

ThermoFisher
SCIENTIFIC

The world leader in serving science

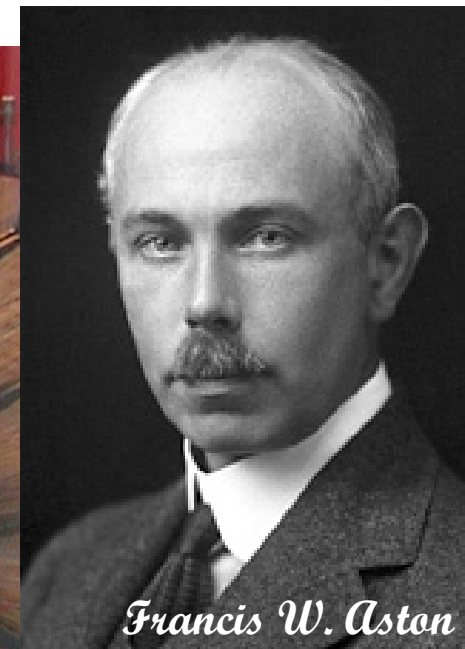
Precision Proteomics

Michaela Scigelova
European LC-MS Marketing

'Promising' Proteomics Technologies ?

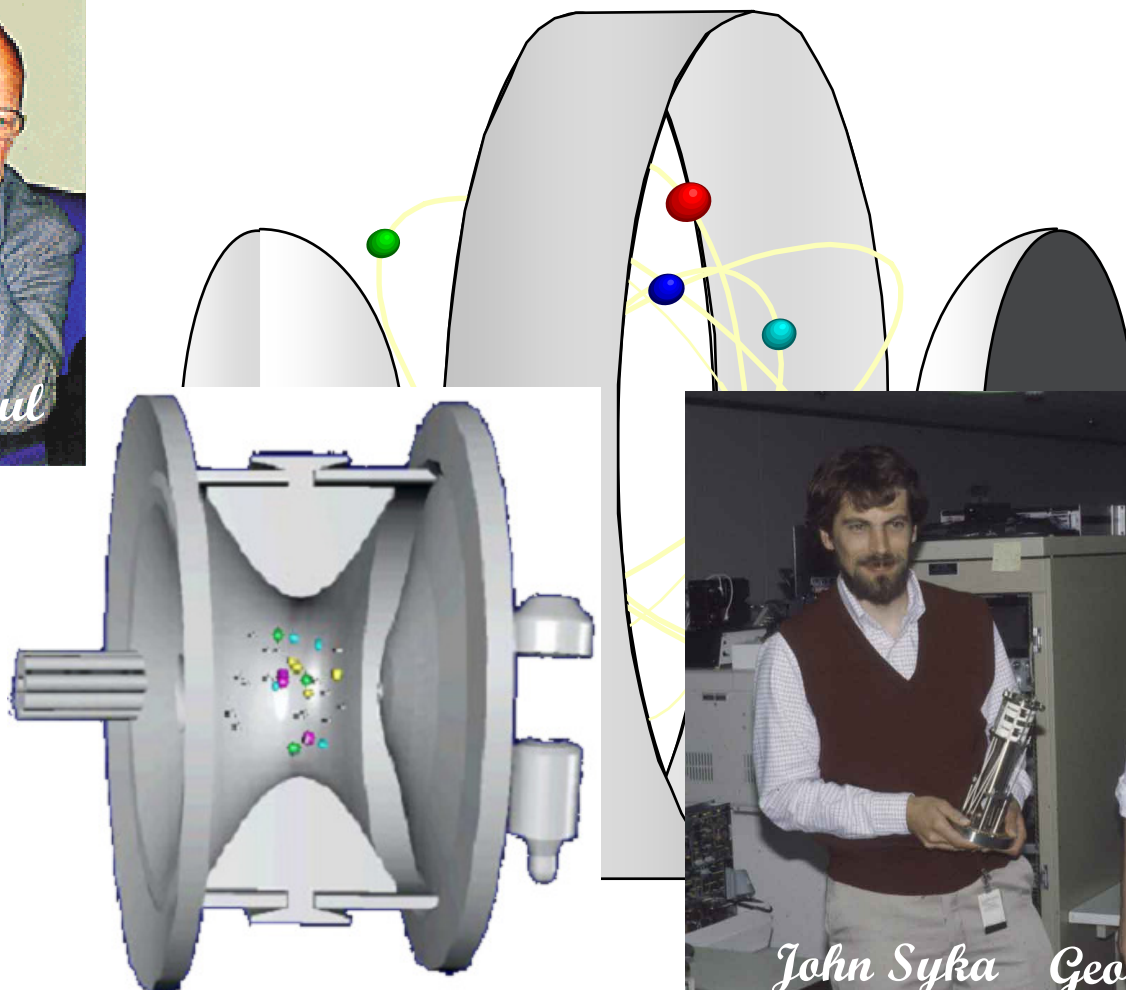
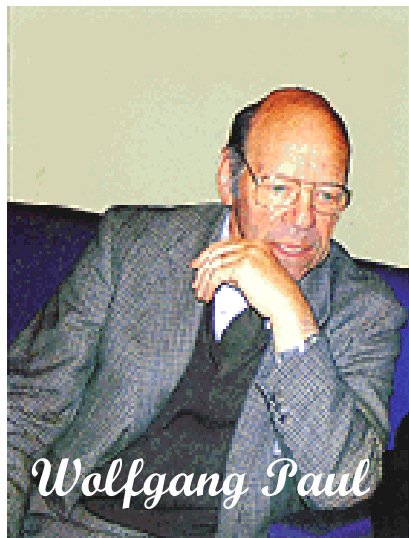
- Two-dimensional gel electrophoresis
- Protein arrays
- SELDI
- MS-based proteomics
 - Quantitative analysis of post-translational modifications
 - Determination of protein interactions
 - Integration of MS technology with other tools of molecular biology
 - Technology improvements in the last 5 years

The birth of a mass spectrometer



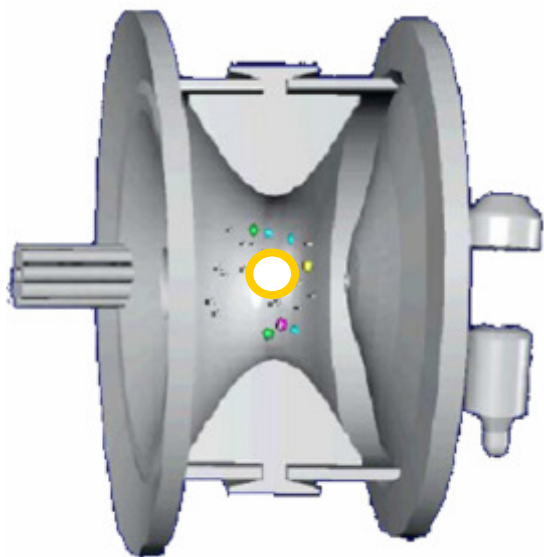
The Science Museum, Birmingham, UK

Ion Trap – does MS/MS and more

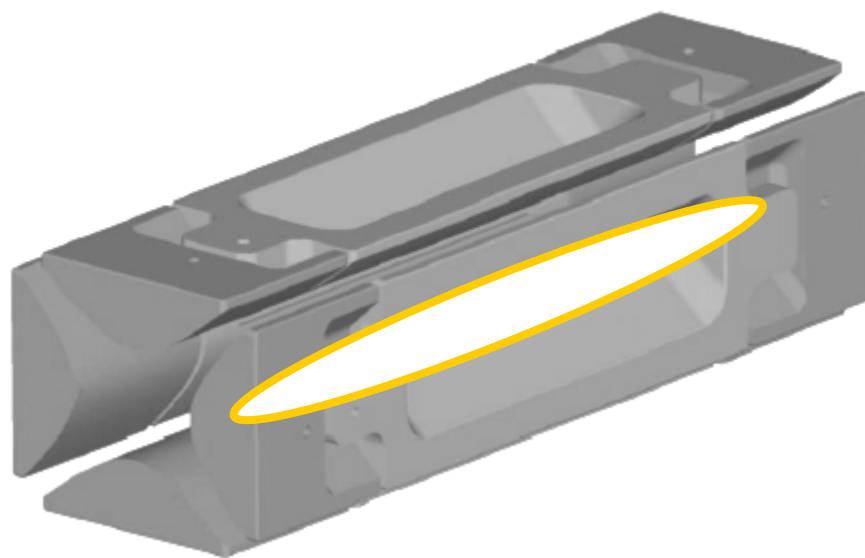


Linear Ion Trap Technology

Classic Ion Trap

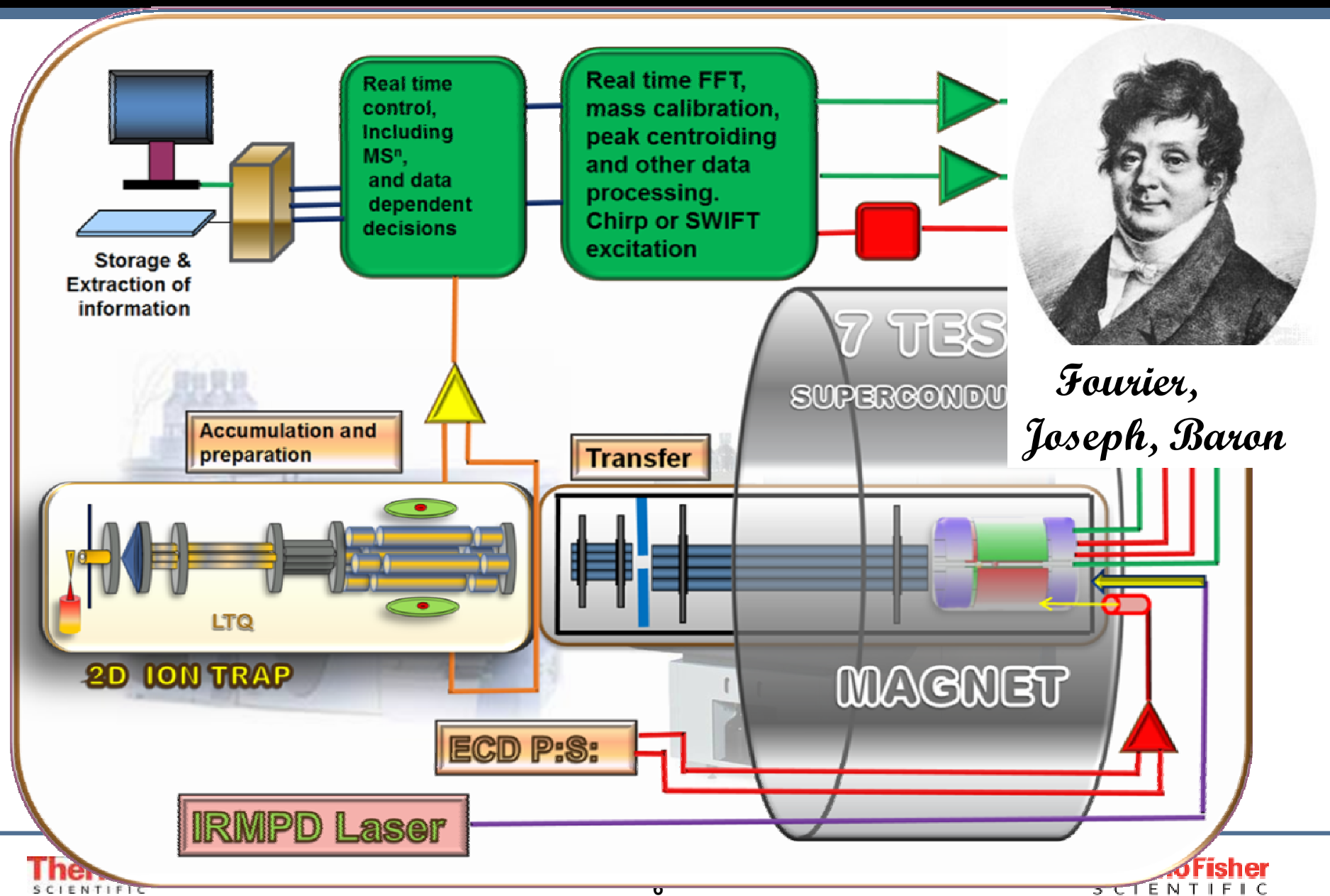


Linear Ion Trap

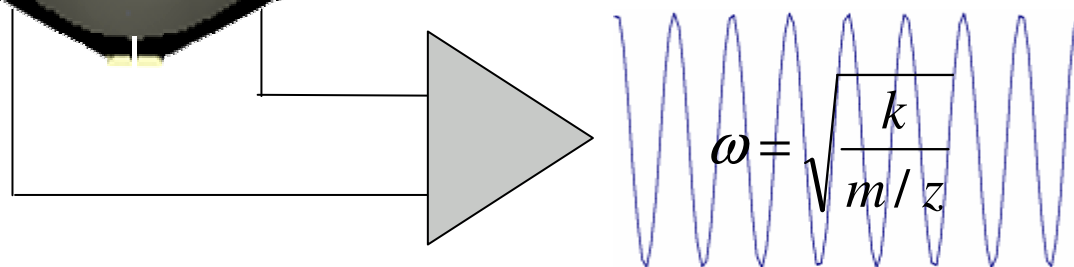
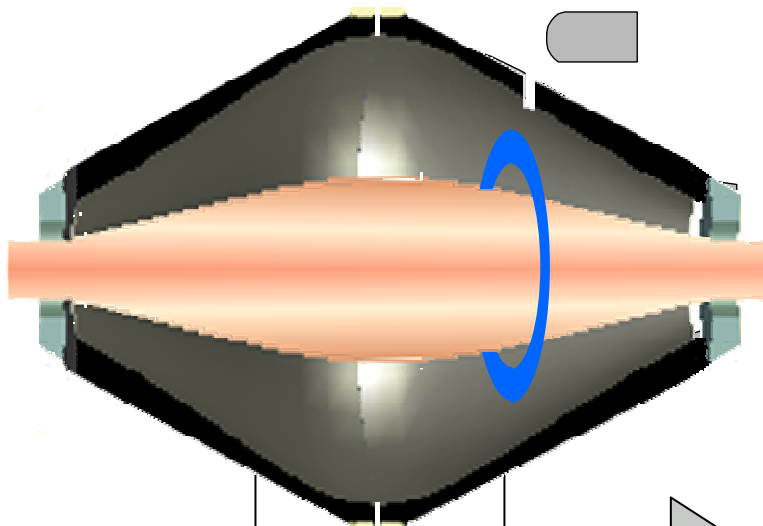


- Advancement in Sensitivity, Speed and Spectral Quality

Powerful hybrid mass spectrometer: LTQ FT



The Orbitrap Analyser



- frequency of oscillation related to m/z

The case for high resolution and high mass accuracy

Goal:

- Precise and comprehensive analysis of complex mixtures
- Best achieved by using high-resolution mass spectrometric technologies

The importance of being highly resolved

Problem:

- Complex mixtures
- Coeluting compounds with similar m/z ratios

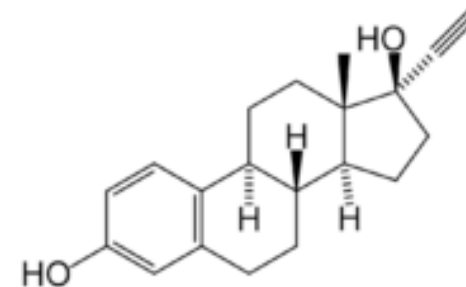
Compromised:

- Accurate mass analysis
- Charge state determination
- Accurate quantitation

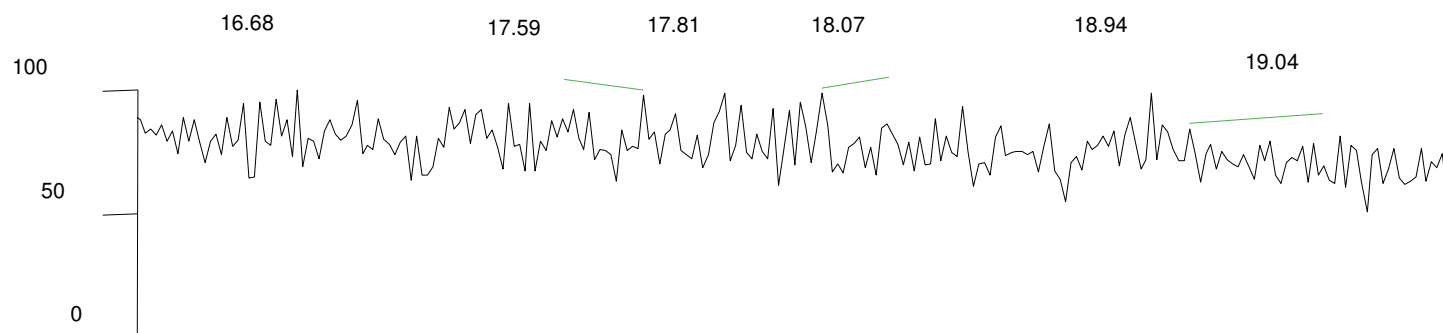
Hormone Analysis

RP = 10,000

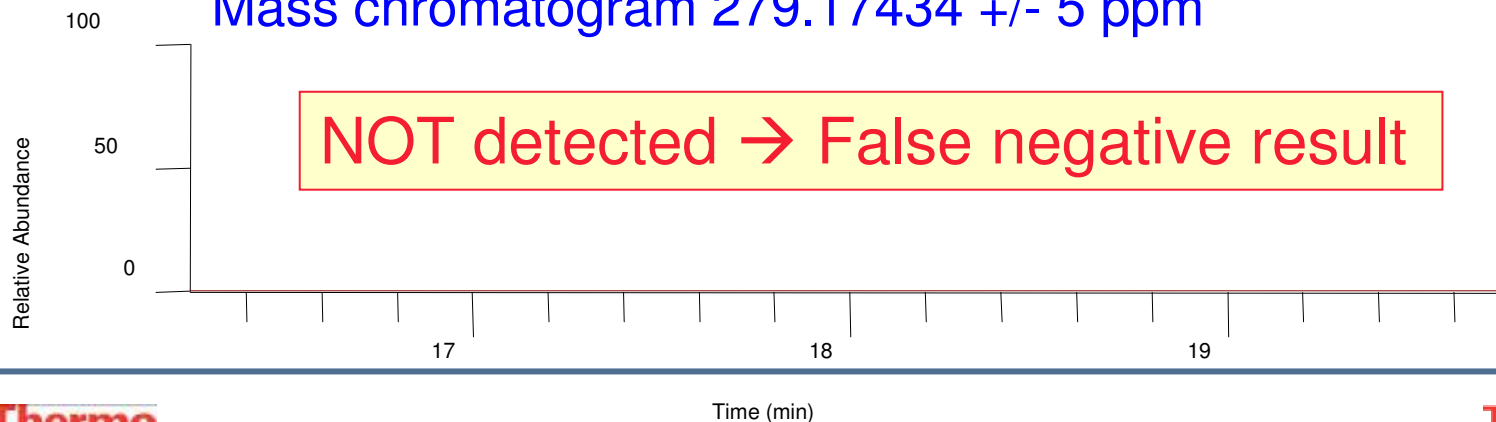
- Ethinyl estradiol in urine matrix
- 100 ppb



