Approaches for Small Molecule Metabolite ID in Discovery

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CPSA (09/28/2016)

Acknowledgements:

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BTx colleagues, Serhiy Hnatyshyn, Petia Shipkova,
Wilson Shu, Tony Paiva, William Humphreys
Bristol-Myers Squibb

Julie Horner, August Specht, Jonathan Josephs
Thermo-Fisher Scientific

Ismael Zamora, Blanca Serra, Fabien Fontaine
Molecular Discovery Ltd.

Bristol-Myers Squibb

Outline

Why discovery biotransformation effort?

The solutions

- Soft-spot ID
- Processing tools for Qual/Quan work-flow
- Hardware–HRMS instruments (multiple fragmentation modes)
- Workflow based solution and Software

Data examples

- From Metabolic Stability Screen and ID Studies
- Structure based approaches and tools

Advances in data processing/reporting

- Data mining and management

Scope of Discovery Biotransformation Functions

- **Determine metabolic soft-spots**
  - In parallel with lead optimization and SAR iterations
  - Overcome high metabolic clearance issue
- **Characterize active metabolites**
  - Determine whether active metabolite plays a role in pharmacological efficacy, especially in the incidence of PK/PD disconnect
  - Strong in-house skillsets in bioassay directed approaches, metabolite generation, isolation and exact structure determination (NMR) are successfully applied to the search for active metabolites
- **Identify genotoxic metabolites**
  - Most issues with latent aryl amine moieties, which will trigger structural alerts and the monitoring for free aryl amine release.

Scope of Discovery Biotransformation Functions-continued

- **Identify reactive metabolites**
  - Recognize reactive metabolites early on as a potential safety risk.
  - Understand/elucidate mechanisms of bioactivation.
  - Guide new synthesis or lead selection to avoid problems whenever possible.
- **Metabolite profiling of advanced compounds**
  - Elucidate metabolic clearance pathways, particularly for those that may present a toxicological issue.
  - Recognize whether a major metabolic pathway is catalyzed by human polymorphic CYPs which may present potential issue of drug-drug interaction in the clinic.
  - Estimate disposition with cold compounds (BDC rats).
  - Increase number of disposition studies in BDC animals and hepatocyte metabolite profiling with radiolabeled compounds.
  - Recognize/predict unique human metabolites.

Soft-Spot ID

Identify sites of metabolism on a compound or series of compounds that are leading to metabolic instability

Determine which system metabolic instability is to be determined in

- ***in vitro?***
  - **Which system?**
    - **Microsomes**
    - **Hepatocytes**
    - **S9**
    - **Plasma**

Microsomes +/- NADPH is typically a good place to start

- ***in vivo?***
  - **Metabolite profiling in plasma**
  - **Requires BDC animals and collection of all excreta**

Where to start?

High substrate concentration microsomal incubations are a good place to start

- **Major metabolites in this system can be quantified**
- **Easy to obtain high quality fragmentation spectra free of interference**
- **Likely not kinetically correct.**

Metabolites observed in the high substrate concentration incubation should be quantitated over a full time course in a low substrate incubation.

- **Look at initial rates of formation to see which metabolites are the primary result of metabolic instability**
- **Assign structures to all the metabolites with high initial rates of metabolism**
  - **Based on the difficulty of doing so and the extent of interest in the compound assigning structures to more minor metabolites may be warranted.**

Quan/Qual Approaches

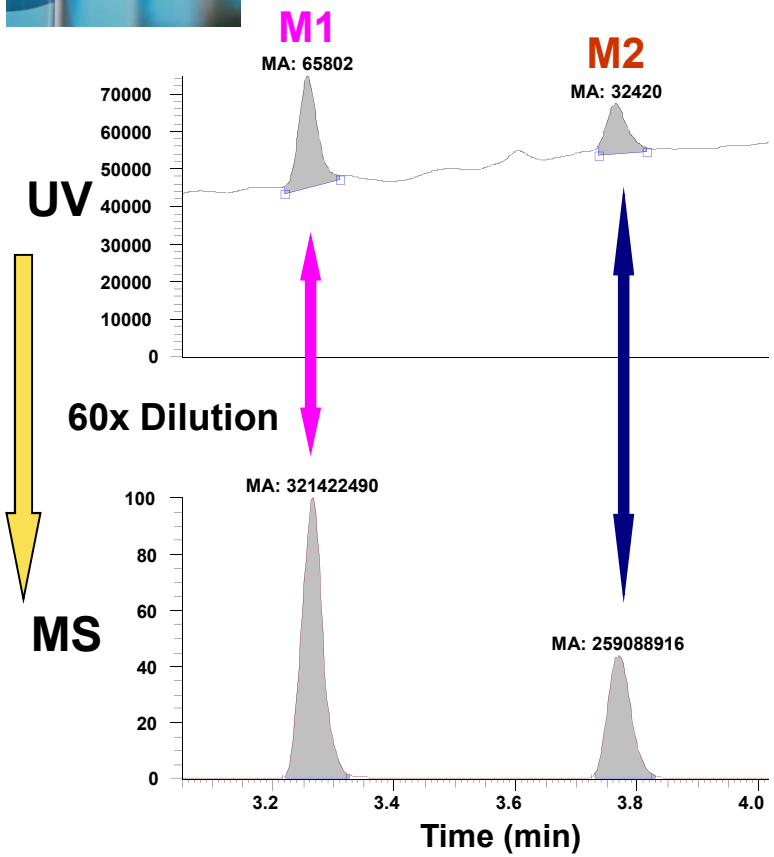
Integrated Qualitative and Quantitative LC-MS

- No single definition of Quan/Qual
  - All data from a single analysis?
  - All data from a single experiment?
  - Reliance on MS response alone?
    - Relative response factors?
    - Correction with “universal detectors”?
- Instrumentation
  - HRMS
    - Q-Tof
    - Orbitrap
    - QE
  - Nominal
    - Q-Trap
    - Linear Trap
- Structural assignments
  - Automated? Or Manual?

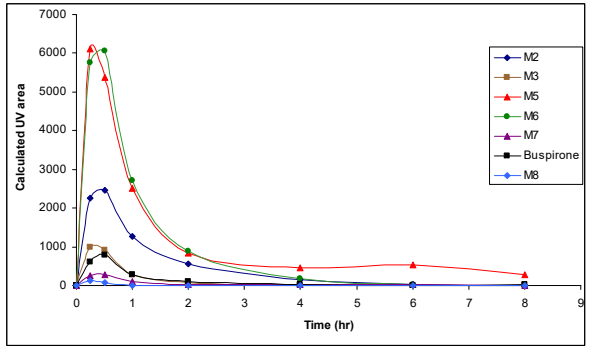
Qual-Quan Work-flow

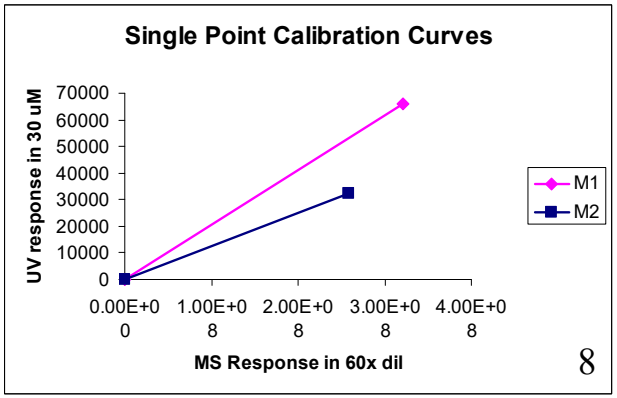
30 uM

High substrate concentration in vitro incubation single time point



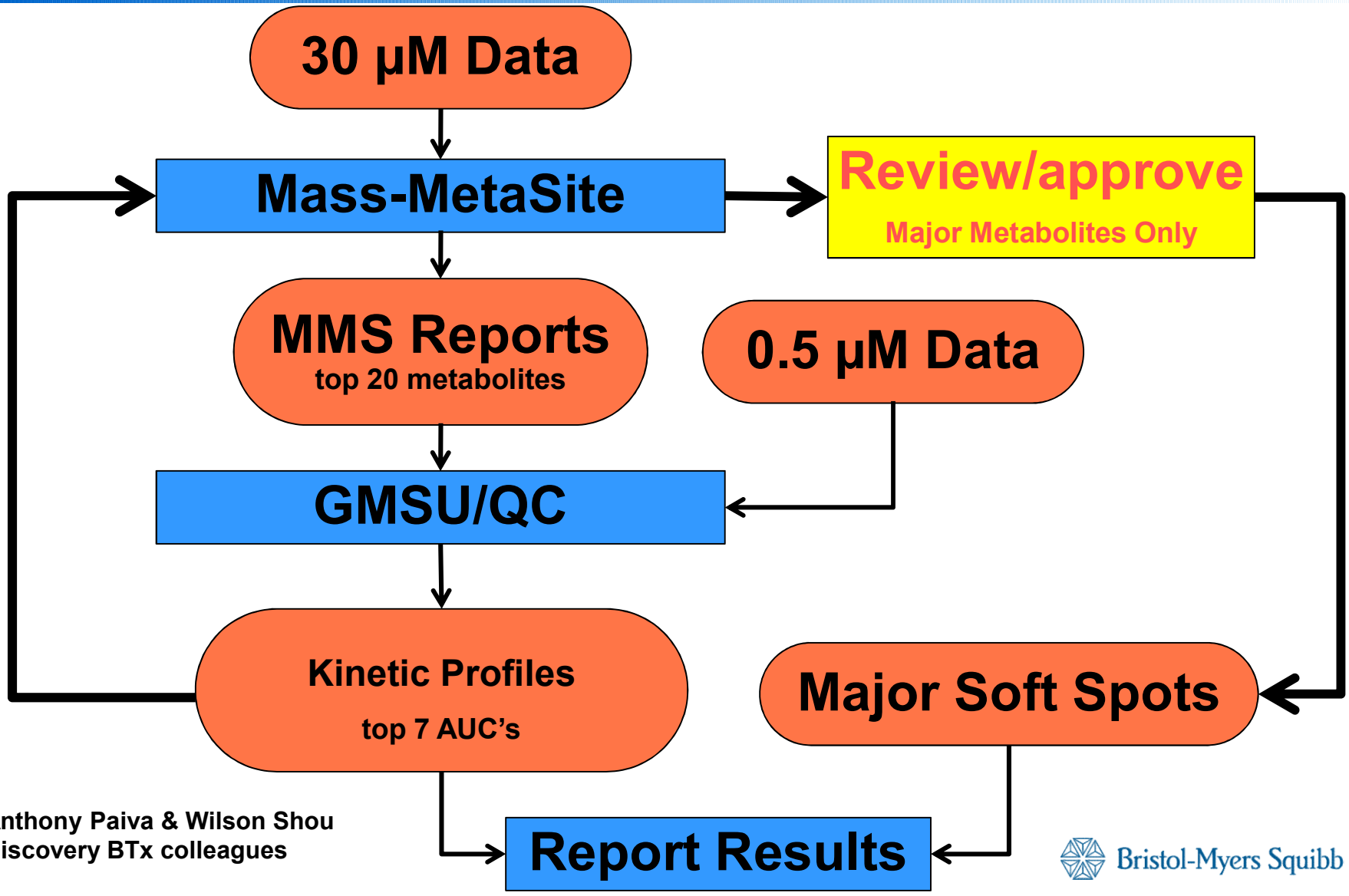
- ***30 uM Incubation***
  - ***Integration of UV areas***
- ***60x dilution***
  - ***Integration of MS areas***
  - ***Concentration range relevant to samples to be quantitated by MS***
- ***Single point calibration curves***
  - ***MS detection at low concentration***
  - ***Readout from curve to "UV corrected area"***





