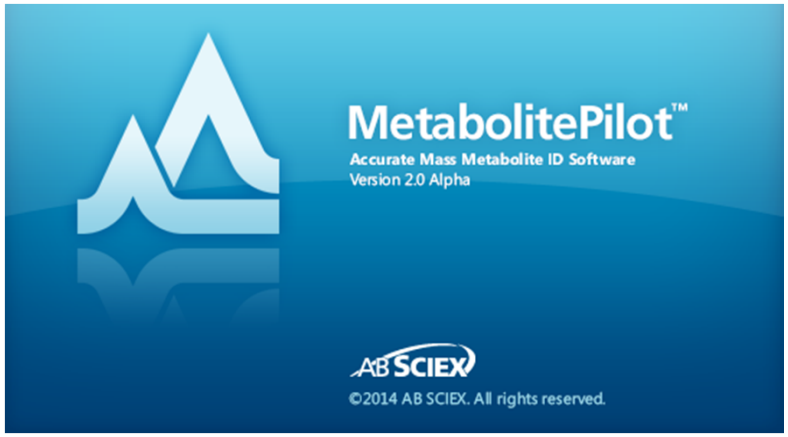
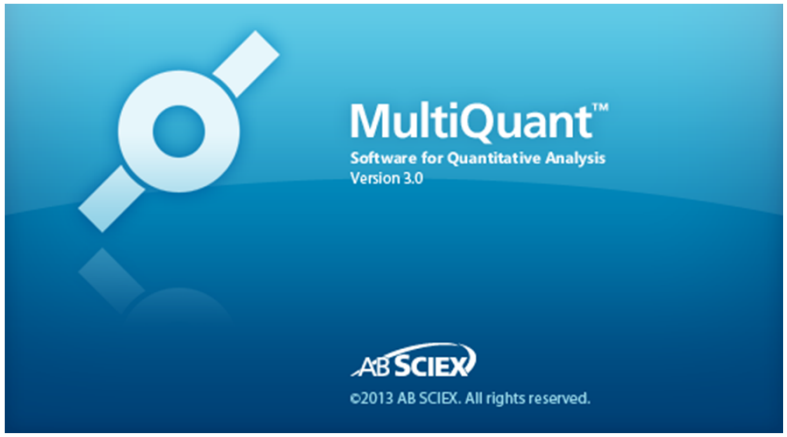
MetabolitePilot™ Software for Metabolite ID



- **Intuitive workspace** for processing accurate mass data
- **High-throughput batch** processing for multiple assay sets
- **Formula prediction** with a high level of chemical intelligence
- **Cleavage Metabolites** – in addition to expected and unexpected metabolites.
- **Integrated MS/MS** fragment interpretation
- **Correlation** across multiple time points for metabolic stability and PK studies and interspecies comparison studies
- **A single solution for comprehensive metabolite identification, structural interpretation and metabolite & parent correlation**

MultiQuant™ Software for Quantitation



- **Intuitive workspace** for processing accurate mass data
- **Multiple analytes** can be compared in a single view
- Metric plots for **quick review** of data
- **Automatic query** for outliers
- **Peak de-convolution** for precise and accurate integration
- **Parameter free integration** toll (MQ4 and SignalFinder™ algorithms)
- **A single solution for quantifying small molecule compounds, biomarkers and biopharmaceuticals**

Metabolite Identification

Two Categories

- Discovery Metabolite Identification: Finding potential candidates
  - In Vitro assays
  - Microsomes or S9 fraction
  - CYP Inhibition studies

- Development Metabolite Identification: Full characterization of candidates
  - In Vitro and In Vivo metabolism
  - Hepatocytes
  - Animal Studies

Challenges for Metabolite ID

Discovery Metabolite Identification

- Lead Generation and Optimization: Identify potential candidates by screening compound library for metabolic stability and soft spots
- Achieving Success Requires: Increased sample throughput and efficiency of data processing

- Obstacles to success:
  - Each compound requires optimization for MRM based analysis
  - Individual methods need to be generated
  - Huge time investment and many compounds fail
  - Implementing separate qualitative and quantitative methods
  - Fast chromatography is desired-reduced cycle time required

Challenges for Metabolite ID

Development Metabolite Identification

- Drug Metabolism and Phamacokinetics: Characterize compound metabolism using *in vitro* and *in vivo* models

- Achieving Success Requires: Detecting, characterizing, and quantifying metabolites with accuracy and efficiency

- Obstacles to success:
  - Accurate structure assignment
  - Ability to detect low level signals in complex matrices
  - Qualitative and quantitative methods
  - Untargeted detection highly desirable

SCIEX
Complete Solution for Metabolite ID

Introducing…
The SCIEX Accurate Mass Met ID Platform

Featuring TripleTOF® 6600 – Our most quantitative discovery system yet!

- **NEW TripleTOF® 6600 System**
  - Hardware innovations
- **NEW SWATH™ Acquisition 2.0**
  - New acquisition and processing strategies
- **NEW MetabolitePilot 2.0 Alpha**
  - Streamlined data analysis and interpretation

