Enhanced Metabolite Identification using Orbitrap™ Tribrid Mass Spectrometer

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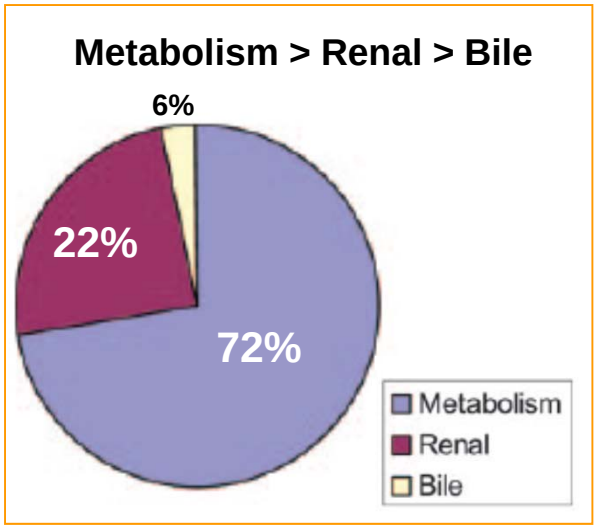
Outline

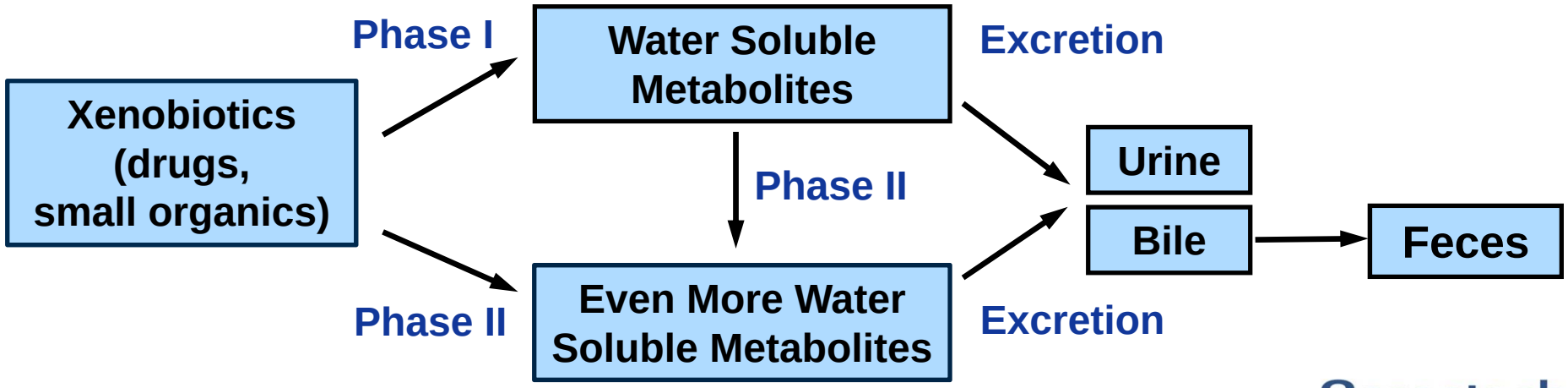
- Introduction
- Orbitrap ID-X with AcquireX Algorithm and Workflow
- Case Studies Using AcquireX DDA Metabolite Identification of 5 Drugs in Liver Microsomal Incubation
- Metabolite Structure ID with Compound Discoverer and Mass Frontier
- Conclusions

Metabolism is a Major Drug Clearance Pathway

- Typically, metabolism converts lipophilic compounds that favor absorption to hydrophilic products for excretion
- Phase I: oxidation, reduction, and hydrolysis
  Phase II: conjugation



Williams *et al.* (2004) *Drug Metab. Dispos.* 32:1201–1208.



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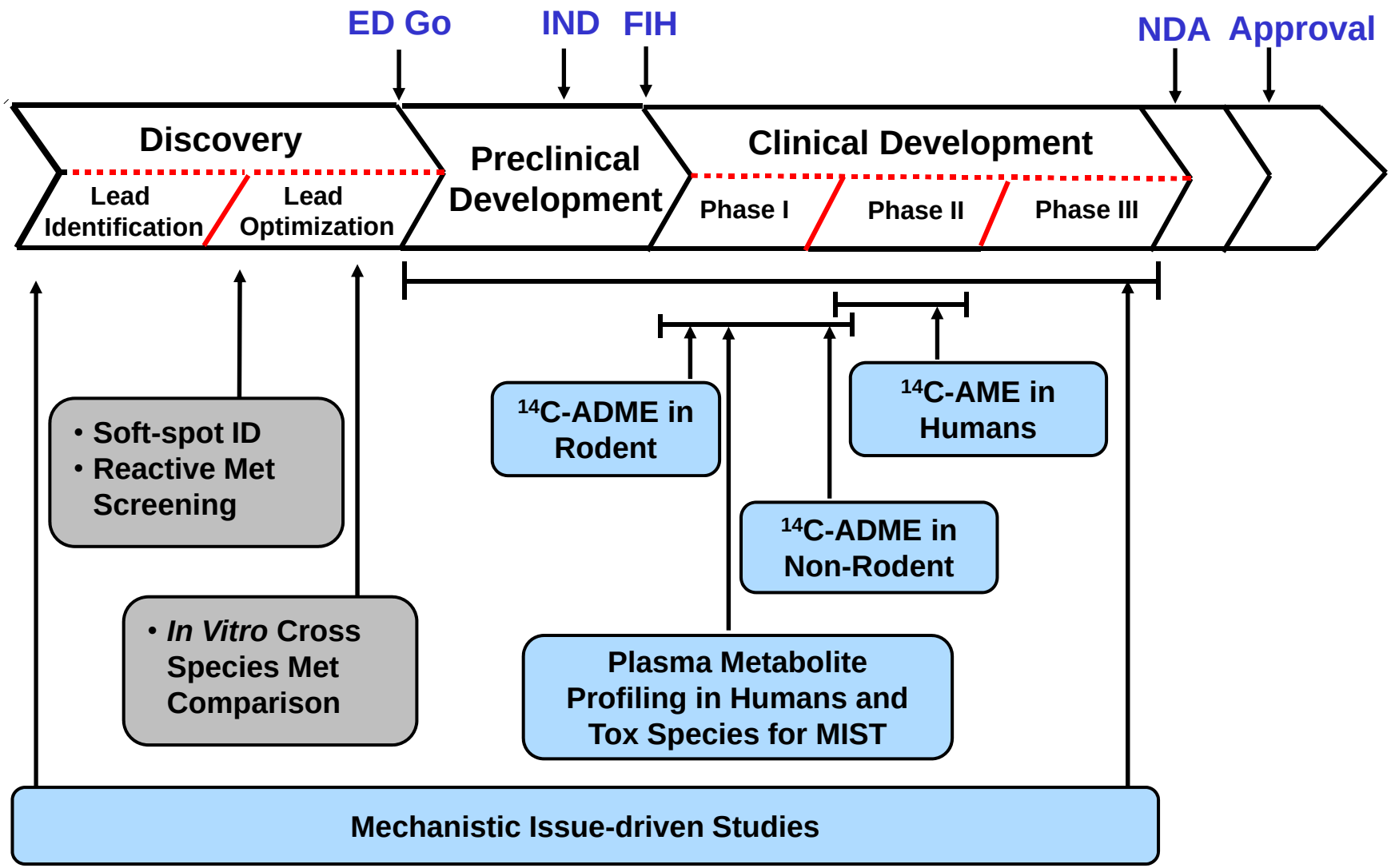
Why Identify Metabolites?

- To determine the site of metabolism.
- To identify active metabolites.
- To identify reactive or toxic metabolites.
- To identify main route of metabolism and clearance mechanism.

