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

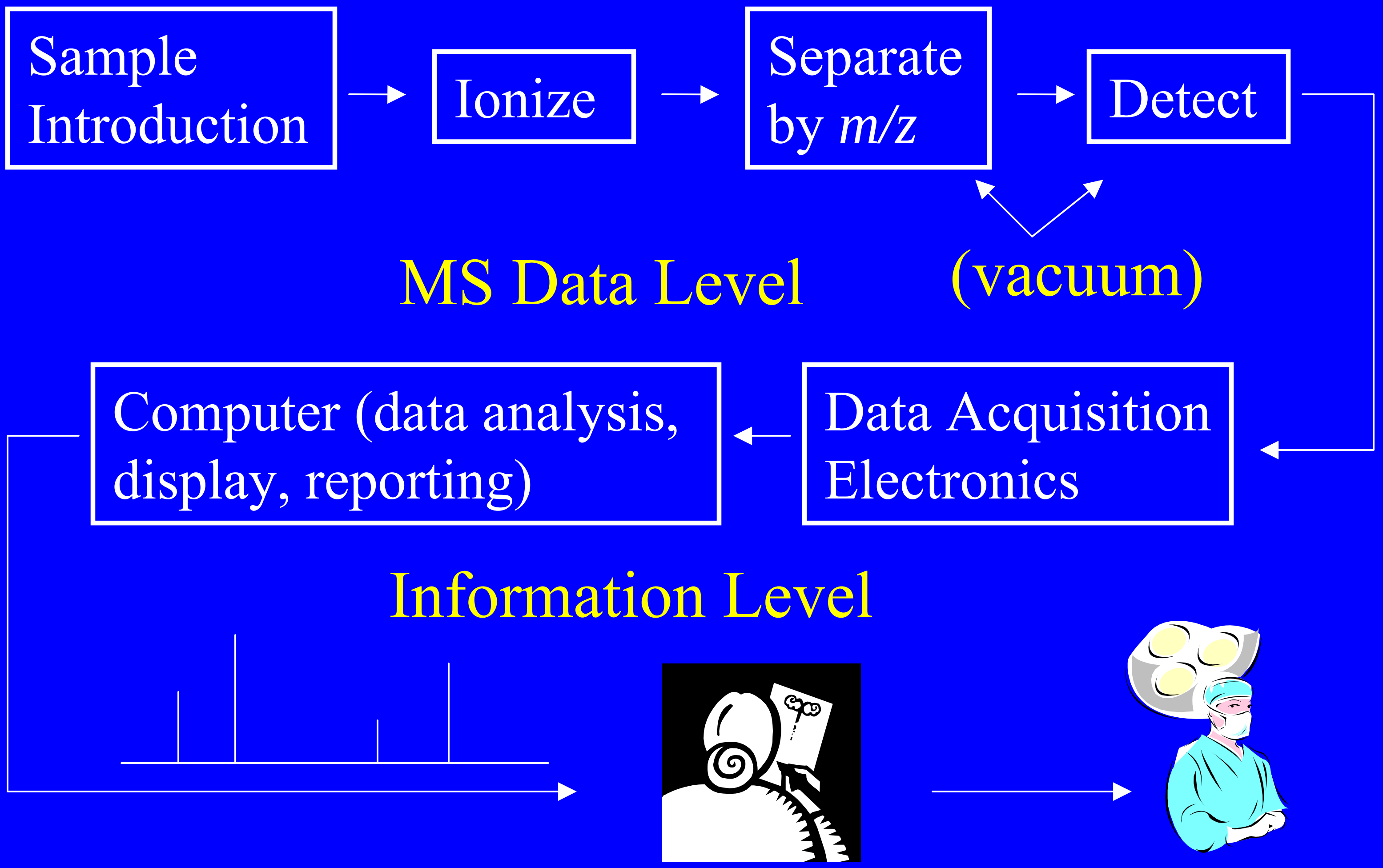
Common Clinical MS Applications

- 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

Sample Introduction → Ionize → Separate by *m/z* → Detect

(vacuum)

MS Data Level

Data Acquisition Electronics → Computer (data analysis, display, reporting)

Information Level

“Ion Sourcery”

- Electron ionization (EI)
- Electrospray ioniztion (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

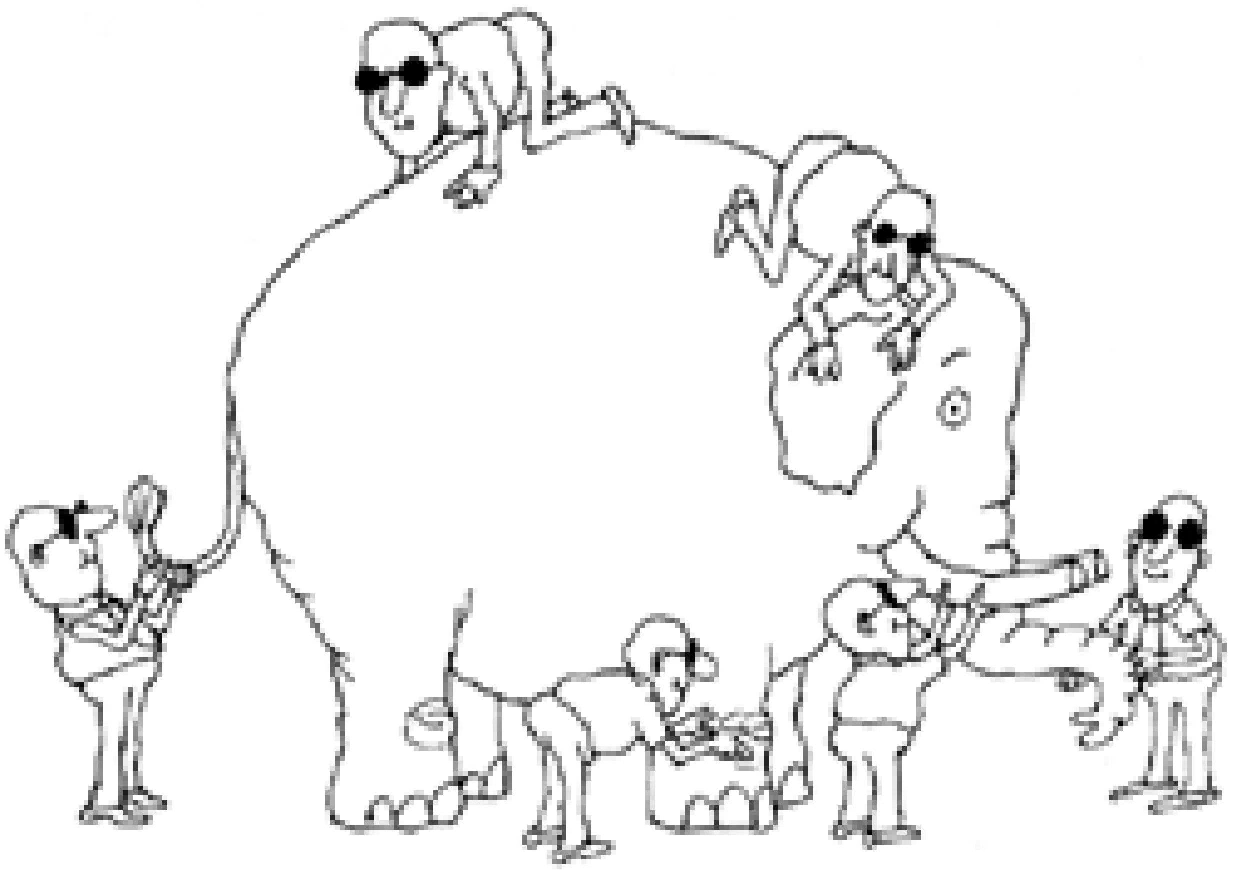
Learn More About
MS Analyzer Types

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

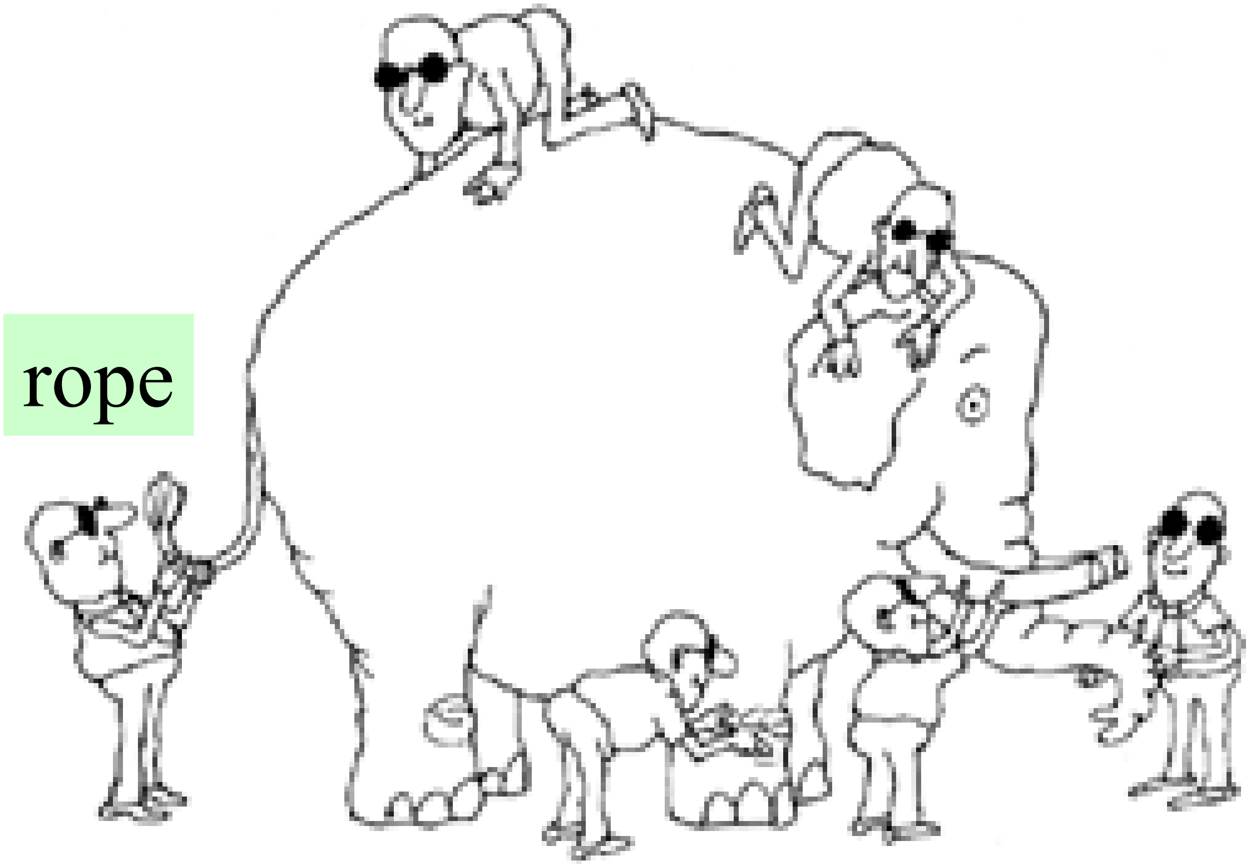
Instrument Configurations

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

The Fundamental Analytical Problem: How to Characterize an Elephant

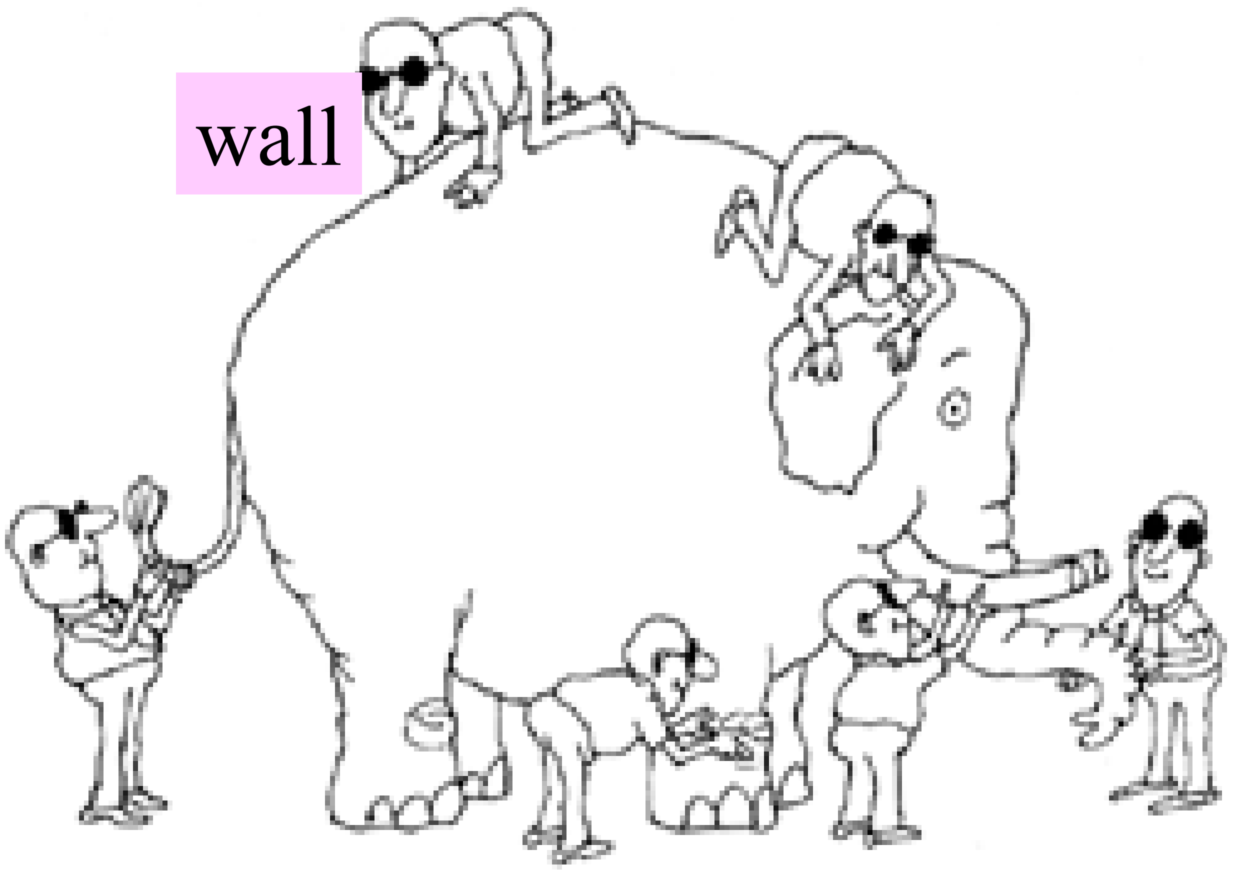


Is it a rope?