TripleTOF® 6600 System

Hardware Improvements

Detector
- 5 orders LDR

Ion Compression Optics

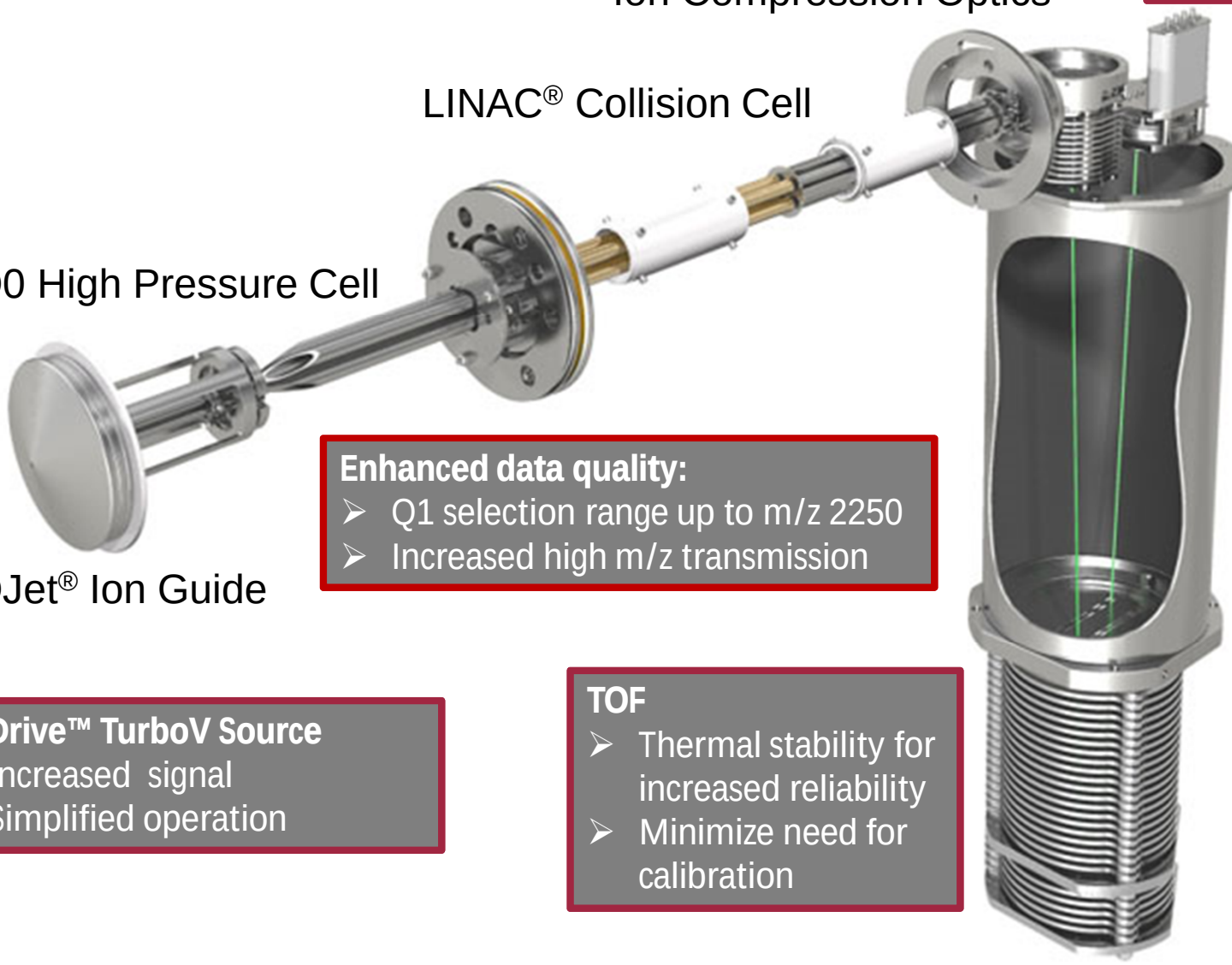
ADC Detector

LINAC® Collision Cell

30 kHz Accelerator

Q0 High Pressure Cell

15 kV Acceleration Voltage



Enhanced data quality:
- Q1 selection range up to m/z 2250
- Increased high m/z transmission

QJet® Ion Guide

IonDrive™ TurboV Source
- Increased signal
- Simplified operation

TOF
- Thermal stability for increased reliability
- Minimize need for calibration

Two Stage Mirror

TripleTOF® 6600 System

What's New!

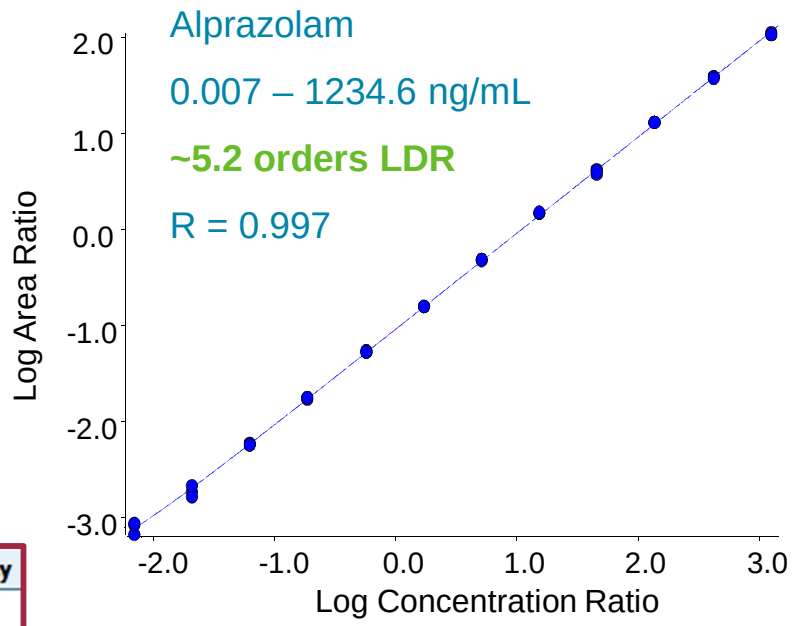
- Broader Dynamic Range
  - Enhanced detector technology **for greater than 5 orders linear dynamic range**
- Improved Coverage and Mass Selection
  - **Extended Q1 mass range up to 2250 m/z**
- Faster Acquisition Rates for comprehensive quant
  - Up to 100 MS/MS per cycle in IDA, up to 100 Hz
  - **Variable windows and up to 200 SWATH windows per cycle**
- Improved Mass Accuracy Stability
  - < 0.5 ppm, internal
  - < 2 ppm RMS, external
- High Resolution
  - > 35,000 in TOF MS
  - > 20,000 or > 30,000 in TOF MS/MS

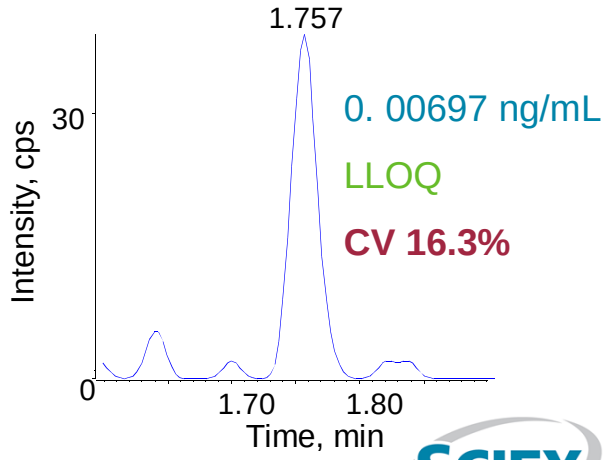
Detector Dynamic Range Extended

MRMHR Workflow

- 5.2 orders observed with Alprazolam using internal standard
- Detector saturation is no longer a limiting factor, source or column are likely to saturate before the detection system

Component N...	Actual Concentra...	Num. V...	Mean	Standard Devi...	Percent CV	Accuracy
Alprazolam 2...	0.00697	3 of 3	7.197e-3	1.171e-3	16.27	103.26
Alprazolam 2...	0.02091	3 of 3	1.889e-2	2.600e-3	13.77	90.33
Alprazolam 2...	0.06272	3 of 3	6.190e-2	1.091e-3	1.76	98.70
Alprazolam 2...	0.18817	3 of 3	1.909e-1	6.110e-3	3.20	101.48
Alprazolam 2...	0.56450	3 of 3	5.818e-1	1.415e-2	2.43	103.07
Alprazolam 2...	1.69351	3 of 3	1.718e0	1.140e-2	0.66	101.47
Alprazolam 2...	5.08053	3 of 3	5.237e0	1.687e-1	3.22	103.08
Alprazolam 2...	15.24158	3 of 3	1.600e1	4.537e-1	2.84	104.97
Alprazolam 2...	45.72474	3 of 3	4.375e1	1.913e0	4.37	95.67
Alprazolam 2...	137.17421	3 of 3	1.406e2	1.065e0	0.76	102.47
Alprazolam 2...	411.52263	3 of 3	4.125e2	4.320e0	1.05	100.24
Alprazolam 2...	1234.56790	3 of 3	1.176e3	1.985e1	1.69	95.26

