Metabolite Identification and Characterization

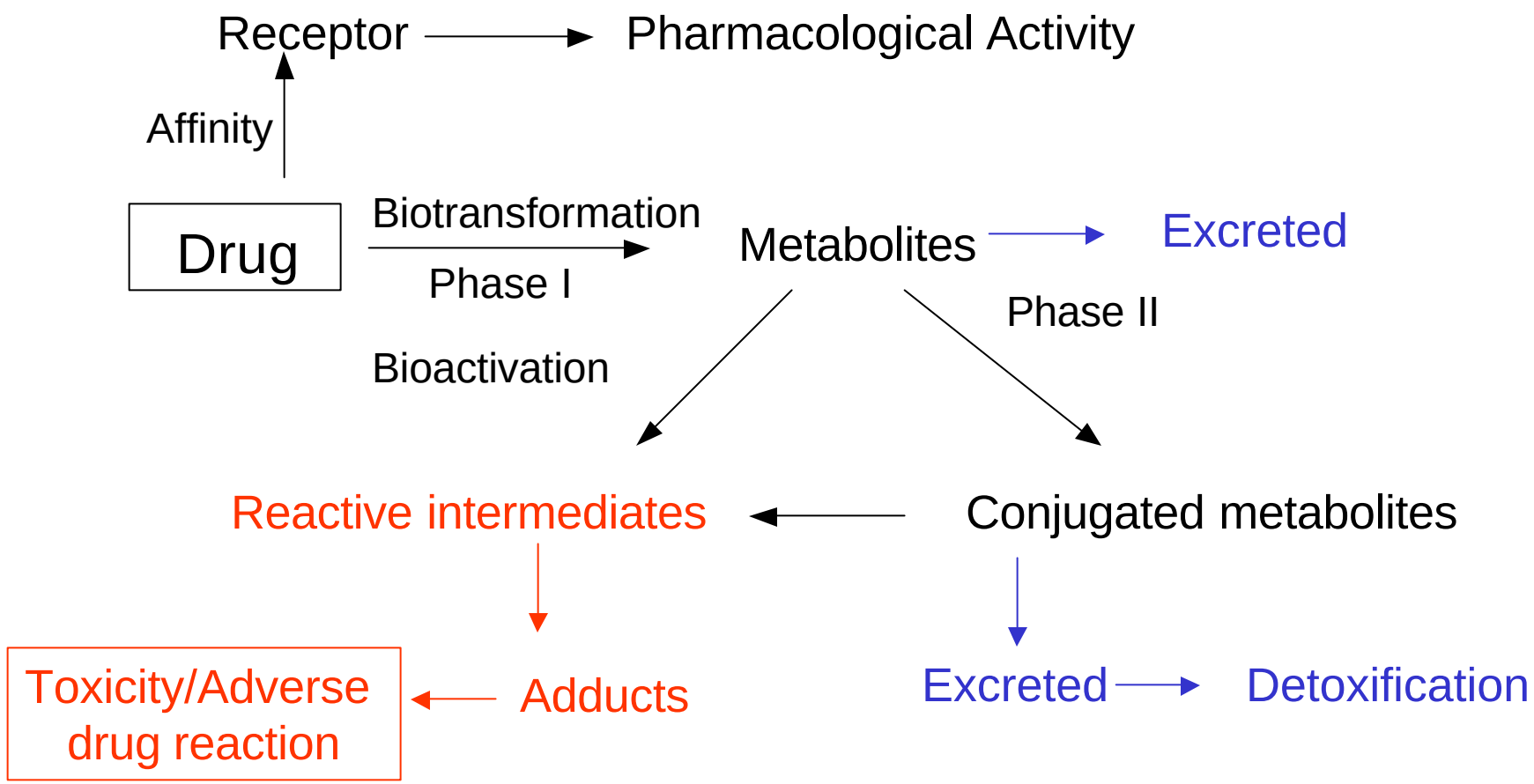
Chandra Prakash, Ph. D.

Pfizer Global Research and Development, Groton, CT

Outlines

- Introduction
- Metabolism Reactions
- LC-MS strategies for metabolite identification
  - Triple Stage Quadrupole (TSQ) LC/MS/MS
  - Ion Traps (LCQ and LTQ)
  - QTOF
- Analytical techniques combined with mass spectrometry for characterization of metabolites
  - Derivatization
  - H/D exchange
- LC-NMR

Fate of Drugs in Living Organisms



Why Identify Metabolites?

- Most of the drugs are eliminated from the body by metabolism: Detoxification process-This is good.
- The metabolites modulate the efficacy of drugs in the treatment of disease.
- The metabolites may possess pharmacological activity.
- The metabolites may be toxic: Bioactivation- bad.
- Pharmaceutical industries are mandated by regulatory agencies to identify metabolites of NCE.
- Metabolites may provide new leads.

Xenobiotic Metabolism

- Phase I (Activation/Detoxification)
  - Polar reactive groups introduced
  - products most often more polar and less lipophilic
  - more water soluble
- Phase II (Detoxification)
  - Covalent "conjugation" to endogenous substances
  - reactions most often abolish biological activity and add to polarity
  - very water soluble

Phase I Metabolism

- Hydroxylation- aliphatic, aromatic
- Epoxidation- aliphatic, aromatic
- O-, N-, S- Dealkylation
- Oxidative Deamination
- N-, S-, P- Oxidation
- Reduction
- Hydrolysis
- Aromatization

Phase II Metabolism

- Glycoside Conjugation
  - Glucuronide
  - other sugars
- Sulfate Conjugation
- Methylation (O-, S-, N-)
- Acylation
- Amino acid Conjugation
- Glutathione Conjugation

Identifying Metabolites- Prerequisite

- Knowledge of *Basic Organic Chemistry*
- Knowledge of *Drug Metabolism and Basic Metabolic Reactions*
- Knowledge of Concepts of Mass Spectrometry
- *Interpretation of Mass Spectra* for Structural Elucidation
- *Interpretation of NMR Spectra* for Structural Elucidation

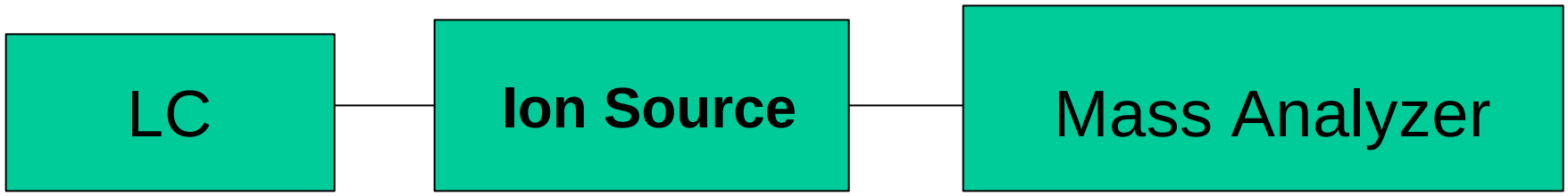
Identifying Metabolites-Basic Needs

- Need the molecular weight
- Need product ions to get structure information
- Need product ion spectrum of the parent drug and metabolites
- Use the nitrogen rule

Techniques for the identification of metabolites

- LC-API MS/MS
  - Single Stage Quadrupole (SSQ) LC/MS
  - Triple Stage Quadrupole (TSQ) LC/MS/MS
  - Ion Traps (LCQ and LTQ)
  - QTOF
- Analytical Techniques combined with MS
  - Derivatization
  - Enzymatic hydrolysis
  - H/D exchange
- LC/NMR

LC/MS



Atomospheric Pressure Chemical Ionization (APCI)

Electrospray (ESI)

Single Quadrupole

Triple Quadrupole

Ion Trap (LCQ)

Q-TOF

General Rules for Choosing Polarity of Ion Detection and pH

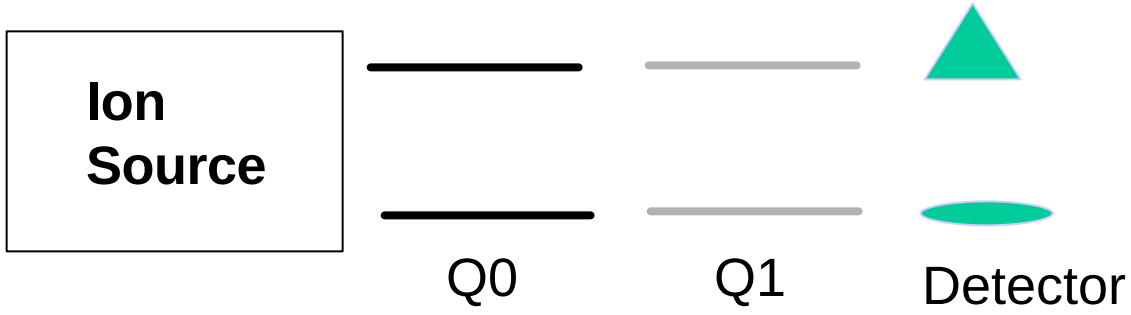
- Positive ion Detection
  - Basic samples
  - Decrease pH →
    - Acetic acid pH (3-5)
    - Formic acid pH (2-3)
    - TFA pH (1-2)
  - pH at least 2 units below pKa of samples

General Rules for Choosing Polarity of Ion Detection and pH

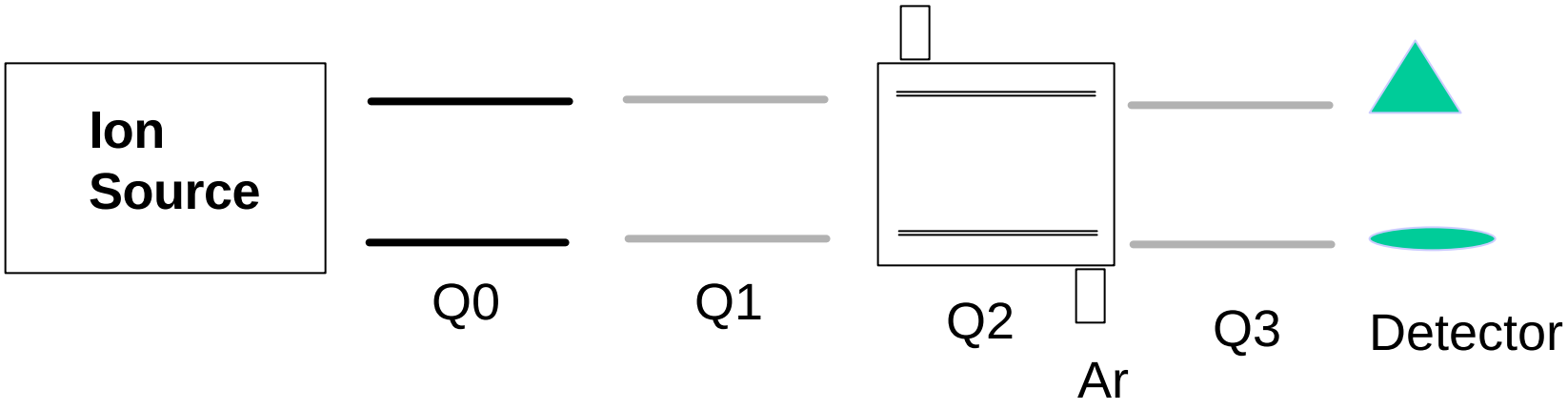
- Negative ion Detection
  - Acidic samples
  - Increase pH
    - Ammonium hydroxide
  - pH at least 2 units above pKa of samples

Quadrupole

Single stage quadrupole (SSQ)



Triple stage quadrupole (TSQ)



Advantages of a TSQ MS

- Renders selectivity due to mass separation at two stages.
- Helps to rapidly identify metabolites in matrices without purification.

MASS SPECTRUM

- Mass Spectrometers Do Not Measure Mass. It is plot of the mass-to-charge ratios (m/z) vs. the % relative intensities of the ions, where base peak is the most abundant ion in the spectrum
- If single charge, z=1 and m/z = m
- Three types of ions in a mass spectrum;
  - Intact molecule± one or more charges⇒Molecular mass
  - Fragment ions⇒Structure information
  - Background ions⇒from non-analyte species

Natural Isotopic Abundance of Common Elements

Element	Isotope Mass	%
Carbon	^{12}C	98.9
	^{13}C	1.1
Hydrogen	^{1}H	99.98
	^{2}H	0.02
Oxygen	^{16}O	99.8
	^{18}O	0.2
Nitrogen	^{14}N	99.6
	^{15}N	0.4
Chlorine	^{35}Cl	75.8
	^{37}Cl	24.2
Sulfur	^{32}S	95.3
	^{33}S	0.76
	^{34}S	4.20

Mass

Element	Nominal Mass	Average Mass	Exact Mass
C	12	12.011	12.0000
H	1	1.00797	1.0078
O	16	15.9994	15.9949
N	14	14.003	14.0031
Cl	35	35.45	34.9689
S	32	32.06	31.972

Average vs. Exact Mass

- Average mass results from occurrence of isotopes. (See below)
  - This is what we weigh
- Exact mass results from non-integer masses of sub-atomic particles.
  - This is what the Mass Spec sees
  - Deviation of exact from nominal is the "Mass Defect"

Examples (C,H,O,N compounds)

Compound	Integer	Avg. Mass	Exact Mass
Caffeine C8H10N4O2			
Xanomeline C14H23N3OS			
Ziprasidone C21H21N4OSCl			

Examples (C,H,O,N compounds)

Compound	Integer	Avg. Mass	Exact Mass
Caffeine C8H10N4O2	194	194.1785	194.0802
Xanomeline C14H23N3OS	281	281.4057	281.1556
Ziprasidone C21H21N4OSCl	412	412.9197	412.1120

Nitrogen Rule

Compound	M .W.	Mass value $[M+H]^+$ or $[M-H]^-$	$M^{+\bullet}$ (EI)
Even number of nitrogens (0, 2, 4)	Even	odd	Even
Odd number of nitrogens (1, 3, 5)	odd	Even	odd

F, W. McLafferty and F. Turecek, Interpretation of Mass Spectra, Fourth Edition (1993)

Mass Changes Associated with Phase I Metabolism

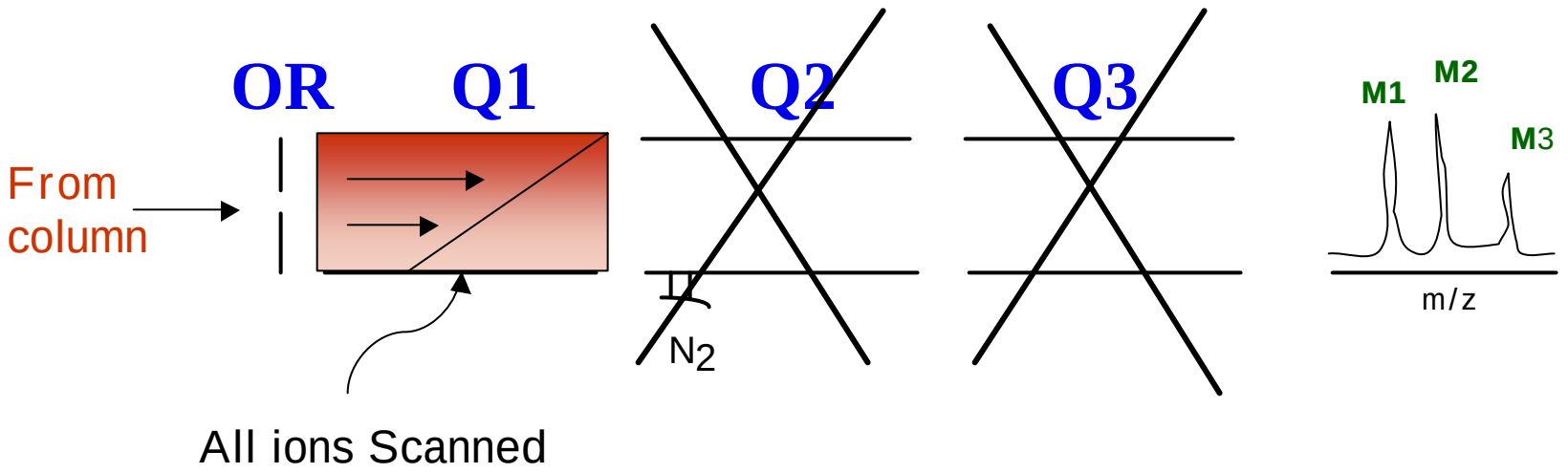
- Hydroxylation- aliphatic, aromatic
  - Lose H & add OH = (M+16)
- Epoxidation- aliphatic, aromatic
  - Lose H & add OH = (M+16)
- O-, N-, S- Dealkylation
  - Add H & lose alkyl (R) = (M+1-R)
- N-, S- Oxidation
  - Add O = (M+16)
- Reduction
  - Add 2H = (M+2)

Mass Changes Associated with Phase II Metabolism

- Glucuronide Conjugation
  - Lose H & add glucuronic acid (M-1+176) = (M+176)
- Sulfate Conjugation
  - Lose H & add HSO_3- (M-1+81) = (M+80)
- Methylation (O-, S-, N-)
  - Lose H & add CH_3– (M-1+15) = (M+14)
- N-acetylation
  - Lose H & add CH_3CO- (M-1+43) = (M+42)
- Amino acid Conjugation
  - Lose H_2O & add amino acid (M-18+R)
- Glutathione Conjugation
  - Various addition of SG-amino acid (M+306-X; X=leaving group)

LC/MS/MS Techniques for the Identification of Metabolites

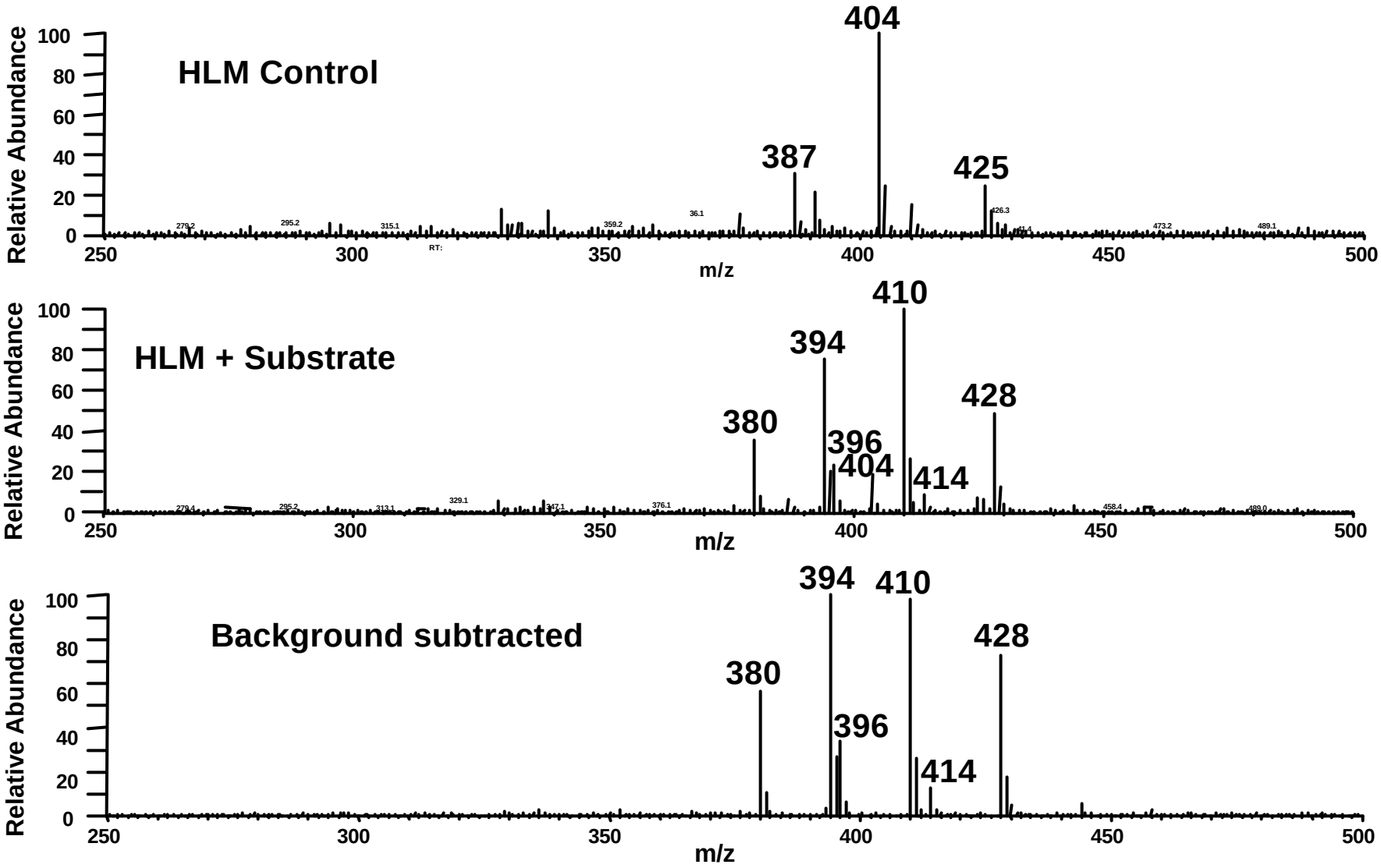
Q1 or Full Scan



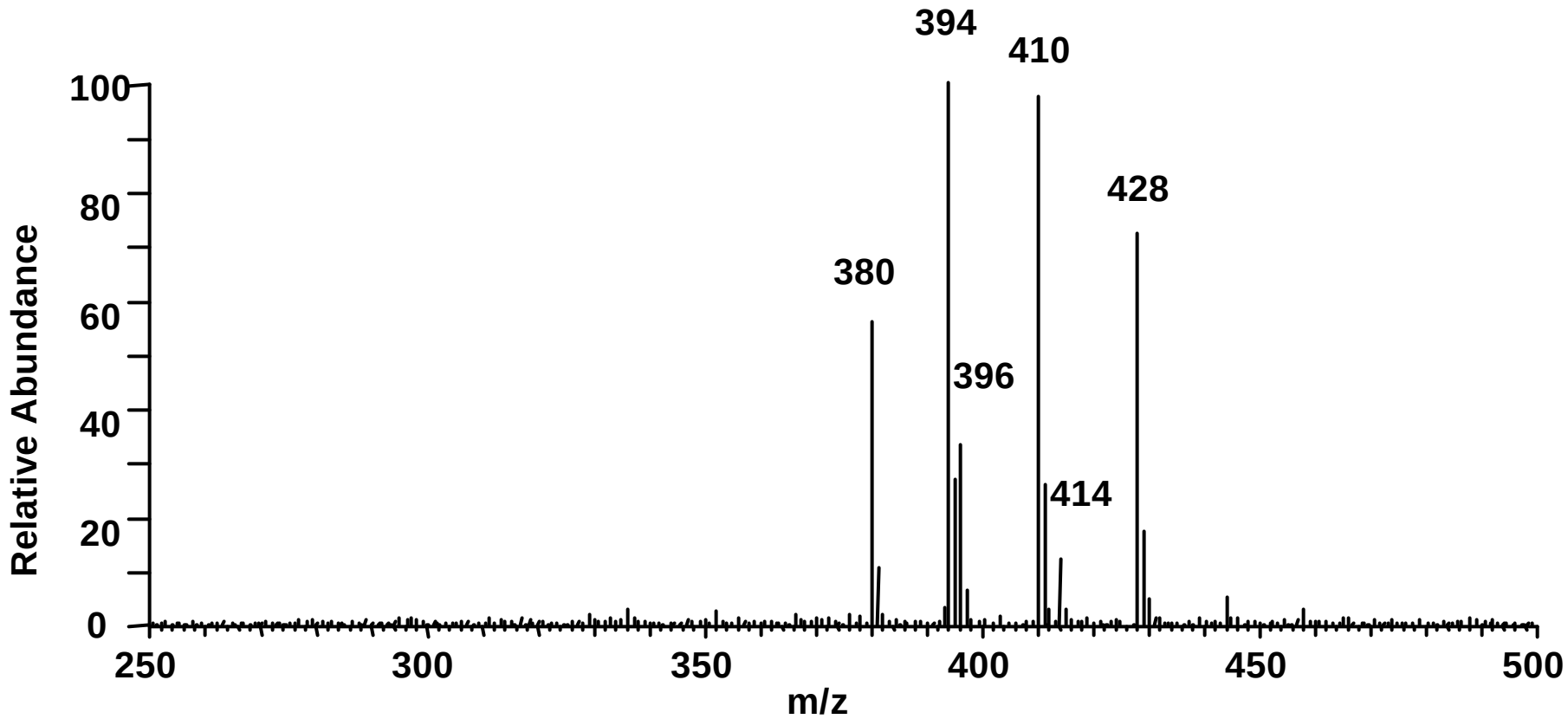
Only Q1 operational (LC/MS mode)

Similar to an LC/MS total ion chromatogram.

