



Search for What's Missing: Unknown Compound Characterization Using LC-MS

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Small molecule structure analysis encompasses broad applications:

Pharmaceutical, metabolomics, food & environmental, clinical, forensic, industrial chemical, etc.

The following are small molecule structure analyses and are crucial for pharma R&D.

- Impurity analysis
 - Drug substance (API)
 - Drug product
 - Genotoxic
 - Stability studies - degradants
- Met ID
 - Drug discovery – pre-clinical development – clinical development
 - *In vitro* (hepatocytes, microsomes, whole blood, plasma)
 - *In vivo* (whole blood, plasma, urine, bile, fecal homogenates)
 - Radio-labeled (^{13}C , ^{14}C , ^3H)
- E&L analysis
 - Extractables
 - Leachables
- Natural products & traditional medicines research
 - Discovery and Identify new medicine

Small Molecule Structure Analyses are Challenging and Complex

- Broad range of chemicals with very diverse structures
 - ChemSpider has 71 M chemical structures.
- Background Interference
 - Complex biologic matrices, excipients, and solvent background
- Sample Limitations
 - Example: ADC drug Met ID, the small molecule “warhead” is only small portion of the drug.
- Unknowns
 - *De Novo* structural determination is not trivial!
- Many small molecule structure analyses are highly regulated.
 - Pharma R&D must follow EPA, FDA, EMEA, countries' regulations and guidelines: compliance and GLP.
 - Toxicity assessments, clinic trails... to ensure drug efficacy and consumer safety

All decisions must be based on solid scientific analysis results.

High Quality Data is Vital!

Reference: Mass Spectrometry Identification Categories in USP Chapter <1663>

Data typically available from GC/MS and LC/MS analyses (see A through E below) are used to designate individual extractables identifications in the categories of **Confirmed**, **Confident**, or **Tentative**

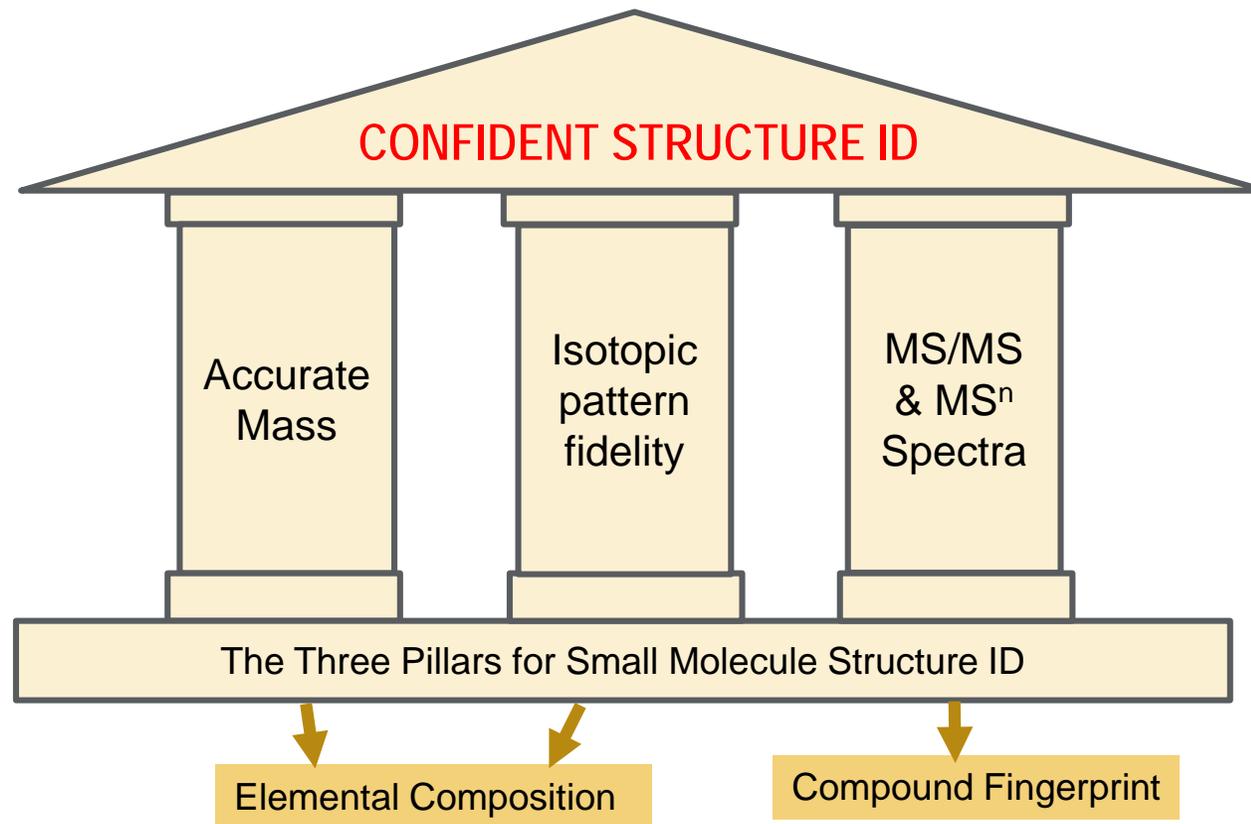
- A. Mass spectrometric fragmentation behavior
- B. Confirmation of molecular weight
- C. Confirmation of elemental composition
- D. Mass spectrum matches automated library or literature spectrum
- E. Mass spectrum and chromatographic retention index match authentic reference compound

Confirmed - A **Confirmed** identification means that A, B (or C), and D (or E) have been fulfilled.

Confident - A **Confident** identification means that sufficient data to preclude all but the most closely related structures have been obtained. The combination of D with any of A, B, or C can be used to provide a confident identification

Tentative - A **Tentative** identification means that data have been obtained that are consistent with a class of molecule only.

Confident Structure ID Requires High Resolution Accurate Mass (HRAM) and MSⁿ Fragments



The Instrument of Choice: Orbitrap™ Mass Spectrometer

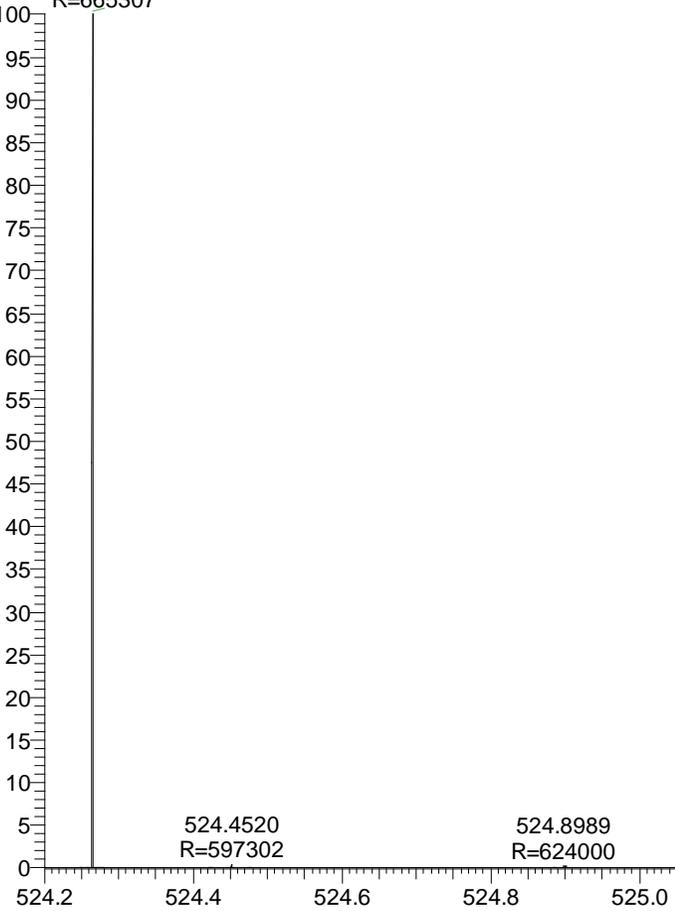
The Power of High Resolution MS: Fine Isotope Structure MRFA

MRFA

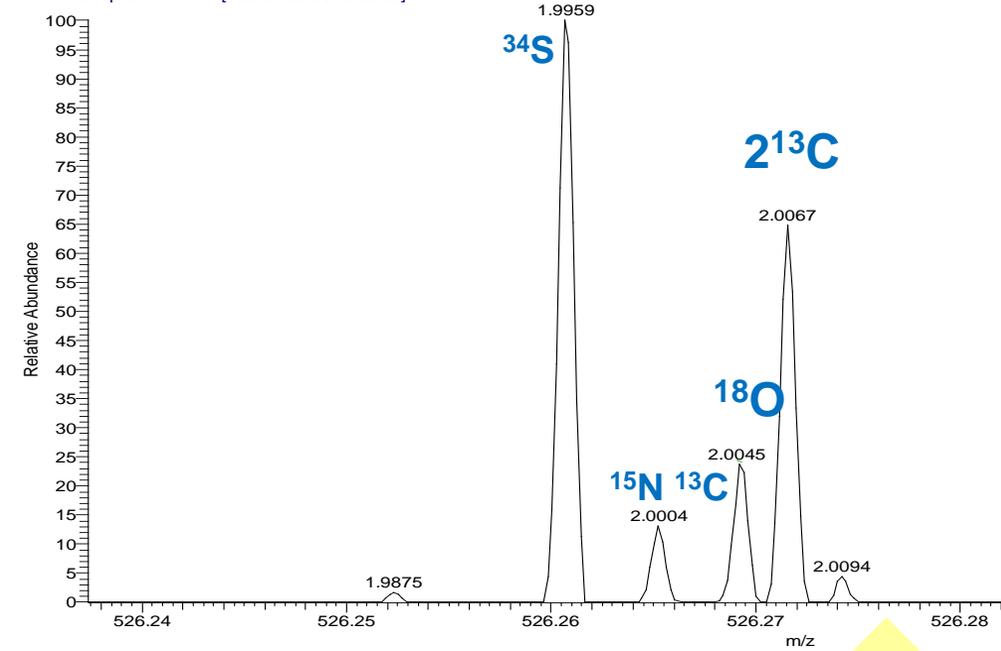
SS_calmix_5e4_1M_stdpress #2 RT: 0.07 AV: 1 NL: 2.82E7
T: FTMS + p ESI Full ms [150.0000-2000.0000]

524.2648
R=665307

Relative Abundance

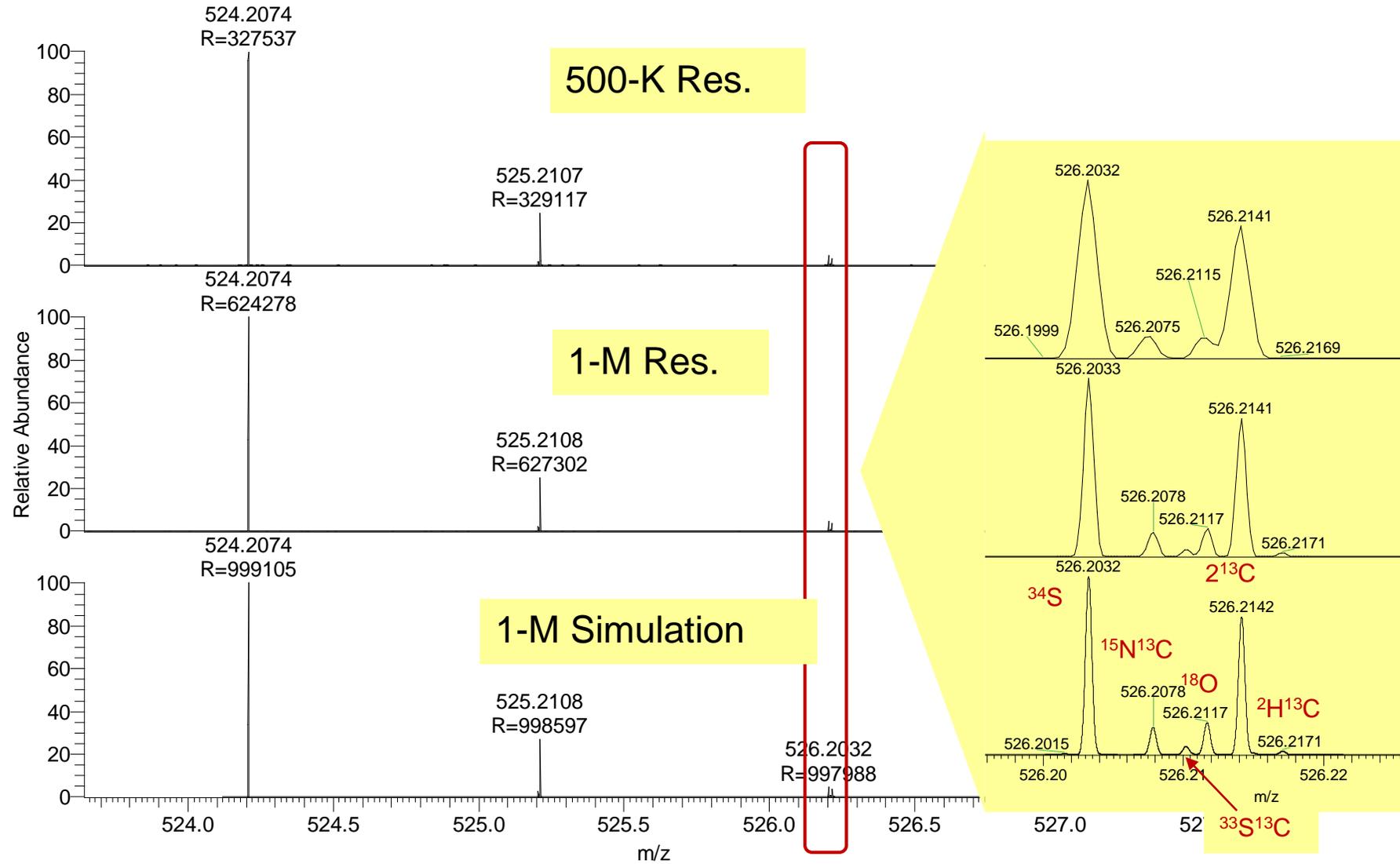


SS_calmix_5e4_1M_stdpress #2 RT: 0.07 AV: 1 NL: 1.25E6
T: FTMS + p ESI Full ms [150.0000-2000.0000]



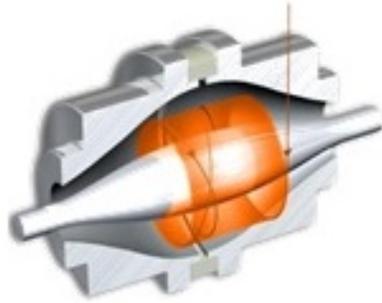
A+2

Ultra High Resolution for Unknown Impurity ID



Orbitrap MS for Small Molecule Structure Analysis

Thermo Scientific LTQ Orbitrap MS – First Generation Hybrid MS



Since 2005 ASMS introduction, Orbitrap MS has become the gold standard for small molecular structure analysis.

Thermo Scientific Q Exactive MS Family – Quan/Qual Workhorse

Thermo Scientific™ Q Exactive™ HF MS



Resolving Power: 240K @ m/z 200
Scan Range: 50-6,000
Scan rate: 18Hz at 15K

Thermo Scientific™ Q Exactive™ Plus MS

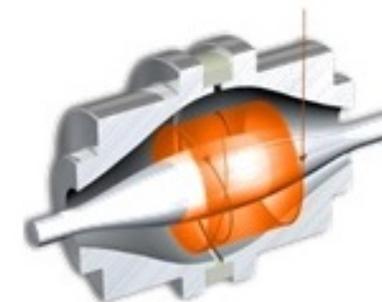


Resolving Power: 140K @ m/z 200
Scan Range: 50-6,000
Scan Rate: 12 Hz at 17.5K
Optional: 280K

Thermo Scientific™ Q Exactive™ MS



Resolving Power: 140K @ m/z 200
Scan Range: 50-6,000
Scan Rate: 12 Hz at 17.5K



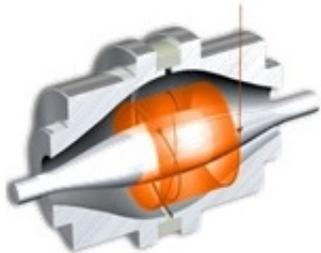
Thermo Scientific™ Q Exactive™ Focus MS



Resolving Power: 70K @ m/z 200
Scan Range: m/z 50-2,000
Scan Rate: 12 Hz at 17.5K

First bench-top Orbitrap MS
High performance
Easy to use, robust
Polarity switching <1 sec
HCD MS2

Transforming Small Molecule Identification and Characterization



- Mass Range m/z 50 – 2000
- Mass Accuracy <1ppm internal, <3ppm external
- Max. Mass Resolution >500,000
- Scan rate 30 Hz OT MS² /40 Hz IT MS²
- Dissociation HCD, CID
- MS/MS and MSⁿ
- Polarity switching on the fly

Thermo Scientific™ Orbitrap ID-X™ Tribrid™ MS – Optimized and dedicated to small molecule structure analysis

- High performance

- Very high resolution (500K at m/z 200) and high scan rate (30 Hz OT/40 Hz IT)
- MSⁿ, CID/HCD multiple dissociation techniques in a single run, OT/IT parallel detection ...