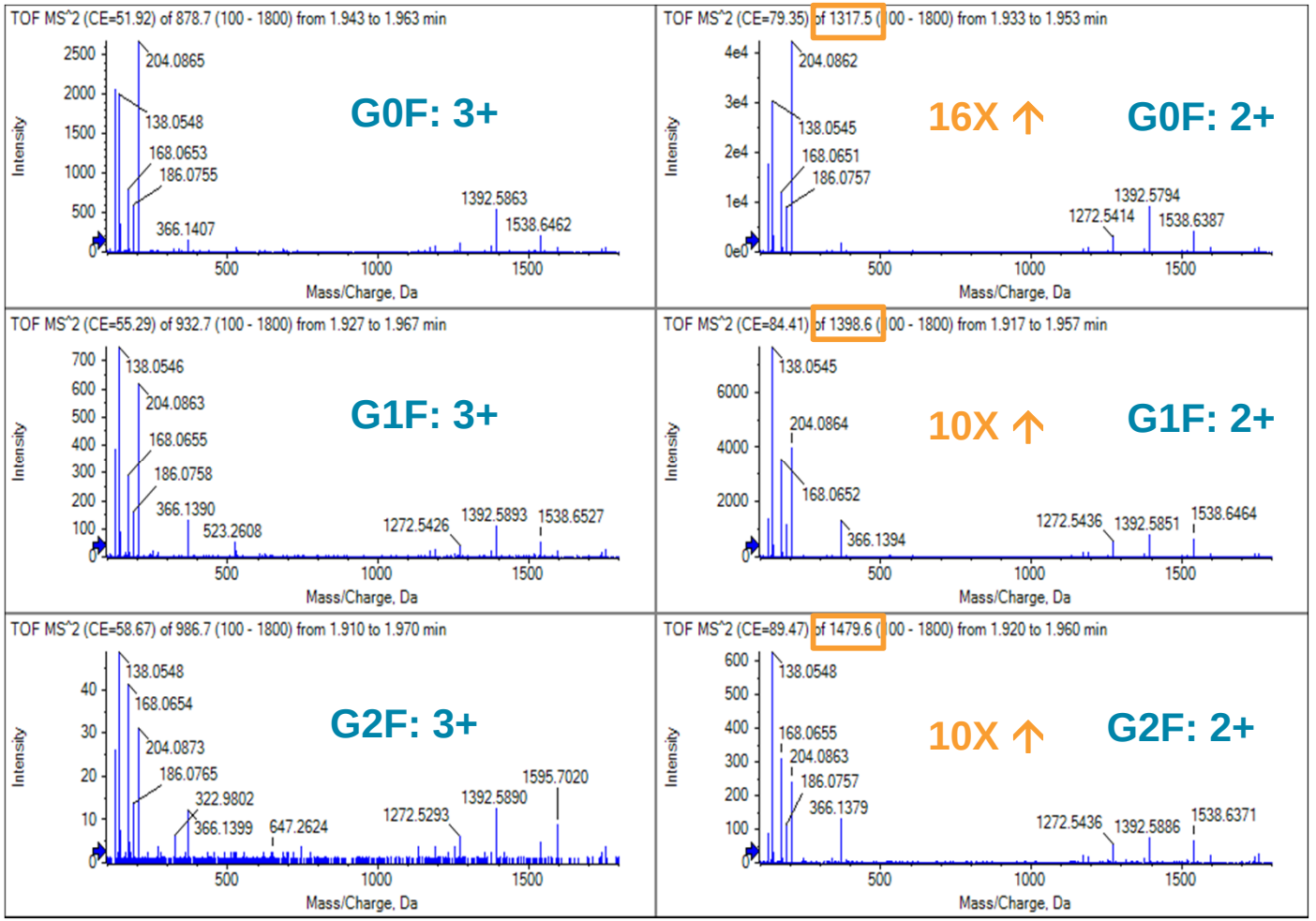




Q1 Transmission up to 2250 m/z

Glycopeptides Example

- Glycosylation an important PTM
- Glycopeptides can be quite large and sugar portion doesn't always take a lot of charge.
- 2+ ions of mAb glycopeptides are now accessible to MS/MS



2+ forms of these glycopeptides are 10-16x greater intensity than their 3+ charged counterparts.

Improved Source Design

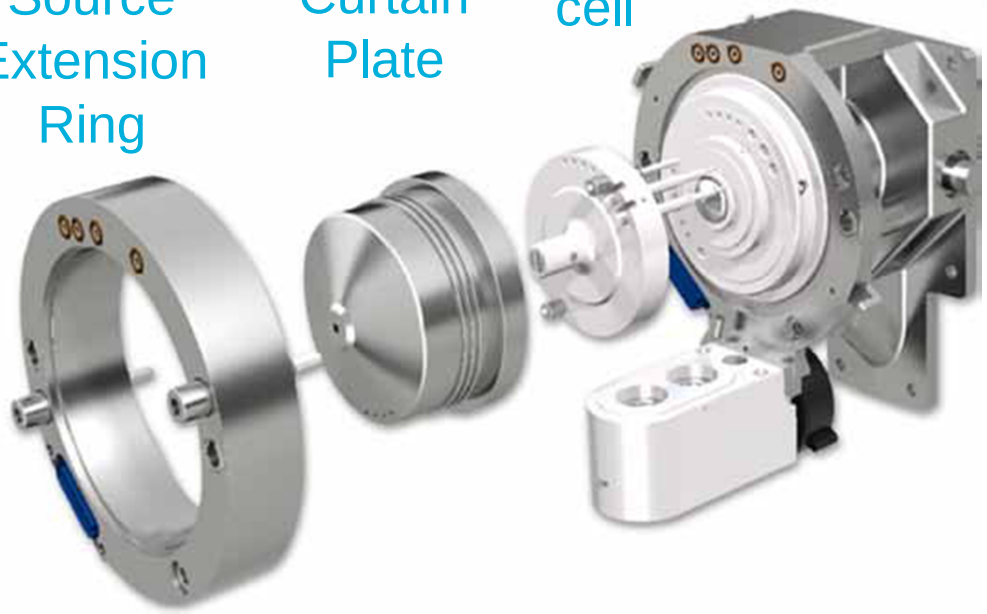
IonDrive™ Turbo V Source

- Larger diameter (11 mm) heaters
- Optimized geometry
- More efficient heat transfer
- Covers a larger cross-section of the spray cone
- Wider “sweet spot” when optimizing probe position
- More robust against fluctuations in gas flow dynamics, and source to source differences

Advances in Selectivity

SelexION™ Technology

- Differential Mobility Separation (DMS)
- Improved selectivity with MRM^{HR} Workflow
- Gas phase fractionation coupled with
  - TOF MS mapping
  - IDA
  - SWATH™ Acquisition



Addressing Metabolite ID Acquisition Challenges

Discovery and Development Met ID

- Information Dependent Acquisition
  - Optimal set up requires prior knowledge of analyte (i.e., m/z, signal)
  - Crowded chromatograms (matrix) and low level analytes can result in missing product ion data
  - Quantitation only with TOF data

- Data Independent Acquisition-SWATH
  - Single method for multiple compounds
  - Product ion spectra
    - Generated for all analytes
    - Retains isotope pattern information
  - Quantitation with TOF or High Resolution Product Ion data