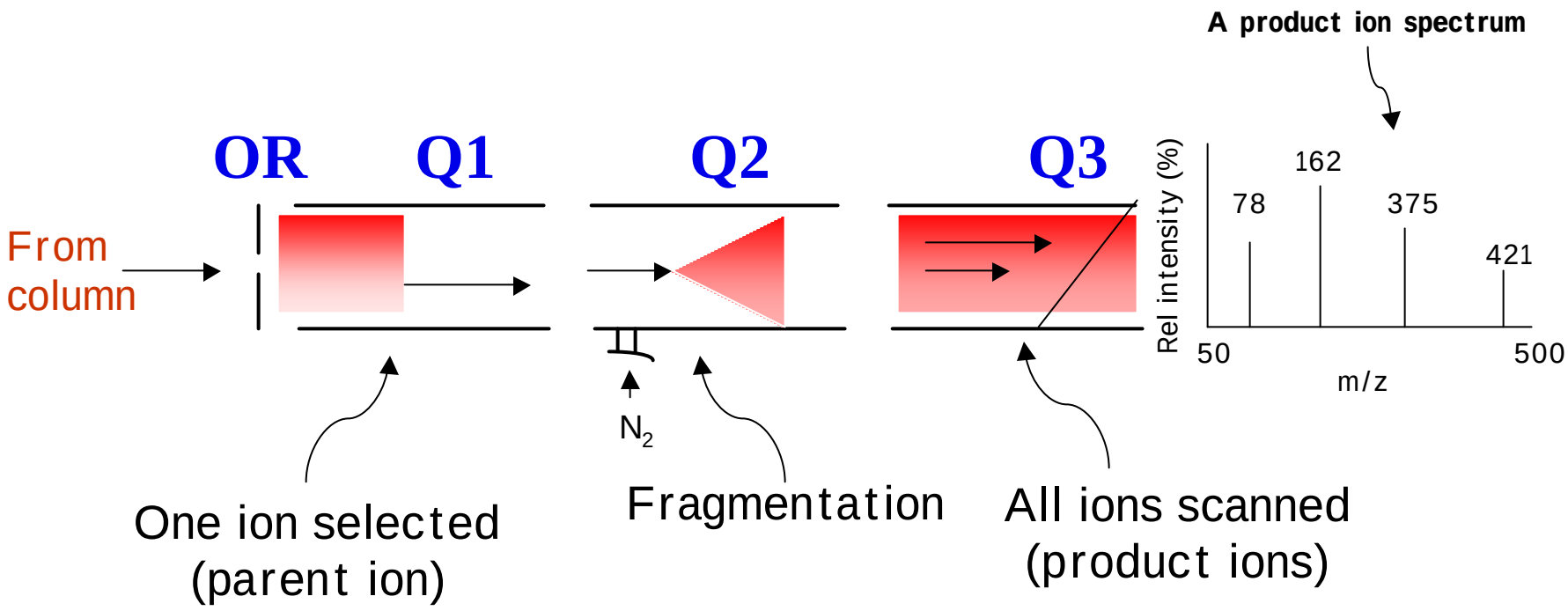
Full Scan MS of Microsomal Incubation of Compound X



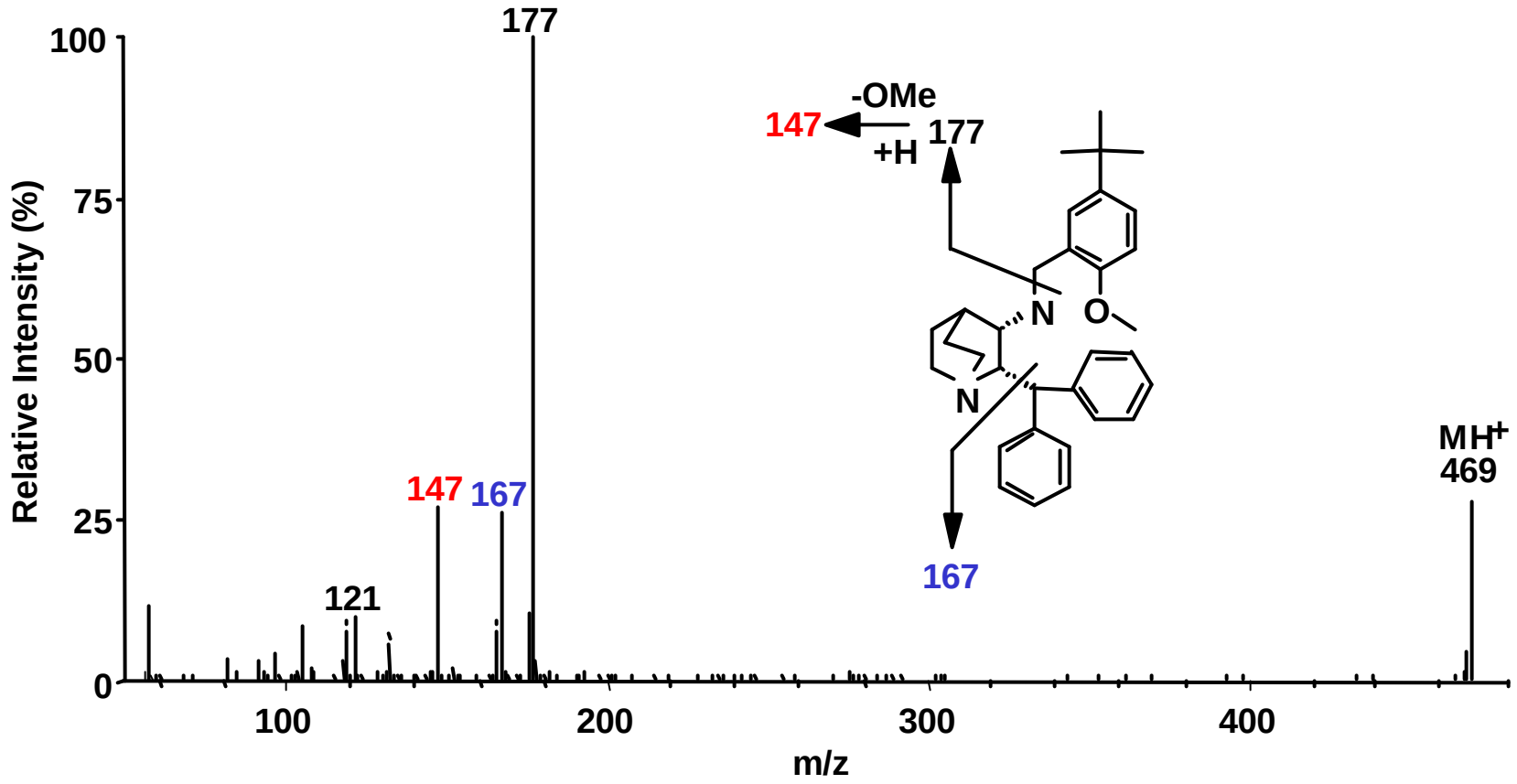
Problem Set:
Full Scan MS of Metabolites of Compound X (MW 394)
Determine possible additions of functionality of metabolites



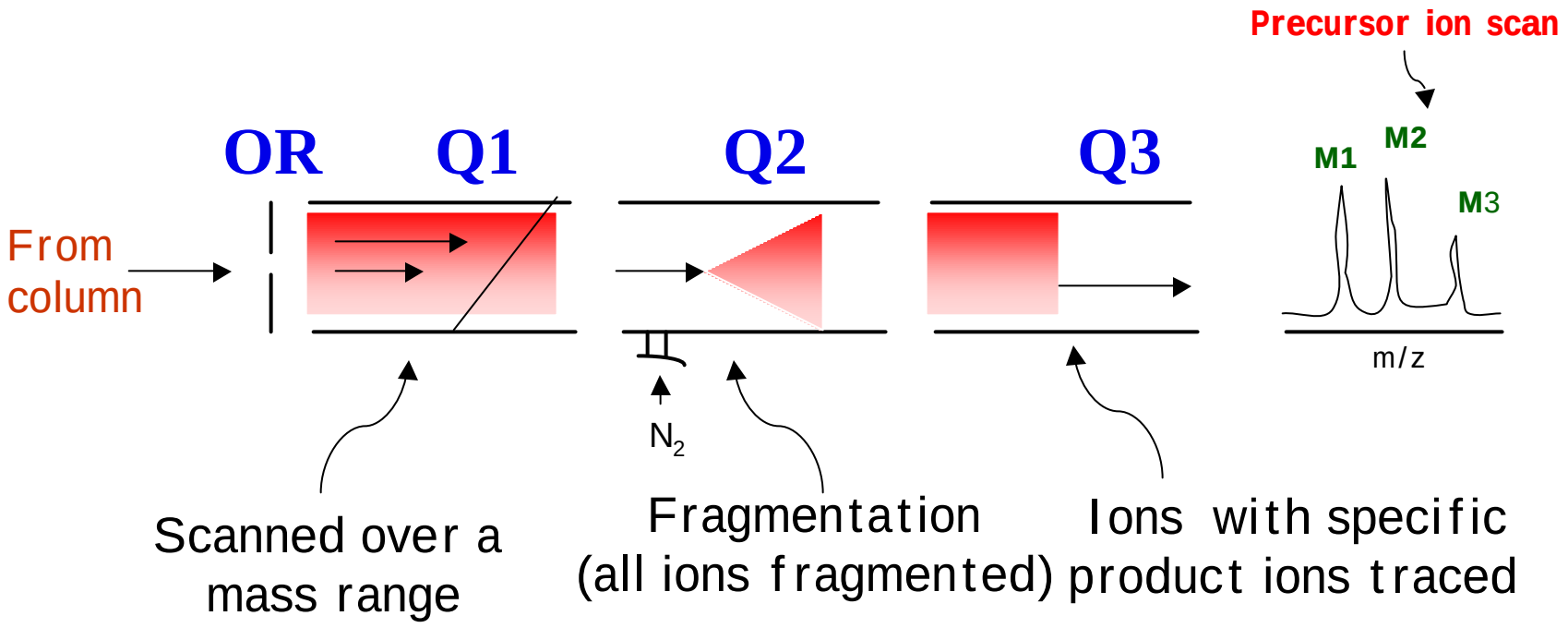
Product Ion Spectrum



CID Product Ion Spectrum of Compound Y

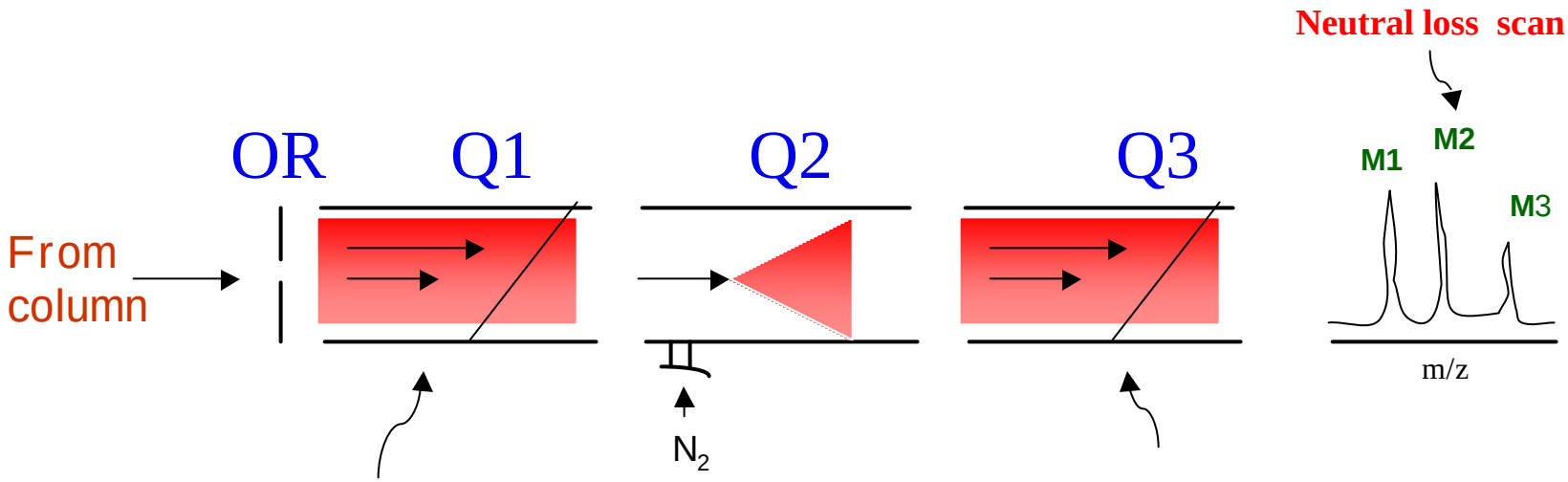


Precursor Ion Scan



Precursor ion experiment yields a spectrum of all parent ions which have the same product ion in their spectrum

Neutral Loss Scan

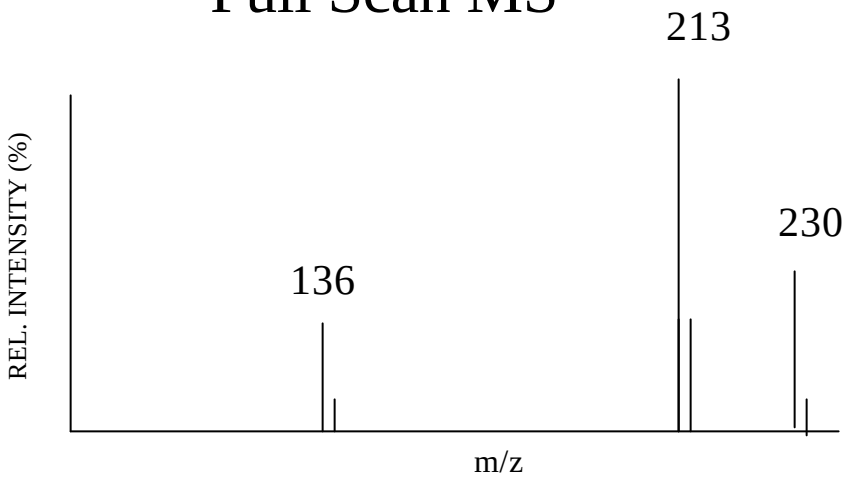


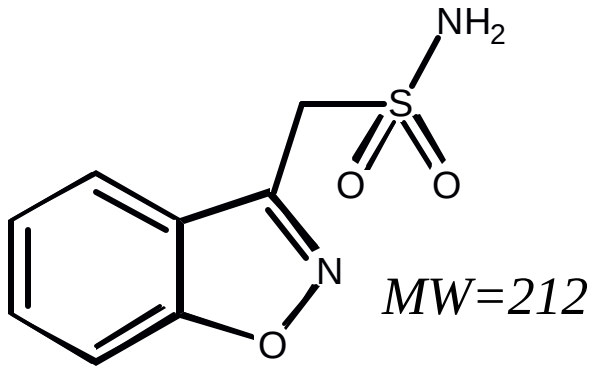
Mass offset corresponds to the mass of neutral fragment loss during fragmentation

Neutral loss experiment yields a spectrum of all parent ions which lose a selected neutral loss fragment

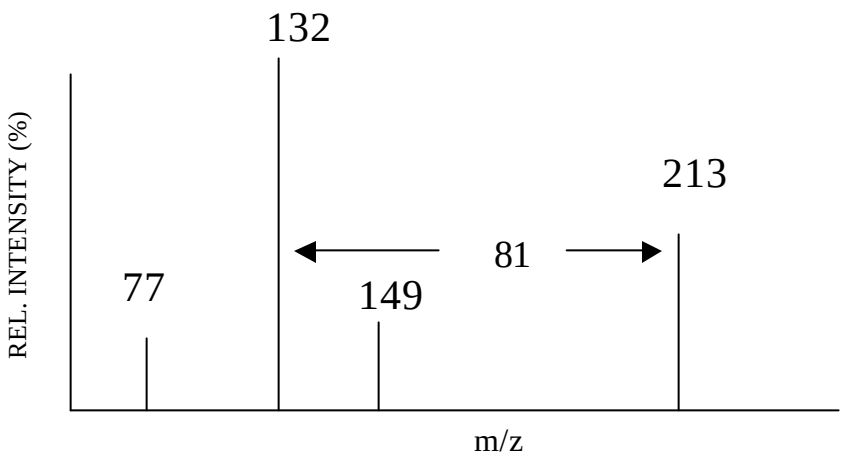
Interpreting Product Ion MS/MS Spectrum

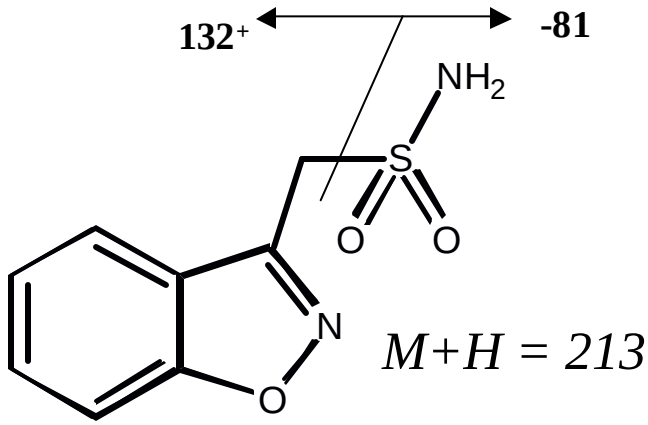
Full Scan MS





Product ion MS/MS

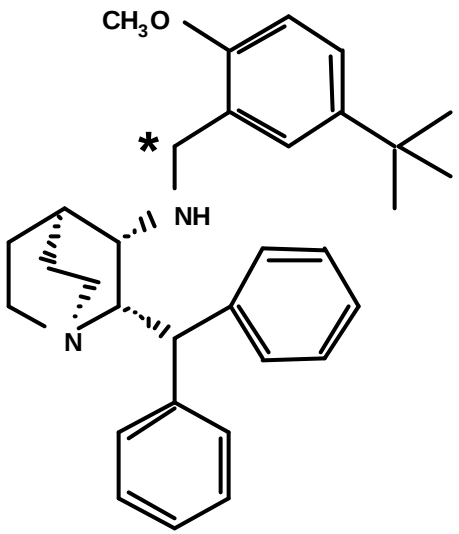




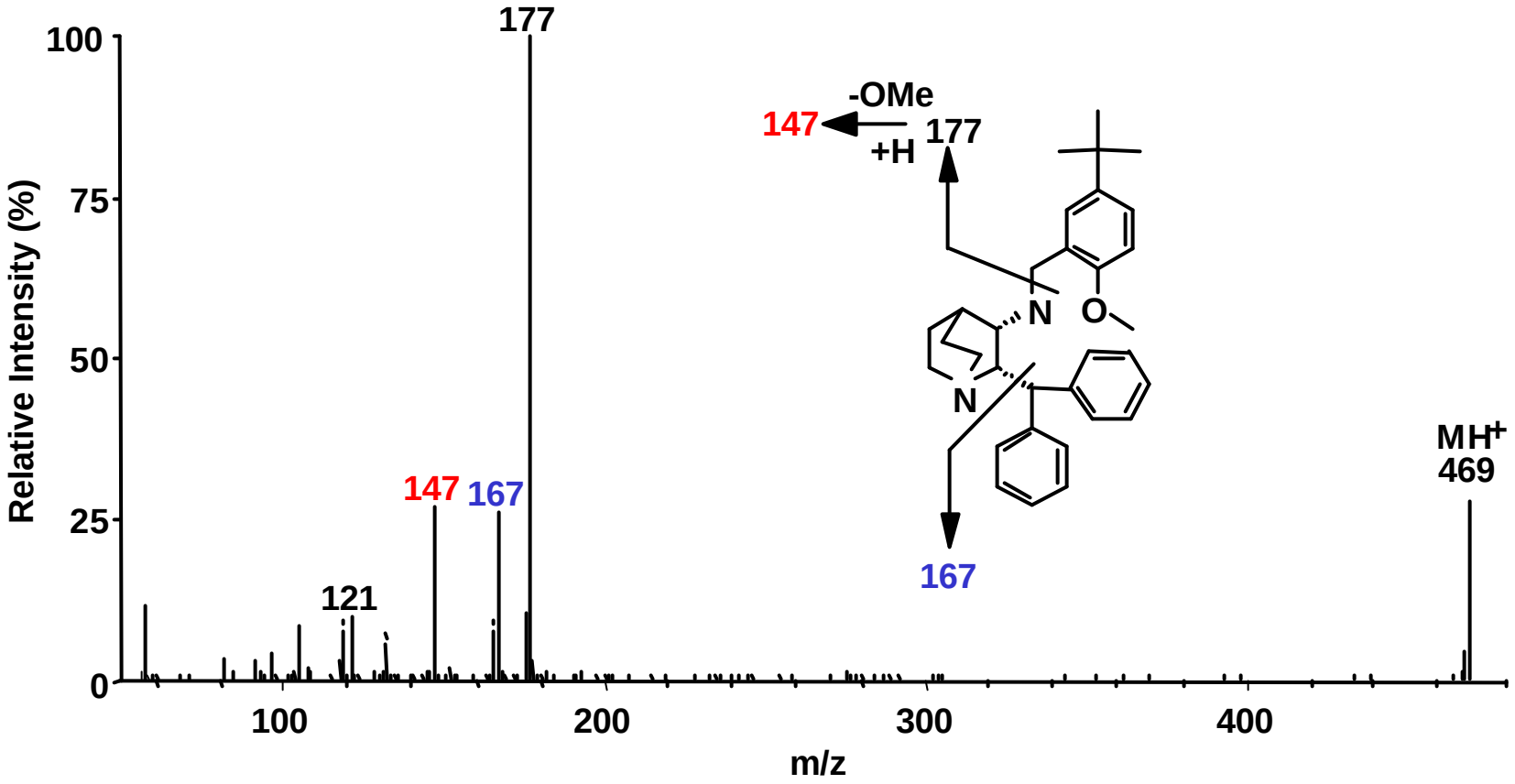
Systematic Approach for the Identification of Metabolites by LC/MS/MS