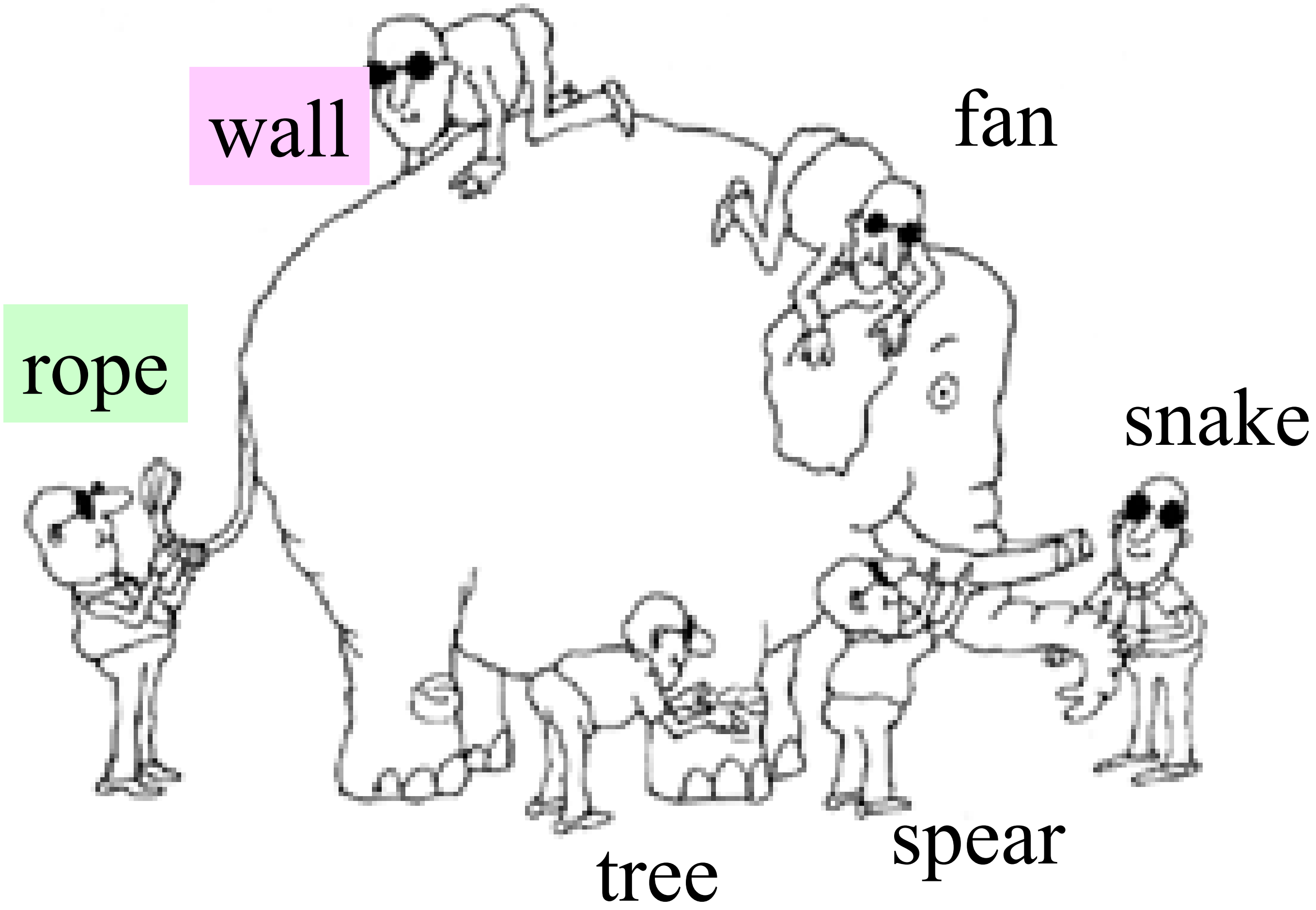


rope

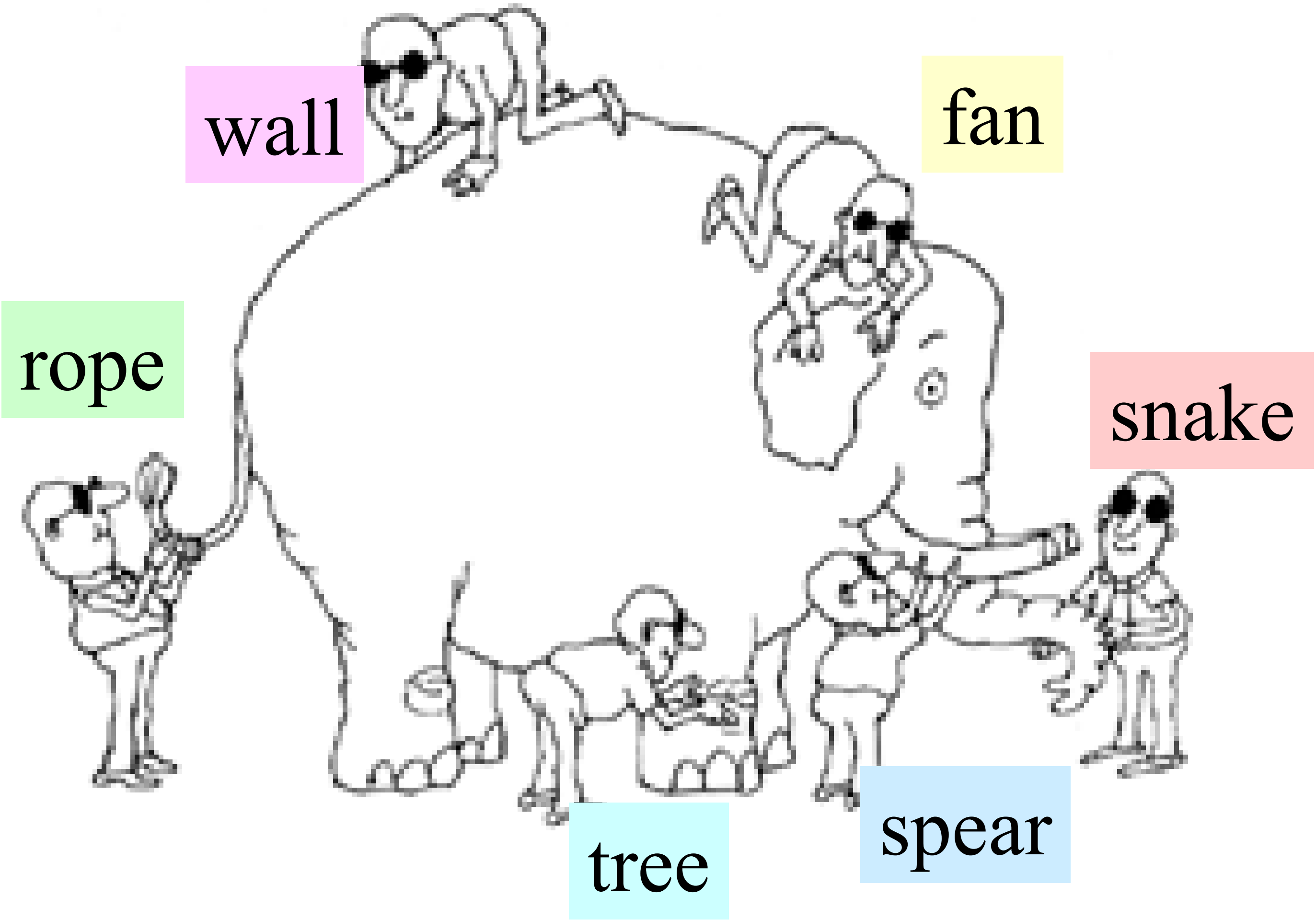
Is it a wall?



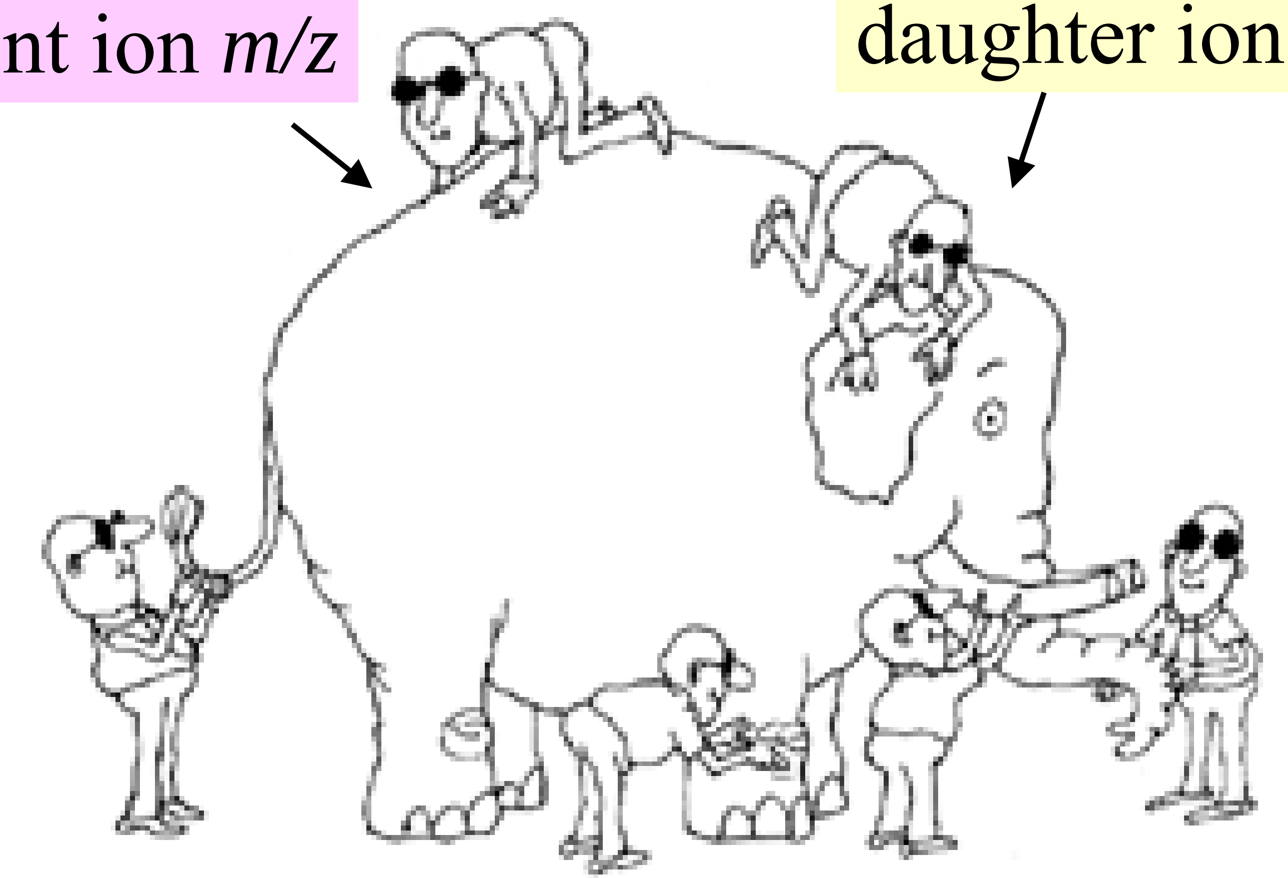
No, it is an elephant!



More Is Better



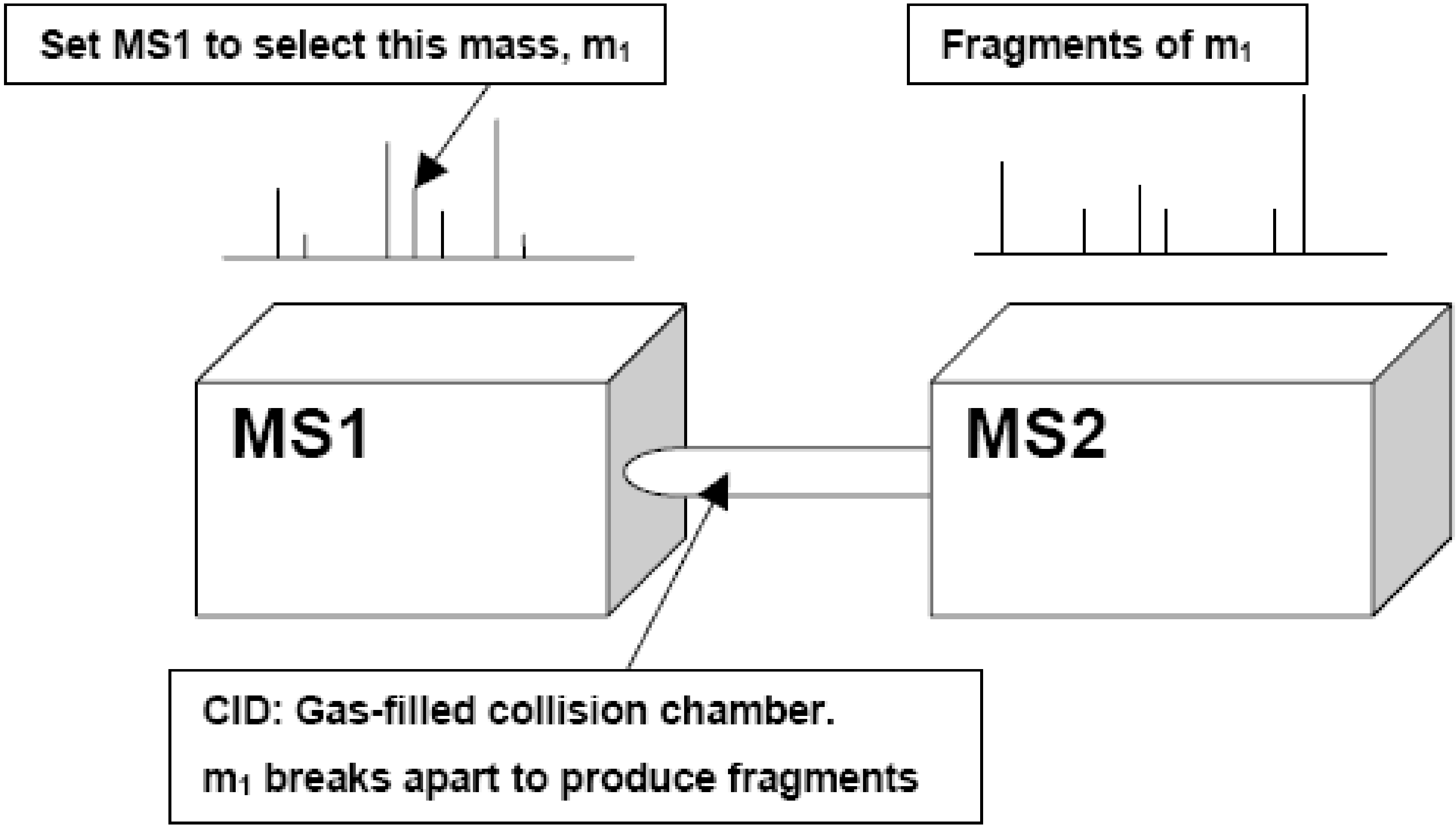
“Hyphenation” = Two or More Physical Properties (MS-MS)



parent ion *m/z*

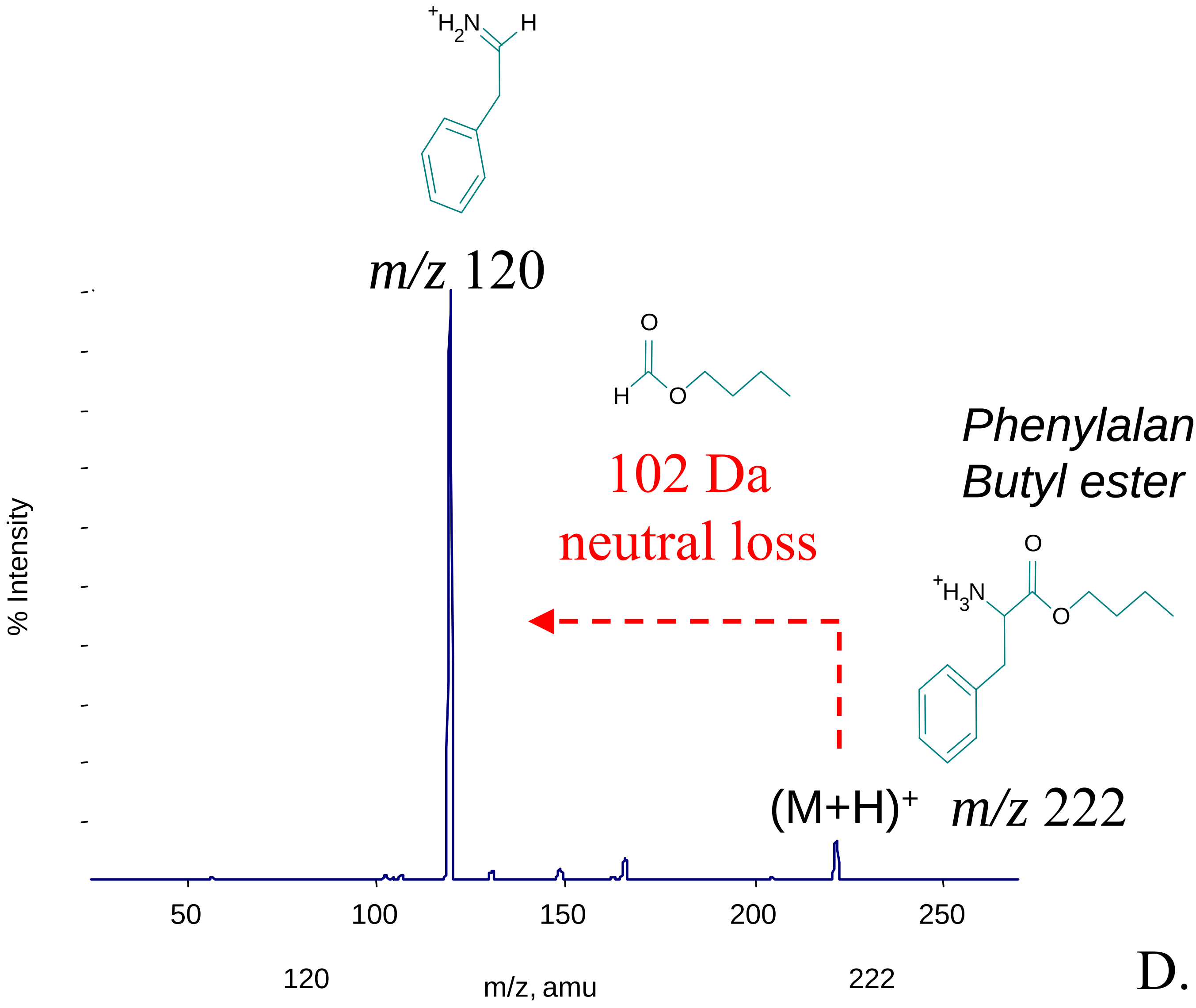
daughter ion *m/z*

MS-MS (Tandem MS)



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

Amino acid MS-MS



m/z 120

102 Da
neutral loss

Phenylalanine
Butyl ester

(M+H)$^+$ *m/z* 222

D. Chace

Application

- Newborn screening by MS-MS
- Donald Chace, Pediatrix

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)

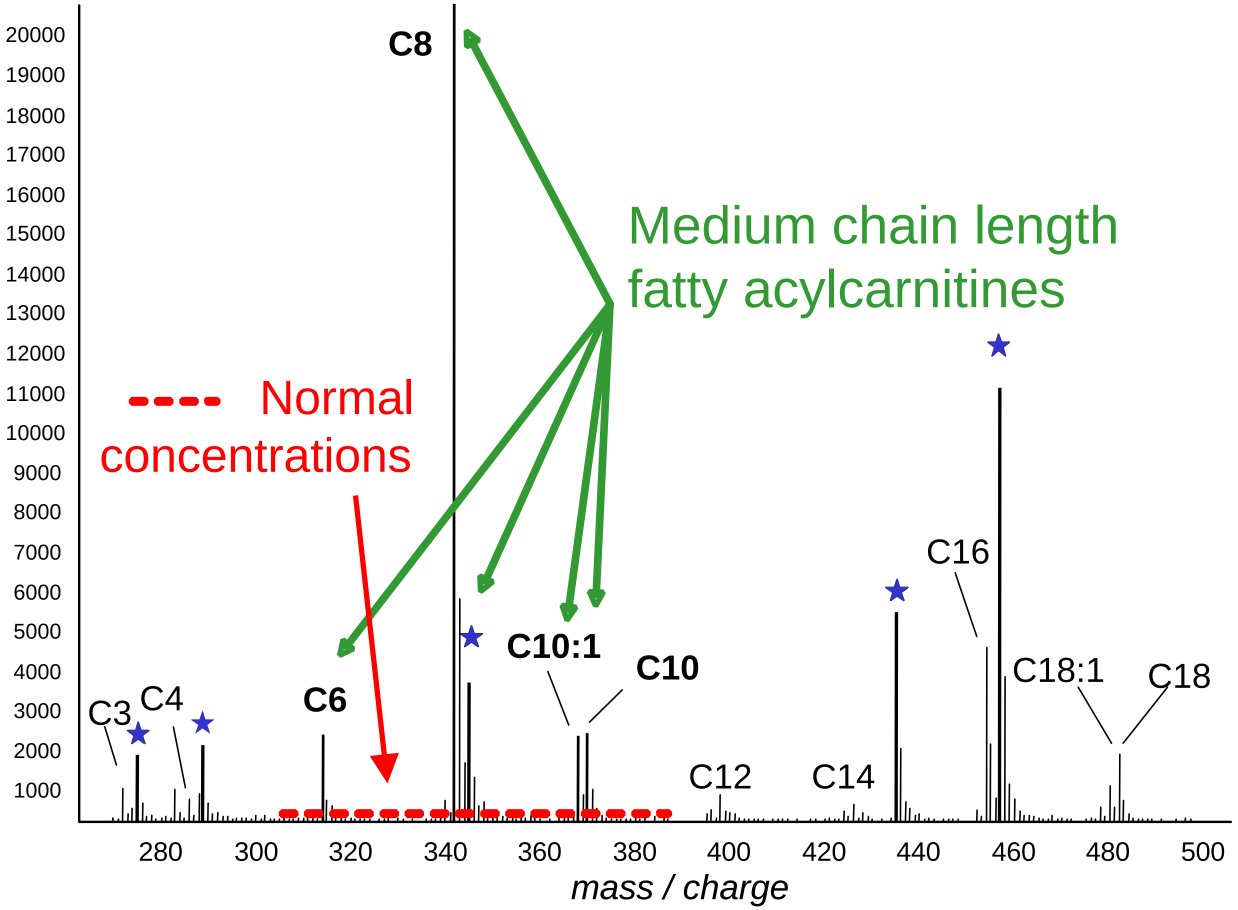
MH^+ → collision cell → neutral product + $C_4H_5O_2^+$