SWATH Acquisition for Met ID Analysis

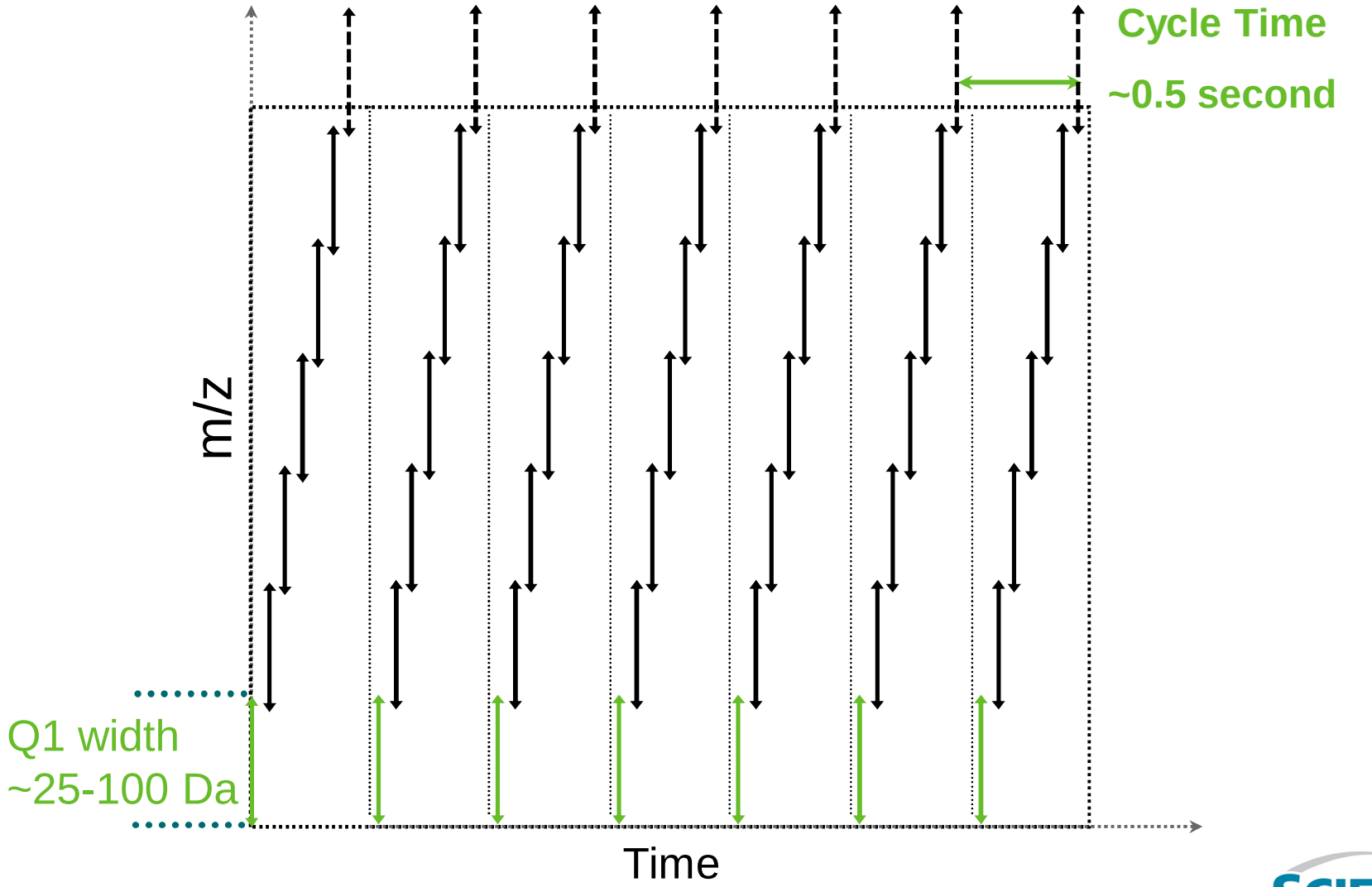
Key benefits and features

- Benefits of SWATH Acquisition
  - Generic method useful for Discovery and Development Phase
  - Comprehensive qualitative and quantitative analysis
  - Ultimate safety net for capturing both predicted and unpredicted metabolites / catabolites
  - Intuitive data processing and broad coverage for metabolite ID

- Unique Features of SWATH Acquisition
  - Speed of TripleTOF allows SWATH on an LC time scale
  - Selective and sensitive MS/MS Quantification
  - Less complex MS/MS spectrum than traditional DIA techniques
  - SWATH Q1 window retains isotope pattern for each fragment
  - Good for ^{14}C/SIL metabolism studies
  - 100% MS/MS for low level metabolite/catabolite identification