- GET A Q1 SCAN OF THE COMPOUND IN QUESTION
- OBTAIN A PRODUCT ION SPECTRUM OF THE COMPOUND: INTERPRET THE SPECTRUM
- IDENTIFY MAJOR FRAGMENT ION AND NEUTRAL LOSS
- IDENTIFY MAJOR FRAGMENT ION AND NEUTRAL LOSS
- RUN PRODUCT ION SCANS FOR ALL POSSIBLE METABOLITES IDENTIFIED FROM STEP 4 PLUS EXPECTED METABOLITES
- INTERPRET THE SPECTRA AND ASSIGN STRUCTURES OF METABOLITES

Example



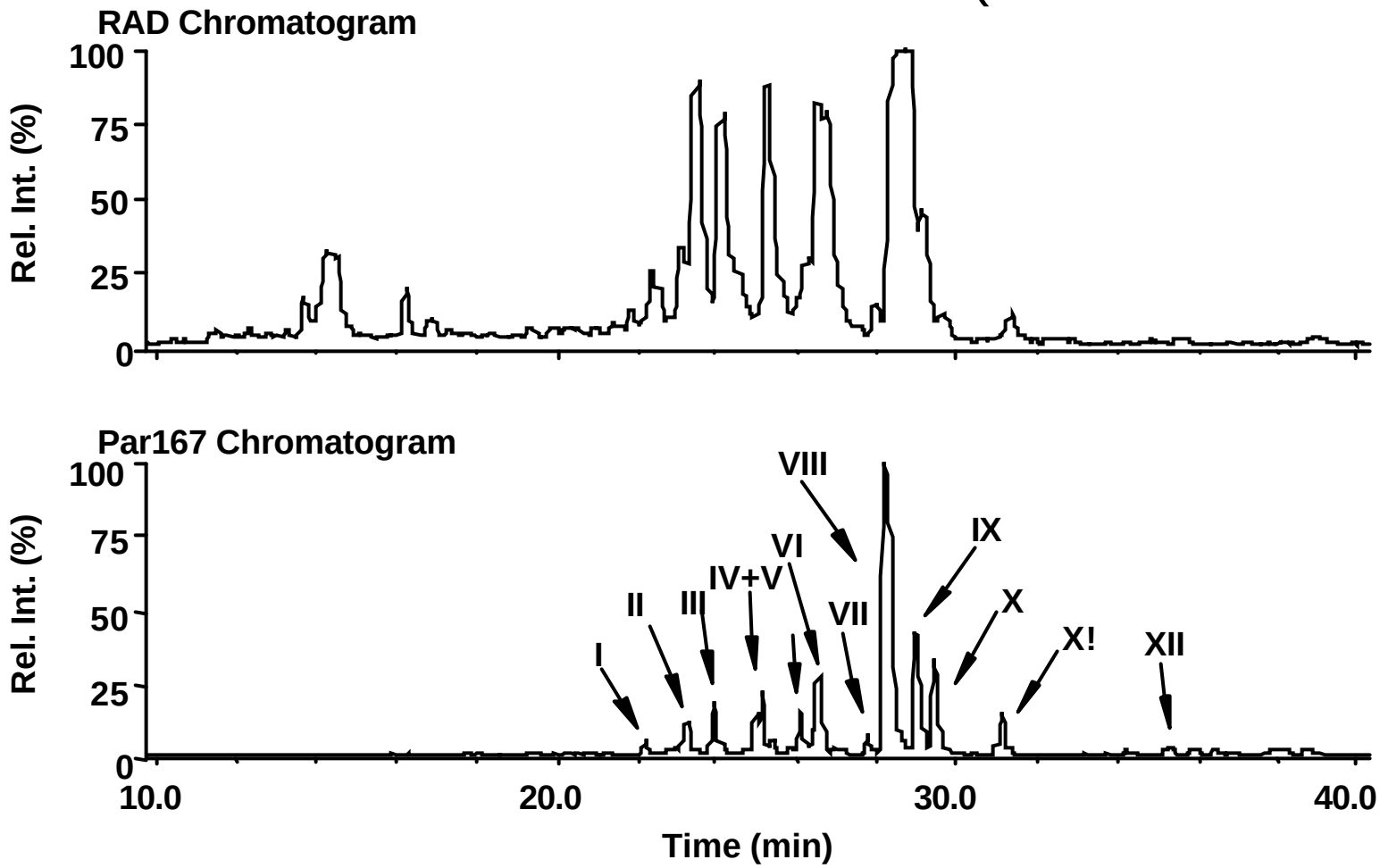
CID Product Ion Spectrum of a Parent Drug



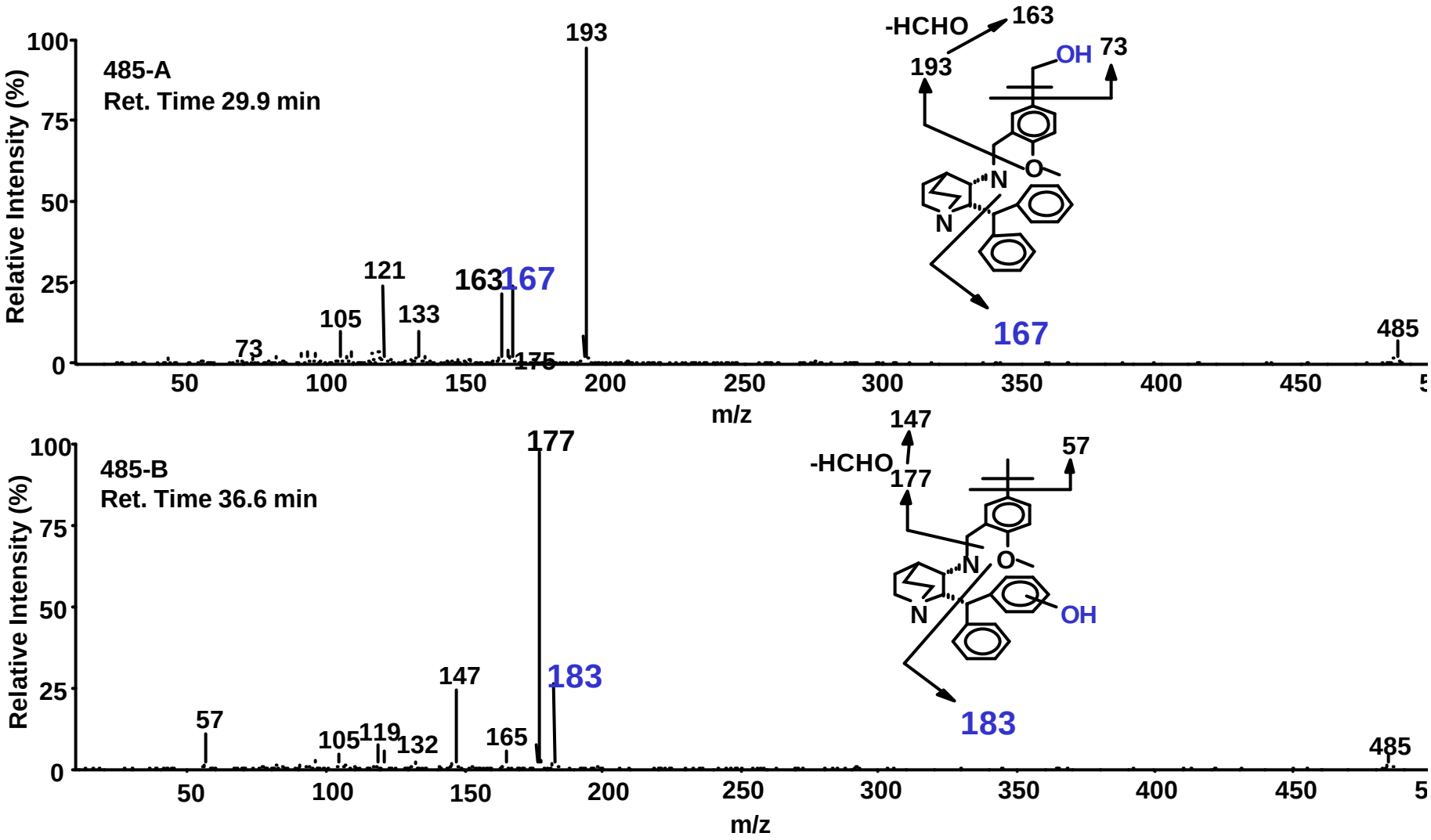
Identify parent scan ions?

HPLC-RAD and TIC Chromatograms for Biliary Metabolites of CJ-11,972

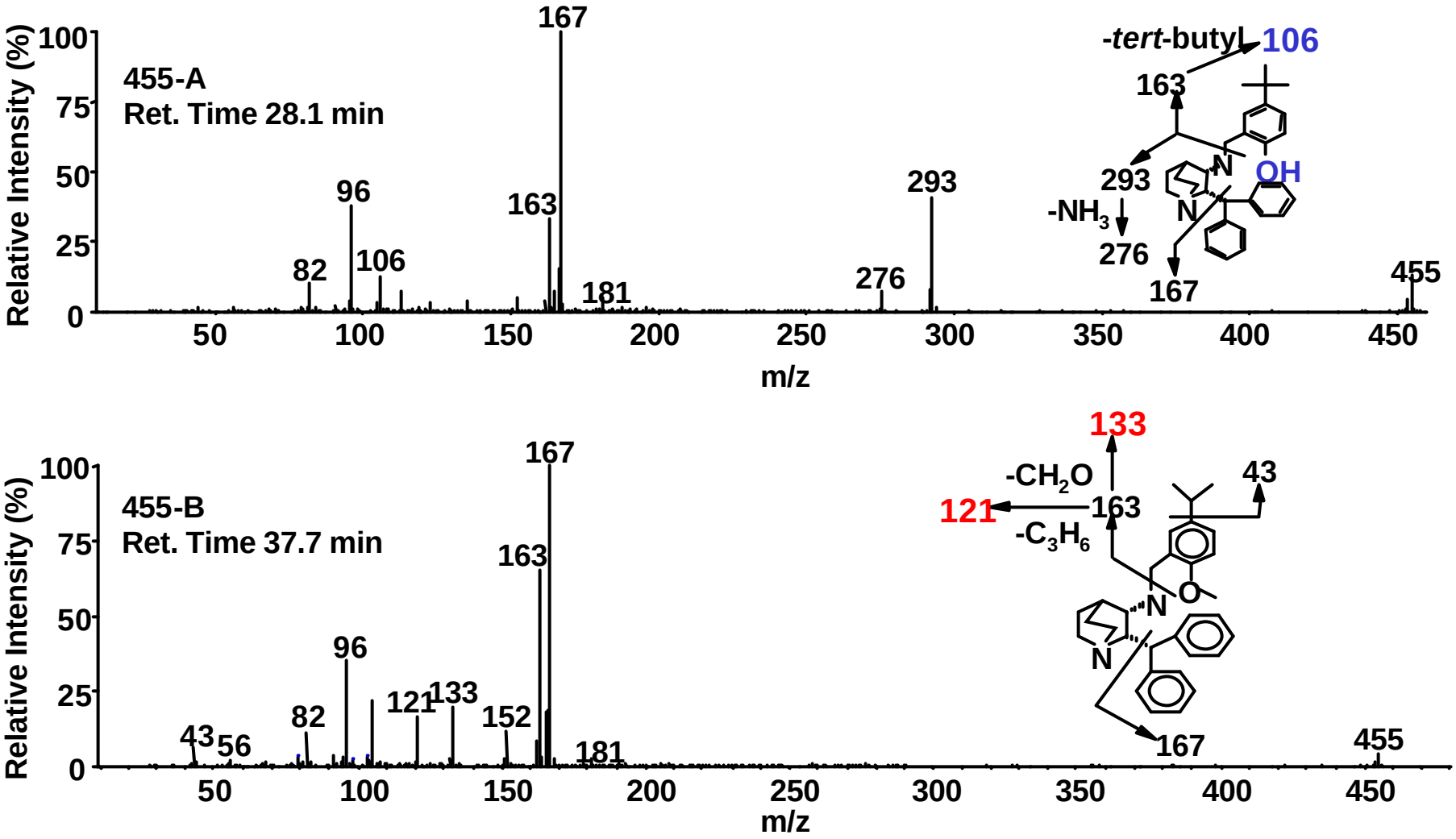
(Parents of m/z 167)



CID Product Ion Spectra of Metabolites 485-A and 485-B



CID Product Ion Spectra of Metabolites 455-A and 455-B

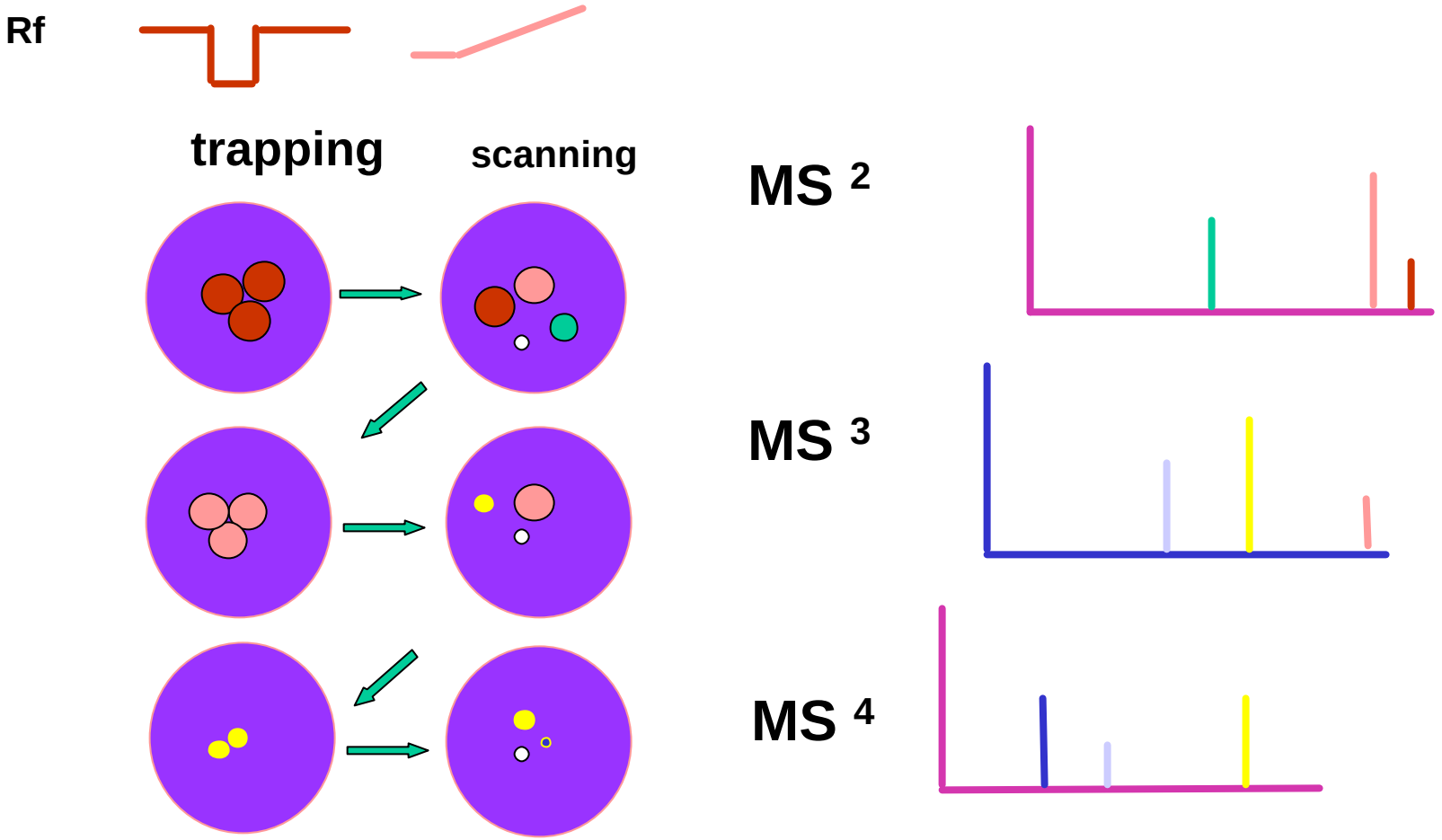


ION TRAP MS

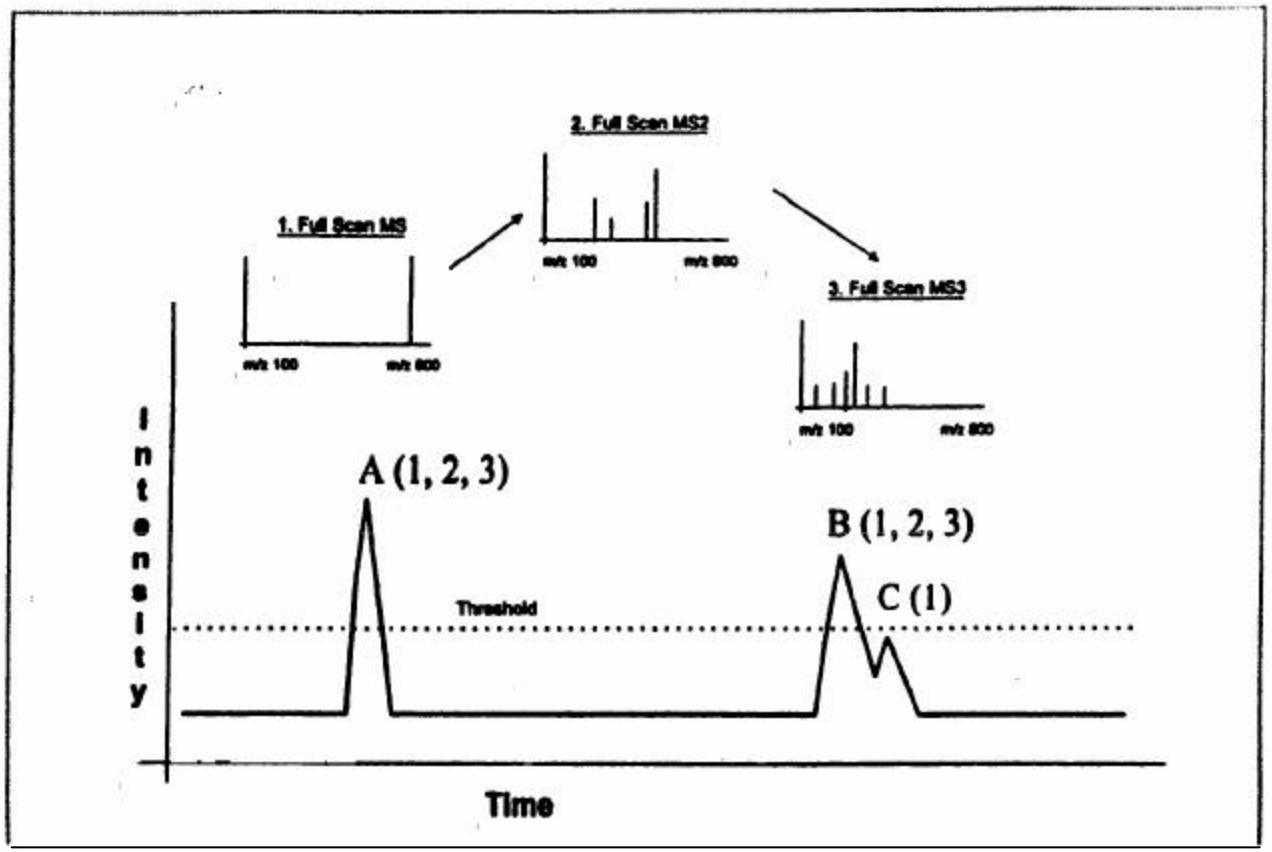
ION TRAP MS

- Sensitivity
  - Ion accumulation
    (10-1000 times better sensitivity than quadrupole MS)
- Specificity
  - Multistage MS capabilities (MSn)
- Speed
  - Can complete an entire scan in 100 ms
- Data Dependent Acquisition
  - Acquire MW information and MSn spectra in the same run
- High value/Cost Ratio