Courtesy: Jonathan Josephs

Data Processing Workflow



Anthony Paiva & Wilson Shou
Discovery BTx colleagues

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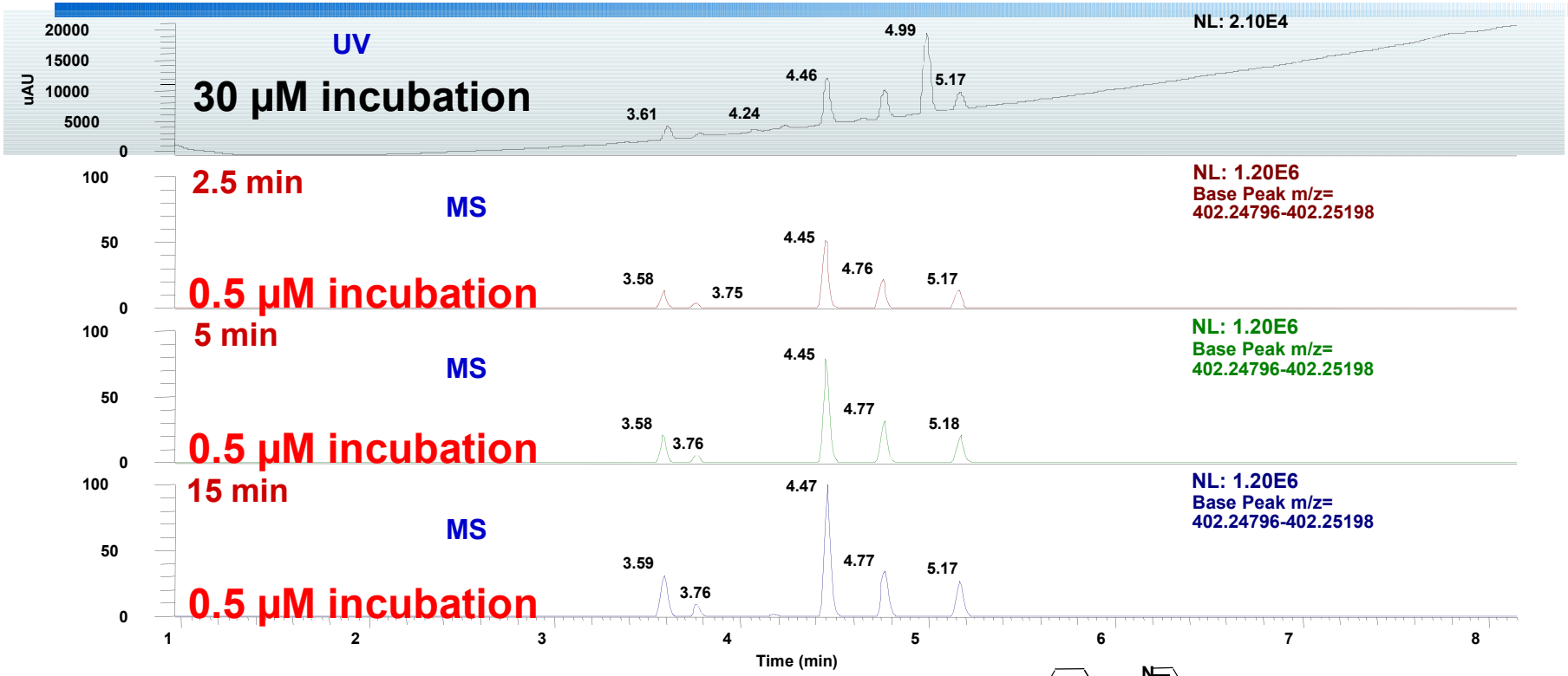
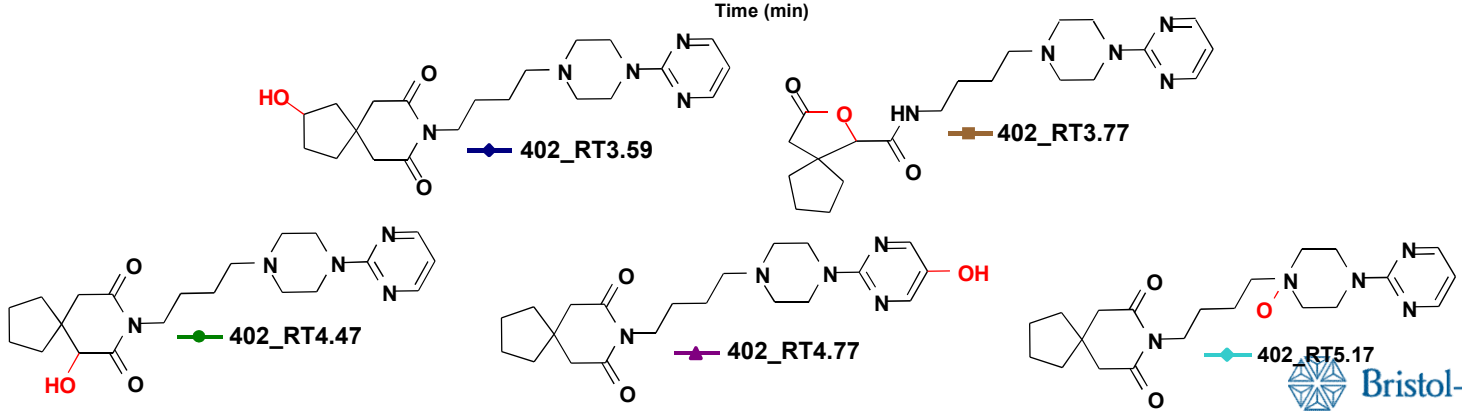
Buspirone *In Vitro* Incubations 0.5 uM HLM +NADPH

Orbitrap Data

0.5 µM Incubation
Time points: 0, 2.5, 5, 10, 15, 20, 30, 60 min
30 µM Incubation
Time points: 0, 45 min





Courtesy: Emily Luk

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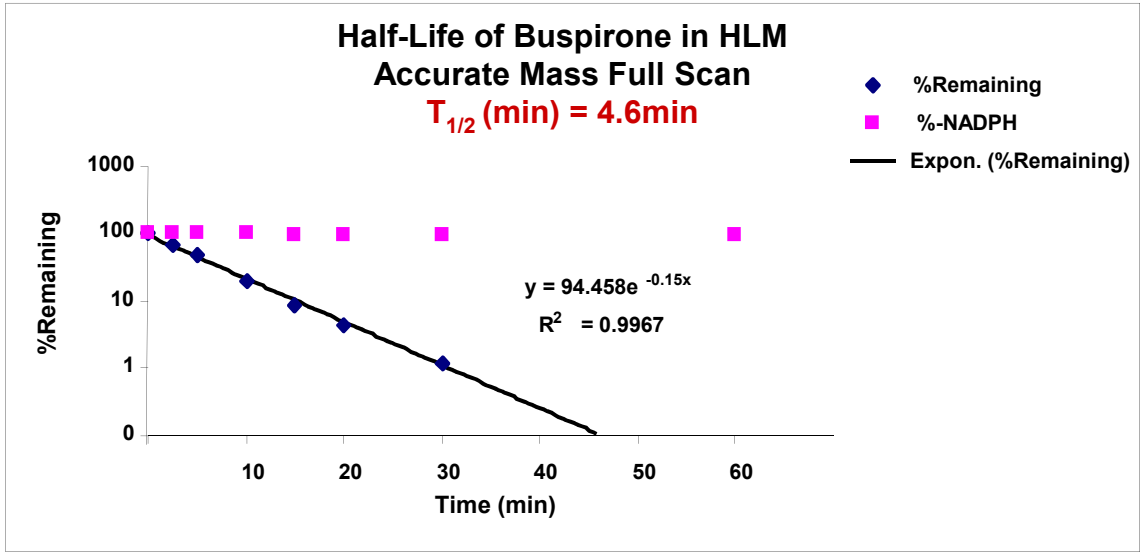
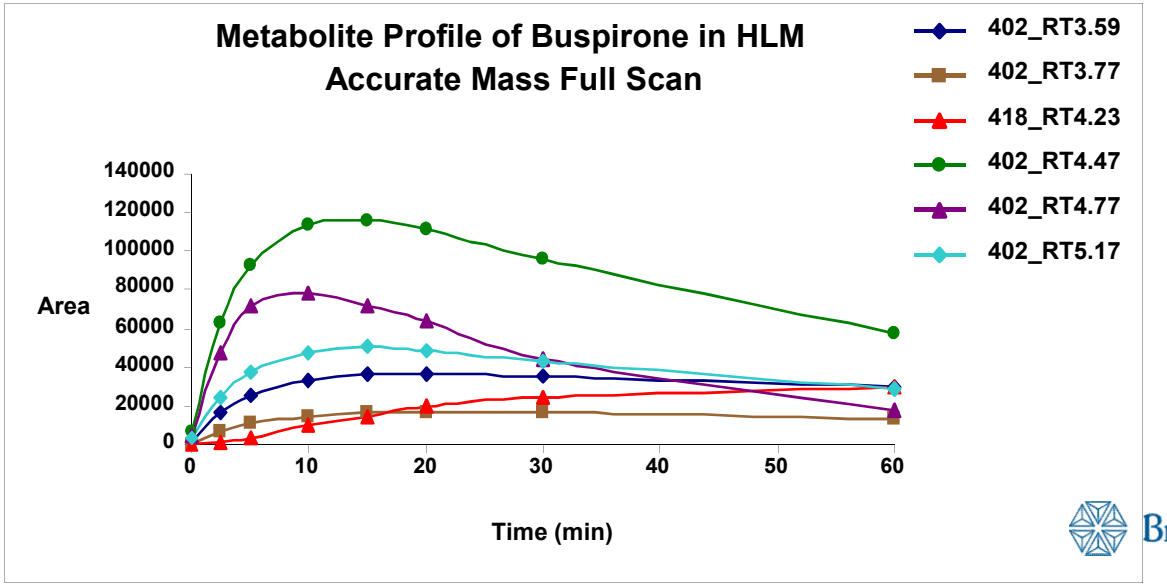
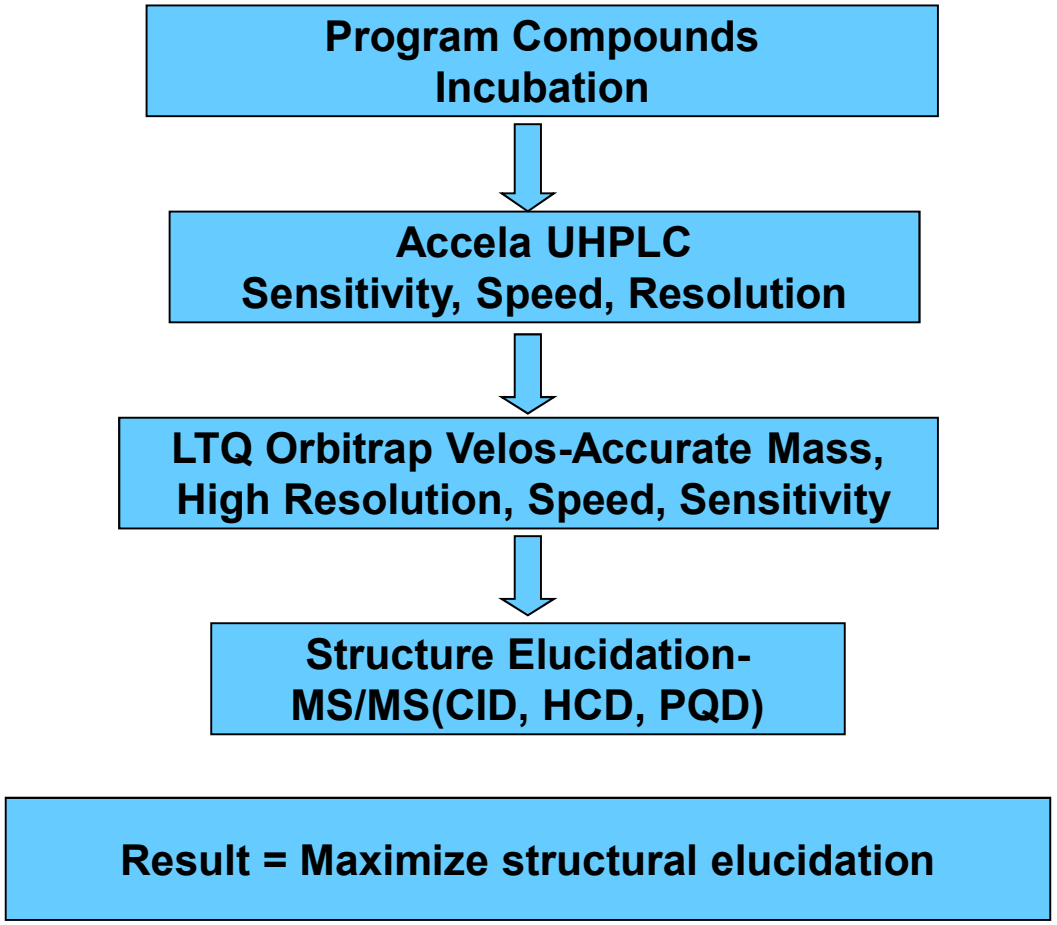
Buspirone 0.5 uM HLM $t_{1/2}$ and Metabolite Formation Plots