Scan MS1 for
m/z of parent ion

Set MS2 for
m/z 85
daughter ion

MCAD Deficiency

Homozygous A985G



Medium chain length fatty acylcarnitines

Normal concentrations

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

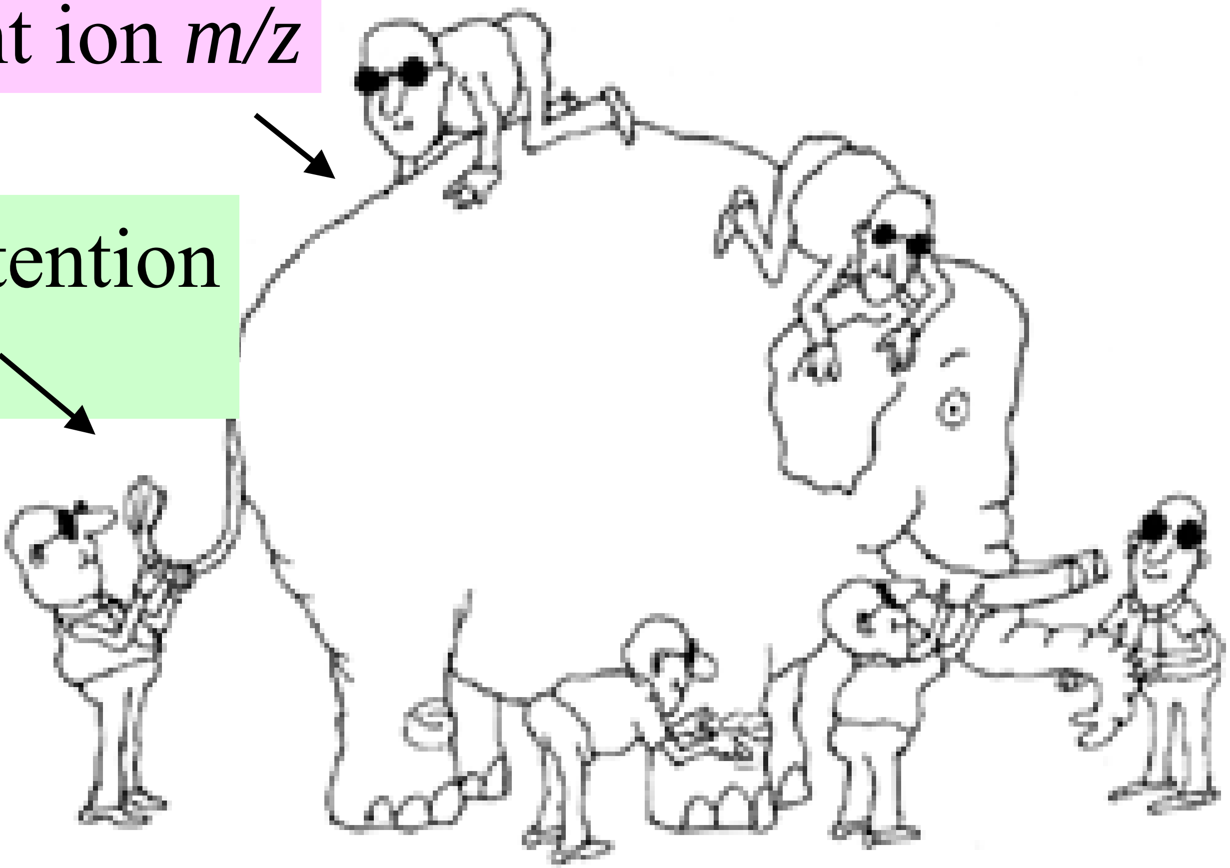
Application

- 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



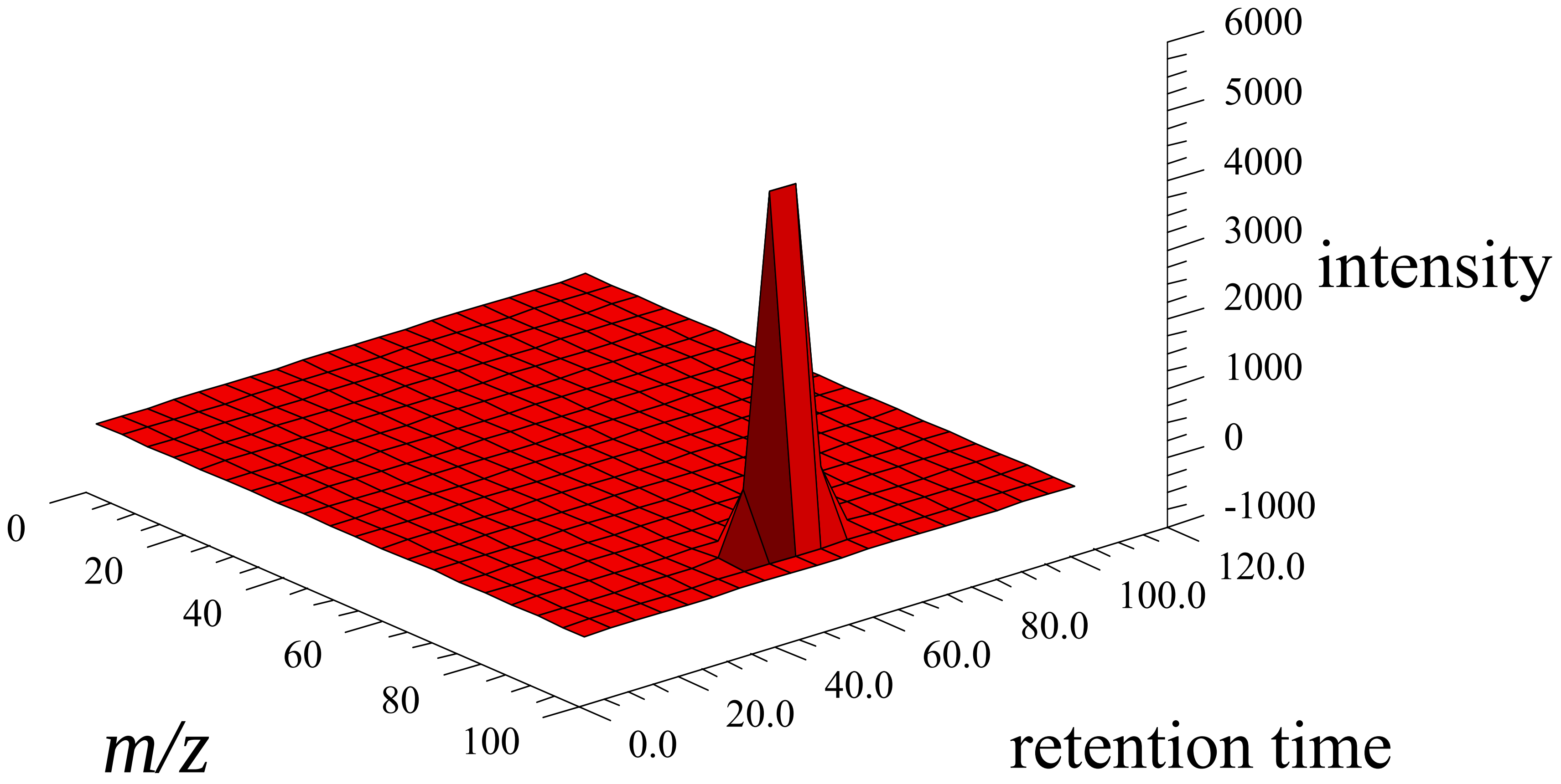
parent ion *m/z*

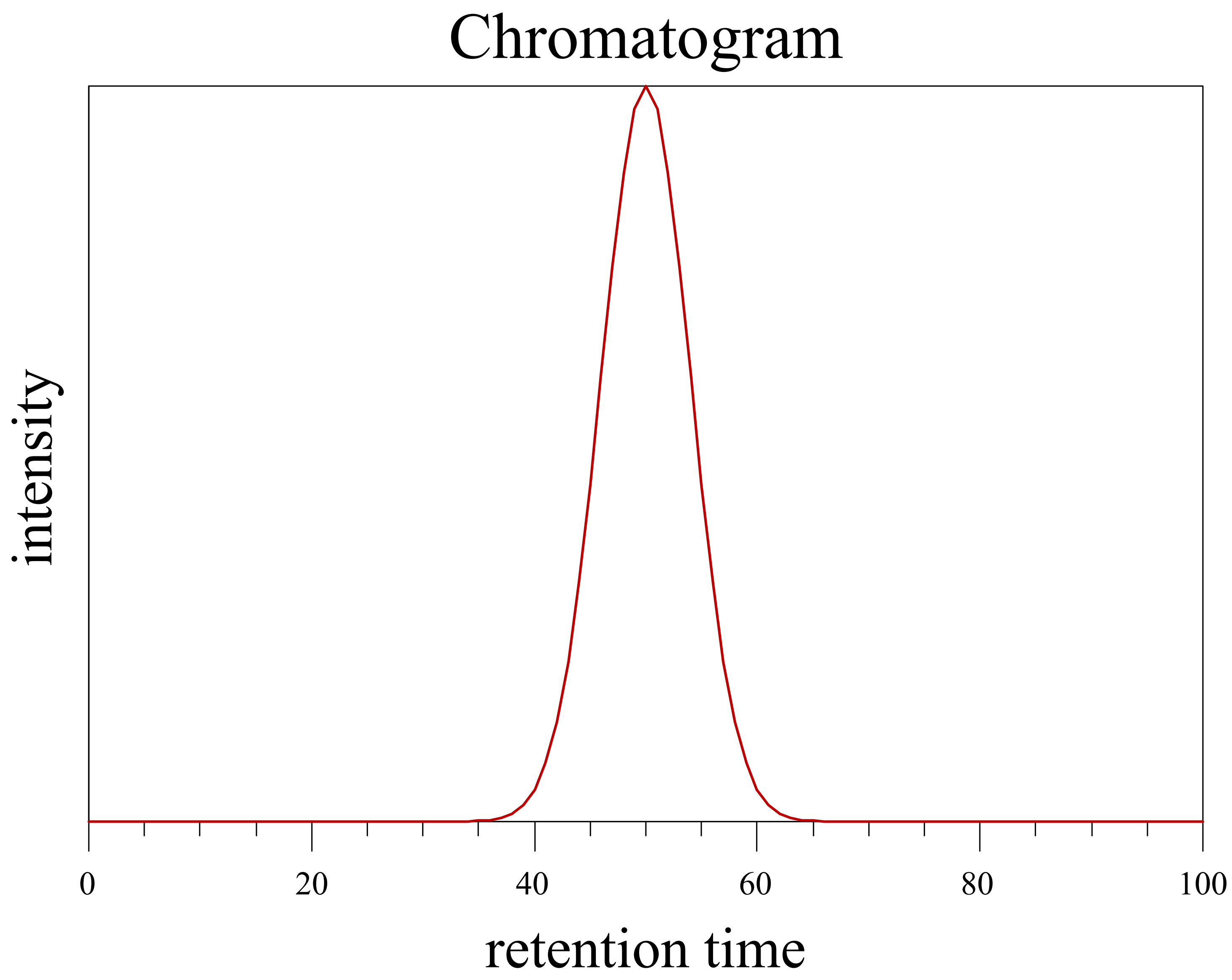
LC retention time

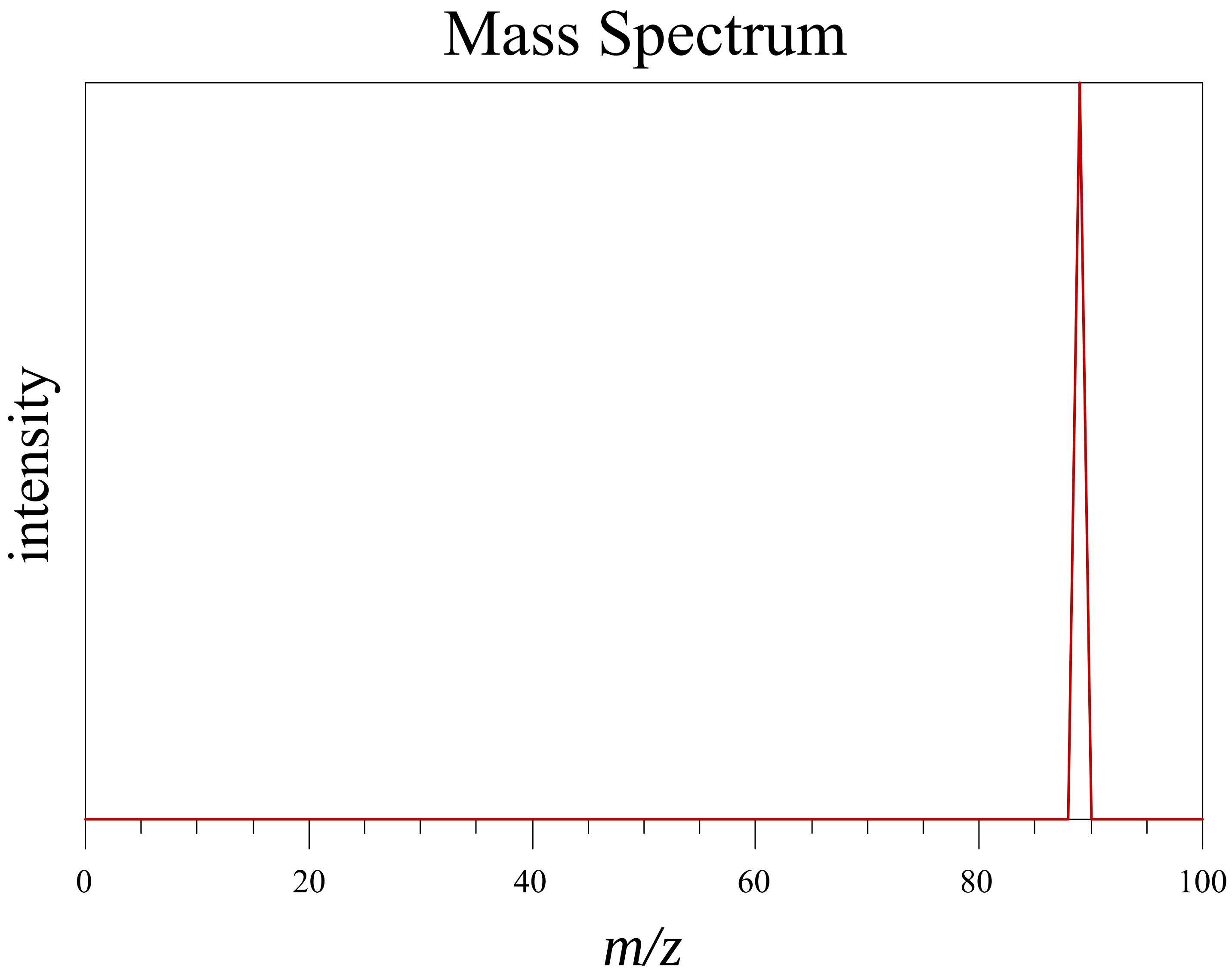
Block diagram of LC-MS

LC → MS

LC-MS Data Concept




Chromatogram
intensity
0
20
40
60
80
100
retention time


Mass Spectrum
intensity
0
20
40
60
80
100
m/z

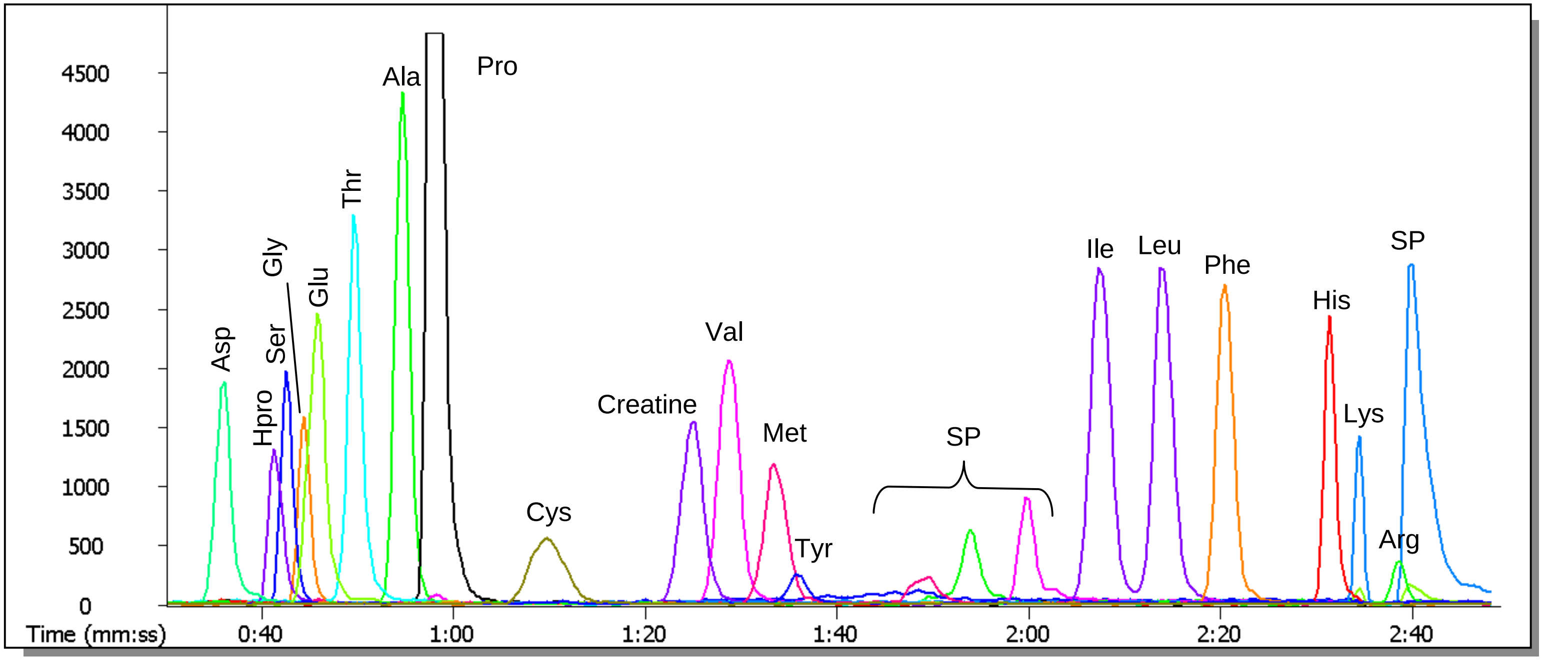
Samples and Method

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


Leco
Unique™ MS
LFN

Fast LC Separation

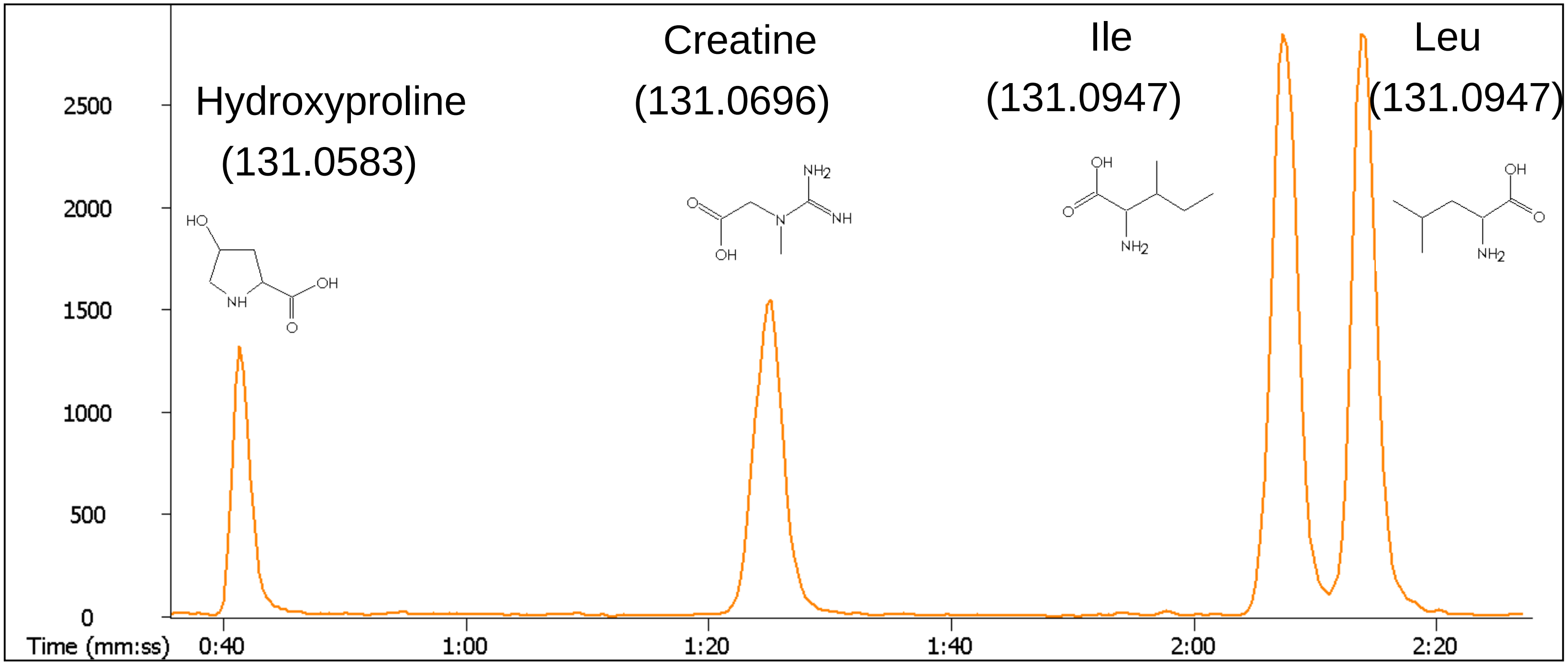
17 amino acids, creatine and hydroxyproline, < 3 minutes



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	$C_4 H_7 O_4 N$	$C_4 H_7 O_4 N$	1
2	00:41.3	HydPro	132.0664	132.0656	0.8	$C_5 H_9 O_3 N$	$C_5 H_9 O_3 N$	1
3	00:42.6	Ser	106.0522	106.0499	2.3	$C_3 H_7 O_3 N$	$C_3 H_7 O_3 N$	1
4	00:44.5	Gly	76.04	76.0393	0.7	$C_2 H_5 O_2 N$	$C_2 H_5 O_2 N$	1
5	00:45.8	Glu	148.0588	148.0605	1.7	$C_5 H_9 O_4 N$	$C_5 H_9 O_4 N$	1
6	00:49.6	Thr	120.0621	120.0656	3.5	$C_4 H_9 O_3 N$	$C_4 H_9 O_3 N$	1
7	00:54.7	Ala	90.0561	90.0550	1.1	$C_3 H_7 O_2 N$	$C_3 H_7 O_2 N$	1
8	00:57.9	Pro	116.0709	116.0707	0.2	$C_5 H_9 O_2 N$	$C_5 H_9 O_2 N$	1
9	01:09.8	Cys	241.028	241.0312	3.2	$C_6 H_{12} O_4 N_2 S_2$	$C_6 H_{12} O_4 N_2 S_2$	2