Metabolite Identification is a Critical Component through all Stages of Drug Discovery and Development



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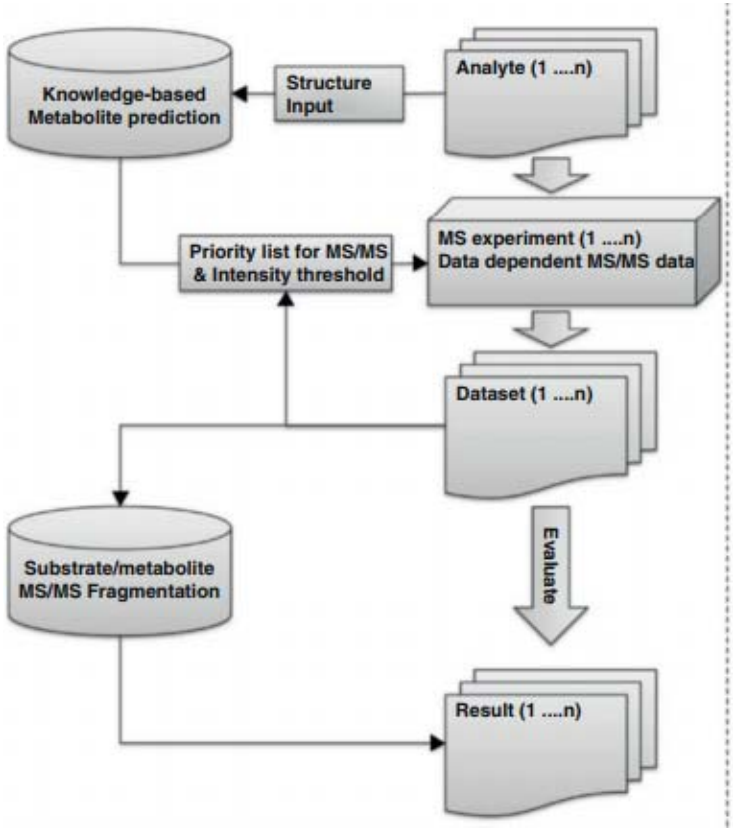
Data Dependent Acquisition of MS/MS Spectra for Drug Metabolites

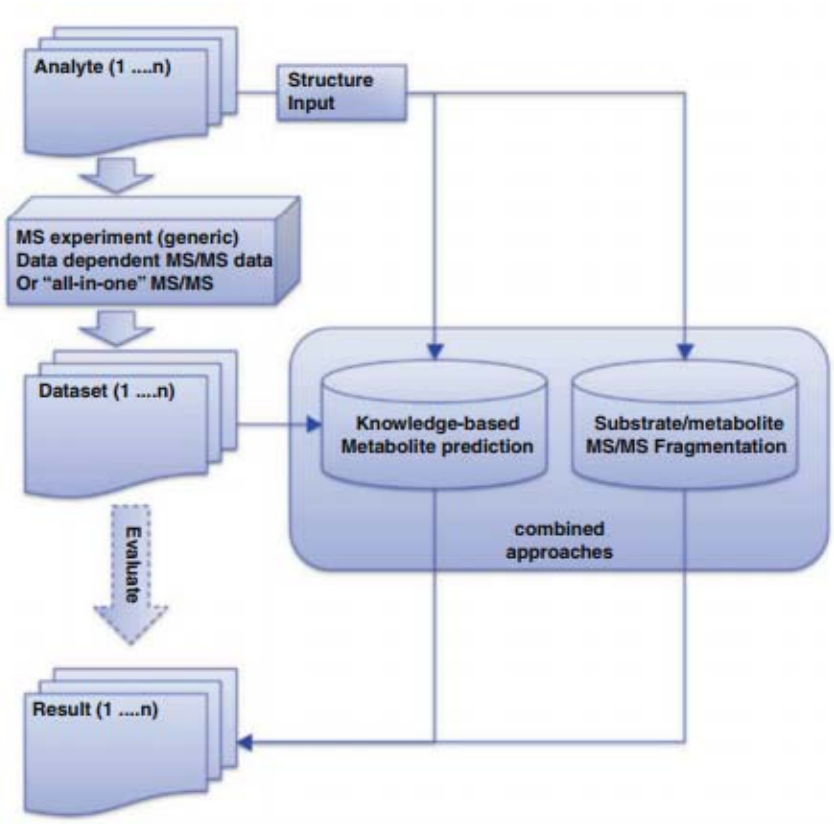
Targeted Approach



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

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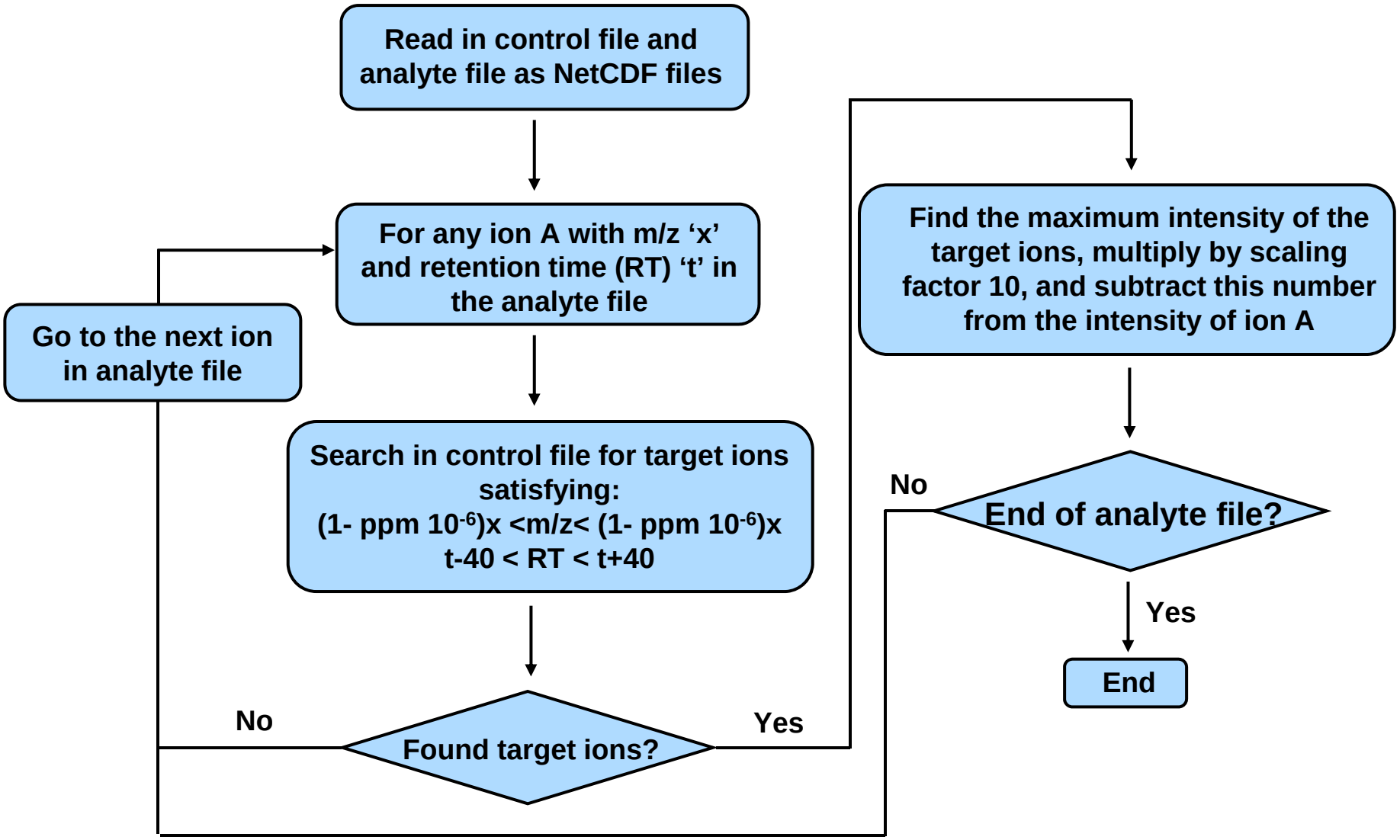
Background Subtraction is a Powerful Post Acquisition Technique for Metabolite Detection



Zhang, H. *et al. J. Mass Spectrom.* **2008**, 43, 1181−1190.
Zhu, P. *et al. Rapid Commun. Mass Spectrom.* **2009**, 23, 1563-1572.

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Orbitrap ID-X with AcquireX Eliminates the Need for User Inputs on Inclusion and Exclusion List

AcquireX Acquisition – a non-targeted data-dependent approach for metabolite identification:

- AcquireX automatically generates an exclusion list from a control sample
  - Excludes the background ions from triggering MS^n
  - Only triggers the ions of interest that are not present in the control
- AcquireX can also automatically generate an inclusion list of all the ions that are present in the sample but not in the control sample

Advantages: Increase the analysis efficiency and confidence

- High quality MS^n data in one run
- No manual post acquisition data processing

Orbitrap ID-X with AcquireX Data Acquisition Workflow

Use a matrix blank or historical matrix data to reduce undesired sample MS^n data

What Xcalibur Does:

- Injects 1 matrix blank sample
- Creates ion exclusion list
- Update MS2 acquisition instrument method
- Acquires a user-defined number of samples

Combine exclusion and inclusion lists to automatically and reliably acquire more relevant MS^n data

What Xcalibur Does:

- Injects 1 matrix blank sample to generate exclusion list
- Injects 1 sample to generate inclusion list
- Automatically applies exclusion and inclusion lists

Use exclusion and inclusion list with multiple ID injections to comprehensively target ion list

What Xcalibur Does:

- Injects 1 matrix blank sample to generate exclusion list
- Automatically re-injects sample multiple times
- Automatically update the inclusion/exclusion list for subsequent injections

Orbitrap ID-X MS with AcquireX

- Orbitrap Tribrid technology –sensitive and versatile MS
- High resolution: 500, 000 resolution (at *m/z* 200)
- High scan speed: Orbitrap MS^n up to 30Hz
- MS^n capability for ultimate structure information
- Multiple dissociations: HCD/CID - sequential or parallel
- **AcquireX**
  - Identify low-level and unknown compounds with confidence (MS^n), even in complex matrices