LC-MS trace



Mass chromatogram 279.17434 +/- 5 ppm



Hormone sample

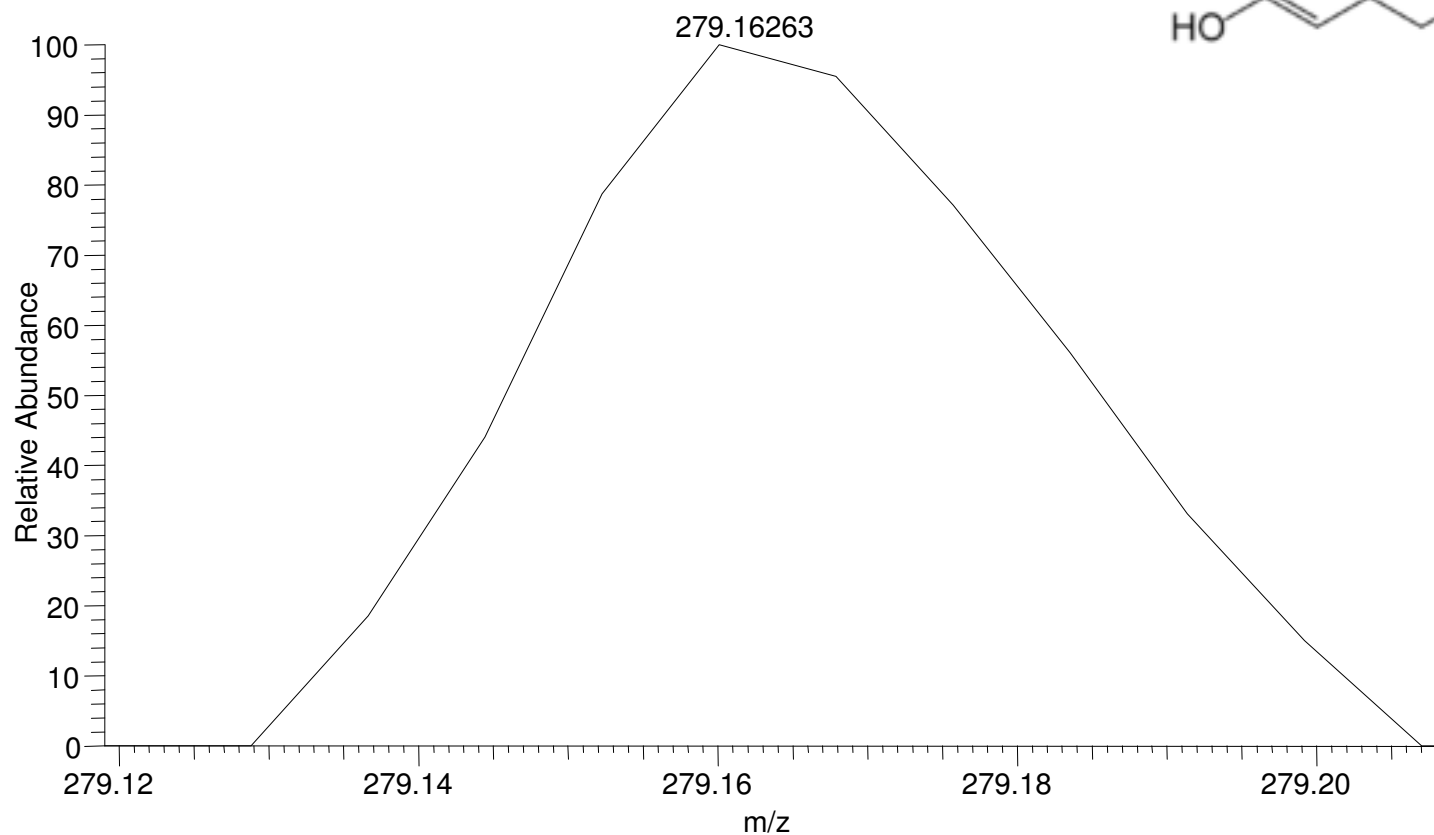
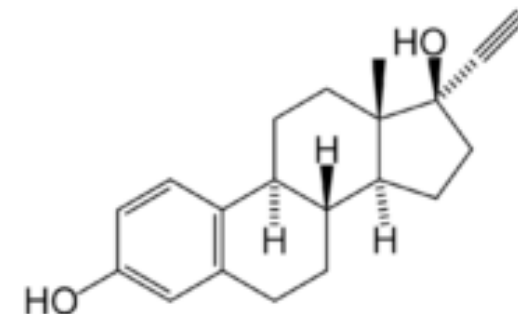
RP = 10,000

Ethinyl Estradiol

Calculated 279.17434

Measured: 279.16263

Error: - 42 ppm



Hormone sample

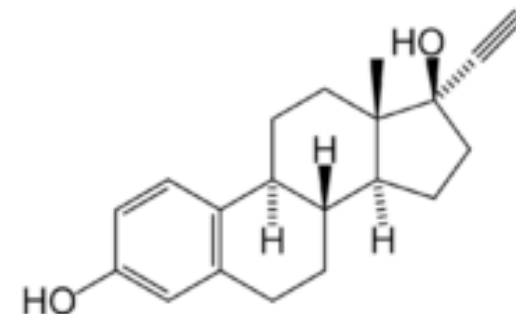
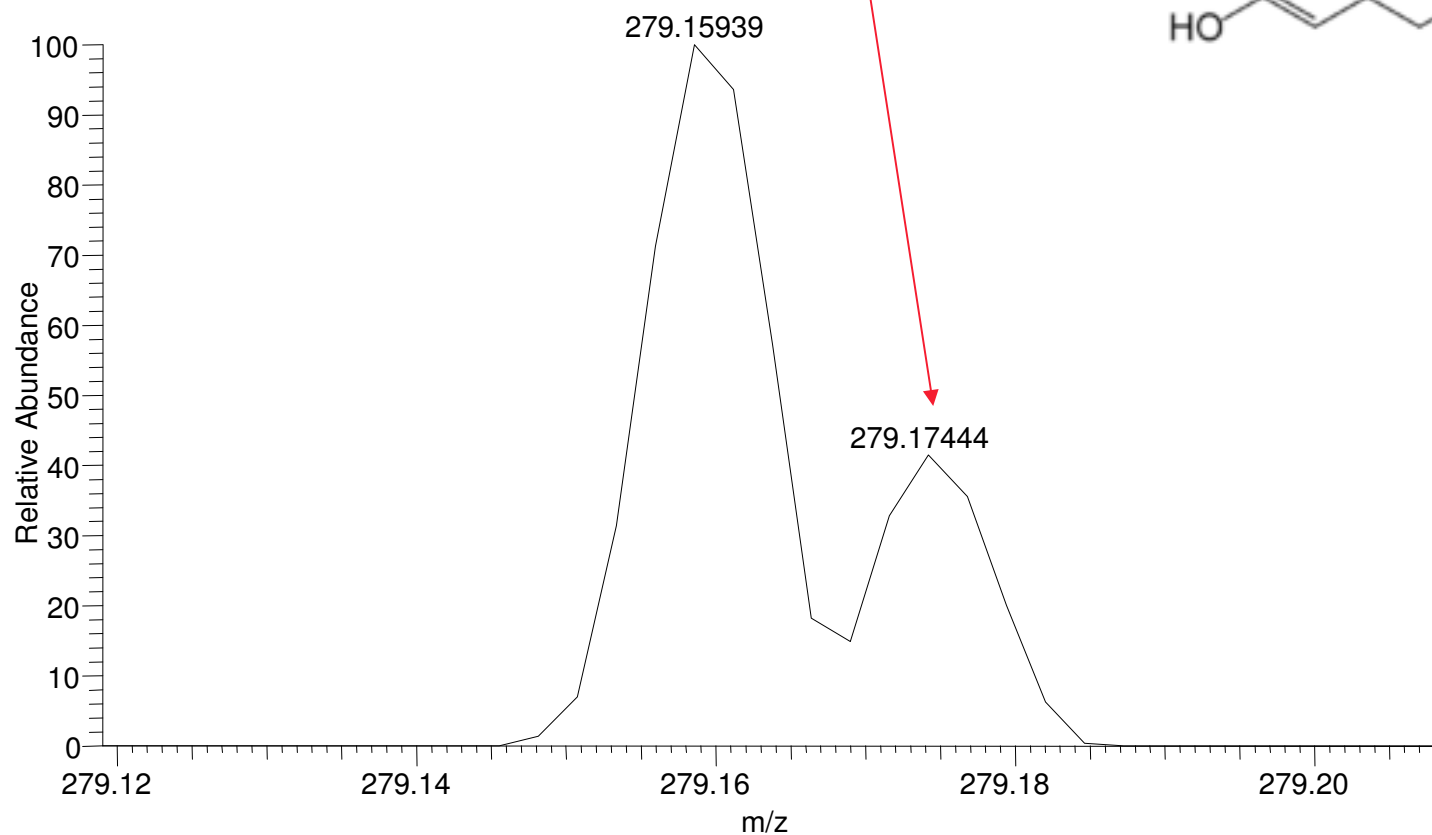
Resolution: 30,000

Ethinyl Estradiol

Calculated 279.17434

Measured: 279.17444

Error < 1 ppm



Hormone sample

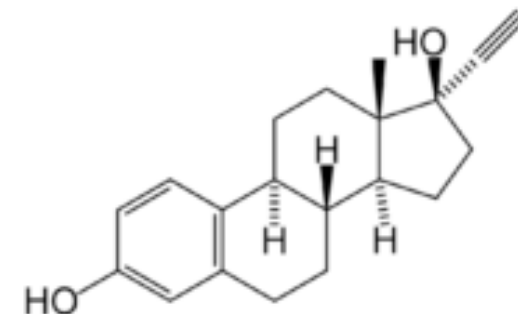
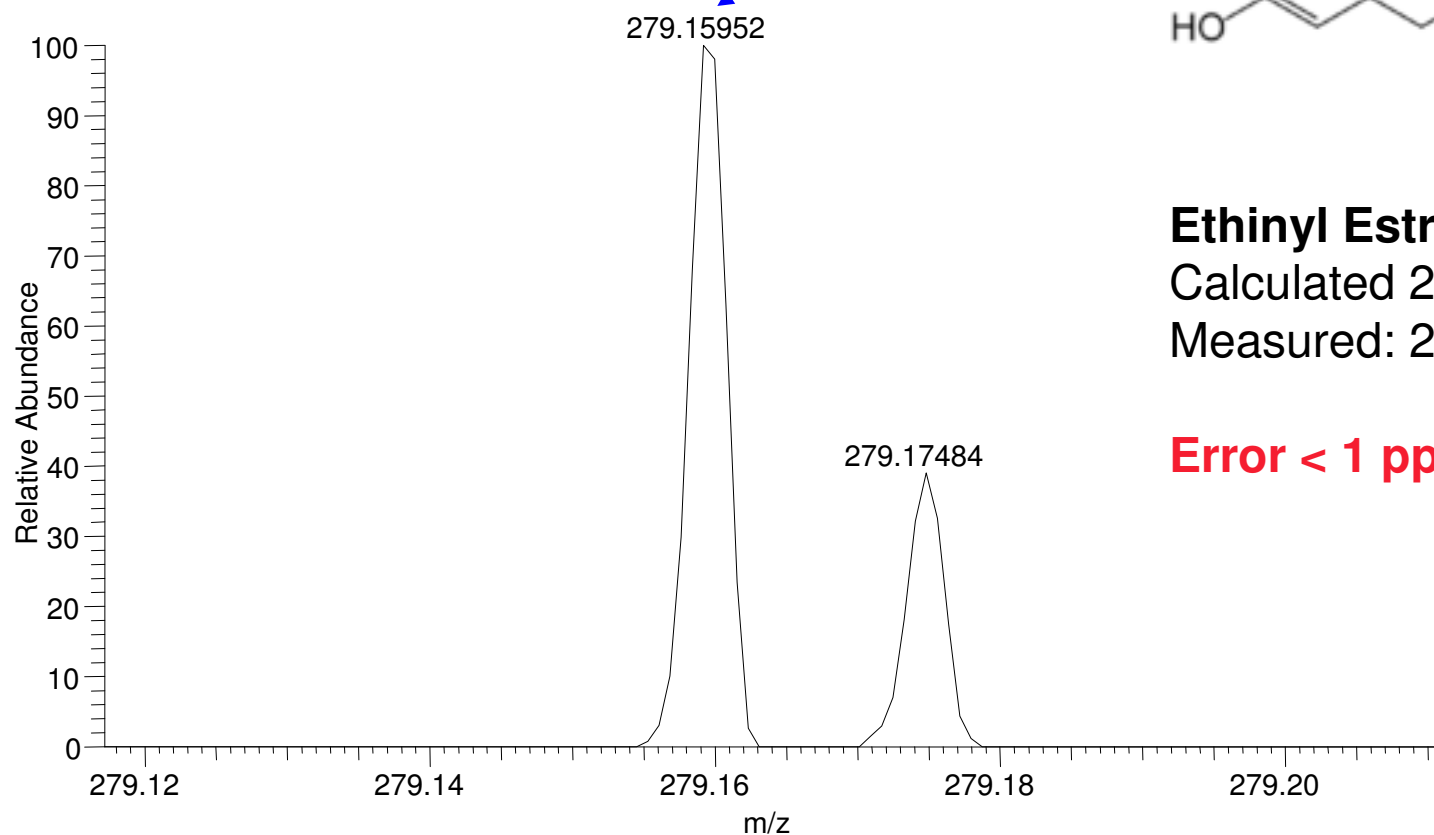
Resolution: 100,000

Butyl Phthalate

Calculated: 279.15909

Measured: 279.15952

Error = 1.5 ppm



Ethinyl Estradiol

Calculated 279.17434

Measured: 279.17484

Error < 1 ppm

Resolution

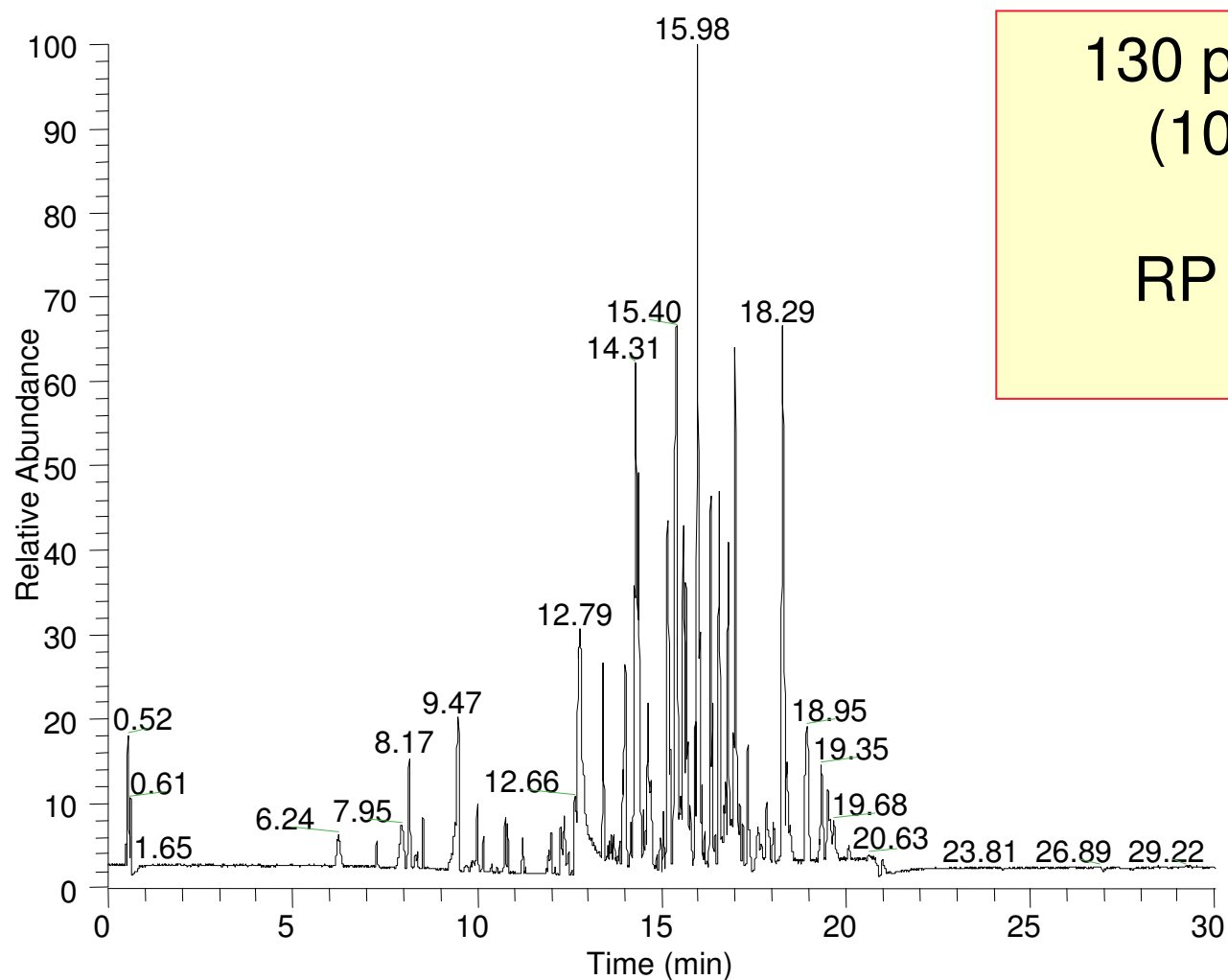
Enables accurate mass



Example

Pesticides in horse feed

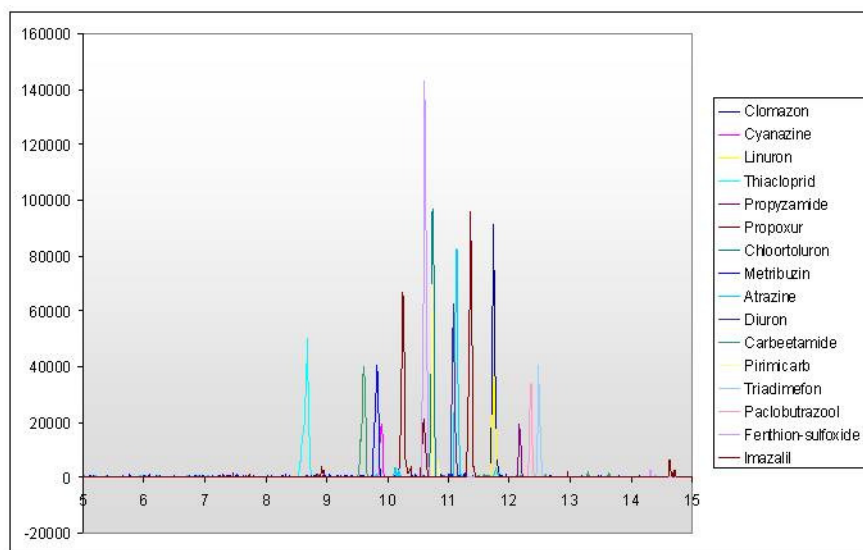
Standard Pesticide Mixture in Horse Feed Matrix