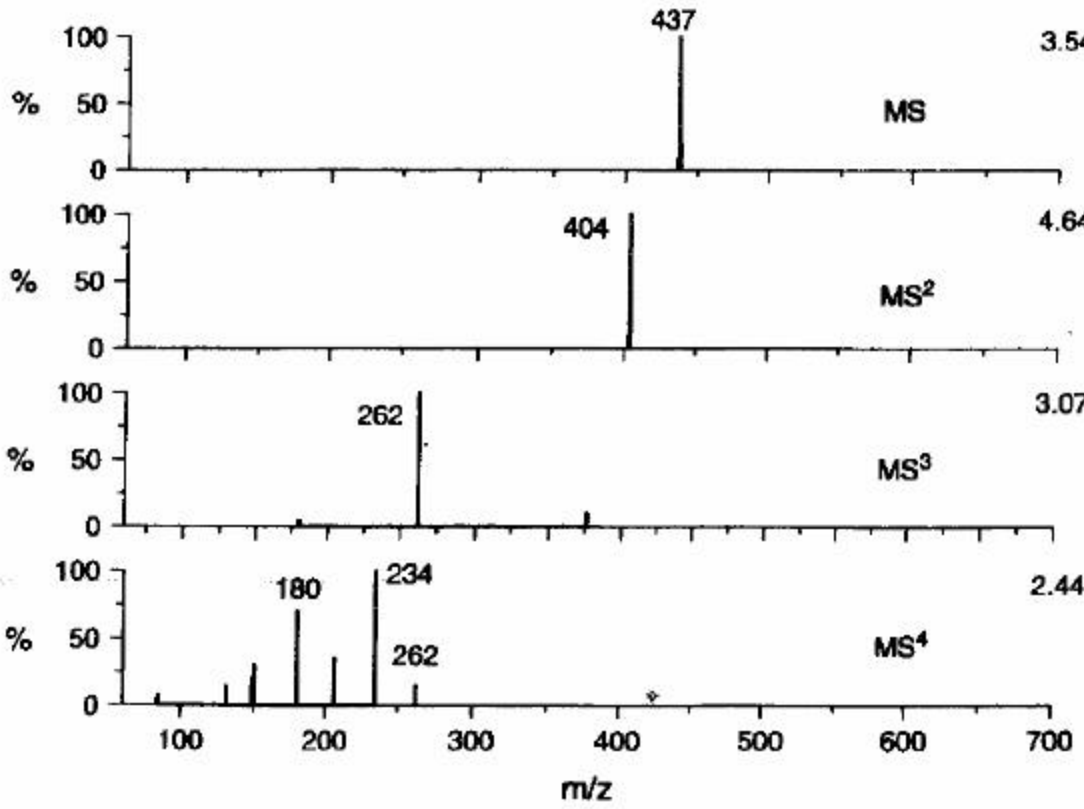
Triple Quad vs LCQ (MS/MS)

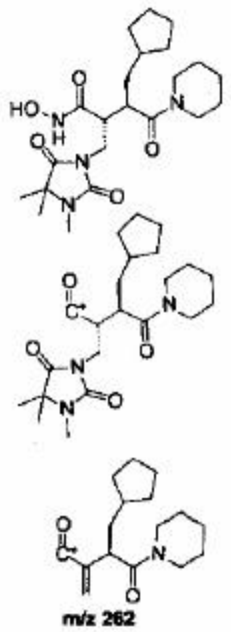


Schematic of data-dependent analysis using LCQ

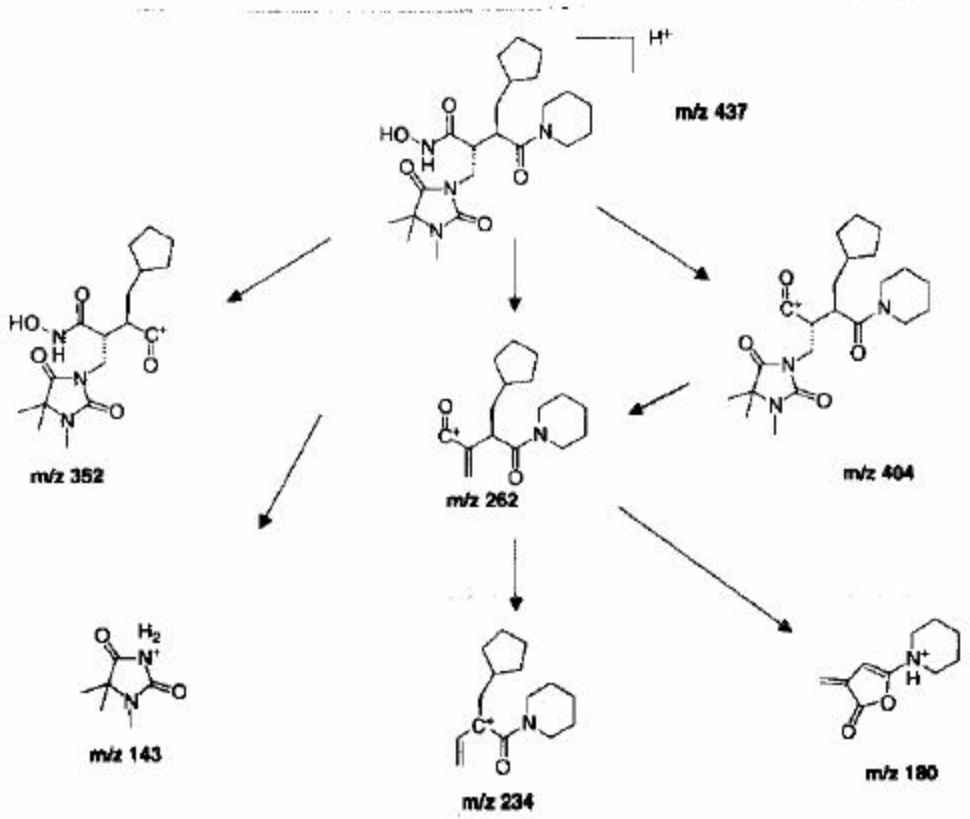


MS, MS^2, MS^3 and MS^4 spectra of trocade on a ion trap





Proposed fragmentation pathways of trocade for the major fragments



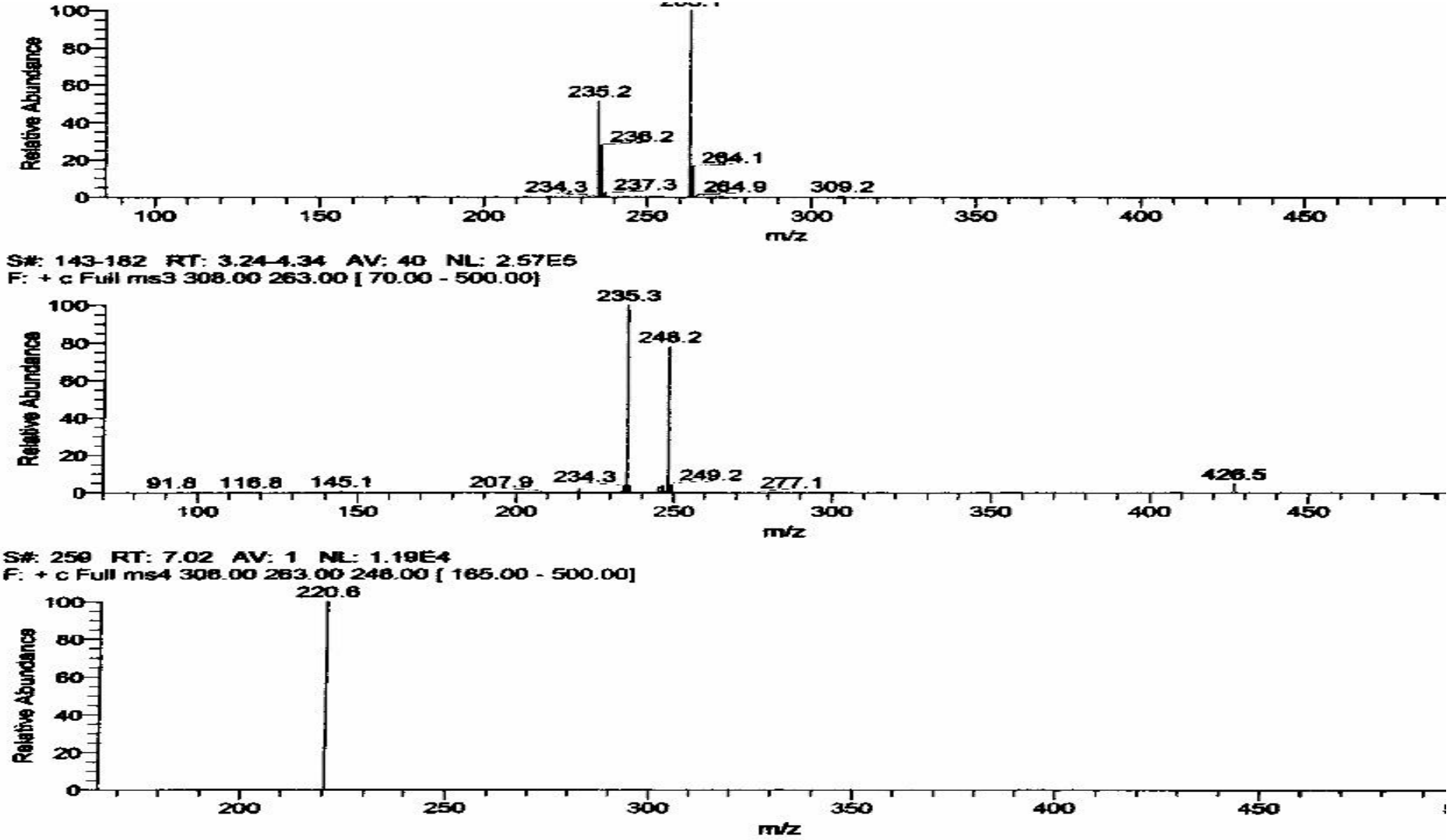


Fig. 2. MS^n behaviour of zolpidem.

Adapted from Smyth et. al., Analytica Chimica Acta 506(2004) 203-214

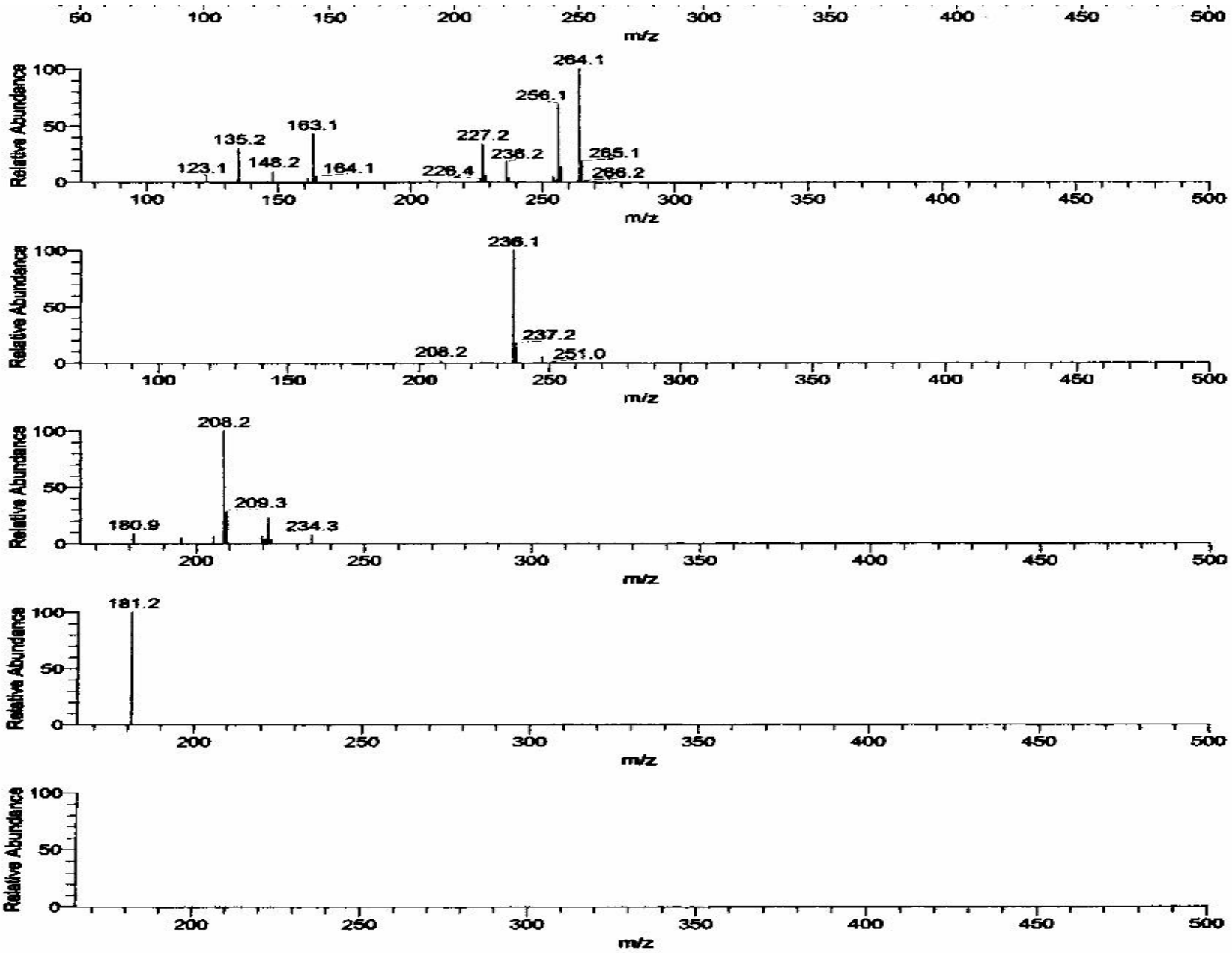


Fig. 4. MS^n behaviour of 7-aminoflunitrazepam.

Adapted from Smyth et. al., Analytica Chimica Acta 506(2004) 203-214

Q-TOF

Why use a Q-TOF ?

Sensitivity

- detection of low level metabolites in complex matrices in vivo

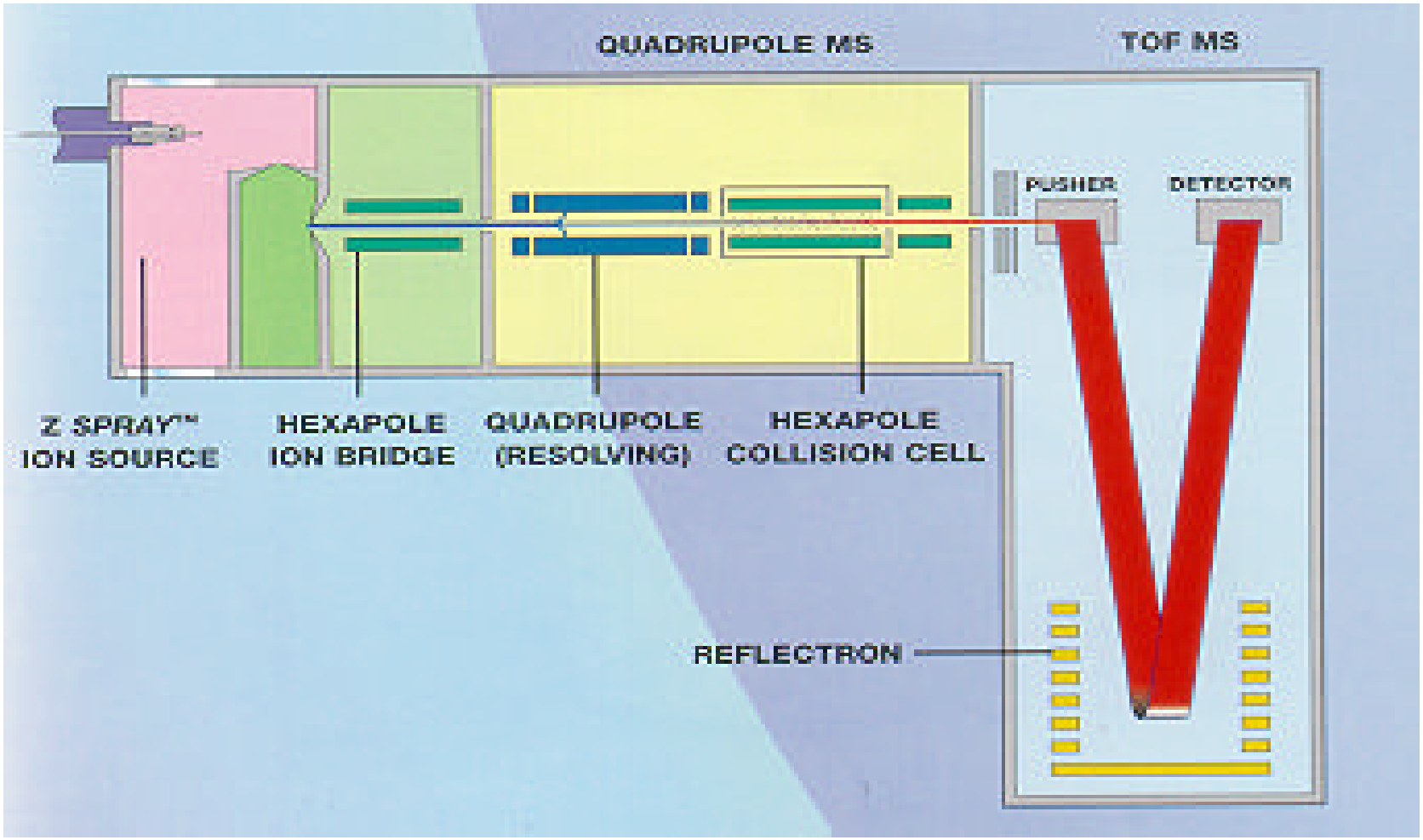
Exact mass (high resolution mass measurement)

- added confidence in confirming expected metabolites (confirm elemental composition for metabolites with the same nominal mass)

LC/MS/MS

- confirmation of metabolites (compare MS/MS spectra)
- data dependent MS --→ MS/MS (time saving, High throughput)

Operating principle of the Q-TOF mass analyzer



MS operation : Quadrupole MS transmits – TOF detects all ions transmitted : full scan mass spectrum

MS/MS operation: Precursor ion selection in quadrupole, collision induced dissociation (CID) in hexapole collision cell – product ion detection in TOF: MS/MS spectrum

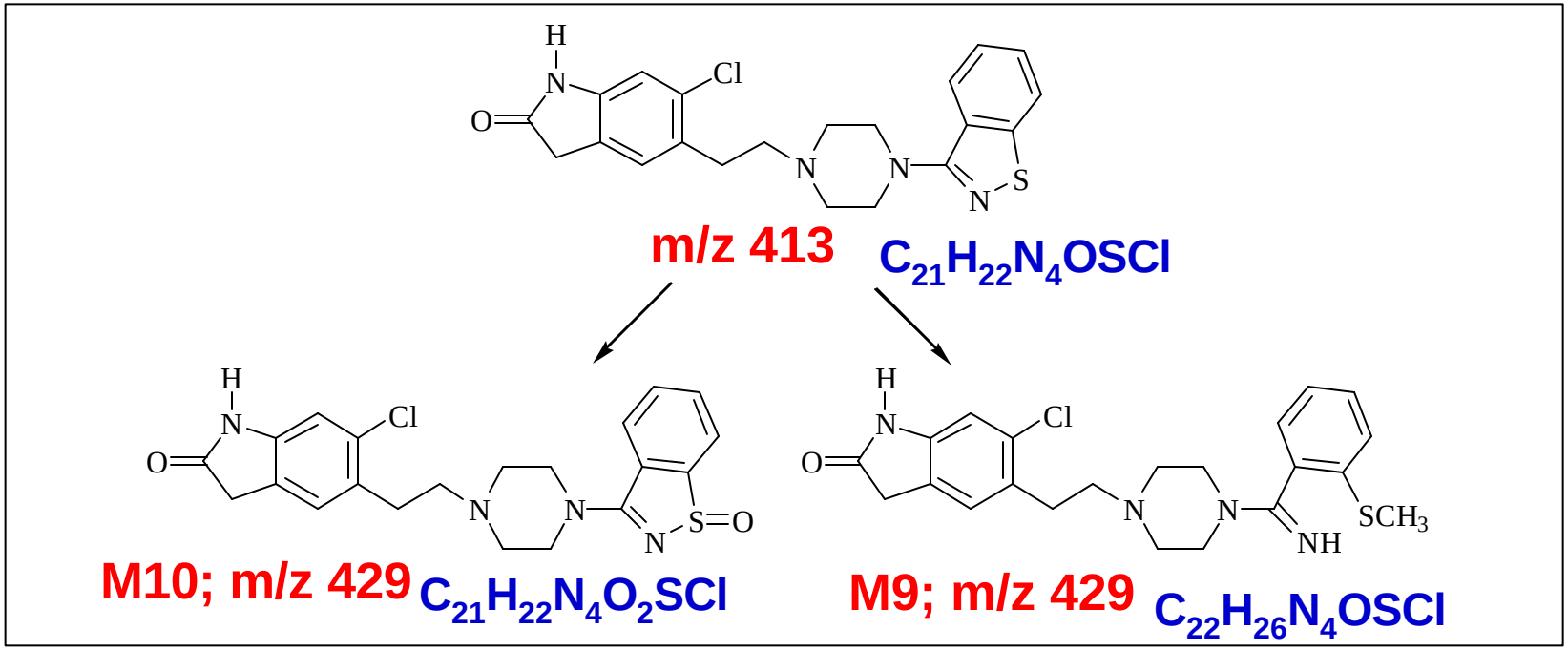
Instrument Capabilities

- High Resolution MS and MSMS
  - 20,000 resolution
  - Peak Width of 0.025 at 500 amu
- High Resolution= High Selectivity
  - Able to easily separate masses that differ in 0.1 amu easily
- TOF allows fast scan speeds without sacrificing sensitivity or scan ranges in MS or MS/MS modes