10	01:25.1	Creatine	132.0762	132.0769	0.7	$C_4 H_9 O_2 N_3$	$C_4 H_9 O_2 N_3$	1
11	01:28.6	Val	118.0864	118.0863	0.1	$C_5 H_{11} O_2 N$	$C_5 H_{11} O_2 N$	1
12	01:33.4	Met	150.0582	150.0584	0.2	$C_5 H_{11} O_2 N S$	$C_5 H_{11} O_2 N S$	2
13	01:35.7	Tyr	182.0837	182.0812	2.5	$C_9 H_{11} O_3 N$	$C_9 H_{11} O_3 N$	1
14	02:07.4	Ile	132.0993	132.1020	2.7	$C_6 H_{13} O_2 N$	$C_6 H_{13} O_2 N$	1
15	02:13.8	Leu	132.1009	132.1020	1.1	$C_6 H_{13} O_2 N$	$C_6 H_{13} O_2 N$	1
16	02:20.5	Phe	166.0843	166.0863	2.0	$C_4 H_{11} O_4 N_3$	$C_4 H_{11} O_4 N_3$	2
17	02:31.4	His	156.0777	156.0769	0.8	$C_6 H_9 O_2 N_3$	$C_6 H_9 O_2 N_3$	1
18	02:34.6	Lys	147.113	147.1129	0.1	$C_6 H_{14} O_2 N_2$	$C_6 H_{14} O_2 N_2$	1
19	02:38.4	Arg	175.1217	175.1191	2.6	$C_6 H_{14} O_2 N_4$	$C_6 H_{14} O_2 N_4$	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

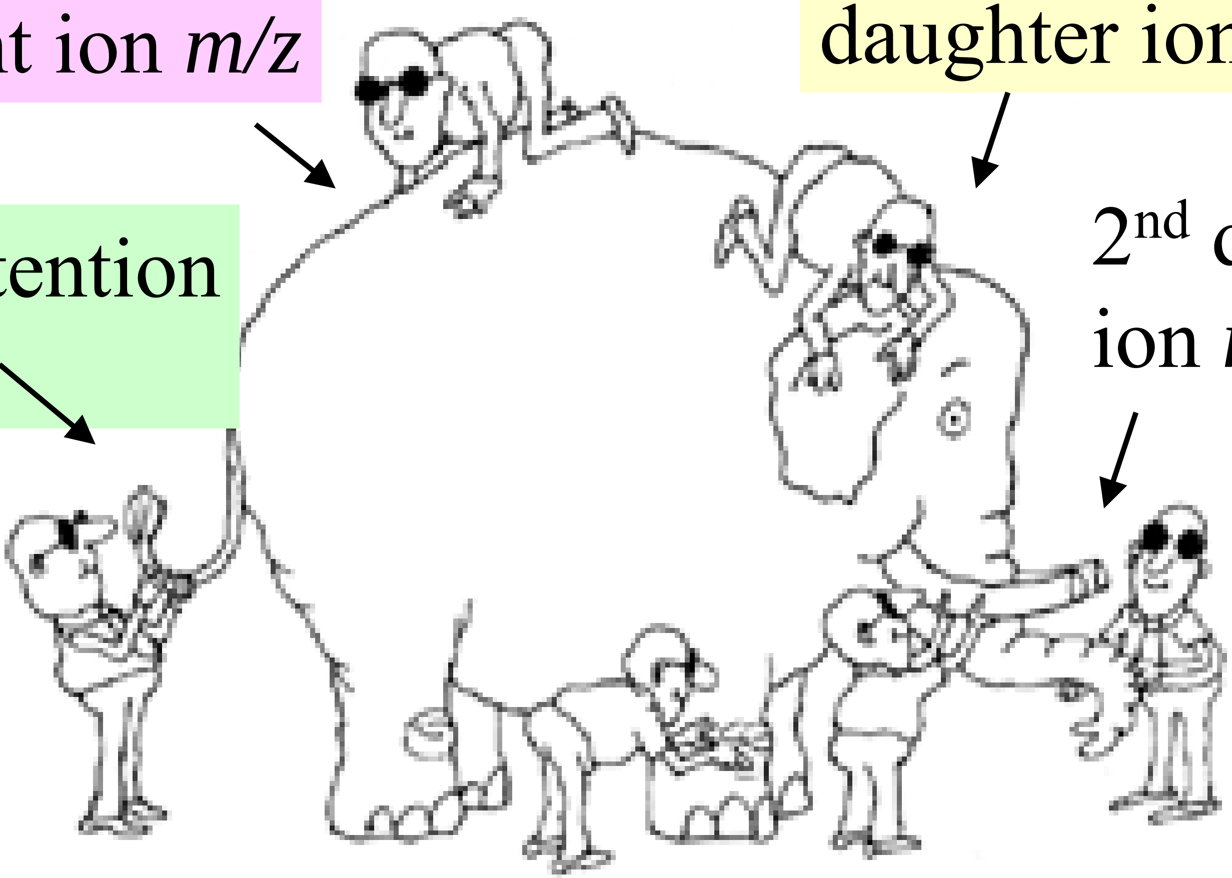
- Sirolimus by LC-MS-MS
  - Triple quadrupole MS-MS
  - Sirolimus

- Mark Kushnir, ARUP

Illustrates

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

LC-MS-MS: Three Physical Properties



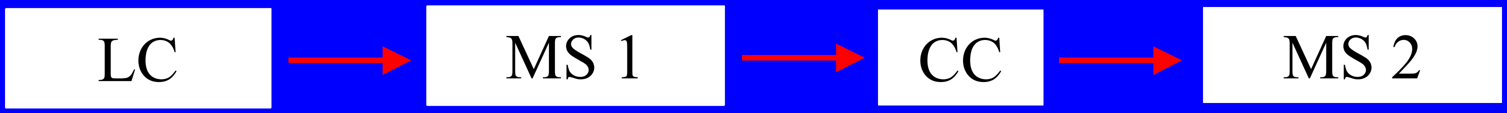
parent ion *m/z*

daughter ion *m/z*

LC retention time

2nd daughter ion *m/z*

Block diagram of LC-MS-MS

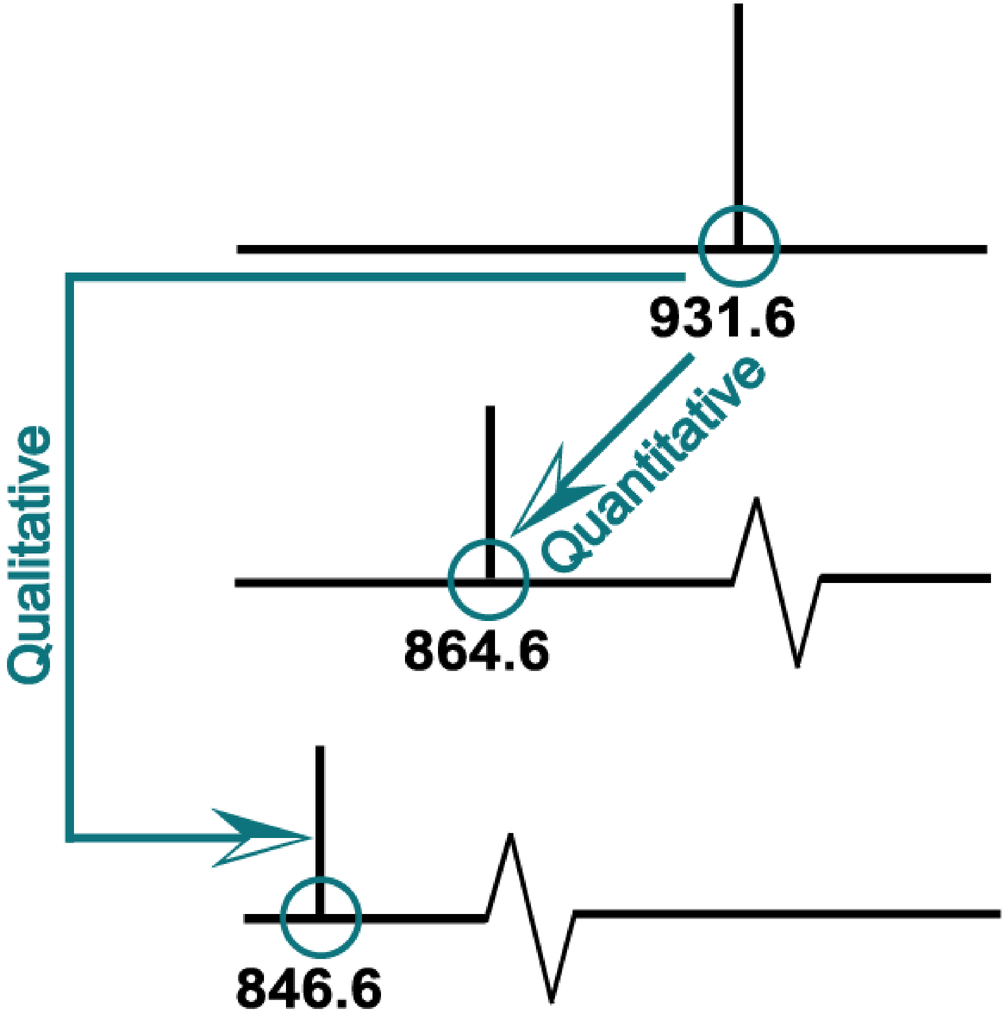

LC → MS 1 → CC → MS 2

MRM = specific parent-daughter ion pairs

Samples and Method

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

Sirolimus: MS-MS transitions



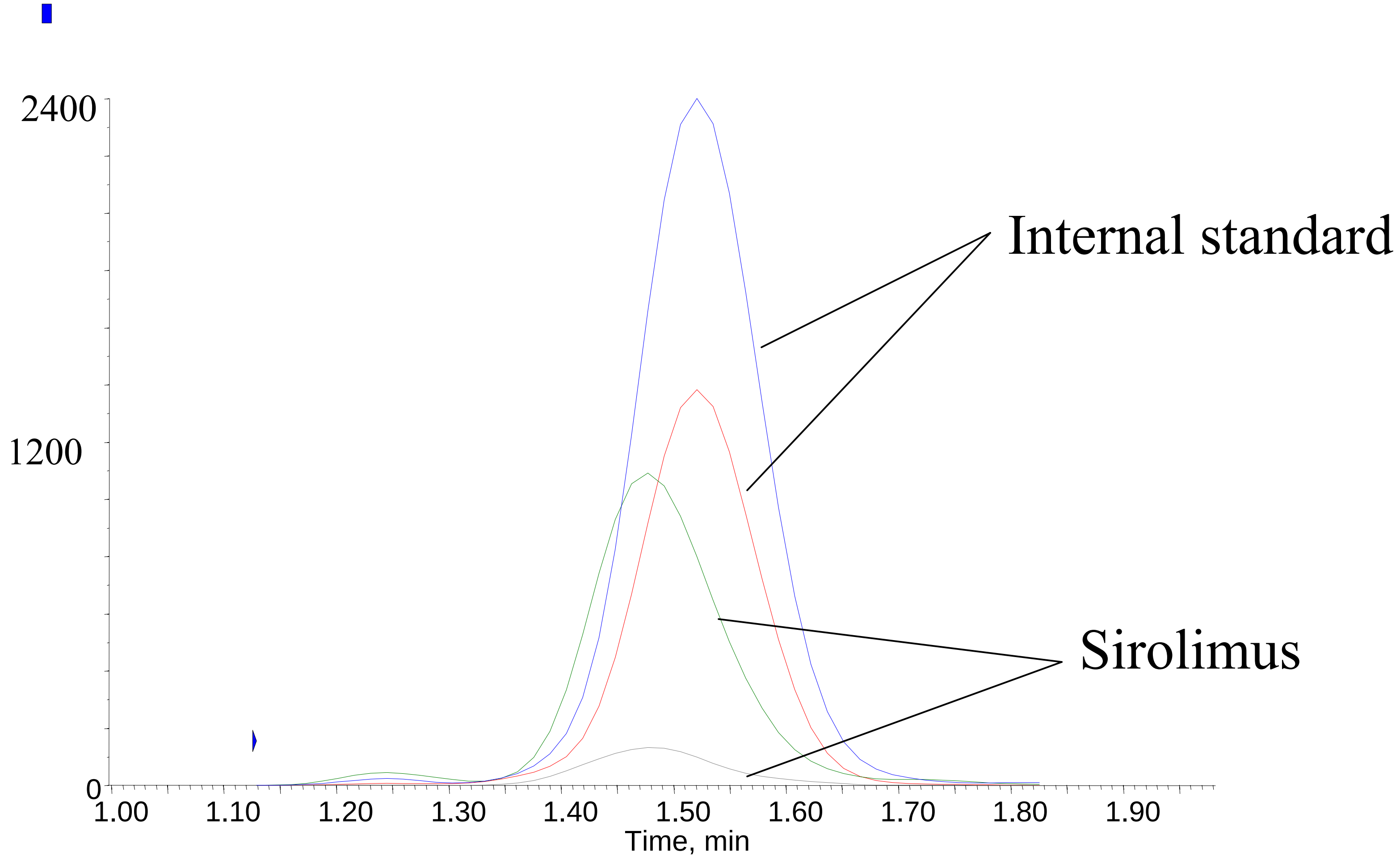
m/z 931 parent ion

m/z 931 → 864

m/z 931 → 846

Sirolimus: Typical Chromatogram

(sample containing 9 ng/mL of sirolimus)