- Ultimate flexibility and capability for data acquisition

- Comprehensive, feature-specific filters enable triggering MSⁿ of low abundant components
- Predefined method templates for quick start

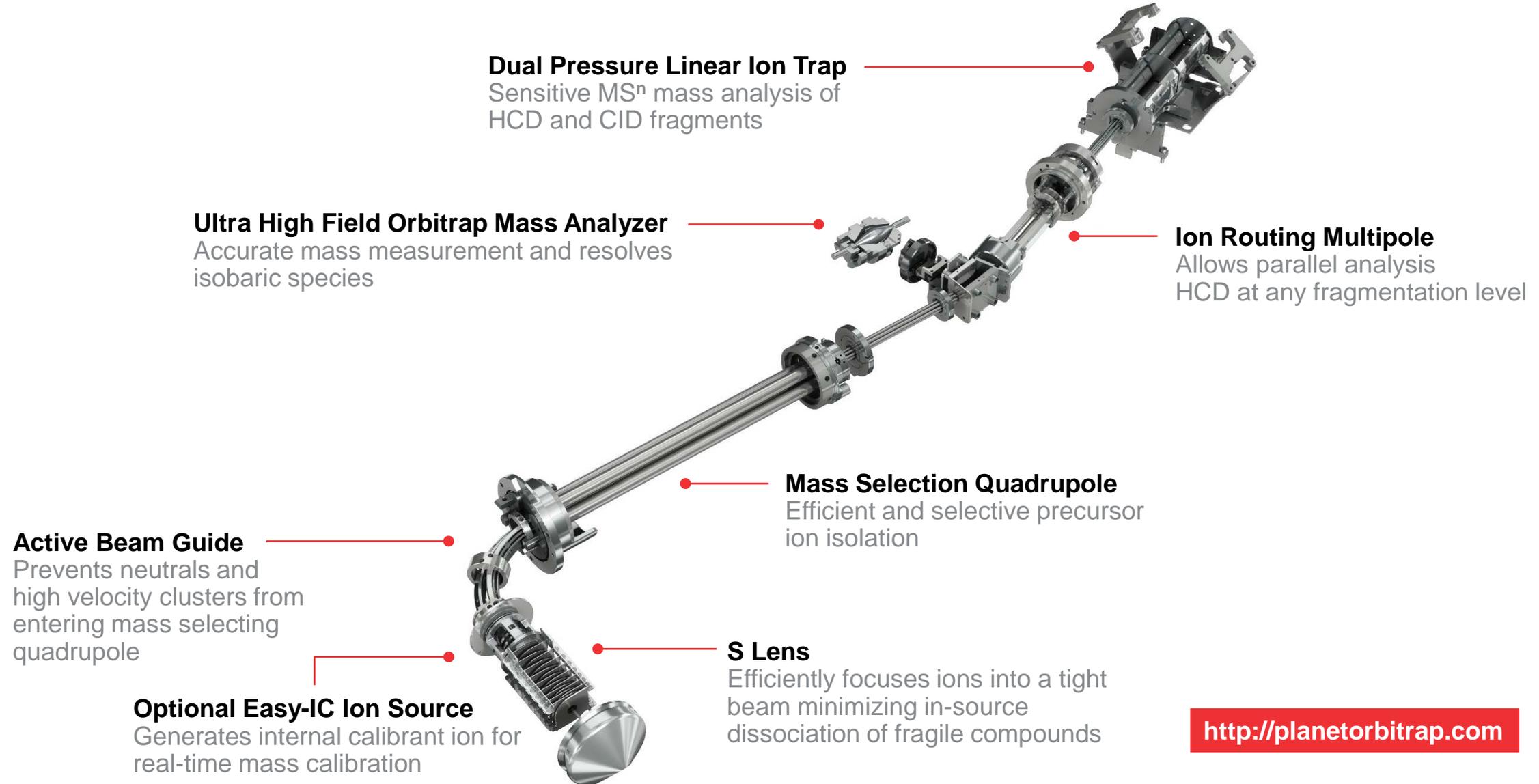
- Novel data acquisition – AcquireX

- Automatic background exclusion, greatly improves efficiency, quality and accuracy of analysis

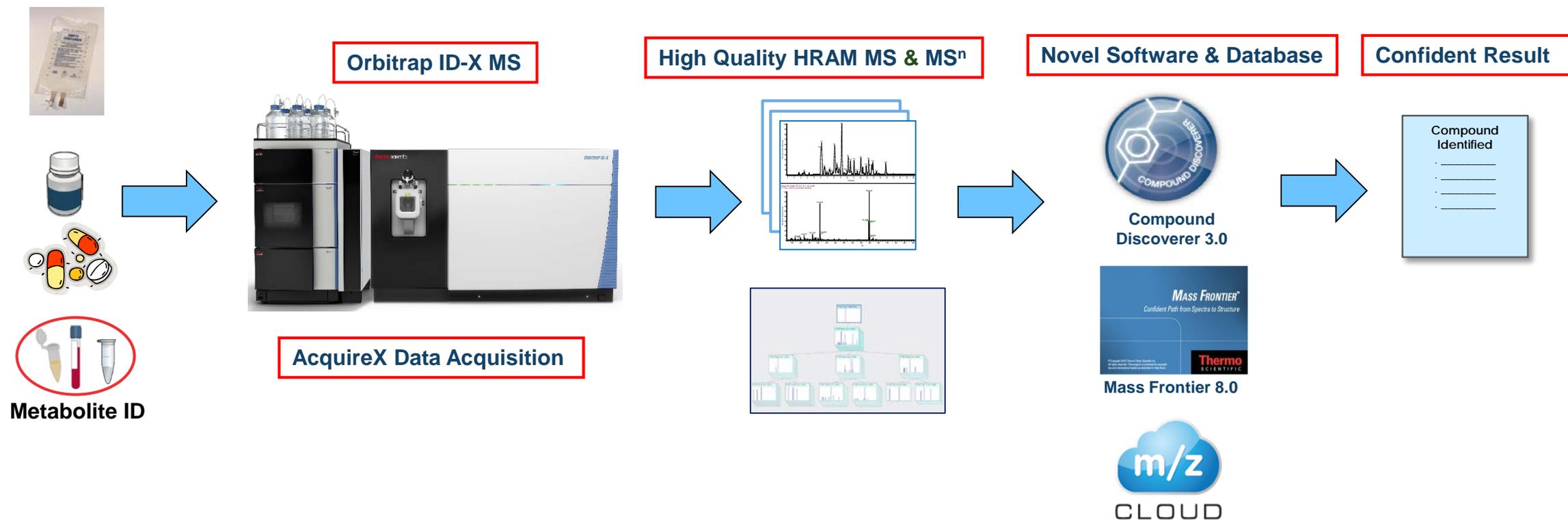
- Advanced data processing SW suite and database

- Thermo Scientific™ Compound Discoverer™ 3.0 software, Thermo Scientific™ Mass Frontier™ 8.0 software, and mzCloud™.
mzCloud is a trademark of HighChem LLC, Slovakia

Schematic for Thermo Scientific Orbitrap ID-X MS – Improved Instrumentation



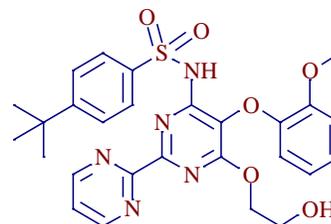
General Workflow for Small Molecule Structure Analysis



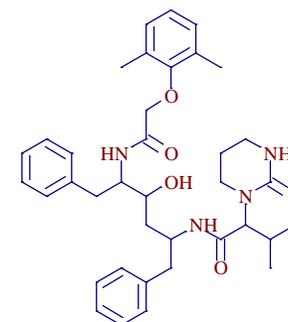
Case Study: Metabolite Identification of 5 Drugs in Human Liver Microsomal Incubation



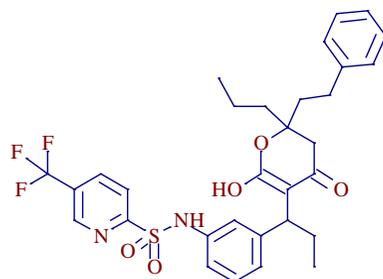
Amprenavir
C₂₅H₃₅N₃O₆S
(M+H)⁺ 506.23193
Cas# 161814-49-9



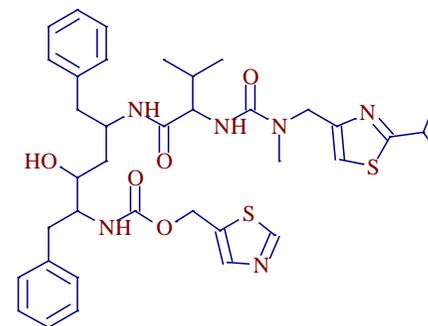
Bosentan
C₂₇H₂₉N₅O₆S
(M+H)⁺ 552.19113
Cas# 147536-97-8



Lopinavir
C₃₇H₄₈N₄O₅
(M+H)⁺ 629.36975
Cas# 192725-17-0



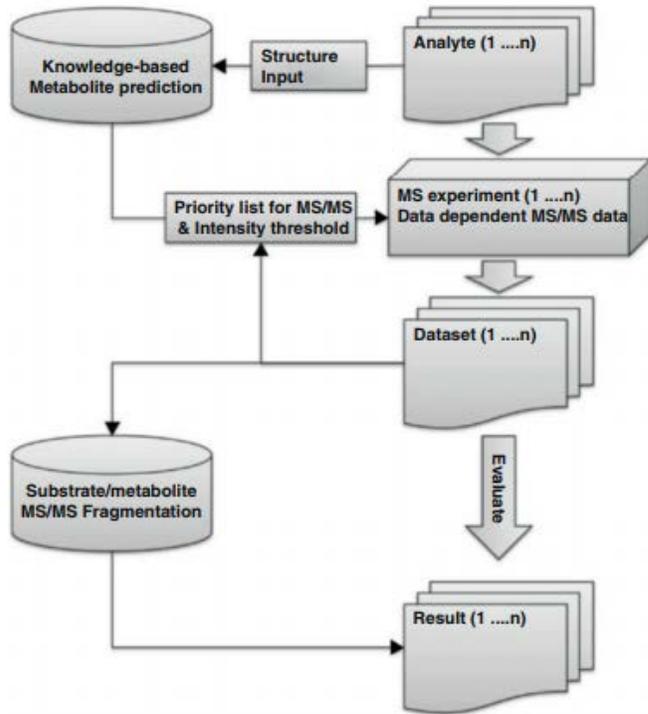
Tipranavir
C₃₁H₃₃F₃N₂O₅S
(M+H)⁺ 603.21350
Cas# 174484-41-4



Ritonavir
C₃₇H₄₈N₆O₅S₂
(M+H)⁺ 721.32004
Cas# 155213-67-5

Traditional Data Dependent Acquisition of MS/MS Spectra for Drug Metabolites

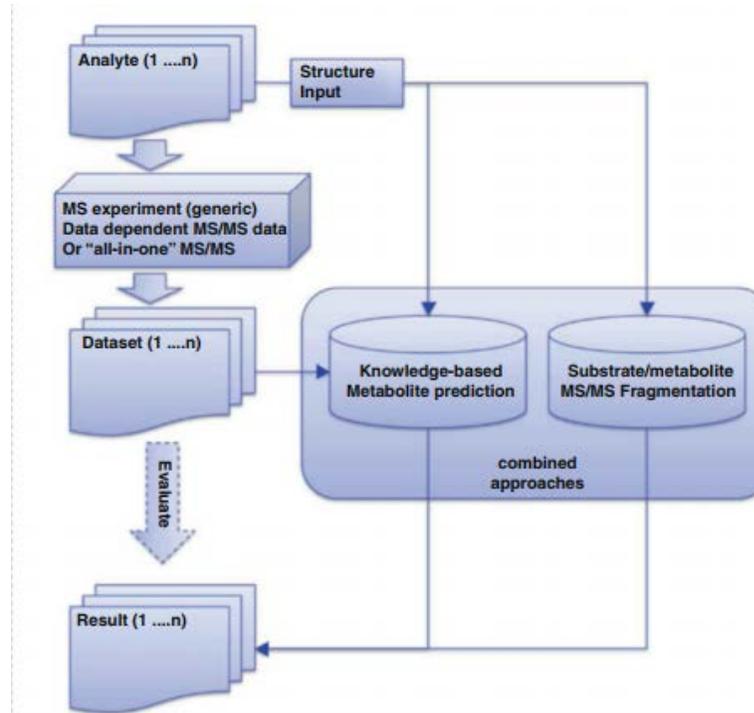
Targeted Approach



DDA Selection Criteria:

- m/z inclusion list based on predicted metabolites
- Isotope pattern
- Mass defect

Non-targeted Approach



DDA Selection Criteria:

- Intensity based ("top N" most intense)
- All ion fragmentation

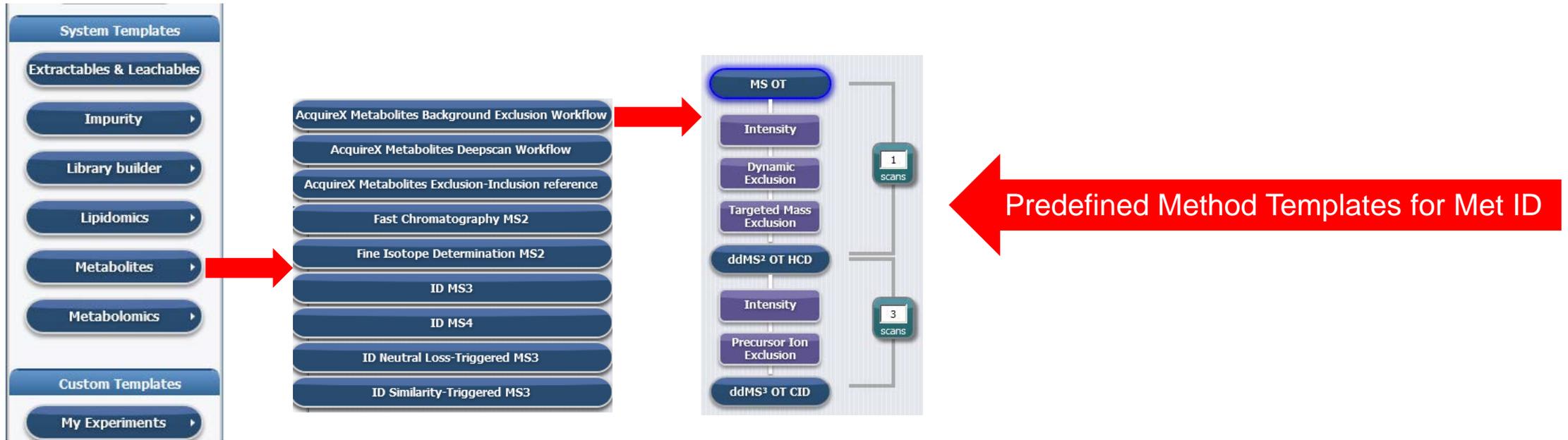
Pähler and Brink, *Drug Discov Today Technol.*, 2013, 10, e207-213

AcquireX with Orbitrap ID-X MS – Tackles the Identification Bottleneck Automatically

- Automatically generates an exclusion list from a control sample.
 - Excludes the background ions from triggering MSⁿ
 - Intelligent data-dependent acquisition - only triggers the ions of interest that are not present in the control.
- Automatically generate an inclusion list of samples for deep scan when needed.
- High quality MSⁿ data in one run and no repeat injections, no need for user to build exclusion and inclusion list offline
- Acquire useful data - better than data-independent acquisition (DIA)

AcquireX greatly improves analysis efficiency, quality, and accuracy!

AcquireX Acquisition Workflow: High Performance and Easy to Use



AcquireX Acquisition Workflow

The diagram shows three panels, each with a small flowchart at the top and a description below. A red arrow points from the 'AcquireX Acquisition Workflow' text to the first panel.