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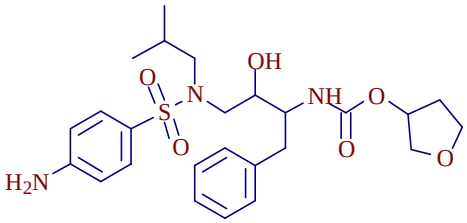
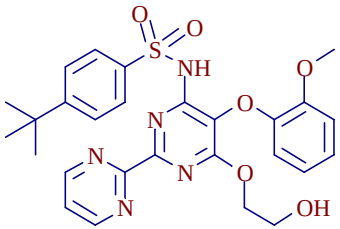
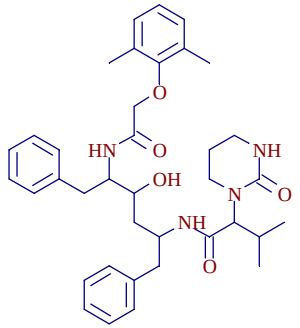
Chemical Structures of Model Compounds Used in the Study



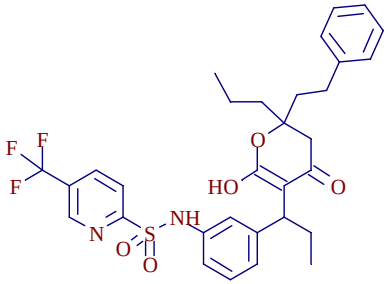
Amprenavir
C25H35N3O6S
$(M+H)^+$ 506.23193
Cas# 161814-49-9



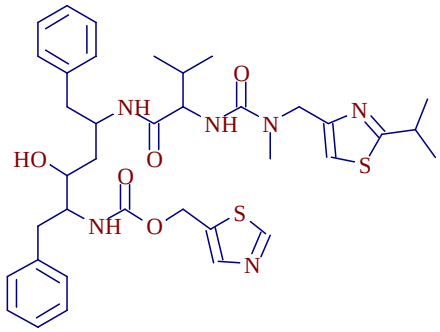
Bosentan
C27H29N5O6S
$(M+H)^+$ 552.19113
Cas# 147536-97-8



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

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Experimental

Sample:

Test compounds (5 µM): Amprenavir, Bosentan, Lopinavir, Ritonavir, and Tipranavir

Human liver microsomes (1 mg/mL) incubation in the presence of GSH and UDPGA and alamethicin at 37 degree for 1 hr

Control:

Human liver microsomes (1 mg/mL) incubation in the presence of GSH and UDPGA and alamethicin without test article at 37 degree for 1 hr

Analytical Method

Chromatography

Thermo Vanquish Flex UHPLC system:

Column: Thermo Hypersil™ GOLD 100 x 2.1 mm, 1.9µm

Gradient: Mobile A: H_2O / 0.1% Formic acid B: ACN

Flow rate: 400µl/min

LC gradient:

Time(min)	0	1.0	2.0	14.0	14.1	16.1	16.2	18.0
B%	5	5	15	70	95	95	5	5

Mass Spectrometry: Thermo Orbitrap ID-X MS

The MS analyses were carried out on Thermo Scientific Orbitrap ID-X™ Tribrid mass spectrometer using electrospray ionization in positive mode. The AcquireX data acquisition was used. High resolution full-scan MS and MS^n data were collected in a data-dependent fashion at resolving power of 120,000 and 30,000 respectively. Stepped HCD collision energy (%): 20, 40, 60 was applied.

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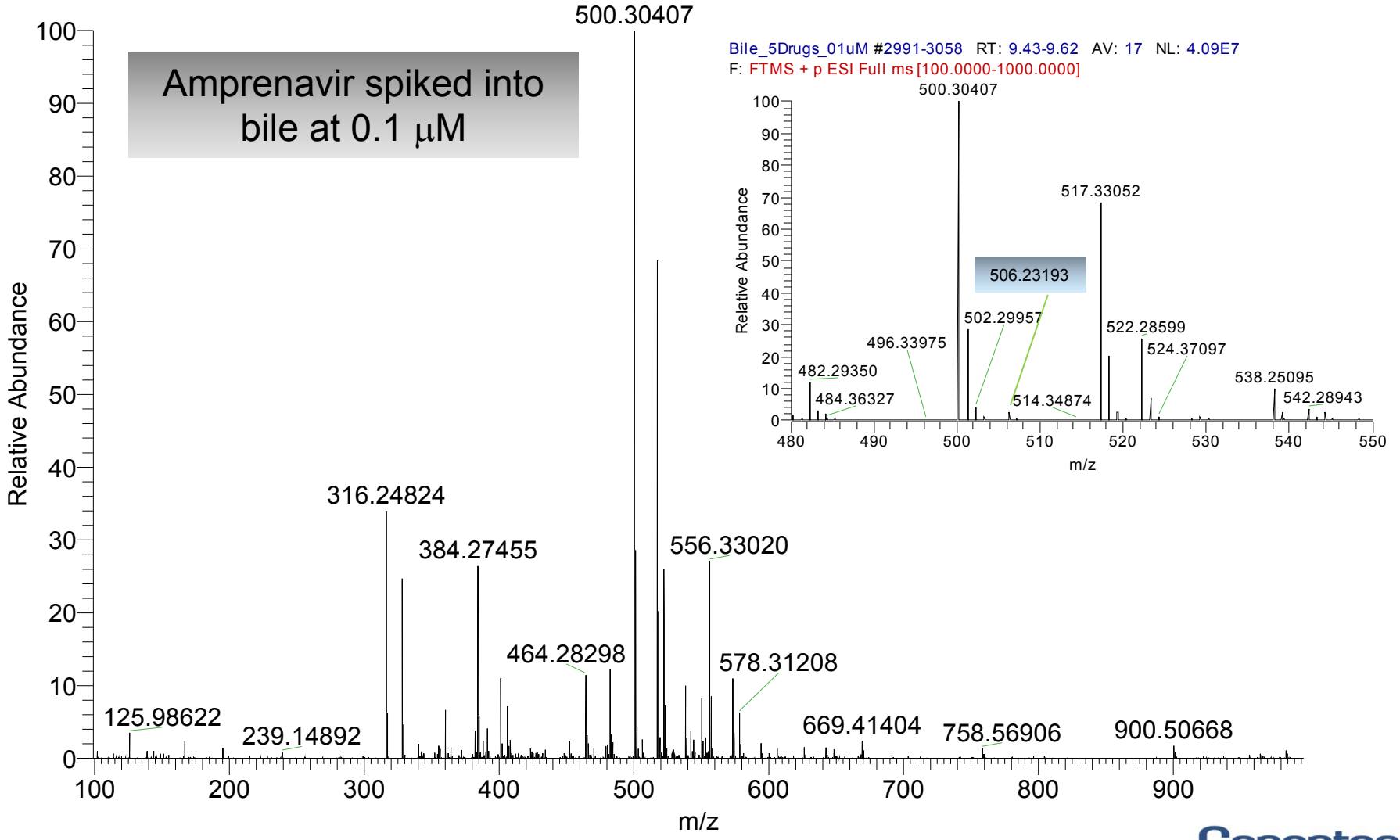
The Molecular Ion of Amprenavir in Bile was Masked by the Abundant Matrix Ions

Bile_5Drugs_01uM #2991-3058 RT: 9.43-9.62 AV: 17 NL: 4.09E7
F: FTMS + p ESI Full ms [100.0000-1000.0000]