Internal standard

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] $ZnSO_4$	CV,%[b] H_2O + $ZnSO_4$	CV,%[b] H_2O + $ZnSO_4$ + 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

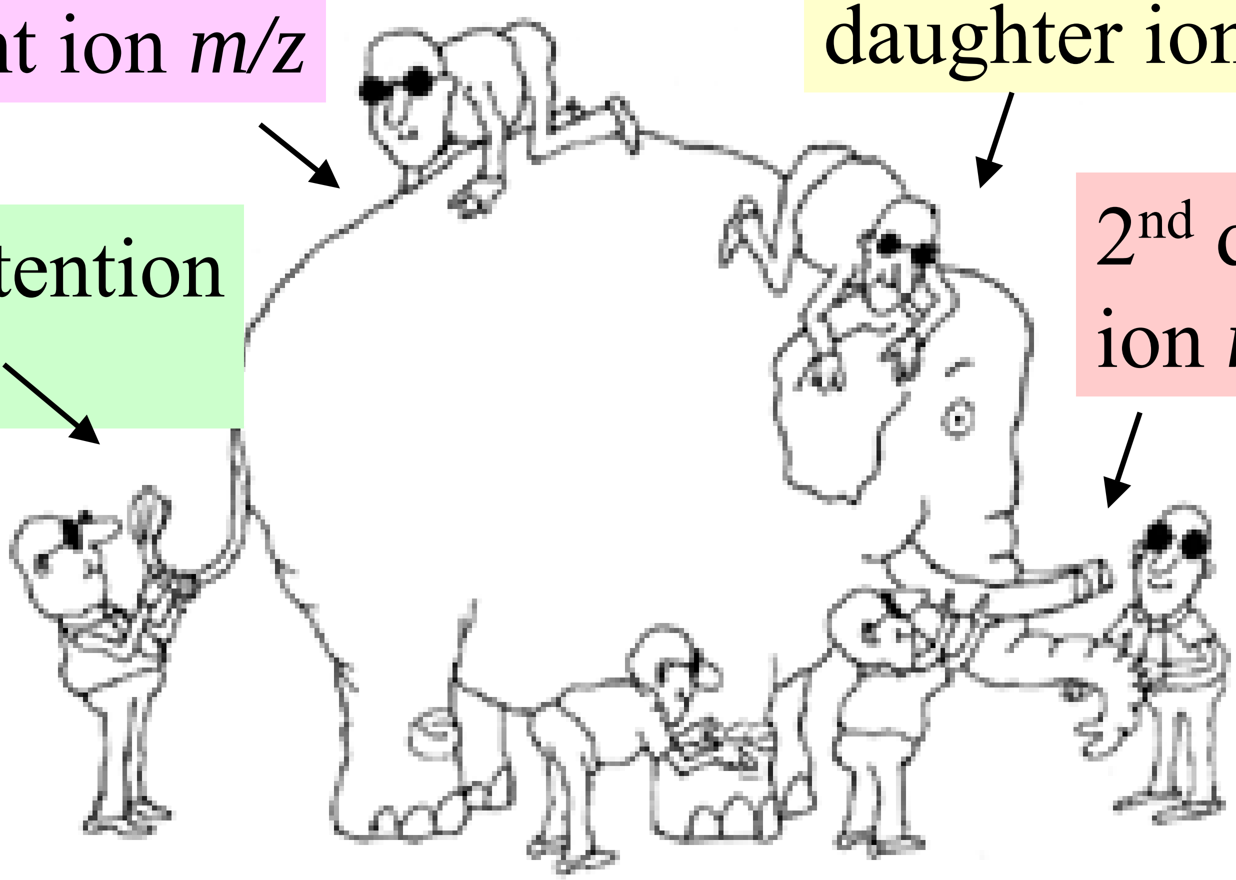
- Urine free cortisol by LC-MS-MS
  - Triple quadrupole

- 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



parent ion *m/z*

daughter ion *m/z*

LC retention time

2nd daughter ion *m/z*

Samples and Method

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

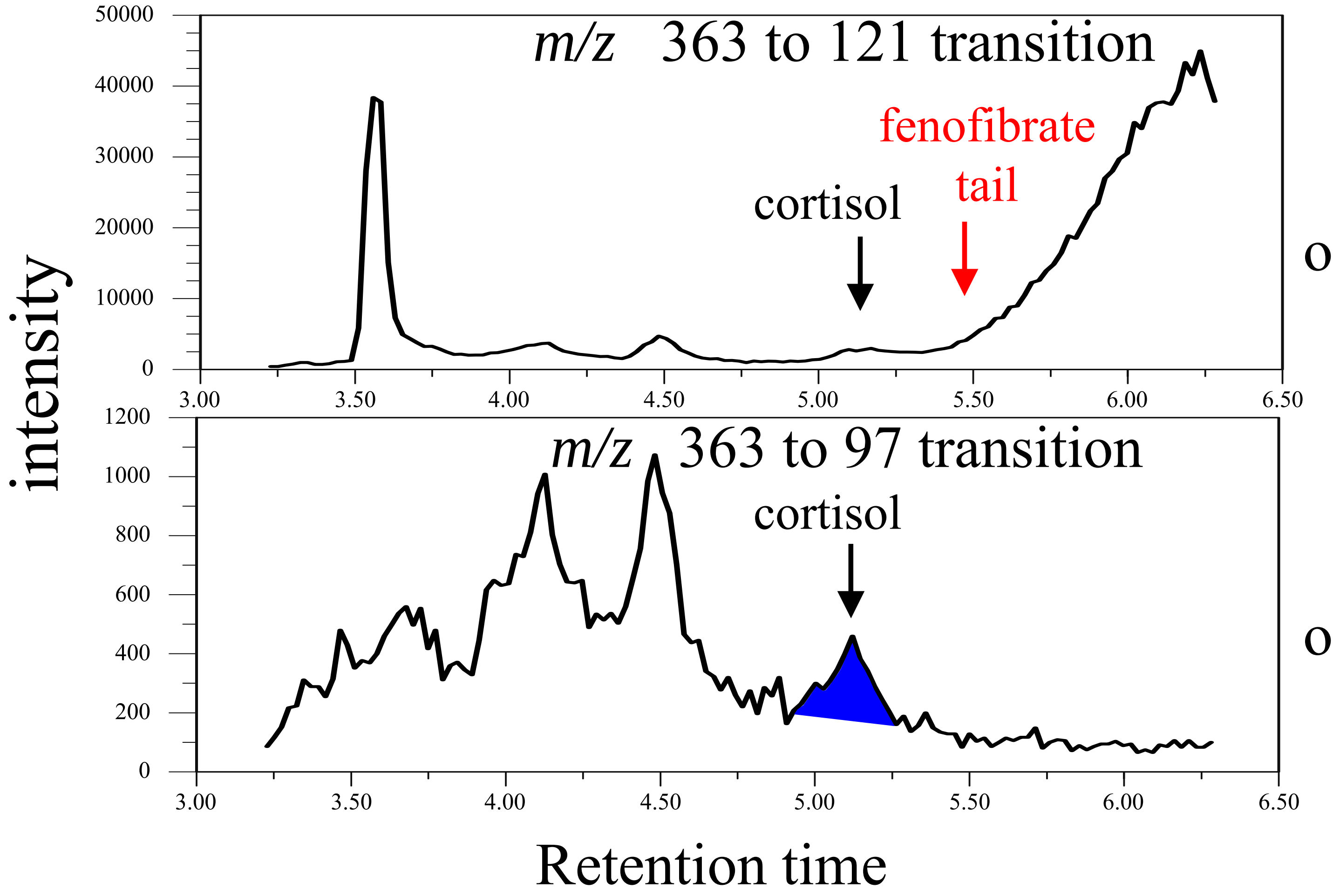
Need to Monitor More Than One MS/MS Transition

- 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)



interference
on transition #1

no
interference
on transition #2

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

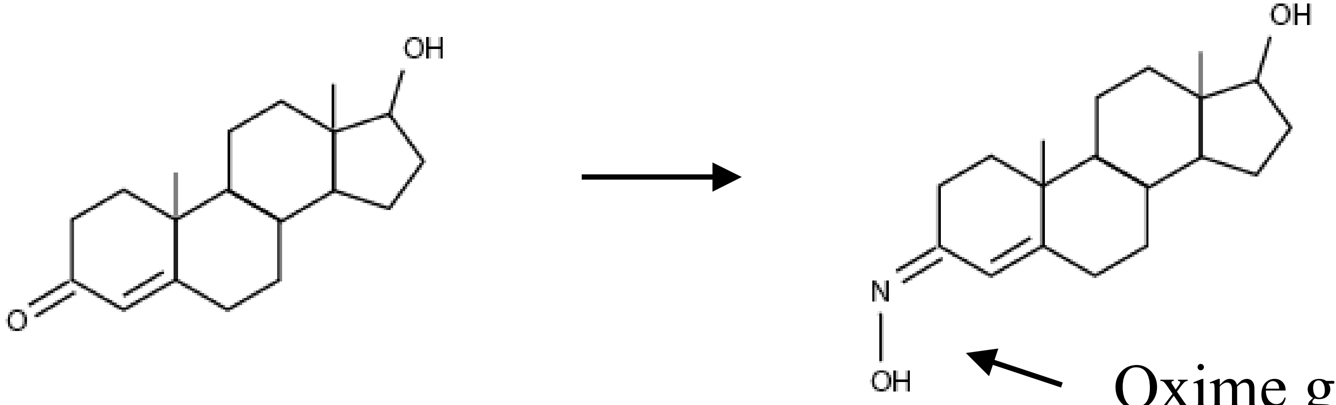
Goals

- 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



Oxime group

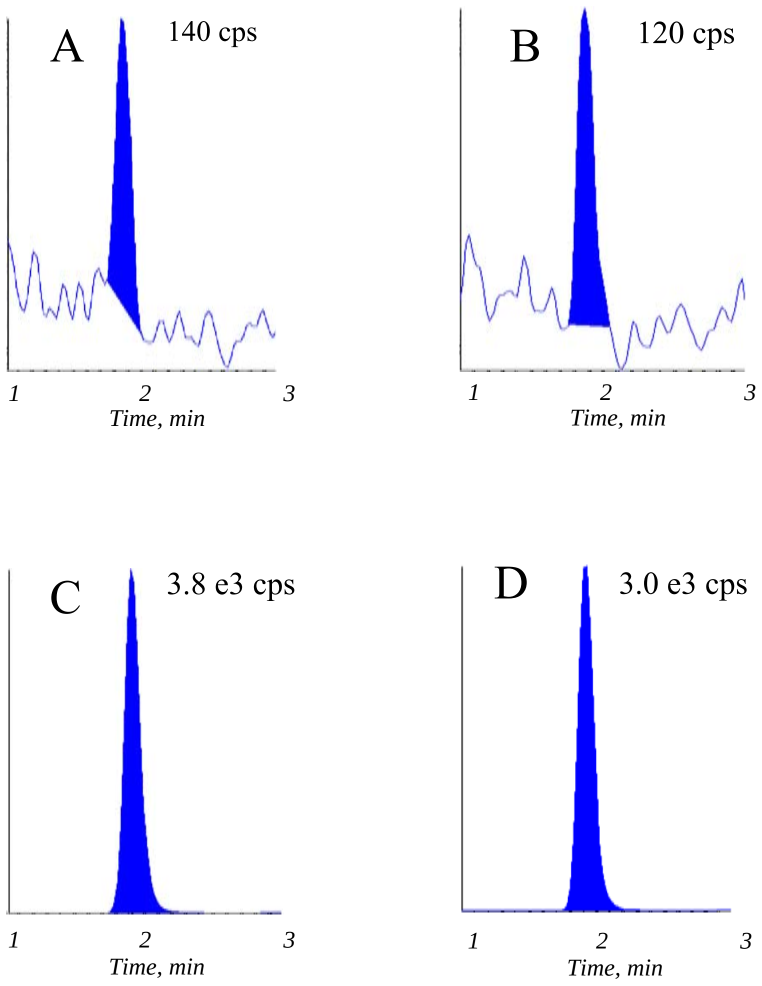
Method Summary

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 → 124
(quantitative)

testosterone
m/z 304 → 112
(qualitative)



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

Internal standard
d_3 testosterone
m/z 307 → 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

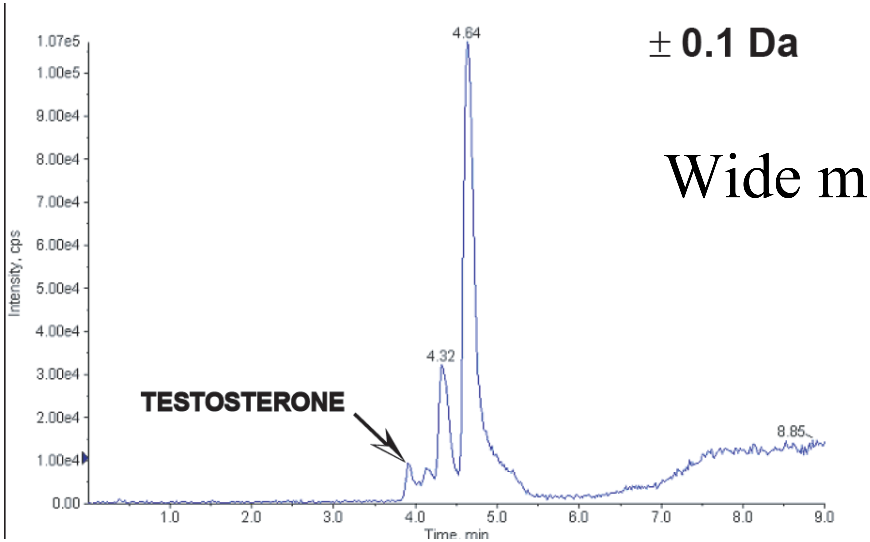
- Paul Zavitsanos, Agilent
- Gordon Nelson, ARUP

Illustrates

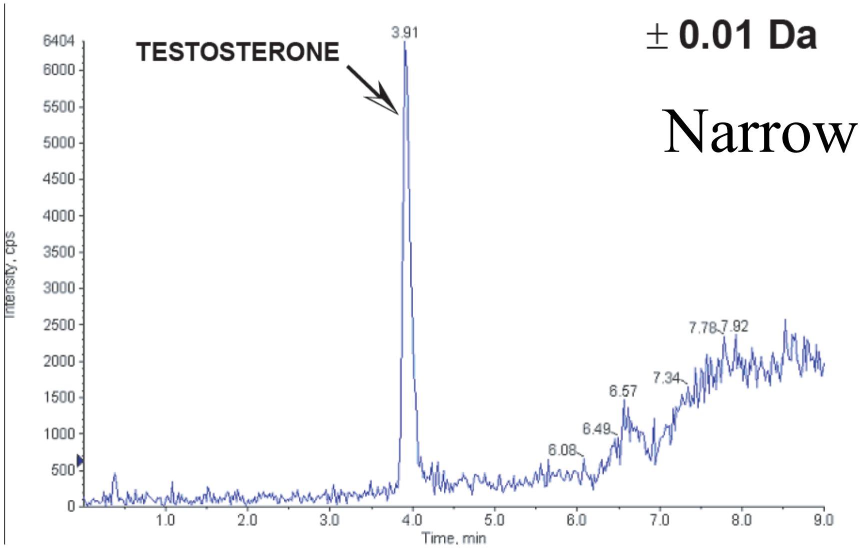
- TOF resolution → Narrow range reconstructed ion chromatograms achieve high selectivity

