

Mass Spectrometry for Steroids, Immunosuppressants, and Other Clinical Applications

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Purpose

- Match MS technology to application

Outline

- MS overview
- Application examples
 - Validated
 - Potential
- Hypothetical decision process

Reasons to Consider MS

- “Universal” detector
- Analytical specificity
- Multiple analytes
- Low consumable cost
- Fast
- Sensitive
- Complementary MS technologies

Reasons Not to Consider MS

- Capital cost
- Maintenance requirements
- Skill requirements
- Unsuitable sample types

Common Clinical MS Applications

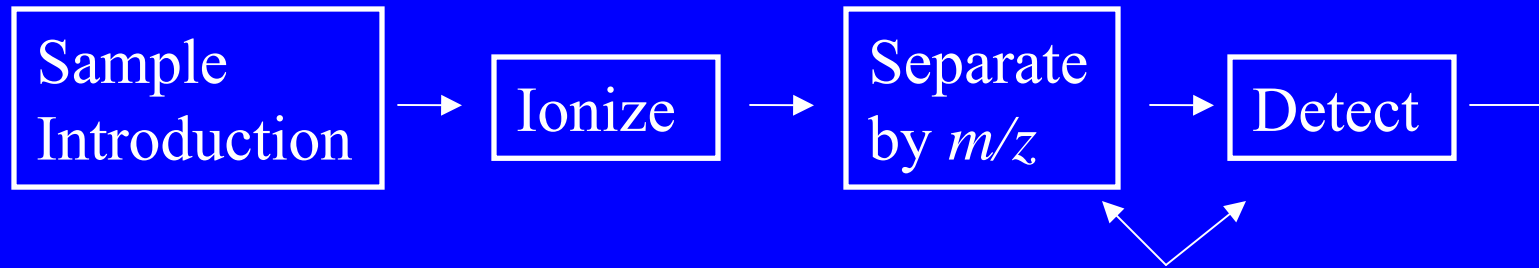
- Newborn screening (MS/MS)
- Therapeutic drug monitoring (LC-MS/MS)
- Drugs of abuse testing (GC-MS, LC-MS/MS)
- Steroids (GC-MS, LC-MS/MS)
- Endogenous compounds (GC-MS, LC-MS/MS)
- Trace elements (ICP-MS)

Less Common or Potential Clinical MS Applications

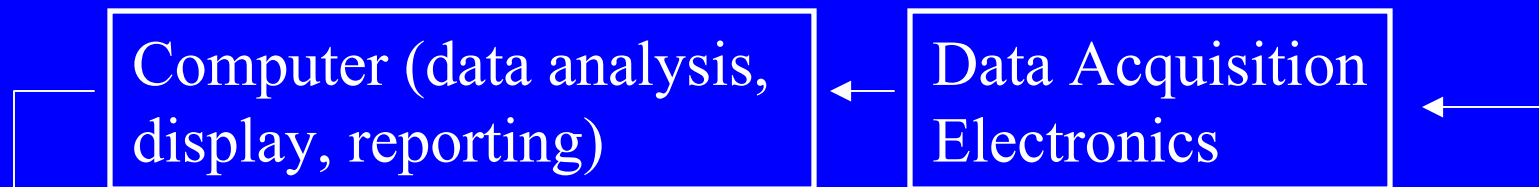
- Other small molecules
- Trace element speciation
- Hemoglobin
- Tumor markers
- Other disease markers
- Genotyping

Mass Spectrometer Block Diagram

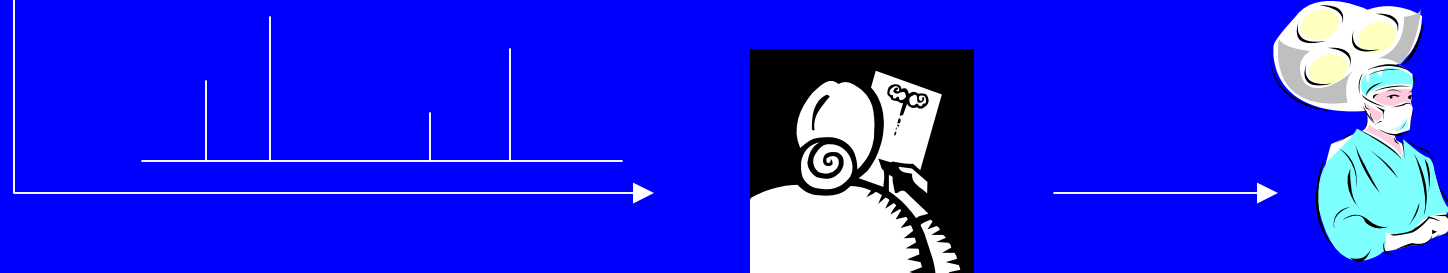
MS Hardware Level



MS Data Level (vacuum)



Information Level



“Ion Sourcery”

- Electron ionization (EI)
- Electrospray ionization (ESI)
- Atmospheric chemical ionization mass spectrometry (APCI)
- Matrix assisted laser desorption ionization (MALDI)
- Inductively coupled plasma (ICP)

EI

- Small molecules - volatile
- Analyte fragmentation (“hard” ionization)
- Analyte “fingerprinting”
- In-vacuum ionization
- Very reproducible and robust
- GC-MS

ESI

- Small to very large molecules
(polar/ionic/non-volatile)
- No fragmentation (“soft” ionization)
- Molecular weight
- Atmospheric pressure ionization
- Somewhat reproducible and robust
- LC-MS/MS

APCI

- Operational properties roughly similar to electrospray
- Better for less polar, low to medium molecular weight molecules

ICP

- Elemental analysis
- Very hard ionization (atomization)
- Atomic weight
- Atmospheric pressure ionization
- Polyatomic interferences

MS Analyzer Technologies

- Quadrupole MS
- Time-of-flight MS (TOF)
- Magnetic sector
- Ion trap (several types)

Quadrupole MS

- Low to medium mass resolution
- Moderate mass accuracy
- High sensitivity - selected ion monitoring (SIM)
- Moderate sensitivity for full spectrum
- User friendly
- Popular

Time-of-flight (TOF)

- Medium to medium-high resolution (2500-15,000)
- Accurate mass
- High sensitivity for full mass spectrum
- Medium sensitivity for SIM
- Highest scan speed

Magnetic Sector

- High resolution (5000-100,000)
- Accurate mass
- High sensitivity for SIM
- Slowest scan
- Big, heavy, old fashioned

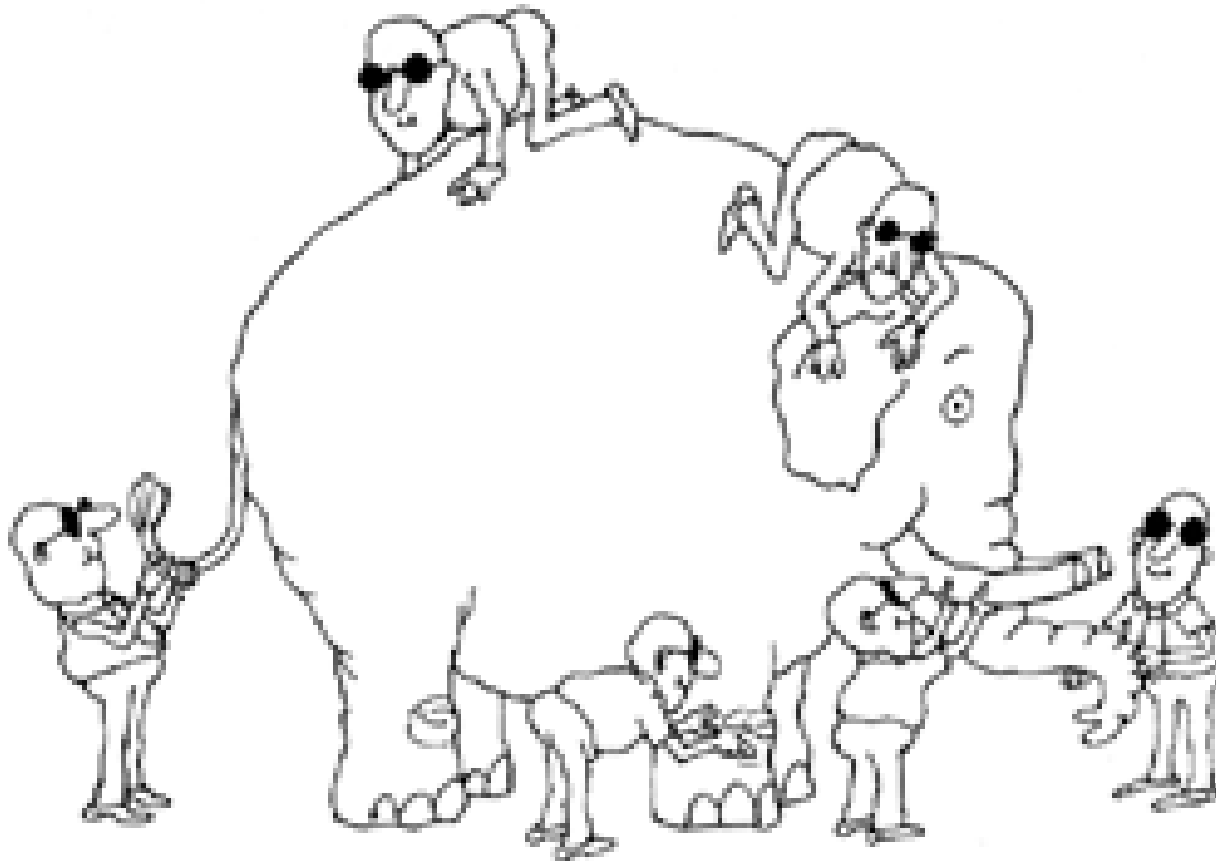
Learn More About MS Analyzer Types

- http://jeolusa.com/ms/docs/ms_analyzers.html

Instrument Configurations

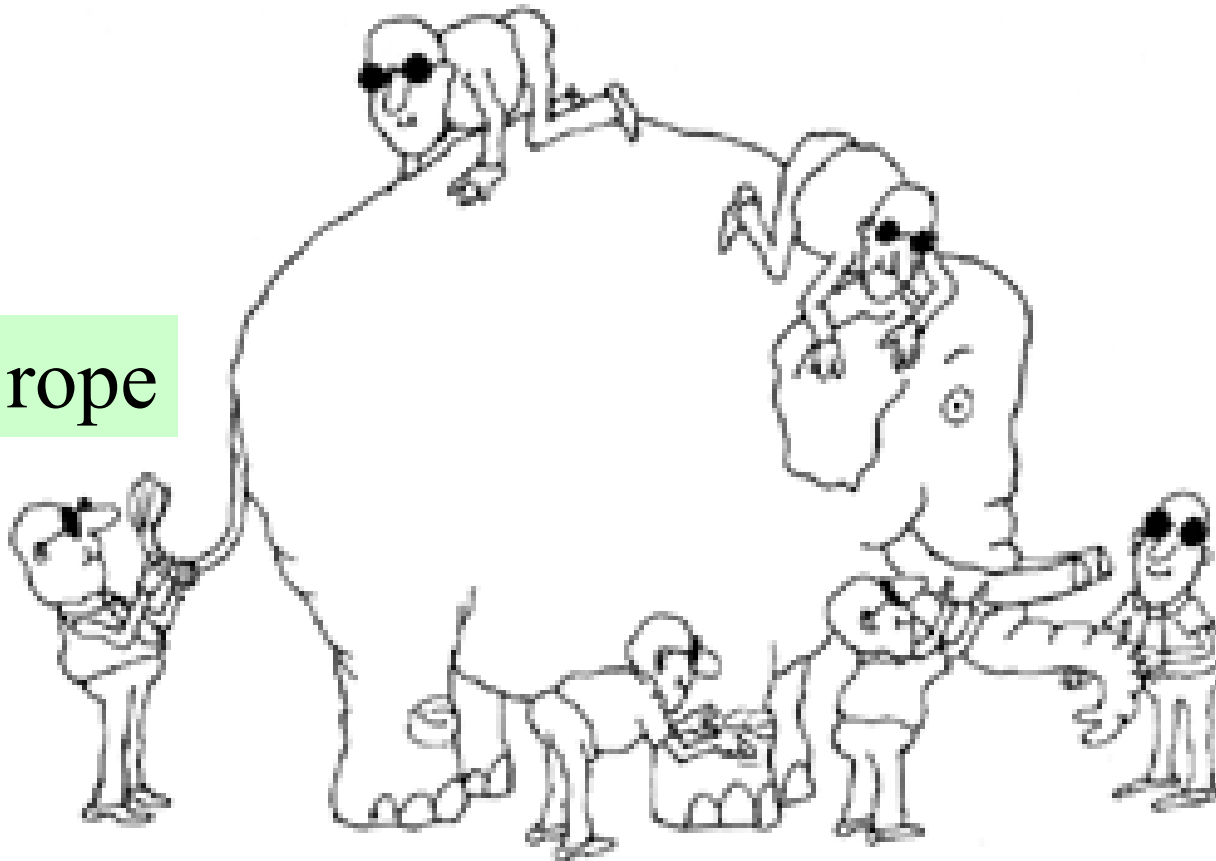
- Stand alone single stage MS
- Chromatography-MS (GC-MS, LC-MS)
- Tandem MS (MS/MS)

The Fundamental Analytical Problem: How to Characterize an Elephant

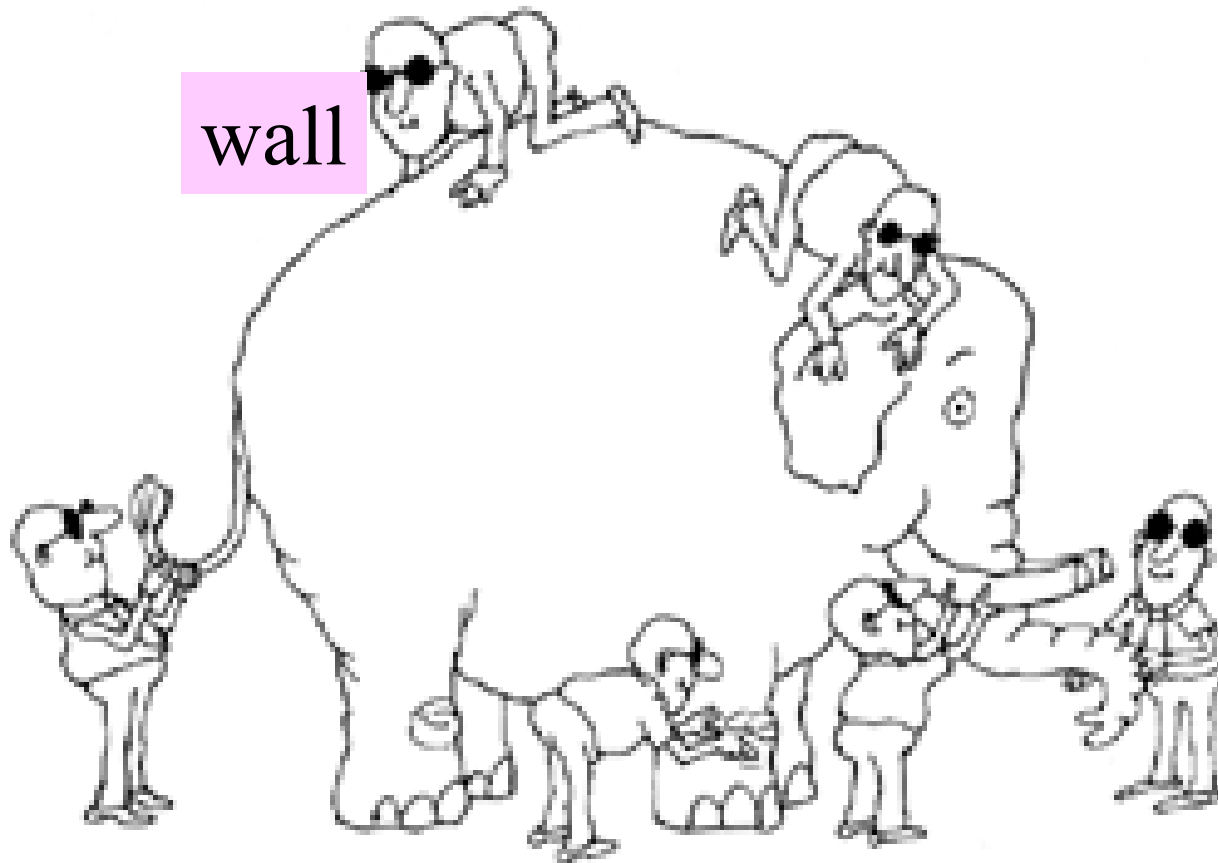


Is it a rope?