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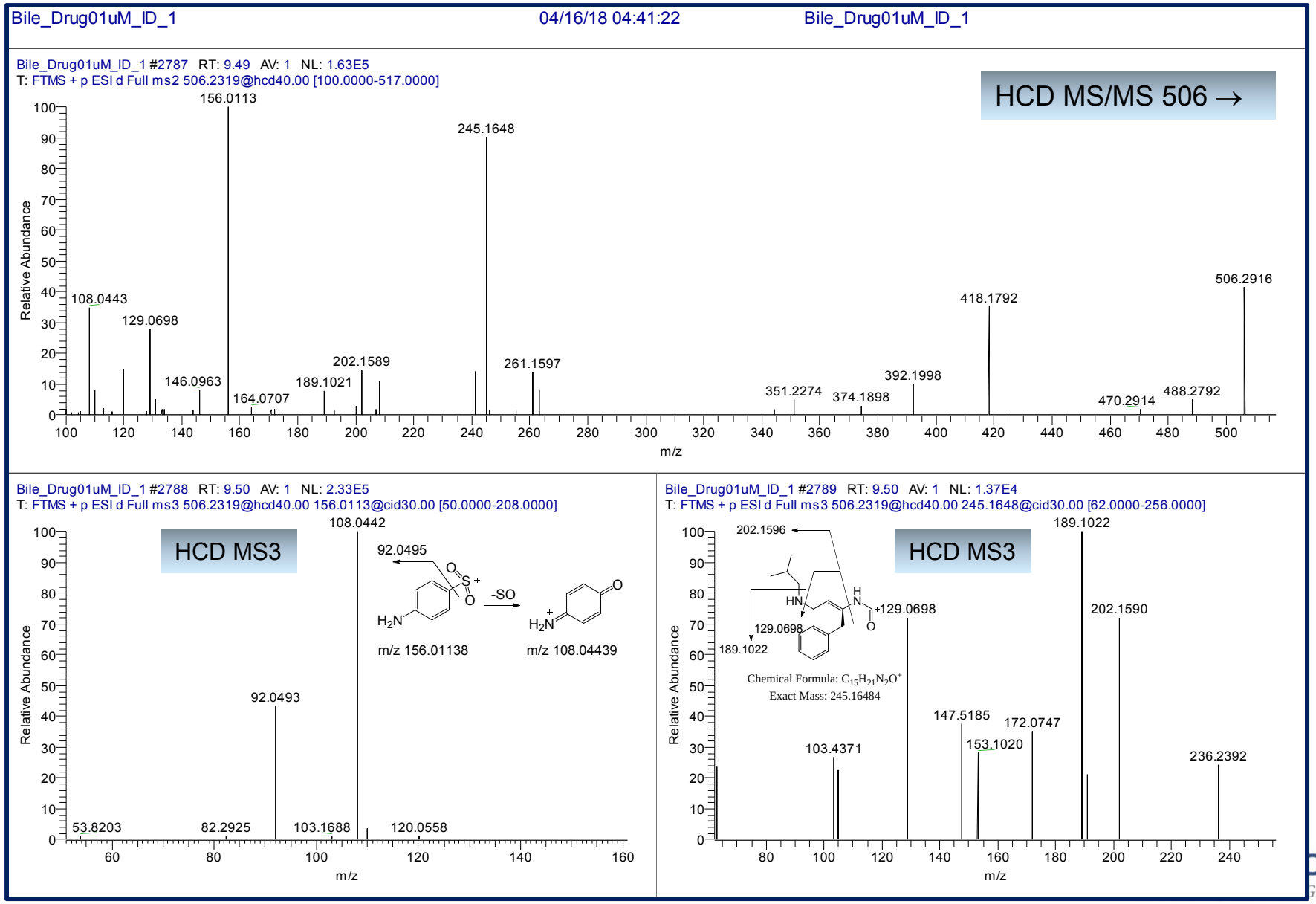
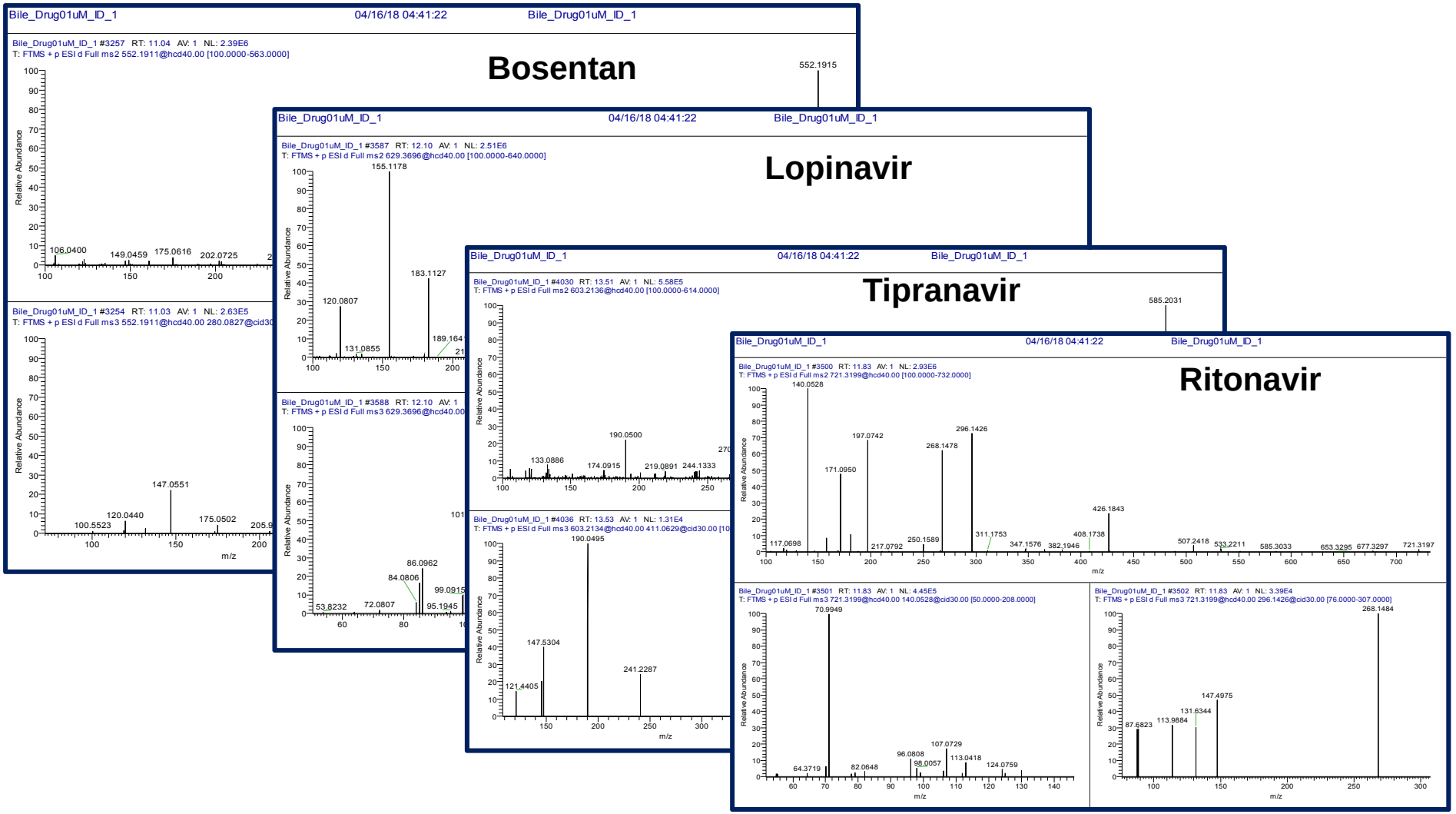
Orbitrap ID-X Successfully Triggered MS^n of Amprenavir in Complex Bile Matrix



Orbitrap ID-X Successfully Triggered MS^n of the other 4 Drug Standards at Low-levels in Complex Matrices