130 pesticides
(100 ppb)

RP 15,000

Standard Pesticide Mixture in Horse Feed Matrix

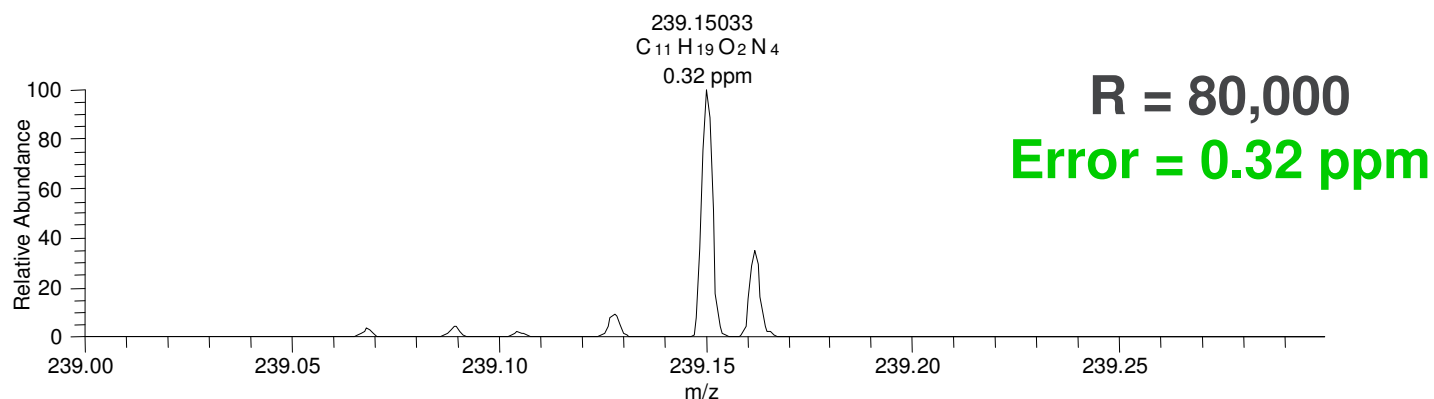
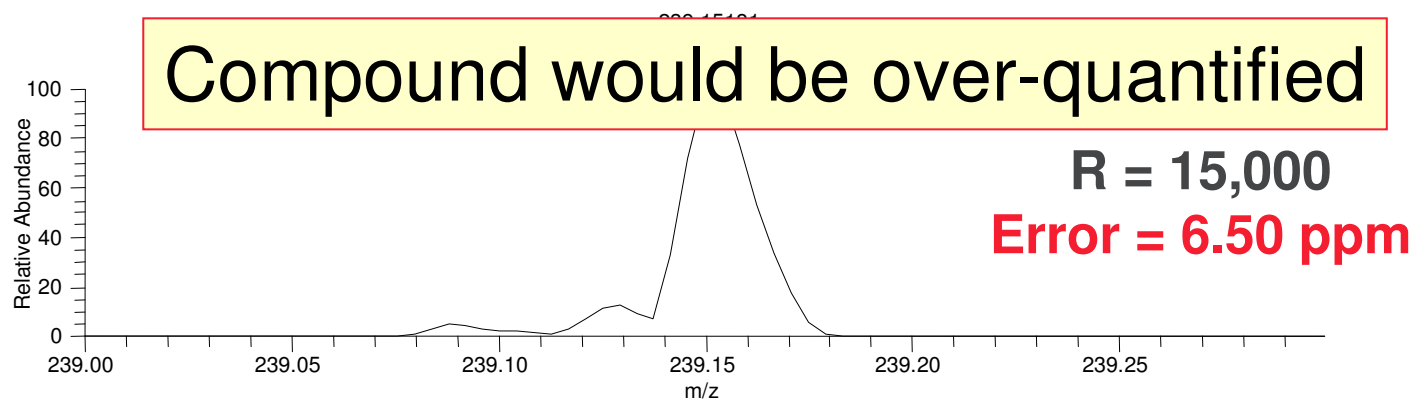


Unusually high error of 6.5 ppm
 → Reanalyze @ higher resolution

Component	Elemental Composition	[M+H]	Error [ppm]
Propoxur	C11H15NO3	210.1125	1.10
Chlortoluron	C10H13ClN2O	213.0789	1.20
Metribuzin	C8H14N4OS	215.0961	2.00
Atrazine	C8H14ClN5	216.1011	2.50
Diuron	C9H10Cl2N2O	233.0243	2.00
Carbetamide	C12H16N2O3	237.1234	1.20
Pirimicarb	C11H18N4O2	239.1503	6.50
Clomazone	C12H14ClNO2	240.0786	0.10
Cyanazine	C9H13ClN6	241.0963	0.90
Linuron	C9H10Cl2N2O2	249.0192	1.20
Thiocloprid	C10H9ClN4S	253.0309	0.70
Triadimefon	C14H16ClN3O2	294.1004	0.30
Paclobutrazol	C15H20ClN3O	294.1368	1.40
Fenthion-sulfoxide	C10H15O4PS2	295.0222	2.00
Triadimenol	C14H18ClN3O2	296.1161	0.20
Imazalil	C14H14Cl2N2O	297.0556	1.70
Spiroxamine	C18H35NO2	298.2741	1.20

Standard Pesticide Mixture in Horse Feed Matrix

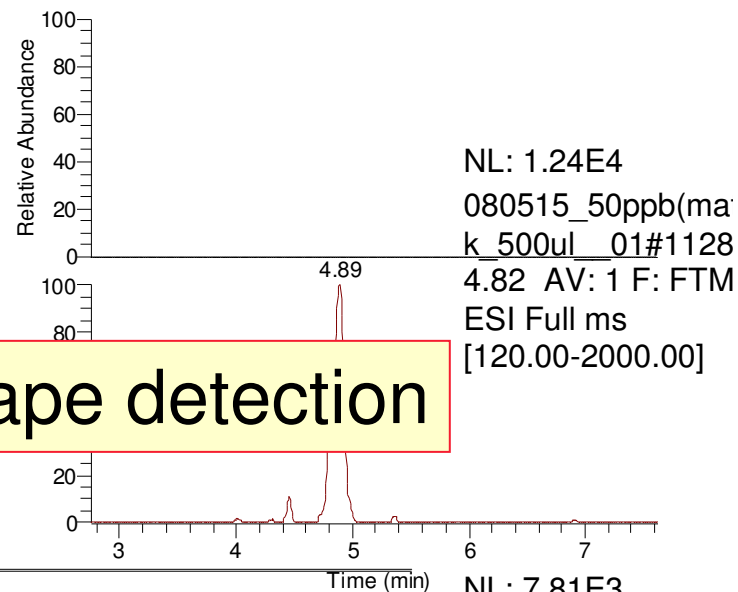
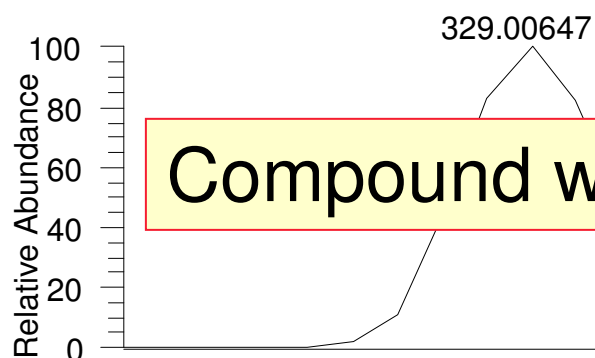
- Unusually high error of 6.5 ppm
- Indicates the need for higher resolution
- Reanalyse at 80,000 resolving power



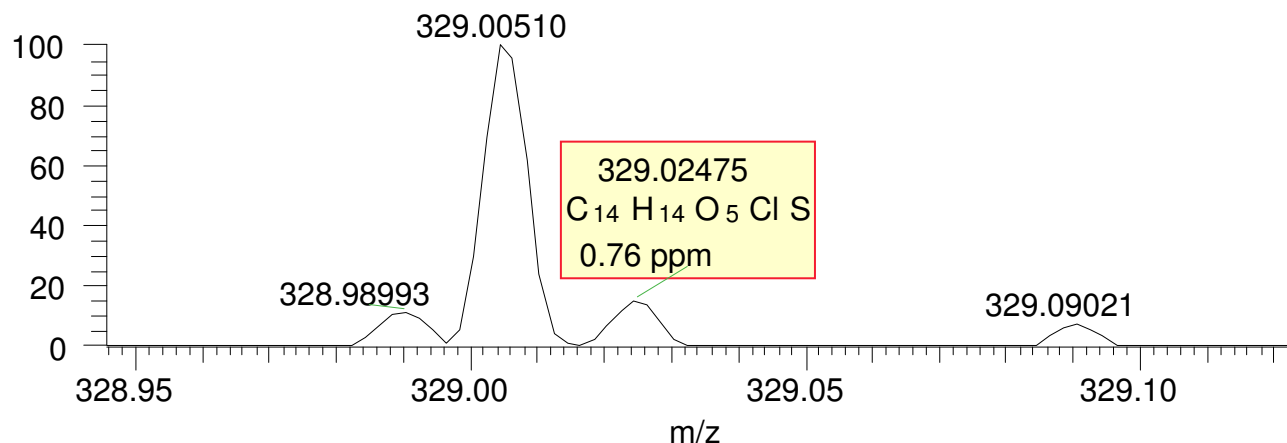
Background Interferences in Pesticide Analysis

- Some pesticides not detected
- Pesticide Sulcotrion (m/z 328.02475)
- Increased resolution needed

RP 15,000



RP 80,000



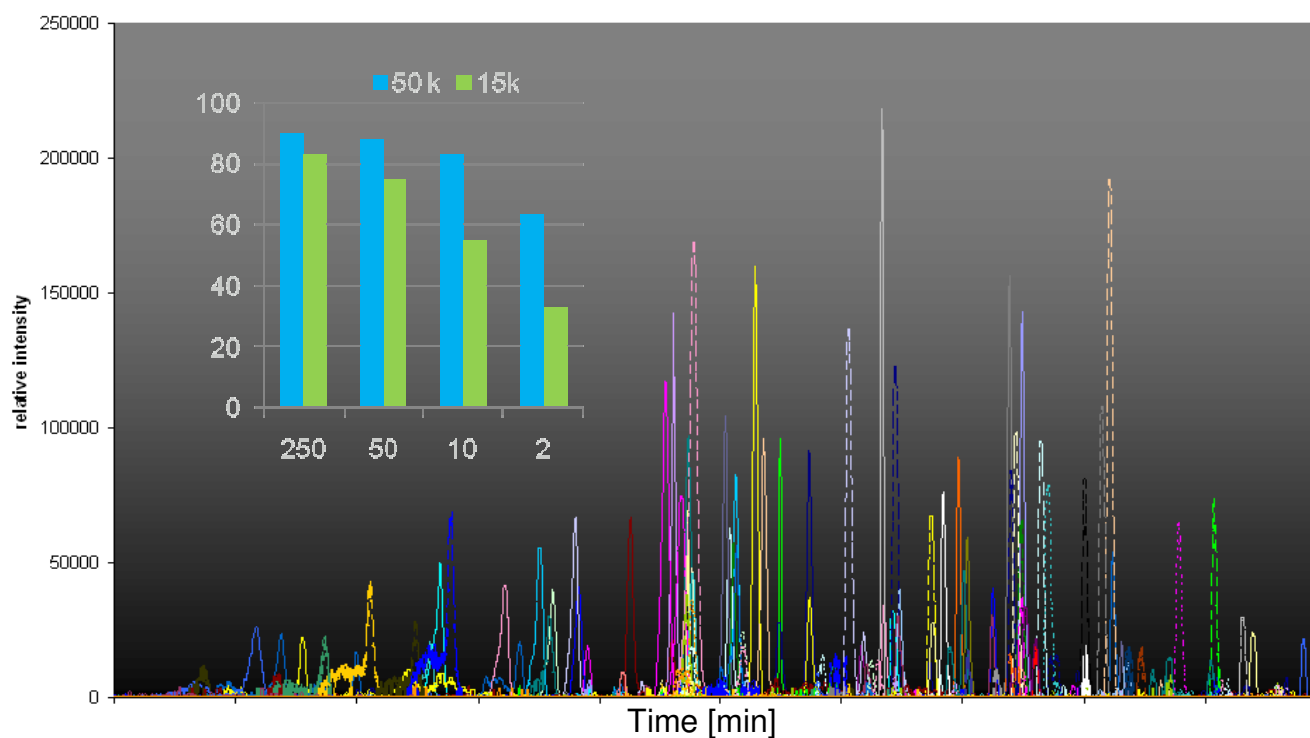
NL: 7.81E3
080515_50ppb(ma: k_500ul_02#665
4.39 AV: 1 F: FTM
ESI Full ms
[120.00-2000.00]

High resolution = more compounds detected

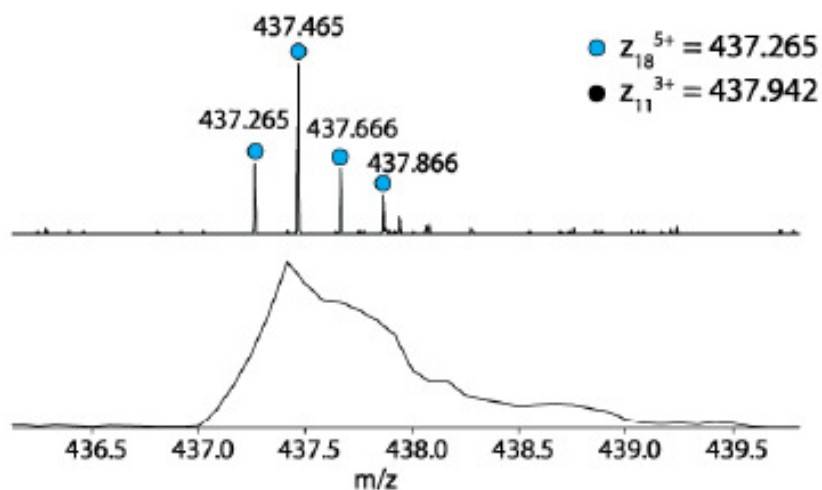
116 pesticides and mycotoxins **in single analysis**