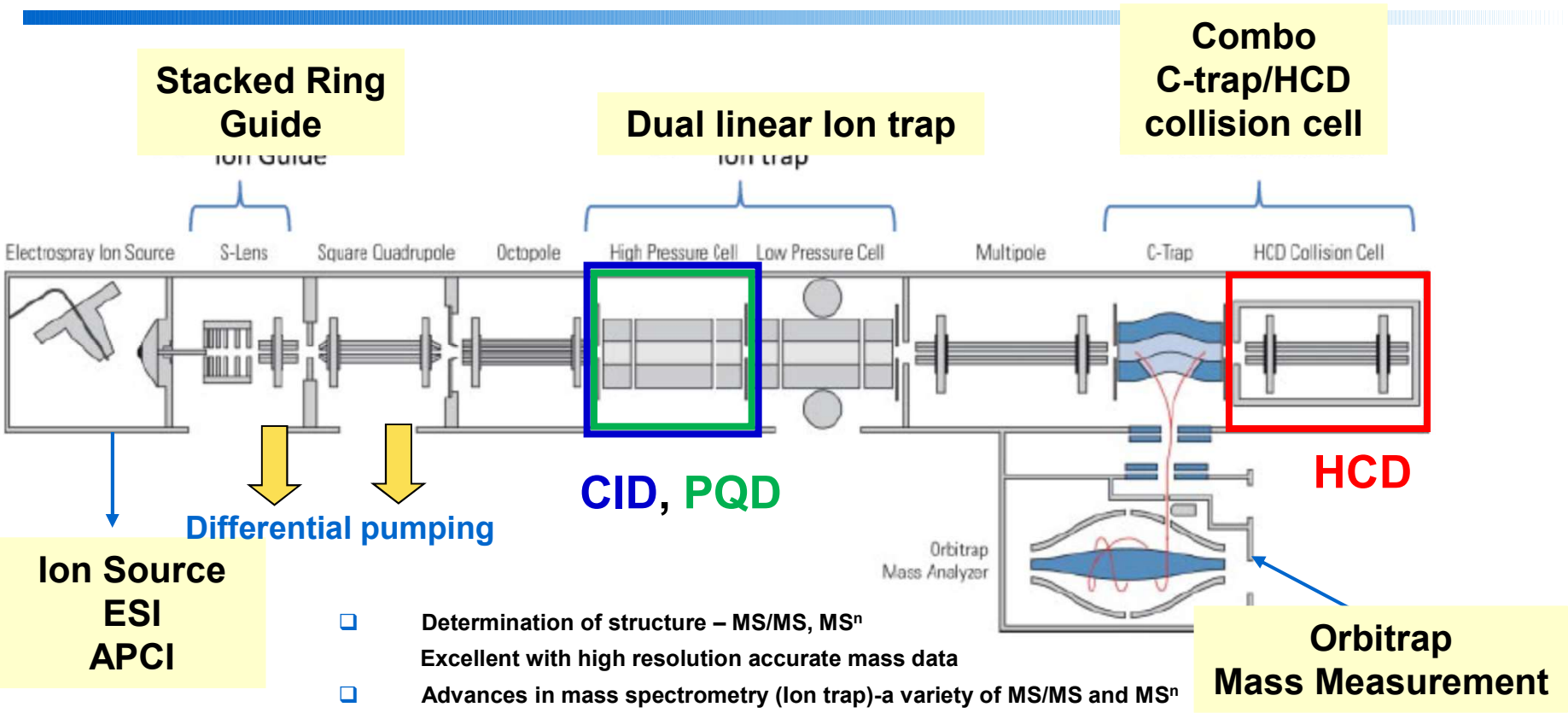


Courtesy: Emily Luk

Workflow for Metabolite ID using Orbitrap Velos to obtain comprehensive structure elucidation



LTQ Velos Orbitrap™



- Determination of structure – MS/MS, MS^n
- Excellent with high resolution accurate mass data
- Advances in mass spectrometry (Ion trap)-a variety of MS/MS and MS^n
  - Better qurehensive fragmentation profile
  - Acquisition of different fragmentation modes within a single analytical run

LTQ Velos Orbitrap™ (hybrid, HRAM – CID, HCD and PQD)

LTQ Velos Pro™ (stand alone, nominal mass – CID & Trap-HCD)

CID: Collision Induced Dissociation
PQD: Pulsed Q Collision Induced Dissociation
HCD: Higher Energy Collisional Dissociation

Makarov, A. *Anal. Chem.* 2000, *72*, 1156-1162.
Makarov, A.; Hardman, M. E.; Schwartz, J. C.; Senko, M. W; WO 2002078046, 2002.
Hardman, M.; Makarov, A. A. *Anal. Chem.* 2003, *75*, 1699-1705.
Hu, Q.; Noll, R. J.; Li, H.; Makarov, A.; Hardman, M.; Cooks, R. G. *J. Mass Spectrom.* 2005, *40*, 430-443.

Fragmentation Modes in LTQ Velos Orbitrap™

Activation Type	CID	HCD	PQD
Precursor Selection	IT	IT/C-Trap	IT
Collision cell	IT	HCD	IT
Collision gas	Helium	Nitrogen	Helium
Collision energy[a]	~35	40-75	35-60
Detection of Ions	IT/Orbitrap	Orbitrap	IT/Orbitrap
Activation, Q value	0.25(constant)	N/A	>0.6
Dissociation	single	single	3-step
Mass range	Low mass cut-off("1/3 rule")	No low mass cut-off	No low mass cut-off
Fragmentation	MS^n	MS^2	MS^n

[a]Normalized
N/A : not applicable

CID: Collision Induced Dissociation
PQD: Pulsed Q Collision Induced Dissociation
HCD: Higher Energy Collisional Dissociation

Compound/Metabolites used for studying CID, HCD and PQD spectra

Compound	Chemical Formula	Exact Mass of Parent (M+H)	Number of Analytes (Parent + Metabolite)
Diclofenac	$C_{14}H_{12}Cl_2NO_2$	296.02	2
Imipramine	$C_{19}H_{25}N_2$	281.20	6
Mirtazapine	$C_{17}H_{20}N_3$	266.16	5
Trazodone	$C_{19}H_{23}ClN_5O$	372.15	4
Coumarin	$C_9H_7O_2$	147.04	2
Warfarin	$C_{19}H_{17}O_4$	309.11	4
Tamoxifen	$C_{26}H_{30}ON$	372.23	5
Verapamil	$C_{27}H_{39}O_4N_2$	455.29	4
Clozapine	$C_{18}H_{20}ClN_4$	327.13	3
Buspirone	$C_{21}H_{32}O_2N_5$	386.25	6

Microsomal incubation in RLM; Substrate concentration: 30uM

The spectra were evaluated separately, based on the following:

- ❑ **range of collision energies required for fragmentation, low mass cut-off, ion intensity**
- ❑ **precursor and fragment mass tolerances were within 3 ppm**

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LC separation and MS analysis

Liquid Chromatography

Accela 1250

Solvent Composition

A: 0.1% formic acid in H_2O

B: 0.1% formic acid in ACN

UHPLC Chromatographic Conditions

Column: ACQUITY UPLC BEH C18, 2.1x100 mm, 1.7 µm

Column Temperature: 55 °C

Injection Volume: 7 µL

Flow Rate: 600 µL/min

Mass Spectrometry

Thermo LTQ-Velos OrbiTrap™

Resolution: Full Scan MS 30,000

MS/MS Scans 7,500

- Data collected: ESI+
- Acq range: *m/z* 100-600
- Acq mode: centroid
- Capillary temperature: 350°C
- Source voltage: 4.0kV

Single injection-three kinds of MS/MS data

Scan 1: Full Scan MS data

Scan 2: MS/MS event for CID data

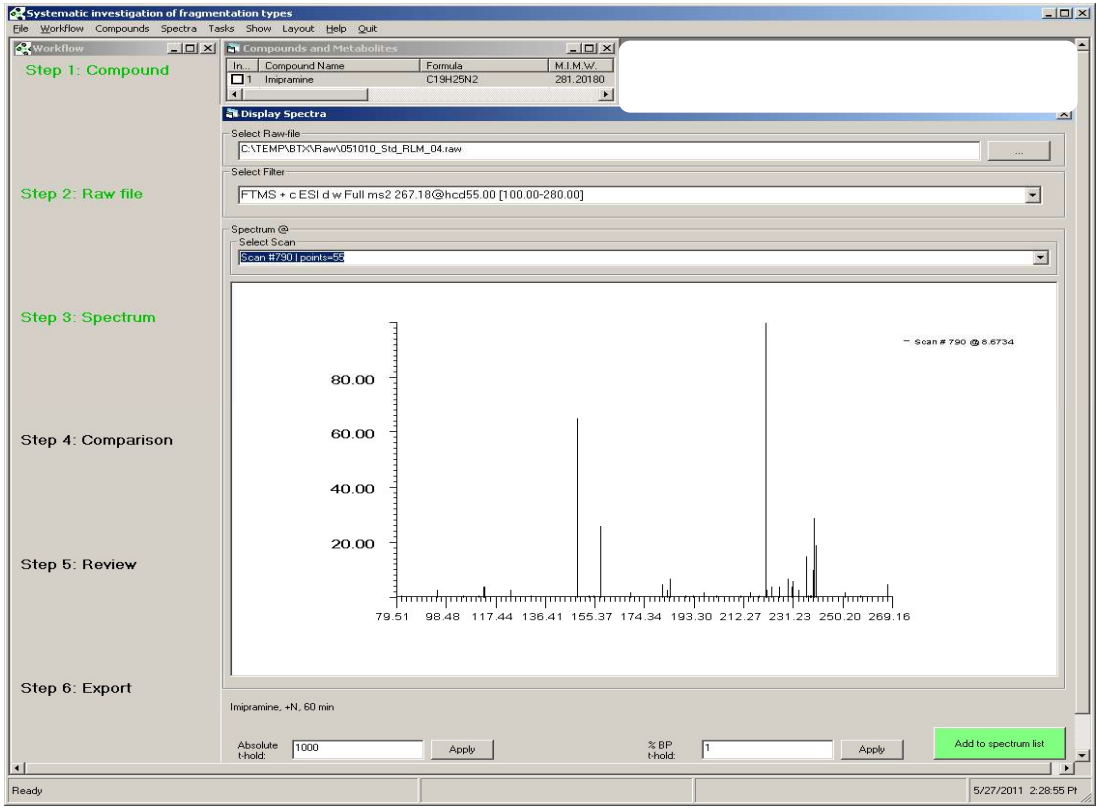
Scan 3: MS/MS event for HCD data

Scan 4: MS/MS event for PQD data

MS/MS parameters

MS/MS	Collision Energy (CE) Optimization	Collision Energy (%)	Activation, Q	Activation Time(ms)
CID	Universal CE	35	0.25	30
HCD	Required	55-75	-	30
PQD	Required	40-50	0.55	0.100

Selected Ion Fragmentation Technique (SIFT) Software



- Easy compare spectra manually for 2-3 compounds
- Proper evaluation requires multiple fragmentation spectra of different chemotypes
- Custom software is needed for comparing spectra
- Help with automated structural assignment