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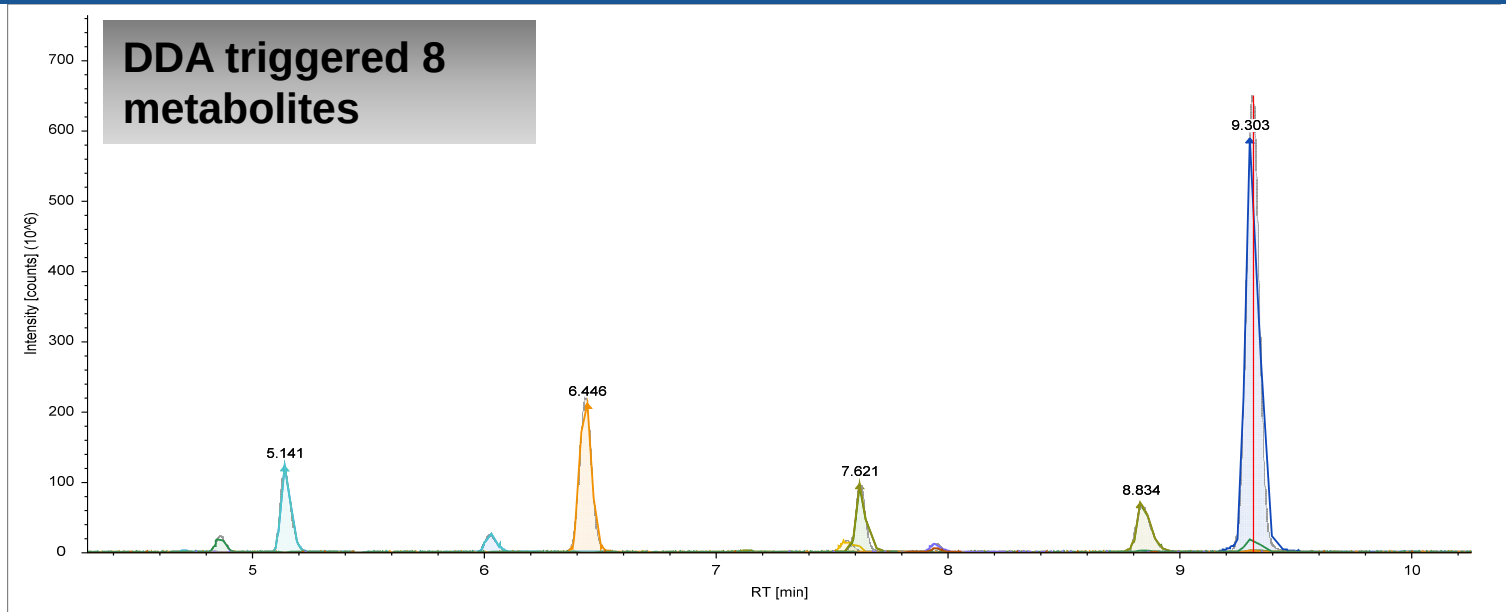
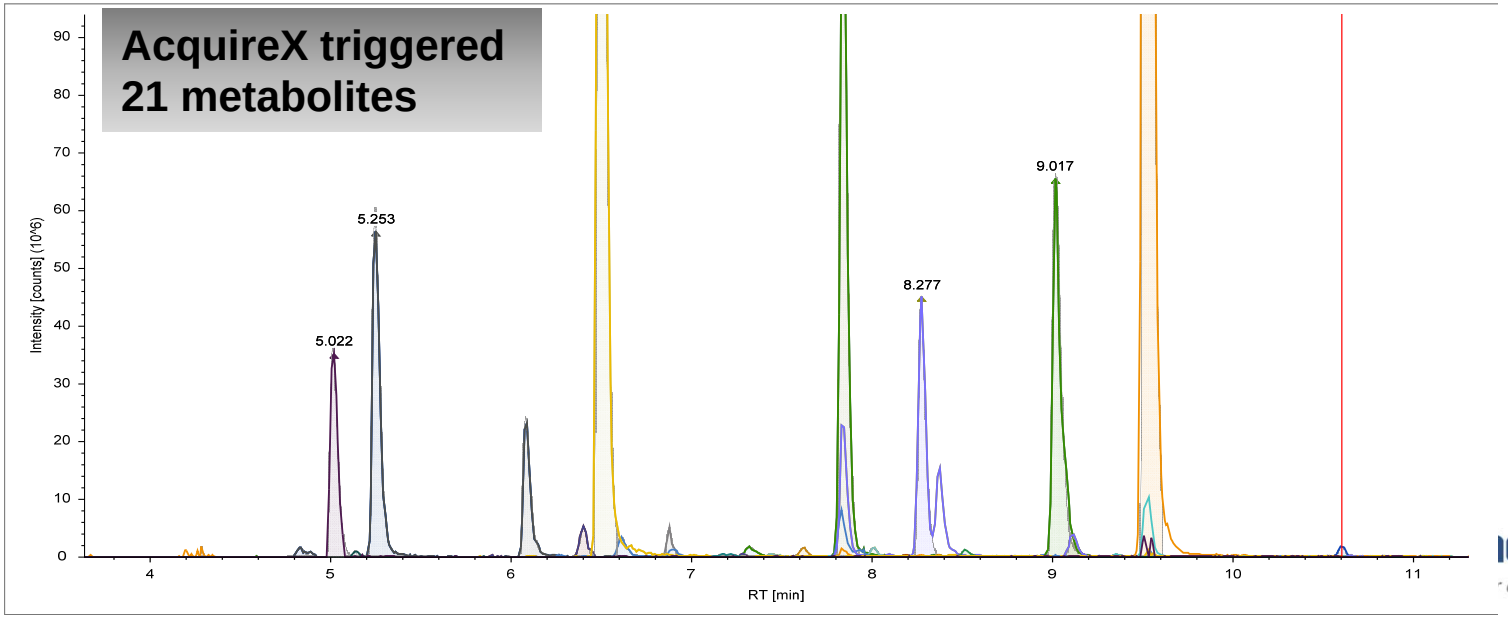
Conventional and AcquireX Workflow Comparison: Amprenavir as an Example





More Metabolites were Triggered for MS^n 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

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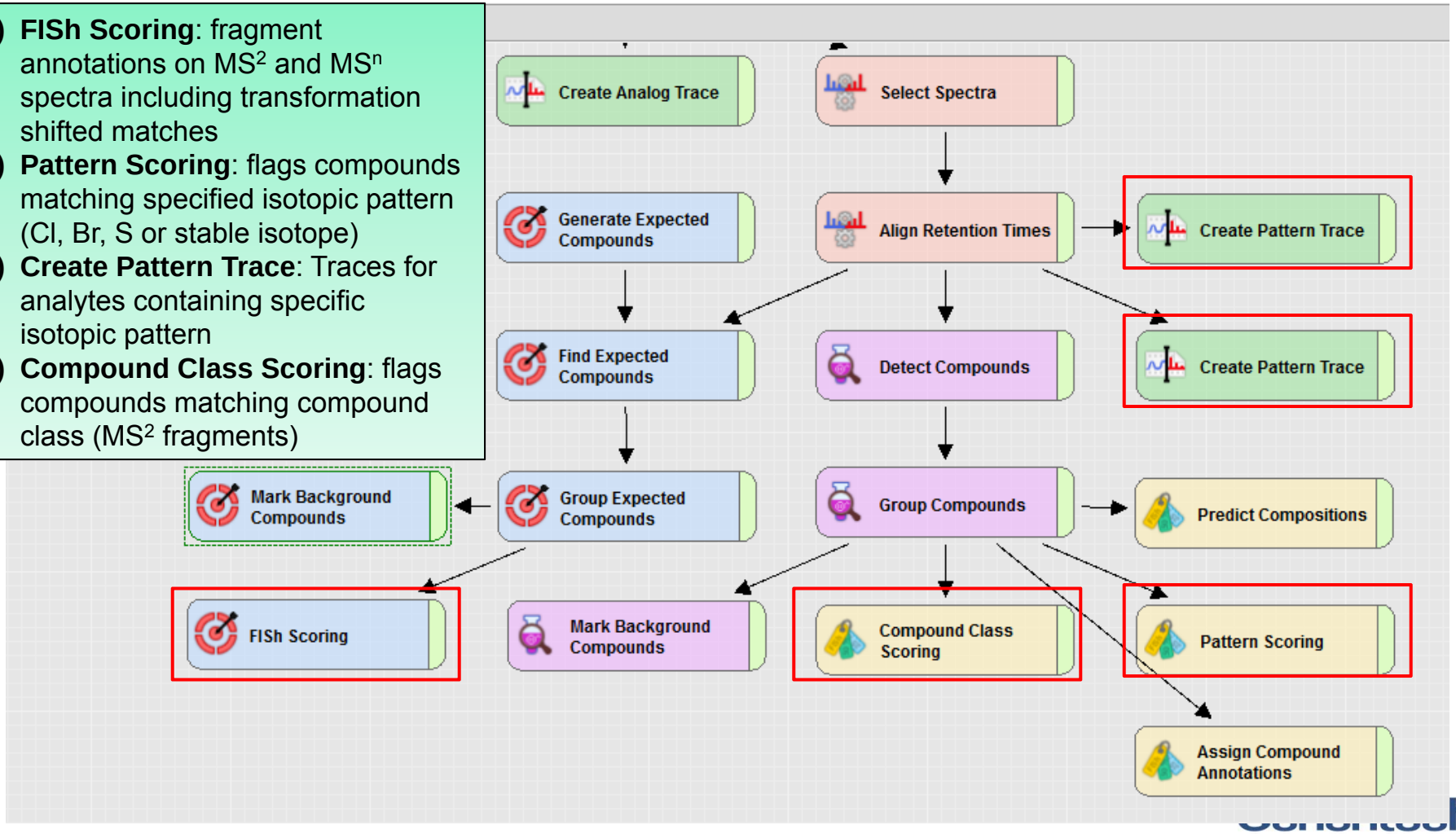
Data Processing Using Compound Discoverer 3.0

Utilizing unique features of CD 3.0, metabolites can be readily identified using expected and unknown workflow with compound class scoring

1) **FISh Scoring**: fragment annotations on MS^2 and MS^n spectra including transformation shifted matches
2) **Pattern Scoring**: flags compounds matching specified isotopic pattern (Cl, Br, S or stable isotope)
3) **Create Pattern Trace**: Traces for analytes containing specific isotopic pattern
4) **Compound Class Scoring**: flags compounds matching compound class (MS^2 fragments)



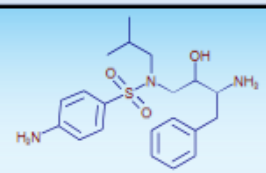
Automatic Dealkylation/Hydrolysis Prediction in Compound Discoverer 3.0

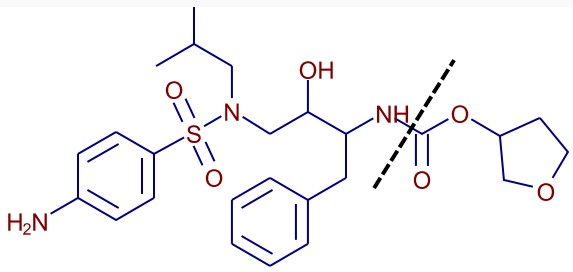
Parameters of 'Generate Expected Compounds'

Show Advanced Parameters

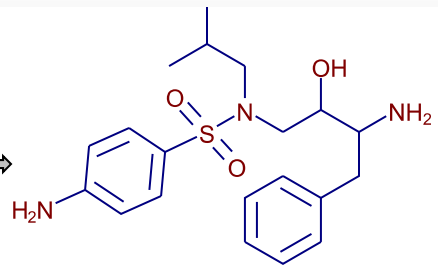
1. Compound Selection	
Compound	161814-49-9 Amprenavir (C25 H35 N3 O6 S)
2. Dealkylation	
Apply Dealkylation	True
Apply Dearylation	True
Max. # Steps	2
Min. Mass [Da]	150
3. Transformations	
Phase I	Dehydration (H2 O ->); Desaturation (H2 ->); Hyc
Phase II	Acetylation (H -> C2 H3 O); Arginine Conjugation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