Experimental Details: SWATH for Discovery Met ID

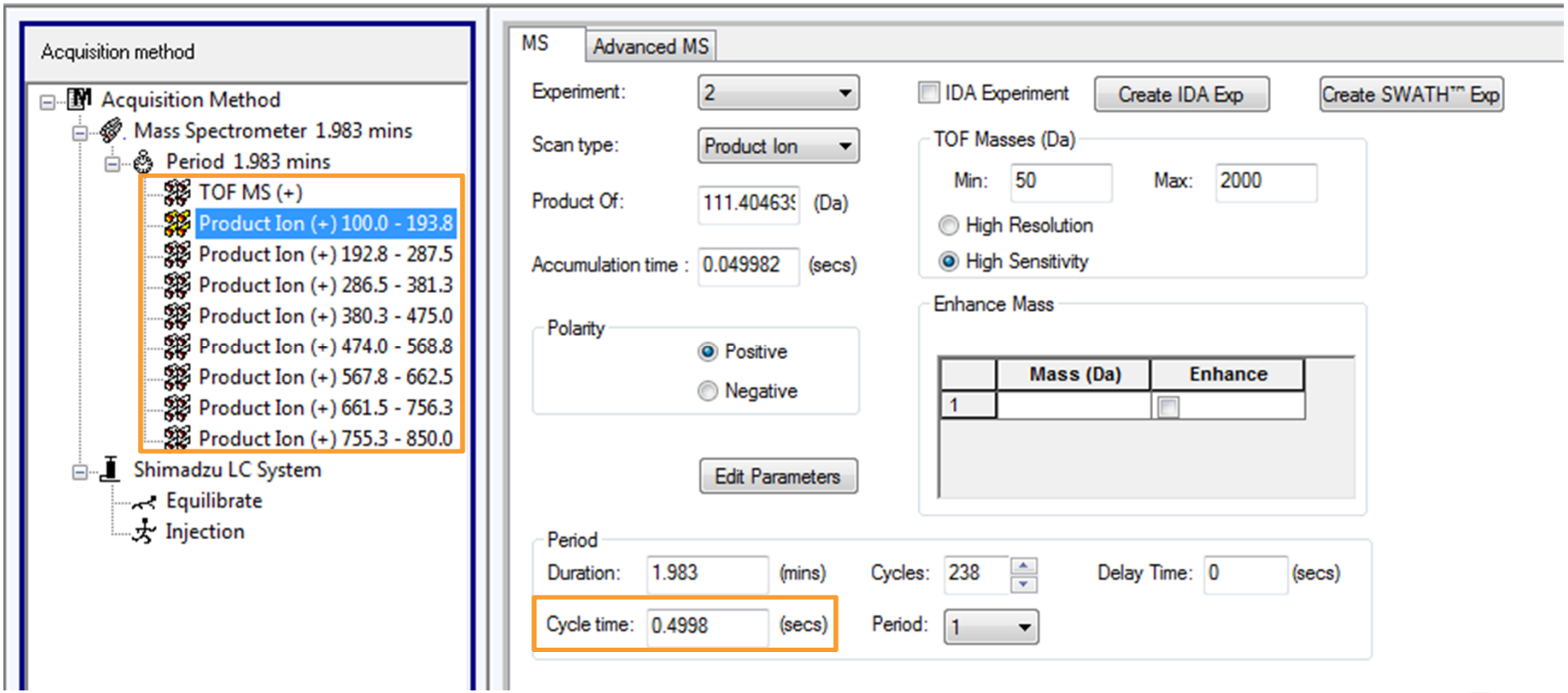
Data Independent Acquisition: SWATH



Experimental Details: SWATH

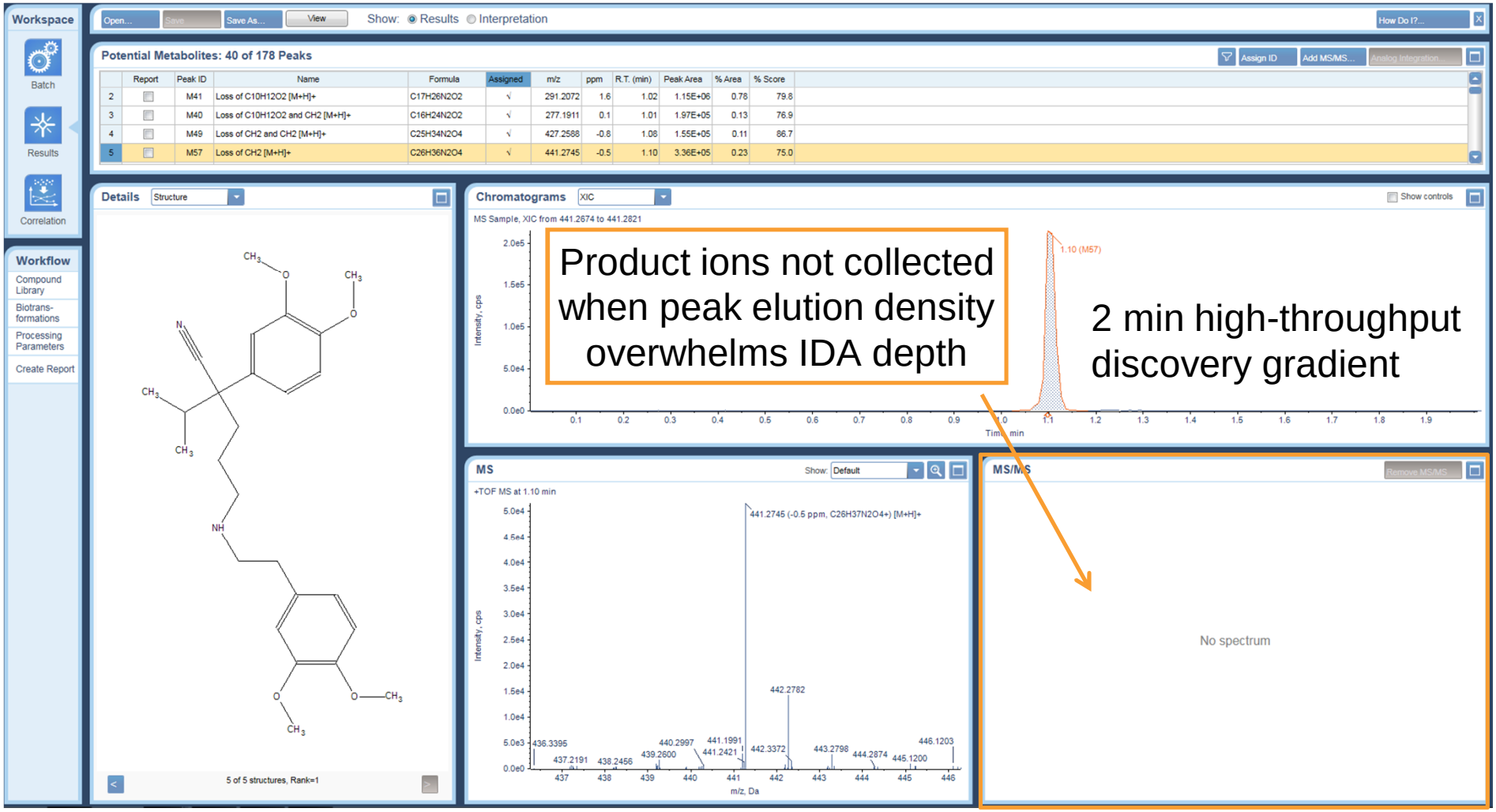
Data Independent Acquisition

- Analyst TF 1.7 helps create a SWATH method.
- Product Ions are collected without regard to decision criteria.