Courtesy:Serhiy Y. Hnatyshyn

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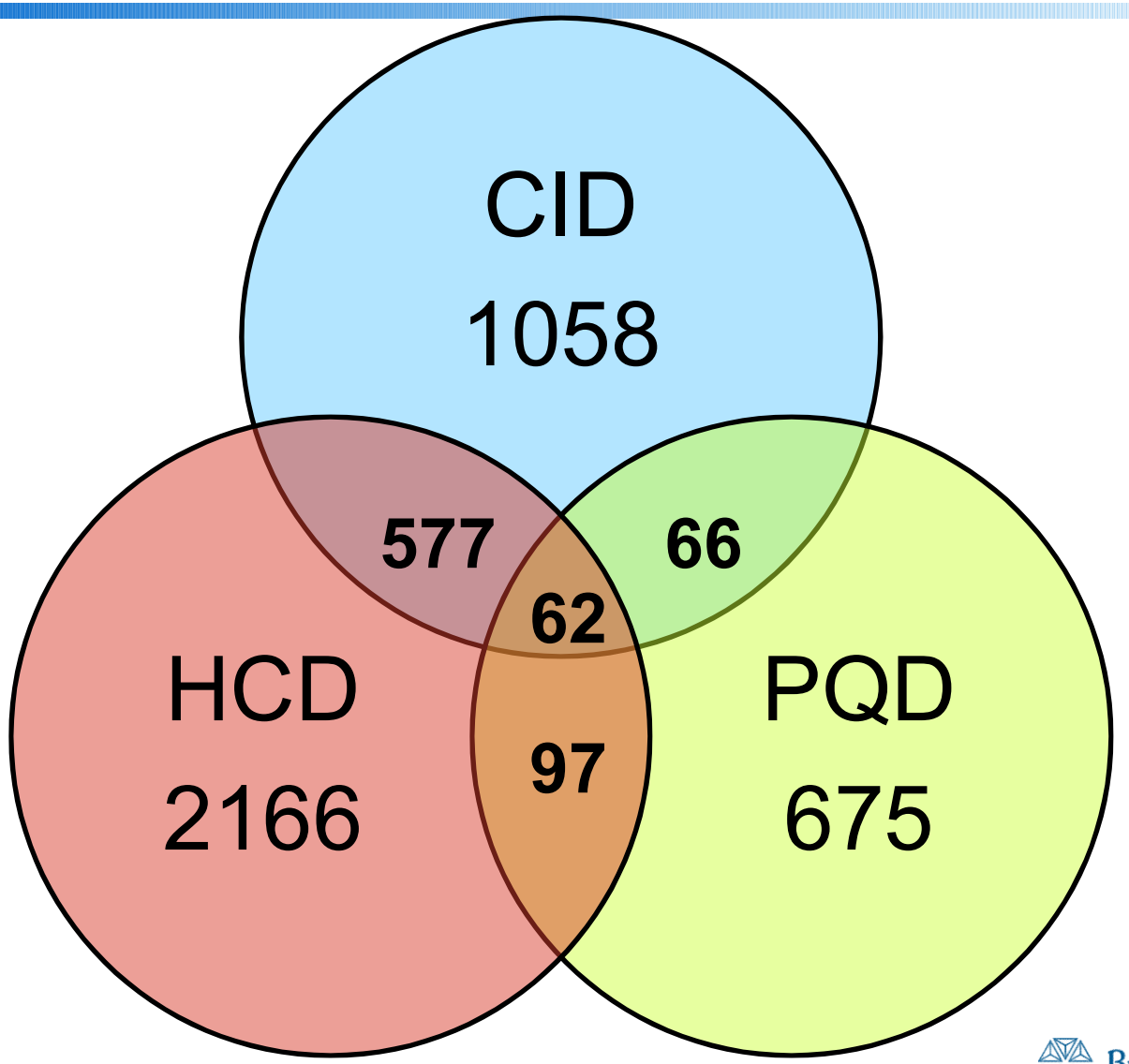
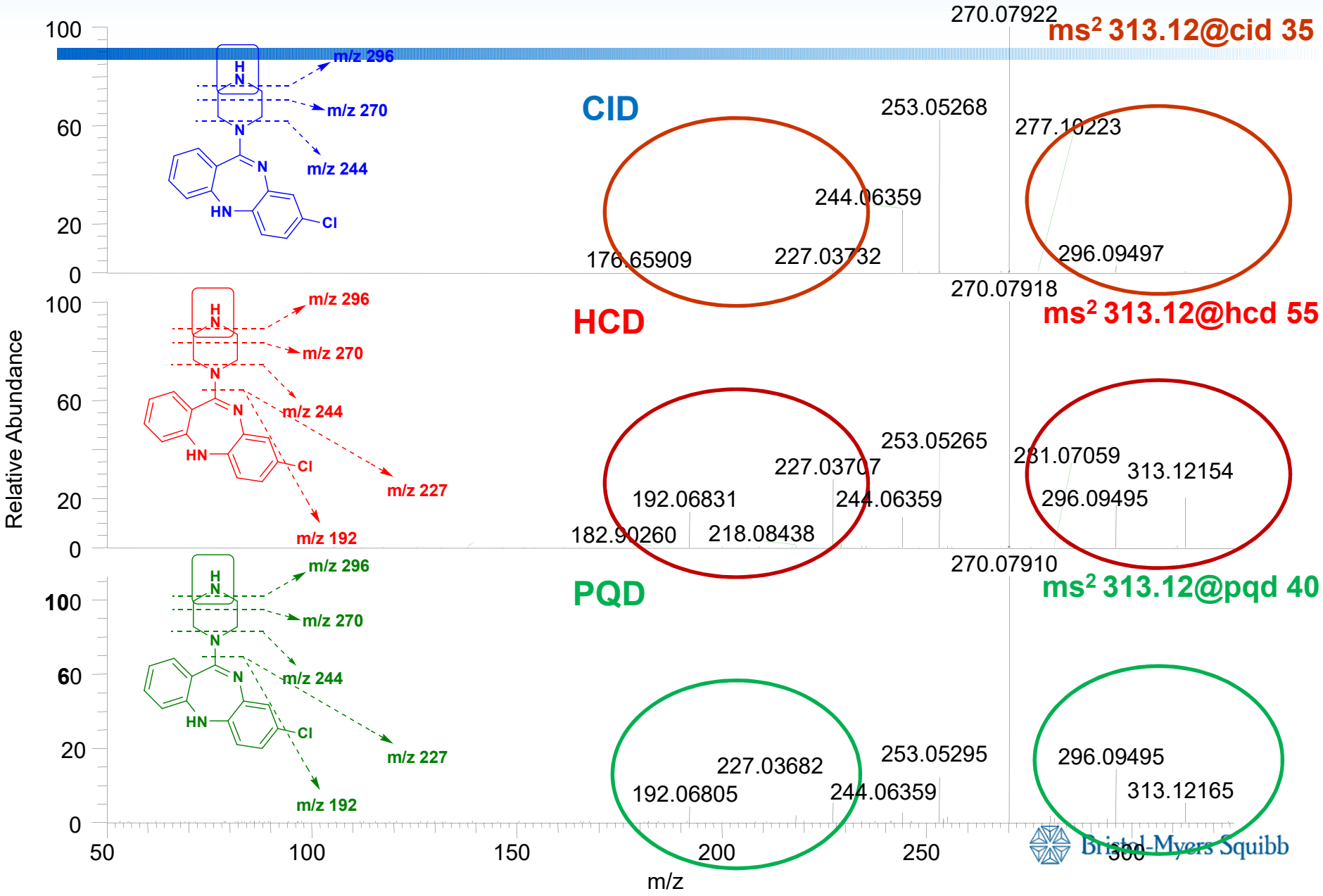
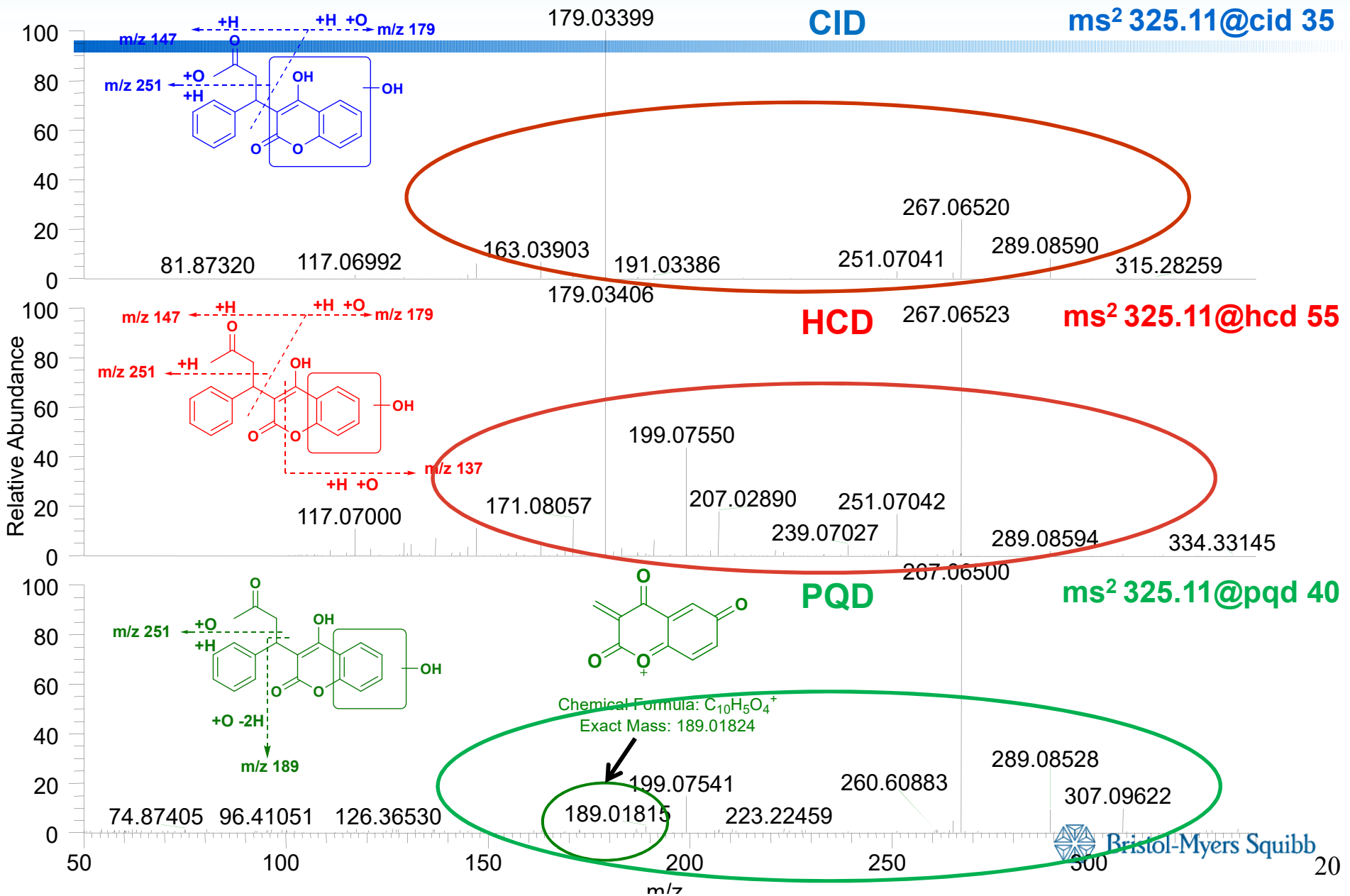
Fragment ions (parent + metabolite)



CID, HCD and PQD spectra for demethylclozapine



CID, HCD and PQD spectra for hydroxywarfarin(metabolite)



Summary for LTQ Velos Orbitrap™ Findings

- A single instrument method can be utilized to obtain all three kinds of MS/MS data -CID, HCD, PQD in a single analytical run—with no loss in data quality.

- Collision energy optimization
  - CID - Follows the "universal collision energy rule"
  - HCD - Some collision energy optimization
  - PQD - More rigorous energy optimization

- HCD and PQD are powerful tools
  - Absence of the low mass cut-off
  - Additional or unique fragments
  - Complementary to CID

- Fragmentation in the HCD cell offers a more triple quadrupole like fragmentation.

