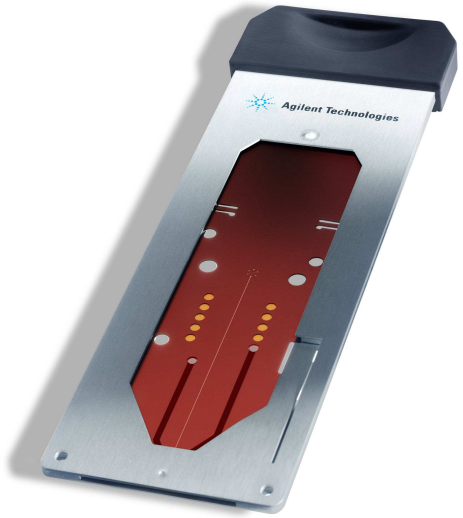
HPLC-Chip/MS

A new microfluidic platform for easy to use, robust and reliable nanospray LC-MS



Agilent Technologies, LSCA

Tom A. van de Goor, Ph.D.
R&D and Marketing GmbH&Co KG
Email: Tom_Vandegoor@Agilent.com

Prague, September 26, 2007

Agilent in Life Sciences and Chemical Analysis

$20 billion total addressable market… ample opportunity for growth

Life Sciences

Pharma, Biotech, CRO, CMO

Academia & Government

Clinical Diagnostics DX

Addressable Market:	$14B
Market Growth Rate:	7-9%
Agilent Projected Grpwth:	12-15%

Chemical Analysis

Petrochemical QA/QC

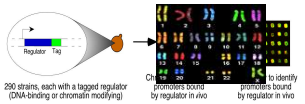
Environmental Testing

Food Testing

Forensics Testing

Addressable Market:	$6B
Market Growth Rate:	5-7%
Agilent Projected Growth:	7-9%

Life Sciences Tools



Spectroscopy Tools

Separations Tools

Consumables

Services & Informatics

AGILENT WORLD TOUR 2007

New Agilent High Performance 1200 Series LC:
Best in Class "Front End" Systems for MS

Preparative LC

Standard LC

Rapid Resolution LC

Capillary & Nano LC

High Sensitivity HPLC-Chip/MS

The industry's most comprehensive LC portfolio to configure an integrated LC/MS system

6000 Series LC/MS Portfolio
New Platforms

6410 Triple Quad LC/MS

- Femtogram sensitivity
- Excellent reproducibility,
- Cost of ownership advantages

Food, Forensic, Environmental, Chemical, Pharmaceutical, Proteomics, Metabolomics

6510 QTOF LC/MS

- 10X sensitivity advantage
- 2X typical mass accuracy
- Attomole MS and MS/MS
- Wide in-scan dynamic range
- Fast scan speeds (20/s)

Proteomics, Metabolomics, Pharmaceutical

6000 Series LC/MS Portfolio Enhanced Platforms

6300 Series Ion Trap LC/MS

Speed, resolution, range, sensitivity

- MS and MS^n Workhorse
- 6340: Electron Transfer Dissociation provides soft ion fragmentation
- Use on chromatographic time scale

Proteomics, Pharmaceutical, Food Safety, Environmental, Clinical

6210 TOF LC/MS

"FTMS Like" Performance

- 2 ppm Mass Accuracy
- High mass resolution
- Wide dynamic range (3-4 orders)
- Fast scan speeds for high throughput screening

Food, Forensics, Environmental, Chemical, Pharma, Metabolomics

Another New LC/MS in the 6000 Series

NEW AT ASMS

6100 Series Single Quads

Higher Performance

6140 handles 1200 Rapid Resolution LC data

Greater Choice and Full Investment Protection

4 Models, Each One Upgradable to the Next

Better Price/Performance on All Models

40% smaller footprint

- 6140:

  1-picogram sensitivity, fast-scan to 10,000 amu/second

- 6130, 6120, 6110:

  High performance to entry level models,

  1 to 10 picogram sensitivity,

  Mass range to 3,000 amu

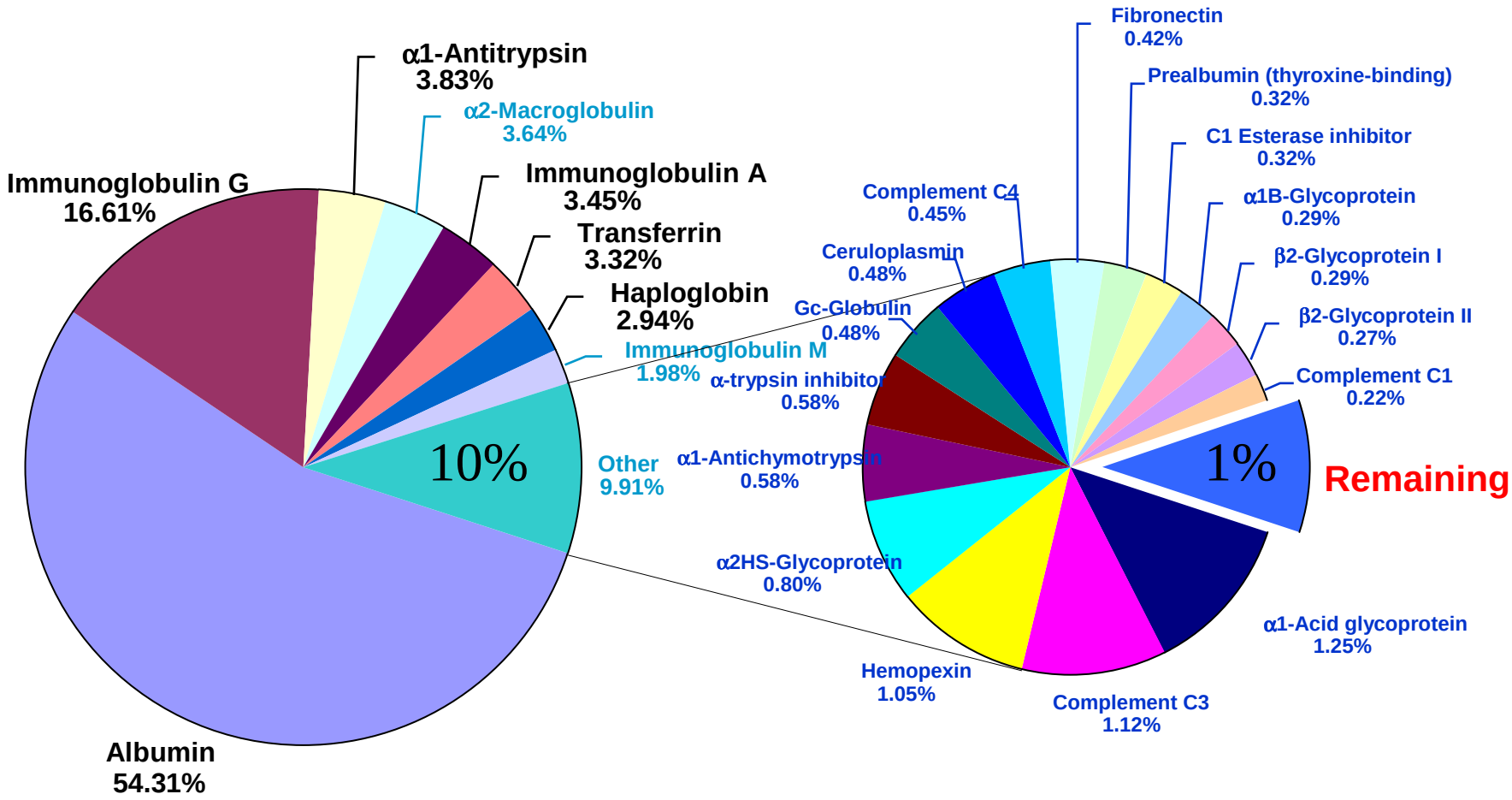
Food, Forensics, Environmental Chemical, Pharmaceutical