- 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

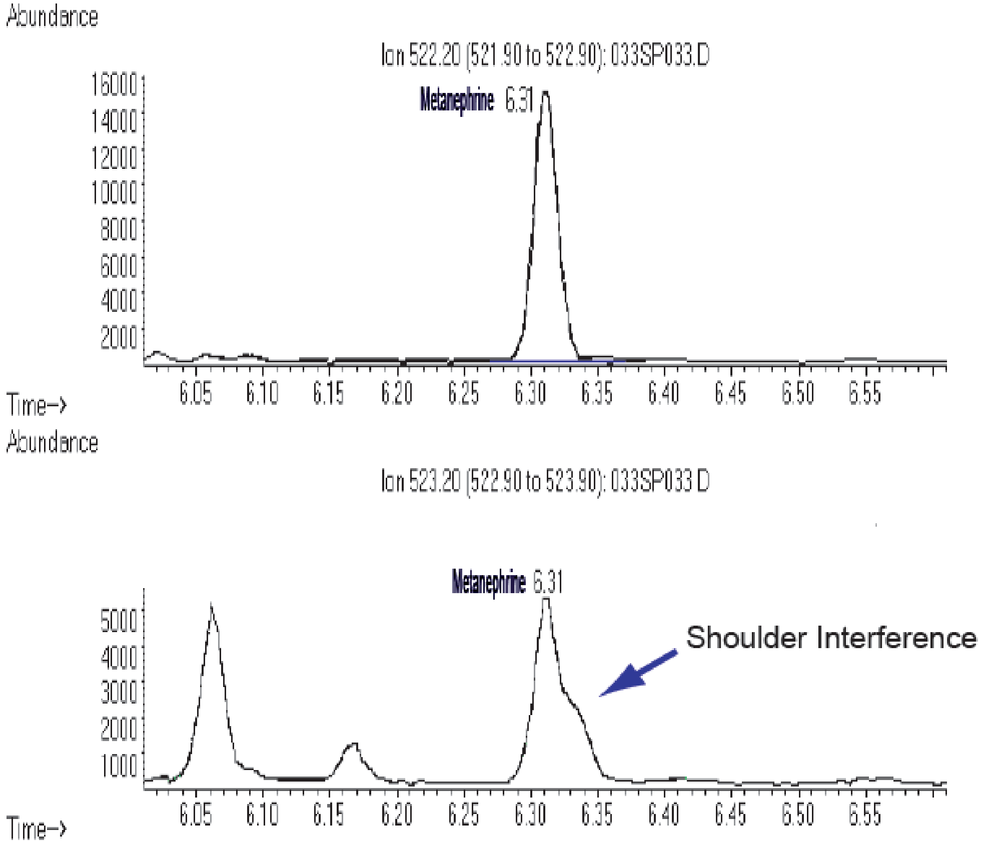
- TOF high resolution → improved selectivity
- TOF accurate mass → analyte confirmation
- Possible alternative to tandem MS

Application

- Metanephrines GC-MS vs. GC-MS-MS
  - Chromatograms
  - Ion ratios

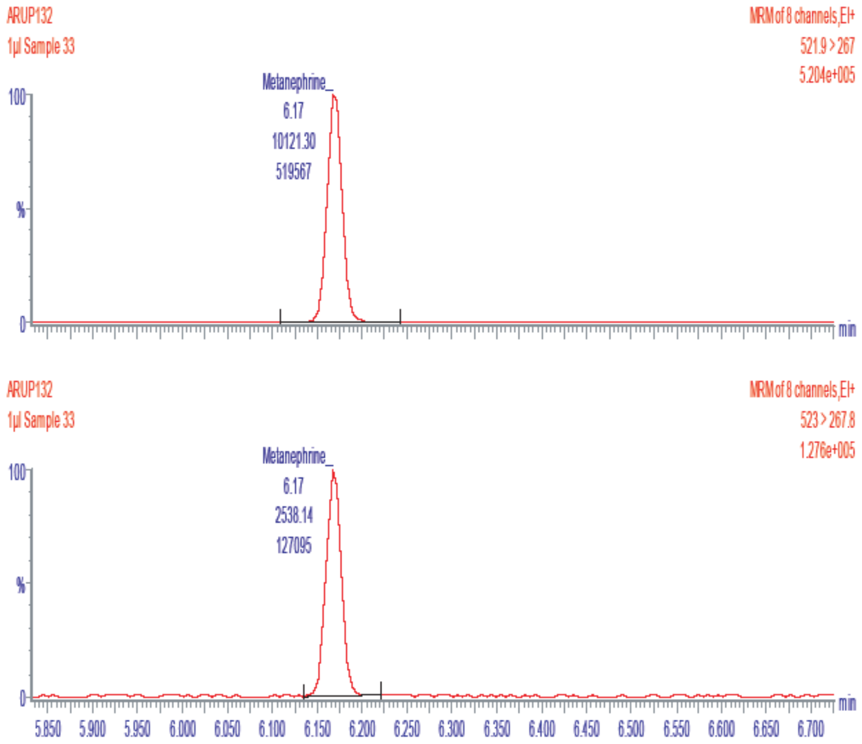
- Peter Hancock, Waters
- Jason Sawyer, ARUP