Detected and Quantified at 100 ppb level

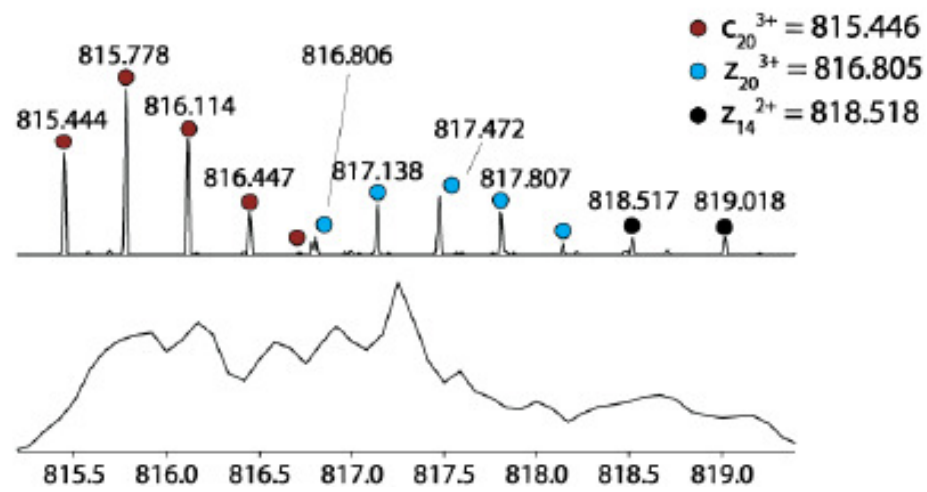
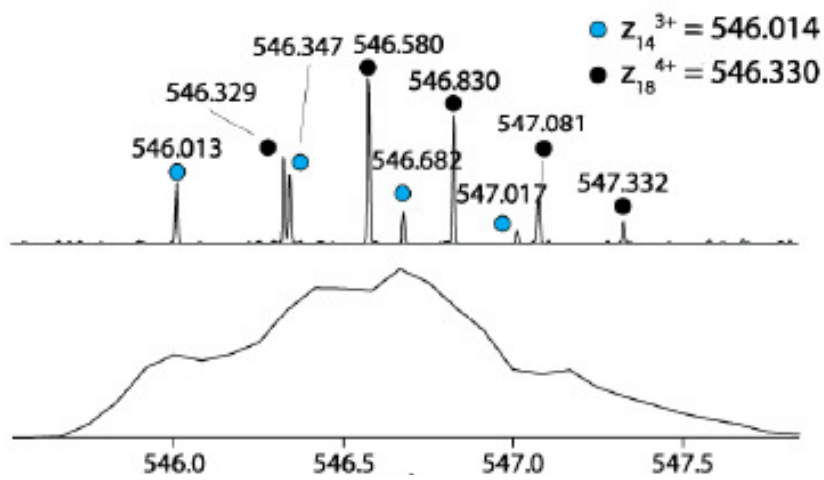
Mass tolerance 3 ppm



ETD Fragmentation Spectrum of ACTH



- Medium size peptide
- MS/MS spectrum
- ETD fragments



McAllister G.C. et al., **Anal. Chem.** 2007

Implementation of Electron-Transfer Dissociation on a Hybrid Linear Ion Trap-Orbitrap Mass Spectrometer

The importance of being highly resolved

Problem:

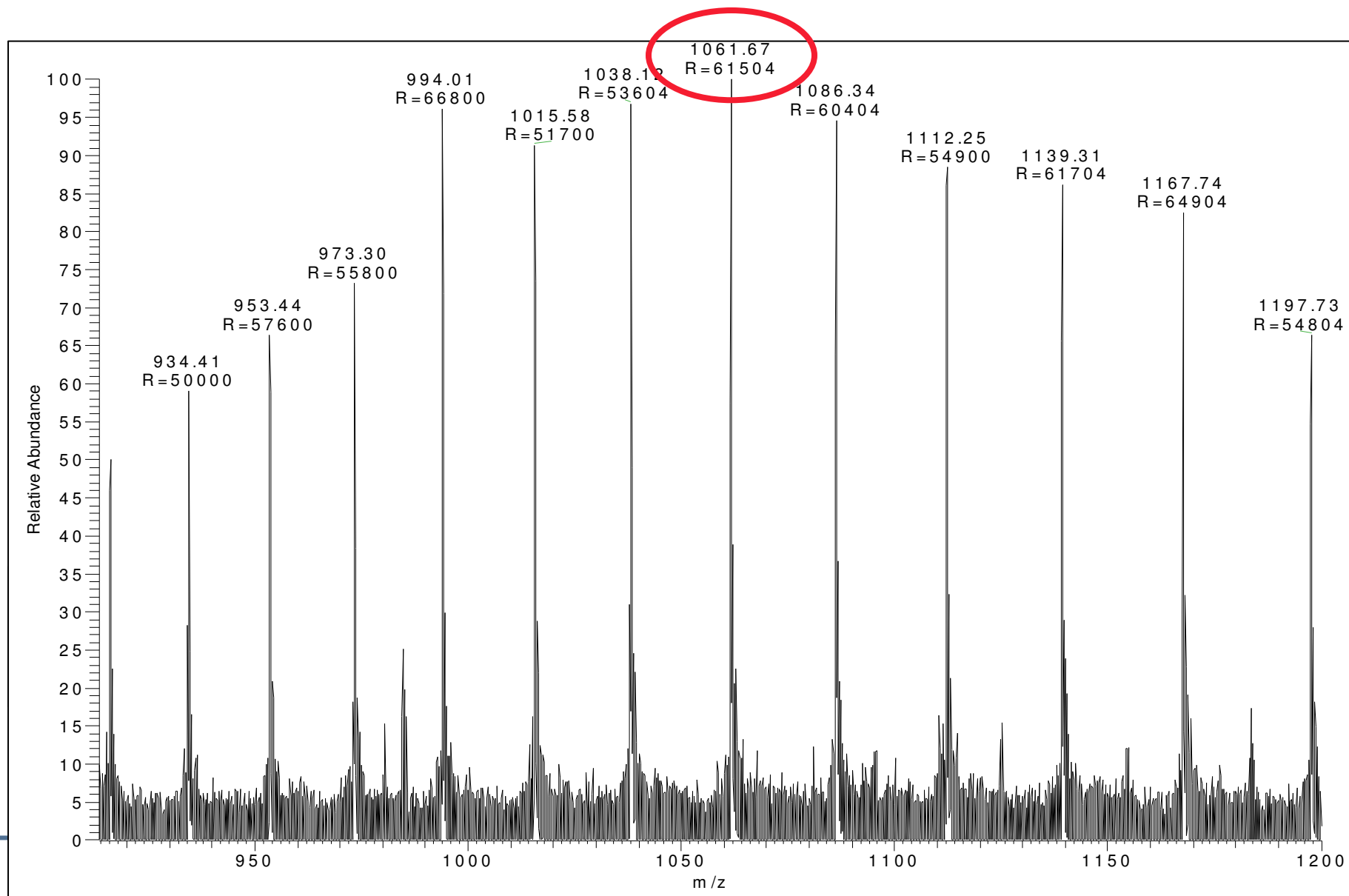
- Large molecules with multiple charges
- Detection of isotopomers

Compromised:

- Charge state determination
- Monoisotopic mass of the macromolecule

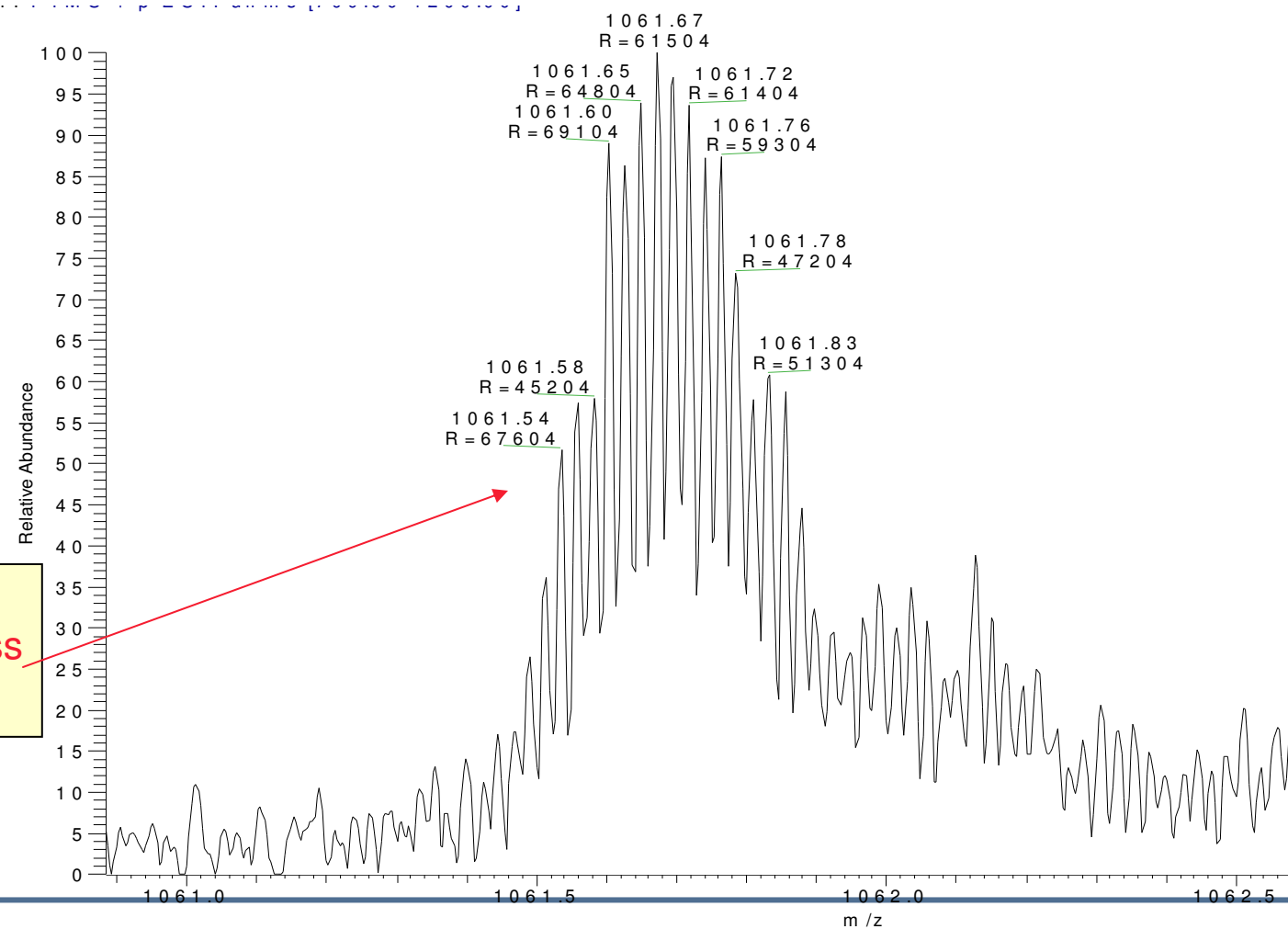
Intact Protein Enolase ~46 kDa

Resolution: 100,000



Intact Protein Enolase ~46 kDa

Resolution: 100,000



Deconvolved
Monoisotopic Mass
1.0 ppm

The importance of being highly resolved

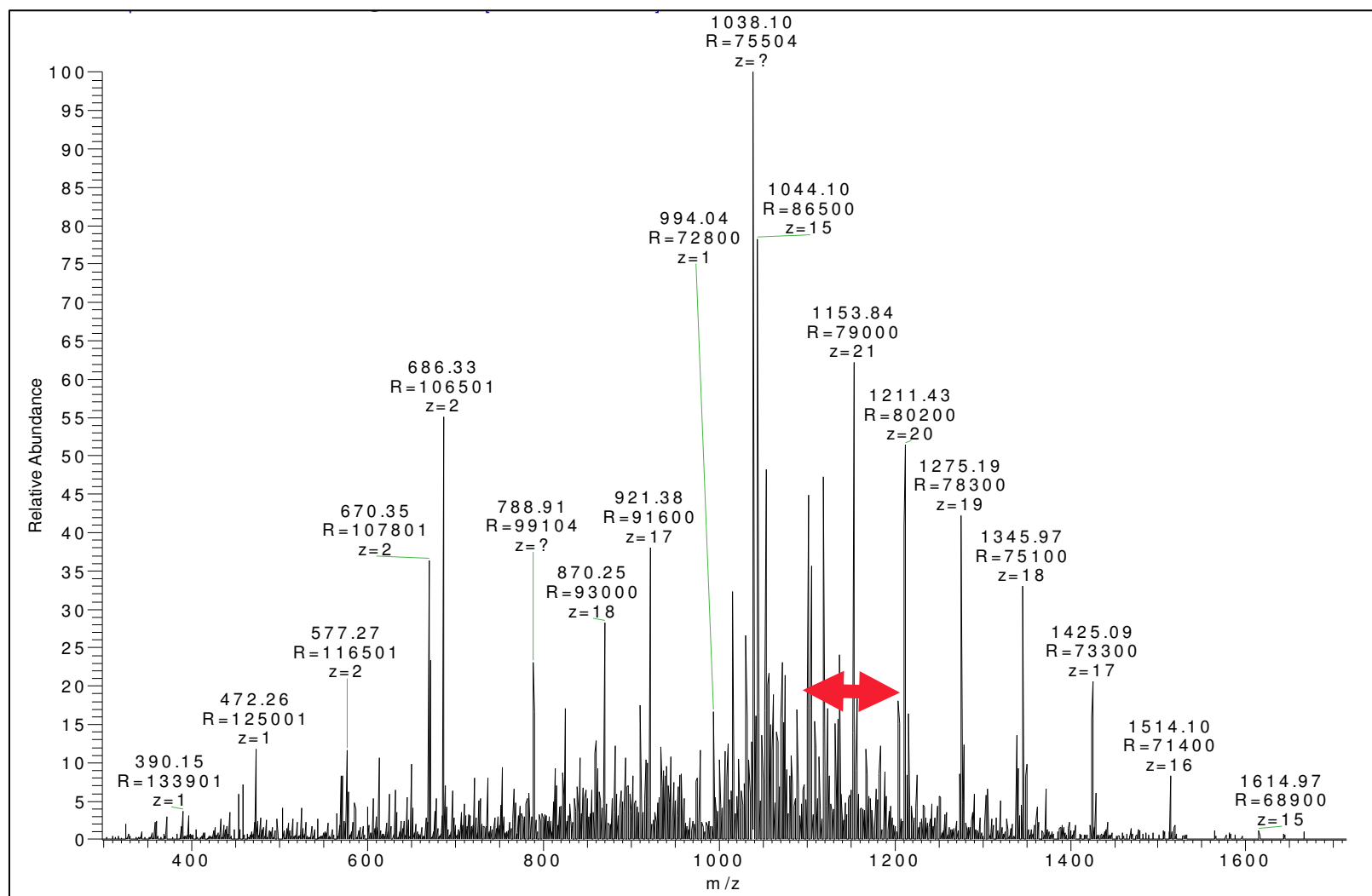
Problem:

- Large molecules with multiple charges
- MS/MS spectra are highly complex
- Overlapping isotope clusters from different fragments

Compromised:

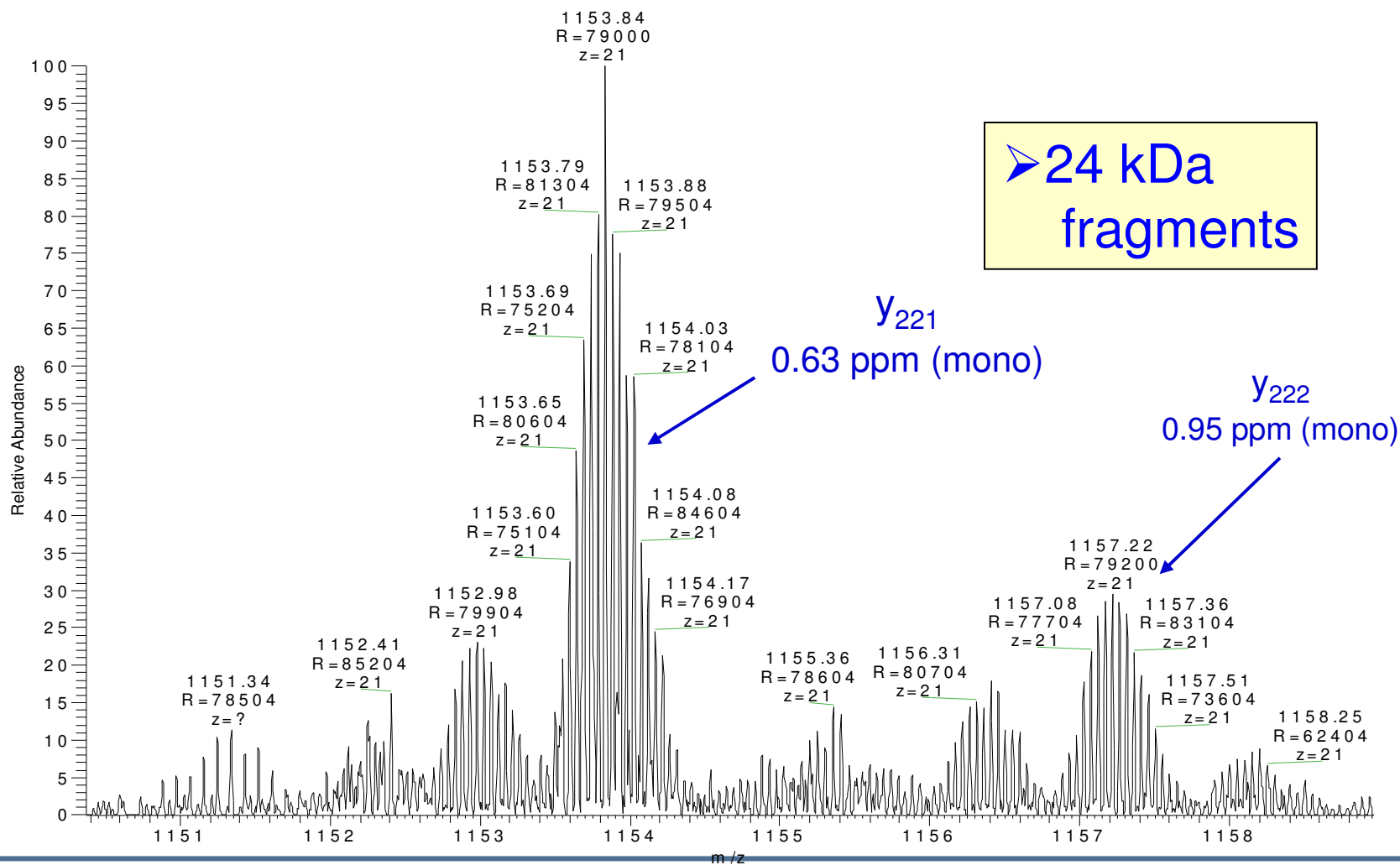
- Maximum sequence coverage
- PTM identification and localisation