- HCD and PQD MS/MS methods
  - Alternative to MS^3 or MS^n spectra

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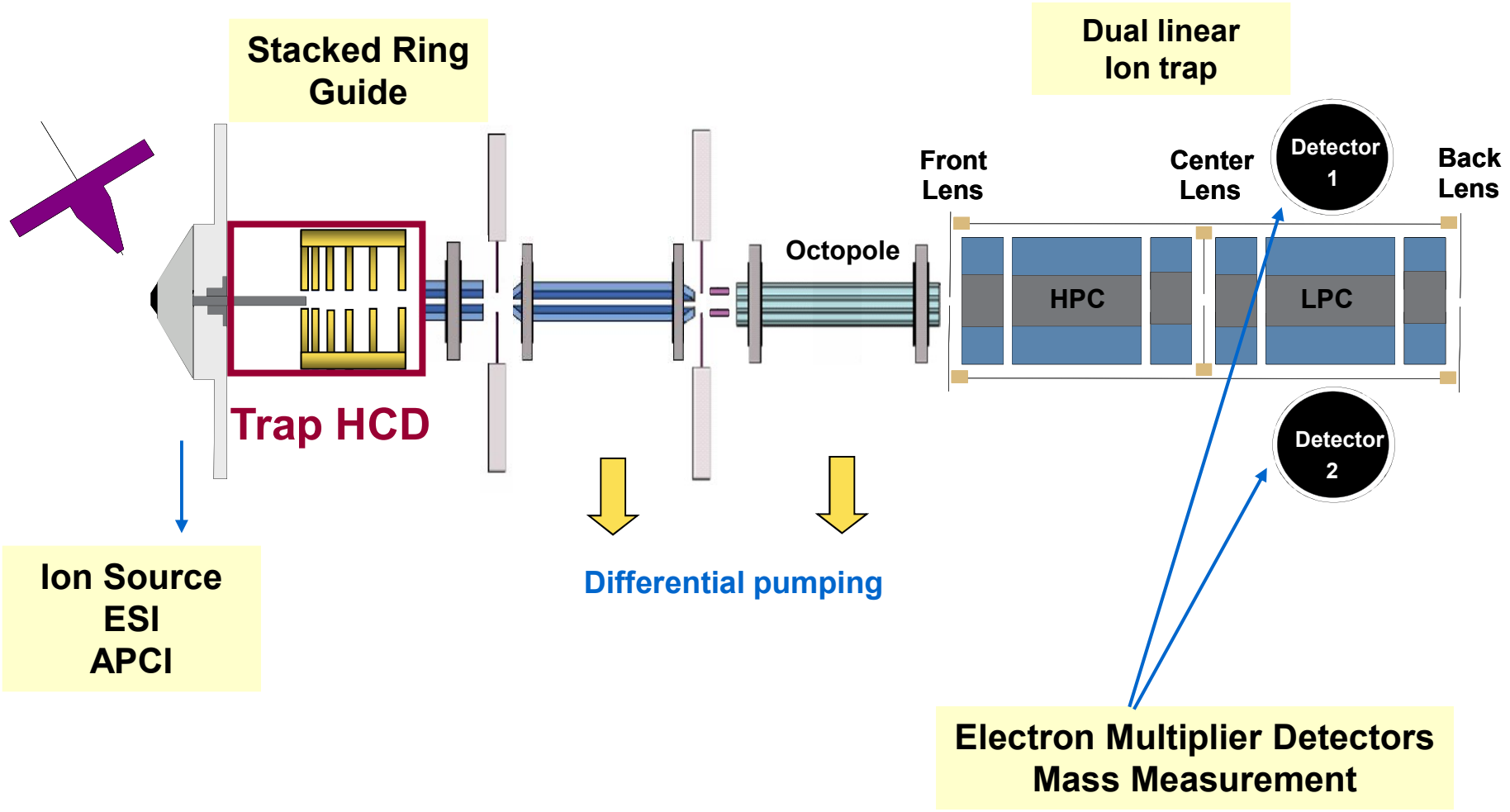
Schematic for a LTQ Velos Pro™



Compounds used for studying CID, HCD & Trap-HCD

Compound	Chemical Formula	Mass of Parent (M+H)
Coumarin	$C_9H_7O_2$	147.04
Phentermine	$C_{10}H_{16}N$	150.12
Amiloride	$C_6H_8ClN_7O$	230.05
Phenytoin	$C_{15}H_{13}N_2O_2$	253.09
Desipramine	$C_{18}H_{23}N_2$	267.18
Sulfamoxole	$C_{11}H_{14}N_3O_3S$	268.07
Imipramine	$C_{19}H_{25}N_2$	281.20
Metoclopramide	$C_{14}H_{23}ClN_3O_2$	300.14
Cinchocaine	$C_{20}H_{30}N_3O_2$	344.23
Methysergide	$C_{21}H_{28}N_3O_2$	354.21
Mefruside	$C_{13}H_{20}ClN_2O_5S_2$	383.04
Pantoprazole	$C_{16}H_{16}F_2N_3O_4S$	384.08
Buspirone	$C_{21}H_{32}N_5O_2$	386.22
Dixyrazine	$C_{24}H_{34}N_3O_2S$	428.23
Tetracycline	$C_{22}H_{25}N_2O_8$	445.16
Verapamil	$C_{27}H_{37}N_2O_4$	455.29
Nefazodone	$C_{25}H_{33}ClN_5O_2$	470.23
Dihydroergotamine	$C_{33}H_{38}N_5O_5$	584.28
Reserpine	$C_{33}H_{41}N_2O_9$	609.28
Erythromycin	$C_{37}H_{68}NO_{13}$	734.47

Concentration of standard: 15 uM

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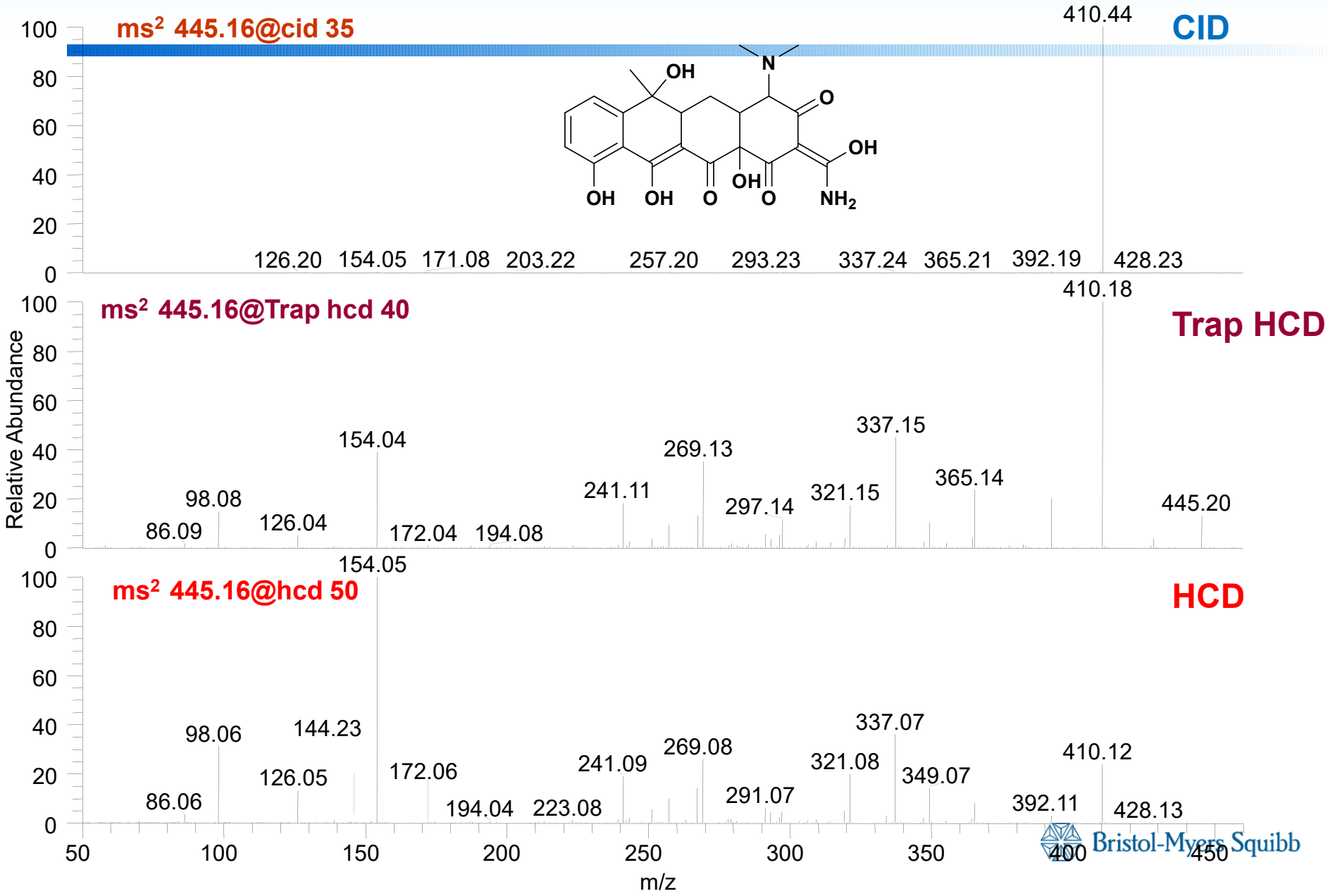
CID, Trap HCD and HCD spectra for tetracycline



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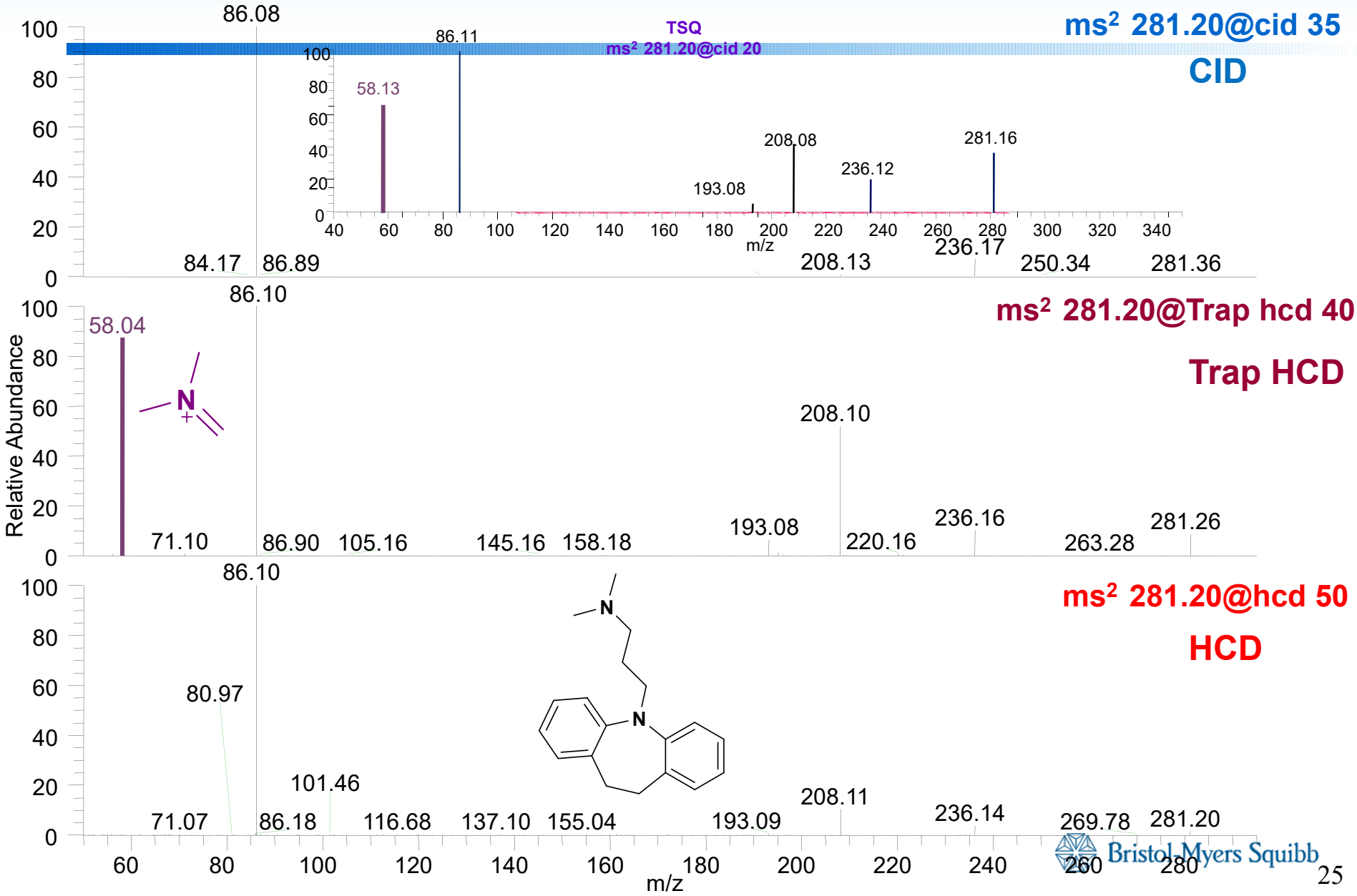
CID, Trap HCD and HCD spectra for imipramine



Summary for LTQ Velos Pro™ Findings

- Two kinds of MS/MS data – CID and Trap-HCD can be obtained in a single analytical run in a data dependent fashion with no loss of data quality.