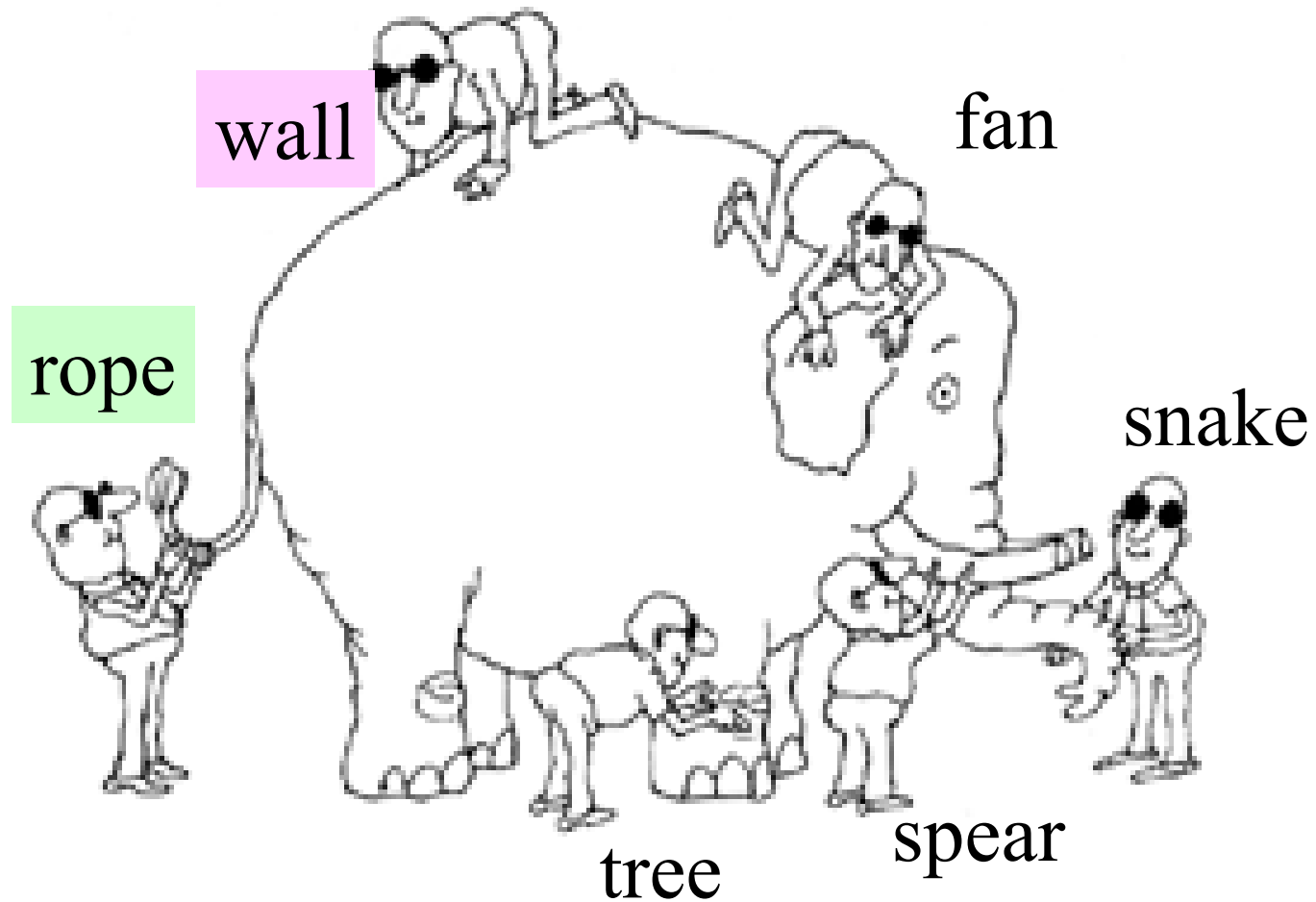
rope



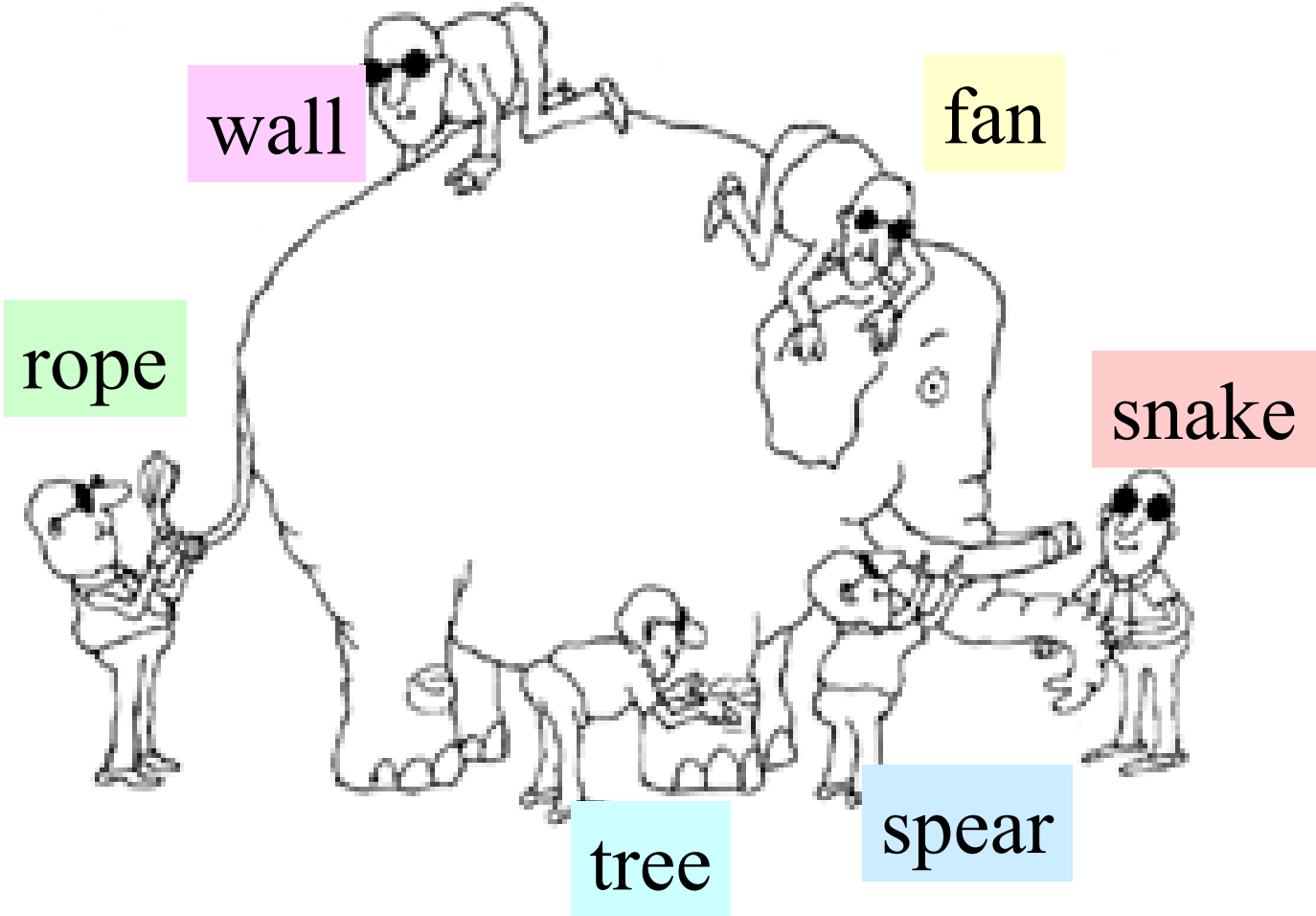
Is it a wall?



No, it is an elephant!



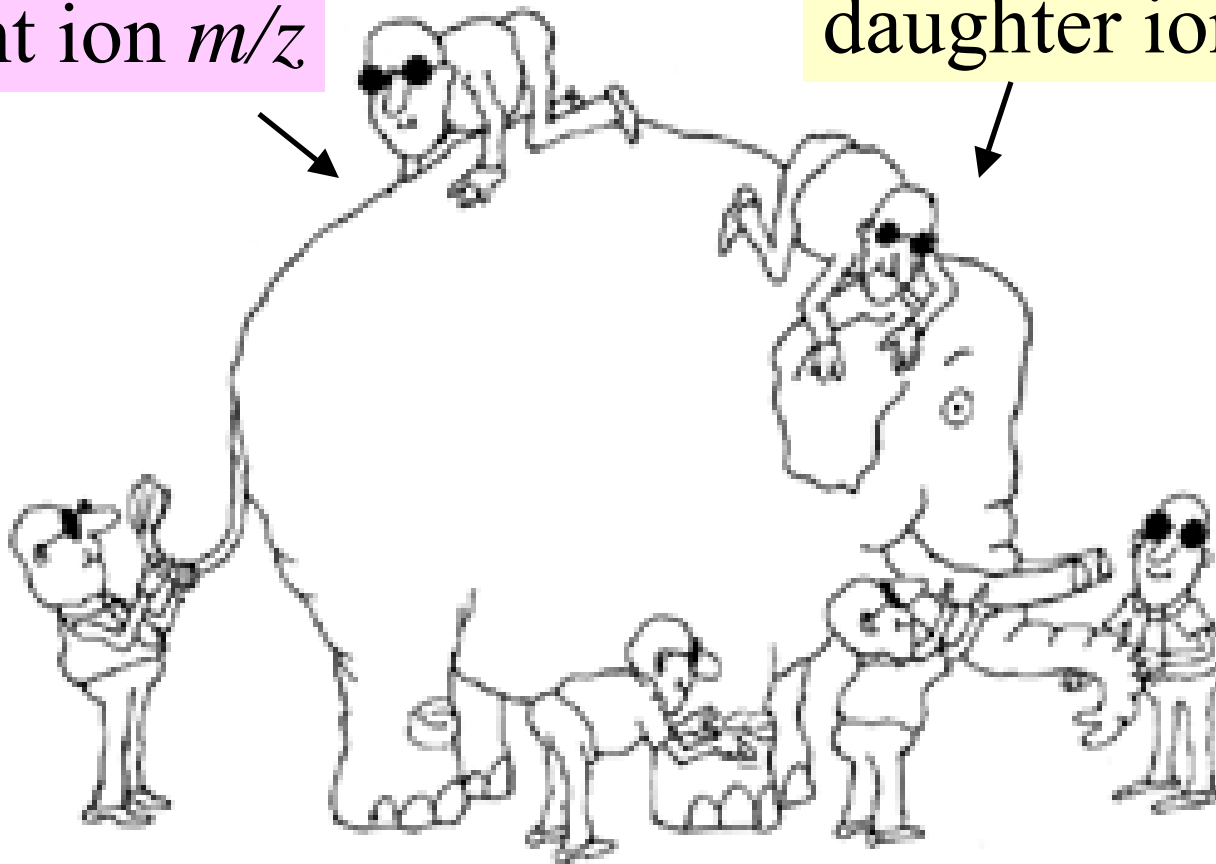
More Is Better



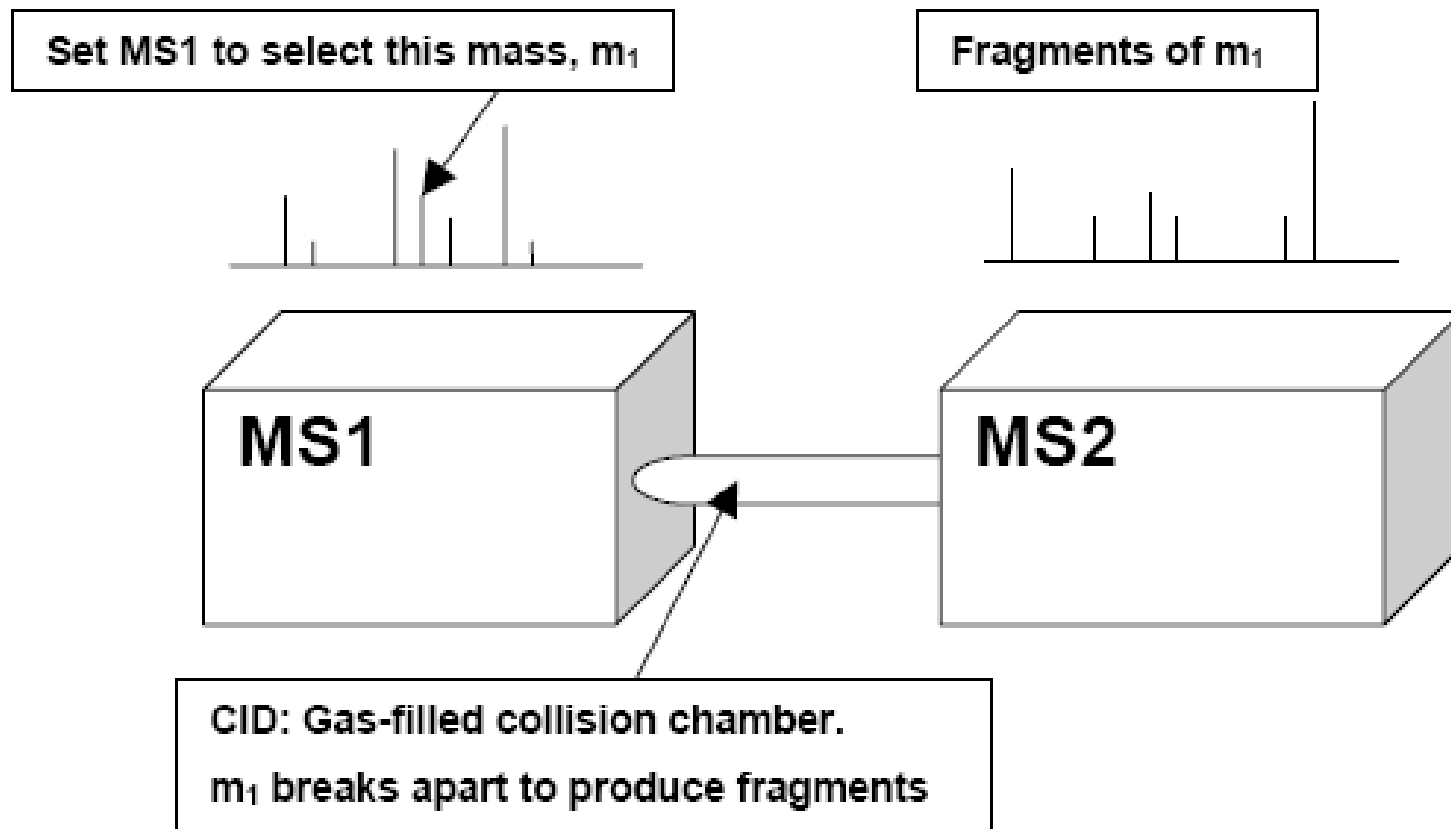
“Hyphenation” = Two or More Physical Properties (MS-MS)

parent ion m/z

daughter ion m/z

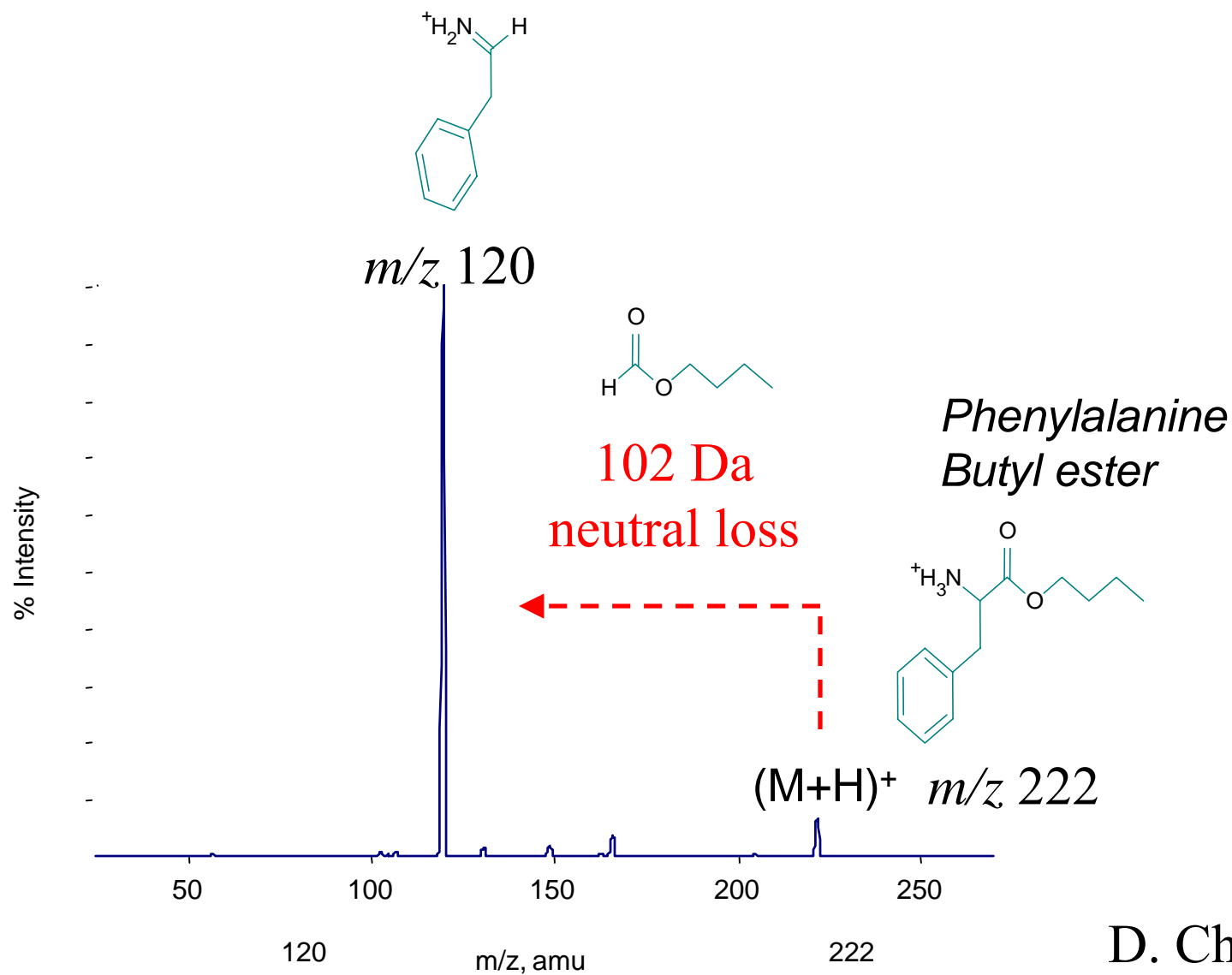


MS-MS (Tandem MS)



<http://jeolusa.com/ms/docs/ms-ms.pdf>

Amino acid MS-MS



D. Chace

Application

- Newborn screening by MS-MS
- Donald Chace, *Pediatrics*

Illustrates

- Multianalyte capability
 - Acylcarnitines (multiple)
 - Amino acids (multiple)
- Selectivity of MS-MS
- Simple sample preparation

Samples and Method

- Dried blood spots
- Solvent extract & dry
- Derivatize (butyl ester)
- Electrospray MS-MS (triple quadrupole)

MS-MS Selective for Aylcarnitine Butyl Esters

(precursor ion scan)