MS/MS of Enolase ~46 kDa



MS/MS of Enolase - Detail

Resolution: 100,000





Resolution

is empowering

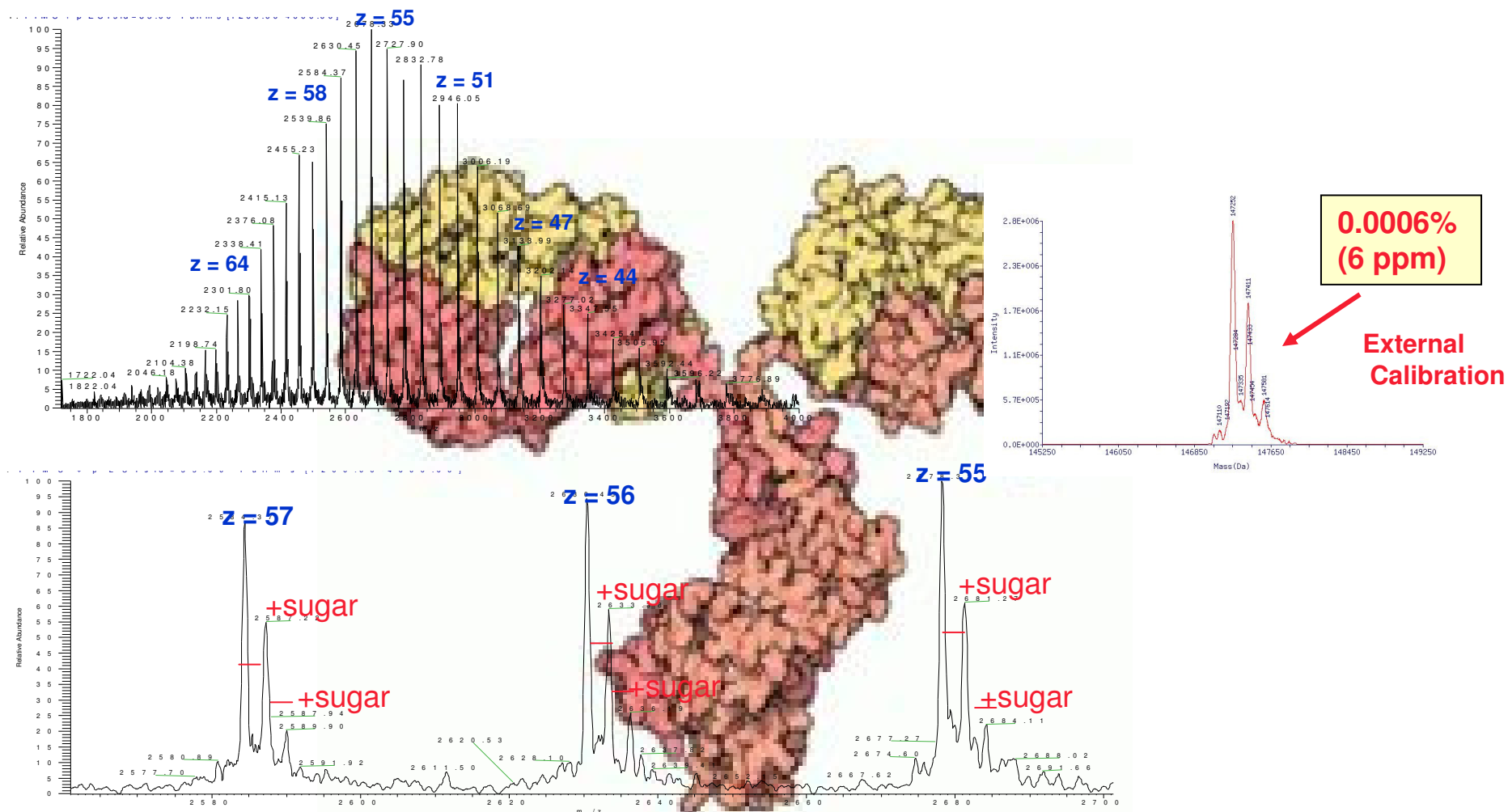
Coverage Map of Enolase Using ProSight PC

b1 -A-V-S-K-V-Y-A-R-S-V-Y-D}S-R-G-N}P-T}V-E}V-E-L-T-T- y412
b26 -E-K-G-V-F-R-S-I-V}P-S-G-A-S-T-G-V-H-E-A-L-E}M-R-D- y387
b51 -G-D}K-S-K-W-M-G-K-G-V-L-H-A-V-K-N-V-N-D-V}I-A-P-A- y362
b76 -F-V-K-A-N-I-D}V-K-D}Q-K-A-V-D-D-F-L}I-S-L-D-G-T-A- y337
b101 -N-K-S-K-L-G-A-N-A-I-L-G-V-S-L-A-A-S-R-A-A-A-A-E-K- y312
b126 -N-V-P-L-Y-K-H-L-A-D-L-S-K-S-K-T-S-P-Y-V}L}P}V}P}F} y287
b151 -L}N}V}L}N-G-G-S-H-A-G-G}A-L-A}L-Q-E}F-M-I-A-P-T-G- y262
b176 -A-K-T-F-A-E-A-L-R-I-G-S-E-V-Y-H-N-L-K-S-L-T-K-K-R- y237
b201 -Y-G-A-S-A-G-N-V}G}D-E}G}G}V}A}P-N-I}Q}T}A}E-E-A}L- y212
b226 }D-L-I}V-D-A-I-K-A-A-G-H-D-G-K-V-K-I-G-L-D-C-A-S-S- y187
b251 -E-F-F-K-D-G-K-Y-D-L-D}F-K-N-P-N-S-D-K-S-K-W-L-T-G- y162
b276 -P-Q-L-A-D-L-Y-H-S-L-M-K-R-Y-P-I-V-S-I-E-D}P-F}A-E- y137
b301 }D-D-W-E-A-W-S-H-F-F-K}T-A-G-I-Q-I}V}A}D-D-L}T-V-T- y112
b326 -N-P-K-R-I-A-T-A-I-E-K-K-A-A-D-A-L-L-L-K-V-N-Q-I-G- y87
b351 -T-L-S}E-S-I-K-A-A-Q-D-S}F}A-A}G}W}G}V}M}V}S-H-R-S- y62
b376 -G-E-T-E-D-T}F}I}A}D}L}V}V}G-L-R-T-G-Q-I-K}T-G-A-P- y37
b401 -A-R-S-E-R-L-A-K-L-N}Q-L-L-R-I-E-E-E-L-G-D-N-A}V}F- y12
b426 }A}G}E}N}F}H}H}G-D-K-L- y1

101 matching fragment ions

Going Really Big

Resolution: 15,000



Bondarenko P, Zhang Z, "LC/MS Top-Down Analysis and Intact Mass Analysis of Recombinant Immunoglobulin Gamma Antibodies on Orbitrap," Amgen Inc, ASMS 2008

ThPL 278

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The importance of being highly resolved

Conclusions

- From a theoretical point of view there is no clear cut-off for desired mass resolving power

BUT

- In our experience 100,000 FWHM is both practical and desirable for complex peptide mixture analysis

How Accurate Is Accurate Enough

Problem:

- False positive identifications
- Unknowns

Compromised:

- Confident identification
- High throughput

Why is accurate mass useful?

- Example: mass 32
- What can it be ??

S

O₂

CH₃OH

N₂H₄



Accurate Mass Is a Powerful Filter

C = 12.0000

H = 1.0078
N = 14.0031

O = 15.9949
S = 31.9721

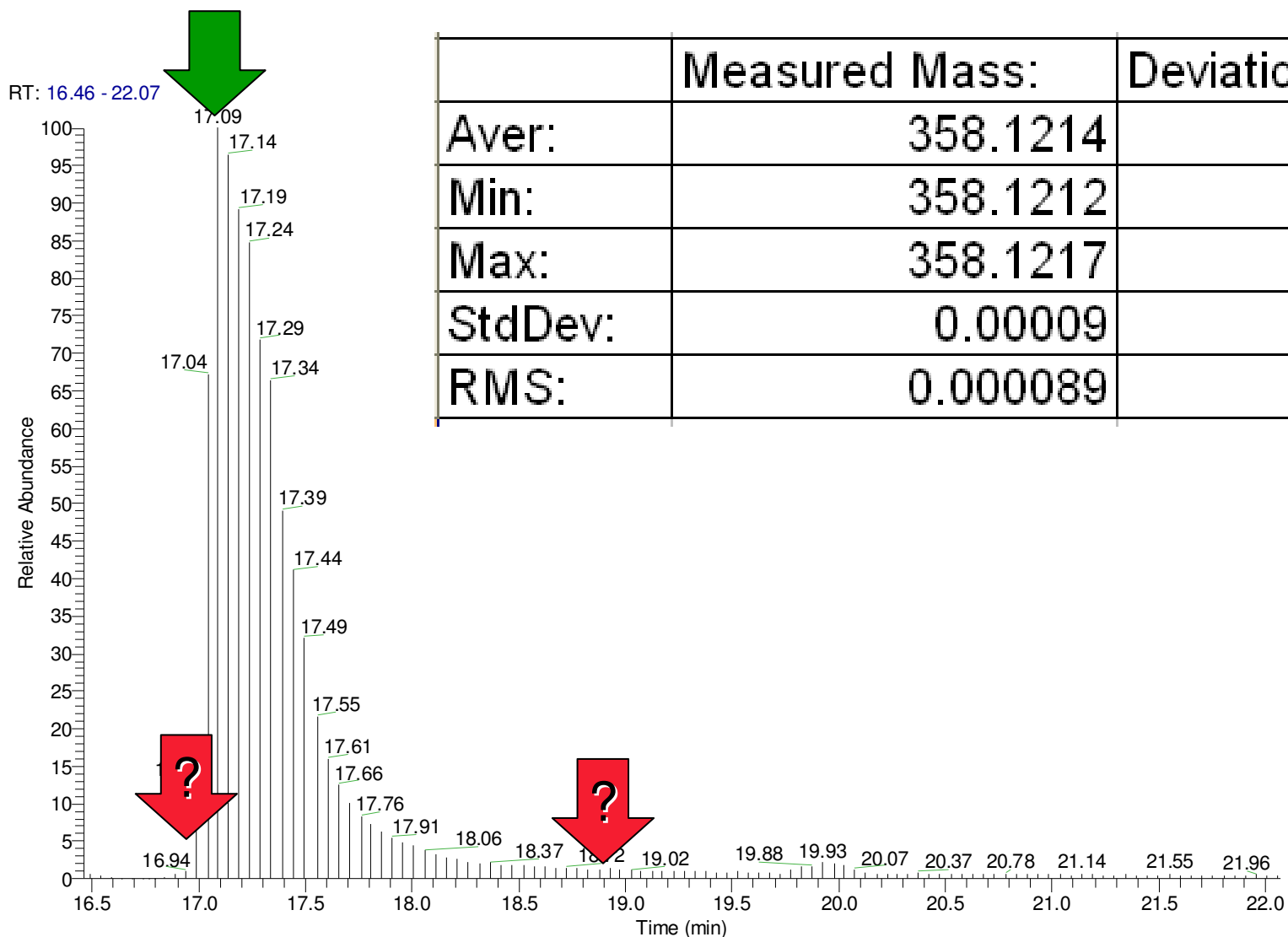
Mass measured	Tolerance [Da]	Suggestions	Calc Mass
32.0	+/- 0.2	O ₂ CH ₃ OH N ₂ H ₄ S	31.9898 32.0261 32.0374 31.9721
32.02	+/- 0.02	CH ₃ OH N ₂ H ₄	32.0261 32.0374
32.0257	+/- 0.002	CH₃OH	32.0261