First Lab-on-a-Chip Product

Caliper Lab-Chip Kits

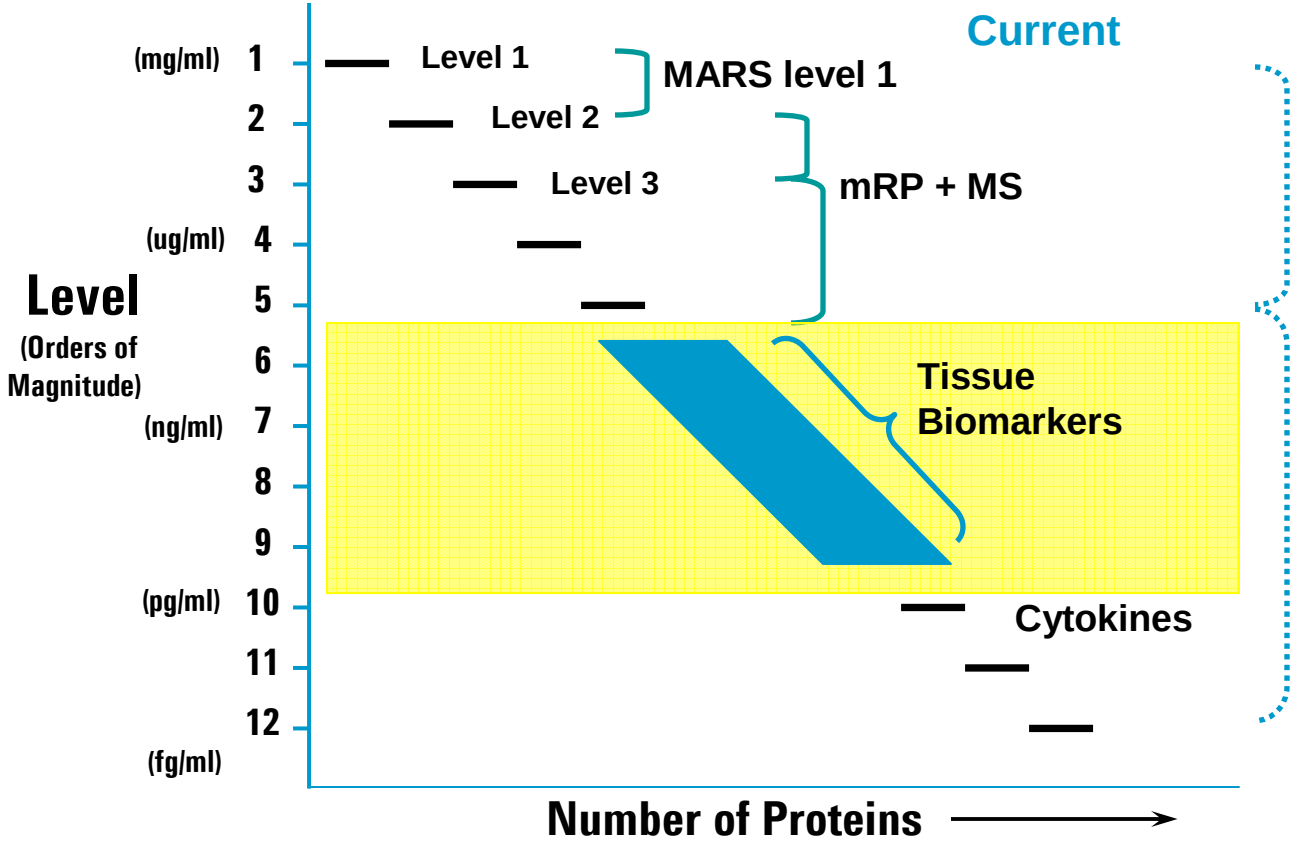
Agilent 2100 Bioanalyzer

Proteins in human serum



A Big Biological Challenge

In human serum the concentrations of proteins range over twelve orders of magnitude



Current technologies permit one to only look at the top layers: High abundance proteins

A major goal is to look at clinically useful proteins (biomarkers) which are typically Moderate to Low abundance proteins.

Outline

Liquid Chromatography and hyphenated techniques

- Liquid chromatography, detection
- Hyphenated techniques
- Miniaturizing HPLC-MS

The Agilent HPLC-Chip/MS system

- HPLC-Chip manufacturing and chip layouts
- HPLC-Chip system hardware components
- Key performance parameters