- BACKGROUND EXCLUSION**
Create and use an exclusion list of constant background ions and peaks to reduce background fragmentation in your ID runs
What Xcalibur Does:
Generates up to 1 exclusion list per sequence
Updates the ddMSn method with the exclusion list
Automatically inject ID samples with updated ddMSn method
SELECT
- BACKGROUND EXCLUSION & COMPONENT INCLUSION**
Combines exclusion and inclusion lists to automatically and reliably acquire more relevant MSn data in a single injection
What Xcalibur Does:
Generates up to 1 exclusion list per sequence
Generates 1 inclusion list per ID injection block
Injects user-defined number of ID injection blocks
SELECT
- DEEP SCAN**
Combines a single exclusion and inclusion list with multiple ID injections to comprehensively fragment relevant precursor ions
What Xcalibur Does:
Generates up to 1 exclusion list per sequence
Generates up to 1 inclusion list per sequence
Injects ID samples until all inclusion list ions are fragmented or user defined number of ID samples are reached
SELECT

AcquireX Data Acquisition Workflow and Sequence Setup

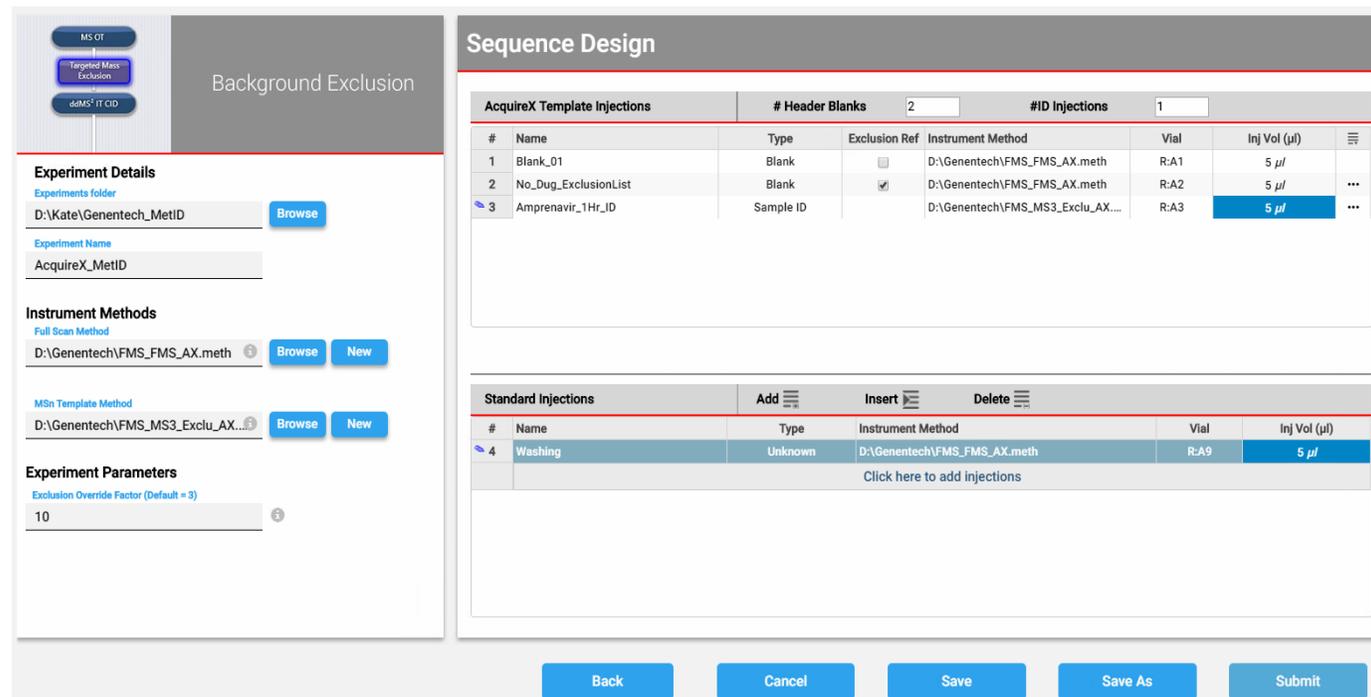
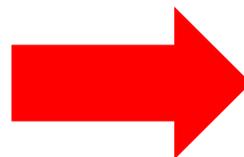


BACKGROUND EXCLUSION

Create and use an exclusion list of constant background ions and peaks to reduce background fragmentation in your ID runs

What Xcalibur Does:

- Generates up to 1 exclusion list per sequence
- Updates the ddMSn method with the exclusion list
- Automatically inject ID samples with updated ddMSn method



Background Exclusion

MS OT
Targeted Mass Exclusion
ddMS² IT CID

Experiment Details

Experiments folder
D:\Kate\Genotech_MetID [Browse](#)

Experiment Name
AcquireX_MetID

Instrument Methods

Full Scan Method
D:\Genotech\FMS_FMS_AX.meth [Browse](#) [New](#)

MSn Template Method
D:\Genotech\FMS_MS3_Exclu_AX... [Browse](#) [New](#)

Experiment Parameters

Exclusion Override Factor (Default = 3)
10

Sequence Design

AcquireX Template Injections # Header Blanks: 2 #ID Injections: 1

#	Name	Type	Exclusion Ref	Instrument Method	Vial	Inj Vol (µl)	
1	Blank_01	Blank	<input type="checkbox"/>	D:\Genotech\FMS_FMS_AX.meth	R:A1	5 µl	
2	No_Dug_ExclusionList	Blank	<input checked="" type="checkbox"/>	D:\Genotech\FMS_FMS_AX.meth	R:A2	5 µl	...
3	Amprenavir_1Hr_ID	Sample ID		D:\Genotech\FMS_MS3_Exclu_AX...	R:A3	5 µl	...

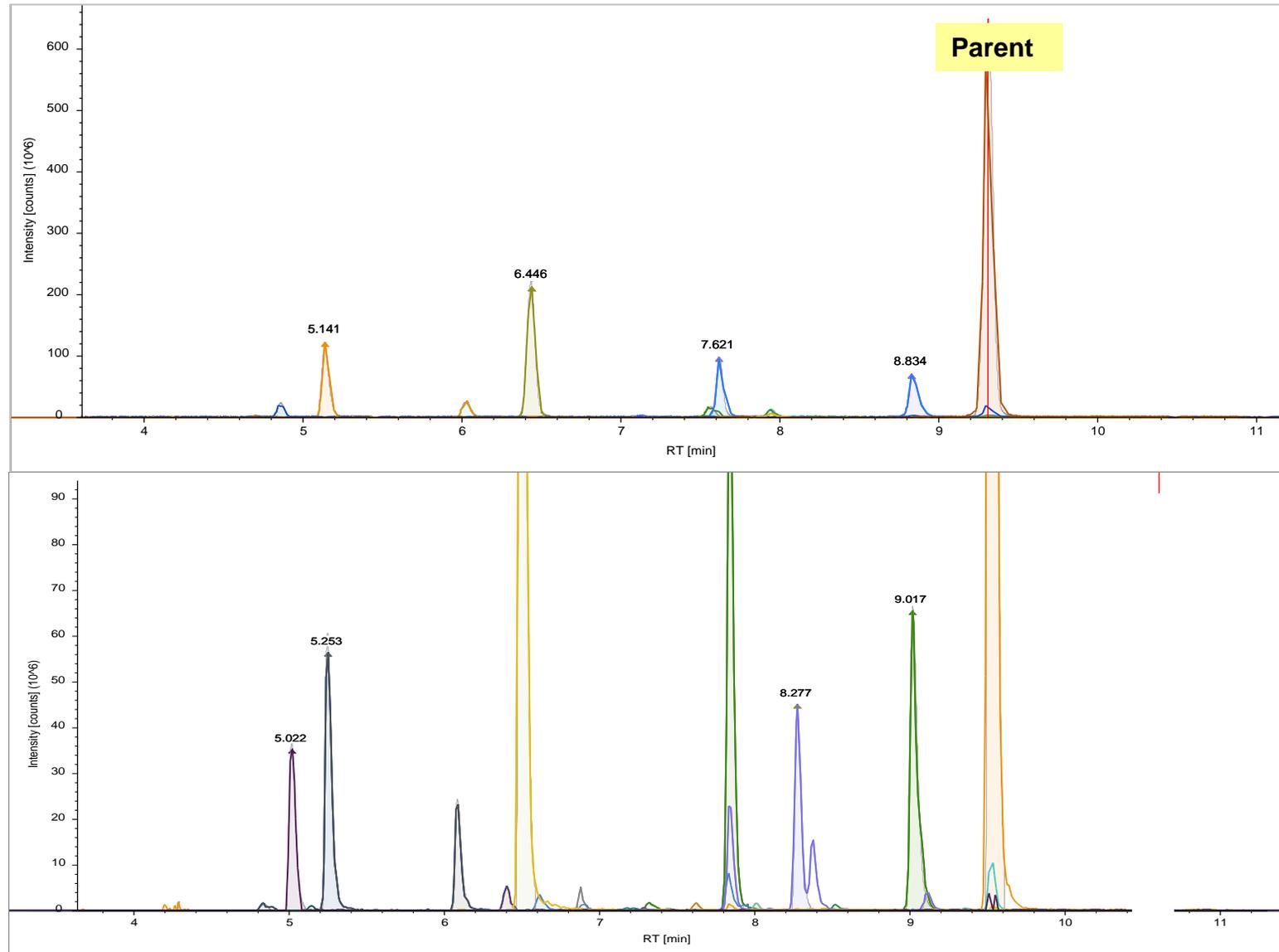
Standard Injections [Add](#) [Insert](#) [Delete](#)

#	Name	Type	Instrument Method	Vial	Inj Vol (µl)
4	Washing	Unknown	D:\Genotech\FMS_FMS_AX.meth	R:A9	5 µl

[Click here to add injections](#)

[Back](#) [Cancel](#) [Save](#) [Save As](#) [Submit](#)

Conventional and AcquireX Workflow Comparison: Amprenavir as an Example

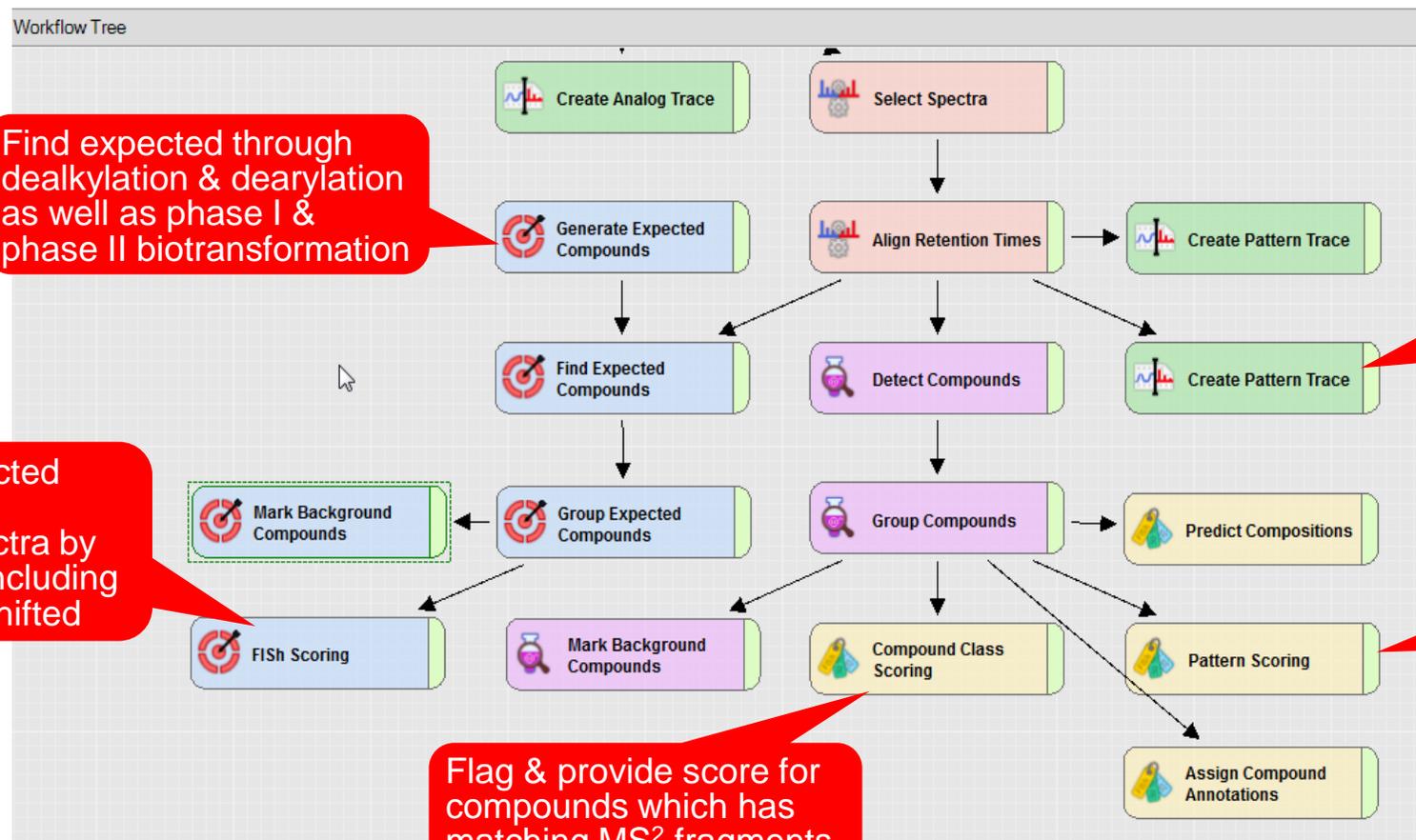


DDA triggered 8 metabolites

AcquireX triggered 21 metabolites

Data Processing Using Thermo Scientific Compound Discoverer 3.0 (CD 3.0) Software

Utilizing unique features of CD 3.0, metabolites both expected and unknown were identified using pre-made “Expected and Unknown” processing workflow



Find expected through dealkylation & dearylation as well as phase I & phase II biotransformation

XIC traces for analytes containing user-defined isotopic patterns

Provide score for detected expected compounds; annotates on MSⁿ spectra by fragment ion search, including transformation mass shifted

Provide score for analytes containing user-defined isotopic patterns

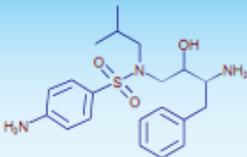
Flag & provide score for compounds which has matching MS² fragments with parent compound

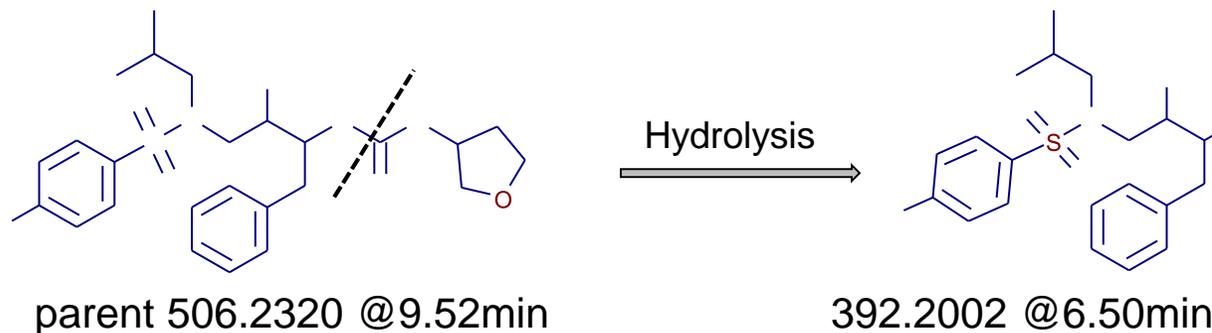
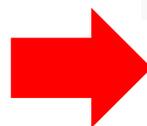
“Generate Expected Compounds” Prediction in CD 3.0

Parameters of 'Generate Expected Compounds'

Show Advanced Parameters

- 1. Compound Selection
 - Compound 161814-49-9 Amprenavir (C25 H35 N3 O6 S)
- 2. Dealkylation
 - Apply Dealkylation True
 - Apply Dearylation True
 - Max. # Steps 2
 - Min. Mass [Da] 150
- 3. Transformations
 - Phase I Dehydration (H2 O ->); Desaturation (H2 ->); Hyc
 - Phase II Acetylation (H -> C2 H3 O); Arginine Conjugation
 - Others
 - Max. # Phase II 1
 - Max. # All Steps 3
- 4. Ionization
 - Ions [M+H]+1; [M-H]-1