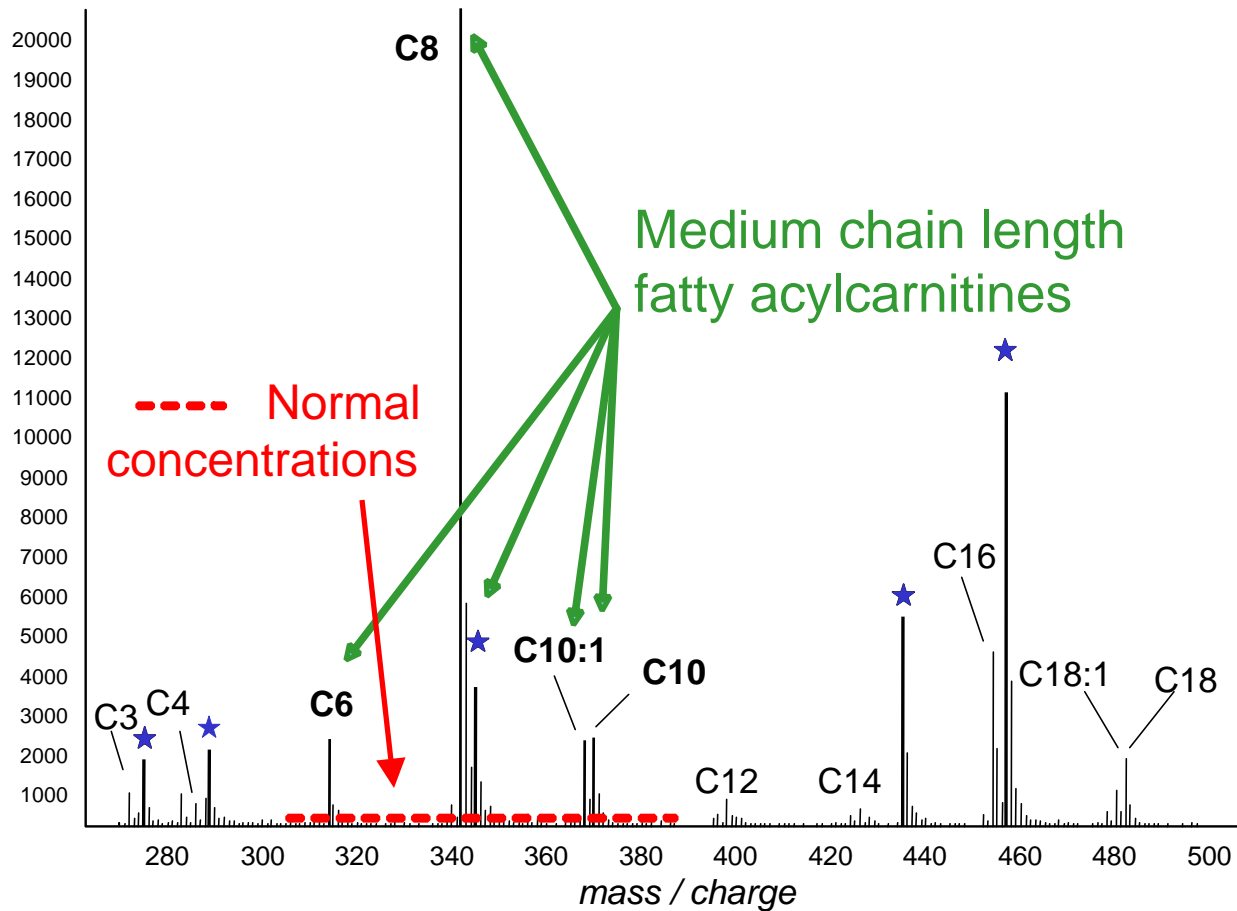
Scan MS1 for
 m/z of parent ion



Set MS2 for
 m/z 85
daughter ion

MCAD Deficiency

Homozygous A985G



Take Home Lesson

- MS-MS specificity → simple sample preparation
- Simple sample prep → low consumable \$
- MS-MS speed → high throughput
- Full spectrum capability → one method, many analytes

Application

- Amino acids by LC-TOFMS
- Paul Kennedy, Leco Corp.

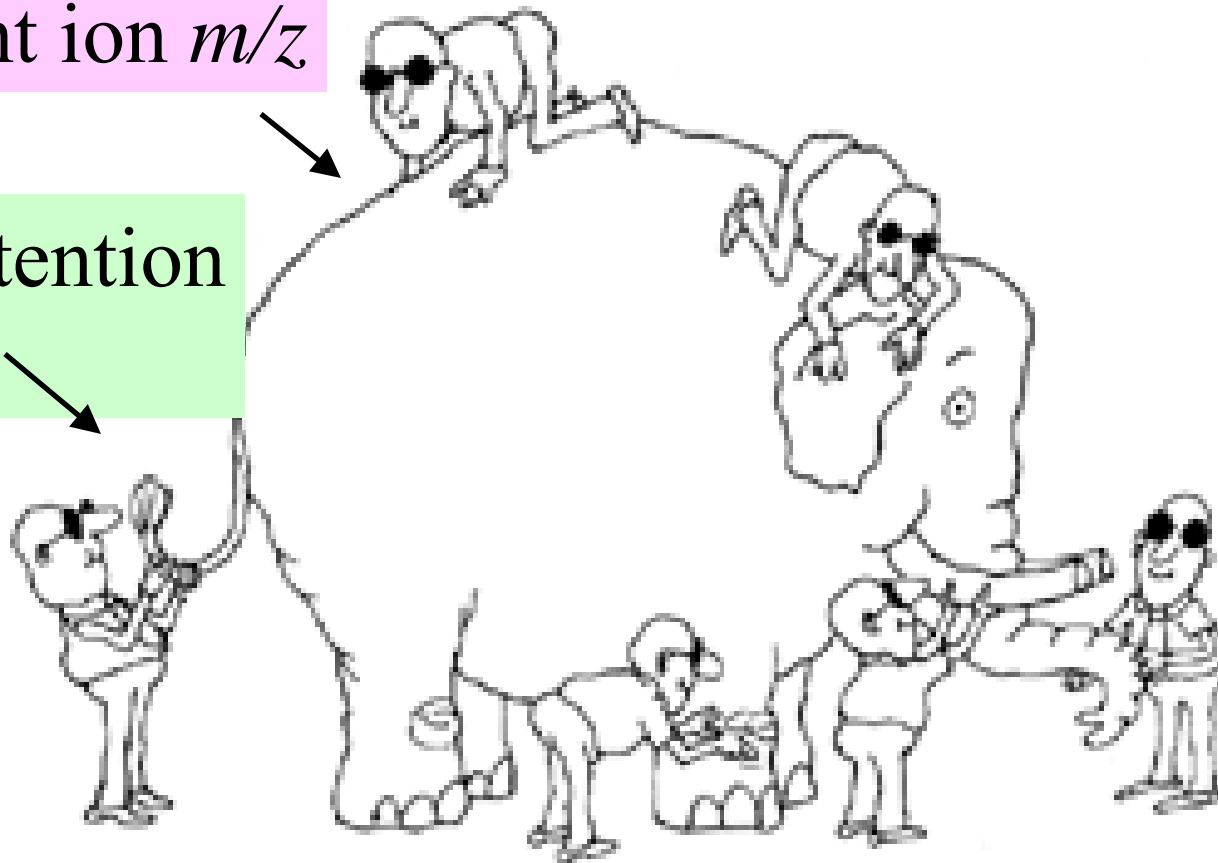
Illustrates

- LC-MS hyphenation
- TOF multianalyte advantage
- Analyte confirmation by accurate mass

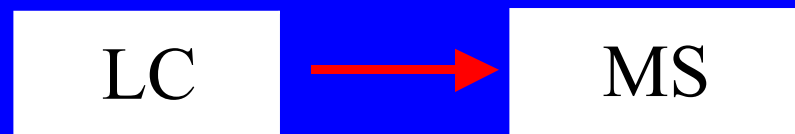
LC-MS: Retention Time & Molecular Weight