GC-MS Interference



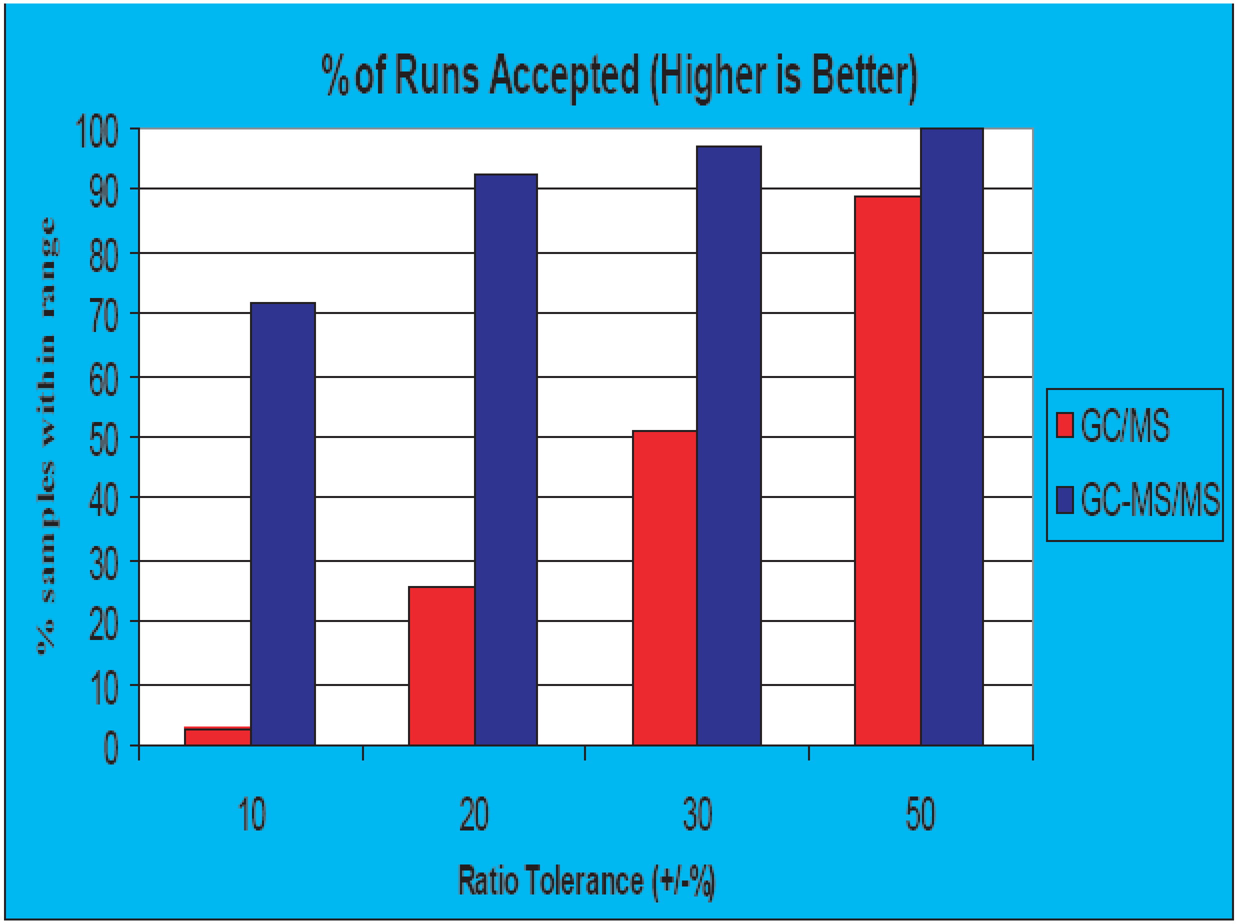
GC-Single Quadrupole Mass Spectrometer

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

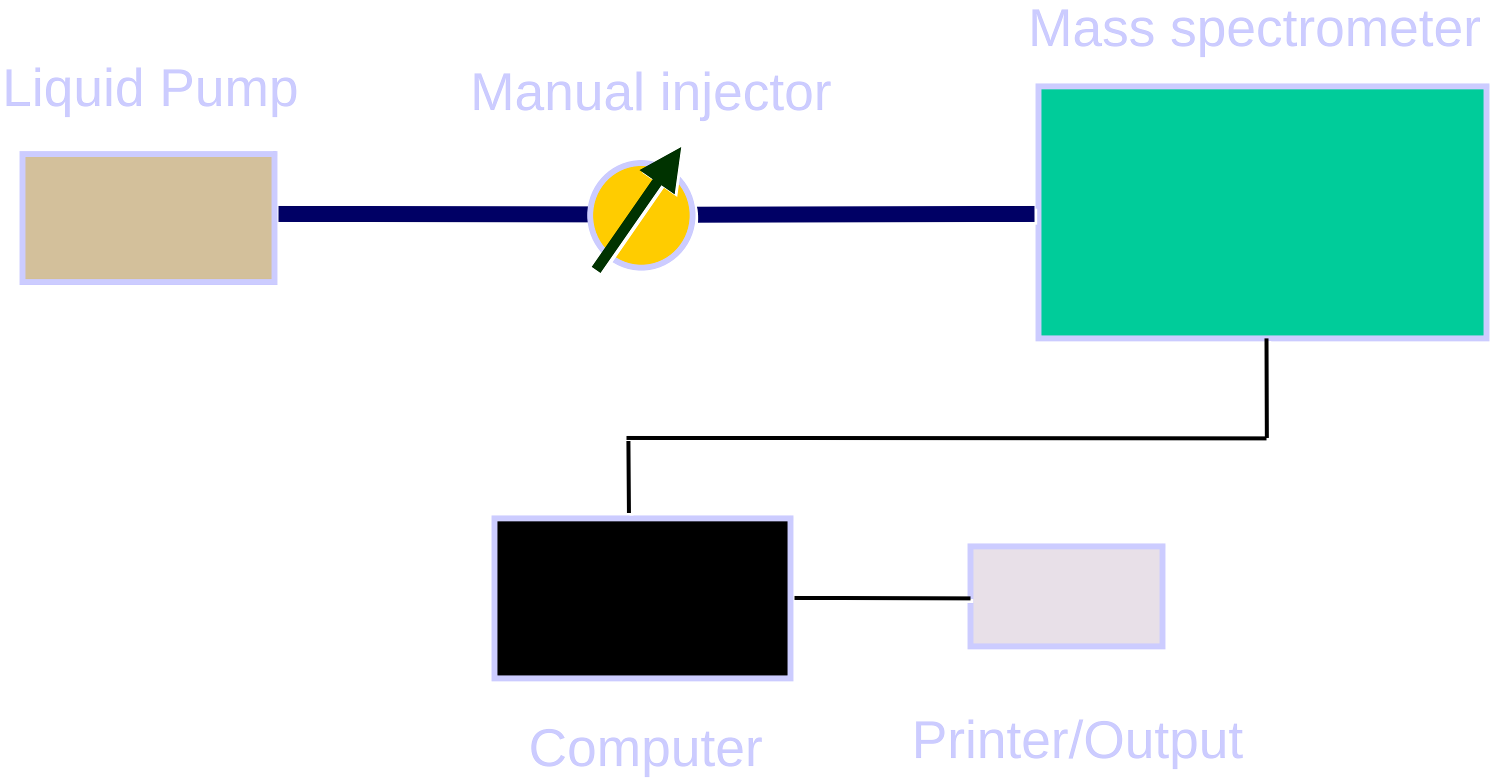
Illustrates

- Protein identification by MS
  - Mass shift identifies mutations

- Multianalyte capability

- Throughput

Hemoglobin Analysis Scheme



Liquid Pump

Manual injector

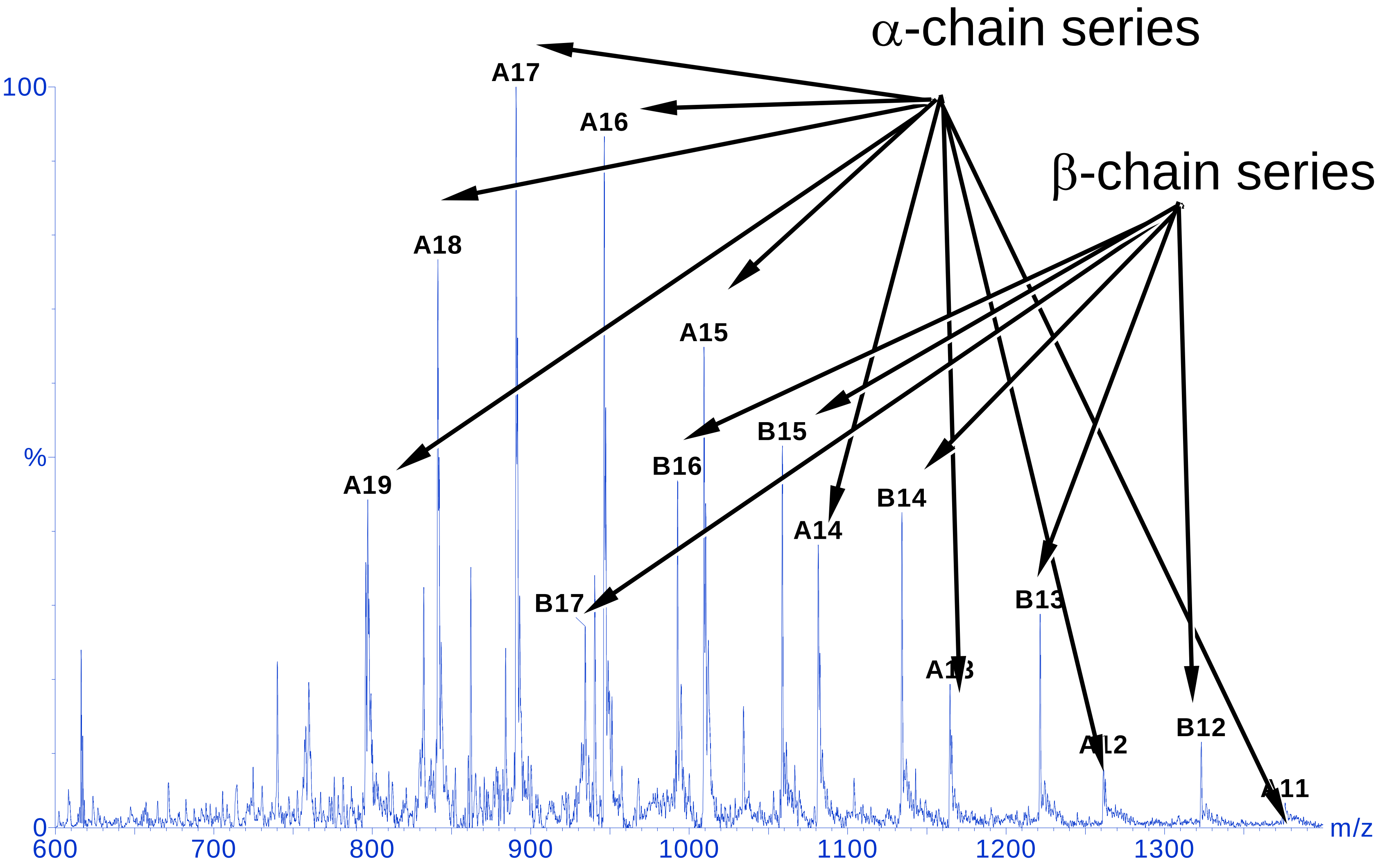
Mass spectrometer

Computer

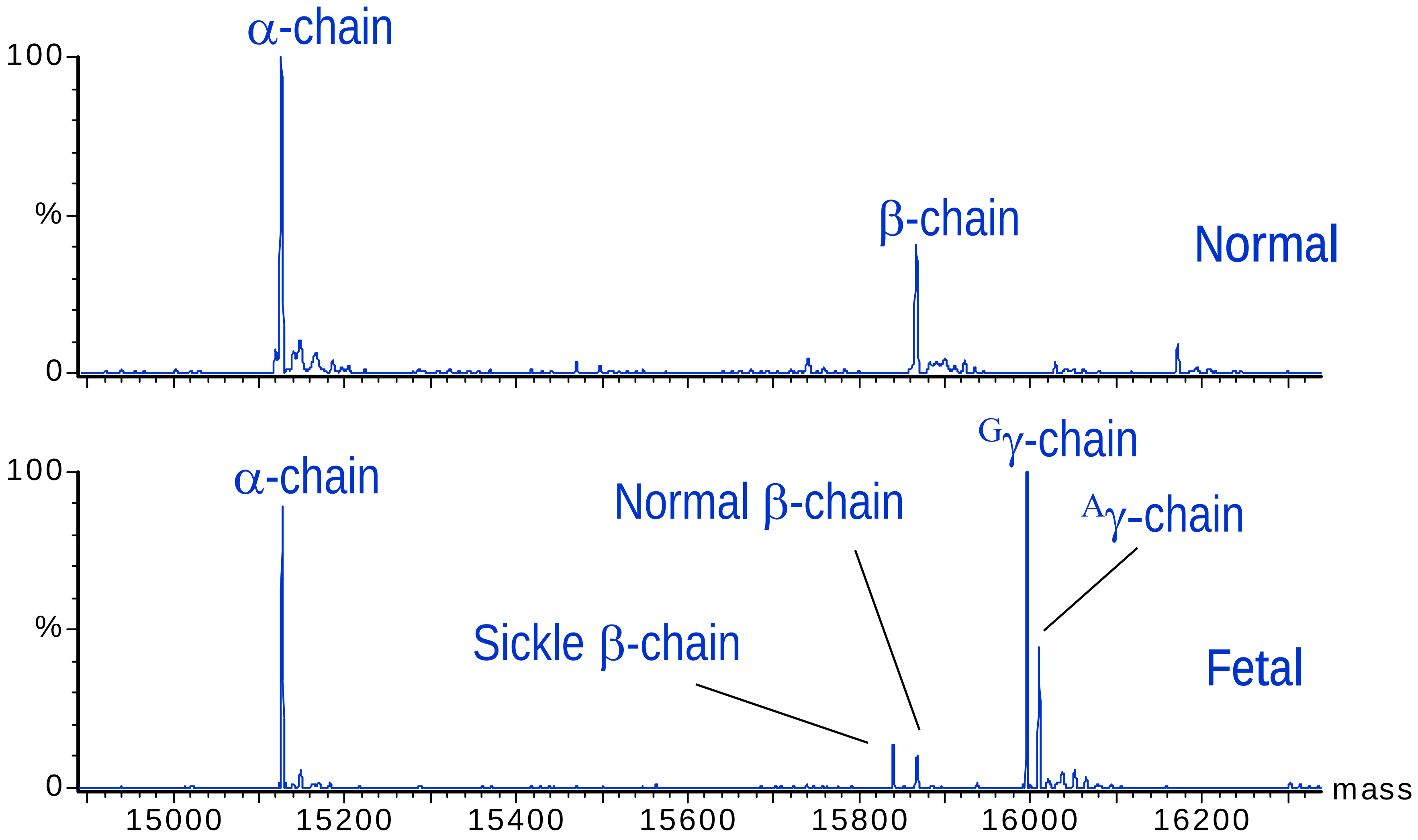
Printer/Output

Application from Brian Green, Waters Corp

Normal Hb ESI Mass Spectrum

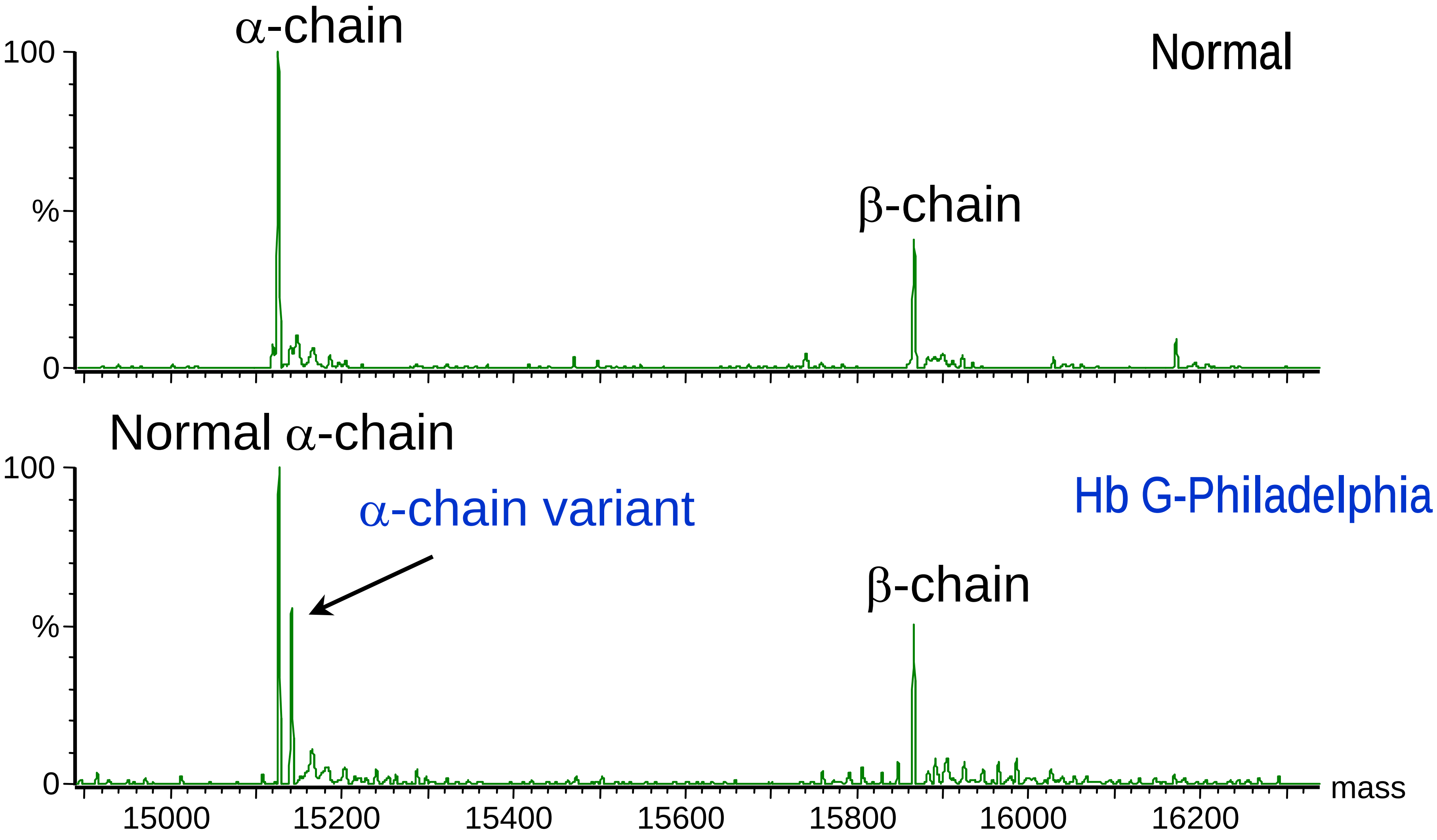


Normal *vs* Sickle Heterozygote (with Fetal Hb)

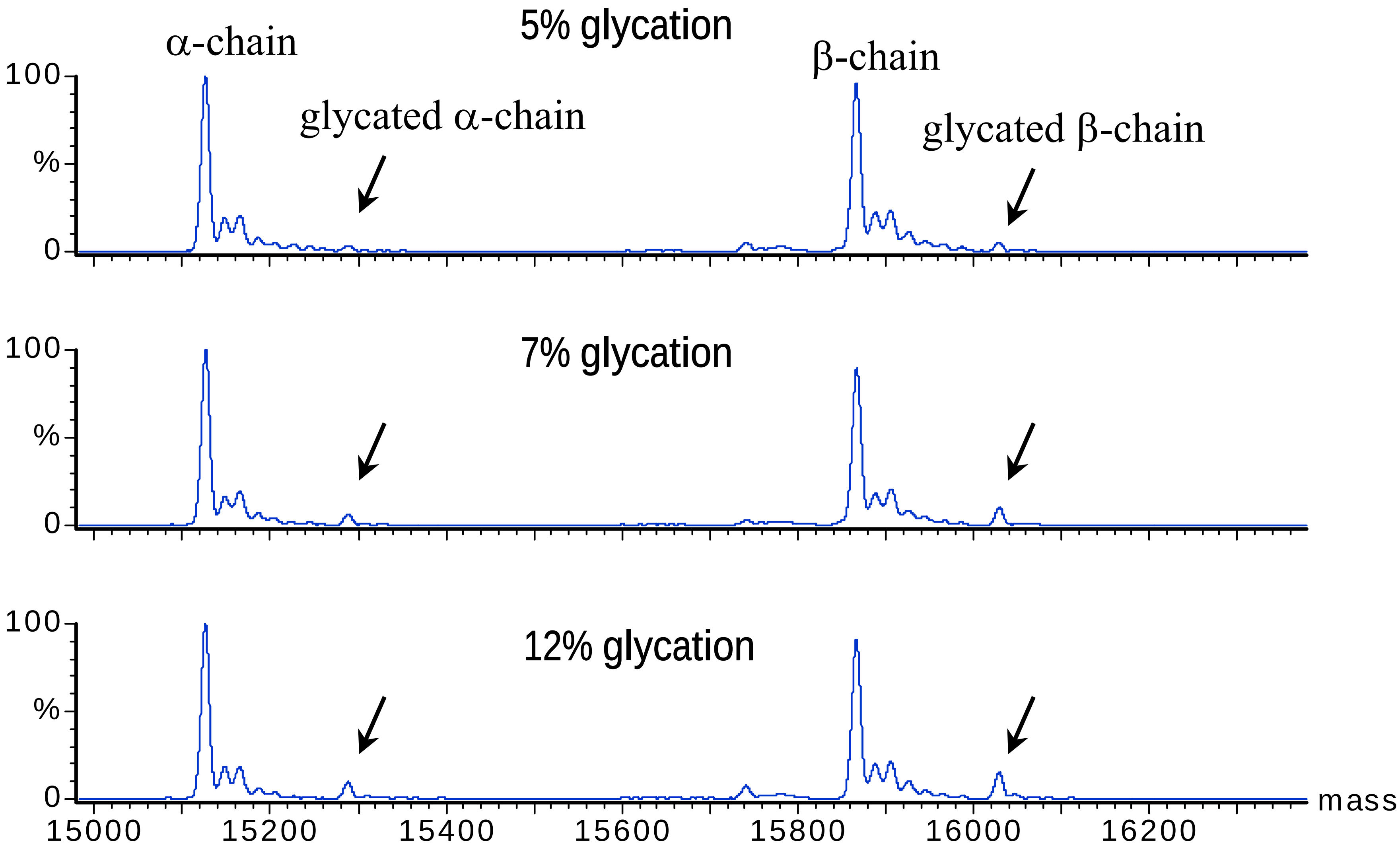


Normal *vs* α-Chain Variant

Hb G Philadelphia (α_{68} Asn→Lys ; Δm=+18Da)



Glycated Hemoglobins



Possible Improvements

- Autosampler (< 1 min/sample)

- TOF-MS
  - Faster, more sensitive for full mass spectrum
  - Higher resolution
  - Accurate mass
    - 60ppm for Δm = 1 Da substitution
    - 2.7ppm for Δm = 0.043 Da (Gln/Lys) substitution

- Enyzme 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

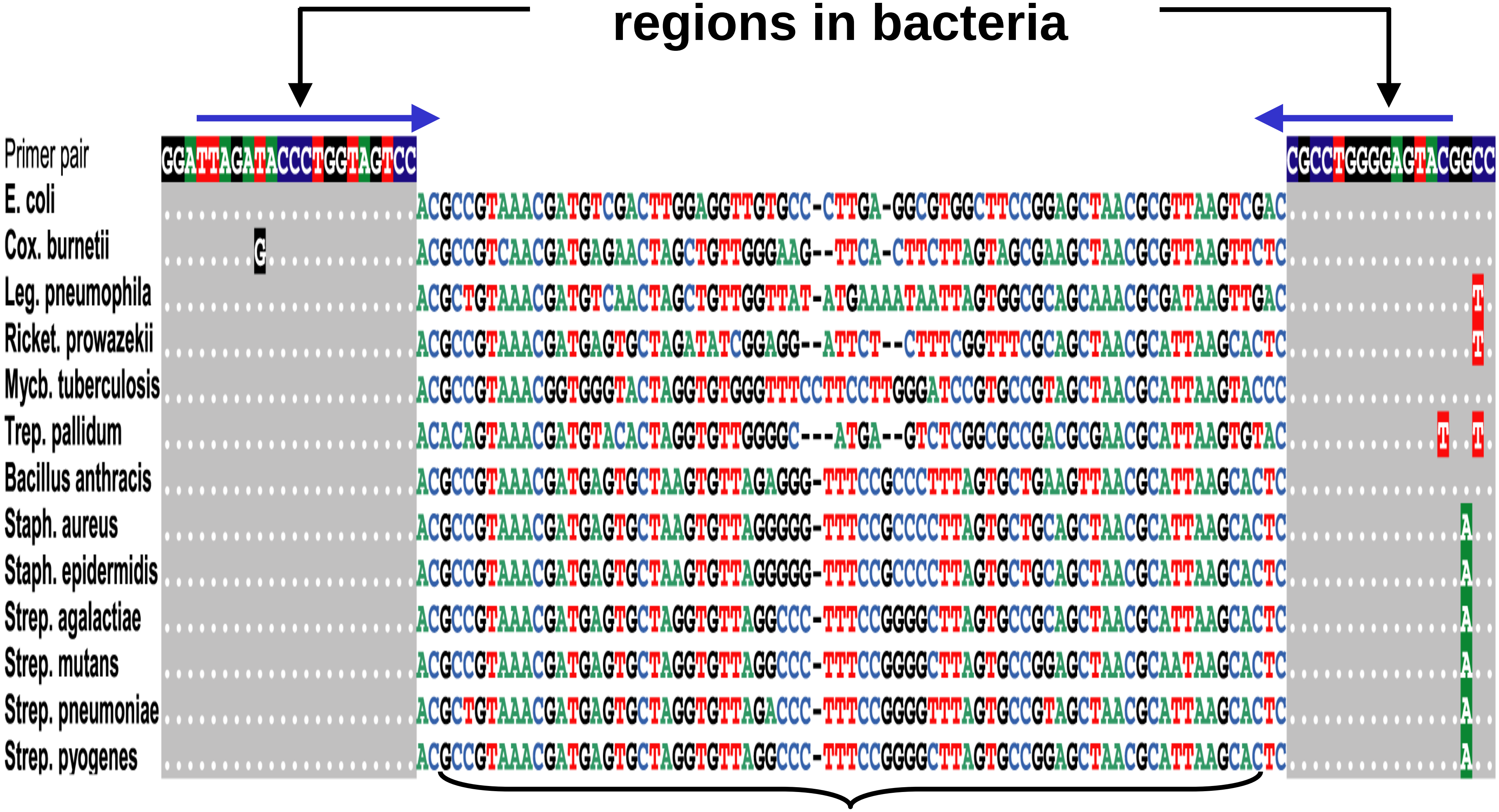
- Genotyping by accurate mass TOF MS
- Steve Hofstadler, Ibis Therapeutics

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

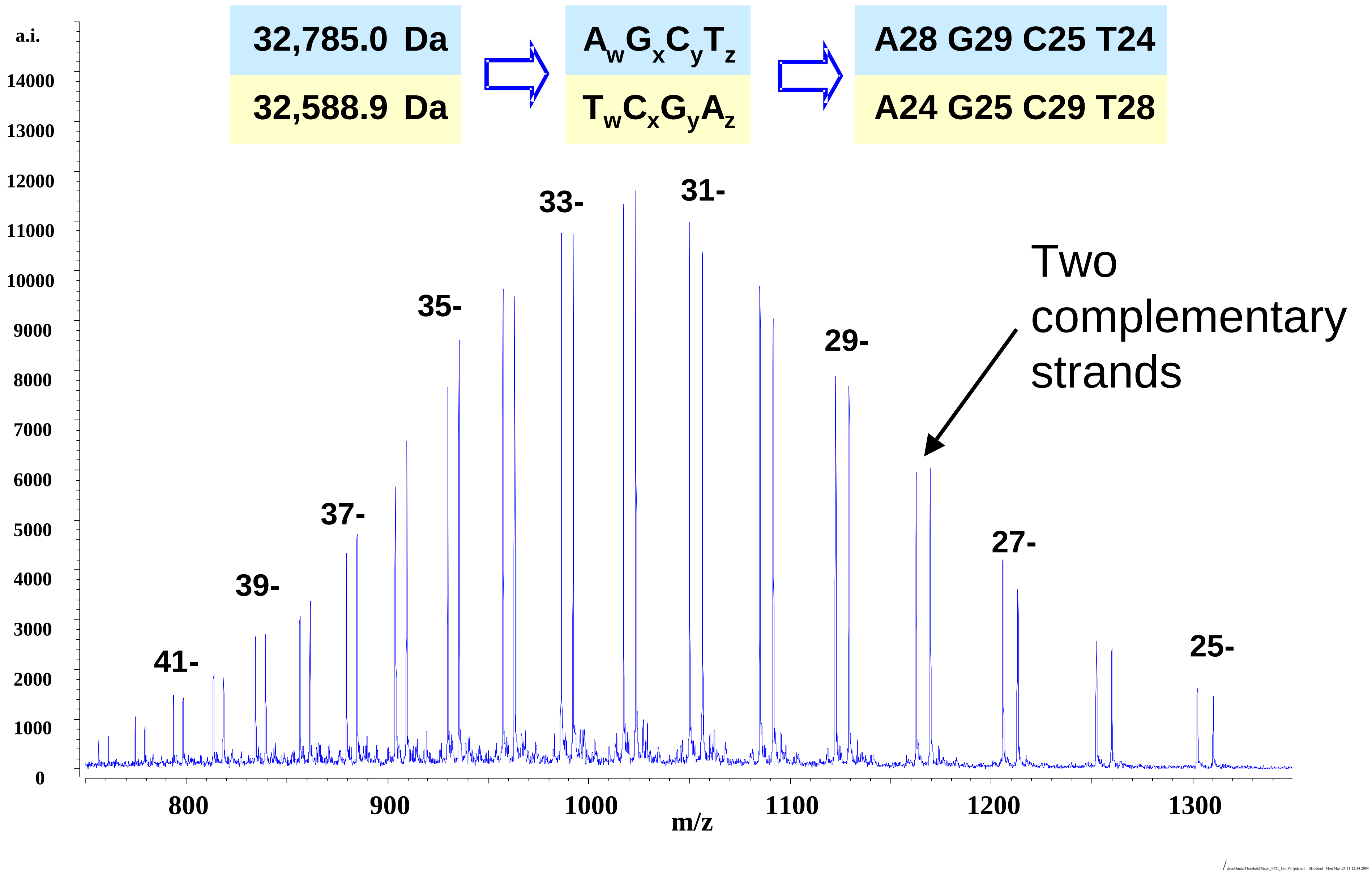


Primer pair	GGATTAGATACCCTGGTAGTCC		CGCCTGGGGAGTACGGCC
E. coli		ACGCCGTAAACGATGTCGACTTGGAGGTTGTGCC-CTTGA-GGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGAC	
Cox. burnetii	G	ACGCCGTCAACGATGAGAACTAGCTGTTGGGAAG--TTCA-CTTCTTAGTAGCGAAGCTAACGCGTTAAGTTCTC	
Leg. pneumophila		ACGCTGTAAACGATGTCAACTAGCTGTTGGTTAT-ATGAAAATAATTAGTGGCGCAGCAAACGCGATAAGTTGAC	T
Ricket. prowazekii		ACGCCGTAAACGATGAGTGCTAGATATCGGAGG--ATTCT--CTTTCGGTTTCGCAGCTAACGCATTAAGCACTC	T
Mycb. tuberculosis		ACGCCGTAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCTTGGGATCCGTGCCGTAGCTAACGCATTAAGTACCC	
Trep. pallidum		ACACAGTAAACGATGTACACTAGGTGTTGGGGC---ATGA--GTCTCGGCGCCGACGCGAACGCATTAAGTGTAC	T T
Bacillus anthracis		ACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGG-TTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTC	
Staph. aureus		ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG-TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC	A
Staph. epidermidis		ACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGG-TTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTC	A
Strep. agalactiae		ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCCC-TTTCCGGGGCTTAGTGCCGCAGCTAACGCATTAAGCACTC	A
Strep. mutans		ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCCC-TTTCCGGGGCTTAGTGCCGGAGCTAACGCAATAAGCACTC	A
Strep. pneumoniae		ACGCTGTAAACGATGAGTGCTAGGTGTTAGACCC-TTTCCGGGGTTTAGTGCCGTAGCTAACGCATTAAGCACTC	A
Strep. pyogenes		ACGCCGTAAACGATGAGTGCTAGGTGTTAGGCCC-TTTCCGGGGCTTAGTGCCGGAGCTAACGCATTAAGCACTC	A