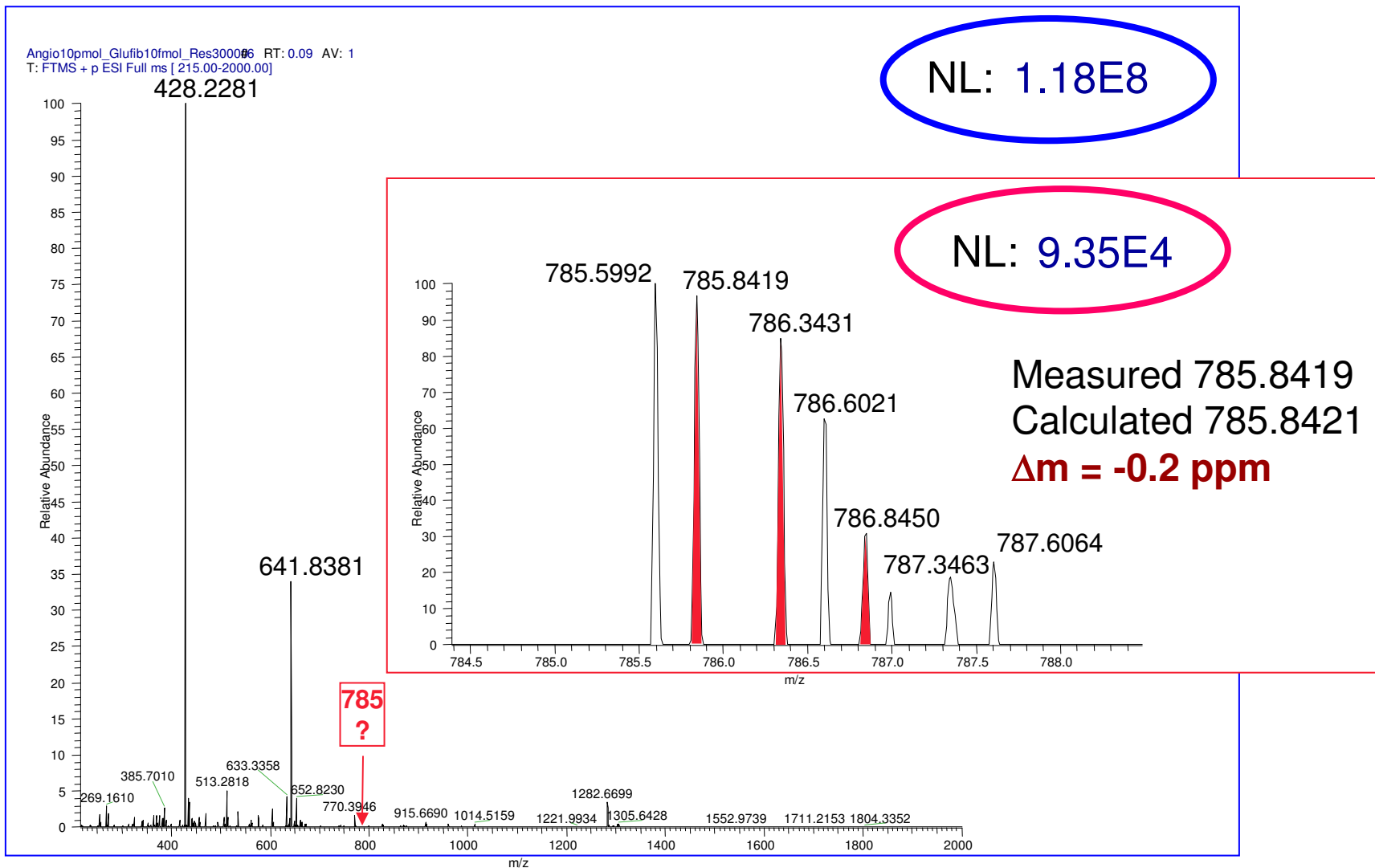
Accurate Mass

Makes Life Easier

Precision of Mass Measurement

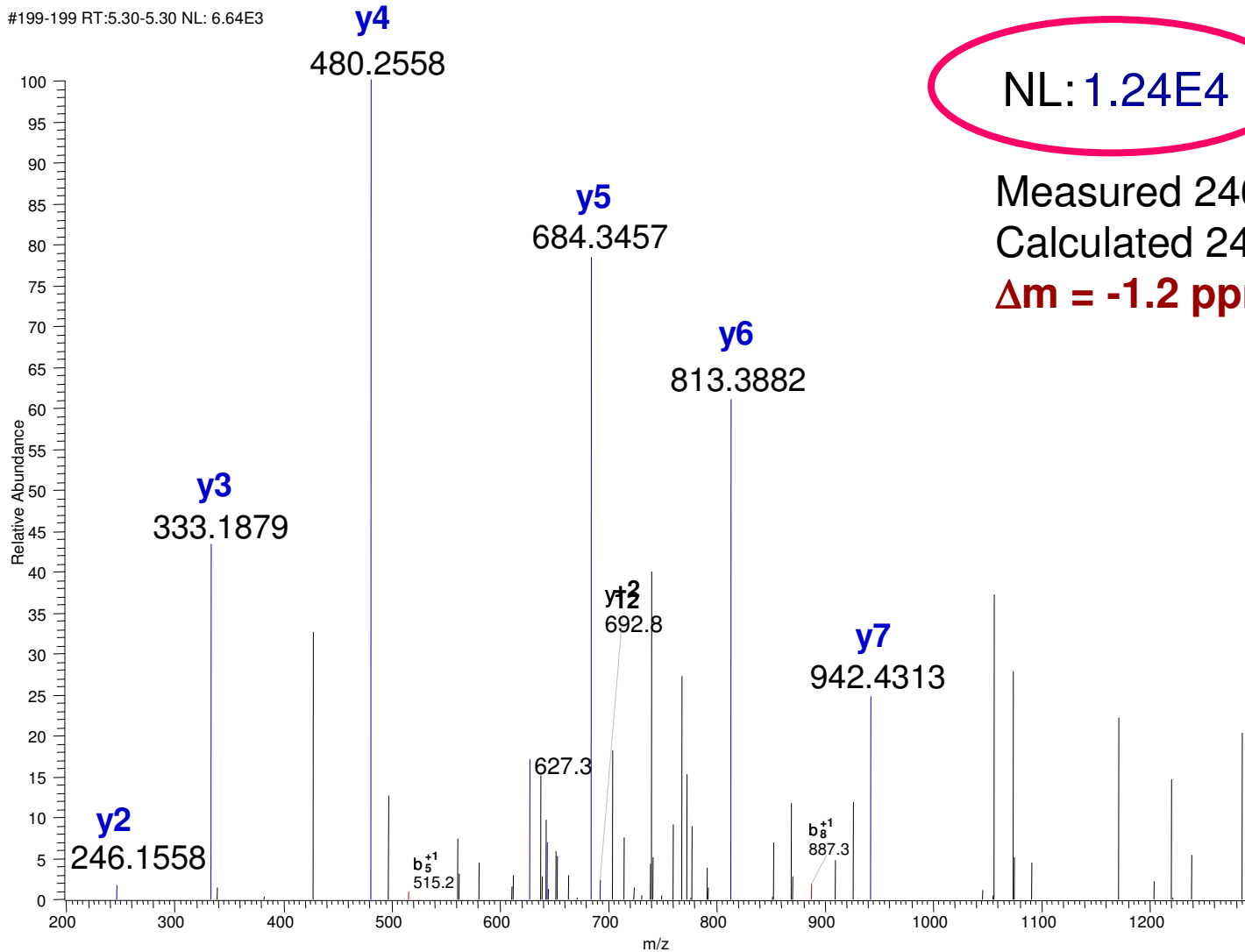


Angiotensin 10 pmol/ul + Glu-fibrinogen 10 fmol/ul



MS/MS Glu-Fibrinogen @10 fmol/ul

#199-199 RT:5.30-5.30 NL: 6.64E3



NL: 1.24E4

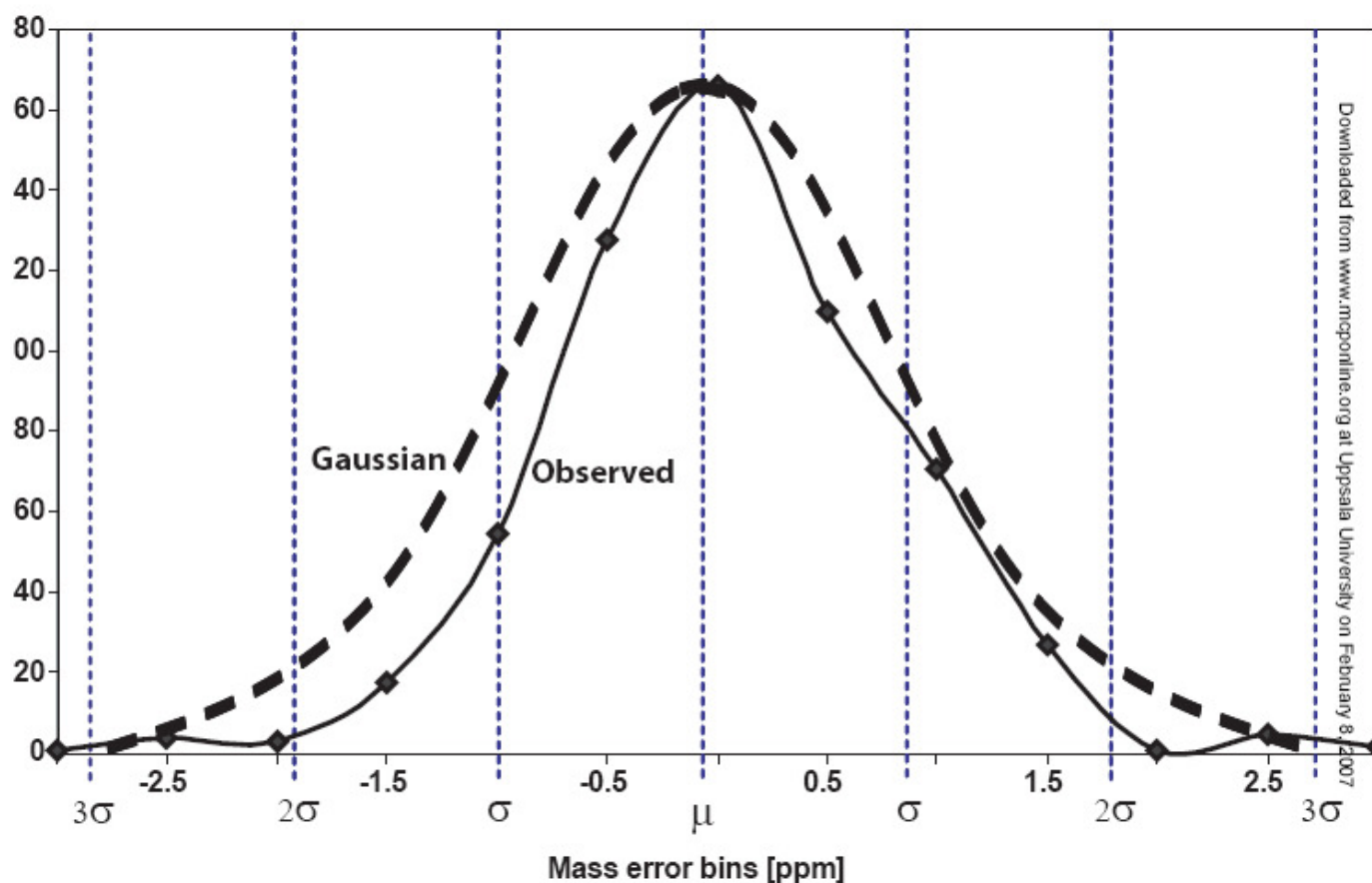
Measured 246.1558

Calculated 246.1561

$\Delta m = -1.2$ ppm

Distribution of Mass Deviations

- Standard deviation 0.8 ppm (σ)
- Maximum deviation can be set to 2σ or 3σ (specificity vs. sensitivity)



Key Points about Accurate Mass

Conclusions

- The measured mass acts as a filter that reduces the number of potential false positive assignments
- Higher MA proportionately increases certainty of identification
- Peptide mass accuracy should be determined individually for each peptide
- Low ppm mass deviation on a chromatographic time scale is now routine with modern LTQ-FT hybrids

State-of-the-art technology:
achievable average absolute mass deviations ~300 ppb

How Accurate Is Accurate Enough

In small-molecule world:

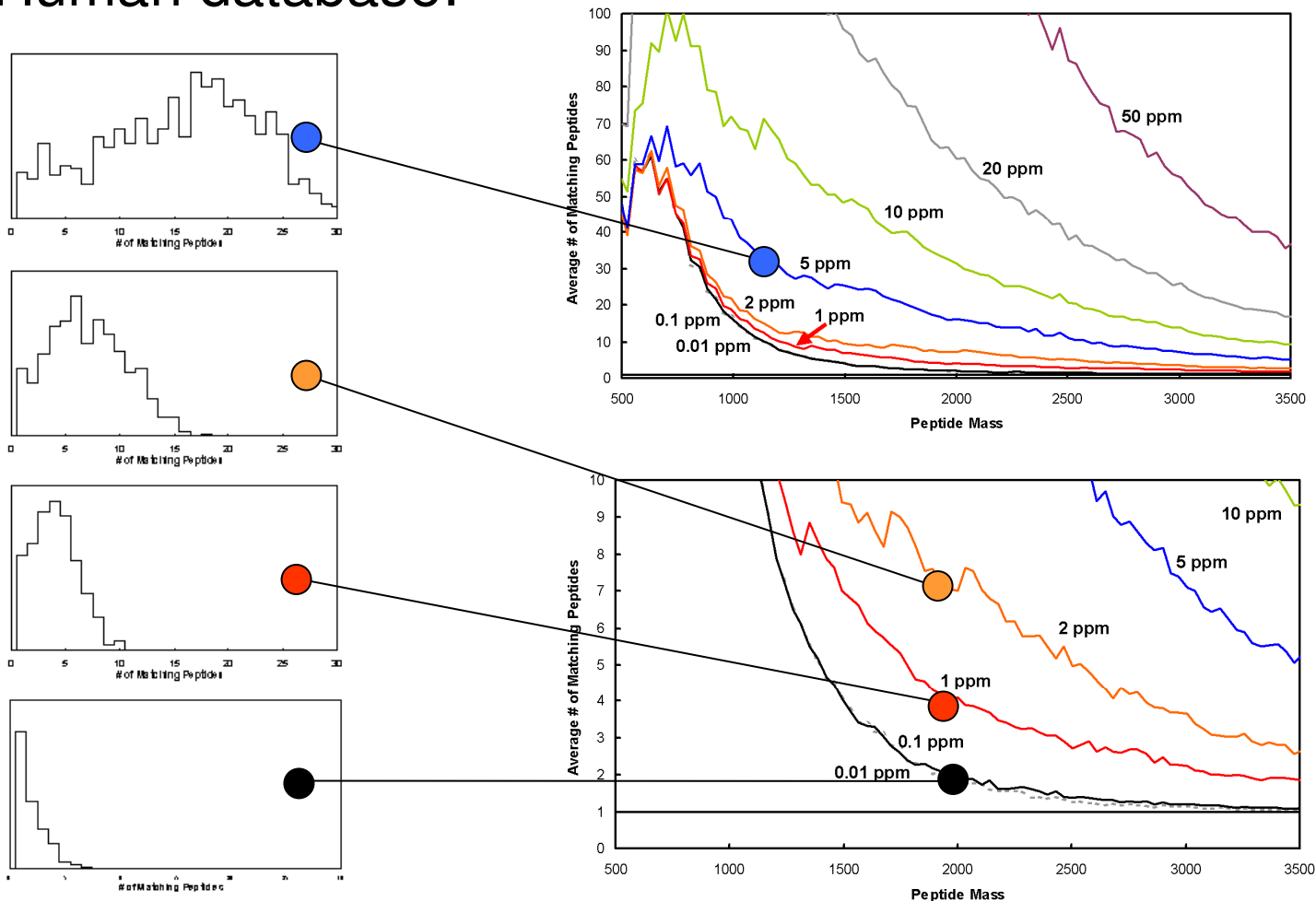
- To provide a unique chemical composition

In shotgun proteomics:

- ~100 ppb for small tryptic peptides
- Chemical composition specified for small peptides
- “Database congestion” eases for higher masses
- Unique peptide candidate for larger peptides is likely

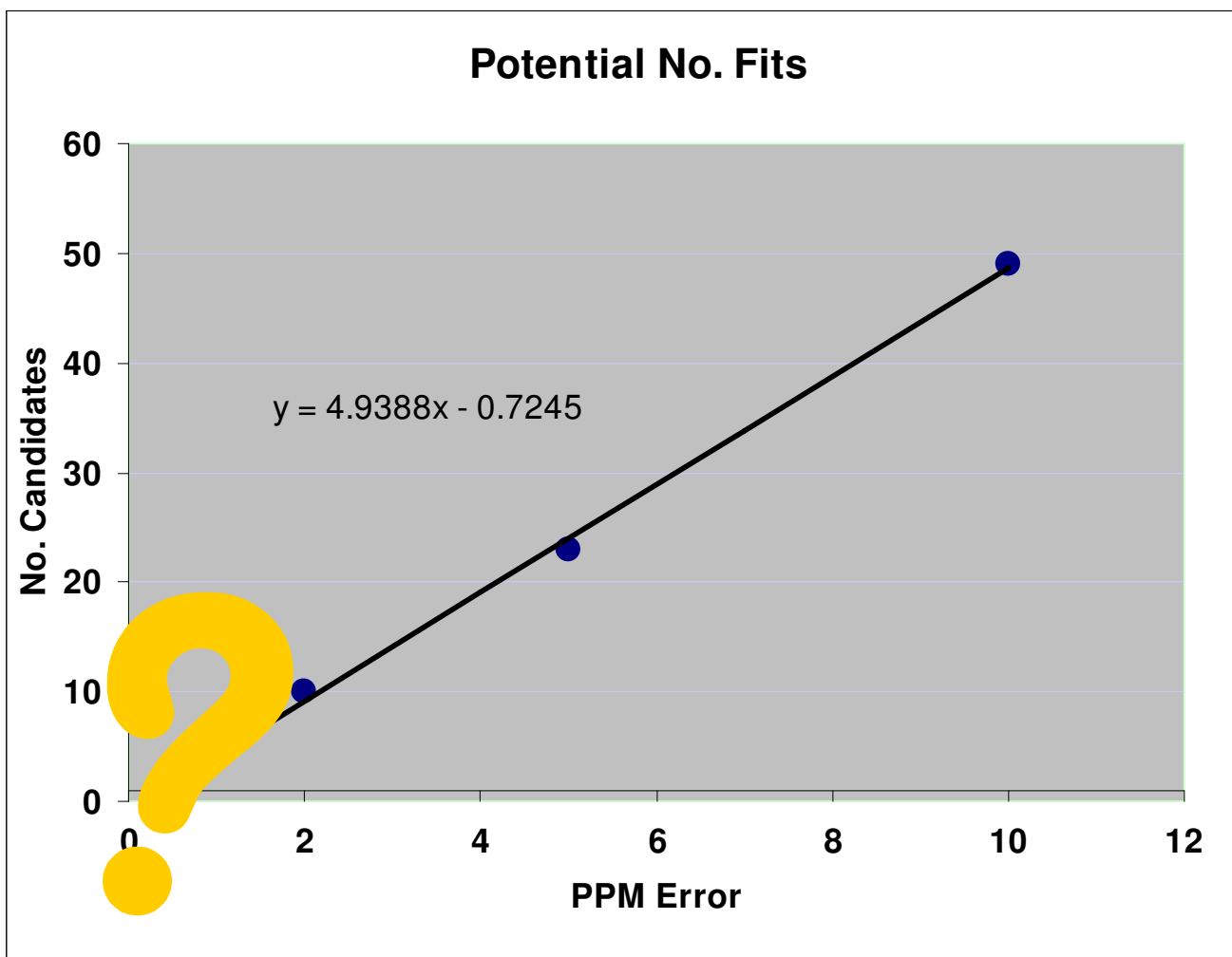
Peptide Identification – Effect of Mass Accuracy

Human database:



Calculations courtesy of Dr. David Fenyo, Rockefeller University

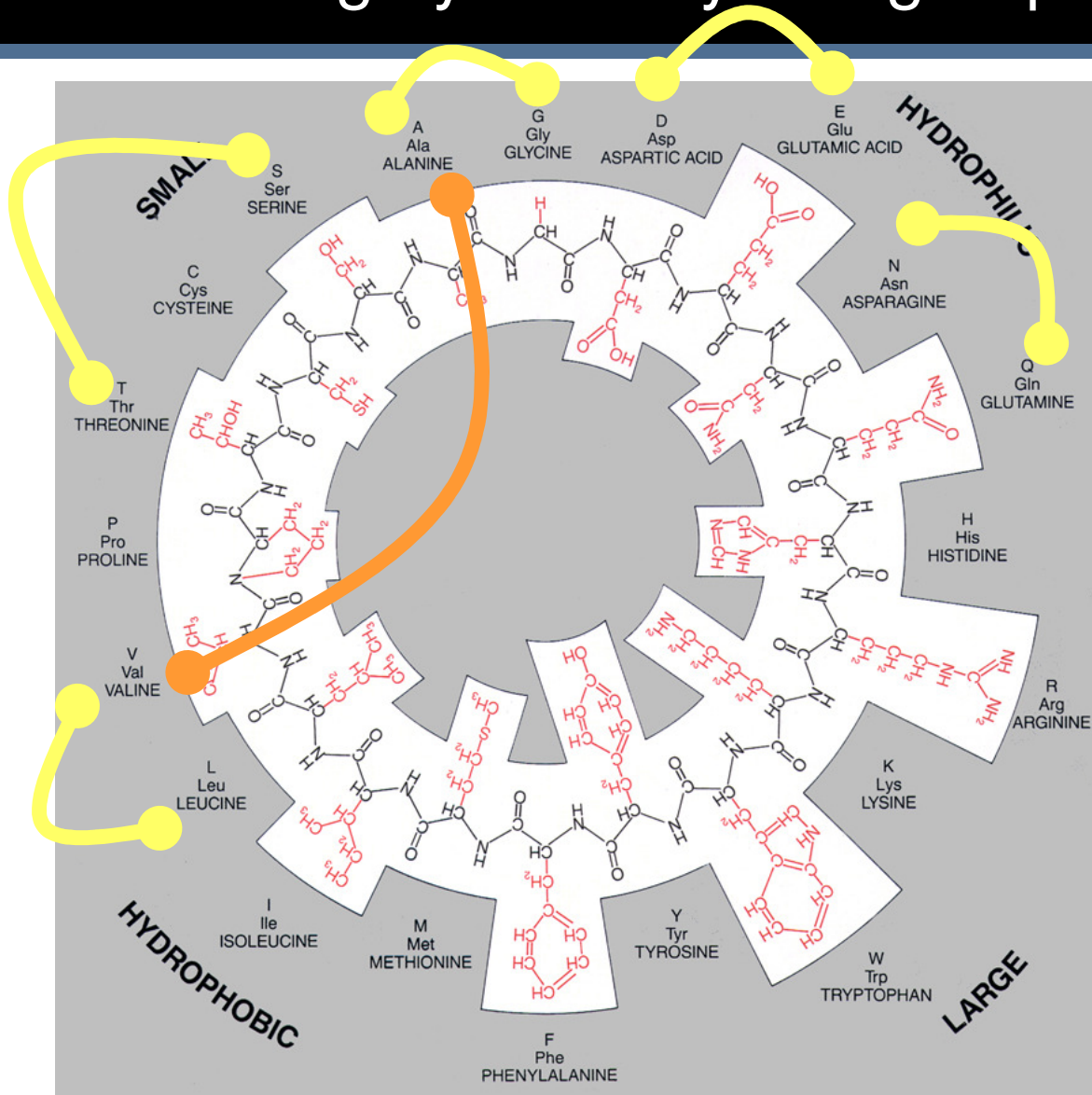
Having a Database Limits the 'Search Space'