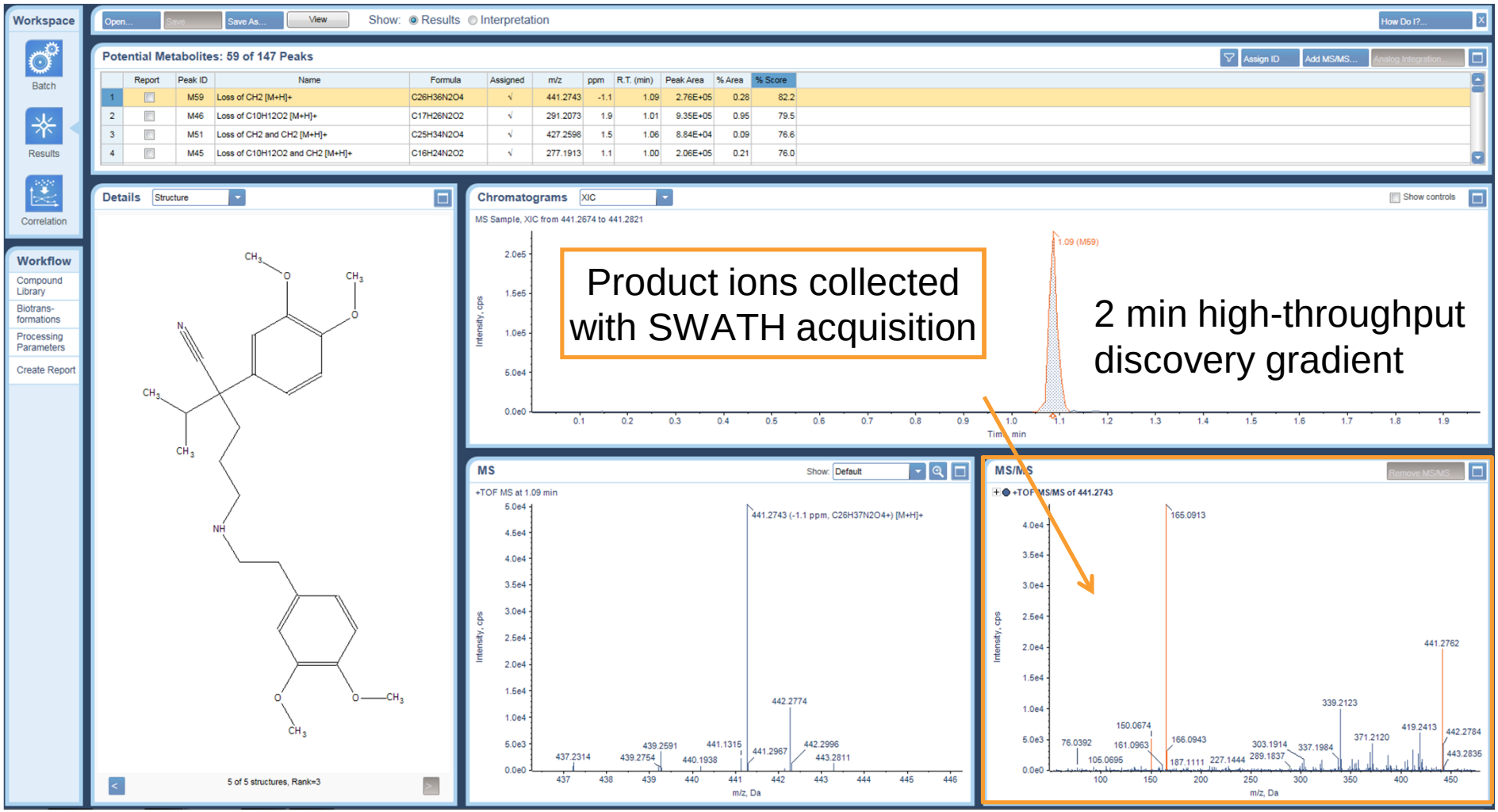
Experimental Details: IDA vs SWATH

IDA results N-Desmethyl verapamil



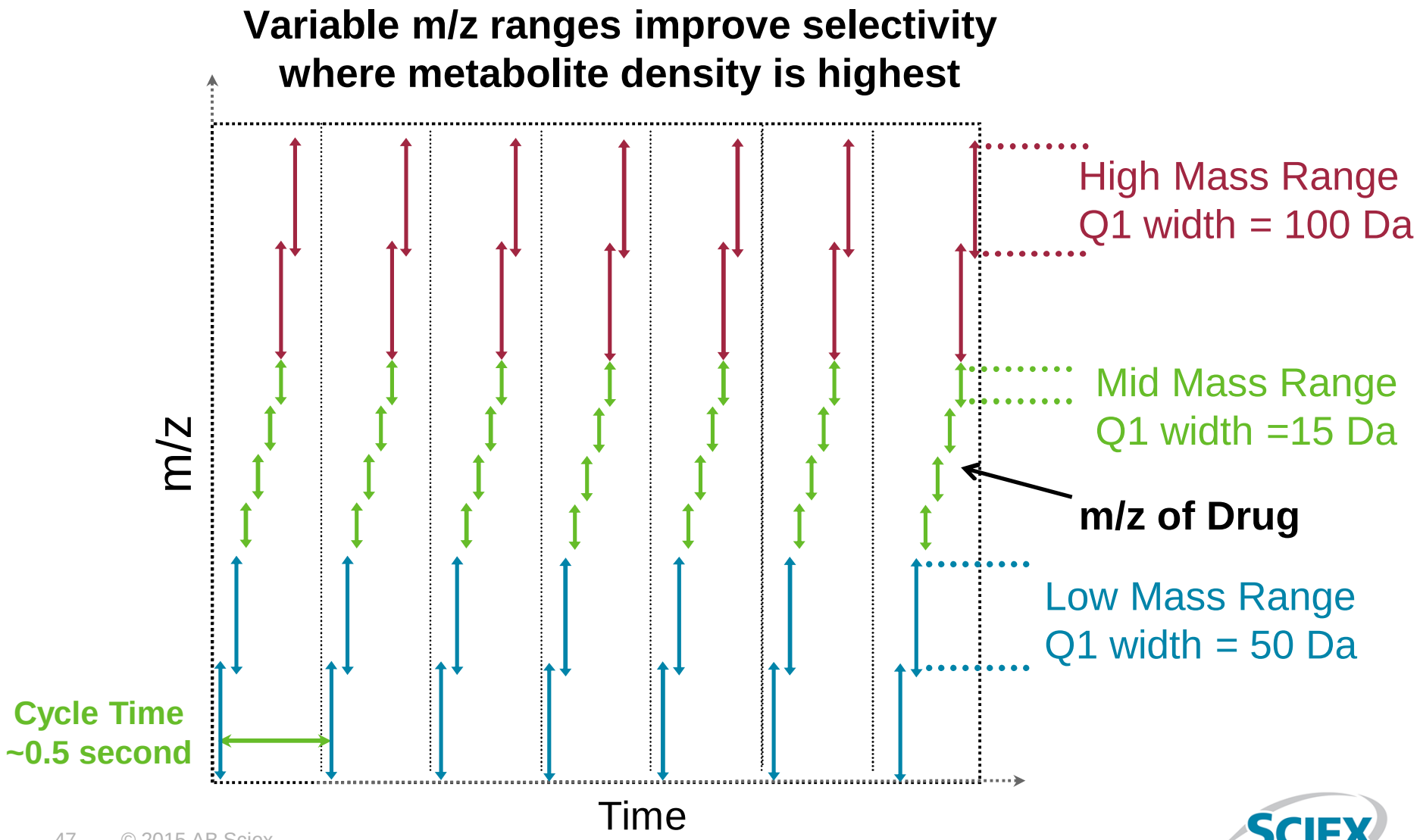
Experimental Details: IDA vs SWATH

SWATH results N-Desmethyl verapamil



Experimental Details: SWATH for Development Met ID

Data Independent Acquisition: SWATH with Variable Windows



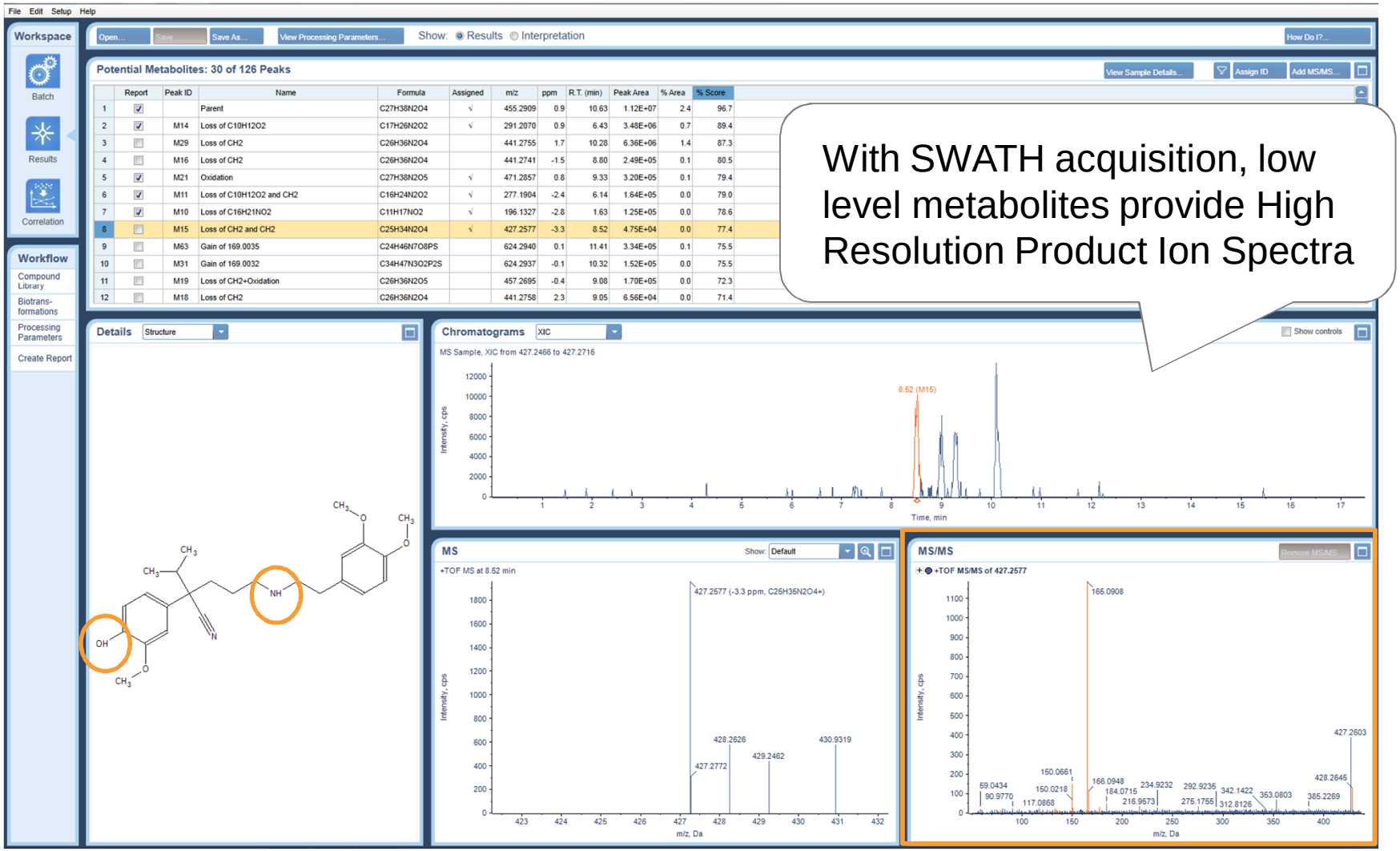
Data Processing

MetabolitePilot 2.0 Alpha

- Process and interrogate accurate mass metabolism data
- Multiple Mass Defect filtering capabilities for cleaner, more relevant data
- Compound Library & Results Database to store & retrieve important project information
- Batch processing for multiple sample sets
- Correlation Workspace
  - Select Multiple Samples (i.e., time points or different species)
  - Correlate Results
- Interpretation View
  - Fragmentation interpretation
  - Structural elucidation of metabolites