parent ion m/z

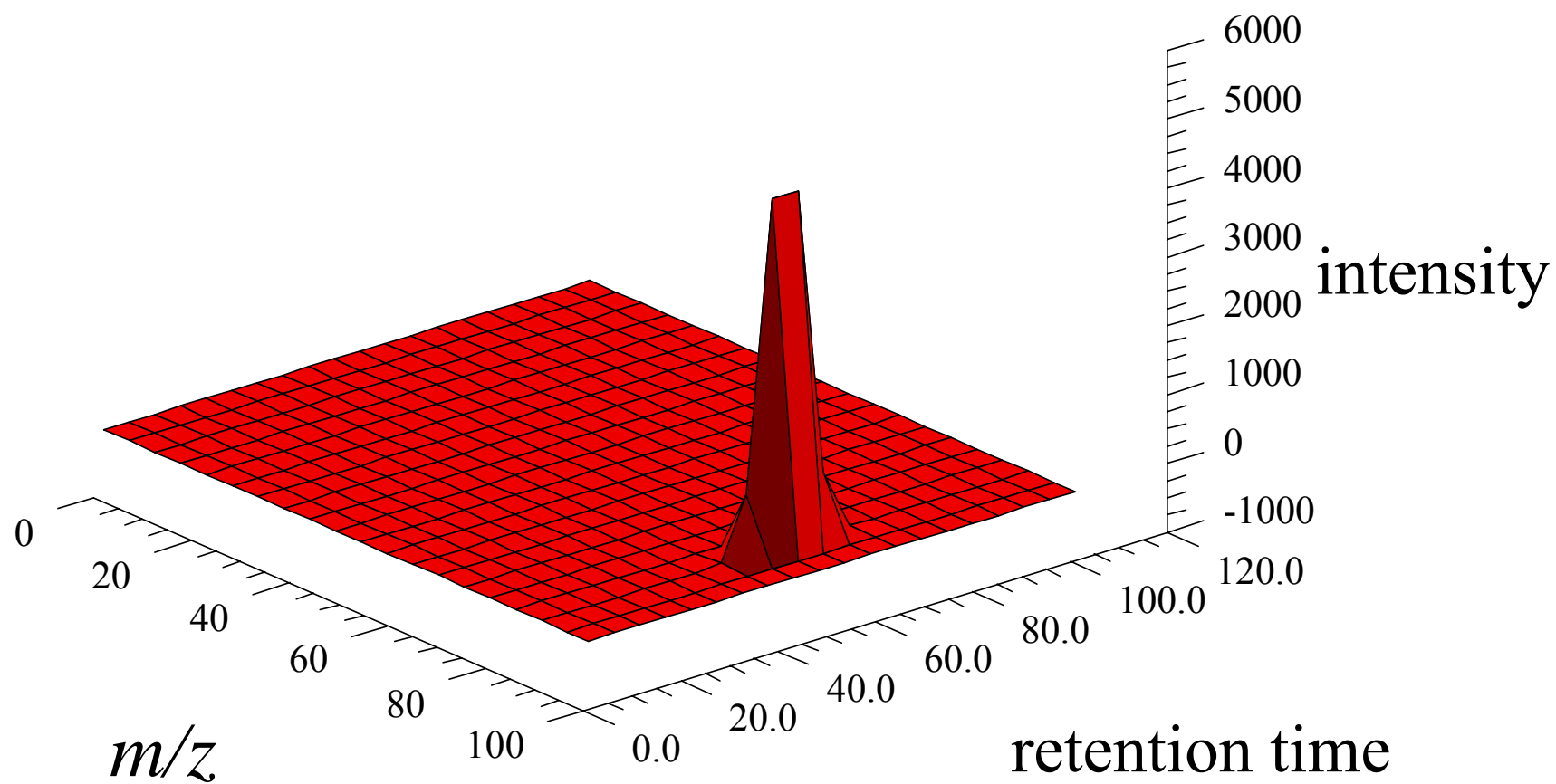
LC retention time



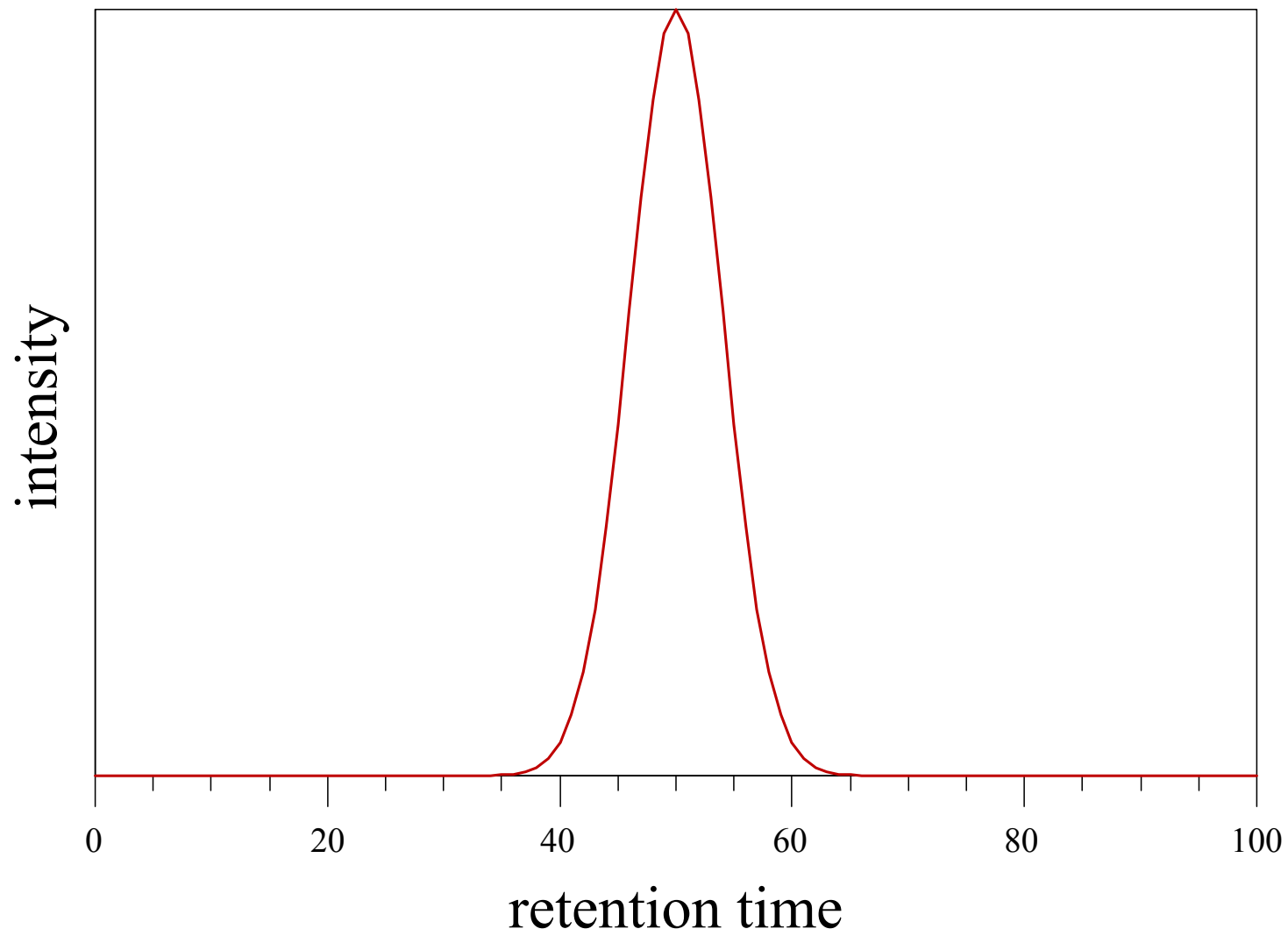
Block diagram of LC-MS



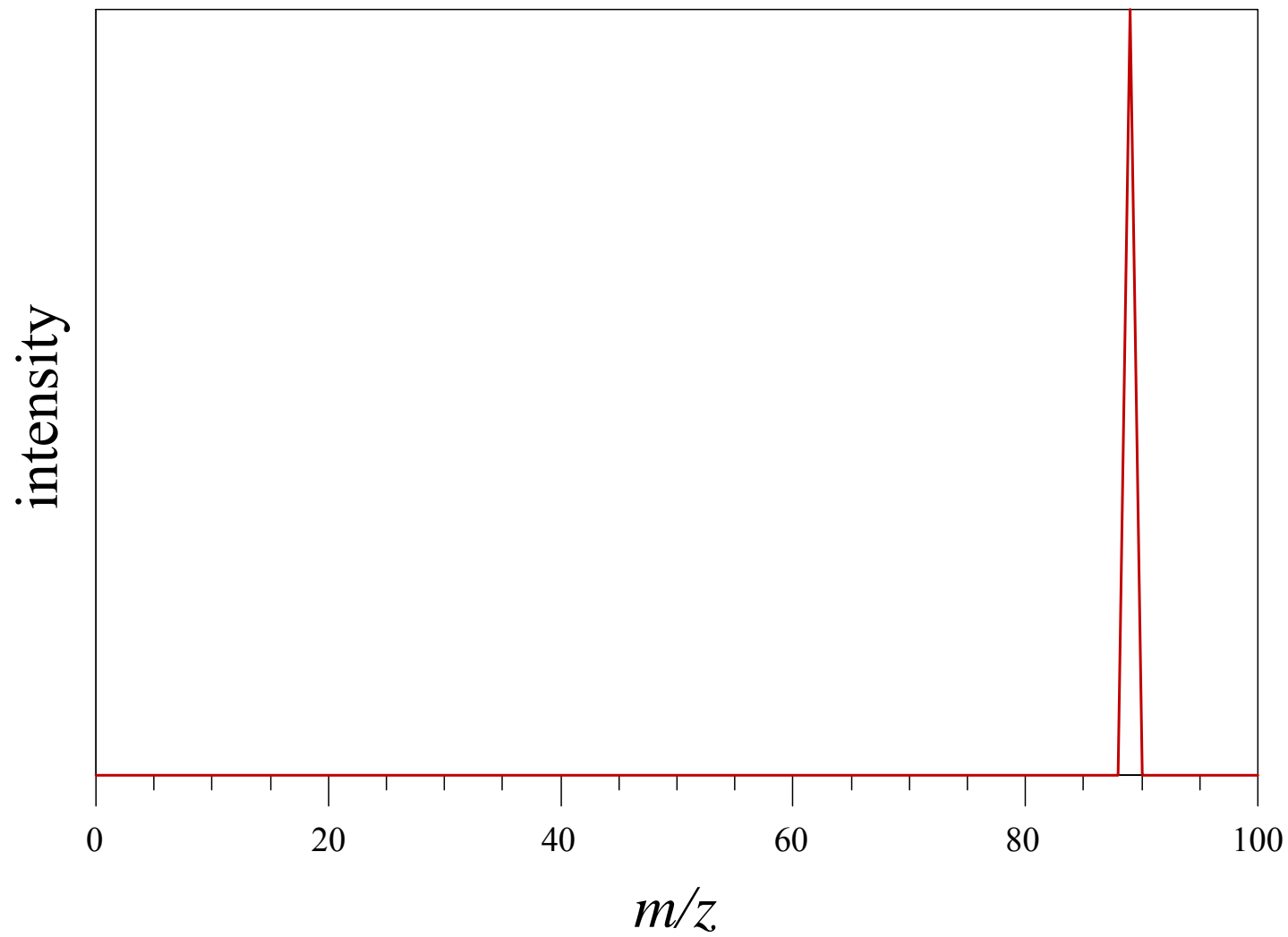
LC-MS Data Concept



Chromatogram



Mass Spectrum



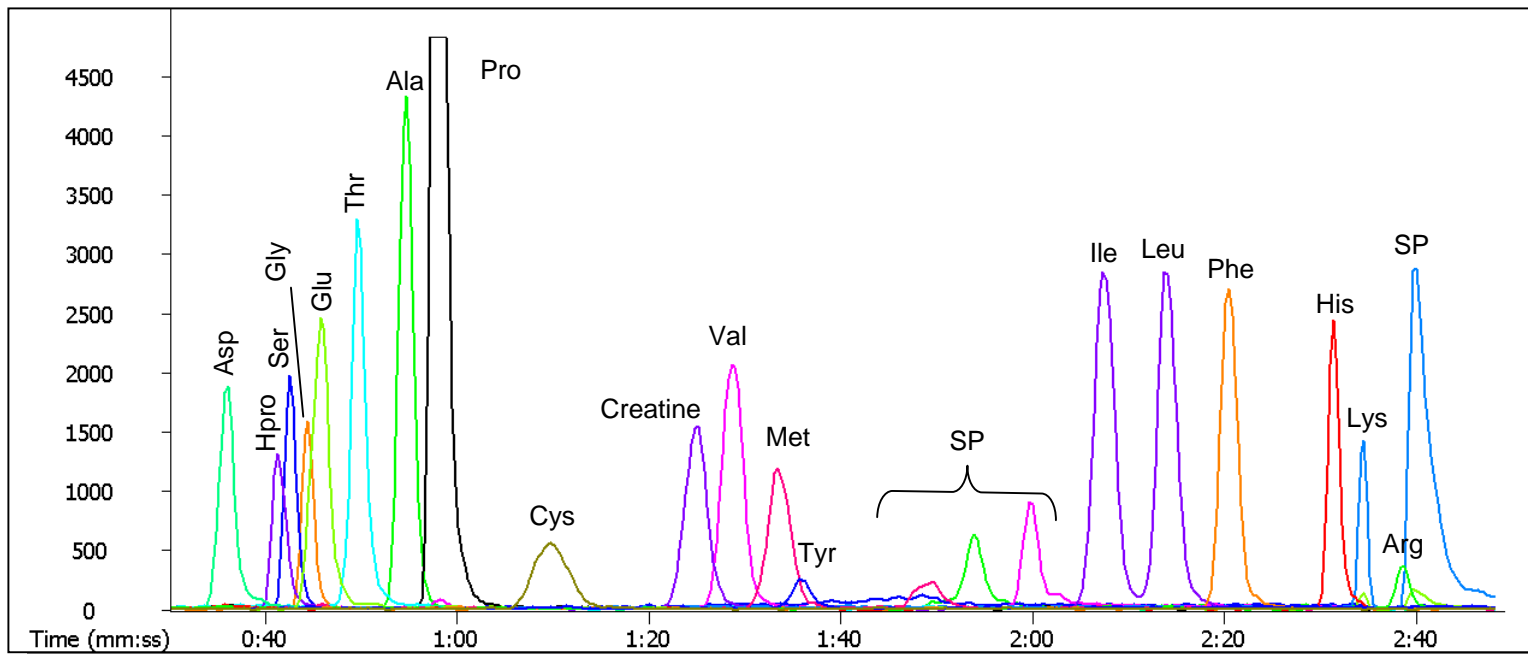
Samples and Method

- Spiked standards, 19 compounds
 - 17 amino acids + creatine + hydroxyproline
- C18 liquid chromatography
- Electrospray Time-of-flight MS



Fast LC Separation

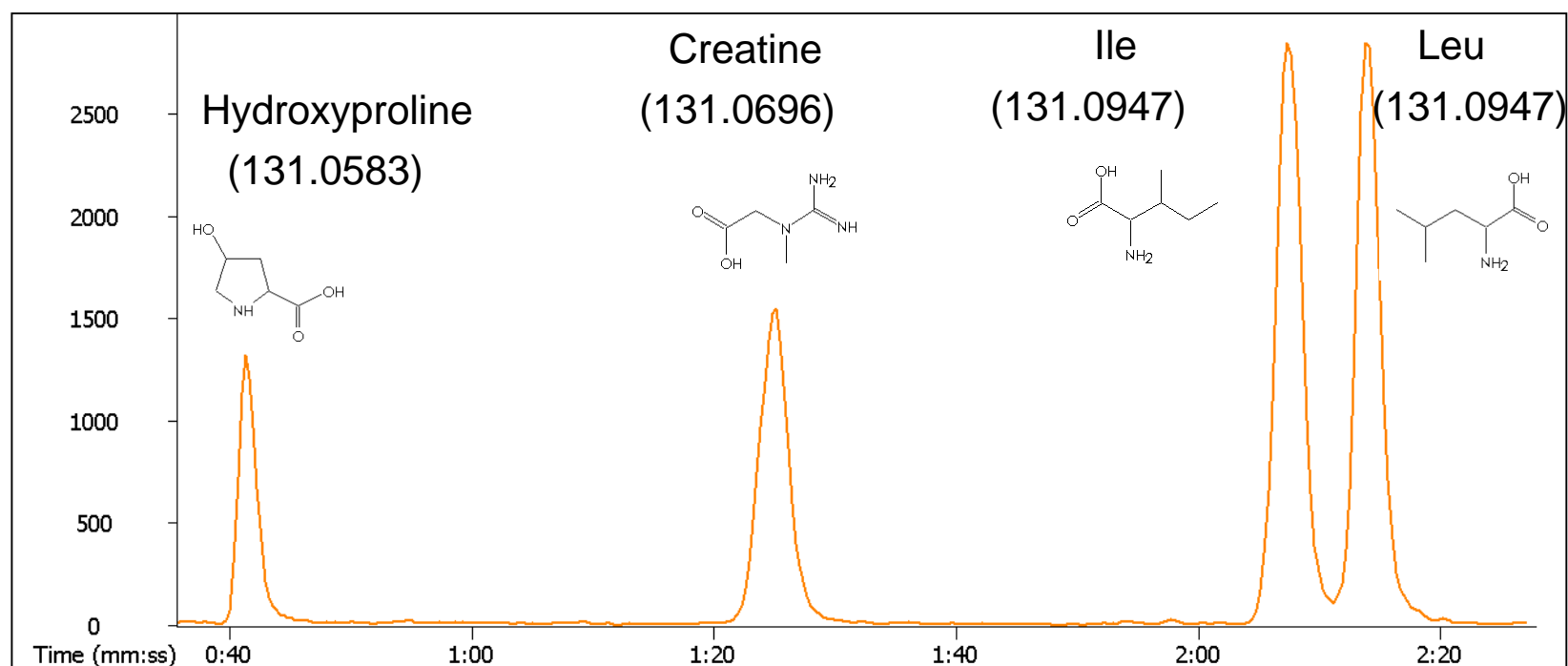
17 amino acids, creatine and hydroxyproline, < 3 minutes



LOD 6 pg for proline to 400 pg for tyrosine

Separation of Common Isobars

Common isobaric interferences hydroxyproline and creatine are easily separated and identified from leucine and isoleucine



Confirmation by Accurate Mass

All 19 components correctly identified by accurate mass measurement

Peak #	R.T. (min:sec)	AA	Unique Mass	Actual	Mass Accuracy (mDa)	Calculated Formula	Actual Formula	Number of Possible Structures
1	00:36.2	Asp	134.0441	134.0448	0.7	C4 H7 O4 N	C4 H7 O4 N	1
2	00:41.3	HydPro	132.0664	132.0656	0.8	C5 H9 O3 N	C5 H9 O3 N	1
3	00:42.6	Ser	106.0522	106.0499	2.3	C3 H7 O3 N	C3 H7 O3 N	1
4	00:44.5	Gly	76.04	76.0393	0.7	C2 H5 O2 N	C2 H5 O2 N	1
5	00:45.8	Glu	148.0588	148.0605	1.7	C5 H9 O4 N	C5 H9 O4 N	1
6	00:49.6	Thr	120.0621	120.0656	3.5	C4 H9 O3 N	C4 H9 O3 N	1
7	00:54.7	Ala	90.0561	90.0550	1.1	C3 H7 O2 N	C3 H7 O2 N	1
8	00:57.9	Pro	116.0709	116.0707	0.2	C5 H9 O2 N	C5 H9 O2 N	1
9	01:09.8	Cys	241.028	241.0312	3.2	C6 H12 O4 N2 S2	C6 H12 O4 N2 S2	2
10	01:25.1	Creatine	132.0762	132.0769	0.7	C4 H9 O2 N3	C4 H9 O2 N3	1
11	01:28.6	Val	118.0864	118.0863	0.1	C5 H11 O2 N	C5 H11 O2 N	1
12	01:33.4	Met	150.0582	150.0584	0.2	C5 H11 O2 N S	C5 H11 O2 N S	2
13	01:35.7	Tyr	182.0837	182.0812	2.5	C9 H11 O3 N	C9 H11 O3 N	1
14	02:07.4	Ile	132.0993	132.1020	2.7	C6 H13 O2 N	C6 H13 O2 N	1
15	02:13.8	Leu	132.1009	132.1020	1.1	C6 H13 O2 N	C6 H13 O2 N	1
16	02:20.5	Phe	166.0843	166.0863	2.0	C4 H11 O4 N3	C4 H11 O4 N3	2
17	02:31.4	His	156.0777	156.0769	0.8	C6 H9 O2 N3	C6 H9 O2 N3	1
18	02:34.6	Lys	147.113	147.1129	0.1	C6 H14 O2 N2	C6 H14 O2 N2	1
19	02:38.4	Arg	175.1217	175.1191	2.6	C6 H14 O2 N4	C6 H14 O2 N4	1

Elements C, H, N, O and S were chosen for formula calculation with a threshold of 5 mDa mass accuracy range.

Take Home Messages

- TOF generally best technology for full spectrum single stage MS
 - Multiple analytes
 - Fastest
 - Most sensitive (for full scan)
- Accurate mass confirms analyte identity

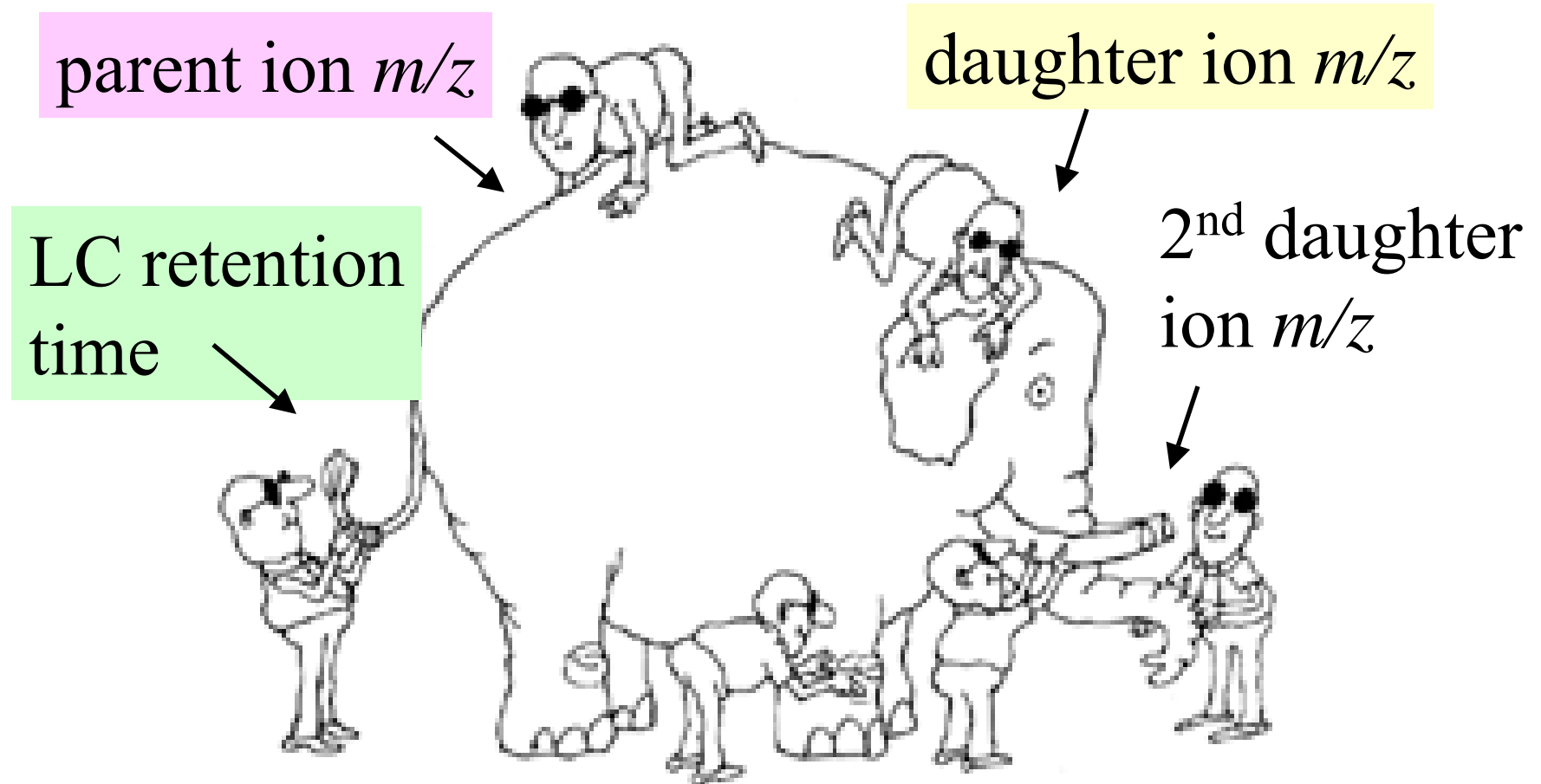
Application

- Sirolimus by LC-MS-MS
 - Triple quadrupole MS-MS
 - Sirolimus
- Mark Kushnir, ARUP

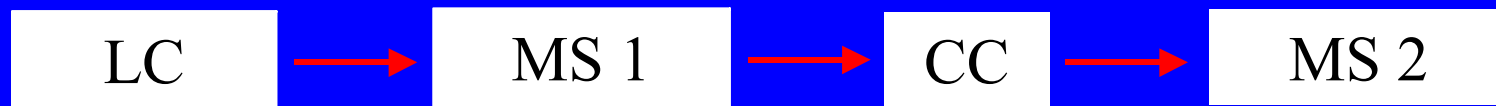
Illustrates

- LC-MS-MS hyphenation
- LC-MS-MS selectivity enables simple sample preparation and method