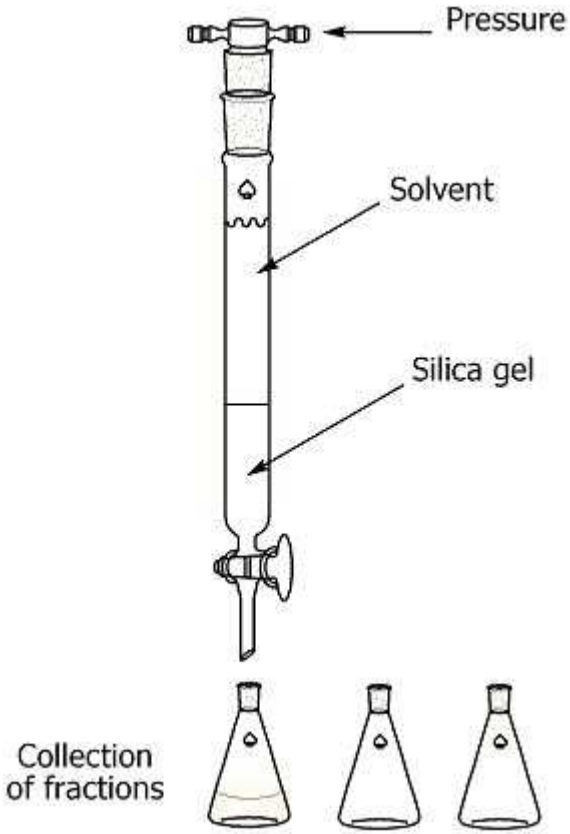
Application examples

Outlook

- Future applications and functionalities

Liquid chromatography

For separation of compounds in solution



How it used to be done

Agilent 1200 Series HPLC

High Performance Liquid Chromatography

HPLC is an analytical technique for the ***separation*** of compound mixtures as a function of time based on *multiplicative distribution* of the sample component between a moving *mobile phase* and a stagnant *stationary phase*

Well established in:

- Environmental analysis
- Pharmaceutical analysis
- Bioanalysis (Genomics, Proteomics and Metabolomics)
- Food analysis
- Quality control
- Process control

HPLC is universal and of superior significance in routine analysis as well as research

Detectors for HPLC

A HPLC detector locates, in the dimension of time, the sample components that have been subjected to a chromatographic process.

Optimal characteristics:

- **High sensitivity**
- Defined response for quantitative analysis
- Broad applicability
- Unaffected response to chromatographic parameters
- No contribution to extra-column band broadening

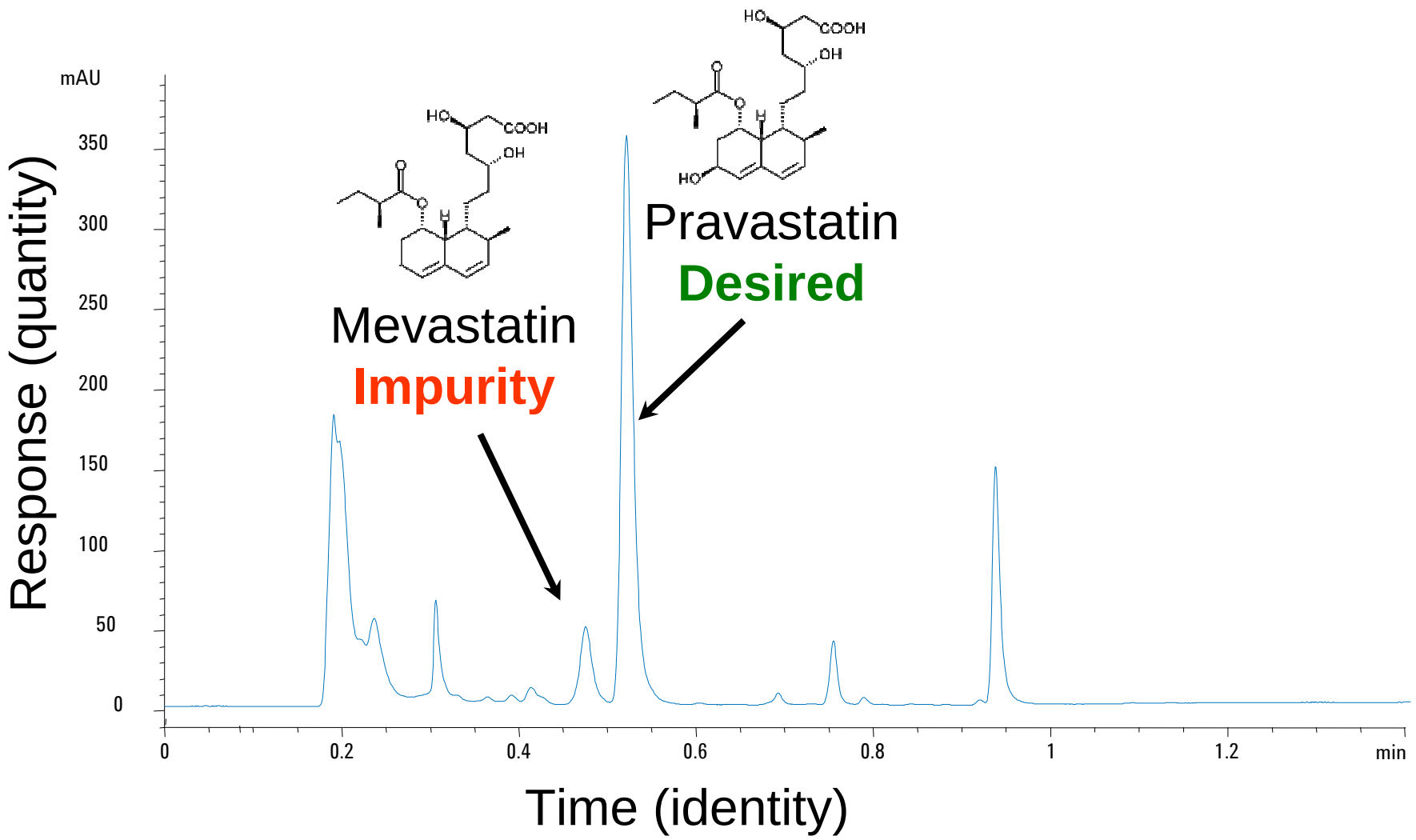
eg. UV detectors
Fluorescence detectors
RI detectors
Conductivity detectors
…