Expected Compounds | Expected Formulas | Expected Features | Related Structures | Input Files

	Checked	Parent Compound	Formula	Molecular Weight	Dealkylated	Composition Cha	Structure
1	☐	Amprenavir	C20 H29 N3 O3 S	391.19296	X	-(C5 H6 O3)	



Hydrolysis



parent 506.2320 @9.52min

392.2002 @6.50min

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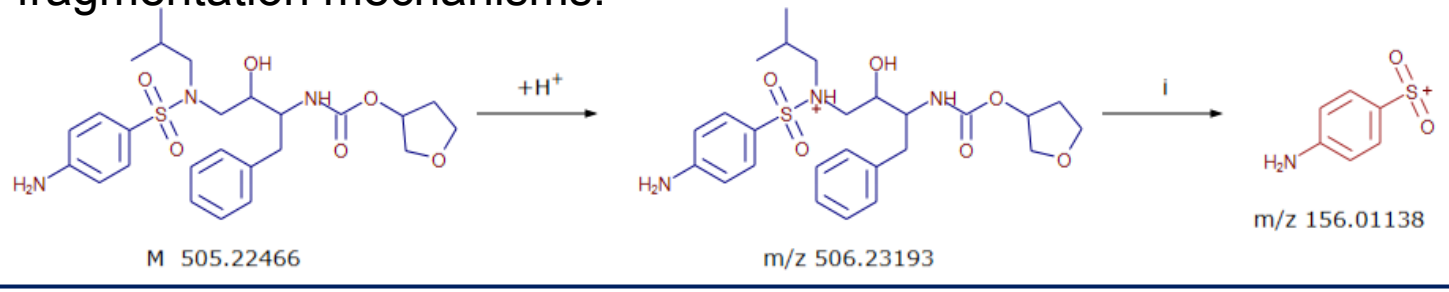
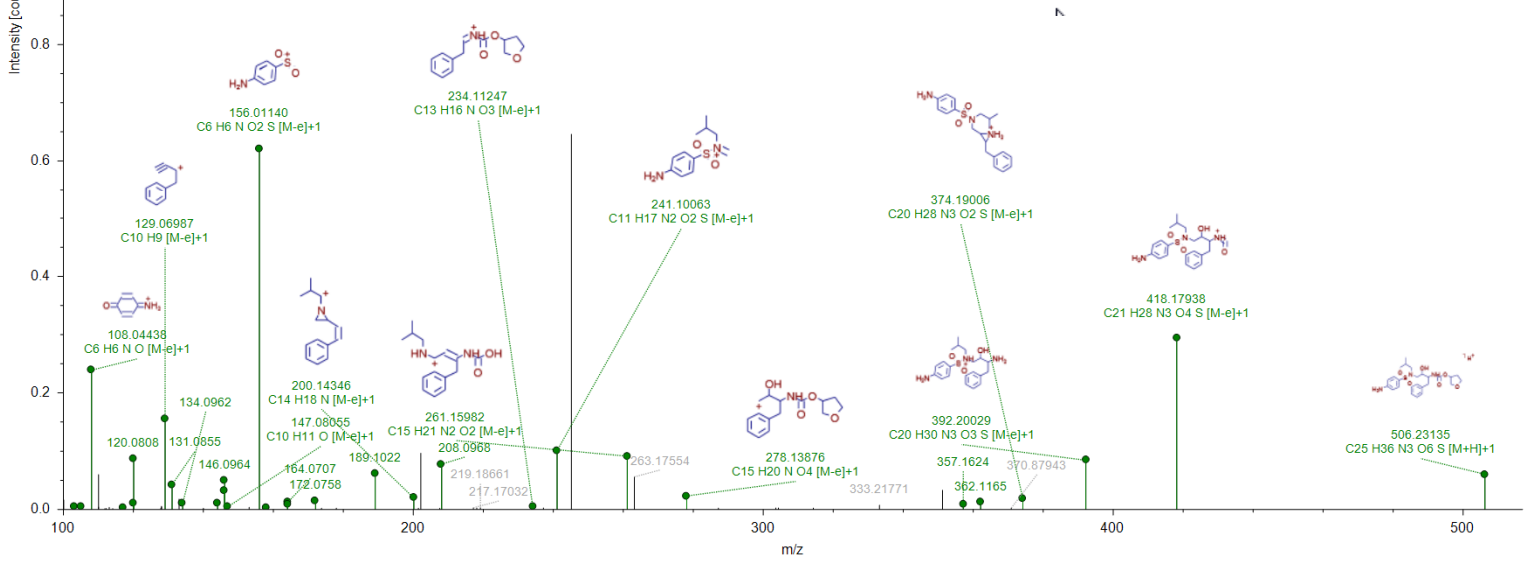
Mass Frontier: Fragment Ion Interpretation Based on Fragmentation Mechanisms

Amp_1Hr_ID (F5) #2867, RT=9.477 min, MS2, FTMS (+), (HCD, DDF, 506.2318@(20;40;65), +1)
Amprenavir C25 H35 N3 O6 S, MW: 505.22466, Area: 32283363
FISh Coverage: 31 Matched, 14 Unmatched, 18 Skipped

Mass Frontier Software predicts comprehensive fragmentation pathways based on a set of general ionization, fragmentation, and rearrangement rules with extensive literature coverage on fragmentation mechanisms.





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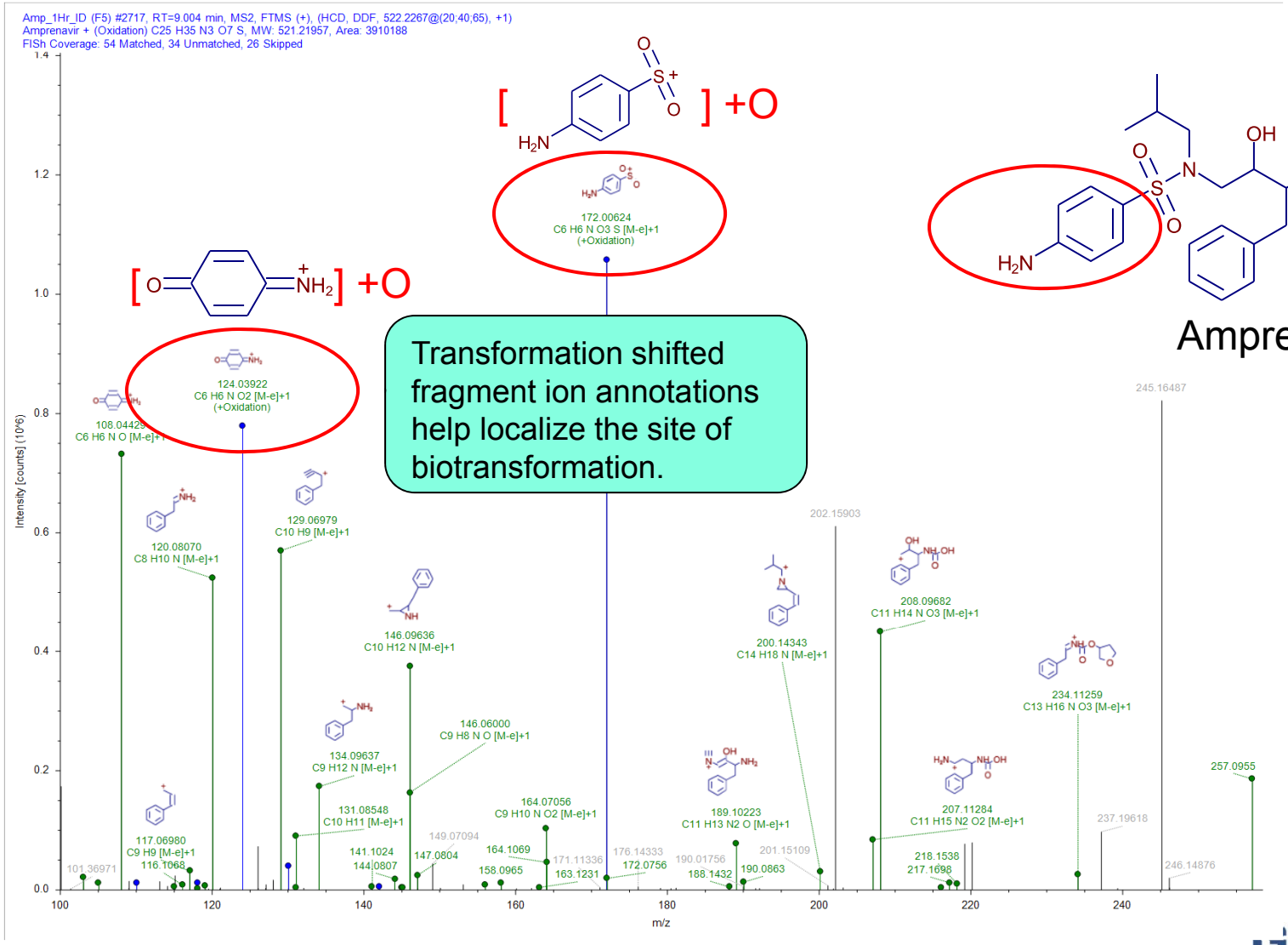
Automatic Fragment Ion Search (FISh) Annotations in CD 2.1 and 3.0