LC-MS-MS: Three Physical Properties



Block diagram of LC-MS-MS

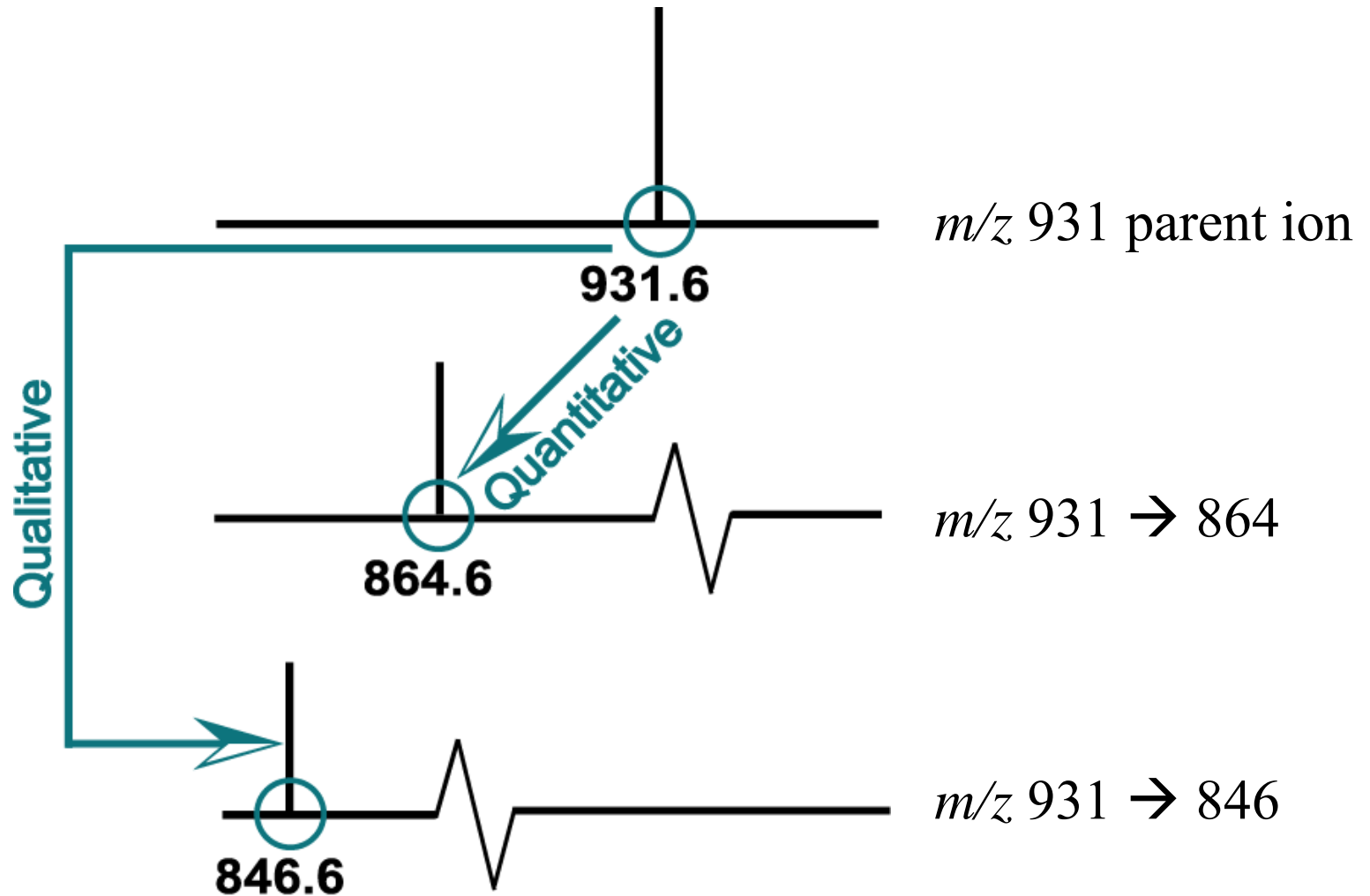


MRM = specific parent-daughter ion pairs

Samples and Method

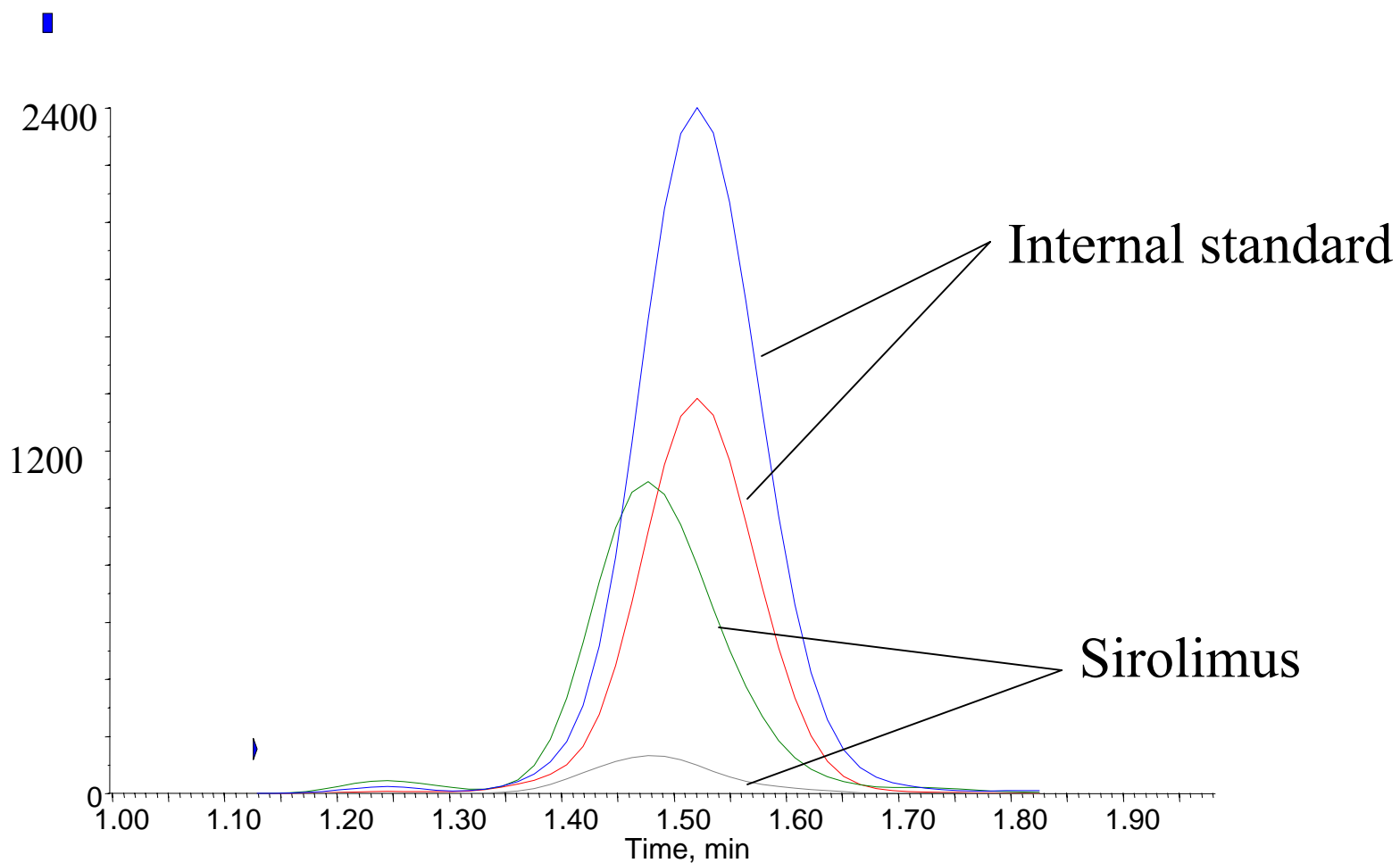
- Lysed Whole blood (250 μ L)
- Protein precipitation
- C18 liquid chromatography (step gradient)
- Electrospray MS-MS (Applied Biosystems Sciex API 3000)

Sirolimus: MS-MS transitions



Sirolimus: Typical Chromatogram

(sample containing 9 ng/mL of sirolimus)



Performance

Limit of detection	1 ng/mL
Limit of quantitation	2 ng/mL
Upper limit of linearity	100 ng/mL
Analysis time	3 min

Take Home Lessons

- LC-MS-MS more specific than LC-MS or MS-MS
- Low interference rate
- Simple sample prep

Application

- Immunosuppressants – revisited
 - Improved sample cleanup
 - Better CV
 - Reduced ion suppression
- Thomas Annesley, U. Michigan

Illustrates

- Benefits of improved sample preparation

Inter-run coefficients of variation - Three sample prep methods -

Drug	µg/L	CV,% ^b	CV,% ^b	CV,% ^b
		ZnSO ₄	H ₂ O + ZnSO ₄	H ₂ O + ZnSO ₄ + SPE
Sirolimus	10	8.6	4.4	4.3
	23	7.6	4.3	4.2
Tacrolimus	9	4.6	2.4	2.7
	17	2.7	1.7	1.4
Cyclosporine	120	4.8	3.8	3.0
	240	4.4	4.0	3.4

^b Tacrolimus (n=10), Cyclosporine (n = 10), Sirolimus (n=12)

T. Annesley

Take Home Lessons

- Sample preparation part of MS technology
- Cleaner extraction → Improved %CV

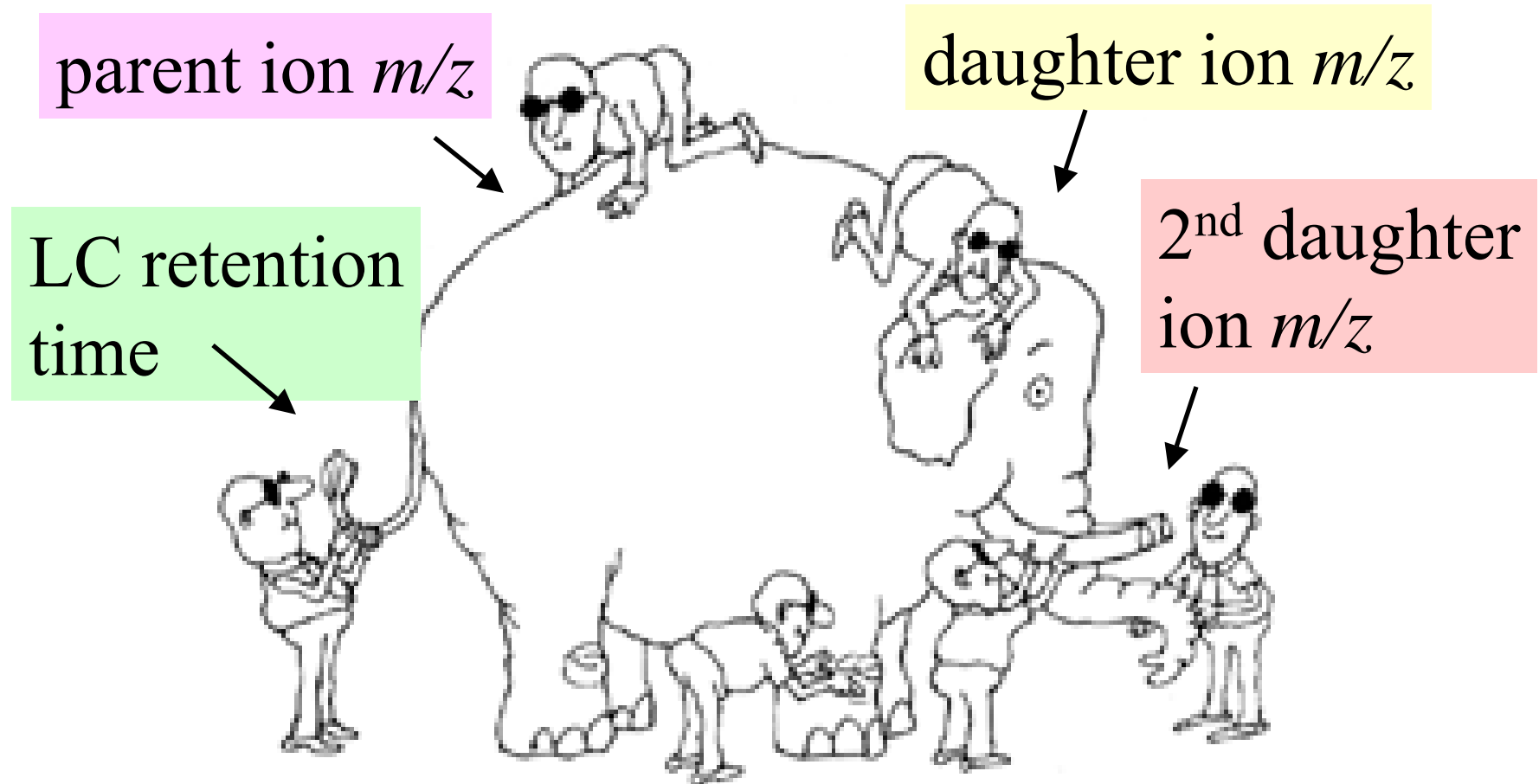
Application

- Urine free cortisol by LC-MS-MS
 - Triple quadrupole
- Mark Kushnir, ARUP

Illustrates

- LC-MS-MS is not interference-free
- Detecting and correcting interferences
 - Multiple MS/MS transitions

LC-MS-MS + Second (Sibling) Daughter Ion



Samples and Method

- Urine (24 hour preferred)
- Liquid/liquid extraction
- Phenyl-Hexyl C18 gradient LC
- APCI MS-MS
 - Quantitative: m/z 363 \rightarrow m/z 121
 - Qualitative: m/z 363 \rightarrow m/z 97
 - Quant./Qual. ratio: $\pm 30\%$

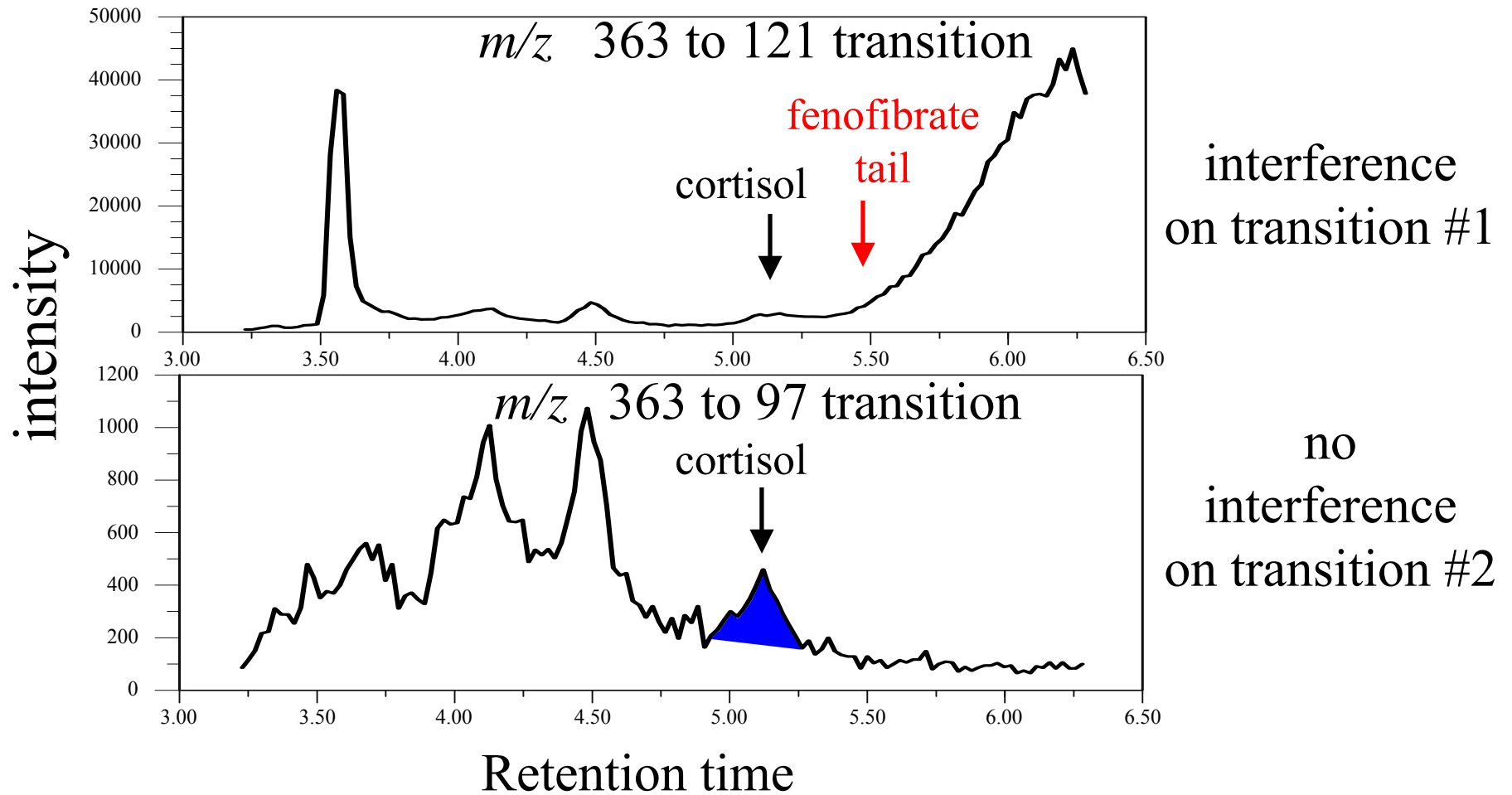
Need to Monitor More Than One MS/MS Transition

- Detect interferences (ratio method)
- Switch to secondary transition for quantitation

Patient Case

- 57 year-old woman
- Cushingoid appearance
- Discordant cortisol analytical results
- Taking **Fenofibrate**

Two MS/MS Transitions (Ratio Out of Tolerance = Interference)



Take Home Lesson

- ESI MS-MS triple quadrupole enables interference detection
 - Not possible with single quadrupole ESI MS
- MS-MS enables correction for interferences
- Great technique – use it!