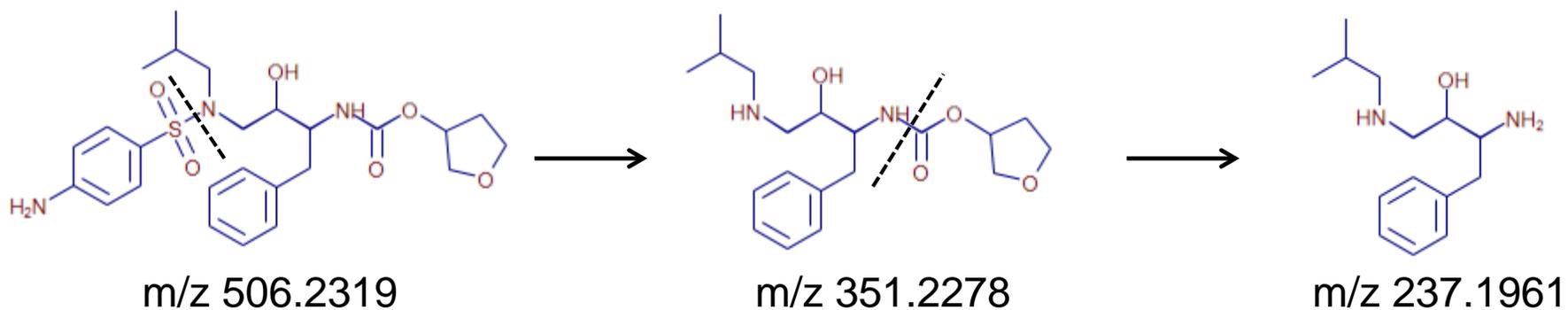
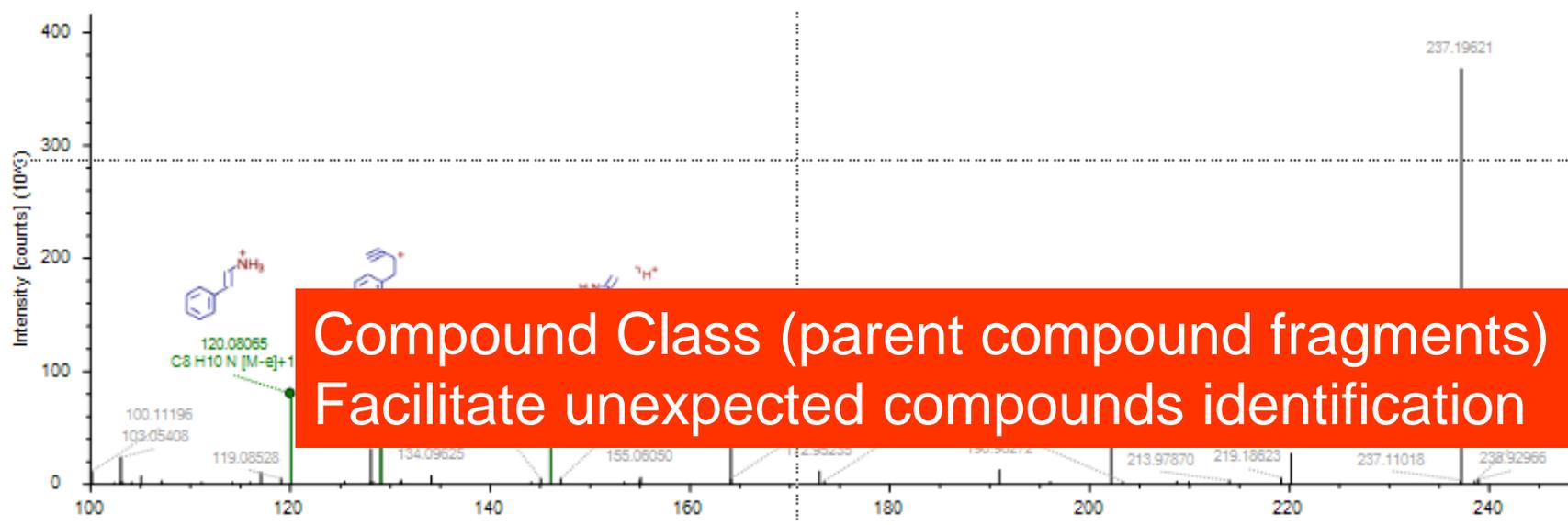
Expected Compounds	Expected Formulas	Expected Features	Related Structures	Input Files			
<input type="checkbox"/>	Checked	Parent Compound	Formula	Molecular Weight	Dealkylated	Composition Cha	Structure
1	<input type="checkbox"/>	Amprenavir	C20 H29 N3 O3 S	391.19296	X	-(C5 H6 O3)	



Dealkylation and Dearylation followed Phase I, Phase II transformation better prediction for expected compounds

Compound Class for Unexpected Metabolite Identification

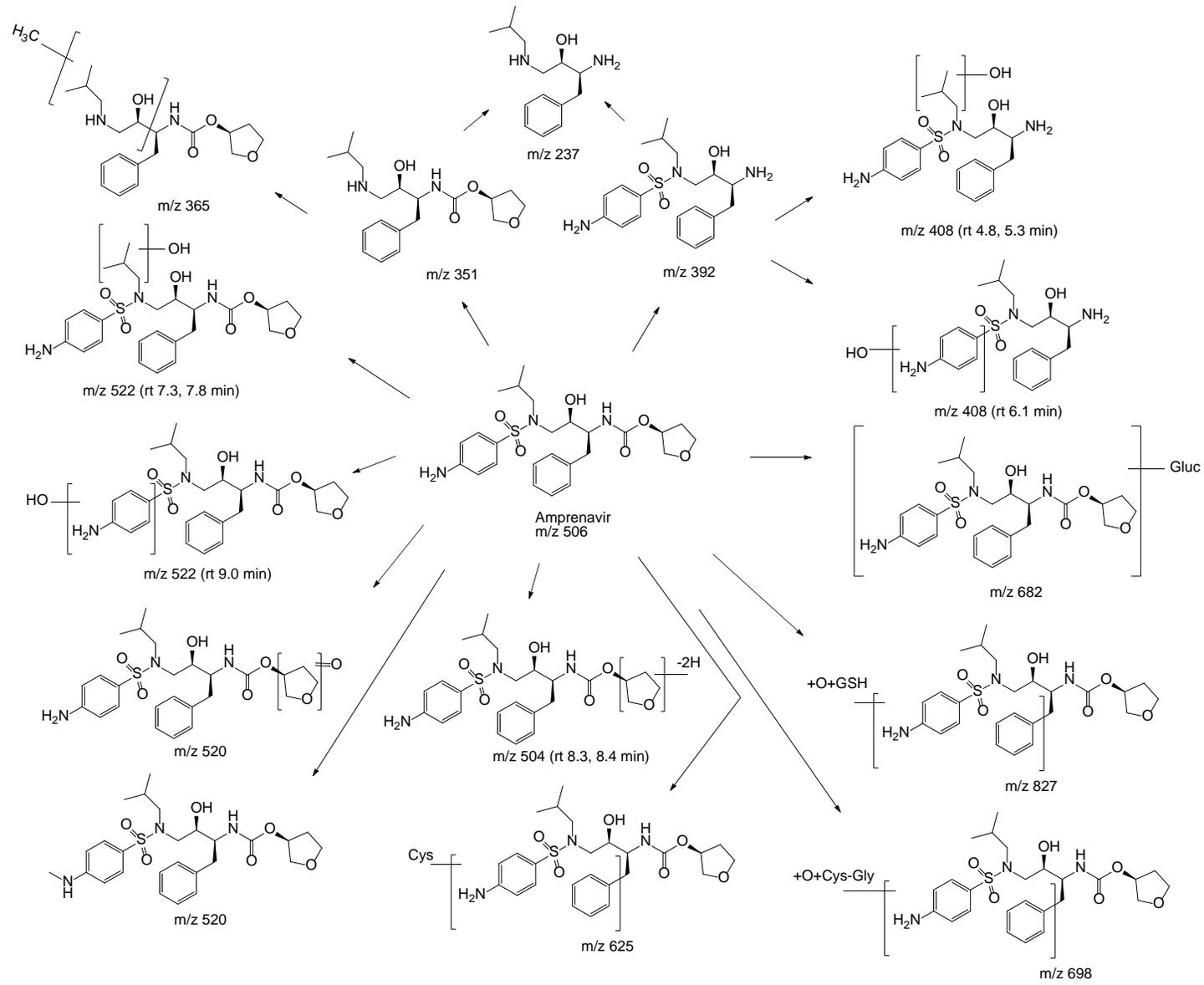
Checked	Name	Formula	Annotation Sc	FISH Coverage	Molecular Weight	RT [min]	Area (Max.)	Class Coverage	MS2	Pattern Matches	Area
<input checked="" type="checkbox"/>		C14 H24 N2 O			236.18892	2.291	183892	27.27	<input checked="" type="checkbox"/>		1.84e5



More Metabolites were Triggered for MSⁿ Using Acquire X than Conventional DDA

RT [min]	Molecular Weight	Formula	Transformations	DDA	AcquireX
2.29	236.1889	C14H24N2O	Sulfonamide hydrolysis + amide hydrolysis		Y
4.84	407.18788	C20H29N3O4S	Amide hydrolysis + oxidation		Y
5.02	350.22056	C19H30N2O4	Sulfonamide hydrolysis	Y	Y
5.14	364.23621	C20H32N2O4	Sulfonamide hydrolysis + methylation		Y
5.25	407.18788	C20H29N3O4S	Amide hydrolysis + oxidation	Y	Y
6.09	407.18788	C20H29N3O4S	Amide hydrolysis + oxidation	Y	Y
6.41	697.24513	C30H43N5O10S2	Oxidation + Cys-Gly-conjugation		Y
6.50	391.19296	C20H29N3O3S	Amide hydrolysis	Y	Y
6.61	519.20392	C25H33N3O7S	Oxidation (+O-2H)	Y	Y
6.88	826.28773	C35H50N6O13S2	Oxidation + GSH Conjugation		Y
7.19	624.22876	C28H40N4O8S2	Cysteine Conjugation		Y
7.32	521.21957	C25H35N3O7S	Oxidation	Y	Y
7.62	405.20861	C21H31N3O3S	Amide hydrolysis + methylation		Y
7.81	681.25674	C31H43N3O12S	Glucuronidation	Y	Y
7.84	521.21957	C25H35N3O7S	Oxidation	Y	Y
8.01	537.21449	C25H35N3O8S	Di-oxidation		Y
8.28	503.20901	C25H33N3O6S	Dehydration		Y
8.37	503.20901	C25H33N3O6S	Dehydration		Y
9.02	521.21957	C25H35N3O7S	Oxidation		Y
9.11	503.20901	C25H33N3O6S	Dehydration		Y
9.52	505.2246	C25H35N3O6S	Amprenavir Parent	Y	Y
10.61	519.24031	C26H37N3O6S	Methylation		Y

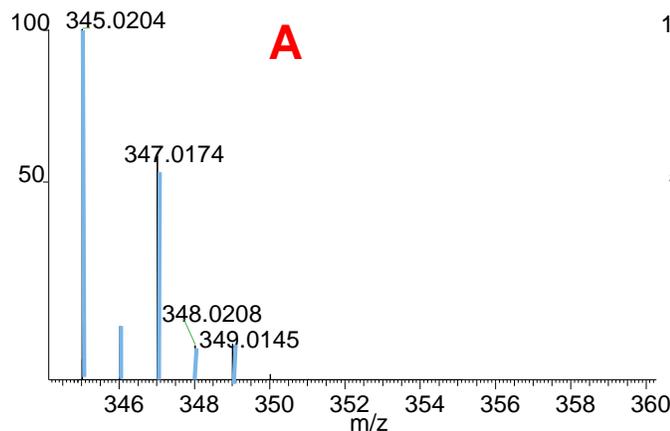
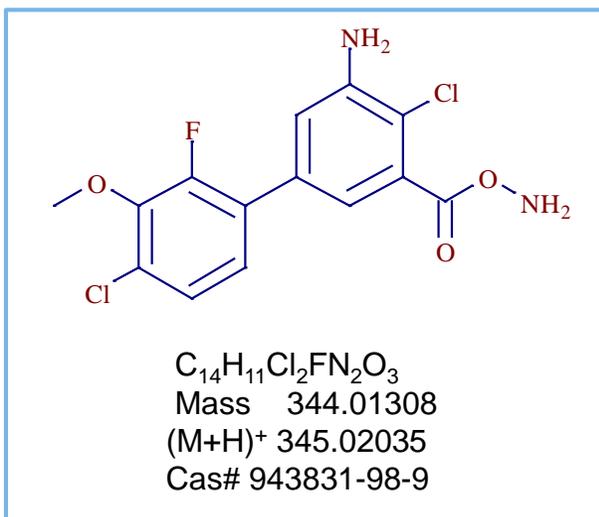
Metabolites Identified from Amprenavir HLM Incubations



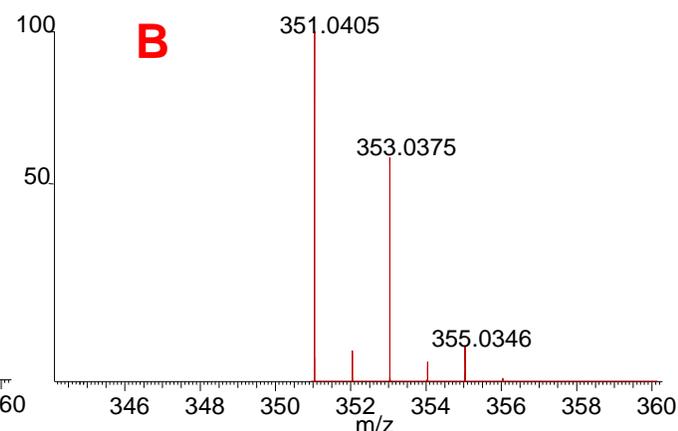
Isotope Triggered Trace Level Metabolite MSⁿ

Use of Isotopically Enhanced Labels for Plant Metabolite Identification

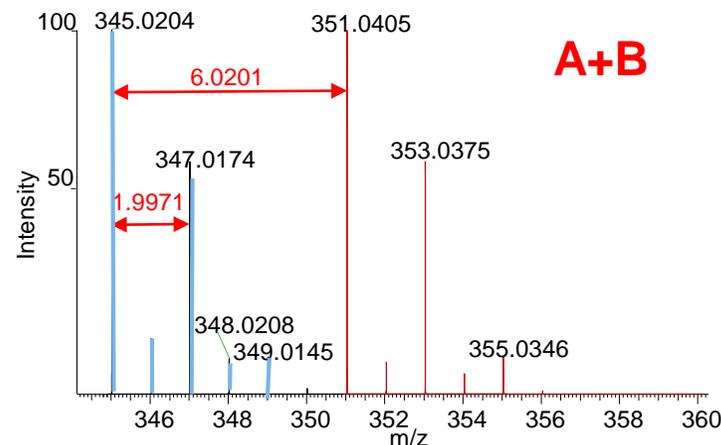
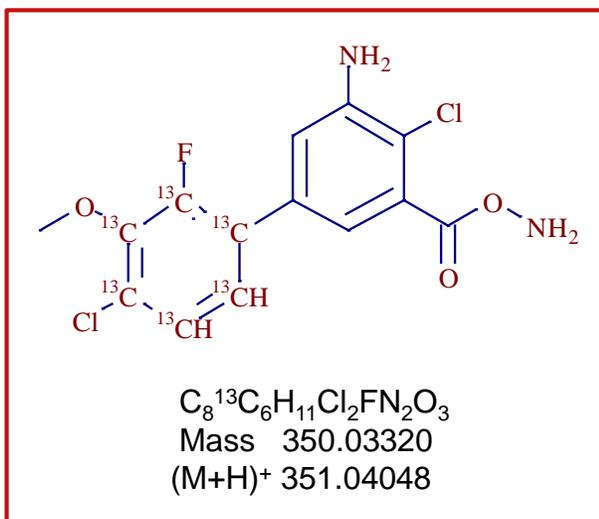
A



B

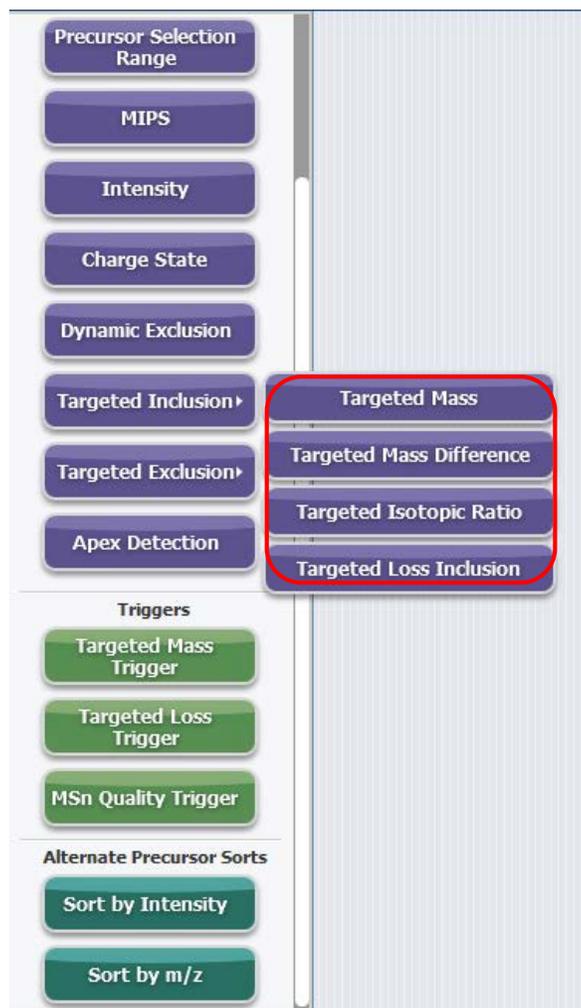


B



Blend of A and B yields significant +2, +6, +8 isotopic pattern

Feature-Specific Filters for Trace Level Labeled Metabolite ID



The feature-specific filters in ID-X method editor allow user to build sophisticated instrument method to capture trace level metabolites present in complex matrices, triggering MSⁿ for confident identification and structure elucidation.