Mass

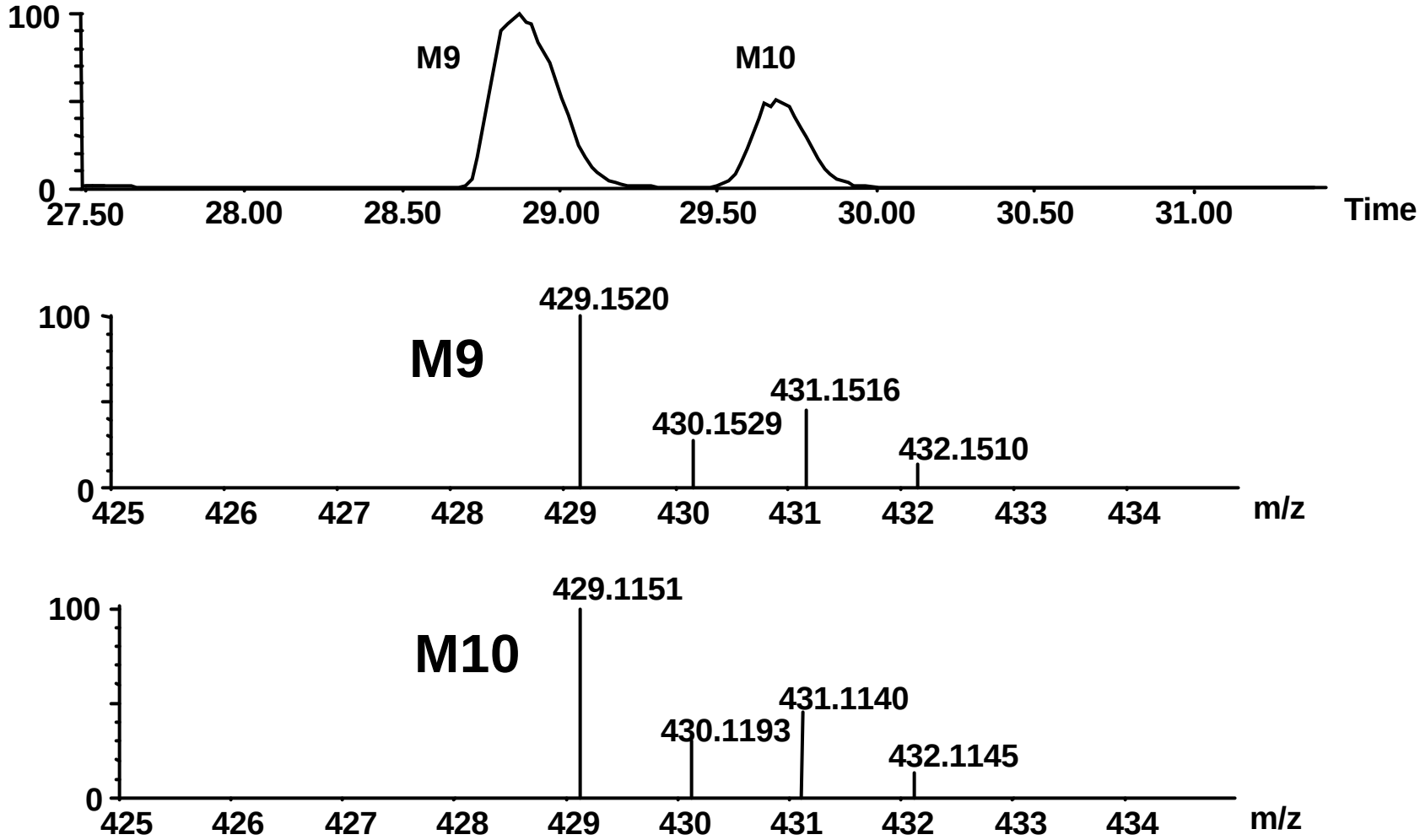
Element	Nominal Mass	Average Mass	Exact Mass
C	12	12.011	12.0000
H	1	1.00797	1.0078
O	16	15.9994	15.9949
N	14	14.003	14.0031
Cl	35	35.45	34.9689
S	32	32.06	31.972

Biotransformation of Ziprasidone



Question: Can we differentiate the structures of these metabolite with m/z 429 by TOF?
Previous assignment: S-oxides or S-methyl (+ 16) .

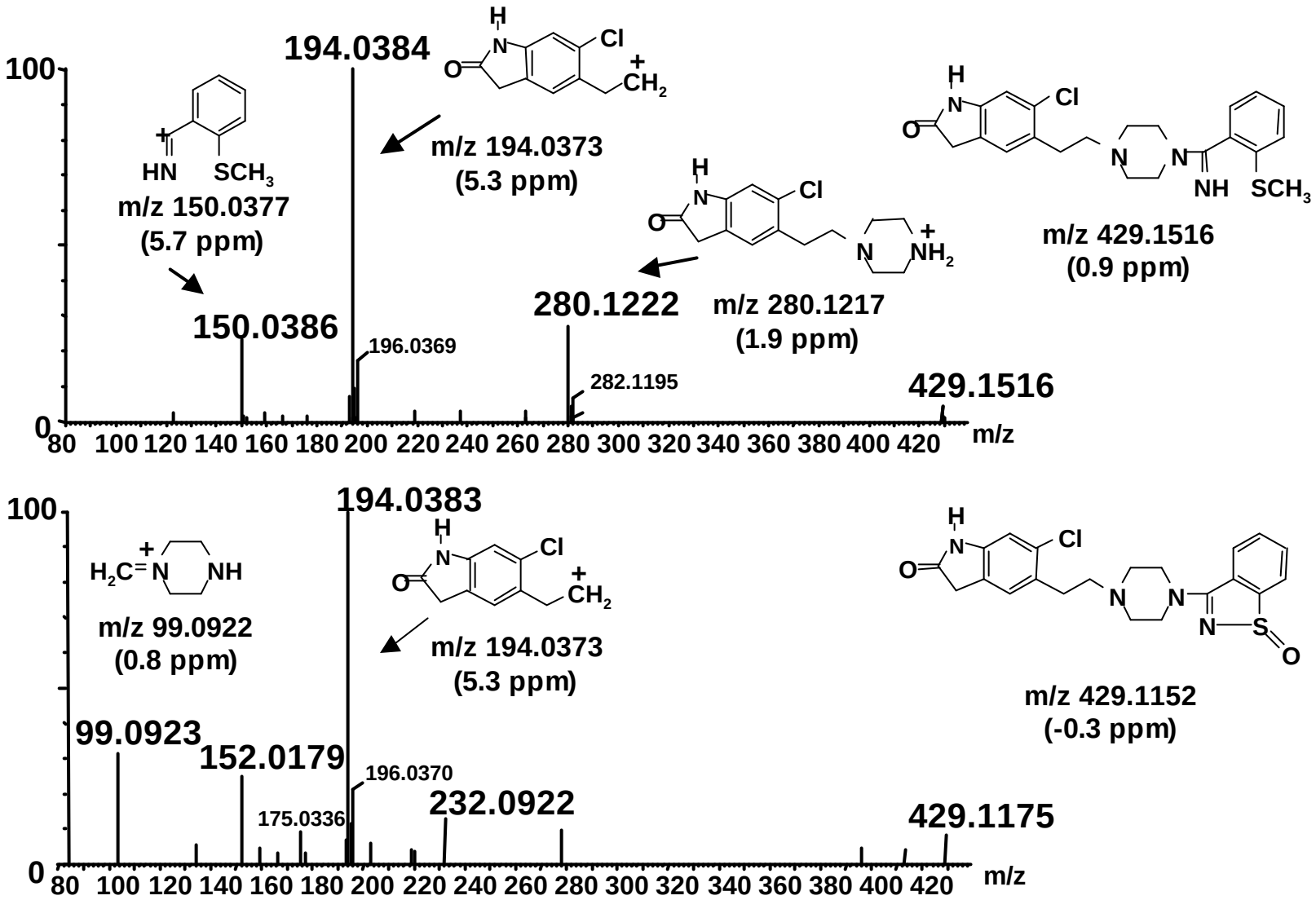
Selected Ion Chromatogram and Full Scan MS of M9 and M10



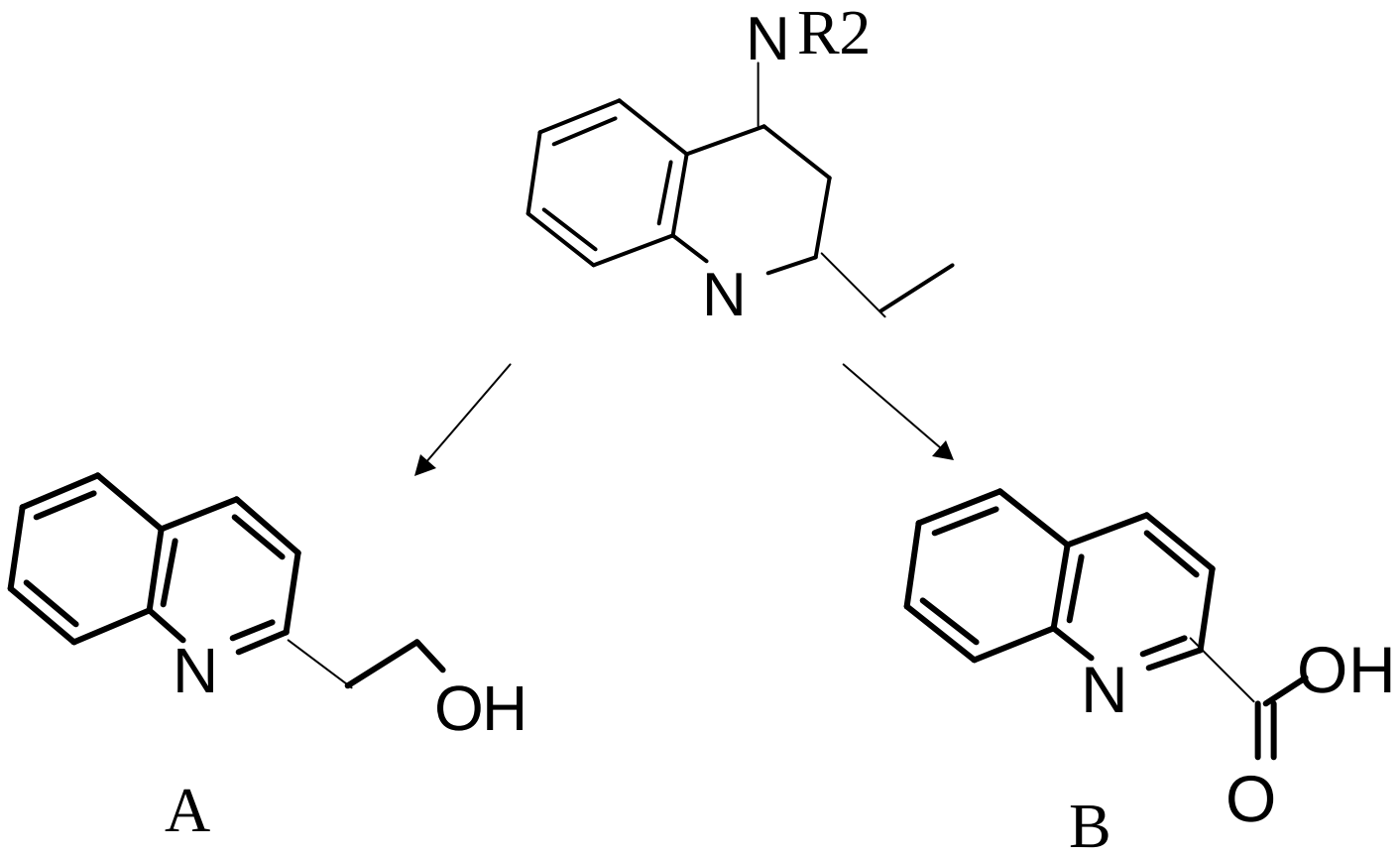
Mass Measurements of M9 and M10

Metab	Cal. Mass	Obs. Mass	+/-mDa	+/-ppm	Mol. Formula
M9	429.1516	429.1520	0.4	0.9	C22H26N4OSCl
M10	429.1152	429.1151	-0.1	-0.3	C21H22N4O2SCl
Parent	413.1203	413.1205	0.2	0.4	C21H22N4OSCl

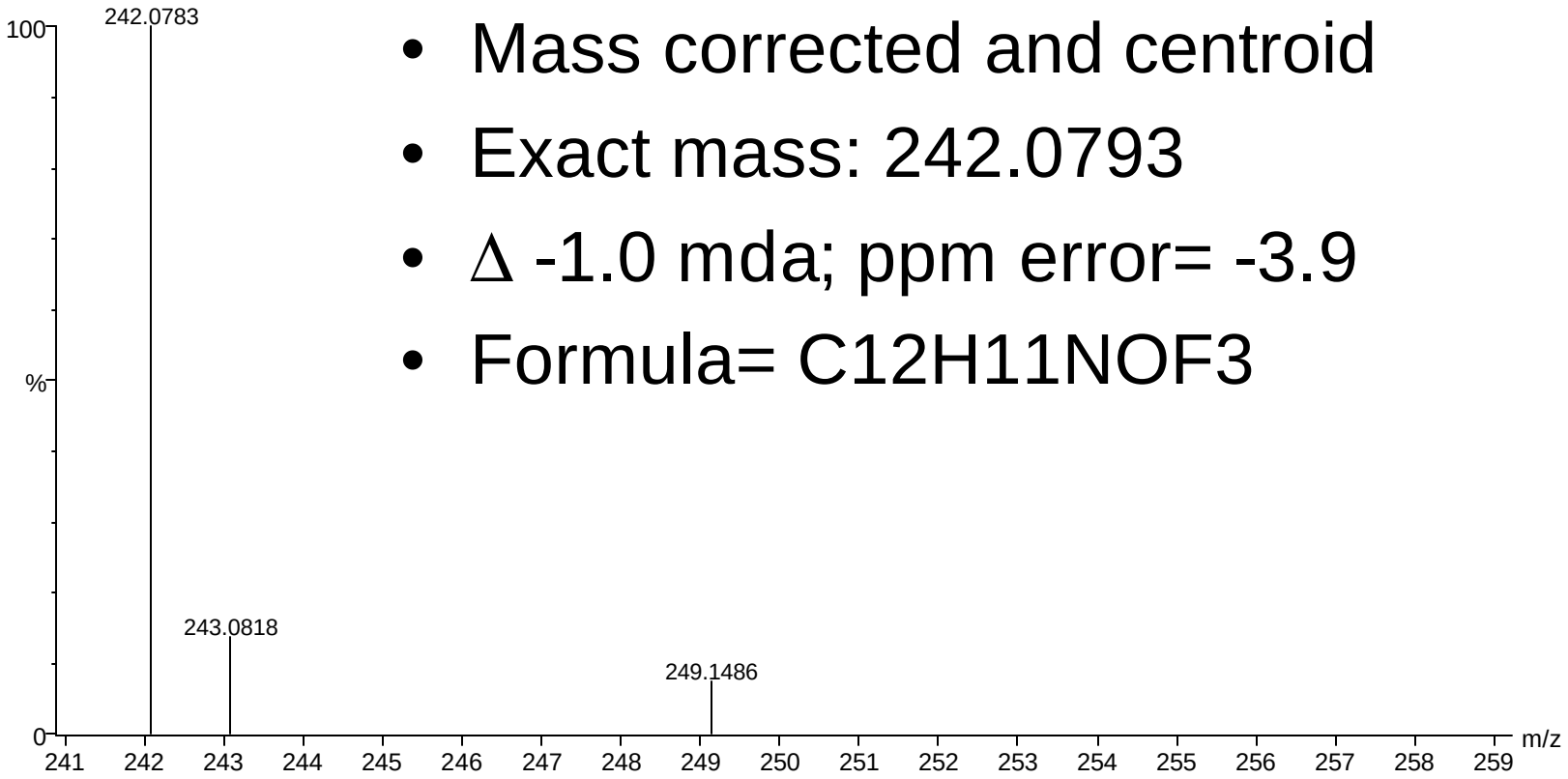
MS/MS Spectra of Metabolites M9 and M10



Two Isobaric metabolites of Compound X

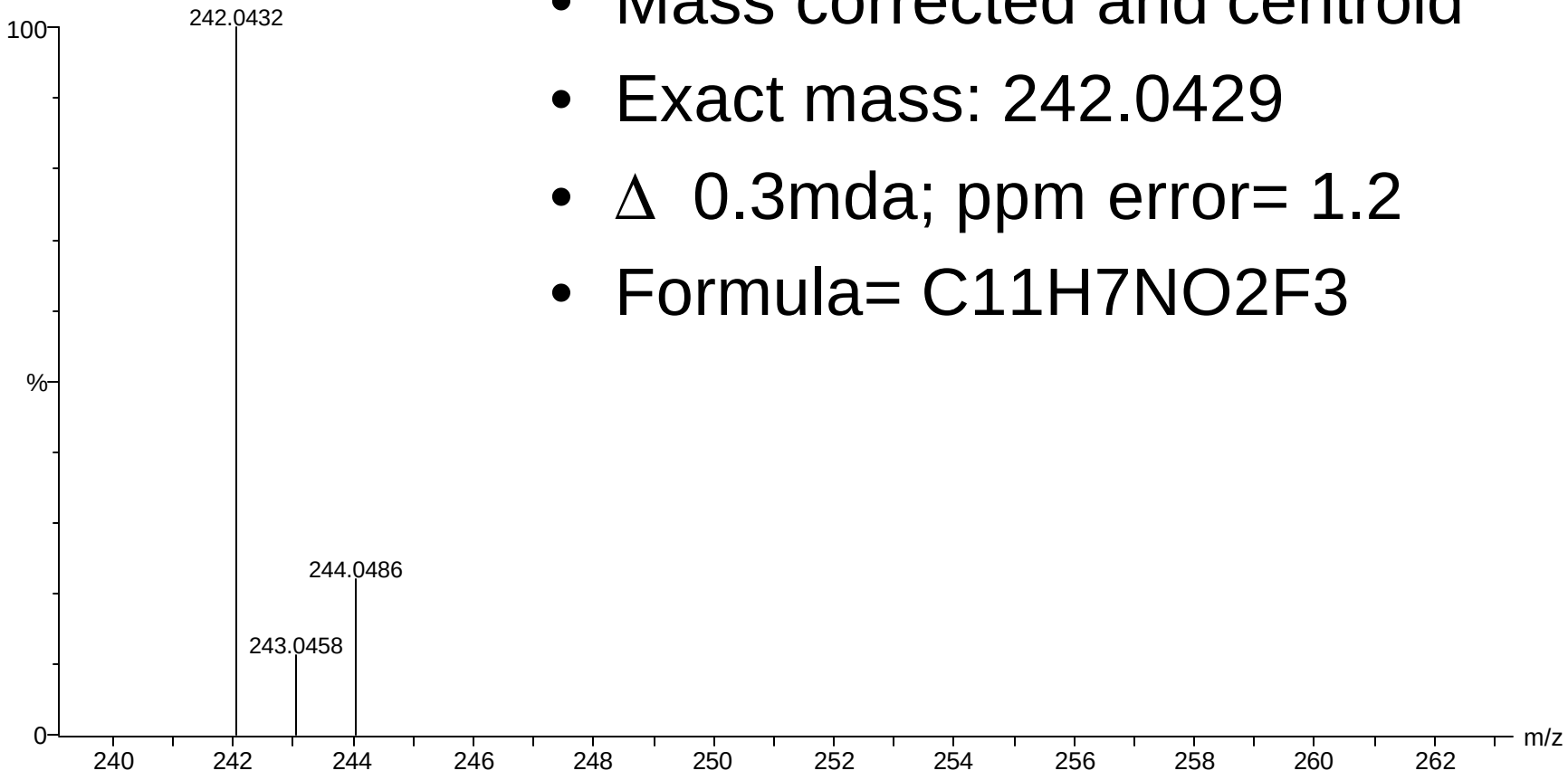


TOF MS Spectrum of Structure A



- Mass corrected and centroid
- Exact mass: 242.0793
- Δ -1.0 mda; ppm error= -3.9
- Formula= C12H11NOF3

TOF MS Spectrum of Structure B



- Mass corrected and centroid
- Exact mass: 242.0429
- Δ 0.3mda; ppm error= 1.2
- Formula= C11H7NO2F3

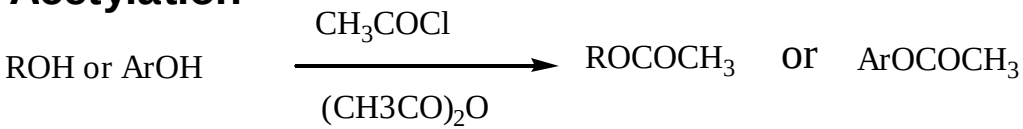
Analytical techniques combined with mass spectrometry for characterization of metabolites