Application

- Testosterone by ESI LC-MS-MS (triple quadrupole)
- Mark Kushnir, ARUP

Illustrates

- Sample prep part of technology
 - Aggressive sample cleanup
 - Derivitization
- High-end instrument (API 4000)
 - Improved sensitivity

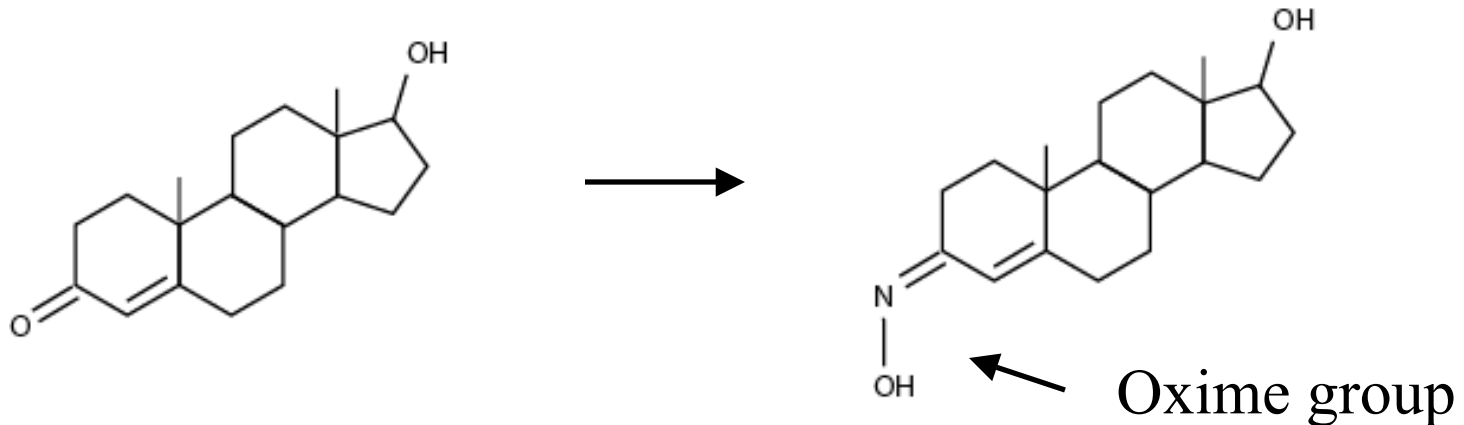
Goals

- 100 μL serum
 - Pediatric patients
 - Allow for re-inject
- 10 pg/mL
 - Women and children

Testosterone Sample Prep

- Two extractions
 - SPE Strata X (Phenomenex)
 - MTBE liquid-liquid extraction

- Derivatize

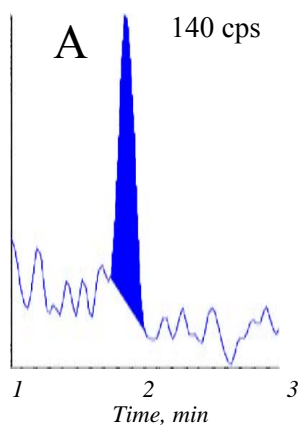


Method Summary

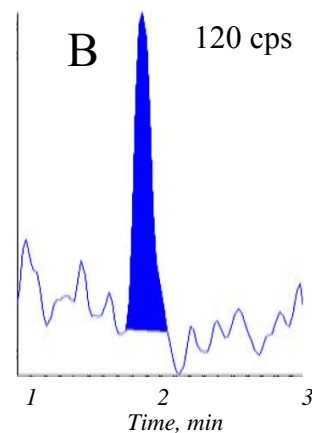
Liquid Chromatogray	C18 isocratic
MS instrument	API 4000
Interface	TurboIonspray (ESI)
MS-MS transitions:	T: m/z 304 → 124 304 → 112
	d ₃ -T: m/z 307 → 124 307 → 112
Analysis time	3 min

Chromatograms, 30 pg/mL testosterone

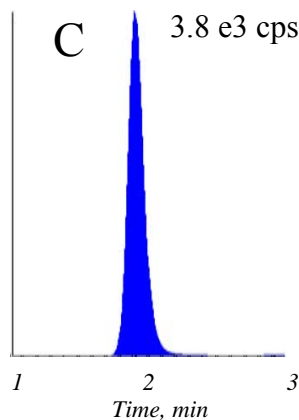
testosterone
 m/z 304 \rightarrow 124
(quantitative)



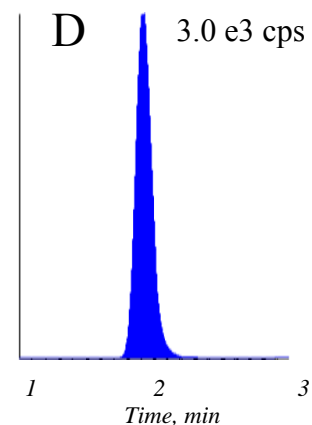
testosterone
 m/z 304 \rightarrow 112
(qualitative)



Internal standard
 d_3 testosterone
 m/z 307 \rightarrow 124
(quantitative)



Internal standard
 d_3 testosterone
 m/z 307 \rightarrow 112
(qualitative)



Outcome

- LOQ improved $> 40X$ (10 pg/mL)
- Low sample volume (100 μL)
- Re-inject possible

Take Home Lesson

- Sample prep is part of MS technology
- Improved LOQ
 - Aggressive sample cleanup
 - Derivatization
 - High end instrument

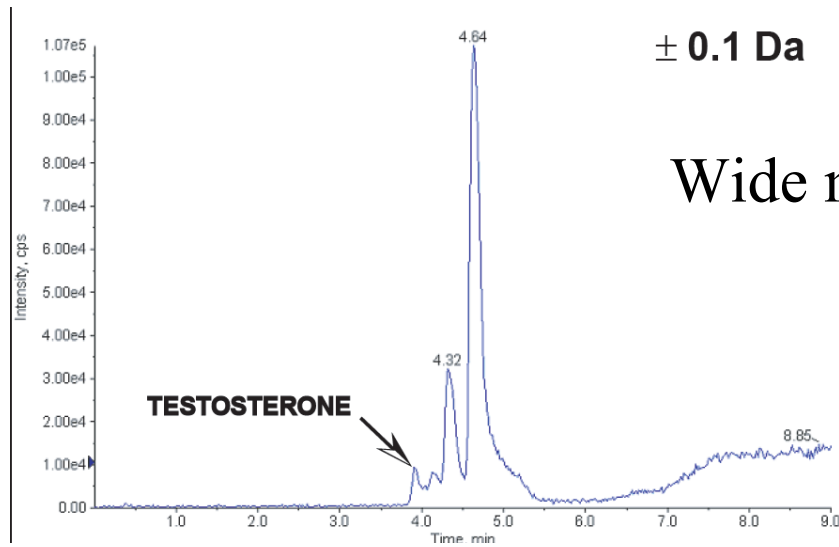
Application

- Testosterone by LC-TOFMS
- Paul Zavitsanos, Agilent
- Gordon Nelson, ARUP

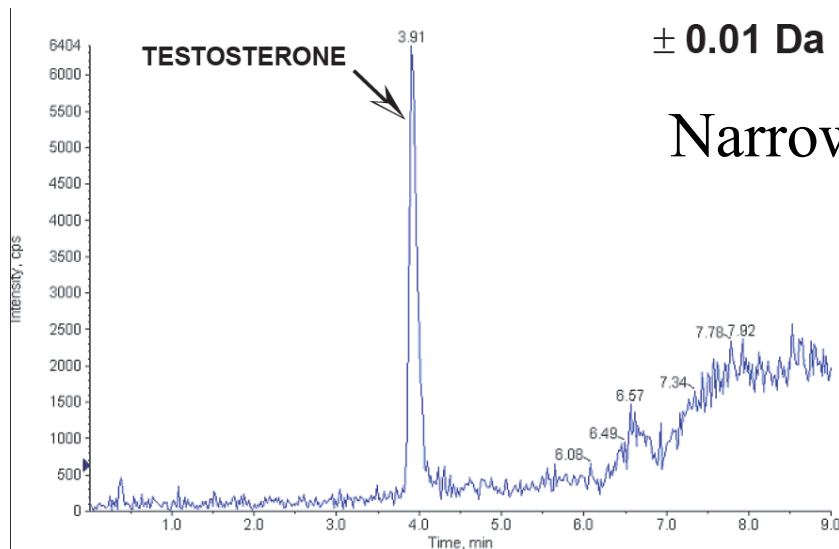
Illustrates

- TOF resolution → Narrow range reconstructed ion chromatograms achieve high selectivity
- Accurate mass confirms compound identity

Reconstructed Ion Chromatograms



Wide mass window = many interferences



Narrow mass window = few interferences

Confirmation by Accurate Mass

Measured mass of three isotopic peaks	ppm mass difference	
	$C_{19}H_{30}NO_2$	$C_{17}H_{28}N_4O$
304.2271	0	-4
305.2301	-1	-5
306.2319	-5	-6

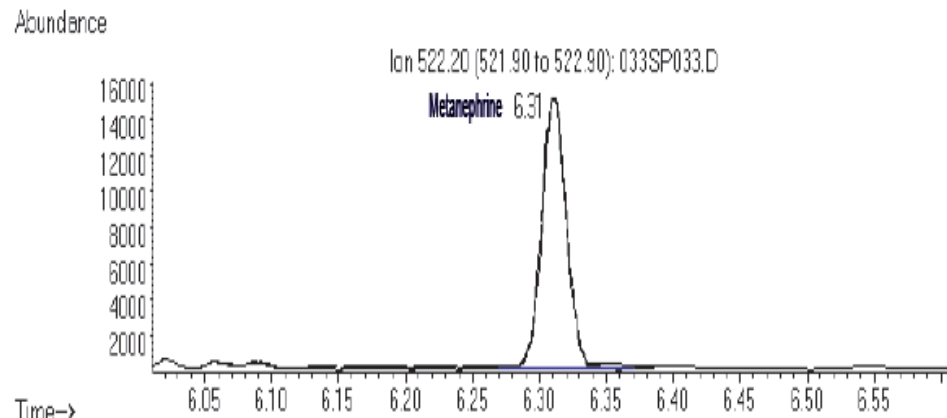
Take Home Lessons

- TOF high resolution → improved selectivity
- TOF accurate mass → analyte confirmation
- Possible alternative to tandem MS

Application

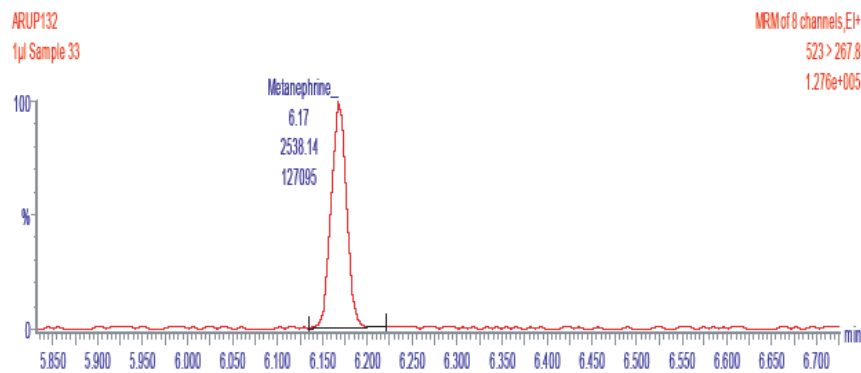
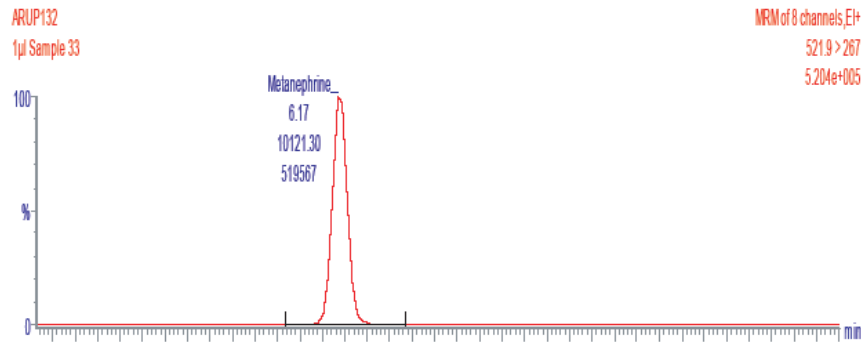
- Metanephrines GC-MS vs. GC-MS-MS
 - Chromatograms
 - Ion ratios
- Peter Hancock, Waters
- Jason Sawyer, ARUP

GC-MS Interference



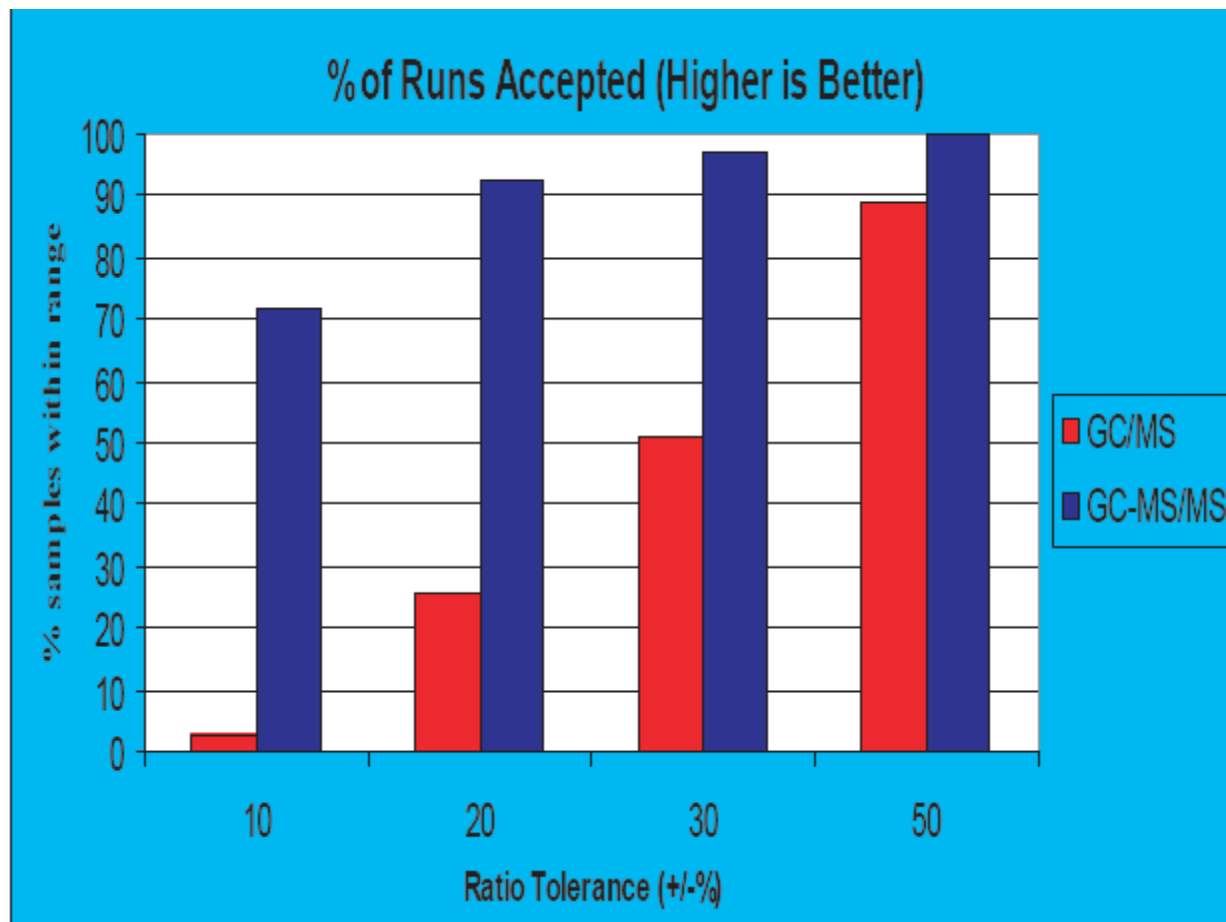
GC-Single Quadrupole Mass Spectrometer

GC-MS-MS No Interferences



GC-Tandem Mass Spectrometer

Less Variation in Ion Ratios



Take Home Lesson

- GC-MS-MS has higher specificity than GC-MS
 - fewer interferences
- Higher cost may be justified by higher quality and lower operating cost (fewer re-runs and rejected samples)

Application

- Hemoglobin by ESI MS (quadrupole)
- Brian Green, Waters Corp.

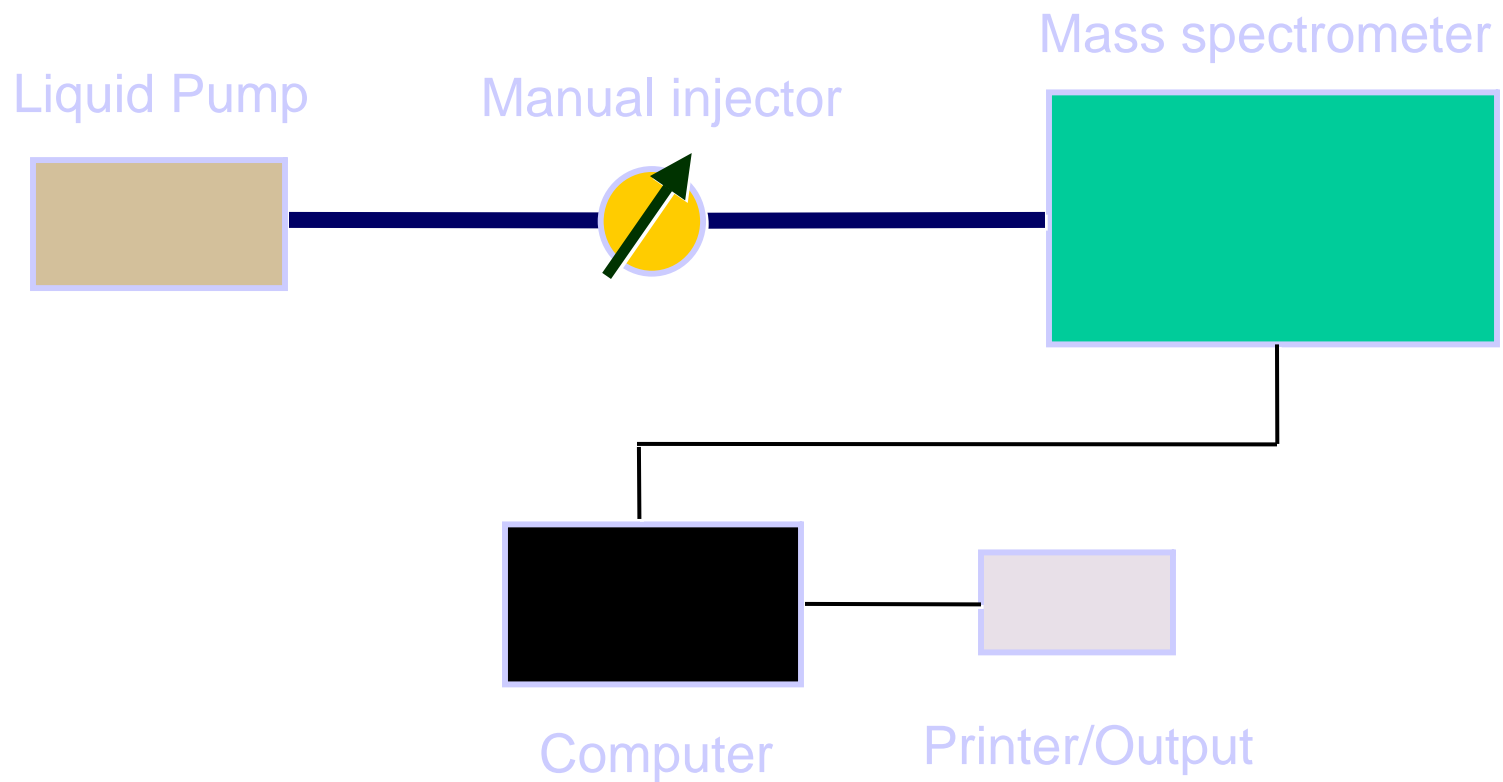
Goals

- Better Hb identification than current LC methods
- Reduce need for confirmation
- Throughput