- HCD and Trap-HCD spectra are very similar. Trap-HCD also displays triple quadrupole like fragmentation.

- HCD and Trap-HCD spectra
  - Absence of a precursor dependent low mass cut-off
  - Generate additional or different fragments as compared to CID
  - Provides important structural information complementary to CID

Software for Metabolite ID

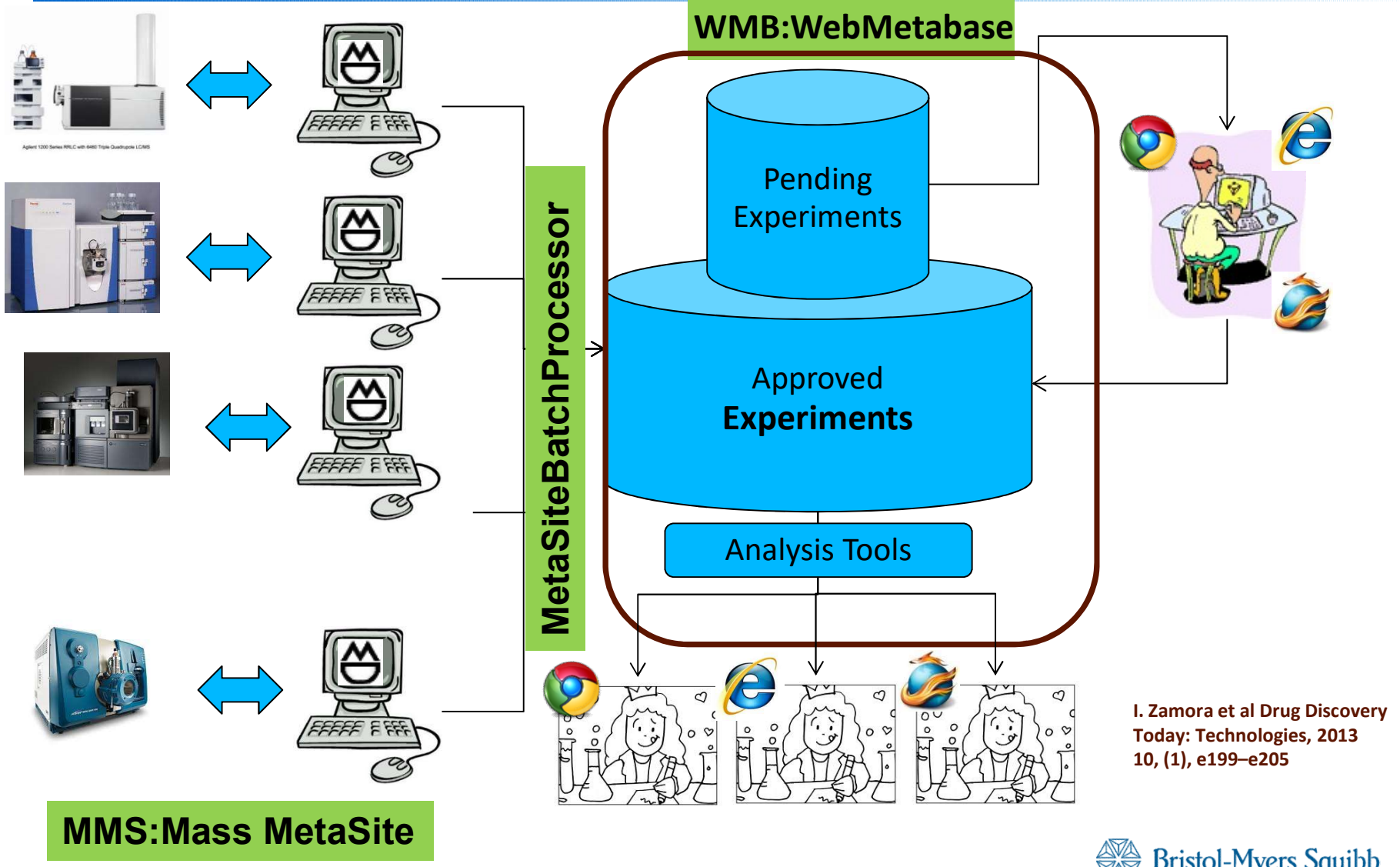
Acquisition Software

- Prior to analysis
  - "Expected" metabolite mass lists
- Data dependent acquisition (DDA)

Post Acquisition Software

- Allows manipulation of known/obvious components
  - Assignment of empirical formula
  - Assignment of structure
  - Quantification of metabolites
- Data mining
  - Detection of minor components that otherwise would be missed
  - Cleans spectra to "pull them out of the dirt"

Mass MetaSite(MMS) & Web Metabase(WMB)



I. Zamora et al Drug Discovery Today: Technologies, 2013 10, (1), e199–e205

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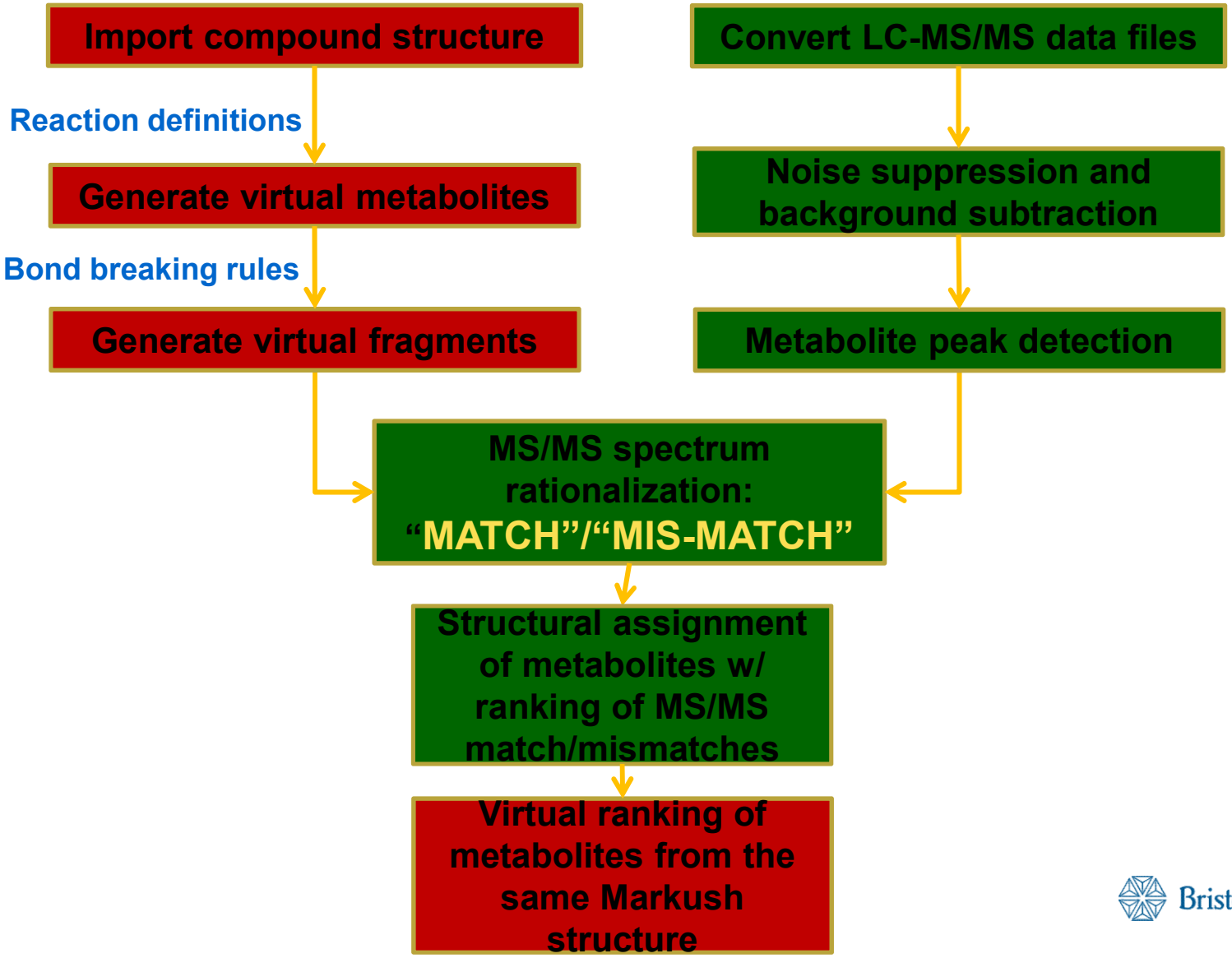
Mass-MetaSite Processing Algorithm



Mass MetaSite

Drug Related Material process

Algorithms

- **Noise suppression**
- **Background subtraction**
- **Isotope pattern analysis**
- **MS/MS Fragmentation**
- **Mass Defect**
- **Retention time analysis**

Expected mass shift based on a set of reactions that are selectable by the user.

Color peak assignment

Background comparison for UV

Additional analysis for GSH

Mass-MetaSite

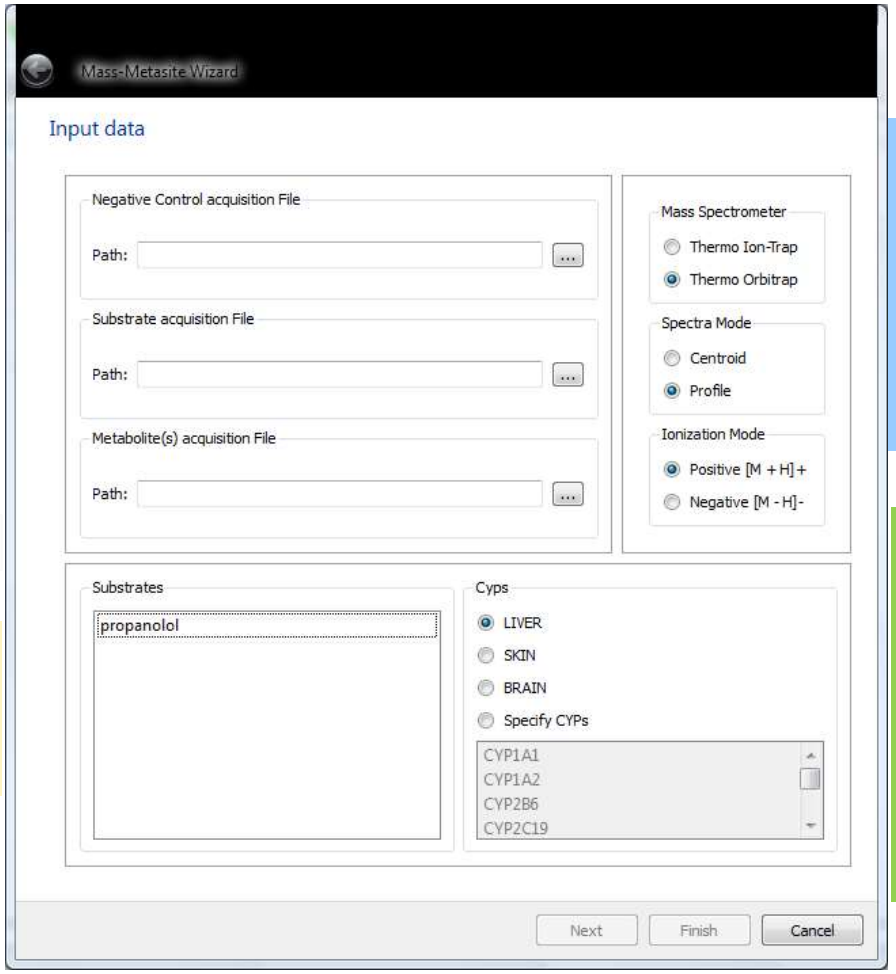
MetaSite LC-MS Methodology: Data Dependent Scan

Input Mass Spec data -List Driven MS acq

Spectra Mode (optional)
- Centroid
- Profile

Will Centroid if profile

Ionization Mode
- Positive
- Negative



3 MS Files as input:
- Blank File (Matrix+/-co-factors)
- Substrate File(T=0)
- Incubation file

Mass Spectometer
- Thermo ion-trap
- Thermo Orbitrap, QE
- ABSciex Triple-TOF
- Agilent Q-TOF
- Bruker Q-TOF
- Waters Q-TOF