Chemical Composition vs. Sequence

■ Peptide	■ Accurate mass (calculated)
ADK	333.1769
DAK	333.1769
GEK (EGK)	333.1769

Amino acids differing by a methylene group (-CH₂-)





The mass itself is

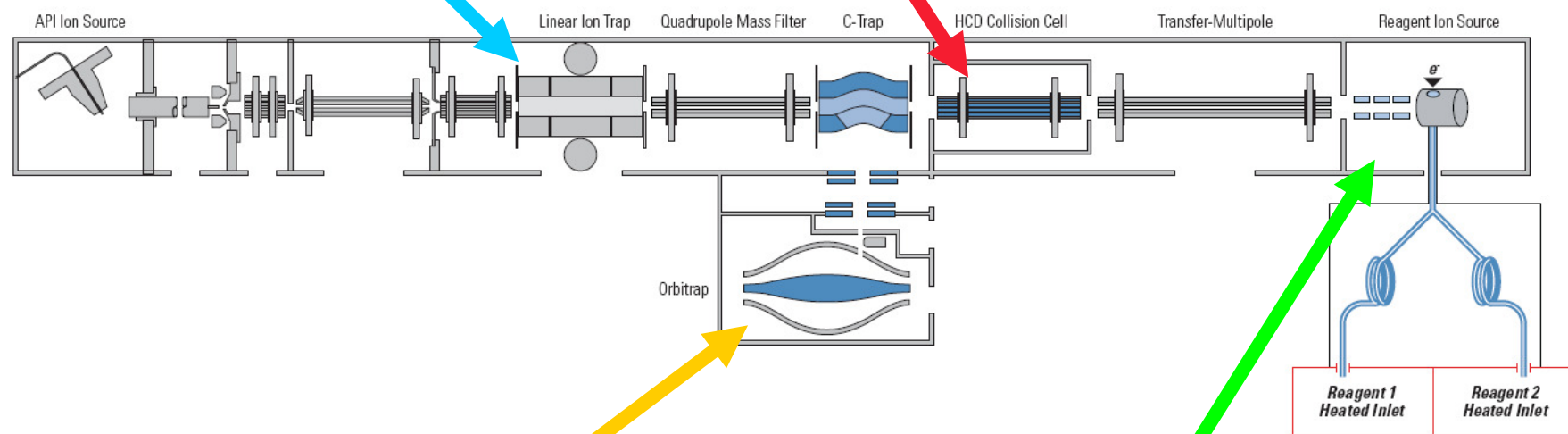
not sufficient

for identification of the peptide

LTQ Orbitrap XL with ETD – *Versatility*

Fragmentation of ions
standard conditions

Fragmentation of ions
Higher energy

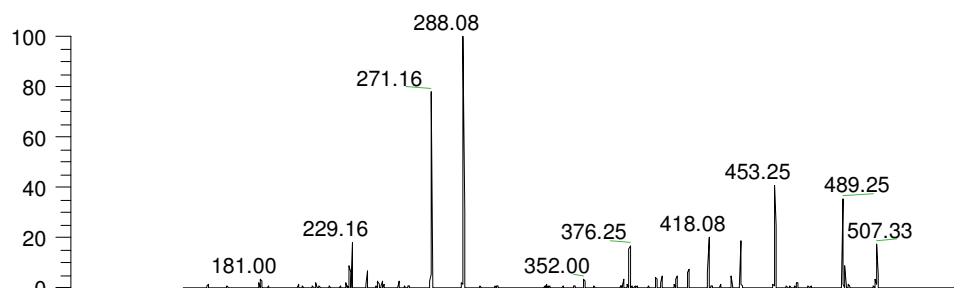


Measurement with high
accuracy and resolution

ETD module for
Peptides with PTM

Different fragmentation techniques

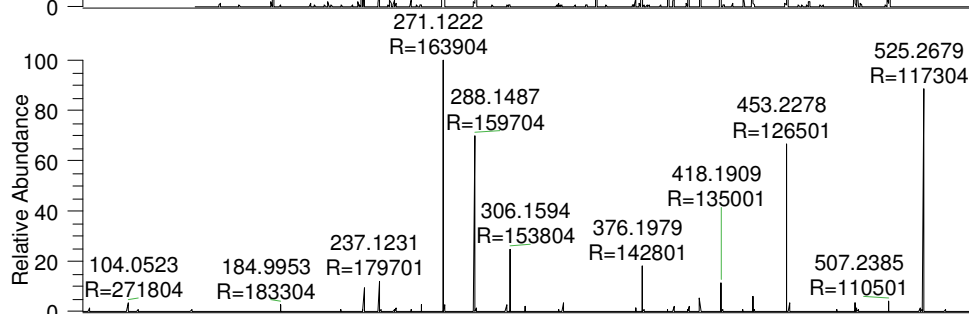
CID



NL: 2.84E3
MRFA_fragmentation1#8
2 RT: 48.35 AV: 1 T:
ITMS + p ESI Full ms2
524.30@cid35.00
[140.00-550.00]

Standard
For peptide ID

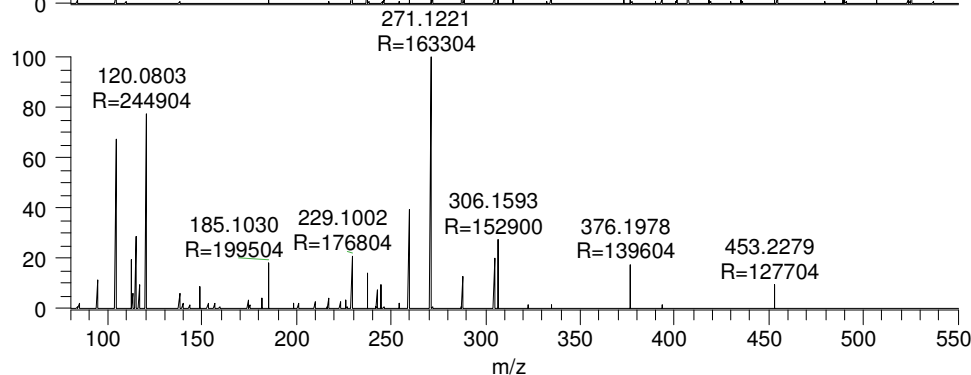
PQD



NL: 6.86E5
MRFA_fragr
7 RT: 50.47
FTMS + p E
524.30@pq
[80.00-550.00]

No low mass cut-off
For iTRAQ quantitation

HCD



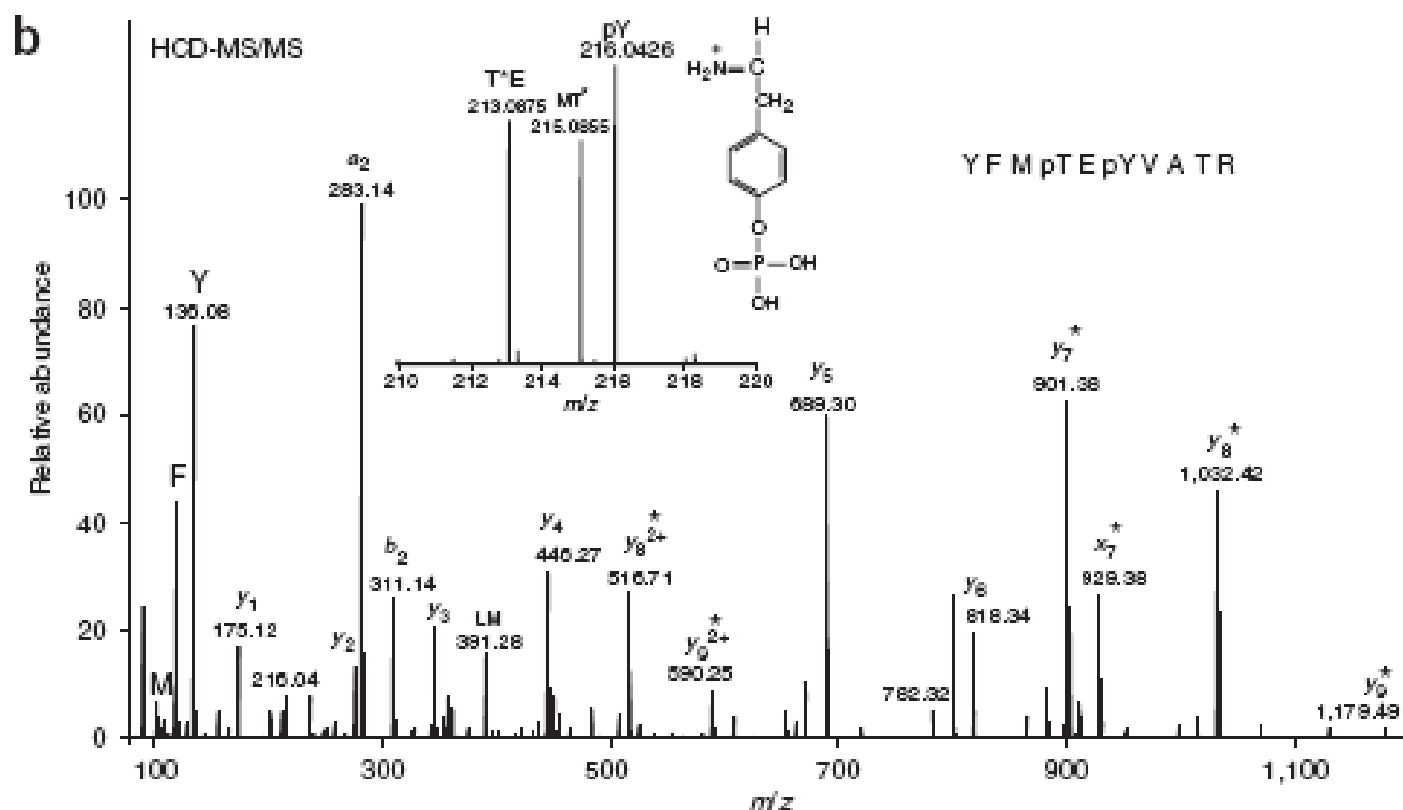
NL: 2.4
MRFA_
8 RT:
FTMS
524.30
[80.00-

No low mass cut-off
Strong immonium ions
Efficient fragmentation of
the precursor (none left)
For iTRAQ quantitation,
de novo sequencing,
phosphoTyr

Higher energy collision useful for phosphotyrosine

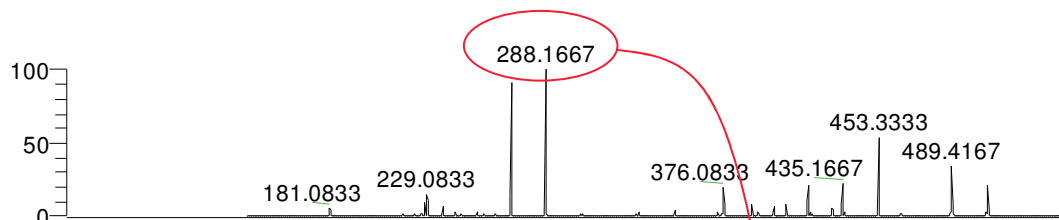
■ Phosphotyrosine diagnostic immonium ion

- Jesper V Olsen, Boris Macek, Oliver Lange, Alexander Makarov, Stevan Horning and Matthias Mann. *Higher-energy C-trap dissociation for peptide modification analysis*. **Nature Methods** 2007, 4, 709-712



Multiple levels of fragmentation – peptide MRFA

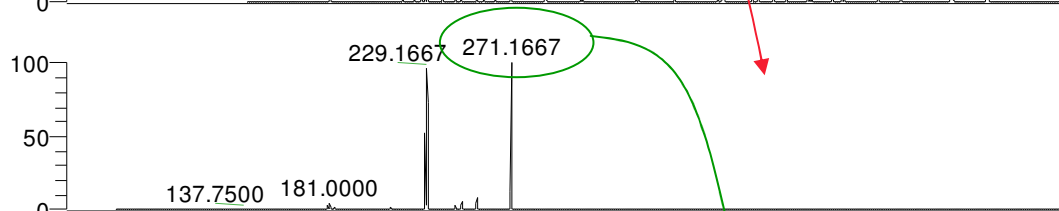
MS2



NL: 2.41E3

MRFA_fragmentation1#84 RT: 48.36 AV: 1 T:
ITMS + p ESI Full ms2 524.30@cid35.00
[140.00-550.00]

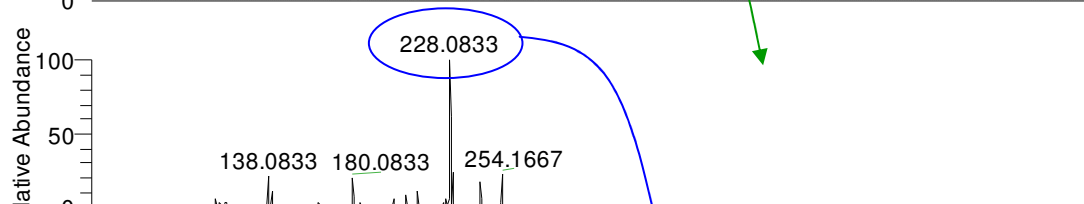
MS3



NL: 2.29E4

MRFA_fragmentation1#94 RT: 53.19 AV: 1 T:
ITMS + p ESI Full ms3 524.30@cid35.00
288.20@cid35.00 [75.00-550.00]

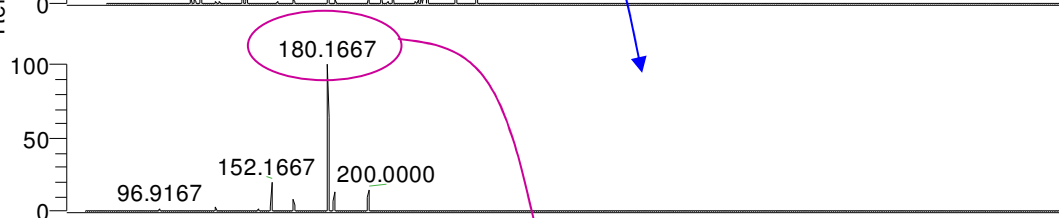
MS4



NL: 7.12E3

MRFA_fragmentation1#104 RT: 54.12 AV: 1 T:
ITMS + p ESI Full ms4 524.30@cid35.00
288.20@cid35.00 271.20@cid35.00
[70.00-550.00]

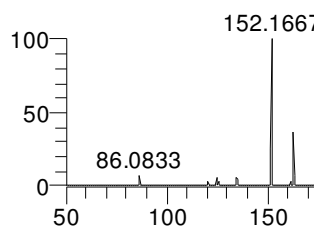
MS5



NL: 5.59E3

MRFA_fragmentation1#113 RT: 55.03 AV: 1 T:
ITMS + p ESI Full ms5 524.30@cid35.00
288.20@cid35.00 271.20@cid35.00
228.20@cid35.00 [60.00-550.00]

MS6

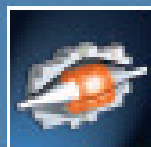


NL: 1.81E3

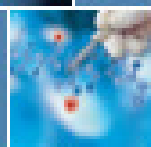
MRFA_fragmentation1#116 RT: 55.64 AV: 1 T:
ITMS + p ESI Full ms6 524.30@cid35.00
288.20@cid35.00 271.20@cid35.00
228.20@cid35.00 180.20@cid35.00
[50.00-550.00]

Excellent **sensitivity** makes multiple levels of fragmentation feasible and useful.

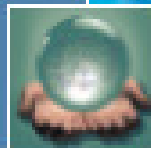
Thermo Scientific LTQ Orbitrap™
 An Overview of the Scientific Literature
 Michaela Szpilajka and David Koel, ThermoFisher Scientific



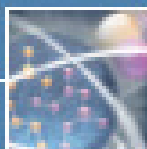
LTQ Orbitrap



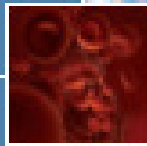
Proteomics



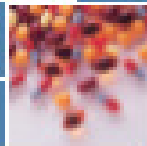
Metabolomics



Metabolism



Lipids



Doping Control



In the three years since its commercial debut (June 2002), the Thermo Scientific Orbitrap mass analyzer has been featured in an unparalleled number of peer-reviewed publications. In this relatively short amount of time, there have been an amazing number of review articles^{1,2,3} as well as publications providing insight into the design and operating principles of the analyzer, focusing primarily on advances in its technological development.⁴⁻⁶

Another major focus of the publications, however, has focused on the mass analysis of various compound classes demonstrating the incredible versatility of the LTQ Orbitrap for both research and routine analysis. This report provides a snapshot of the scientific literature where the Orbitrap mass analyzer has been used.

Probably the most useful attributes of the Orbitrap mass analyzer are the high mass resolution and mass accuracy. They are of general utility for both small and large molecule analysis. It is the analysis of very complex mixtures where the extreme dynamic range and versatility of the LTQ Orbitrap can be truly appreciated. It comes as no surprise then that most scientific papers are from the field of proteomics. The second largest group of published articles deals with the analysis of metabolites including metabolite identification, structural characterization, and quantitation in the context of metabolite studies, environmental analysis and doping control. Research focused specifically on metabolomics and lipid analysis has also embraced this new technology.

The Popularity of the LTQ Orbitrap



Representation of individual applications areas using the Orbitrap mass analyzer in published peer-reviewed journals with the number of publications noted.