Isotope triggering

- By delta mass: Sulfur, Chlorine
- By isotope peak relative intensity
- By customer-defined mass difference

Comprehensive, feature-specific filters to trigger low abundant components MSⁿ

Feature Specific Filters Capture Ions of Interest for MSⁿ

Targeted Mass Difference

Targeted Mass Difference Properties

Number of precursors in the targeted group: 2

MASS LIST

Compound	Formula	Delta M1
1		6.02

Partner Intensity Range Relative to the Most Intense Precursor (%): 64-96

Mass Tolerance: ppm

Low: 5

High: 5

Perform subsequent scan on: lowest m/z ion in the pair

Charge state requirement: Ions must be the same charge

Targeted Isotopic Ratio

Targeted Isotopic Ratio Properties

MASS LIST

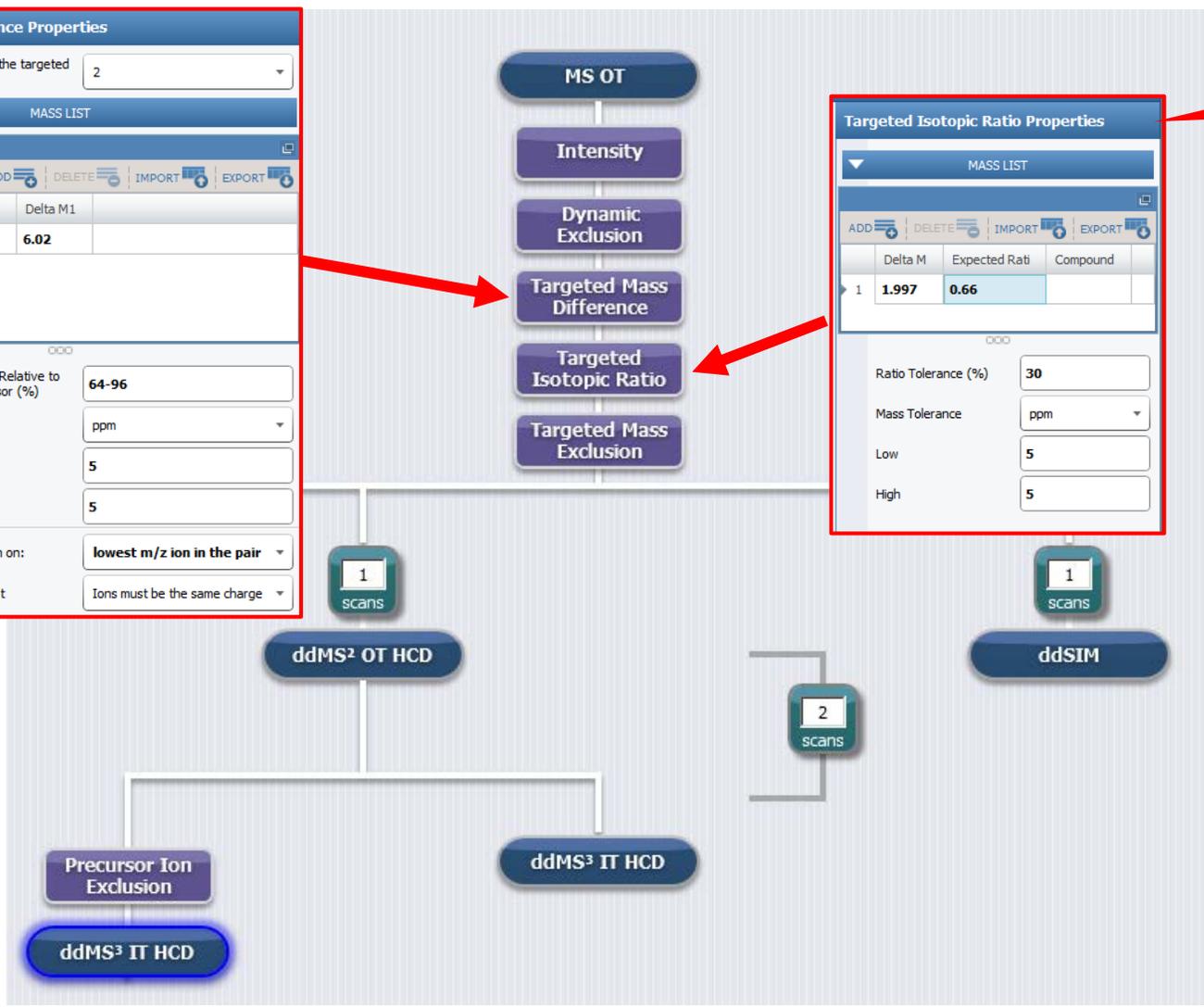
Delta M	Expected Rati	Compound
1	1.997	0.66

Ratio Tolerance (%): 30

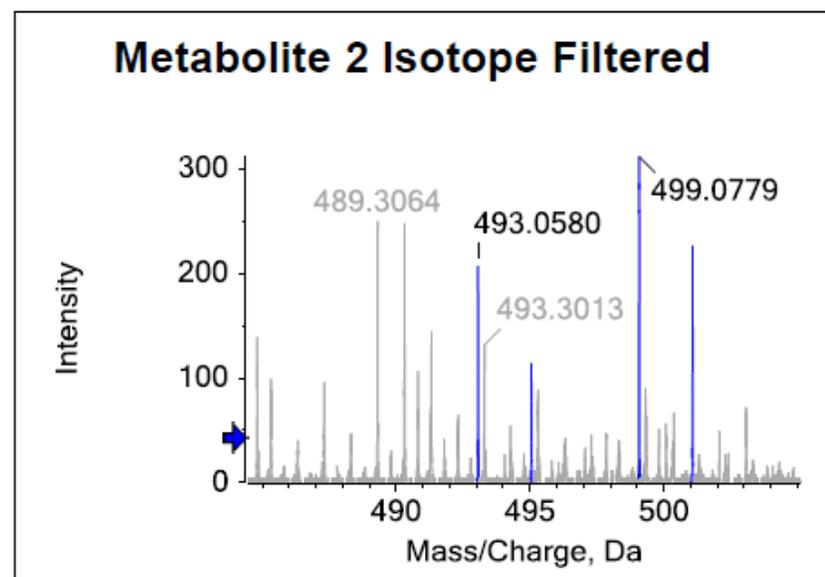
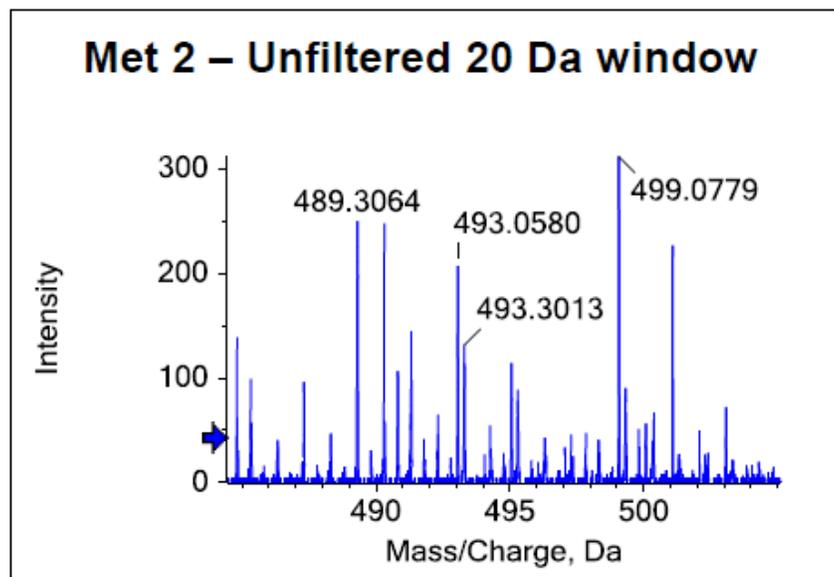
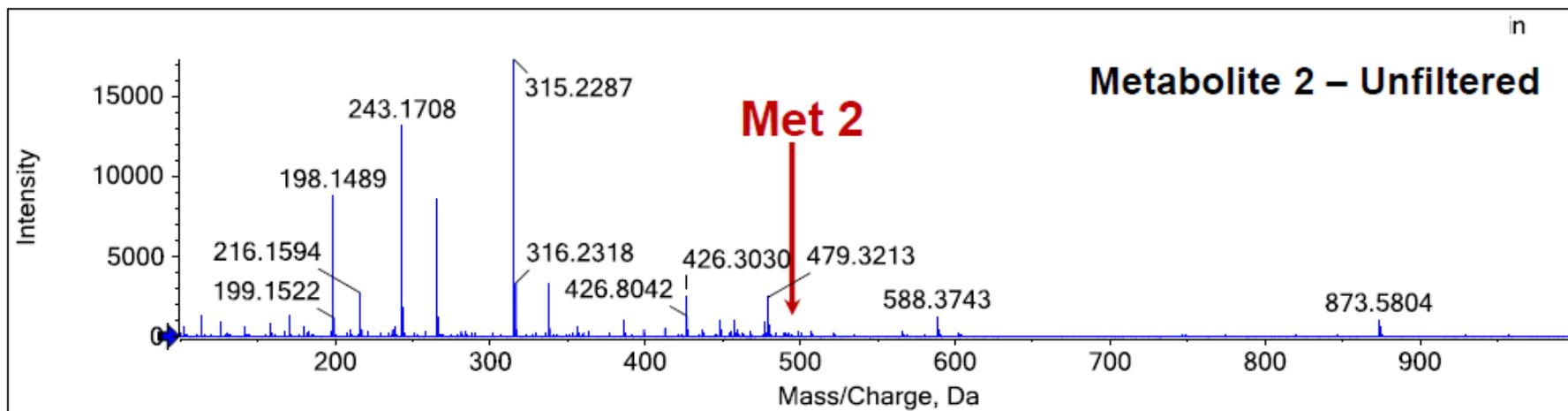
Mass Tolerance: ppm

Low: 5

High: 5

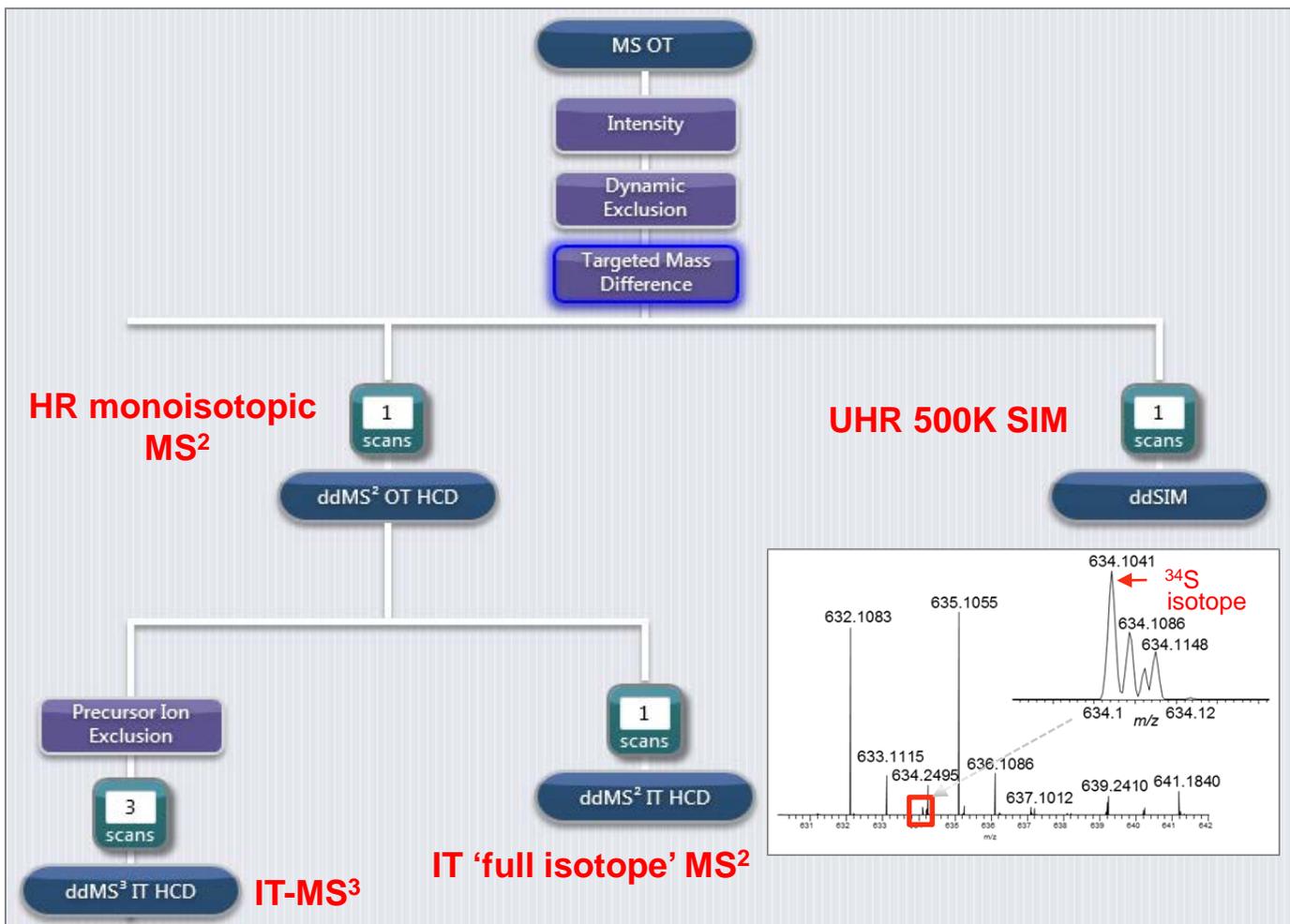


Isotopic Filtering of Mass Spectra



Xenobiotics: Labeled Metabolite Identification in Complex Matrices

Ultra High Resolution, High Specificity Experimental Design



Data courtesy of Dr. Jeff Gilbert, Dow AgroSciences

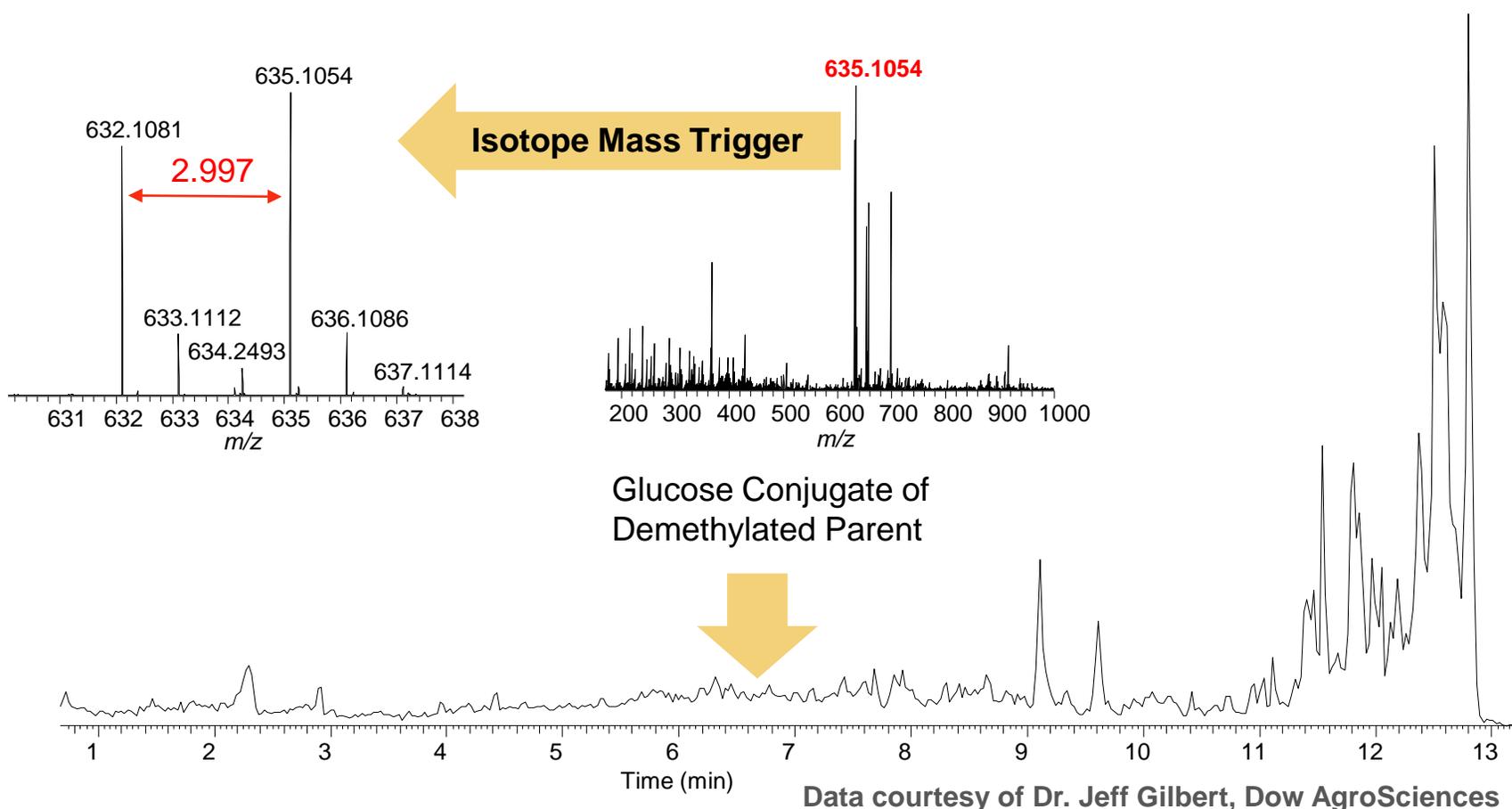
Structure Elucidation Workflow

- Xenobiotic material is applied as mixture of natural and a stable isotope-labeled material to create an 'un-natural' isotope cluster in resulting metabolites
- 'Mass-Difference' filtered data dependent method acquires OT-MS/MS, (monoisotopic), Ultra High Resolution OT-SIM (Selected Ion Monitoring), IT-MS³ and IT-MS² (full isotope window)
- Unique workflow provides comprehensive spectral data that enables full structural elucidation of unknown metabolites