Signal and scan filtering

Comparison of manual integration *vs.* MMS

Nefazadone: Phase 1 metabolites

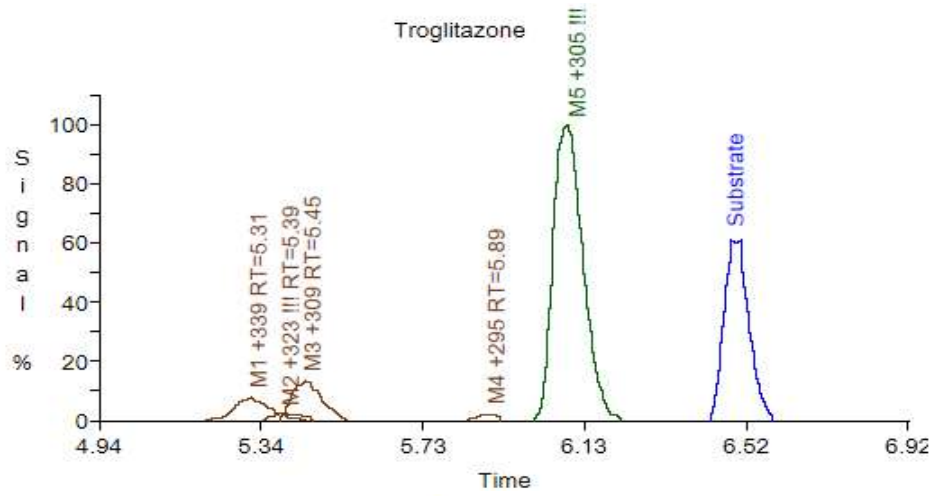
PEAK LIST
20151218_Nefazodone_HLM_Qual_04.raw
RT: 1.78 - 8.53
Number of detected peaks: 14

Top 6 metabolites matched!

Apex RT	Start RT	End RT	Area	%Area(manual)	m/z	MMS, RT(min)	MMS(Area)	MMS(met)
2.16	2.09	**2.23**	**15155.78**	**21.25**	**213(-257)**	**2.19**	**18.48**	**-257**
3.71	3.69	**3.79**	**6295.421**	**8.83**	**360(-110)**	**3.71**	**10.17**	**-110**
3.85	3.83	**3.93**	**4550.283**	**6.38**	**374(-96)**	**3.88**	**3.96**	**-96**
4.73	4.69	**4.81**	**10657.17**	**14.94**	**502(+32)**	**4.76**	**11.10**	**+32**
5.25	5.2	**5.32**	**7352.332**	**10.31**	**486(+16)**	**5.27**	**8.73**	**+16**
5.48	5.45	5.51	2042.288	2.86	486(+16)			
5.54	5.54	**5.59**	**3399.045**	**4.77**	**486(+16)**	**5.59**	**4.59**	**+16**
5.71	5.66	5.76	8761.728	12.28	486/502(small)(+16/+32))			
6.04	6.02	6.08	1939.359	2.72	470(P)	**6.07**	**3.46**	**P**
6.12	6.09	6.13	771.561	1.08	484(+14)			
6.19	6.17	6.2	362.69	0.51	486(+16)	**6.21**	**5.46**	**+16**
6.27	6.23	6.33	2869.616	4.02	486(+16)			
6.44	6.38	6.5	1807.748	2.53	486(+16)			
7.47	7.43	7.57	5364.388	7.52	235, 271, 616			

Troglitazone:5 GSH adducts detected

Troglitazone-GSH adducts: m/z's (737, 781, 747, 751, 765)



Name	RT	m/z	m/z diff (ppm)	Area ABS	Area %	ion formula	Max score
M1 +339 RT=5.31	5.31	781.2430	-1.41	2.65E07	4.76	$[C_{34}H_{44}N_4O_{13}S_2 + H]^+$	130.9
M2 +323 !!! RT=5.39	5.39	765.2483	-1.66	7.08E06	1.27	$[C_{34}H_{44}N_4O_{12}S_2 + H]^+$	121.5
M3 +309 RT=5.45	5.45	751.2325	-1.53	4.04E07	7.27	$[C_{33}H_{42}N_4O_{12}S_2 + H]^+$	2.2
M4 +295 RT=5.89	5.89	737.2527	-0.78	4.59E06	0.83	$[C_{33}H_{44}N_4O_{11}S_2 + H]^+$	173.1
M5 +305 !!! RT=6.08	6.08	747.2375	-1.37	3.22E08	57.97	$[C_{34}H_{42}N_4O_{11}S_2 + H]^+$	319.2
Substrate	6.49	442.1687	-1.03	1.55E08	27.90	$[C_{24}H_{27}NO_5S + H]^+$	

Present in T=0

Detected by SAC also

Courtesy: Savannah Mason, Abbvie, CPSA 2015

Samples analyzed so far by MMS…

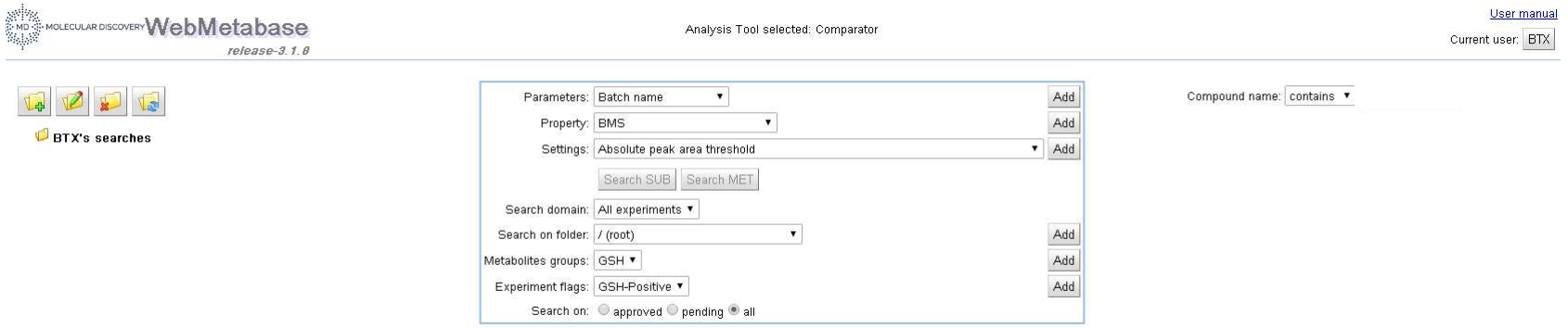
Matrix	Experiment	Compound(Comments)
Liver Microsomes	Phase 1 & 2	Low, medium, fast turnover
Hepatocytes	Suspension	Medium turnover
Hepatocytes	Long-term	Low turnover
Plasma	Profiling	Low and medium levels of metabolites
Serum	Stability	Low turnover
Urine	Profiling	Medium turnover

WMB: Analysis Tools

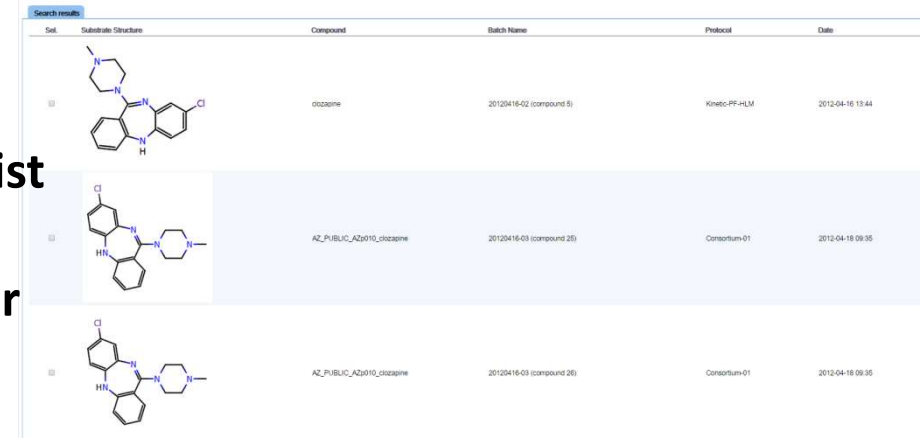
Comparator: Compares two or more MS files (multi-timepoints/cross-platform)

Comparator search result

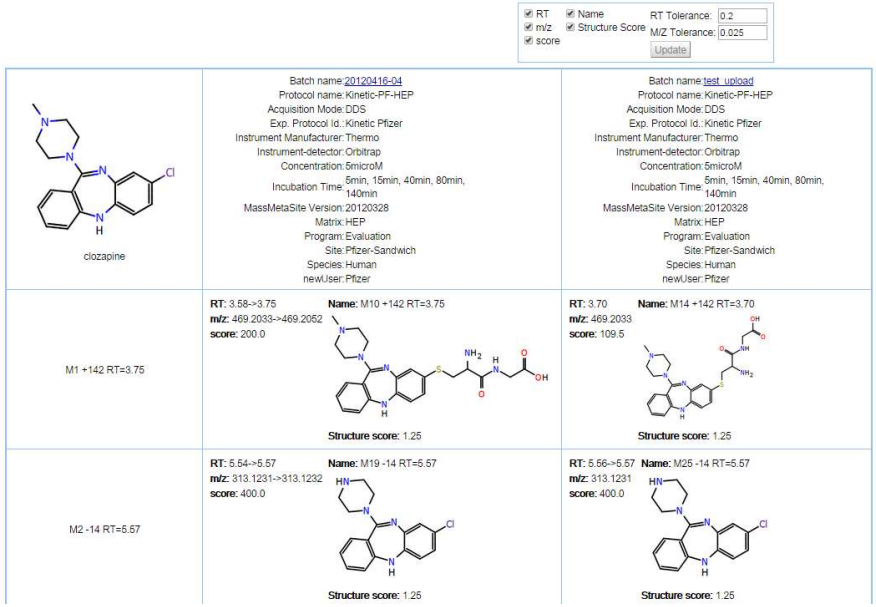
The first action in the analysis is to define the search options to select the experiments to compare: Comparator search panel



Once the compound name added and other options of the search defined a list of selected experiments is shown to allow the selection of the ones the user wants to compare.