Illustrates

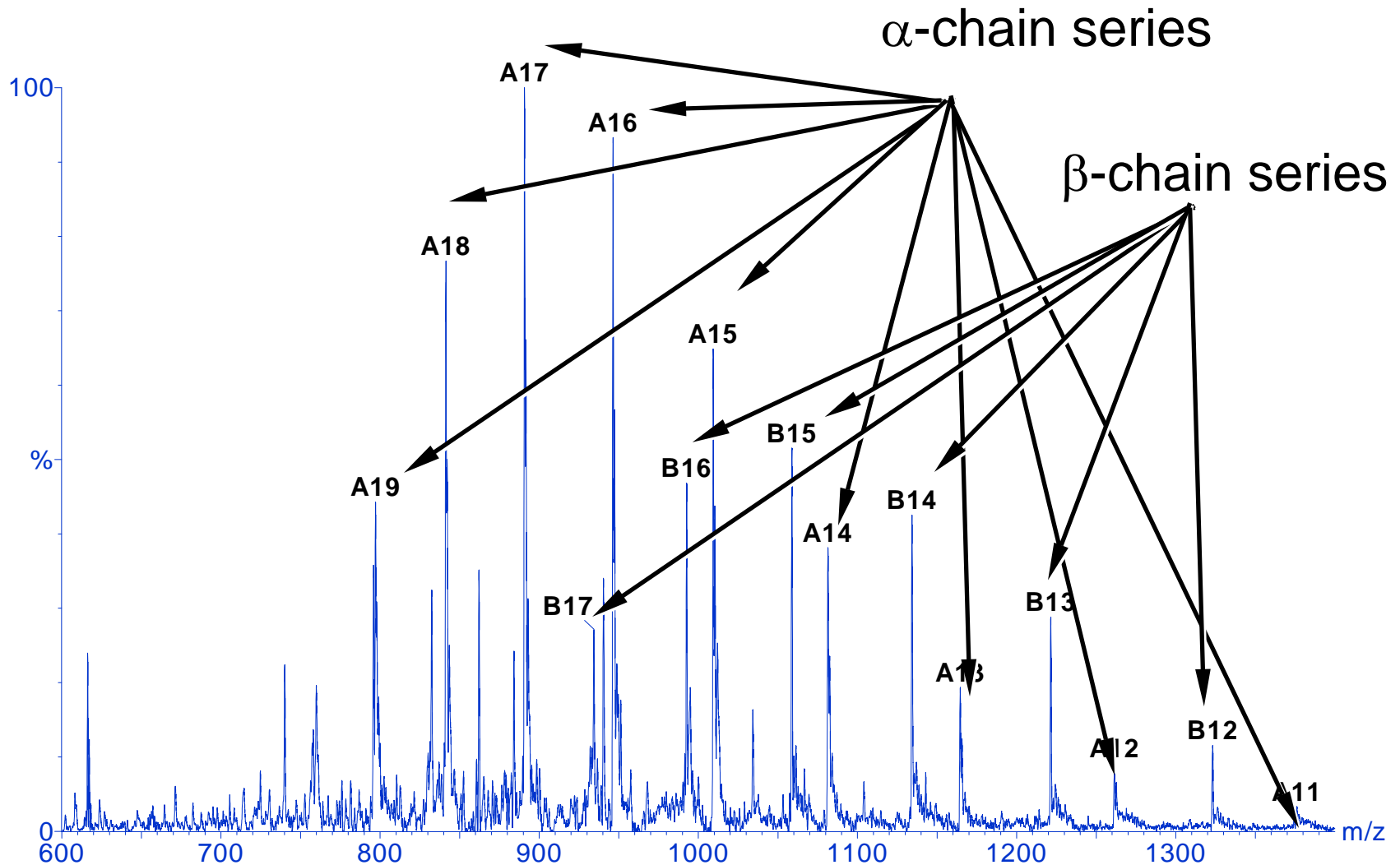
- Protein identification by MS
 - Mass shift identifies mutations
- Multianalyte capability
- Throughput

Hemoglobin Analysis Scheme

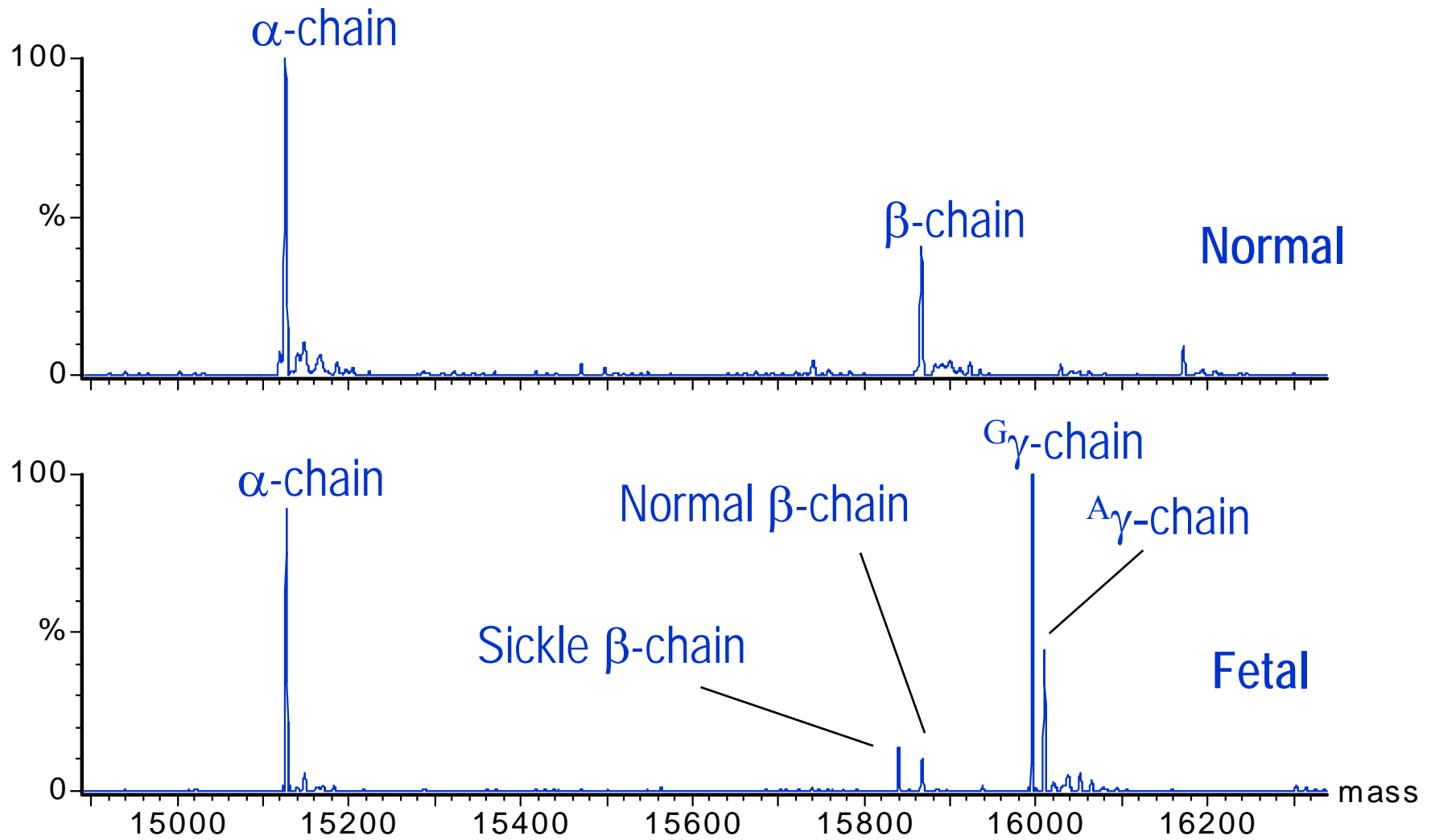


Application from Brian Green, Waters Corp

Normal Hb ESI Mass Spectrum

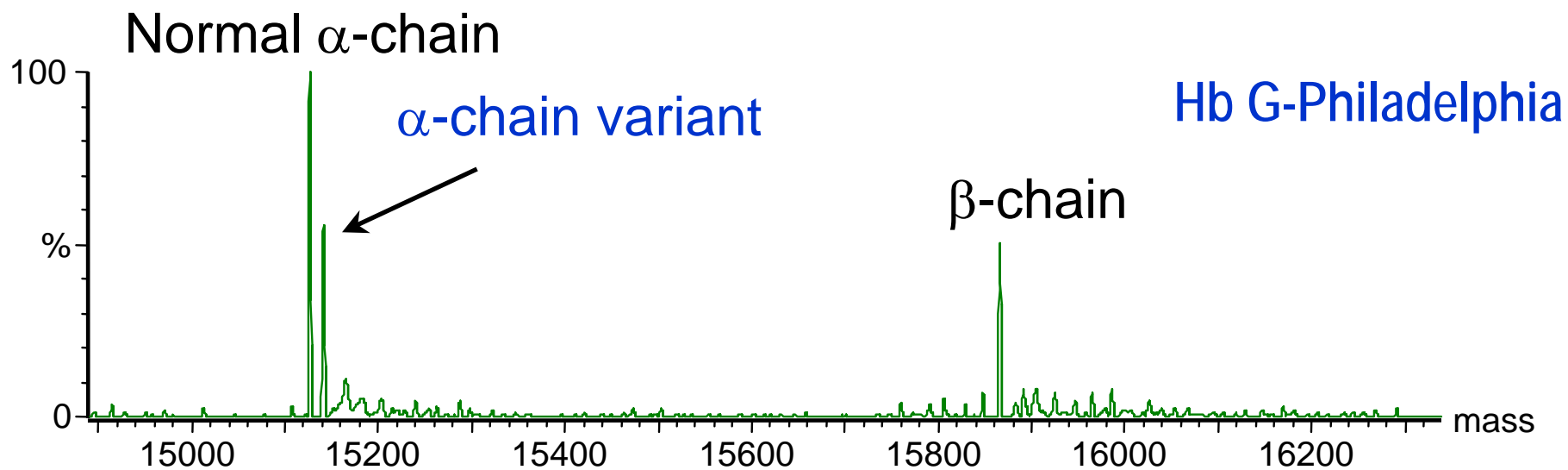
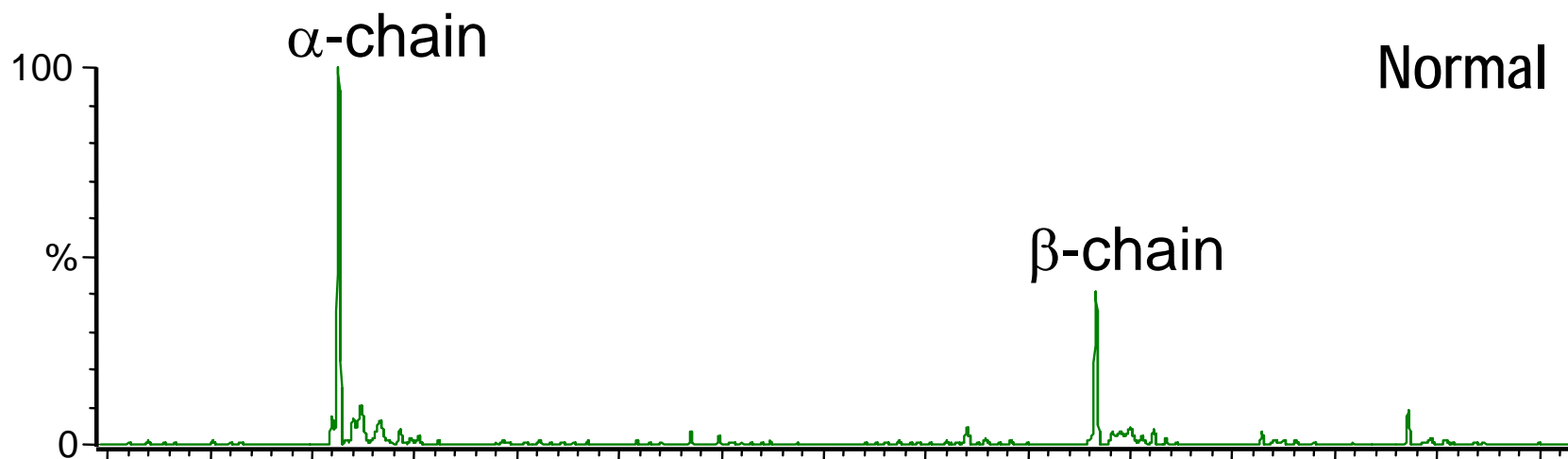


Normal vs Sickle Heterozygote (with Fetal Hb)

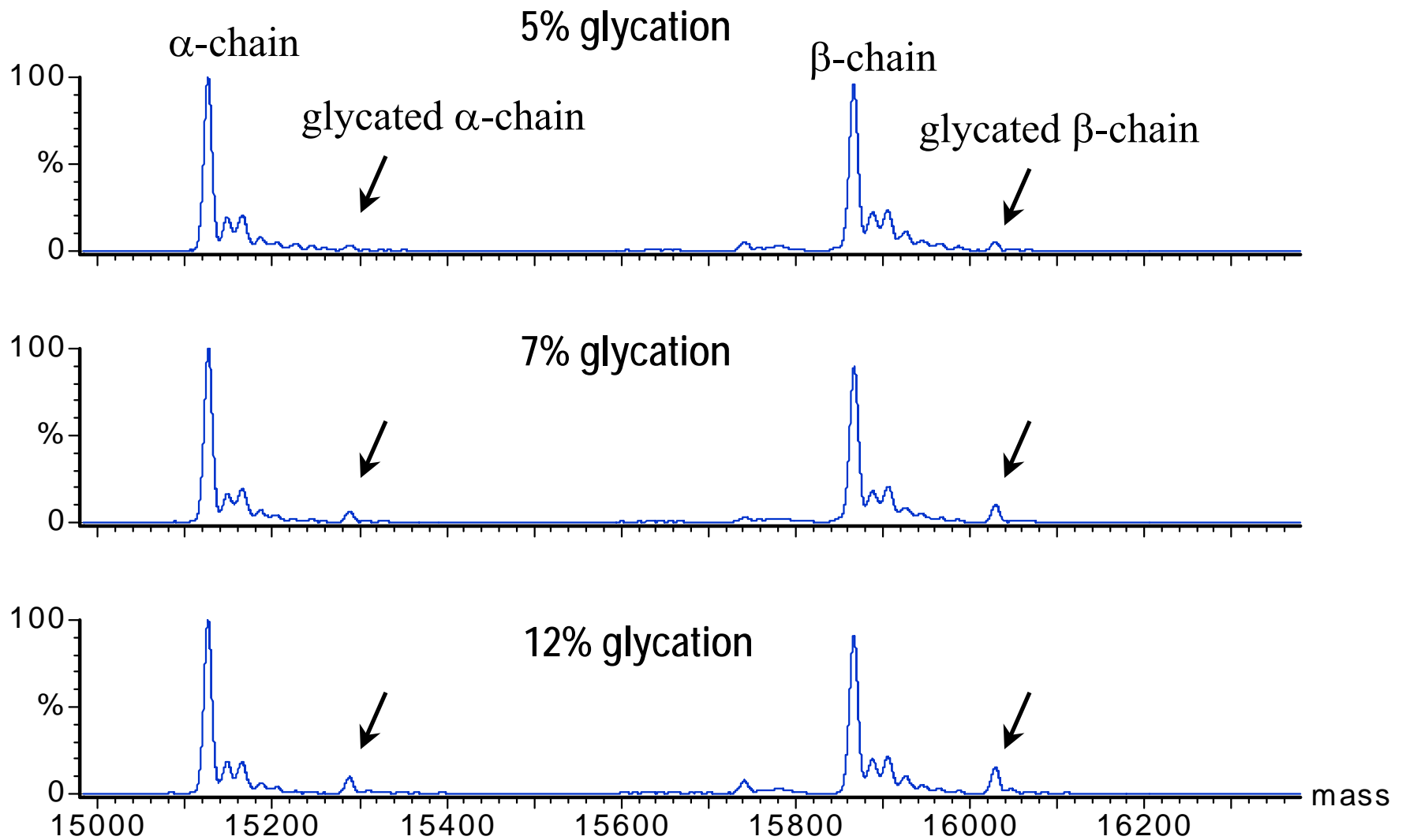


Normal vs α -Chain Variant

Hb G Philadelphia (α_{68} Asn \rightarrow Lys ; $\Delta m=+18Da$)



Glycated Hemoglobins



Possible Improvements

- Autosampler (< 1 min/sample)
- TOF-MS
 - Faster, more sensitive for full mass spectrum
 - Higher resolution
 - Accurate mass
 - 60ppm for $\Delta m = 1$ Da substitution
 - 2.7ppm for $\Delta m = 0.043$ Da (Gln/Lys) substitution
- Enzyme digest + MS/MS or LC-MS-MS
 - Additional confirmation
 - Already been done

Take Home Lesson

- Several MS technologies feasible
- Multiple analyte capability
 - Full spectrum scan
 - Can see all hemoglobin variants in sample
- Mass shift tells what variants are possible (even for unknown variants)

Application

- Genotyping by accurate mass TOF MS
- Steve Hofstadler, Ibis Therapeutics

Analytical Approach

- Amplify bacterial ~100-mer DNA
 - Universal primers
 - Variable regions (identify species/strain)
- Accurate TOF mass measurement
- DNA base composition calculation
- Database matching to identify bacteria