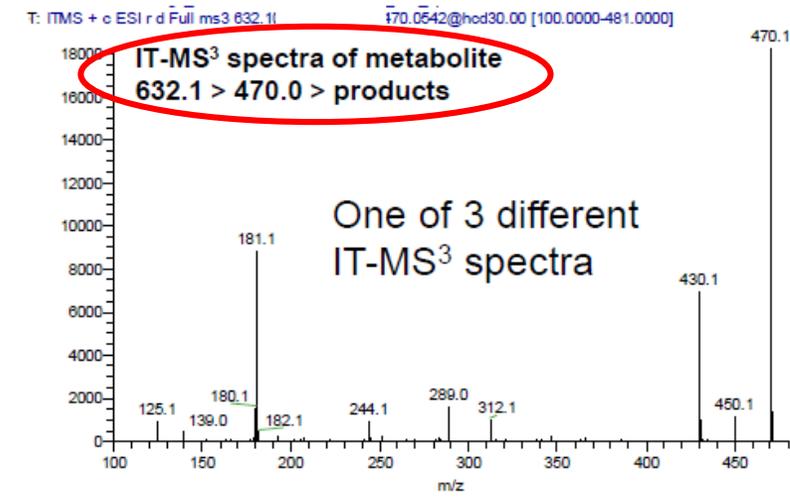
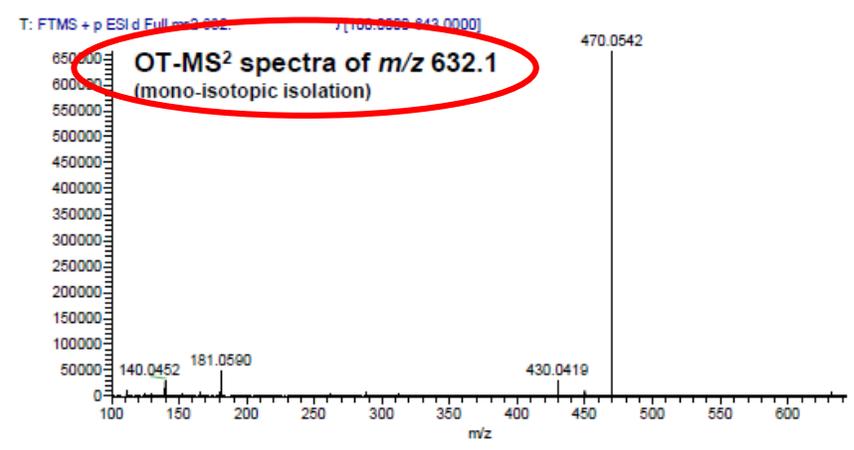
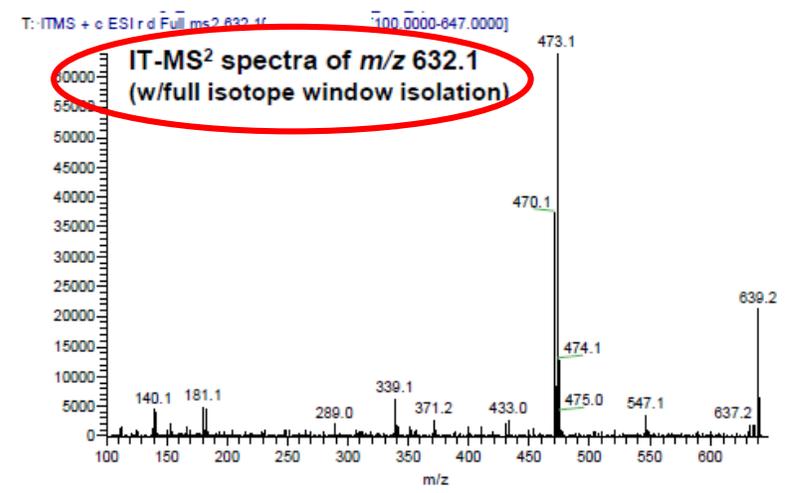
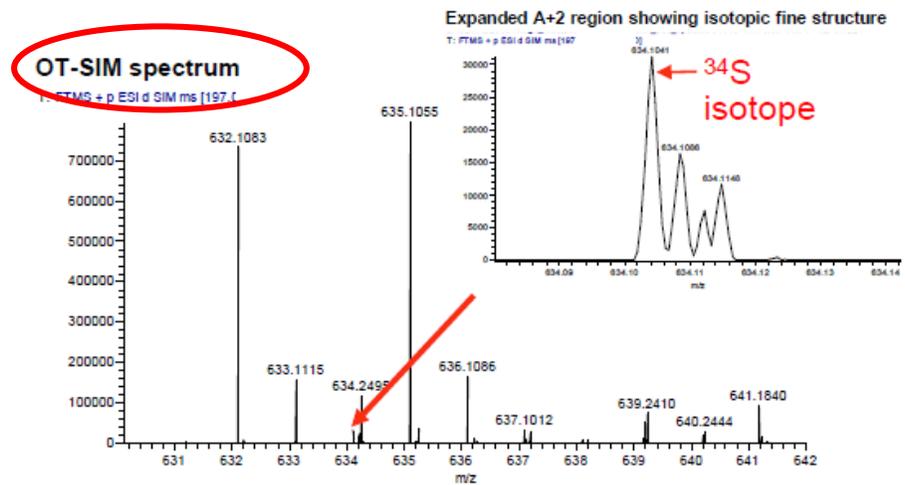
High Specificity MS³ Fragmentation of Metabolites

Customized Workflow

- 'Targeted Mass Difference' filter allows specific triggering of MS/MS scans on precursors that have peaks with a specified mass difference
- High specificity in experimental design allows the detection of trace components with 500K resolution for elemental composition determination
- High sensitivity MS³ analysis provides fragmentation spectra allowing structure validation
- This highly selective workflow increases throughput by 2X vs. a traditional targeted workflow



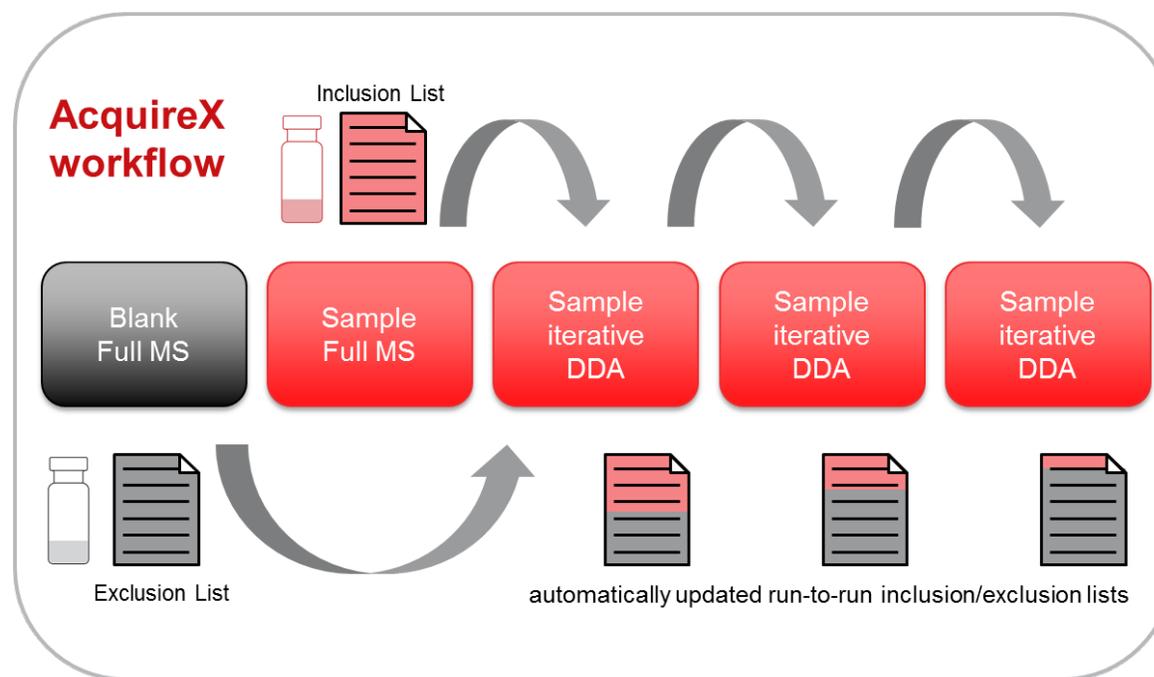
Mass Difference Triggered MSⁿ of Plant Metabolites in Complex Biomatrix



Orbitrap ID-X MS AcquireX Deep Scan Workflow for MetID and Metabolomics Research

		
<h3>BACKGROUND EXCLUSION</h3>	<h3>EXCLUSION & COMPONENT INCLUSION</h3>	<h3>DEEP SCAN</h3>
<p>Create and use an exclusion list of constant background ions and peaks to reduce background fragmentation in your ID runs</p>	<p>Combines exclusion and inclusion lists to automatically and reliably acquire more relevant MSⁿ data in a single injection</p>	<p>Combines a single exclusion and inclusion list with multiple ID injections to comprehensively fragment relevant precursor ions</p>
<p>What Xcalibur Does:</p>	<p>What Xcalibur Does:</p>	<p>What Xcalibur Does:</p>
<ul style="list-style-type: none">• Generates up to 1 exclusion list per sequence• Updates the ddMSⁿ method with the exclusion list• Automatically inject ID samples with updated ddMSⁿ method	<ul style="list-style-type: none">• Generates up to 1 exclusion list per sequence• Generates 1 inclusion list per ID injection block• Injects user-defined number of ID injection blocks	<ul style="list-style-type: none">• Generates up to 1 exclusion list per sequence• Generates up to 1 inclusion list per sequence• Injects ID samples until all inclusion list ions are fragmented or user defined number of ID samples are reached
<p>SELECT</p>	<p>SELECT</p>	<p>SELECT</p>

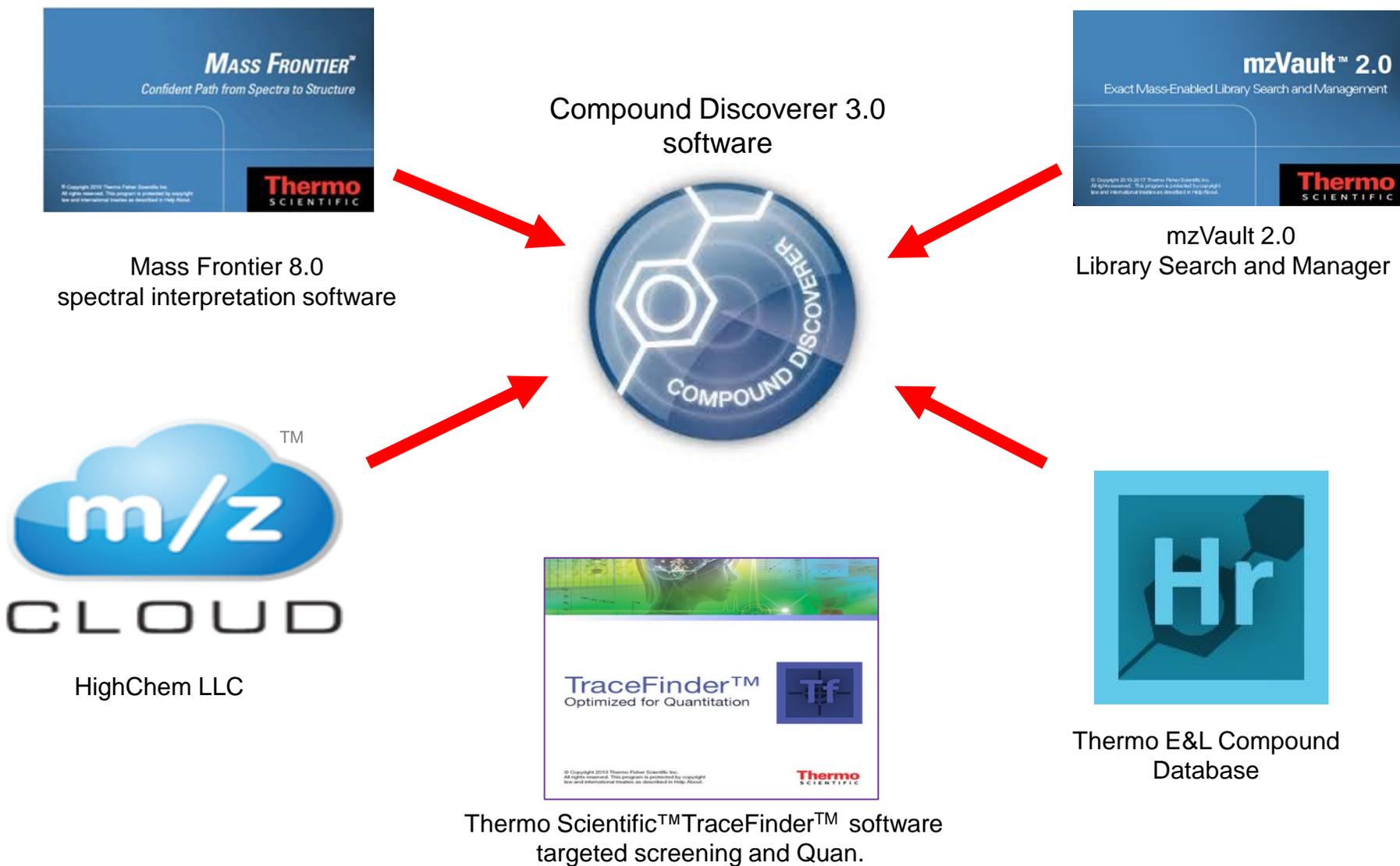
Collect more meaningful data, not just more data to maximize productivity



Compared with Traditional DDA, AcquireX Deep Scan Workflow Significantly Increases ID by Triggering More MS/MS and MSⁿ

Compound Discoverer 3.0 for Data Processing

Data Analysis Software, Database and Spectral Library Suite



Thermo Scientific Compound Discoverer Software (CD 3.0)



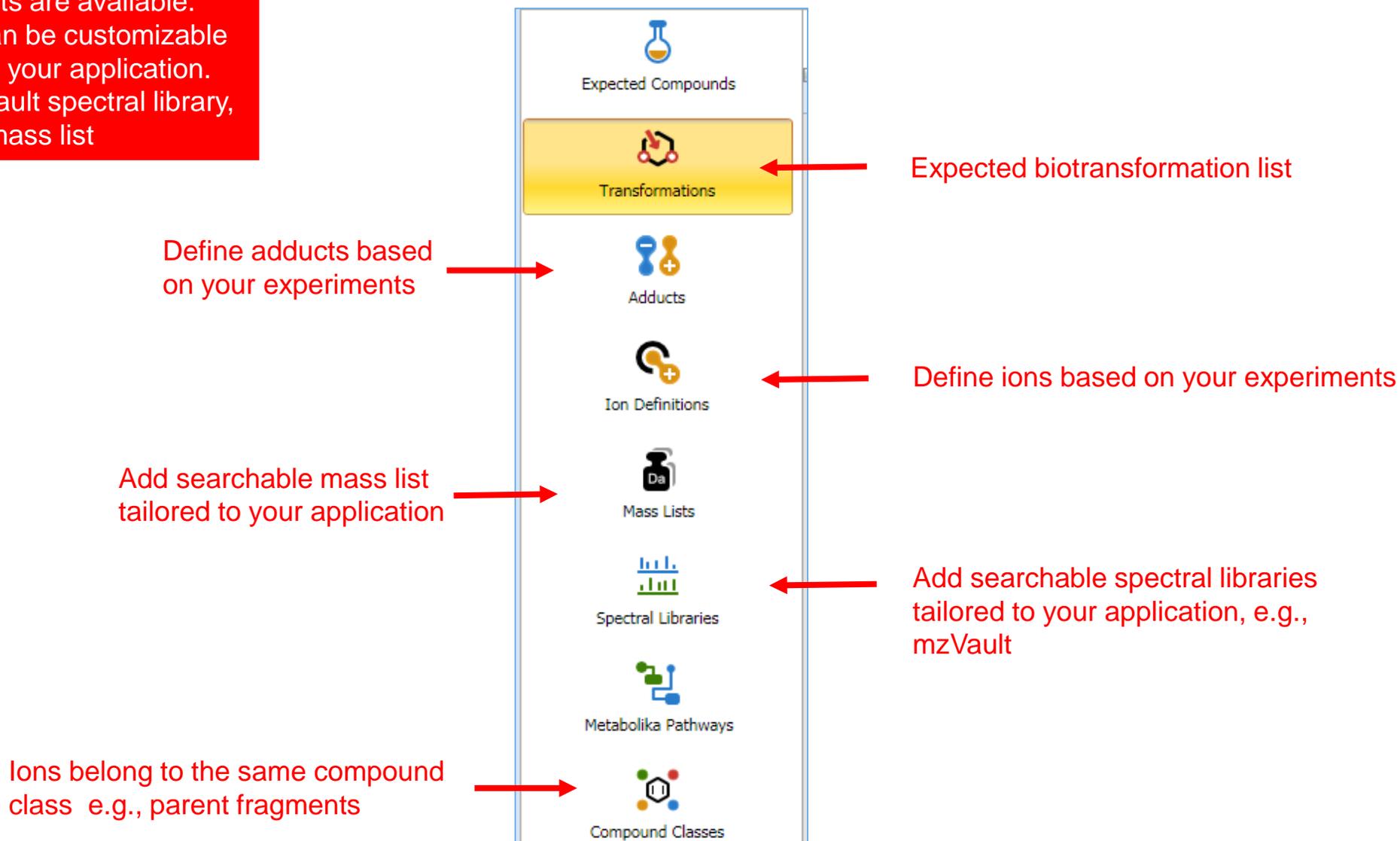
<https://mycompounddiscoverer.com/>

- ❑ Component extraction based on HRAM and isotope pattern
- ❑ Elemental composition prediction
- ❑ Customizable feature set for advanced data processing
- ❑ Searching online/offline spectral libraries and multiple databases for ID
- ❑ Statistics and differential analysis