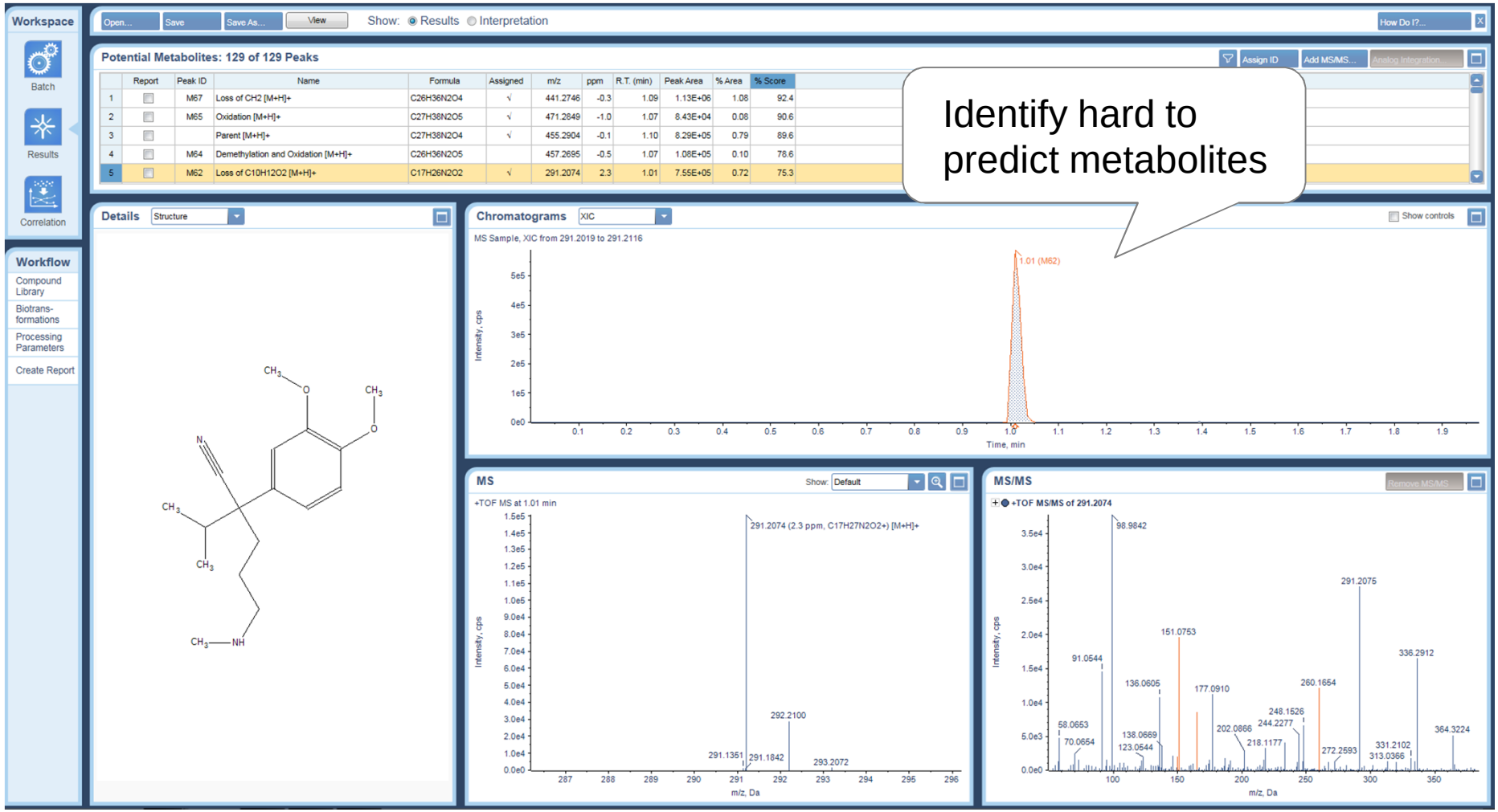
Data Processing: Results

MetabolitePilot 2.0 Alpha: Verapamil 10uM HLM incubation



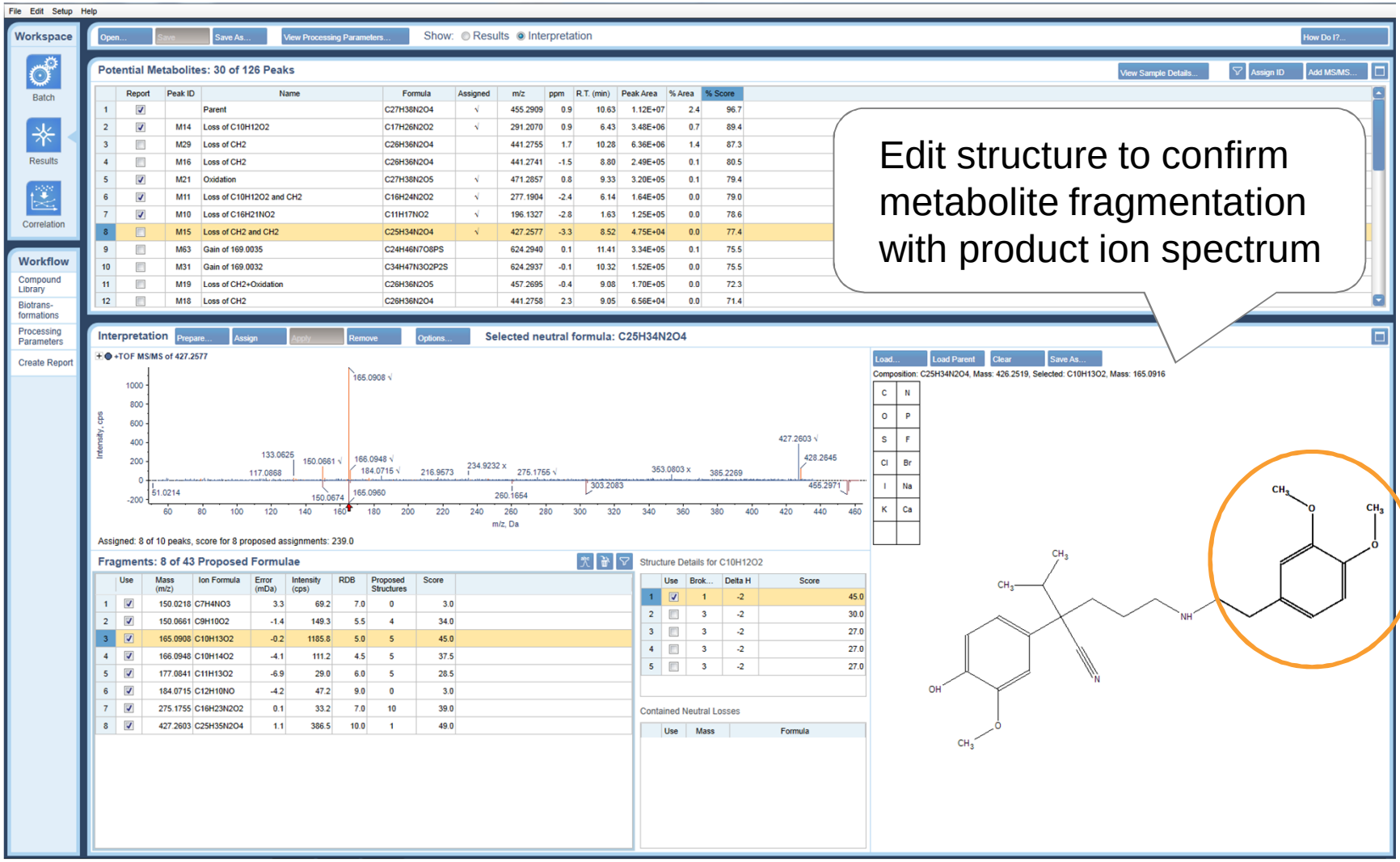
Data Processing: Results

MetabolitePilot 2.0 Alpha: Verapamil 1uM HLM incubation



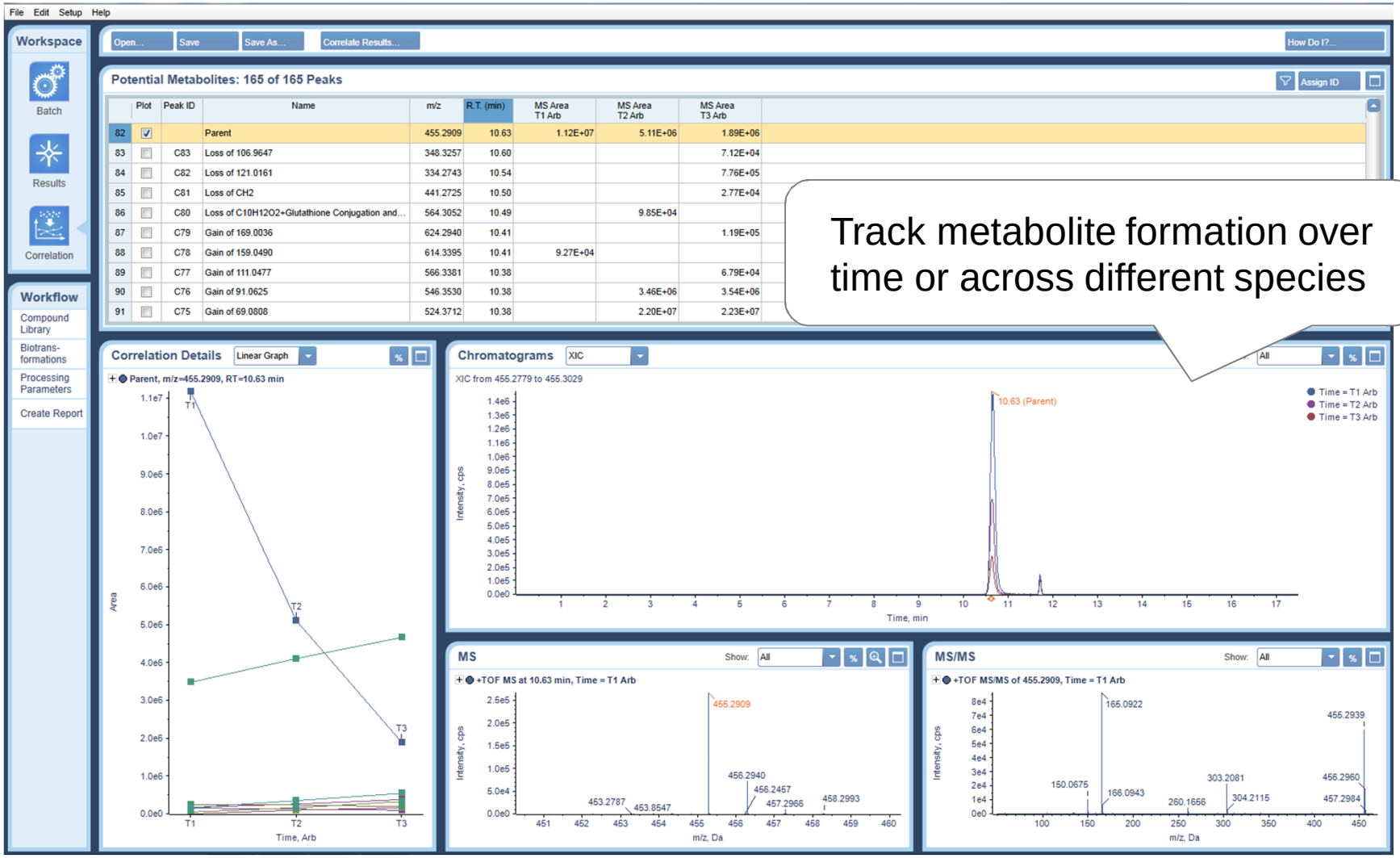
Data Processing: Interpretation

MetabolitePilot 2.0 Alpha: Verapamil 10uM HLM incubation



Data Processing: Correlation

MetabolitePilot 2.0 Alpha: Verapamil 10uM HLM incubation



Summary

- The TripleTOF® 6600 System and SWATH™ Acquisition 2.0 for Discovery and Development Metabolite ID provides
  - A digital MS & MSMS record of a sample/time point/species study. Allowing for retrospective data mining of the data without performing re-incurred analysis