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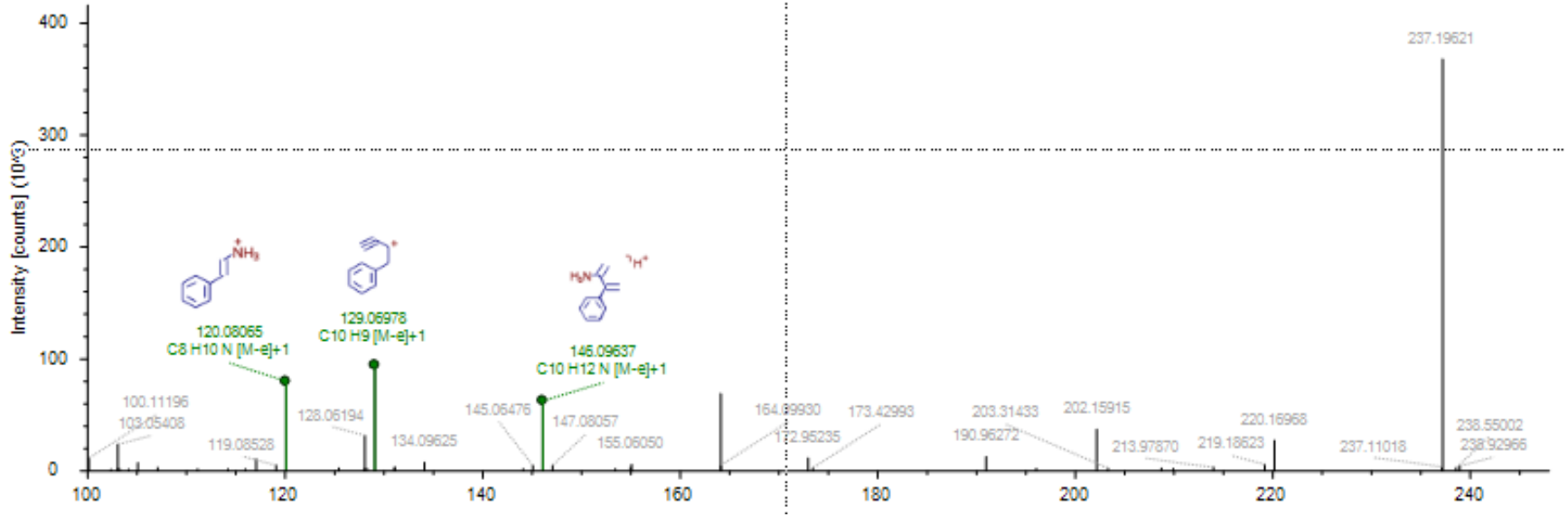
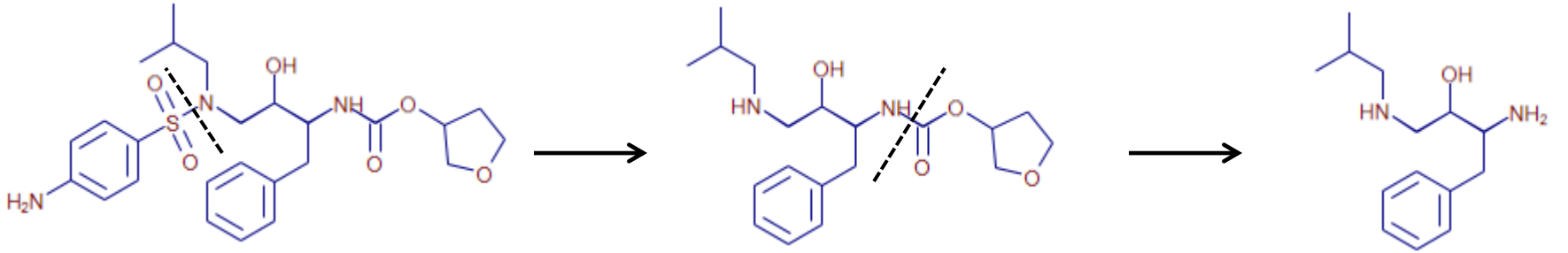
Unexpected Metabolite Found By Compound Class

Compounds | Compounds per File | Expected Compounds | Expected Compounds per File | Expected Formulas | Features | Expected Features | Input Files | Specialized Traces

	Checked	Name	Formula	Annotation Sc	FISh Coverage	Molecular Weight	RT [min]	Area (Max.)	Class Coverage	MS2	Pattern Matches	Area
17	☑		C14 H24 N2 O			236.18892	2.291	183892	27.27			1.84e5





m/z 506.2319 m/z 351.2278 m/z 237.1961

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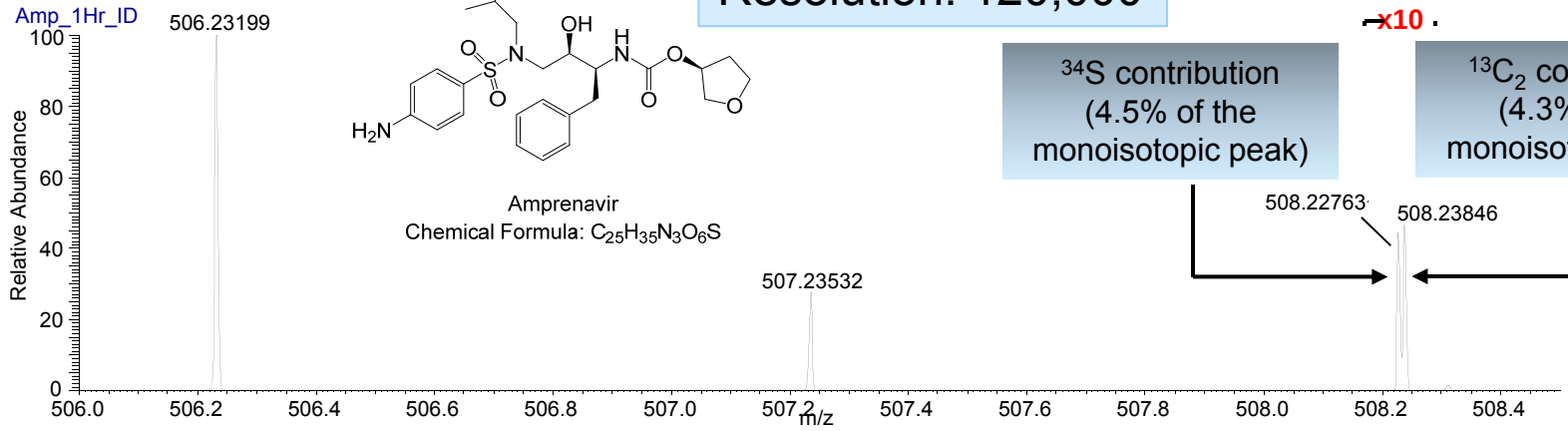
Ultra High Resolution and Isotopic Pattern Searching Algorithm in CD Allowes Selective Metabolite Detection

Resolution: 120,000



^{34}S contribution (4.5% of the monoisotopic peak)

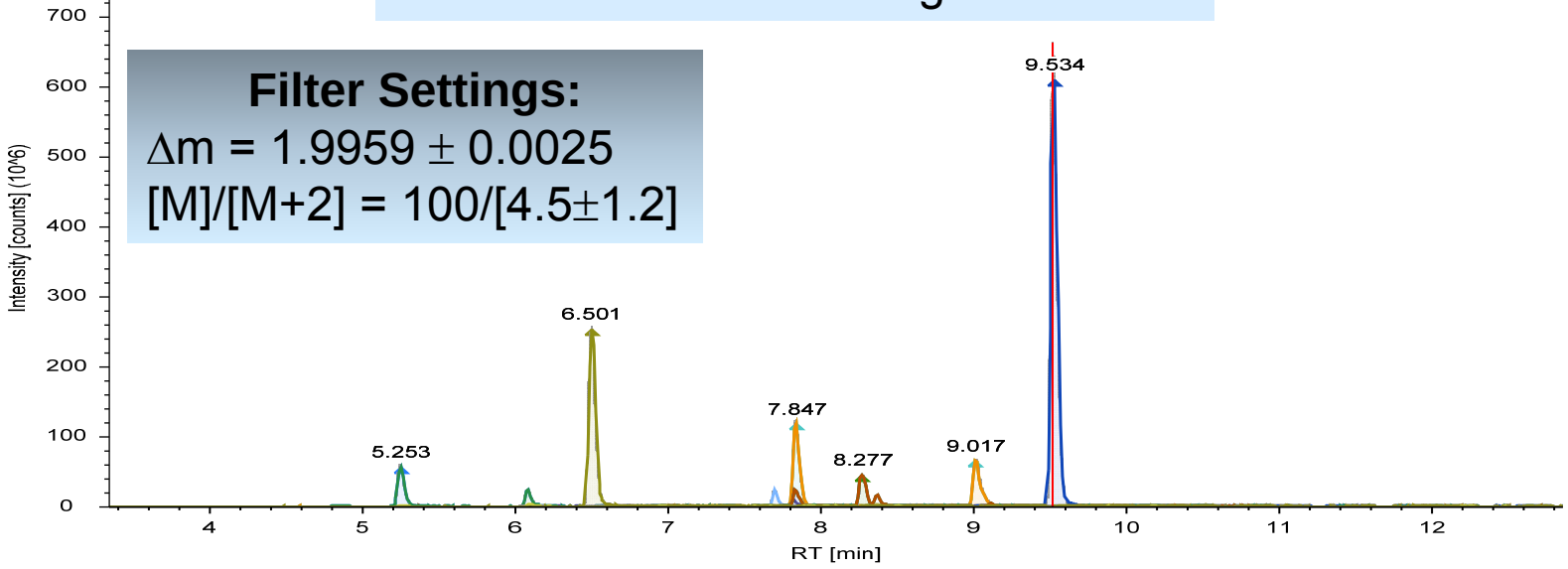
$^{13}C_2$ contribution (4.3% of the monoisotopic peak)