- Provides reliable qualitative information upon the separated analytes

Mass Spectrometry detectors

Pharmaceutical example

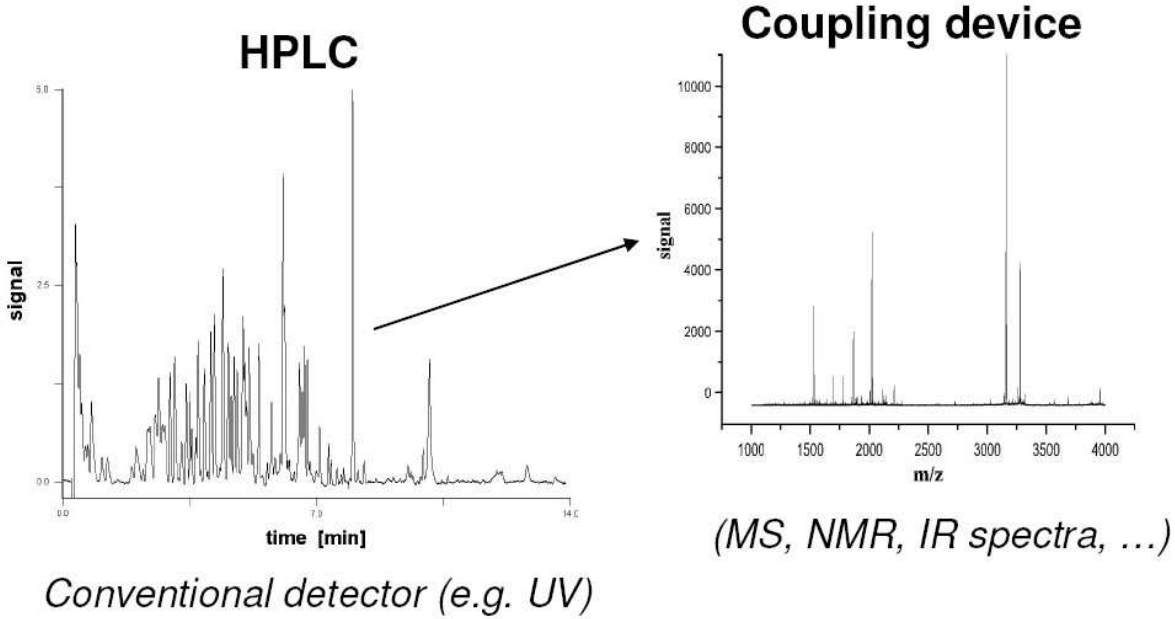
Identify and quantify impurities in drug manufacturing