  - Increased sample throughput and efficiency of data processing
  - Advances for detecting, characterizing, and quantifying metabolites with accuracy and efficiency

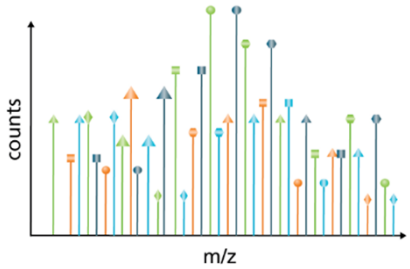
SWATH™ Acquisition to MRM Workflow

Pathway for a Complete Solution

Discovery → **Development** → **GLP Bioanalysis**

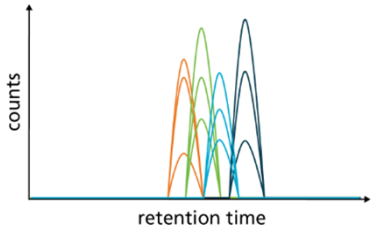
TripleTOF® System

SWATH™ Acquisition



High Res XICs

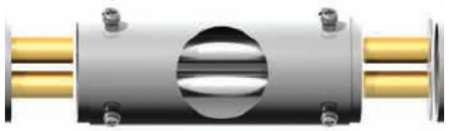
Multiple Reaction Monitoring (MRM)



High Sensitivity Quant

QTRAP® System

Q2



Easy Method Transfer with LINAC® Collision Cell

SCIEX

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Knowledge for Life.™

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