When Derivatization is Useful

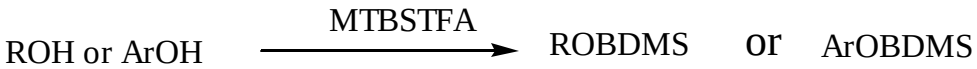
- Metabolite is unstable
- Metabolite is very polar
- Metabolite is volatile
- To characterize the functional group
- To prove MS fragmentation
- To improve sensitivity when metabolite is available in only trace amounts

Derivatizations of Hydroxyl Groups

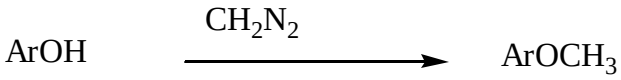
-Acetylation



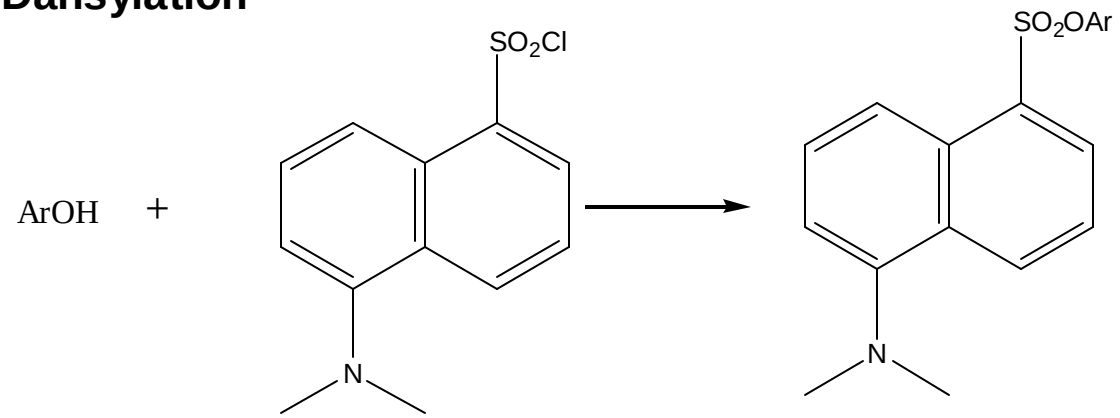
-Silylation



-Methylation



-Dansylation



MTBSTFA; *Tert*-butyldimethylsilyl-N-methyltrifluoroacetamide

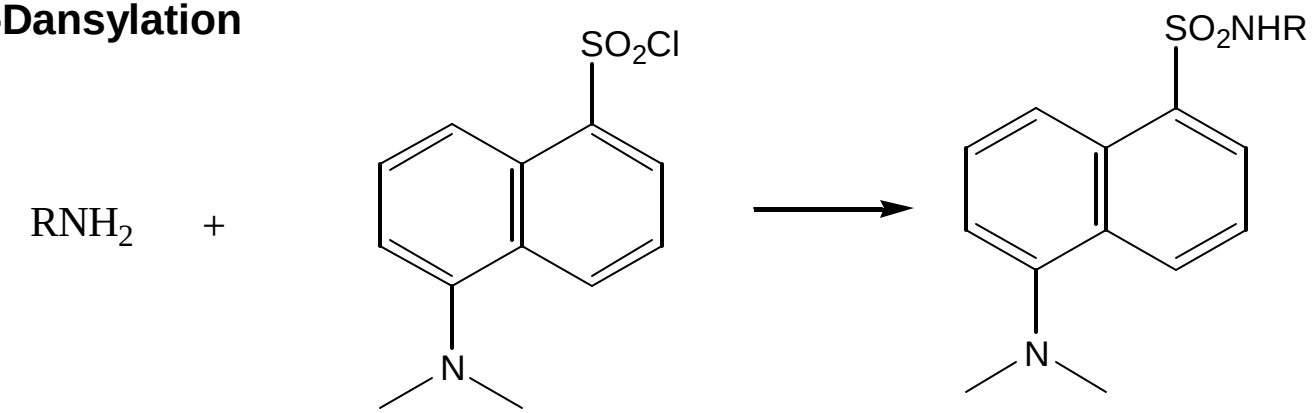
Derivatizations of Amines

-Acetylation

$RNH_2 \xrightarrow[(CH3CO)_2O]{CH_3COCl} RNHCOCH_3$

$RNH_2 \xrightarrow{MBTFA} RNHCOCF_3$

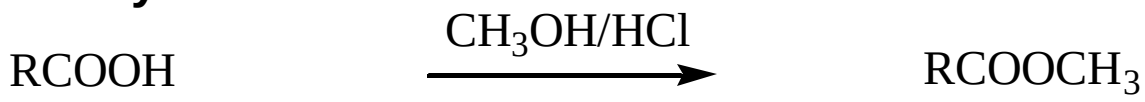
-Dansylation

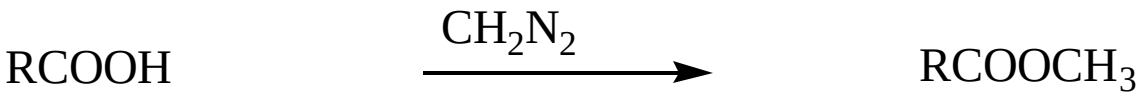


MBTFA; N-methylbis(trifluoroacetamide)

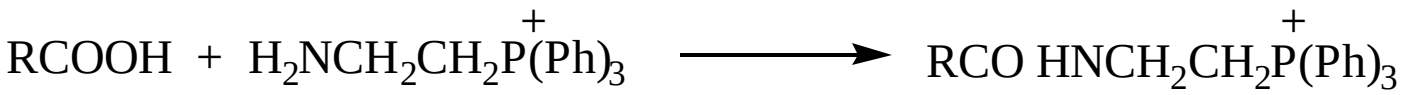
Derivatization of Carboxyl Group

-Methylation

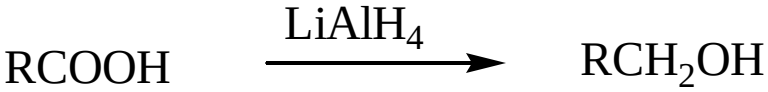




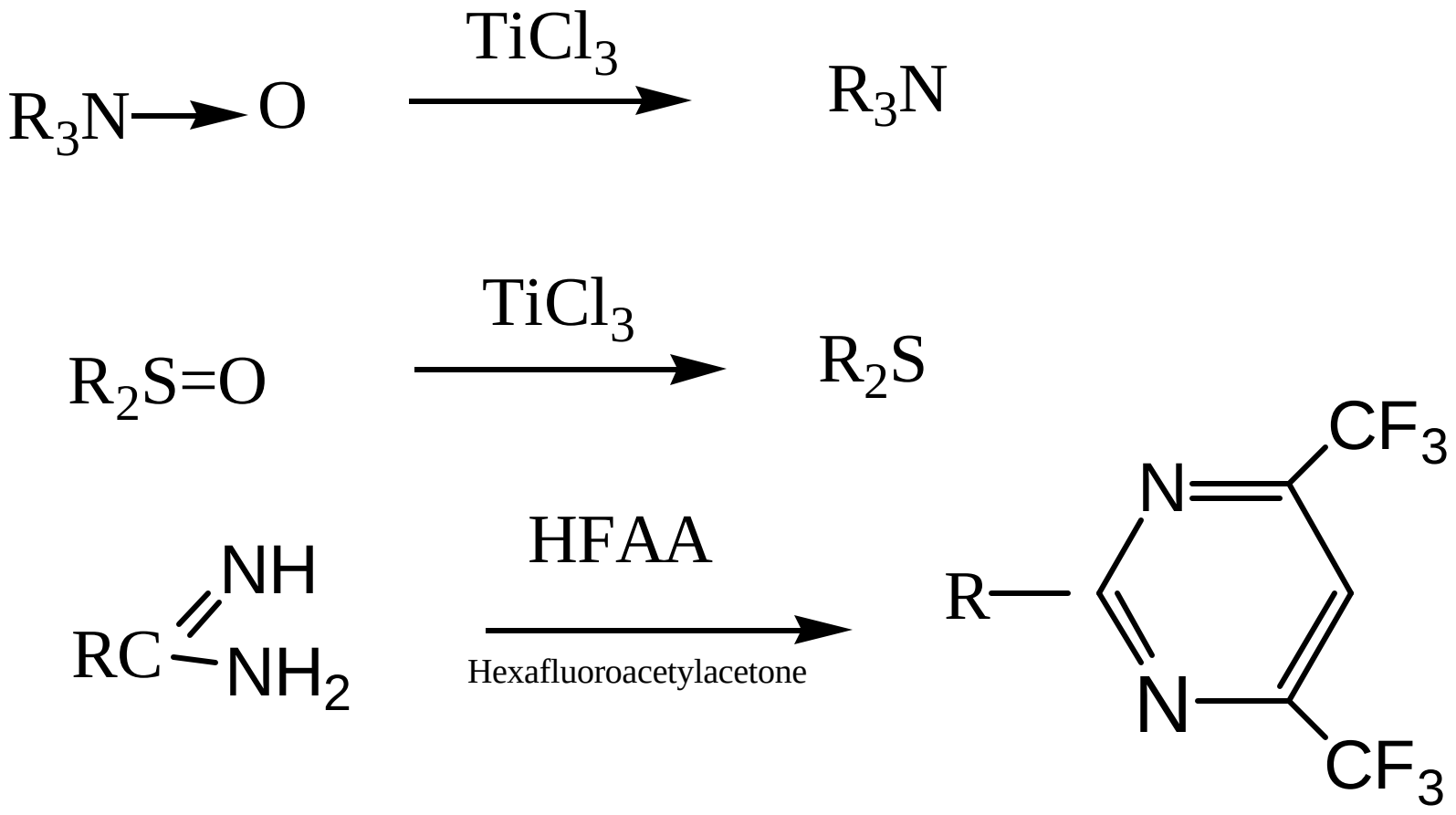
-Triphenylphosphonium Derivative



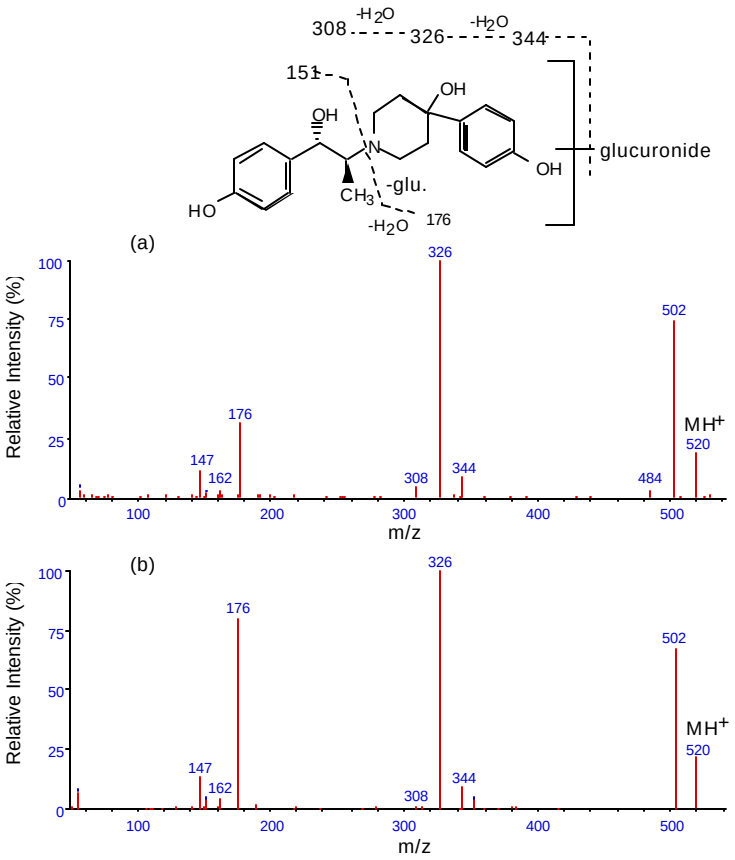
-Reduction



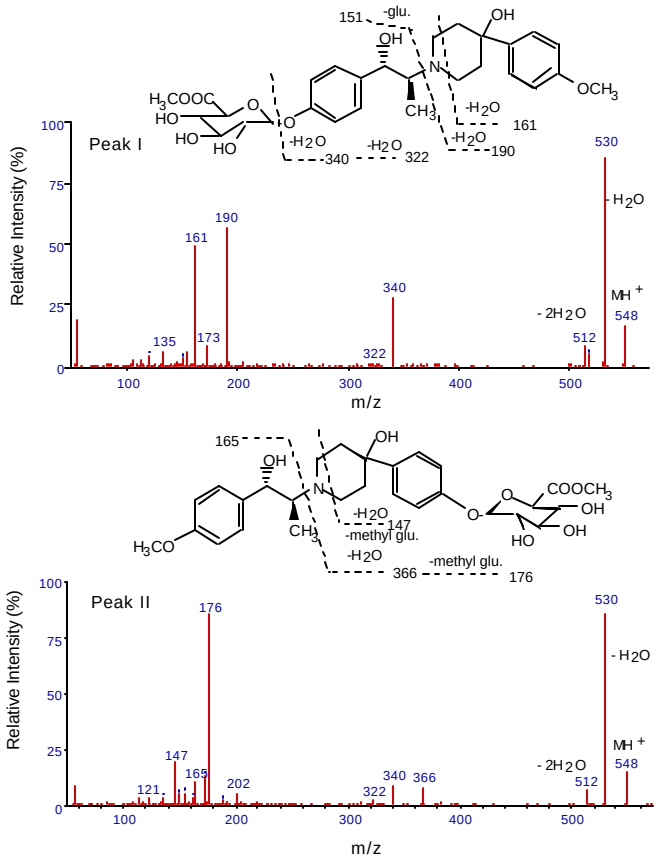
Functional group-specific chemistry



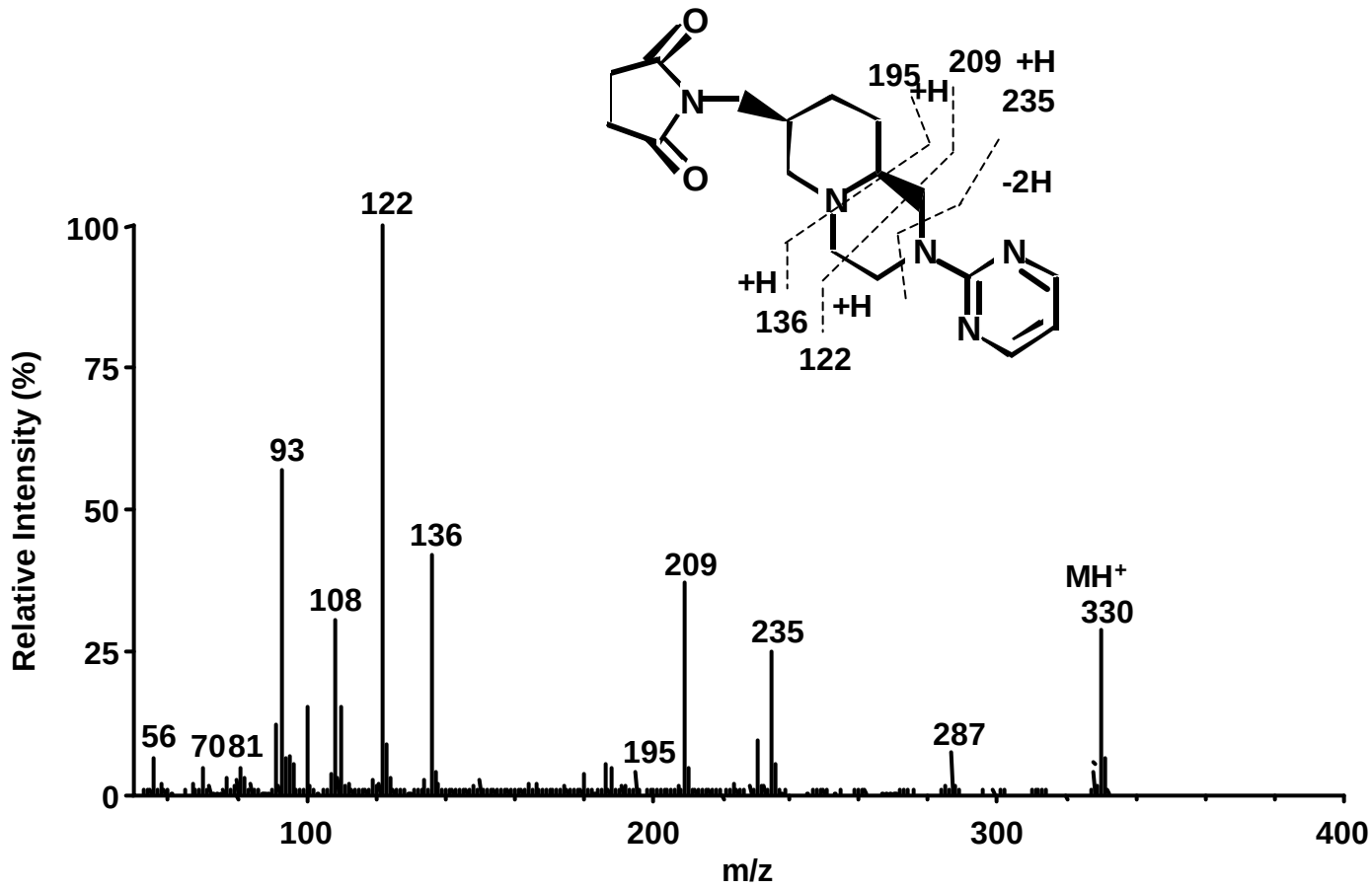
CID product ion spectra of glucuronide conjugates