Complete Small Molecule Structure Analysis Platform

Customizable Feature Set for Data Processing

- Default lists are available.
- All lists can be customizable to tailor fit your application. e.g., mzVault spectral library, E&L mass list

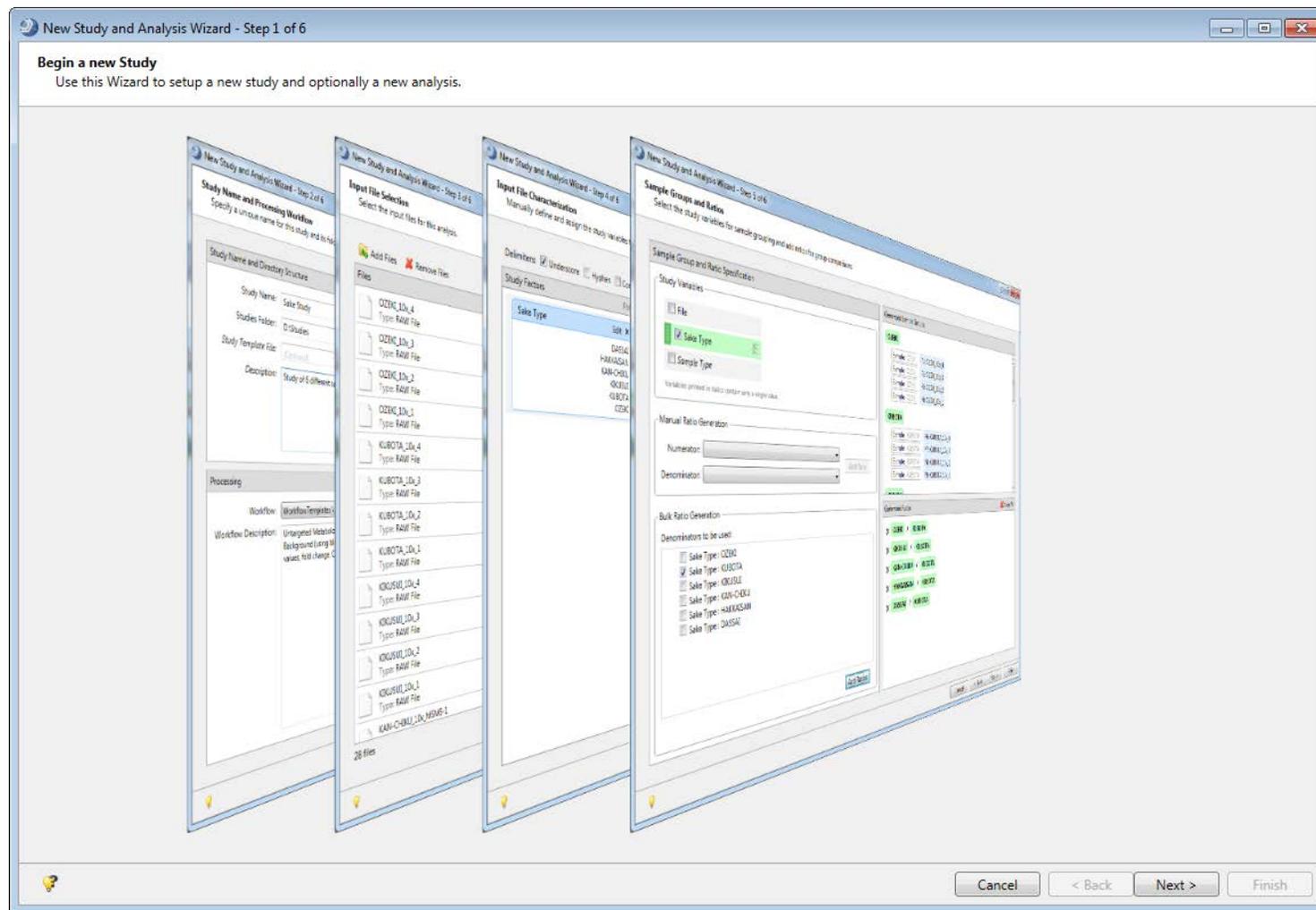


Fragment Ion Search - FISh Algorithm

- Fragment Ion Search – A means of detecting related components in complex samples
- Data is scanned using fragmentation knowledge from a known structure
- Components that share common fragments are detected
- Fragments can be modified (metabolically or otherwise) and still be detected

The Wizard Allows You to Build Processing Workflow

Compound Discoverer 3.0 Software



Description
for each step

Creating a study and analysis use the guided “New Study and Analysis wizard” and build-in workflow templates

Node-based Processing Workflow

The screenshot displays the Compound Discoverer 2.1.0.398 interface. The main window shows a workflow titled "Pharma Label IPA-Extract-one" with a description: "Untargeted E&L workflow without statistics: Find and identify unknowns-Performs retention time alignment, unknown compound detection, and compound grouping across all samples. Predicts elemental compositions for all compounds, and hides chemical background (using Blank samples). Identifies compounds using mzCloud".

The workflow is visualized in a "Workflow Tree" diagram. The nodes are:

- Input Files
- Create Analog Trace (left branch)
- Select Spectra
- Create Analog Trace (right branch)
- Align Retention Times
- Detect Unknown Compounds (left branch)
- Create Mass Trace (left branch)
- Create Mass Trace (right branch)
- Group Unknown Compounds
- Merge Features
- Search Mass Lists (left branch)
- Search mzCloud (left branch)
- Search ChemSpider (bottom branch)
- Predict Compositions (right branch)
- Mark Background Compounds (right branch)

The "Post-Processing Nodes" section at the bottom contains:

- Assign Compound Annotations

The left sidebar shows a "Workflow Nodes" list with categories: 1. Input / Output, 2. Data Processing, 3. Tracer, 4. Expected Compounds, 5. Unknown Compounds, and 6. Comparison. The "Study Definition" tab is highlighted in red, and the "Workflow Tree" label is also circled in red.

Result View – Data Interpretation

Compound Discoverer 2.1.0.398

File Reporting Libraries View Window Help

Start Page x Pharma Label-IPA-a x Pharma Label IPA Extract x Pharma Label Extractables x **Pharma Label H2O -IPA-1** x

Chromatograms

Group By:

- solvent (3/3)
- Sample Type (2/2)
- File (6/6)

Filter By:

- solvent
- Sample Type
- File

solvent: H2O
Sample Type: Sample

solvent: IPA
Sample Type: Sample

solvent: n/a
Sample Type: Blank

7.618

Mass Spectrum

F6 #3373, RT=7.575 min, MS1

F6 #3375, RT=7.580 min, I

F6 #3393, RT=7.618 min, MS1

F6 #3394, RT=7.623 min, I

F6 #3413, RT=7.662 min, MS1

F6 #3414, RT=7.666 min, I

LF_IPA_Extract-2-Pos-2 (F6) #3393, RT=7.618 min, MS1, FTMS (+)
C15 H30 N6 O6 as [M+H]⁺1

Intensity [counts] (10⁹)

m/z

Compounds

Checked	Name	Formula	Annotation Sc	FISH Coverage	Molecular Weight	RT [min]	Area (Ma)	# ChemSpider Re	# mzCloud F	mzCloud Best Mat	Mass List	mzCloud
<input type="checkbox"/>	Oleamide	C18 H35 N O	■■■		264.24510	17.335	146255525	112	4	98.5		
<input type="checkbox"/>	Melamine	C3 H6 N6	■■■		126.06560	0.472	106680600	7	5	100.0		
<input type="checkbox"/>	Hexamethoxymethyl melamine	C15 H30 N6 O6	■■■		390.22225	7.621	106255412	3	3	98.7		
<input type="checkbox"/>	Eicosapentaenoic acid	C20 H30 O2	■■■		302.22429	16.490	96918439	710	10			
<input type="checkbox"/>	6-Propyl-2-naphthol	C13 H14 O	■■■		186.10445	9.903	89008975	397	12			
<input type="checkbox"/>	Methamphetamine	C10 H15 N	■■■		149.12048	1.070	87923353	297	4	93.7		
<input type="checkbox"/>	pro-ser-arg	C14 H26 N6 O5	■■■		358.19619	7.621	82384299	4	6			

Hide Related Tables

Structure Proposals

Checked	Molecular Weight	RT [min]	FWHM [min]	Max. # MI	# Adducts	Area	Study File ID
<input type="checkbox"/>	390.22219	7.619	0.045	4	2	106255412	F6
<input type="checkbox"/>	390.22232	7.624	0.048	4	2	98027607	F3

sub-tables →

Multiple Database Searching in Parallel to Identify Known Unknowns

The screenshot displays the Compound Discoverer 2.1.0.398 interface. The top section shows a chromatogram with a peak at 7.618 minutes. The middle section shows a mass spectrum with a base peak at m/z 177.08813. The bottom section shows a table of search results.

Checked	Name	Formula	Annotation Sc	FISH Coverage	Molecular Weight	RT [min]	Area (Ma)	# ChemSpider Re	# mzCloud f	mzCloud Best Mat	Mass List	mzCloud Bes	Group Areas	Group CV [%]
<input type="checkbox"/>	Oleamide	C18 H35 N O	■■■		264.24510	17.335	146255525	112	4	98.5			1.23e4 1.38e8	29 9
<input type="checkbox"/>	Melamine	C3 H6 N6	■■■		126.06560	0.472	106680600	7	5	100.0			1.06e8 4.17e4	1 10
<input type="checkbox"/>	Hexamethoxymethyl melamine	C15 H30 N6 O6	■■■		390.22225	7.621	106255412	3	3	98.7			1.93e3 1.02e8	1 6
<input type="checkbox"/>	Eicosapentaenoic acid	C20 H30 O2	■■■		302.22429	16.490	96918439	710	10			83.0	2.50e3 9.34e7	1 5
<input type="checkbox"/>	6-Propyl-2-naphthol	C13 H14 O	■■■		186.10445	9.903	89008975	397	12			84.9	4.01e7 8.40e7	22 8

Checked	Compound Match	Structure	Name	Formula	Molecular Weight	ΔMass [Da]	ΔMass [ppm]	Type	Scan #	Match	Best Match	Best Sim. Match	mzCloud ID
<input type="checkbox"/>	■		Hexamethoxymethyl melamine	C15 H30 N6 O6	390.22268	0.00043	1.11	Identity	3394	98.5	98.7	98.2	2645

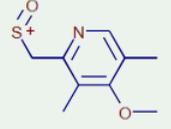
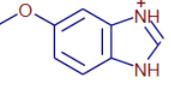
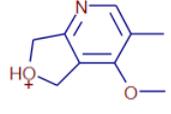
Mirror plot for each mzVault hit:
observed MS2 vs. library MS2
• Matches collision energy between raw file MS/MS and reference MS/MS

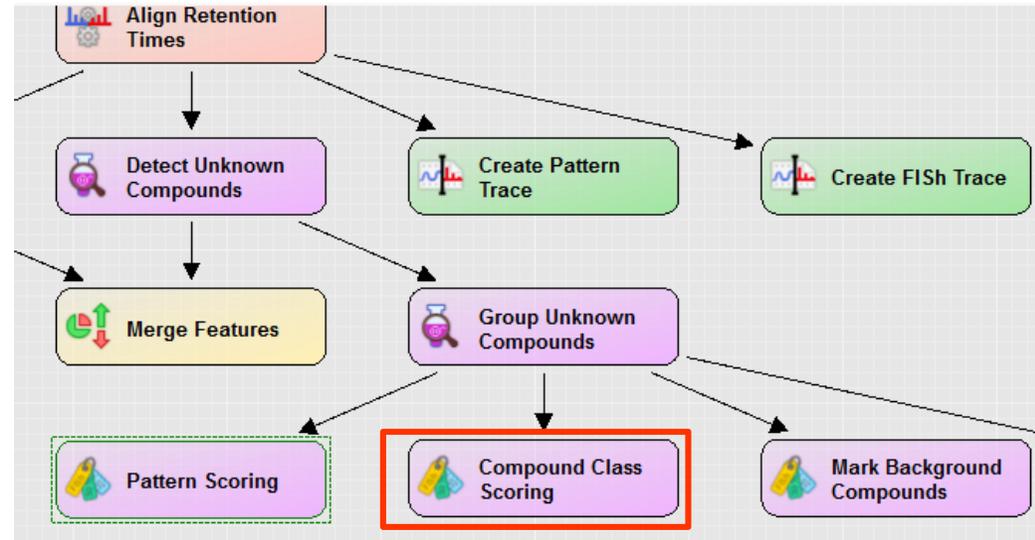
Sub table show detailed info for compound

Compound Class Scoring: Tool to Find Structurally Related Compounds

Edit 'Omeprazole Compound Class' (9 fragments)

New... Edit... Delete Import

	m/z	Structure	Formula	Charge
1	198.05833		C9 H12 N O2 S	1
2	149.07094		C8 H9 N2 O	1
3	138.09134		C8 H12 N O	1
4	166.08626		C9 H12 N O2	1



- Create compound class using common fragments from parent and known metabolites
- Use Mass Frontier™ to predict fragments from parent mol file. Copy and paste the fragment structures to construct compound class.

“Structure Proposal & Apply FISH” for Unknown ID

The screenshot displays the Compound Discoverer 2.1.0.398 interface. The top panel shows a chromatogram with a peak at 35.312 minutes. The middle panel shows a mass spectrum for the peak at 35.342 minutes, with a base peak at m/z 508.80409. The bottom panel shows a table of structure proposals. A red box highlights the 'Add to Structure Proposals and Apply FISH Scoring' option in the context menu. A red arrow points from this option to the 'Specify FISH Scoring Settings' dialog box, which is also highlighted with a red box. The dialog box shows settings for FISH scoring, including 'Annotate full spectrum tree', 'Use general rules', 'Use fragmentation libraries', 'Allow aromatic cleavage', 'Max. Depth' (set to 5), 'High accuracy mass tolerance' (set to 2.5), 'Low accuracy mass tolerance' (set to 0.5), and 'S/N threshold' (set to 3). A second red arrow points from the dialog box to the 'FISH Scoring Queue' panel, which shows the progress of the scoring process for the selected structure proposal.

Chromatograms

Group By:

- Solvent Type (2/2)
- Sample Type (1/1)
- File (2/2)

Filter By:

- Solvent Type
- Sample Type
- File

Mass Spectrum

IP A_H2O_Extract_2 (F1) #9355, RT=35.342 min, MS1, FTMS (+)
C32H64N2O2 as [M+H]⁺

F1 #9363, RT=35.371 min, M:
F1 #9364, RT=35.375 min

Intensity [counts] (10⁶)

m/z

Specify FISH Scoring Settings

- Annotate full spectrum tree
- Use general rules
- Use fragmentation libraries
- Allow aromatic cleavage
- 5 Max. Depth
- High accuracy mass tolerance: 2.5 mmu
- Low accuracy mass tolerance: 0.5 Da
- S/N threshold: 3

OK Cancel

FISH Scoring Queue

Name: N,N'-1,8-Octanediyldidodecanamide
MW [Da]: 508.49678
Processing Since: 5 ms
State: Processing

Found Match	Structure	Formula	Molecular Weight	ΔMass [Da]	ΔMass [ppm]	CSID	# References
	N,N'-1,8-Octanediyldidodecanamide	C32H64N2O2	508.49676	-0.00007	-0.13	2523028	6

FISH stands for “Fragment Ion Search”

mzCloud - Advanced High Resolution Mass Spectral Database



mzCloud is a trademark of HighChem LLC, Slovakia

The screenshot displays the mzCloud website interface. At the top left is the logo with the text "m/z CLOUD". To its right is the text "Advanced Mass Spectral Database". Below the logo is a paragraph describing the database as a state-of-the-art mass spectral database for various fields like life sciences, metabolomics, and pharmaceutical research. It mentions a freely searchable collection of high-resolution mass spectra and notes that online access is free and requires no registration. A blue button labeled "Enter Database" is present, along with a "New mzCloud" app icon for Google Play. A search bar is labeled "Search for Compounds by Name or ID". At the bottom of the main content area, statistics are shown: 16,470 (+244) compounds and 23,185 (+343) trees, with a note that 4,282,600 (+) were added in the last 14 days. On the right side, a vertical list of "Compound classes" includes: Therapeutics/Prescription Drugs, Drugs of Abuse/Illegal Drugs, Sports Doping Drugs, Steroids/Vitamins/Hormones, Endogenous Metabolites, Natural Products/Medicines, Natural Toxins, Counterfeit Drug (Therapeutic), Extractables/Leachables, Pesticides/Herbicides, Excipients/Additives/Colorants, Illegal Additives, Personal Care Products/Cosmetics, Textile Chemicals/Auxiliary/Dyes, Industrial Chemicals, Perfluorinated Hydrocarbons, and Nanomaterials. A search bar on the right side of the page has a "Search" button. A "view more statistics" link is also visible.