Broad Range Priming in Bacteria

Primers to conserved regions in bacteria

Primer pair

GGATTAGATACCCCTGGTACTCC

CGCCCTGGGGAGTACGGCC

E. coli

ACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC-CTTGA-GGCGTGGCTTCCGGAGCTAACCGGTTAAGTCGAC

Cox. burnetii

C

ACGCCGTCAACGATGGAAC TAGCTGTTGGGAAG--TTCA-CTTCTTAGTAGCGAAGCTAACCGGTTAAGTTCTC

Leg. pneumophila

ACGCTGTAAACGATGTCCAACTAGCTGTTGGTTAT-ATGAAAA TAATTAGTGGCGCAGCAAACCGGATAAAGTTGAC

Ricket. prowazekii

ACGCCGTAAACGATGAGTGCTAGATATCGGAGG--ATTCT--CTTTCGGTTTCGCAGCTAACGCATTAAGCACTC

Mycb. tuberculosis

ACGCCGTAAACGGTGGGTACTAGGTGTTGGGTTTCCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCC

Trep. pallidum

ACACAGTAAACGATGTACACTAGGTGTTGGGGC---ATGA--GTCTCGGCGCCGACGGAACGCATTAAGTGAC

Bacillus anthracis

ACGCCGTAAACGATGAGTGCTAAGTGTAGAGGG-TTTCGCCCTTGTAGTGCCTGAAGTTAACGCATTAAGCACTC

Staph. aureus

ACGCCGTAAACGATGAGTGCTAAGTGTAGGGGG-TTTCGCCCTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Staph. epidermidis

ACGCCGTAAACGATGAGTGCTAAGTGTAGGGGG-TTTCGCCCTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Strep. agalactiae

ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCC-TTTCGGGGCTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Strep. mutans

ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCC-TTTCGGGGCTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Strep. pneumoniae

ACGCTGTAAACGATGAGTGCTAGGTGTTAGACC-TTTCGGGGTTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Strep. pyogenes

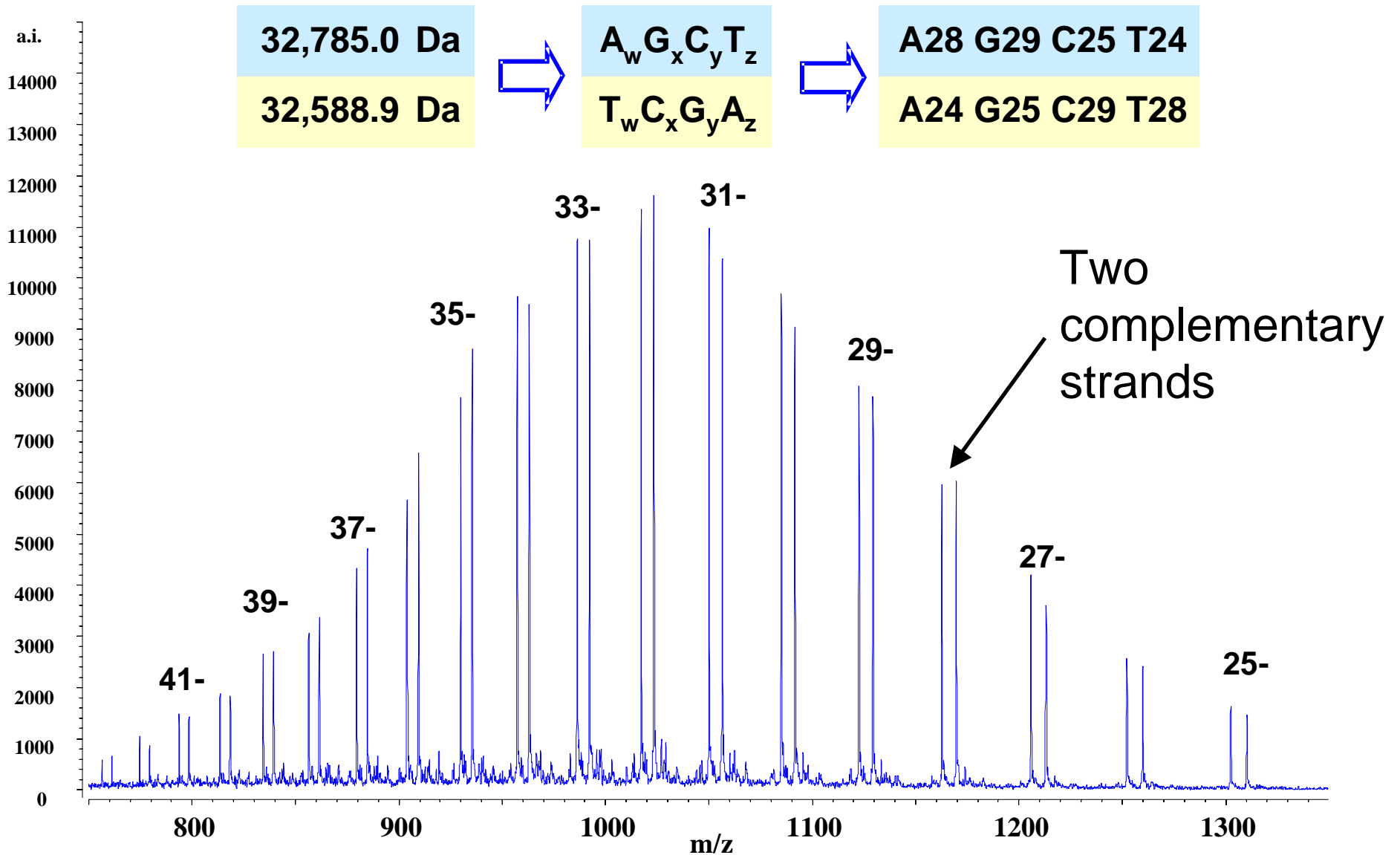
ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCC-TTTCGGGGCTTGTAGTGCCTGCAGCTAACGCATTAAGCACTC

Variable region

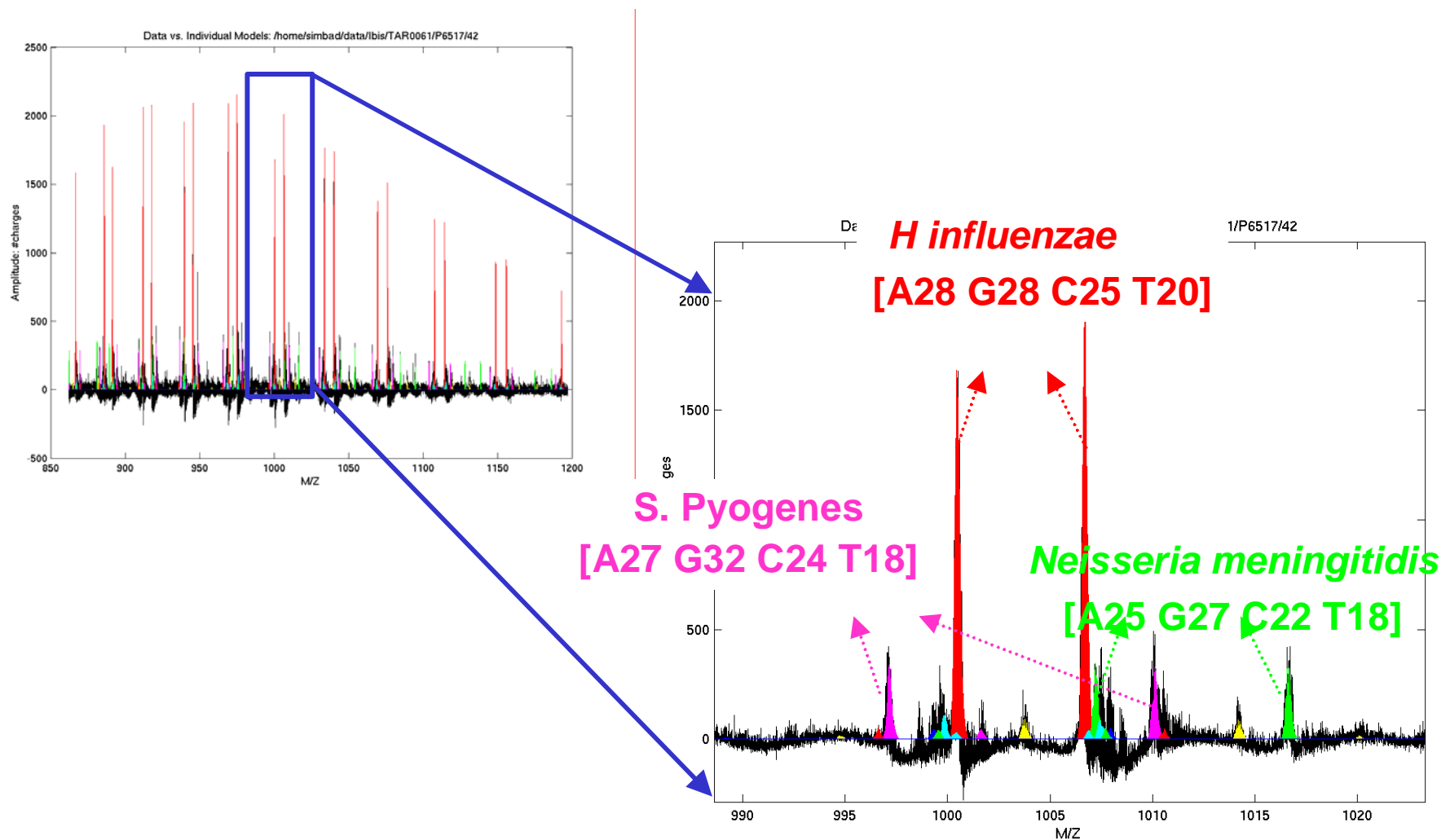
$\Rightarrow \Delta [A_w G_x C_y T_z]$

ESI-TOF Spectrum of *Staph. aureus* amplicon

Measured MW's are within 10 ppm of theoretical



Primer 349: 23S rDNA 1826-1924 From an Interesting Swab



Ecker et al. *Proc. Nat. Acad. Sci. USA*, (2005) 102(22), 8012-17

Take Home Lesson

- TOF → accurate mass → unambiguous base composition → bacterial identification
- TOF → full MS → universal and specific bacterial identification (strain level)
- TOF → full MS → identify multiple species in mixed samples

Hypothetical Decision Process

- Iron by ICP-MS
 - $^{56}\text{Fe}^+$
- Complication: polyatomic interferences
 - $^{40}\text{Ar}^{16}\text{O}^+$

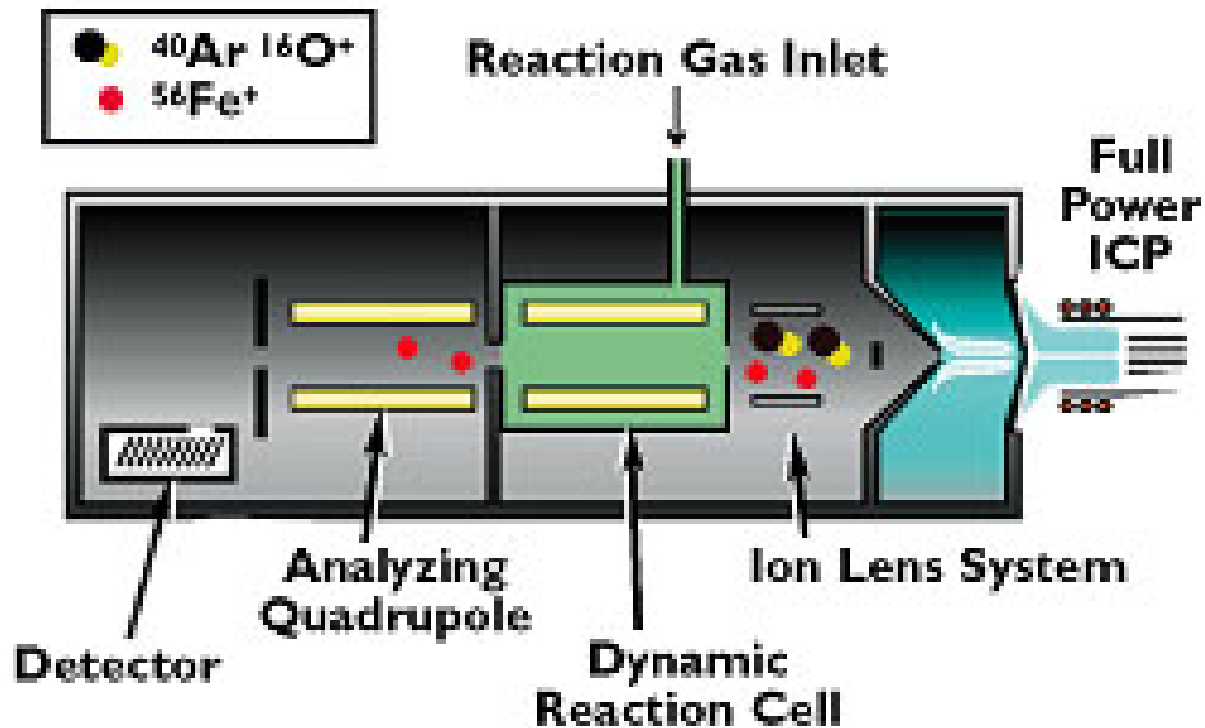
Typical ICP Interferences

<u>Analyte</u>	<u>Interference</u>
^{56}Fe	$^{40}\text{Ar}^{16}\text{O}$
^{80}Se	$^{40}\text{Ar}_2$
^{39}K	$^{38}\text{Ar}^1\text{H}$
^{40}Ca	^{40}Ar
^{51}V	$^{35}\text{Cl}^{16}\text{O}$
^{52}Cr	$^{40}\text{Ar}^{12}\text{C}$
^{63}Cu	$^{23}\text{Na}^{40}\text{Ar}$
^{75}As	$^{40}\text{Ar}^{35}\text{Cl}$

Possible Technologies

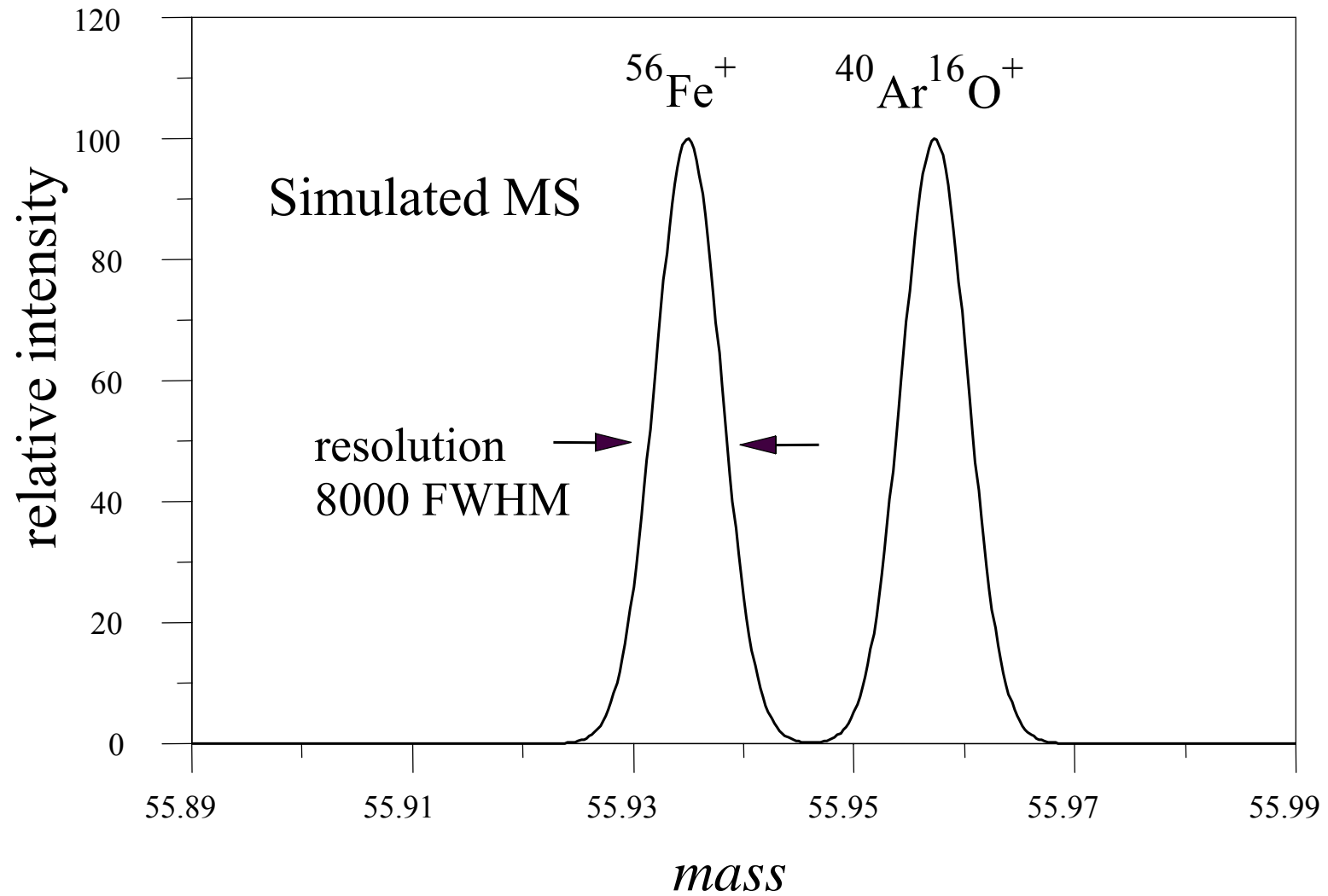
- “Ordinary” ICP-MS
- Dynamic Reaction Cell ICP-MS
 - e.g. PerkinElmer Sciex ELAN 6100 DRC
- High resolution magnetic sector MS
 - e.g. Thermo Finnigan Element 2

Dynamic Reaction Cell (Chemistry)



http://www.wcaslab.com/tech/icpms_drc.htm

High Resolution MS (Physics)



Which Technology to Choose?

- Robustness (physics beats chemistry?)
- Ease of use (quadrupole > sector?)
- Interferences (range)
- Versatility (other analytes)
- Sensitivity (sector > quadrupole?)
- Dynamic range (sector > quadrupole?)
- Price (sector > quadrupole)
- Service
- Competition

Important topics not discussed

- Other MS techniques (especially MALDI, ion trap, and ICR-related)
- Proteomics
- Isotopic methods (e.g. *H. pylori*)
- Structure determination

Bottom Line

- Not just LC-MS/MS
- Many complementary MS technologies
- Match technology to application

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