All Metabolites Containing One Sulfur

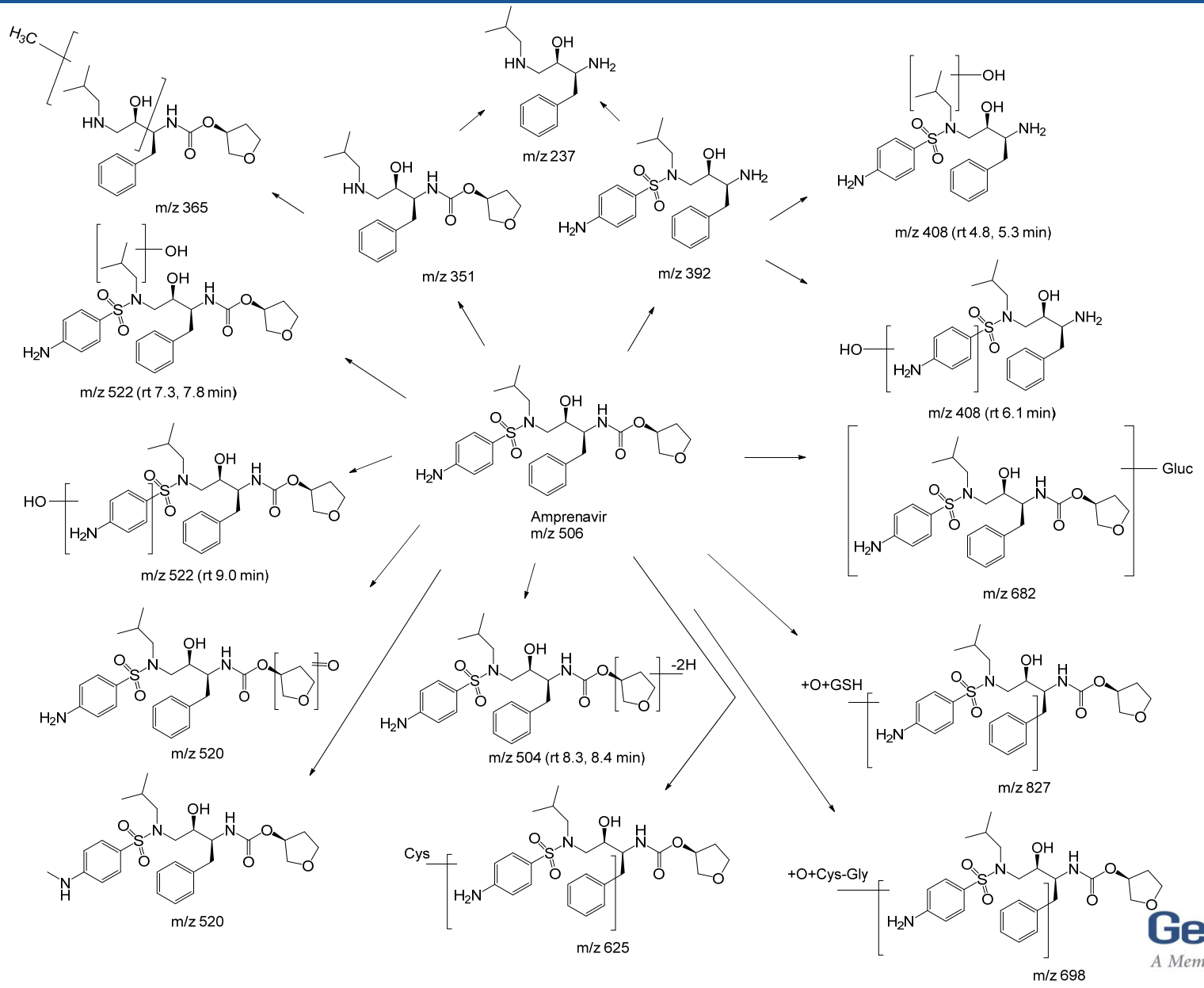
Filter Settings:

$\Delta m = 1.9959 \pm 0.0025$

$[M]/[M+2] = 100/[4.5 \pm 1.2]$



Metabolites Identified from Amprenavir HLM Incubations



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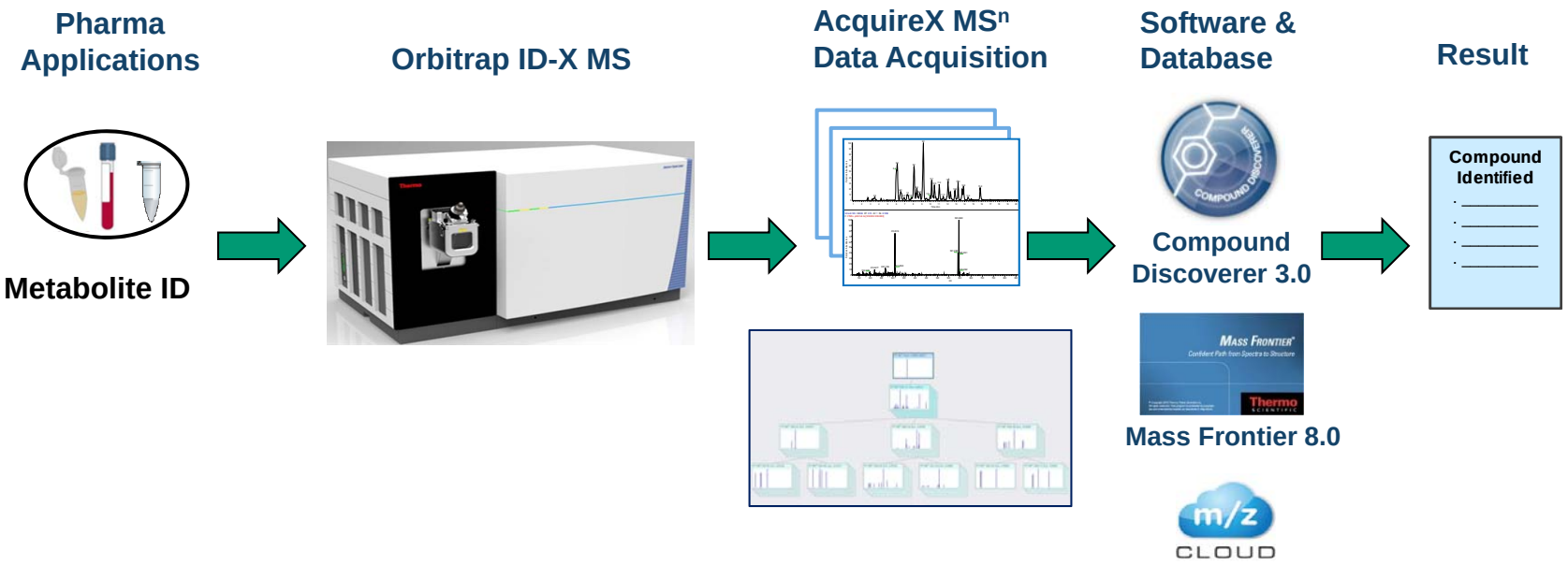
An integrated Data Acquisition and Processing Suite for Metabolite Identification



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Conclusions

- High resolution mass spectrometry has gained wide acceptance as a tool of choice for drug metabolite identification due to its high mass resolving power and mass accuracy.
- New data acquisition methods that selectively trigger drug-related ions for data-dependent MS^n acquisition or to automatically generate mass inclusion list and exclusion list have been introduced to facilitate drug metabolite identification.
- Significant progress has been made in software development (such as Compound Discoverer and Mass Frontier) for automatic structural assignment from high resolution MS/MS dataset to improve the throughput of metabolite identification.

Acknowledgement

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DMPK, Genentech

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