<https://www.mzcloud.com>

Free, Available to everyone, everywhere

mzCloud Spectral Library

mzCloud is a trademark of HighChem LLC, Slovakia

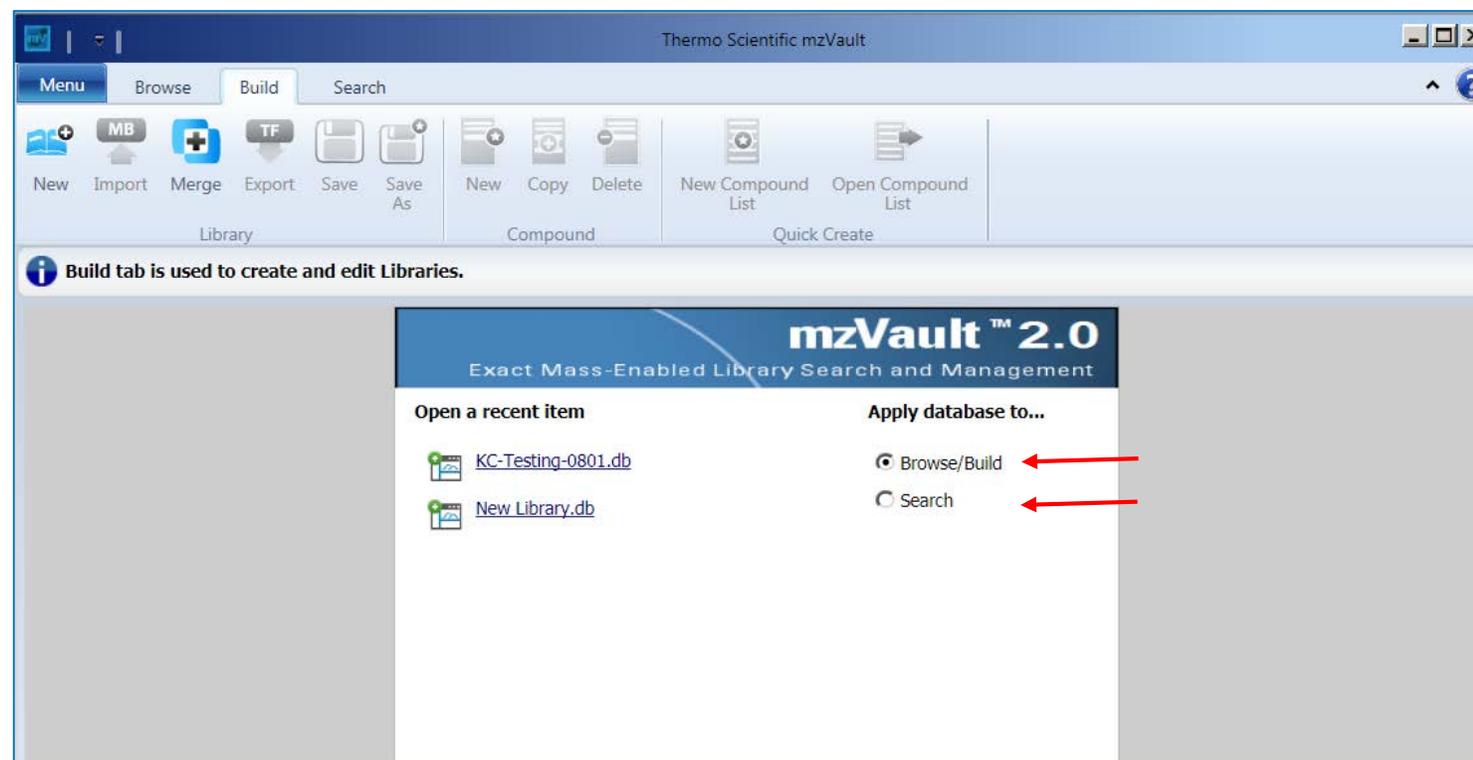
The screenshot displays the mzCloud Spectral Library interface. On the left, a navigation menu is visible with a red box around the 'Search' section, which includes options like 'Spectrum', 'Tree', 'Structure', 'Monoisotopic Mass', 'Peak', 'Precursor', and 'Name'. A callout 'Search by different terms' points to this menu. The main area shows a 'Reference Library' with search results for 'Irganox 1035', 'Accelerator BBTS', '5-Chloro-2-hydroxy-4-methylbenzophenone', and '[(4-Methylphenyl)sulfonylamino]urea'. A callout 'Metadata available for selected library entry' points to the details of the '[(4-Methylphenyl)sulfonylamino]urea' entry. The 'Spectral Tree' section shows a stack of spectra, with a callout 'Spectral tree' pointing to it. The 'Recalibrated Spectrum' section displays a mass spectrum with chemical structures and peak labels (e.g., 57.06988, 133.06479, 203.14304, 219.17434, 231.13796). A callout 'Recalibrated spectra' points to this section. The 'Breakdown Curves' section shows a plot of relative intensity versus m/z, with a callout 'Breakdown curves' pointing to it. At the bottom, a callout 'Permanent citable links for entry, tree, and spectra' points to the 'Metadata' section.

Thermo Scientific mzVault 2.0



Exact Mass-Enabled Library Search and Management

- Searching library – local mzCloud
- Creating customer library



- High resolution mass spectrometry is the necessary tool for drug metabolite identification. Only Orbitrap MS delivers high quality HRAM data with high mass accuracy, isotopic pattern fidelity, and MSⁿ for confident small molecule structure characterization.
- Orbitrap ID-X MS AcquireX data acquisition workflows generate background exclusion lists and inclusion lists automatically, which minimizes matrix interferences and increase triggering MSⁿ of drug-related ions. AcquireX improves overall metabolite ID and small molecule structure analysis efficiency, quality, and accuracy!
- Compound Discoverer 3.0, Mass Frontier 8.0, and mzCloud suite facilitate confident small molecule structure analyses.

Analysis of Chinese, Korean, and American Ginseng Roots by LC-MS² and LC-MS³ by Orbitrap ID-X

Stephanie Baran, Kate Cornejo, Caroline Ding, Rafi Talerstein, Seung Shwam, Thermo Fisher Scientific, 380 River Oaks Pkwy, San Jose, CA, USA, M134

ABSTRACT

Purpose: Investigate the differences between ginseng roots of varying quality and variety by LC-MS² and LC-MS³. Advanced excitation tools available in Compound Discoverer 3.0 are highlighted including the newly implemented sign algorithm for making candidate structures.

Methods: Ginseng roots of varying quality and variety from Chinese, Korean, and American origin were purchased from a TCM pharmacopoeia and were investigated by LC-MS² and LC-MS³. Samples were finely ground to powder and peaks followed by an MS/MS excitation. Samples were chromatographically separated by a 22 minute reverse phase gradient using a Thermo Scientific™ Acquity C18 LC (1 x 100 mm, 2.5 μm) column and then analyzed on the newly released Thermo Scientific™ Orbitrap ID-X Hybrid Mass Spectrometer. Samples were analyzed by Full MS 3 (Triple and a postion sample was analyzed by Acquire with the dynamic data dependent MS³ options for increased compound detection and excitation. Acquire was used to function based on level of confidence, highest priority being in-Cloud match and lowest being excitation made with Fragment for search (FFS).

Results: Chinese (high quality and high quality) and Korean (high and low quality) samples in the original compound (PCO) library regarding structure differences between these two groups. Differences were observed with 37 Chinese (high and low quality) ginseng, 33 of 1,000 compounds had an MS/MS match with 5. Chinese with an MS/MS match with the compound 3,000 and with a similar or exact match to spectra present in in-Cloud. For results that had either an exact or similar match, multiple were applied to new candidate structures found in the library based on a Classification Database. The highest ranked candidate structures were analyzed by MS/MS to verify fragmentation.

INTRODUCTION

Traditional Chinese Medicine (TCM) has been in practice for thousands of years, incorporating natural herbs and plants as forms of medical treatment. Ginseng is a classic medicine. TCM ingredients include ginseng that is not well understood or accepted by Western medicine. One such root incorporated into many TCM medicines is ginseng, originating from the genus Panax. This can be administered orally or purchased from health store from a TCM pharmacopoeia of a reliable root depending on the origin, variety, and quality of the root.

MATERIALS AND METHODS

Sample Preparation: Ginseng samples were purchased from a local Chinese pharmacopoeia and were finely ground to powder and peaks. 2 mg of 80% methanol was added to 200 mg of ground ginseng samples and were extracted overnight, followed by 1 hour of extraction, solvent for extraction and then 1 mL was transferred to a 1.5 mL vial, centrifuged. Samples were then centrifuged at 10000 rpm for 5 minutes and the supernatant was transferred to a 1.5 mL vial. A control sample was prepared with each sample added from ginseng #1 through #5 to separate LC run for the injected experiments.

Table 1. Ginseng Sample Analysis. Samples of different origin, variety, and quality were purchased from a local Chinese pharmacopoeia. Samples #1 through #5 were run to optimize by Full MS only, while the control sample was injected by Acquire.

Ginseng #	Origin (Quality or variety)	MS/MS Method
Ginseng #1	American	Full MS
Ginseng #2	Korean	Full MS
Ginseng #3	Chinese (High quality)	Full MS
Ginseng #4	Chinese (Low)	Full MS
Ginseng #5	Chinese (High)	Full MS
Ginseng Pool	Mix of #1 through #5	Applied MS/MS Deep Scan

Figure 1. Ginseng Samples ground to powder and peaks. Samples were finely ground to powder and peaks before they were analyzed.



Liquid Chromatography Method

System: Versar™ Hybrid UPLC
 Column: Acquity C18 2.1 x 100 mm, 2.5 μm
 Mobile Phase: A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid
 Column Temp: 45 °C
 Auto Sample Temp: 4 °C
 Injection Volume: 5 μL
 Gradient: Figure 2



Figure 2. Liquid Chromatography Gradient



Table 2. MS/MS Source Parameters. Source conditions used for analysis of LC for rate of 200 μL/min.

Parameter	Positive	Negative	Electro-Spray	ESI
Source Voltage	3.0 kV	3.0 kV	3.0 kV	3.0 kV
Capillary	300 °C	300 °C	300 °C	300 °C
Temperature	300 °C	300 °C	300 °C	300 °C
Flow Rate	0.5	0.5	0.5	0.5

Mass Spectrometry Method

Table 3. Mass Spectrometry Parameters

Parameter	MS1	MS2	MS3
Scan Range	70-700	100-700	100-700
Resolution	30K	30K	15K
MS/MS	Yes	Yes	Yes
Scan Type	MS	MS/MS	MS/MS
Scan Range	100-700	100-700	100-700
Scan Rate	10K	10K	10K
Scan Time	100	100	100
Scan Delay	100	100	100
Scan Delay	100	100	100
Scan Delay	100	100	100
Scan Delay	100	100	100

Figure 3. Acquire Data Acquisition Workflow

Acquire workflow diagram showing the process from sample injection to data acquisition and processing.

RESULTS

Method Differences

Figure 4. Principle Component Analysis. Scatter plot showing the separation of samples based on origin and quality.

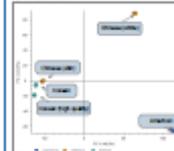
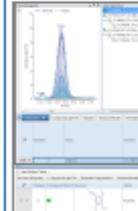


Figure 5. Investigating the Statistically Significant Compounds in the Results Table

Color reference to chemical compound names (left) and the MS/MS scores collected, and Full MS scan collected with candidate structure label below.



Compound Annotation Strategies (In Order of Confidence)

- in-Cloud Search - Exact match to spectral library (> 95 Compounds, > 2M spectra). The highest confidence annotation are those that have an exact spectral match to compounds in the in-Cloud.

Figure 6. in-Cloud Search to Carapiglylene while

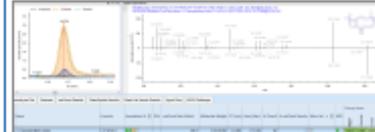


Figure 3. Mass Spectrometry Method Parameters



Figure 4. Principle Component Analysis

Figure 5. Investigating the Statistically Significant Compounds in the Results Table

Figure 6. in-Cloud Search to Carapiglylene while

Figure 7. Fragmentation Database Search

Figure 8. Reference Plot of American vs. Korean

Figure 9. Search with LeadRank, Precursor and Molecular Formula match with TCM Reference

Figure 10. Classification Database Search

Figure 11. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 12. Total of Annotations Assigned to List of Compounds

Figure 13. Number of MS/MS Compounds of the 5776 Compounds

Figure 14. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 15. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 16. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 17. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 18. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 19. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 20. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 21. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 22. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 23. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 24. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 25. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 26. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 27. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 28. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 29. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 30. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 31. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 32. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 33. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 34. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 35. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 36. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 37. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 38. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 39. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 40. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 41. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 42. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 43. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 44. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 45. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 46. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 47. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 48. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 49. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 50. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 51. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 52. Fragmentation of MS/MS and MS/MS explained by Acquire

in-Cloud Search - Exact match to spectral library (> 95 Compounds, > 2M spectra)

The search results can be obtained from the spectra available in in-Cloud Search and additional information available in the spectra available in in-Cloud Search. This means the experimental spectra obtained from the search are more complete in in-Cloud Search than the spectra obtained from the search.

Figure 3. in-Cloud Search match to Compound 16

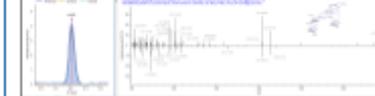


Figure 4. Fragmentation Database Search with in-Cloud Applied (MS/MS Parameters with structure)

This list was obtained from the list table as a reference. The list contains structures which enables an in-Cloud search to new candidate structures. In-Cloud search by connecting the fragmentation data and compound structures of lower compounds in in-Cloud to that of the higher structures.

Figure 5. Search with LeadRank, Precursor and Molecular Formula match with TCM Reference



Figure 6. Classification Database Search with in-Cloud Applied (precise structure)

A precursor and molecular formula search of Classification was conducted in in-Cloud search to new candidate structures with in-Cloud. For example, precise. See Figure 10 for more details on how to use in-Cloud search.

Figure 7. MS/MS and MS/MS. Candidate structures submitted to MS/MS (exact structure information of the fragments observed based on MS/MS observed in-Cloud)



Summary of in-Cloud Search and MS/MS Match

Figure 12. Total of Annotations Assigned to List of Compounds

Figure 13. Number of MS/MS Compounds of the 5776 Compounds

Figure 14. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 15. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 16. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 17. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 18. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 19. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 20. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 21. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 22. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 23. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 24. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 25. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 26. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 27. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 28. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 29. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 30. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 31. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 32. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 33. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 34. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 35. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 36. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 37. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 38. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 39. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 40. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 41. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 42. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 43. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 44. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 45. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 46. Fragmentation of MS/MS and MS/MS explained by Acquire

Figure 47. Fragmentation of MS/MS and MS/MS explained by Acquire

CONCLUSIONS

- Acquire enabled on the new Orbitrap ID-X Hybrid Mass Spectrometer improved data acquisition for integrated analysis with unknown structures.
 - Utilization of Acquire allowed for 5,776 compounds MS/MS gathered out of 1,700 compounds by 30 minute method.
 - Of the 5,776 compounds defined as MS/MS gathered 37 had an exact match to in-Cloud match > 95, whereas 3,739 had an exact or similar match to in-Cloud.

- The differences in quality and variety observed between American, Korean, and American ginseng were investigated closely using statistical tools in Compound Discoverer 3.0 to identify the compounds of interest.
 - No major differences observed with high and low quality ginseng.
 - Sub-Korean (low and high quality) closely resembled Chinese (high quality) whereas Chinese (high) and American (high quality) differed significantly.

- Additional advanced excitation tools available in Compound Discoverer 3.0 led to more compounds with positive identifications.
 - Compounds were identified with either a direct or similar match to spectra in in-Cloud.
 - The spectra that did not have a direct or similar match the in-Cloud algorithm was applied to new candidate structures by connecting experimental spectra gathered in compounds found in in-Cloud.

REFERENCES

See P138 for more information on Acquire and how it improves depth of coverage for data dependent MS/MS experiments.

TRADEMARKS/LICENSING

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