Variable region $\Rightarrow \Delta\,[A_wG_xC_yT_z]$

ESI-TOF Spectrum of *Staph. aureus* amplicon

Measured MW's are within 10 ppm of theoretical

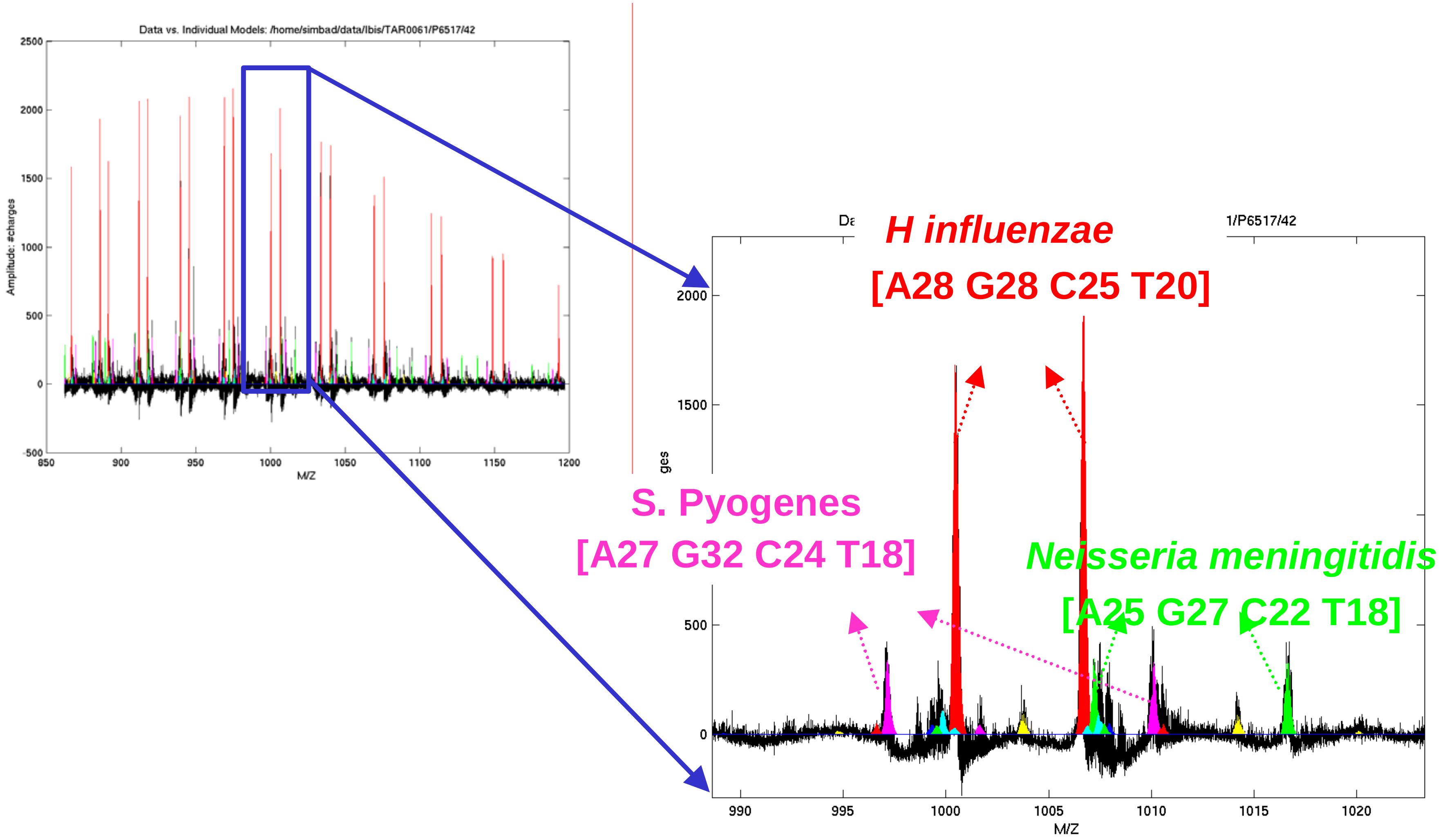


32,785.0 Da → **$A_wG_xC_yT_z$** → **A28 G29 C25 T24**

32,588.9 Da → **$T_wC_xG_yA_z$** → **A24 G25 C29 T28**

Two complementary strands

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



H influenzae

[A28 G28 C25 T20]

S. Pyogenes

[A27 G32 C24 T18]

Neisseria meningitidis

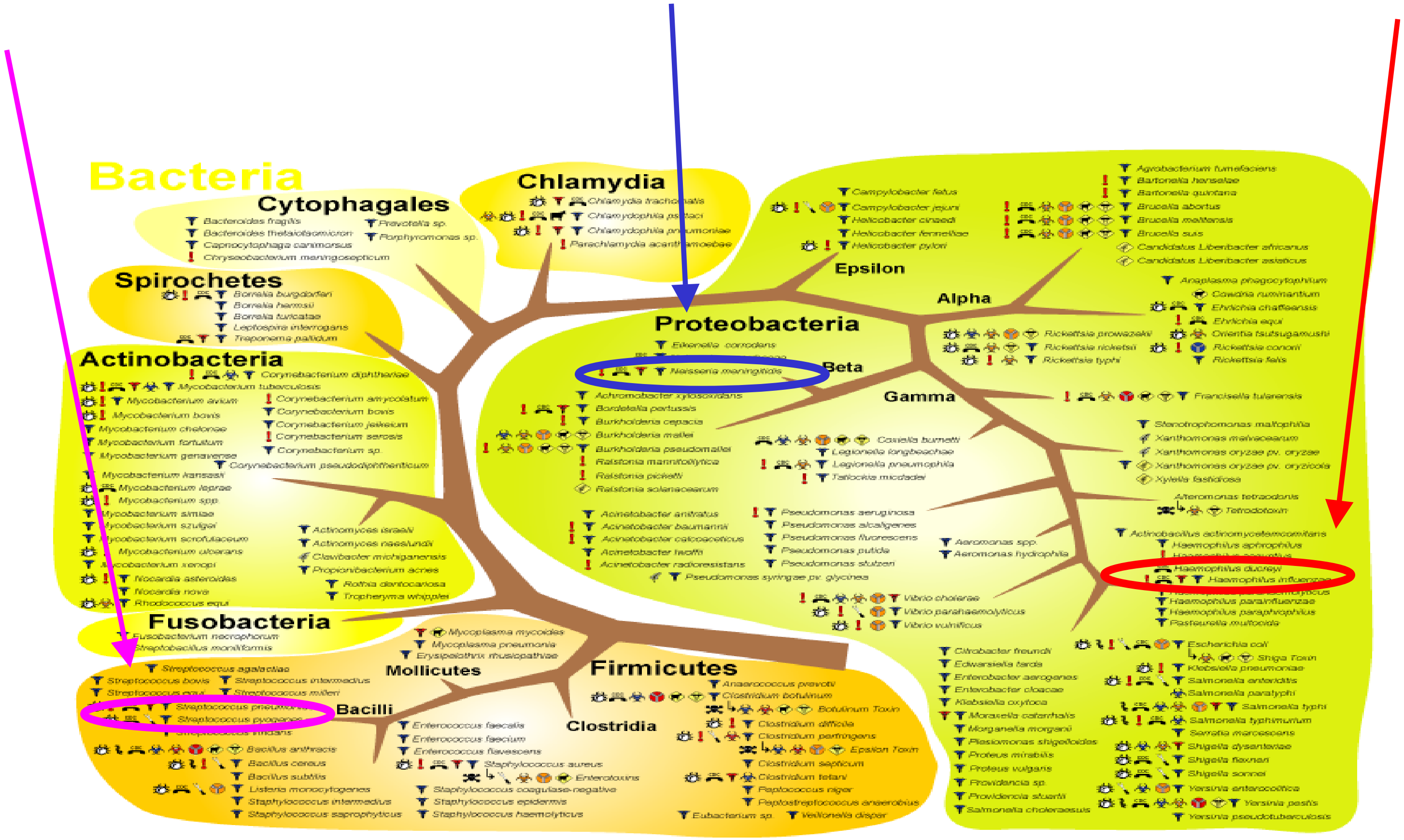
[A25 G27 C22 T18]

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

Streptococcus pyogenes

Neisseria meningitidis

Haemophilus influenzae



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
  - $^{56}Fe^+$
- Complication: polyatomic interferences
  - $^{40}Ar^{16}O^+$

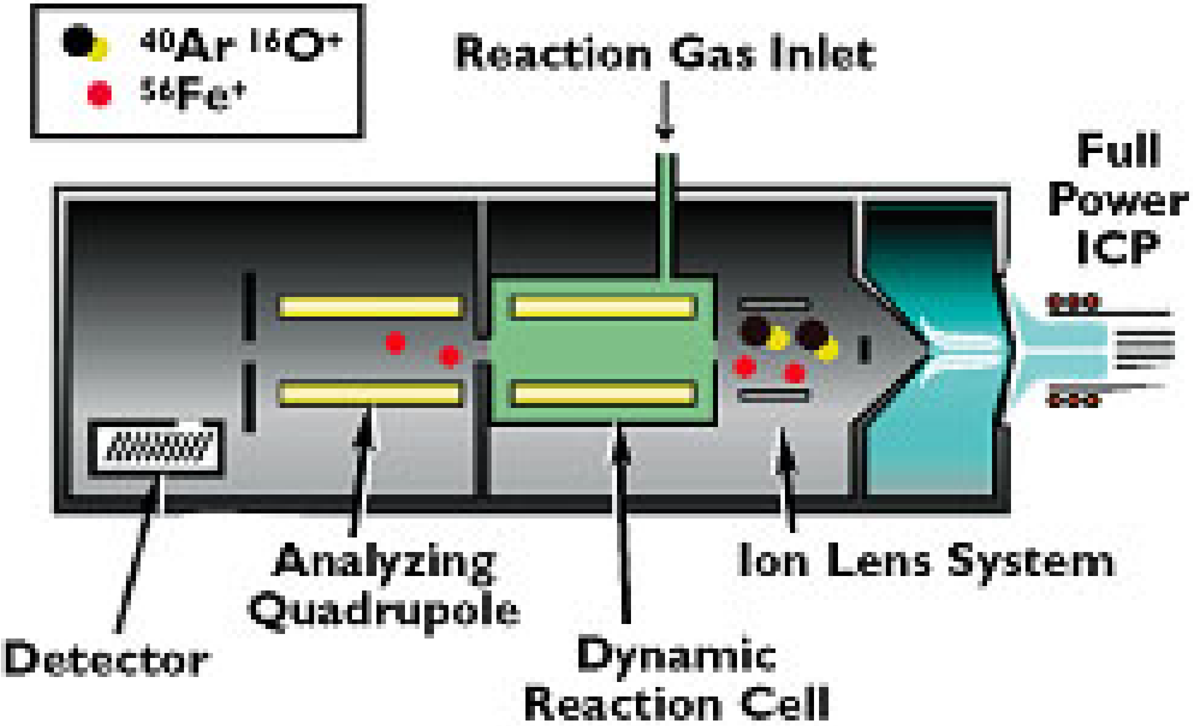
Typical ICP Interferences

Analyte	Interference
^{56}Fe	$^{40}Ar^{16}O$
^{80}Se	$^{40}Ar_2$
^{39}K	$^{38}Ar^{1}H$
^{40}Ca	^{40}Ar
^{51}V	$^{35}Cl^{16}O$
^{52}Cr	$^{40}Ar^{12}C$
^{63}Cu	$^{23}Na^{40}Ar$
^{75}As	$^{40}Ar^{35}Cl$

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

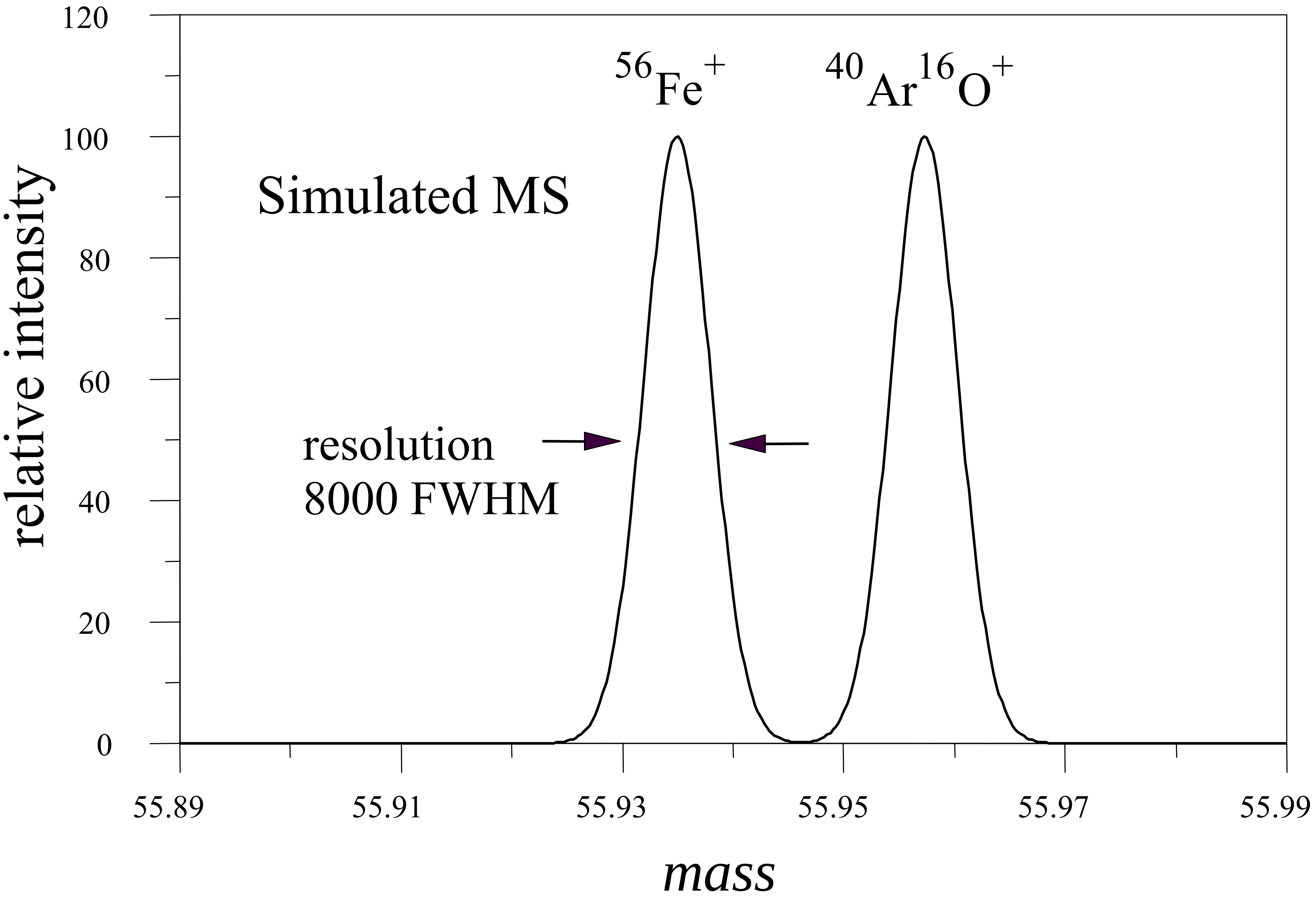
Dynamic Reaction Cell (Chemistry)



$ArO^+ + NH_3$ -----> $ArO + NH_3^+$

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

Acknowledgements

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