CID product ion spectrum of glucuronide conjugates after methylation

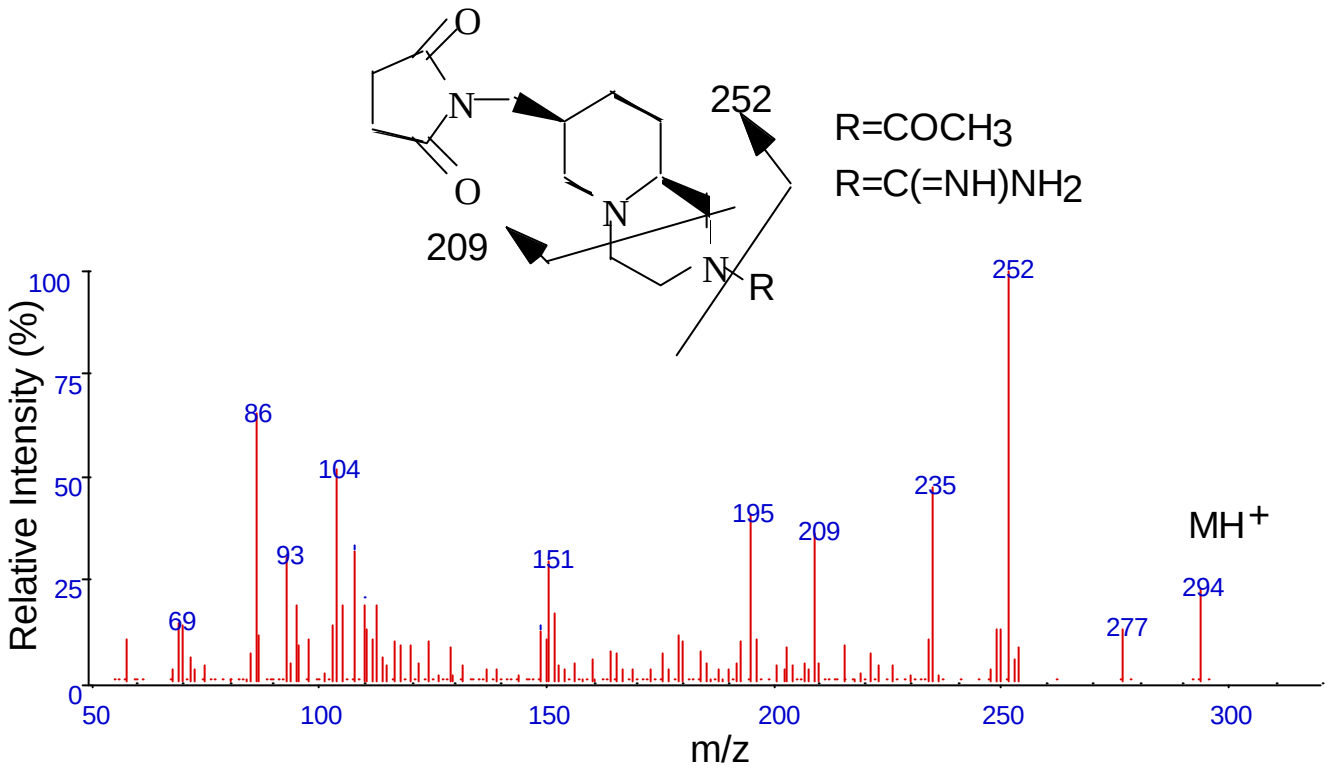


CID Product Ion Spectrum of Sunepitron

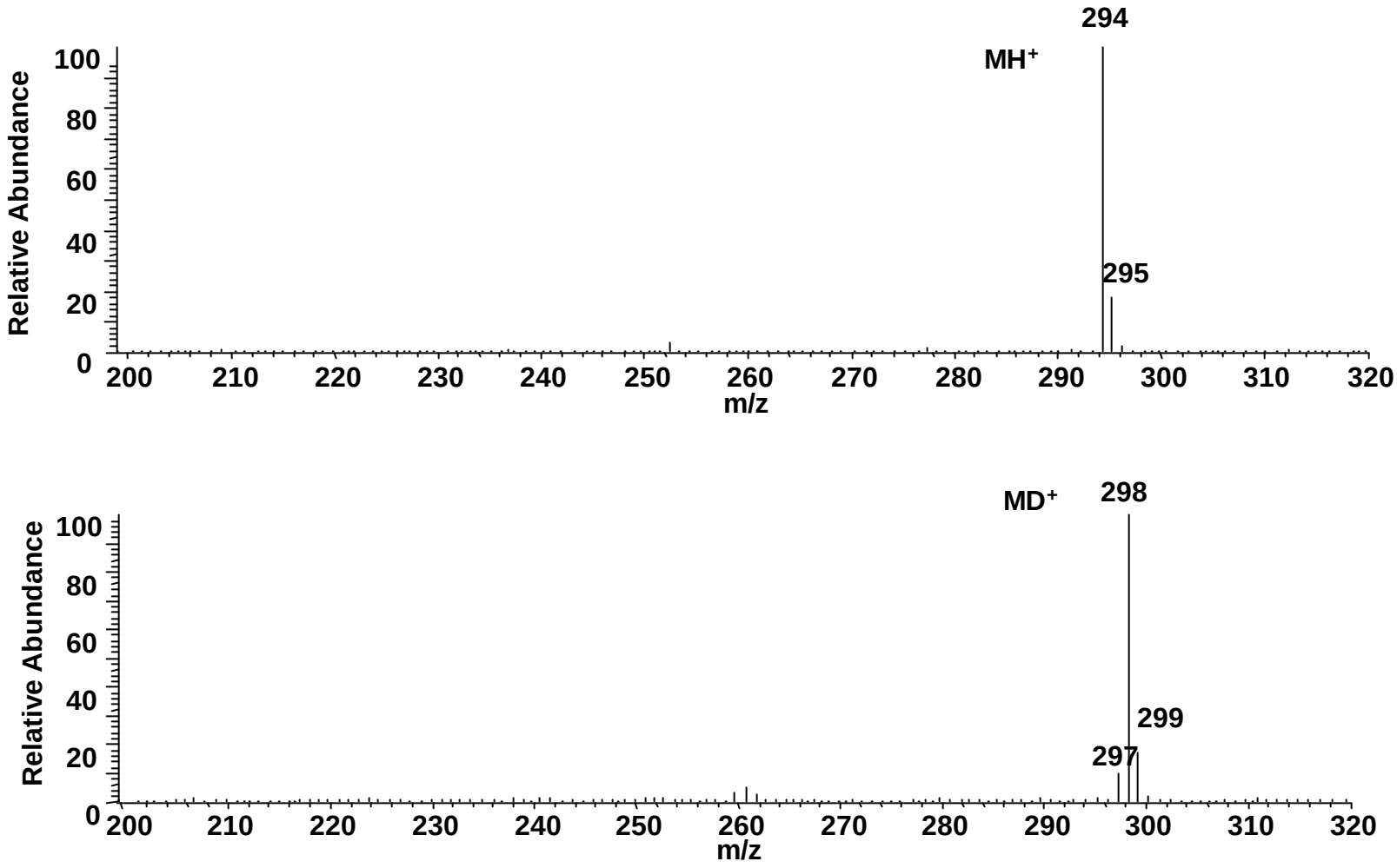


C. Prakash, et al. Drug Metab Disp. (1997) 26, 206-218

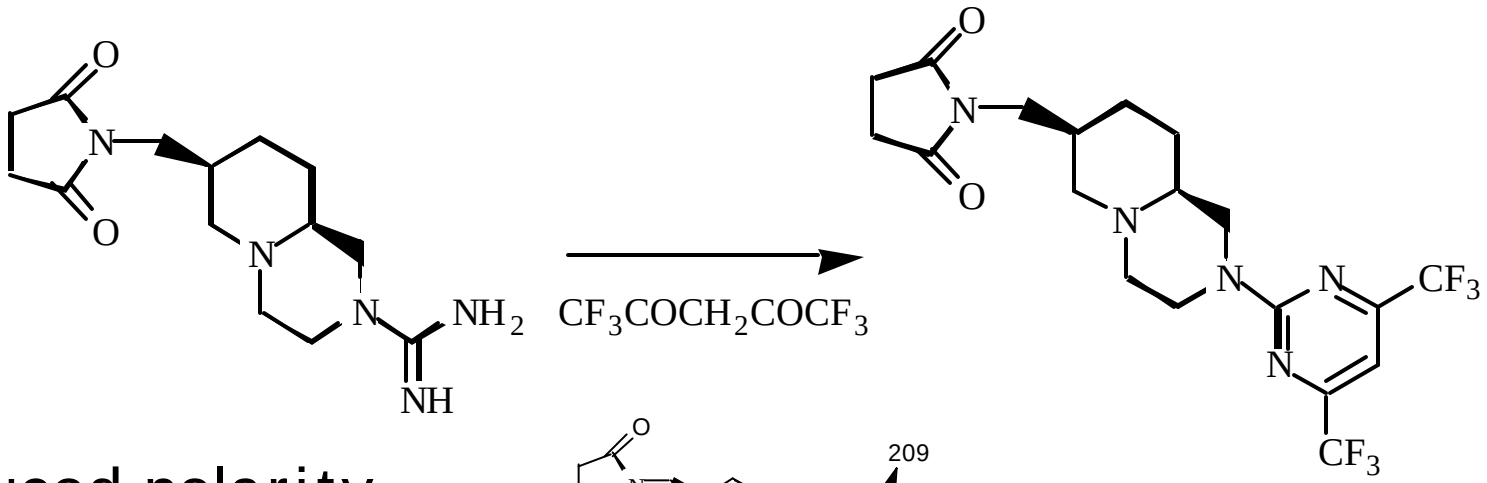
CID product ion spectrum of M18



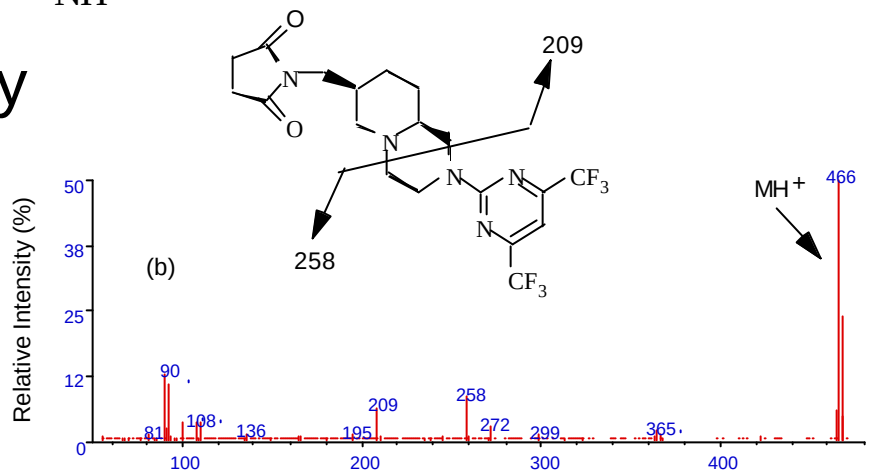
Mass Spectra of M18 Before and After Deuterium Exchange



CID product ion spectrum of M18 after treatment with HFAA



- Reduced polarity
- Diagnostic mass increase



Identification of Drug Metabolites
LC-NMR

ADVANTAGES

- LC-NMR (Continuous flow or stopped flow)
- Fast
- Reportedly sensitive (50 - 200 ng)
- Amenable to automation
- Negate the need for isolation
- Sample Stability
- Cleaner Spectra

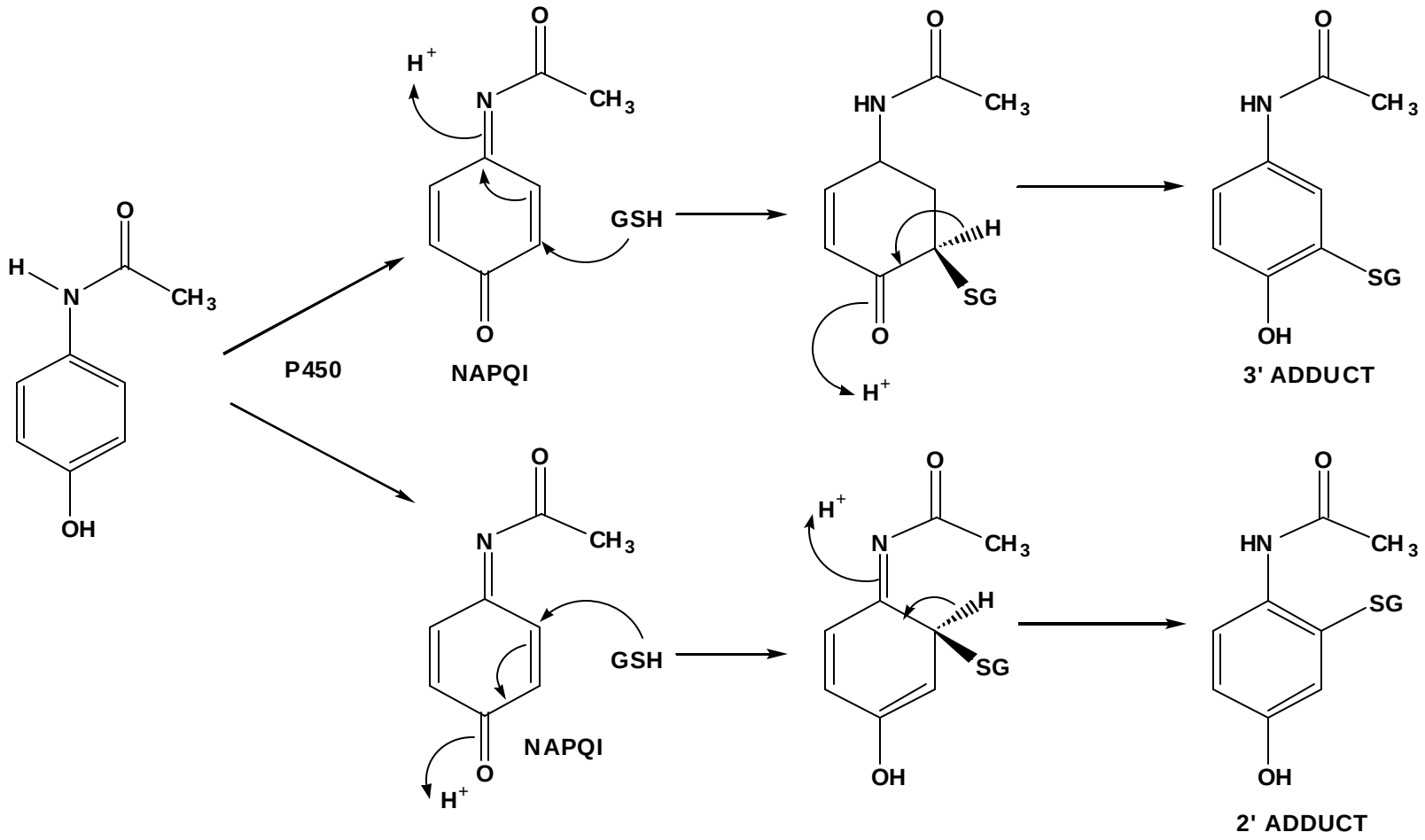
Disadvantages and Limitation of LC-NMR

- Sensitivity
  - Nearly eliminates quantitative application
- The Chromatograph
- Solvent Suppression
- Expensive deuterated mobile phase and buffers
- Shimming problems introduced by LC-gradient methods

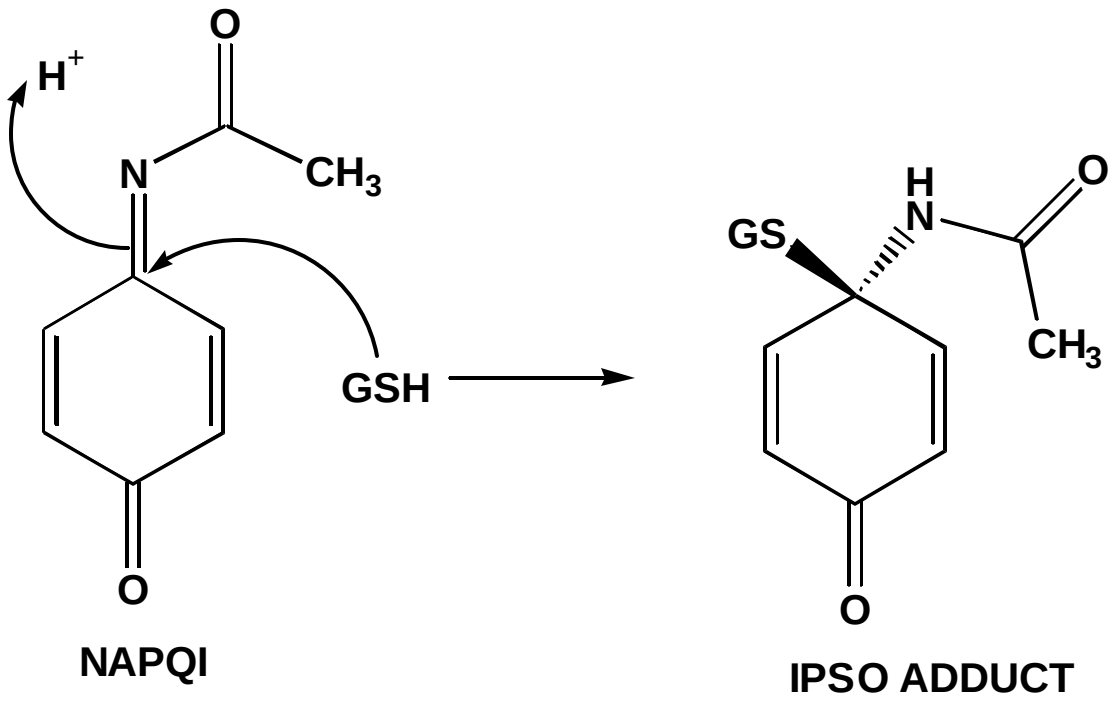
What information does NMR provide

- Each proton (or carbon atom) in a molecule typically has a different resonant frequency (chemical shift)
  - Thus, NMR spectrum is a fingerprint of a molecule
  - Chemical shifts are governed by nuclear environment, e.g. CH_3O, CH_3CN, CH_3 NH_2
- Adjacent NMR-active nuclei 91-4 bonds apart) in a molecule may couple to one another
  - Coupling constants can be identified by inspection of 1D spectra

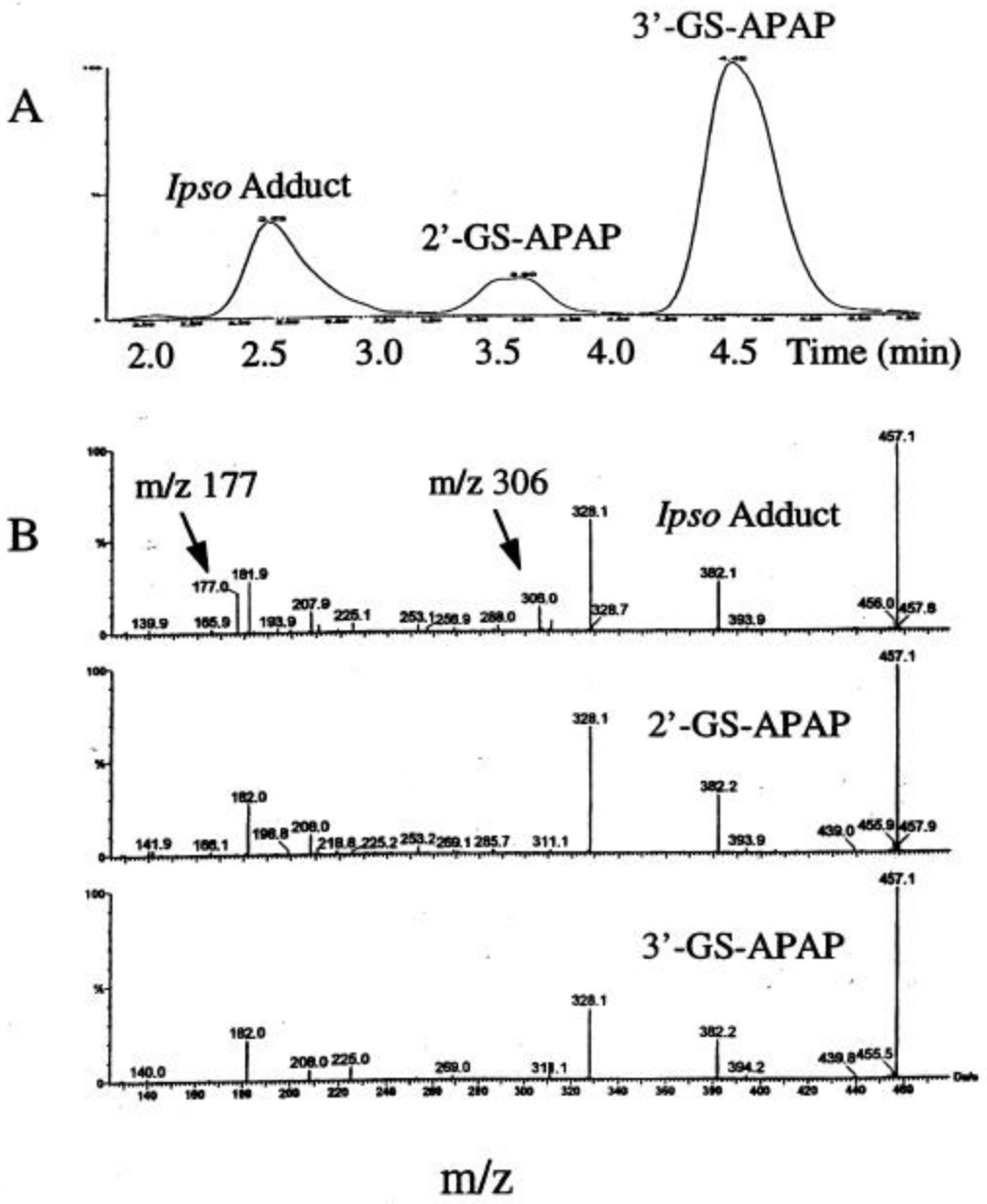
Activation of Acetaminophen by Cytochrome P450 to N-acetyl-p-benzoquinonimine (NAPQI) and subsequent conjugation with Glutathione



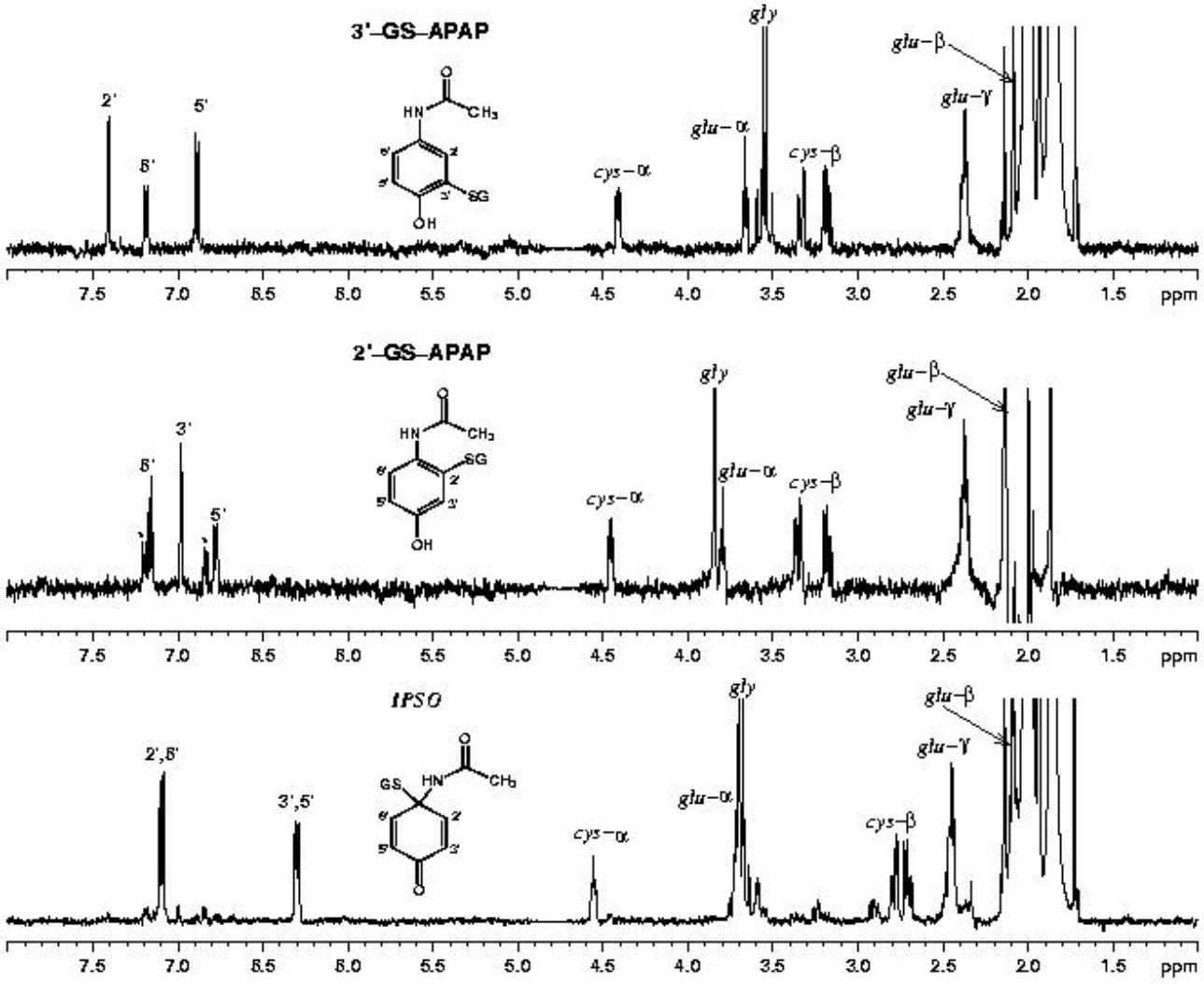
Proposed Reaction Product of N-acetyl-p benzoquinone imine(NAPQI) with Glutathione



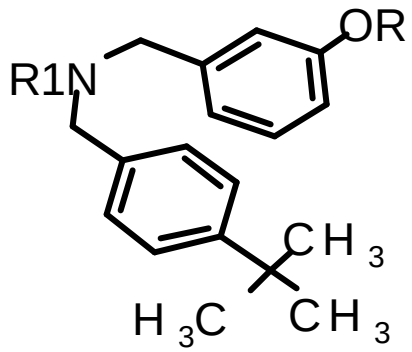
CID Product Ion Mass Spectra of Reaction Mixture