Overview of published literature LTQ Orbitrap

How Accurate Is Accurate Enough – Quantitation perspective

Problem:

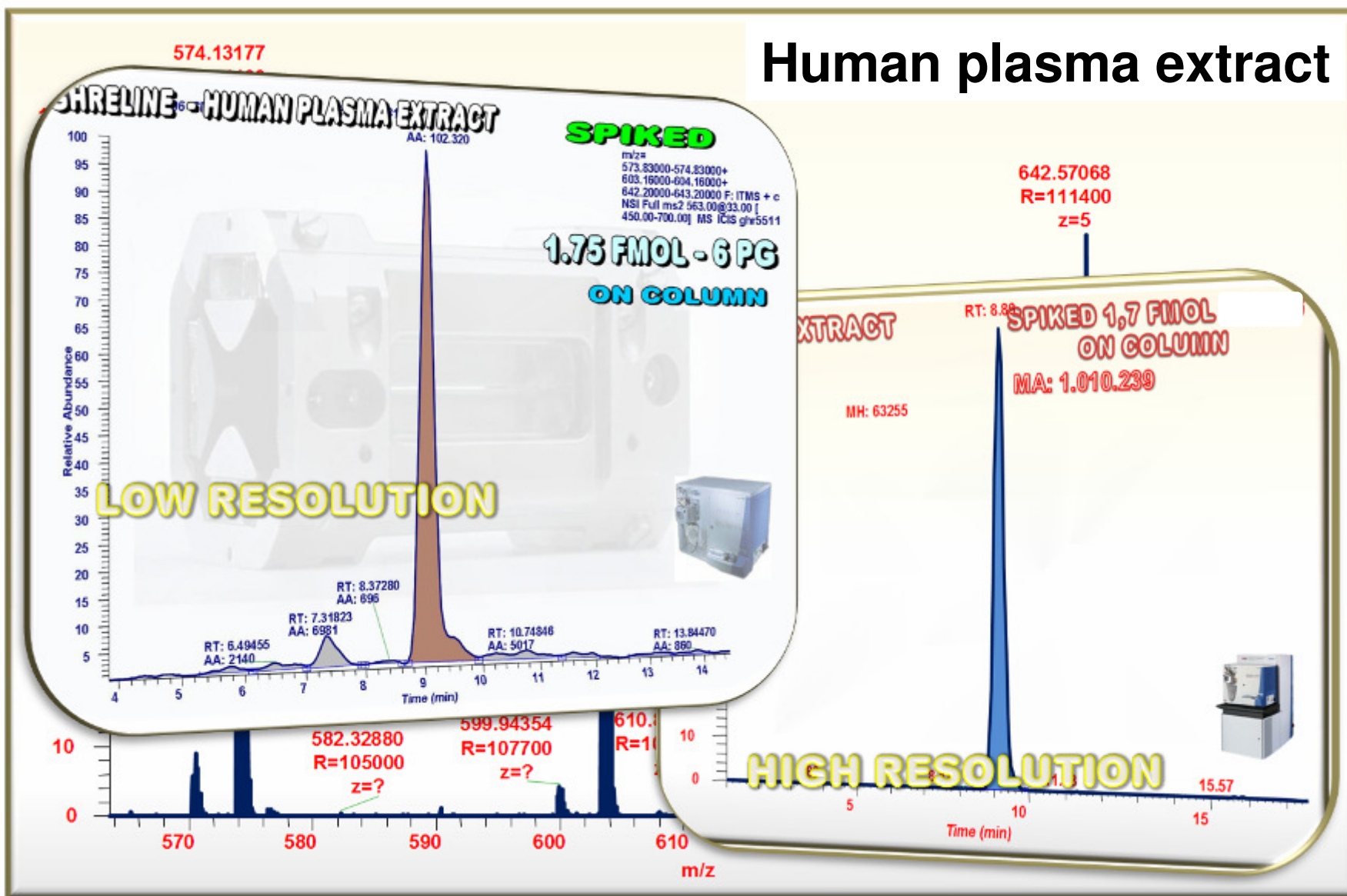
- Complex mixtures
- Interferences of nearly isobaric compounds

Compromised:

- Detection limits
- Accurate quantitation

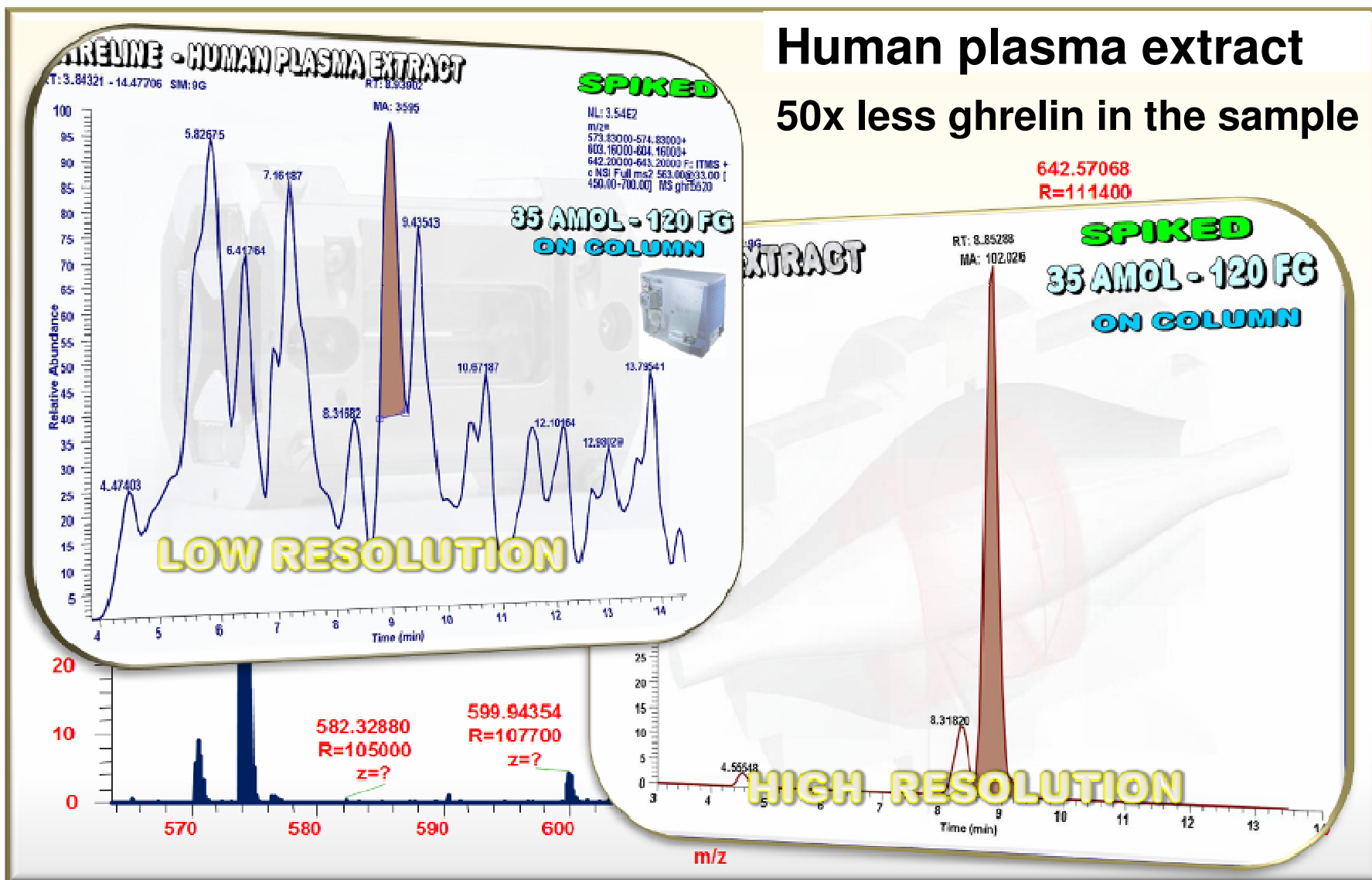
Ion Trap vs. Orbitrap Detection: 6 pg on column

Human plasma extract



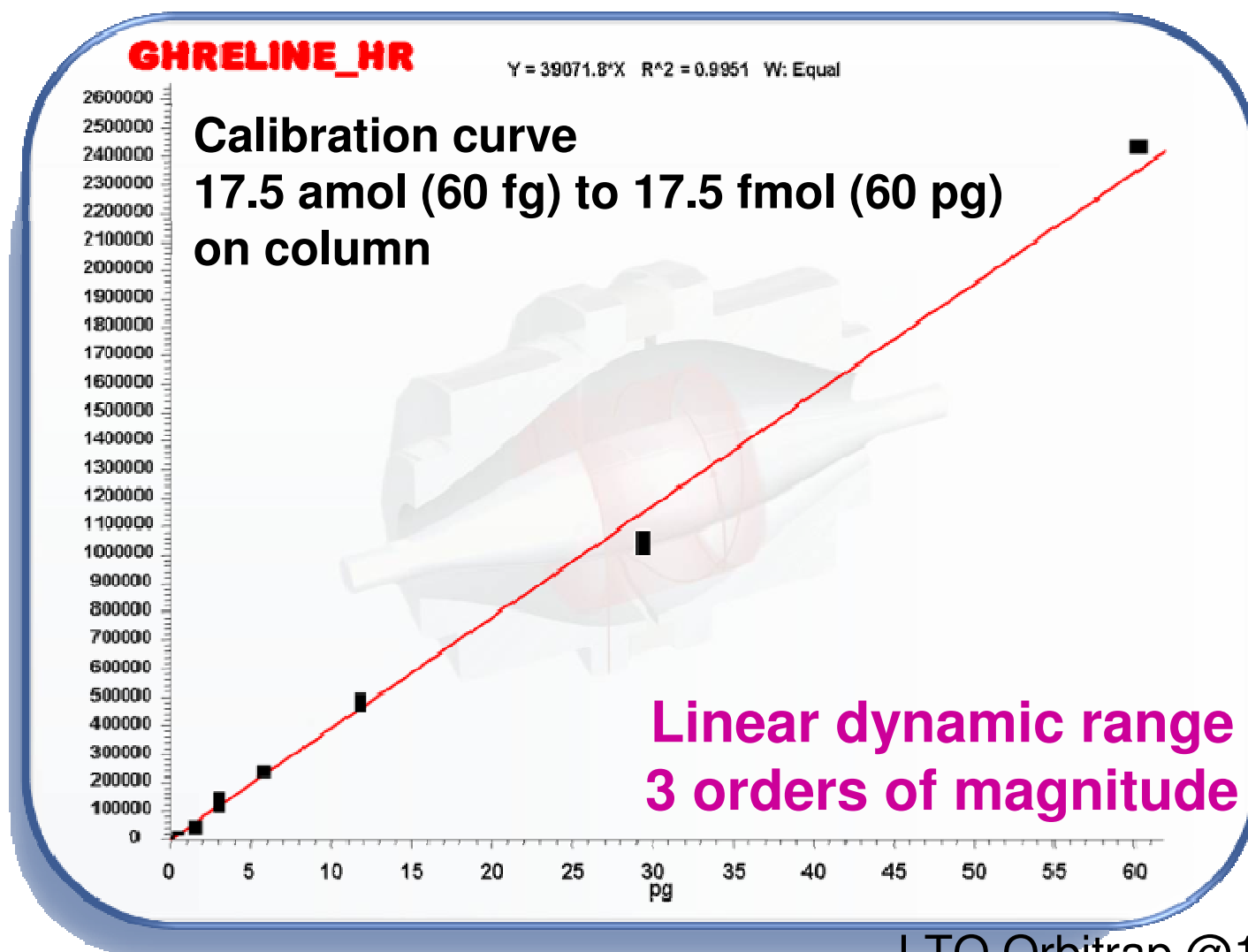
Data courtesy of Giorgio Vago, Thermo Italy

Ion Trap x Orbitrap Detection: 120 fg on column



Data courtesy of Giorgio Vago, Thermo Italy

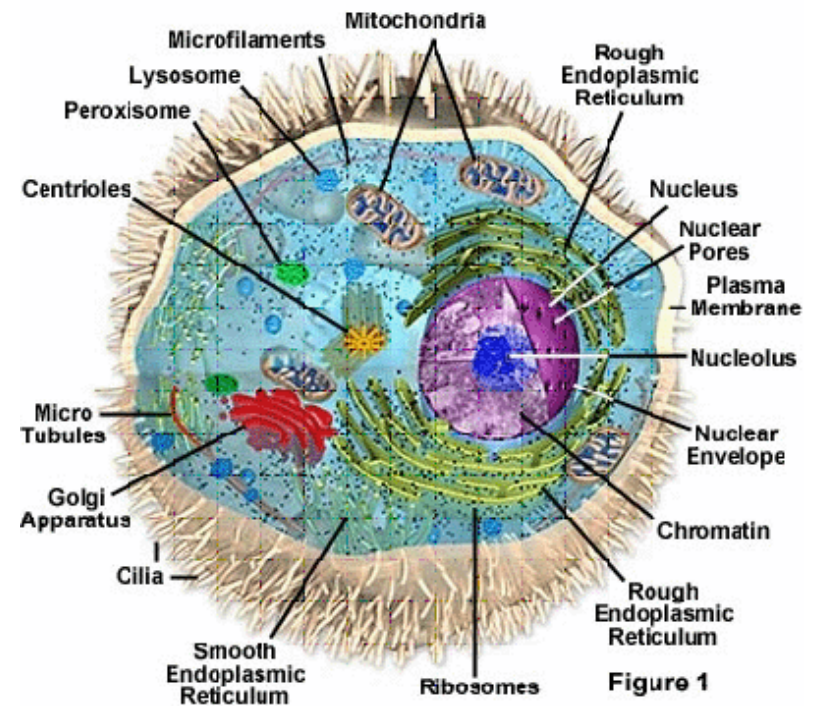
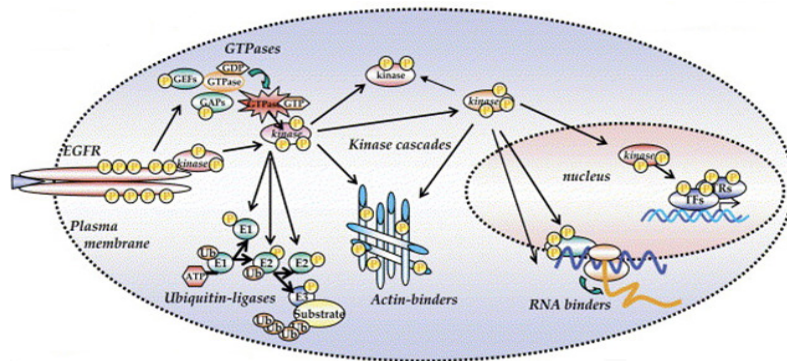
Quantitation of Ghreline in Human Plasma Extract



LTQ Orbitrap @100,000 RP

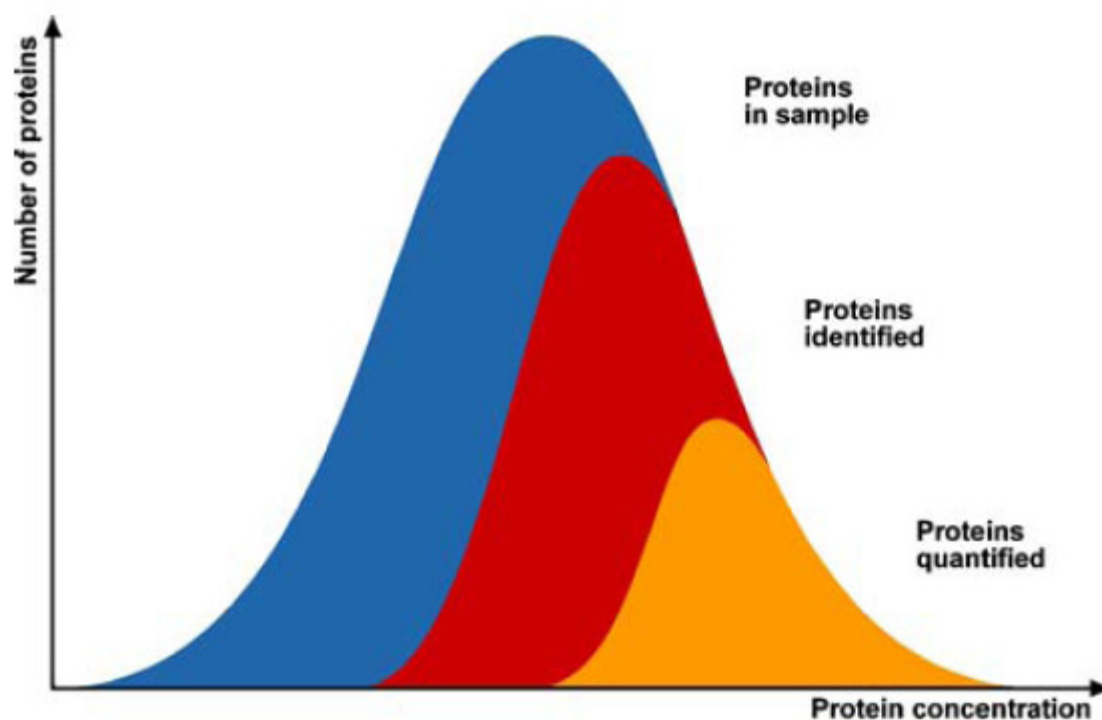
The Second Generation Proteomics

- The comprehensive, **quantitative** and **spatially resolved** analysis
 - Expansion in scale
 - Protein interactions
 - In time and space



MS-Based Proteome Identification and Quantitation

- Schematic representation of the fraction of a proteome that can be identified or quantified by mass-spectrometry-based approaches.



- Cellular proteins span a wide range of expression
- Current mass spectrometers typically sample only a fraction of all proteins present
- Due to limited data quality, only a fraction of all identified proteins can also be reliably quantified

Conclusions

- Resolution is empowering
- Resolution enables accurate mass
- Mass accuracy is a powerful filter
- Mass accuracy enables confident identification
- Both are indispensable for accurate quantitation

Acknowledgements

- Matthias Mann, MPI Martinsried
- Neil Kelleher, University of Illinois Urbana-Champaign
- Pavel Bondarenko, Amgen
- David Fenyo, Rockefeller University
- Giorgio Vago, Thermo Scientific Italy

Prophecies...

***Ultimately, ALL proteomics
will be quantitative***

—Ruedi Aebersold Dec. 17, 2004