Comparator Results

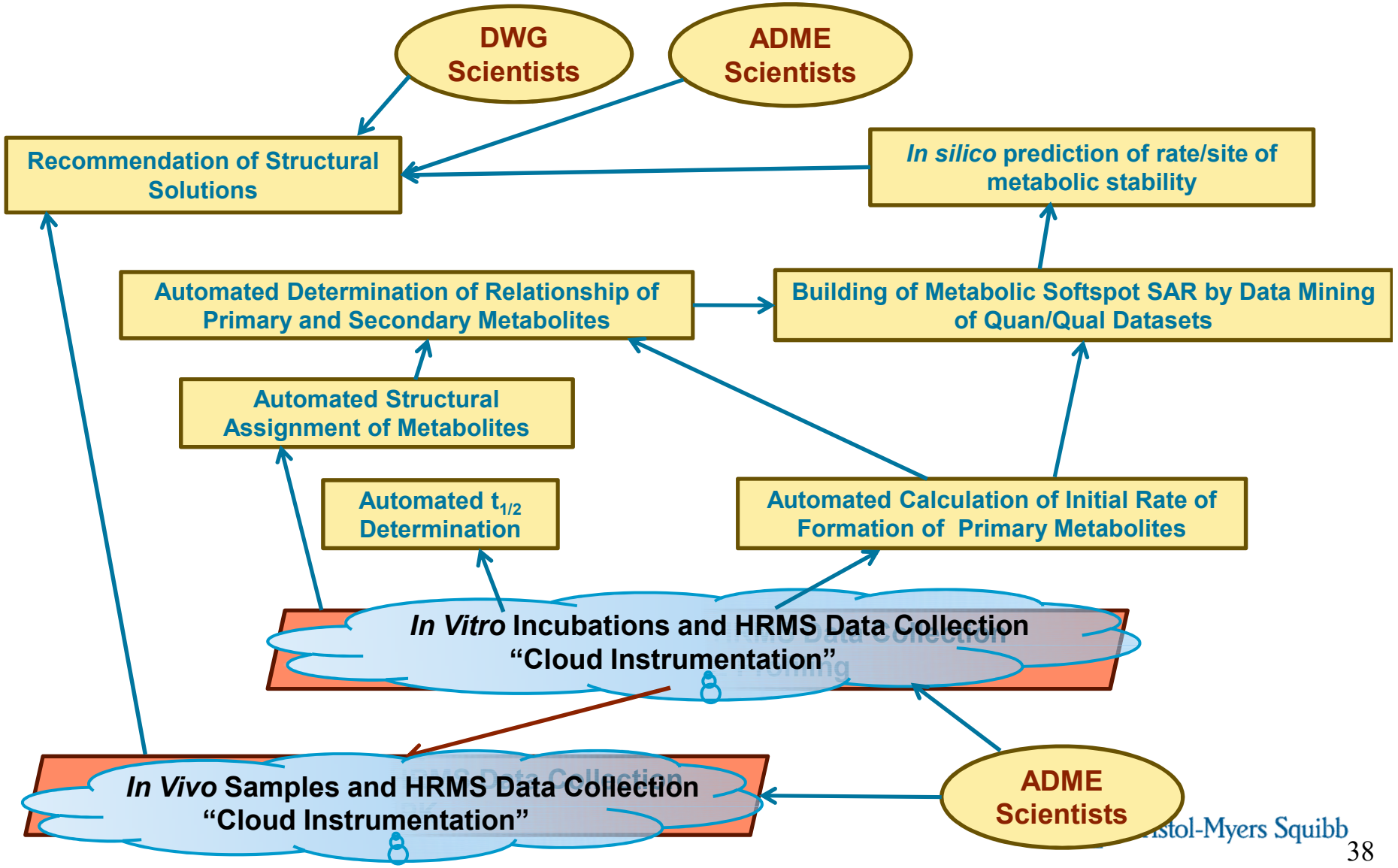


In each Metabolite row, the structures of the metabolites in each experiment are shown, and a scoring is assigned. If the structures metabolites are in total agreement, the scoring is 2 (max value), if one of the structures is included in the other because there are markuses defined then the scoring descends, and if there is no matching metabolite in the other experiments, the scoring is 0.

The result of the Comparator analysis can be saved and it will be available in the Historic ATools area of each of the experiments considered in it.

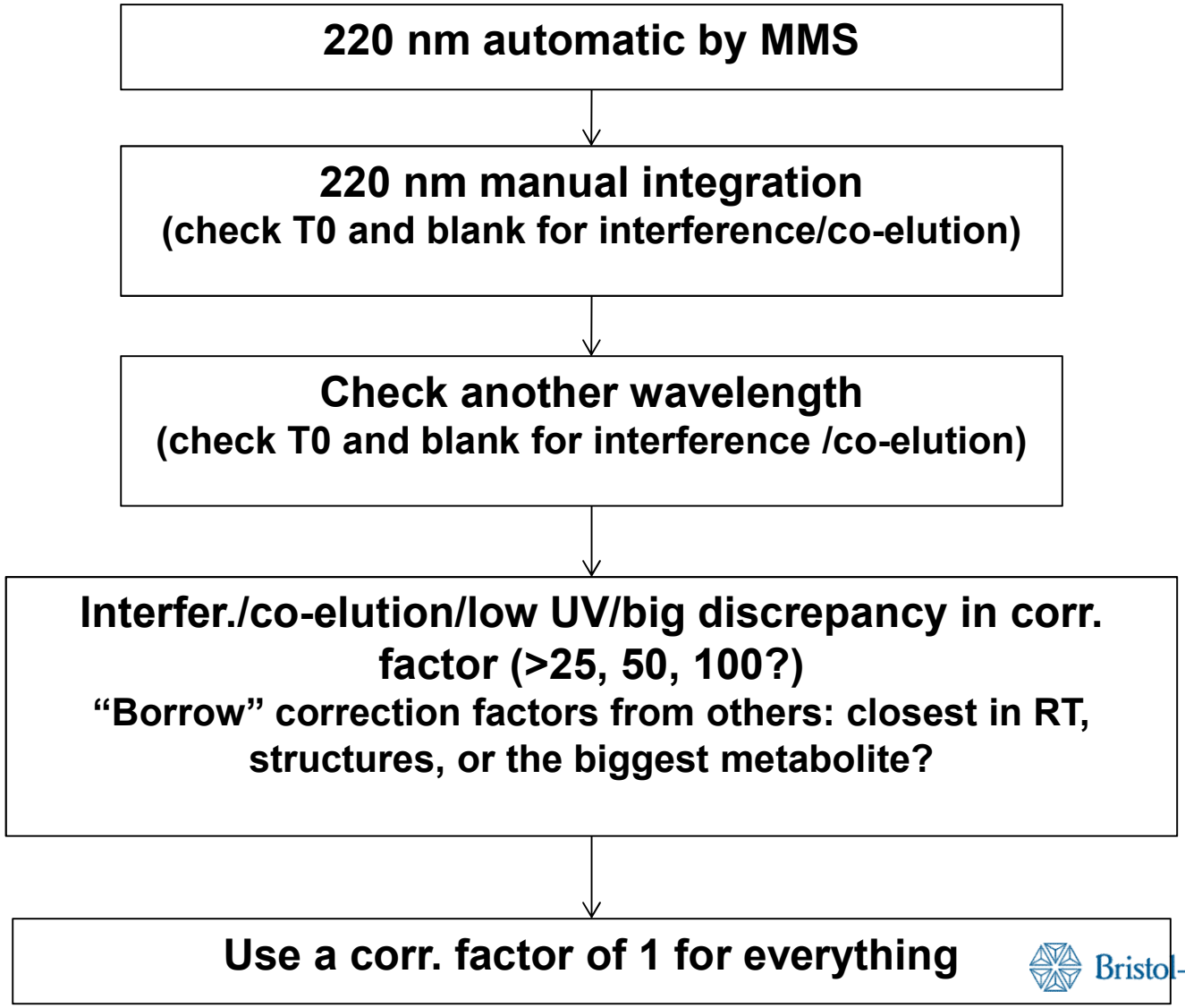
Easily searchable database for compounds, metabolites, fragmentation data…

Future Vision of HRMS Quan/Qual Integration



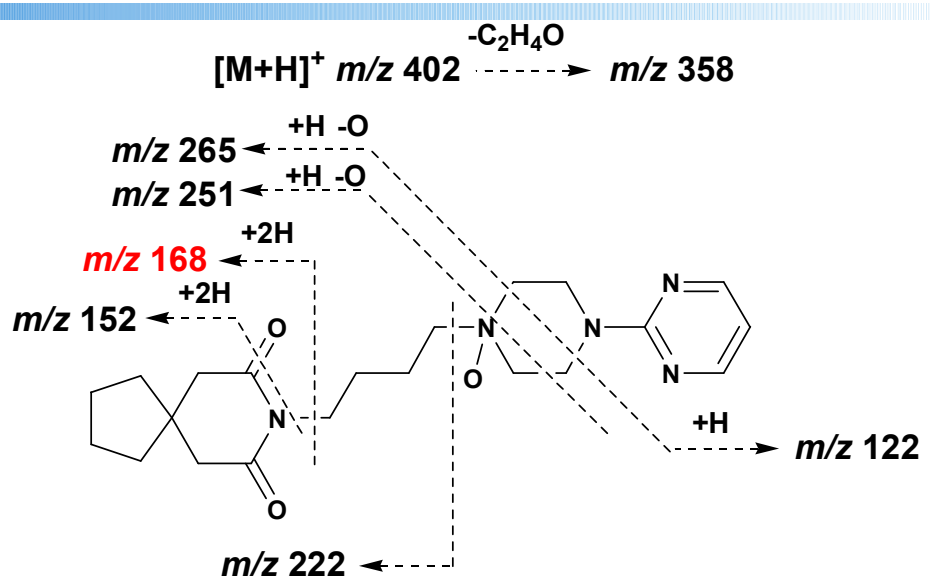
BU

Current UV Procedures



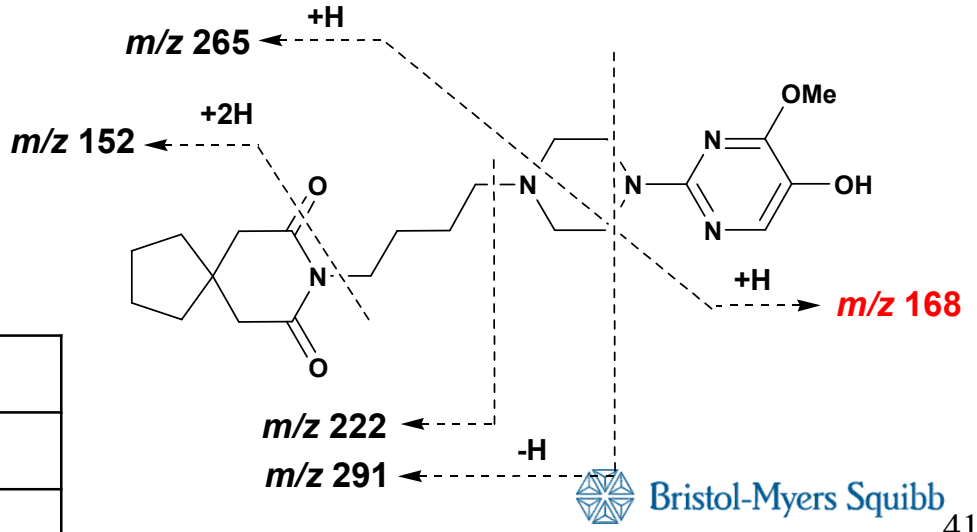
Buspirone Metabolites MS/MS Assignments
Value of accurate mass

Buspirone-N-oxide (Bus-N-Oxide)



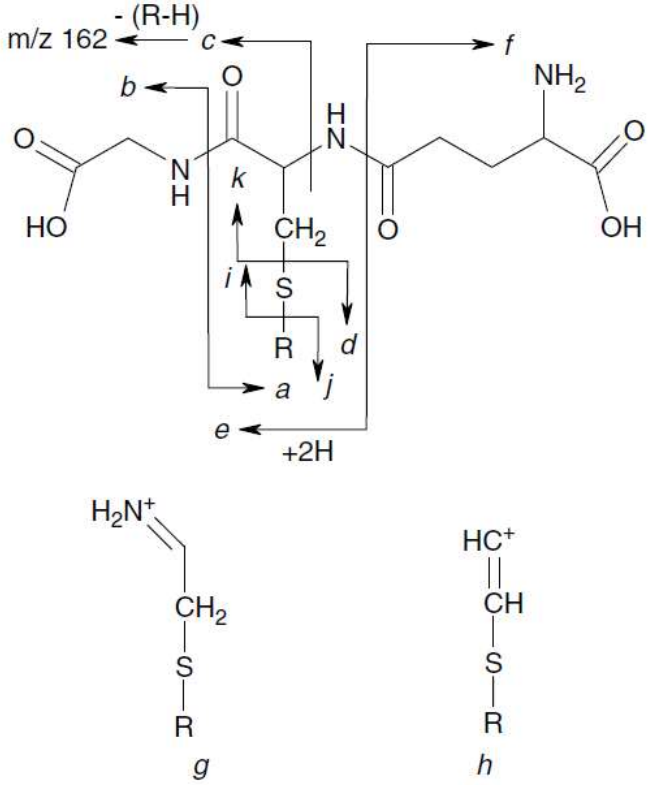
[M+H]$^+$ *m/z* 432

5-OH, 4-OMe Buspirone (5-OH, 4-OMe)



Formula	Theoretical m/z
$C_9H_{14}NO_2$	168.10191
$C_7H_{10}N_3O_2$	168.07675

Identification and detection of GSH adducts



Fragment Ion	*m/z*
a	$MH^+ - 75$
b	76
c	$MH^+ - 146$
c – (R–H)	162
c – H_2O	$MH^+ - 164$
d	$MH^+ - 273$
e	$MH^+ - 129$
e – H_2O	$MH^+ - 147$
f	130
g	$MH^+ - 232$
g – H_2O	$MH^+ - 250$
h	$MH^+ - 249$
i	308
j	$MH^+ - 305/307$
k	274

Characteristic fragment ions of glutathione conjugates using CID.

Bristol-Myers Squibb

J. Mass Spectrom. 41, 1121, 2006

Mass MetaSite

MetaSite LC-MS Methodology: Flow Chart