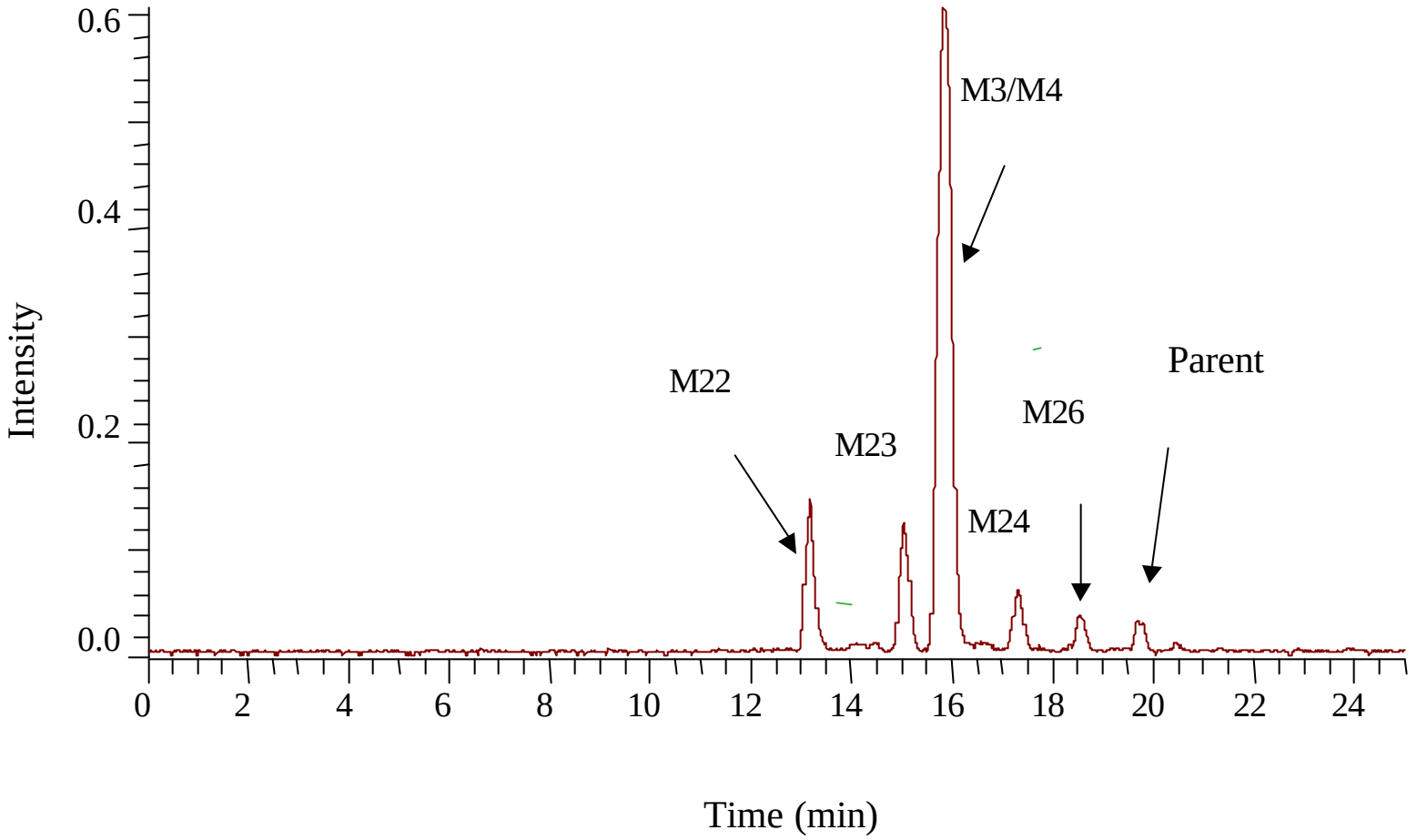
^{1}H NMR Data Obtained on The Reaction Mixture



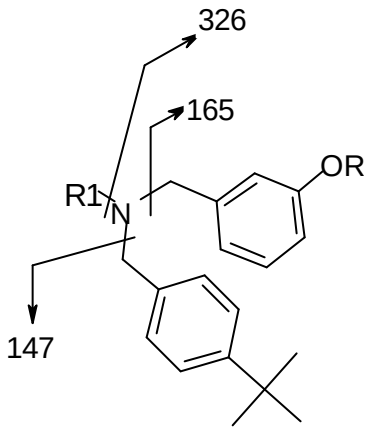
Characterization of Metabolites of Compound X

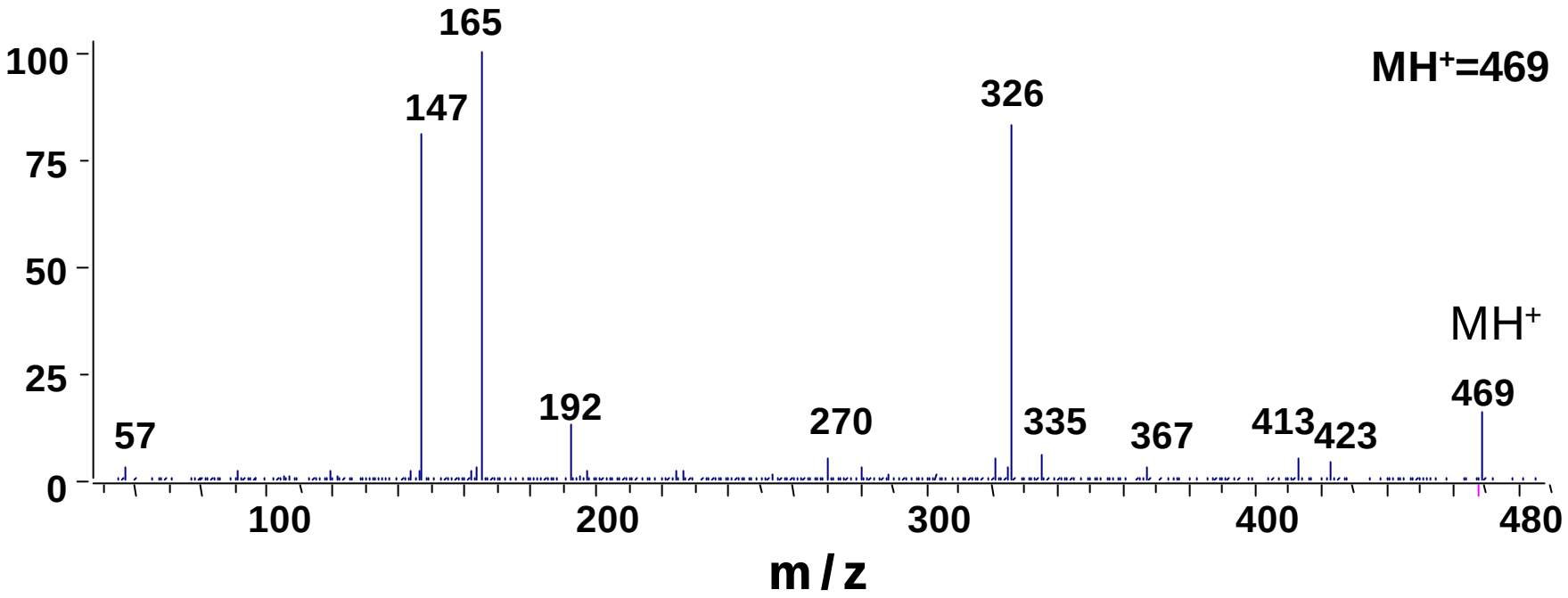


HPLC Radiochromatograms of Compound X Metabolites

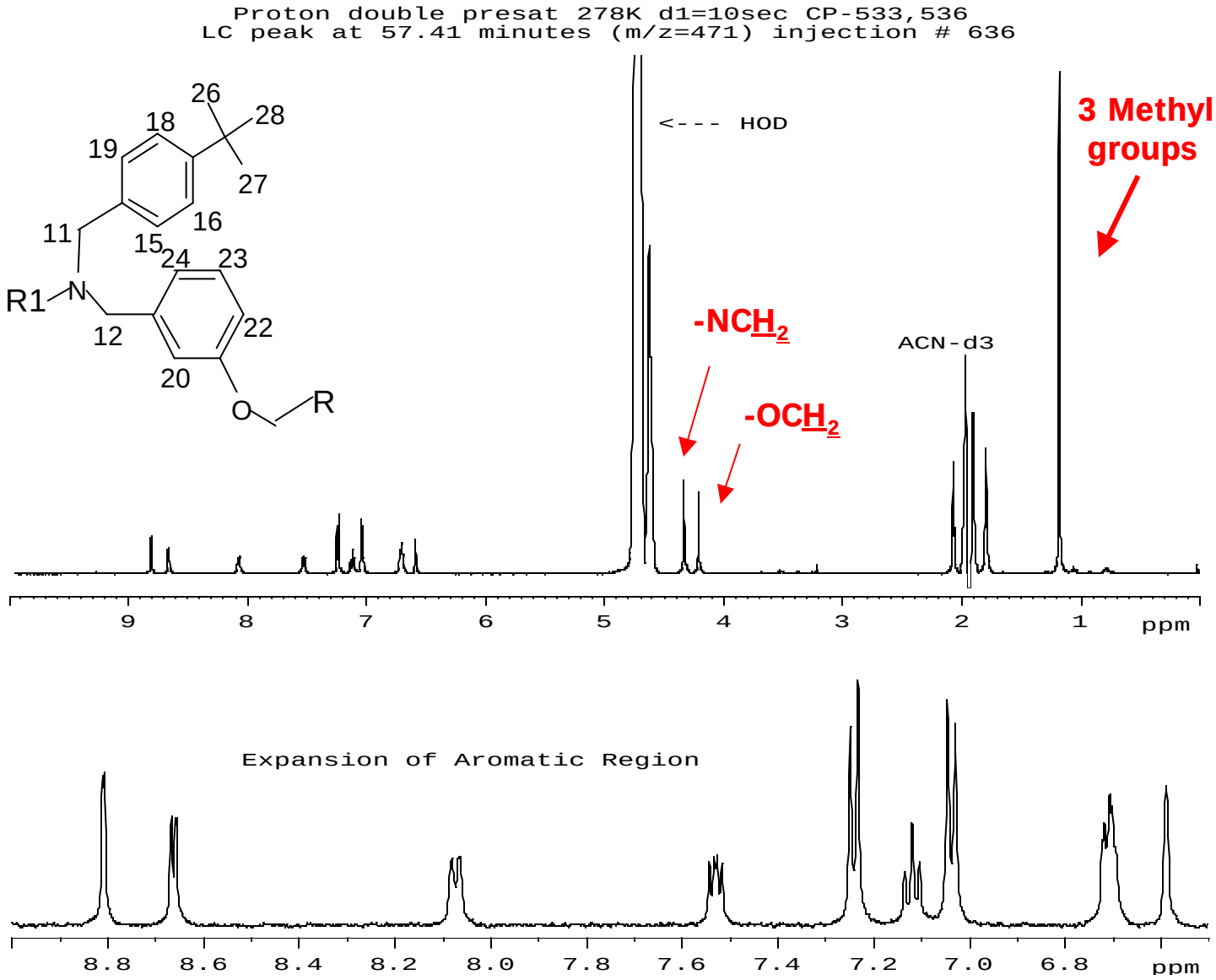


CID Product Ion Spectrum of compound X

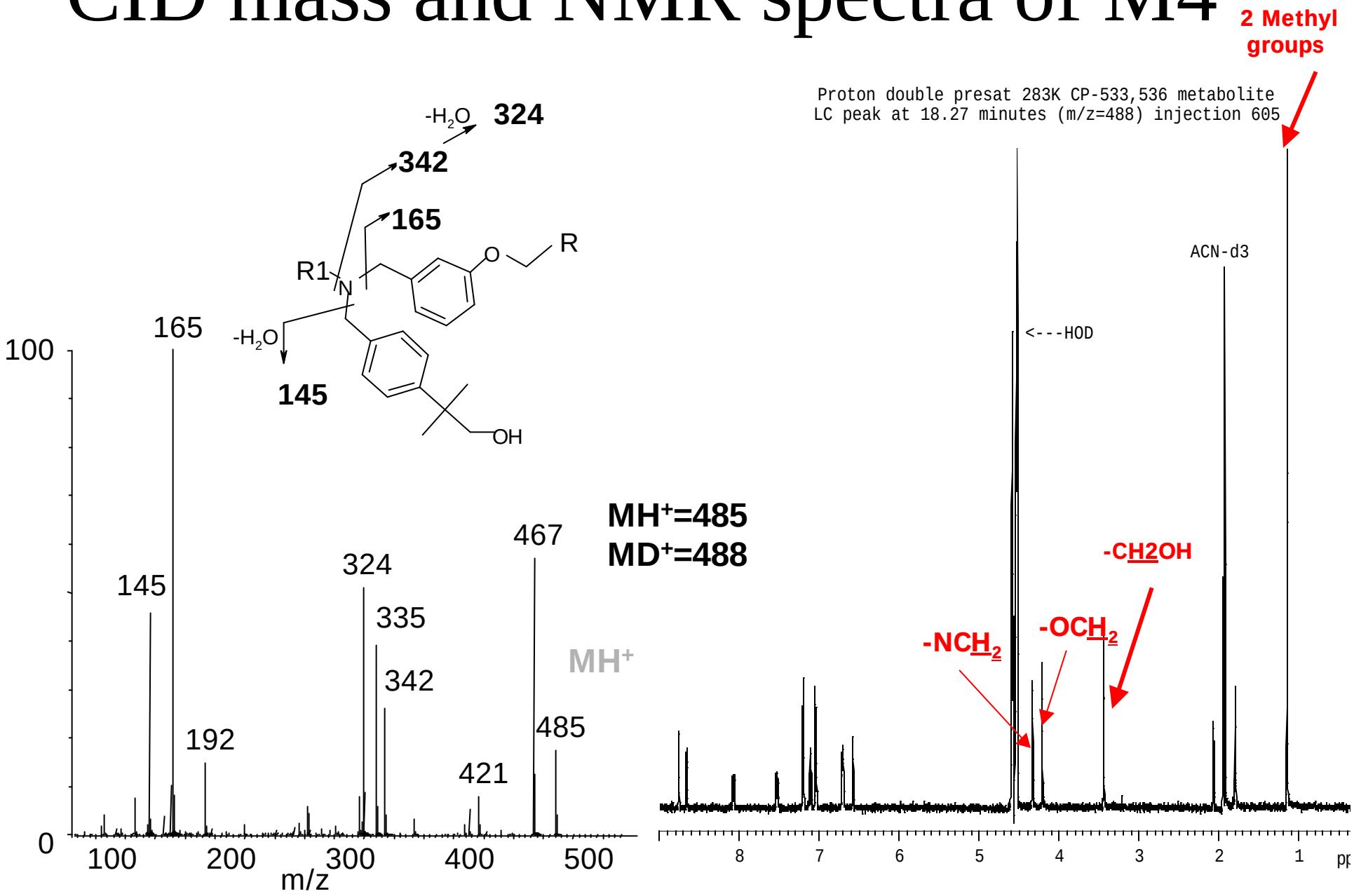




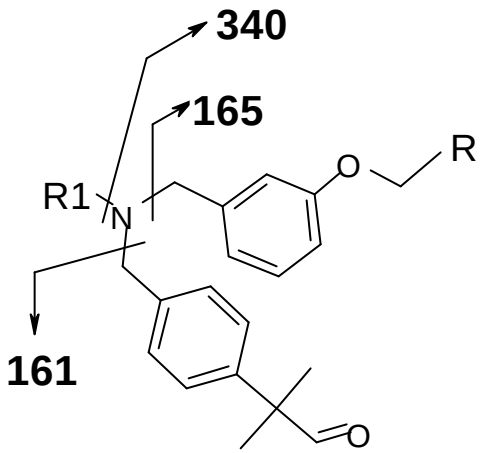
^{1}H NMR of Compound X



CID mass and NMR spectra of M4

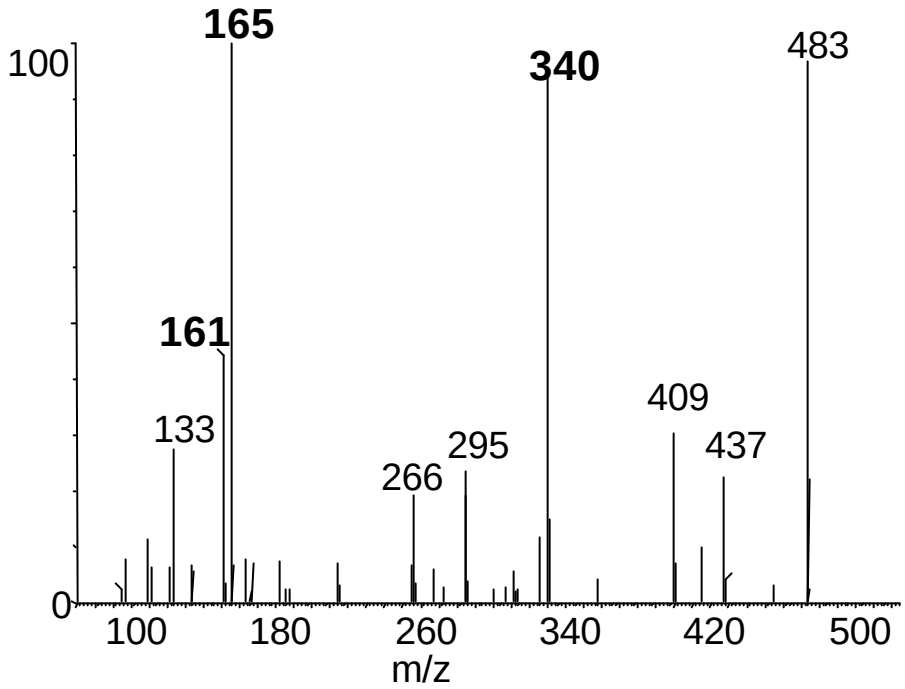


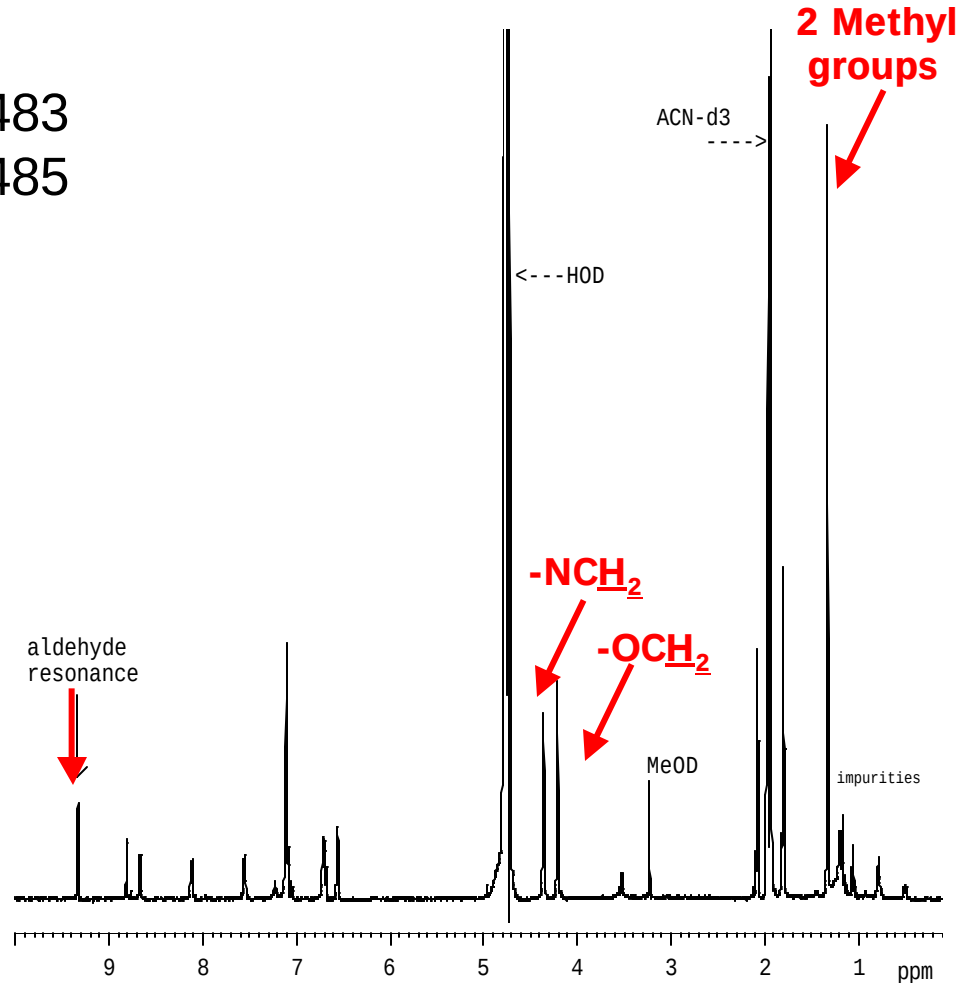
CID mass and NMR spectra of M24



$MH^+ = 483$

$MD^+ = 485$





Conclusions

- Combination of LC/MS/MS with other analytical approaches is a powerful tool for solving difficult problems encountered in the analysis of drug metabolites.

SOME REFERENCES

- *Biochemistry of reactions by Bernard Testa.*
- *Biotransformation of Xenobiotics - Andrew Parkinson - in Casarett and Doull's Toxicology, 5th edition.*
- *Drug Biotransformations - Neal Castagnoli - in Burgers medicinal chemistry 4th edition.*
- *Drug Metabolism -Bernard Testa- in Burgers Medicinal Chemistry, 5th edition.*
- *Metabolism of Heterocycles by L. A. Damani in Comprehensive Heterocyclic Chemistry*