Hyphenated techniques: HPLC - MS

Hyphenation techniques comprises all methods for ***coupling*** HPLC with ***detection units***, which provide any ***qualitative information*** upon the eluting sample components, eg:

- **HPLC-MS**
- HPLC-NMR
- HPLC-ICP-MS
- …

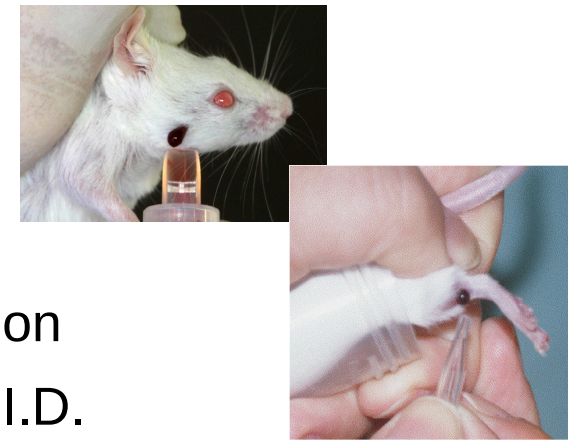


Conventional detector (e.g. UV)

(MS, NMR, IR spectra, …)

The HPLC system and predominately the separation column properties have to be adapted to the respective detection device.

Why miniaturize HPLC/MS?



- Radically reduces sample volume
- Decrease solvent and stationary phase consumption
- Increase mass sensitivity with decreasing column I.D.
- Increase column efficiency with decreasing column I.D.
- Faster reaction time in smaller volumes (surface to volume ratio)

AND

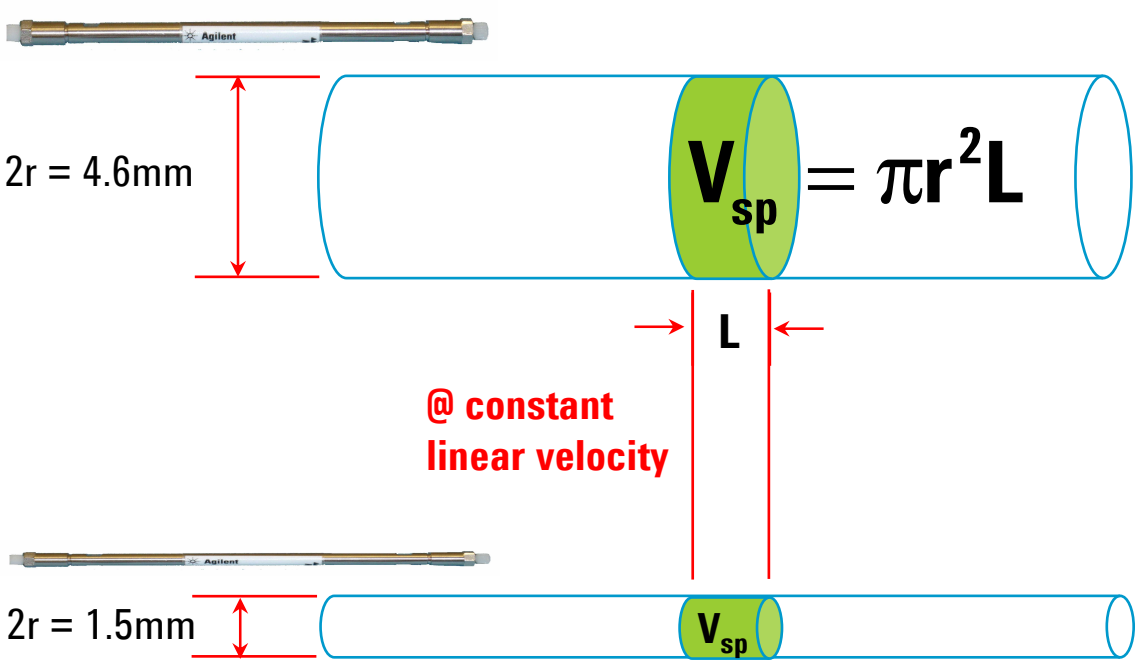
there is a clear trend in the (Bio)Pharmaceutical industry and Life Science community to put advanced equipment like LC-MS, in the hands of research workers who are not liquid chromatography or MS specialists

➔ Strong need for easy-to-use & robust analytical tools

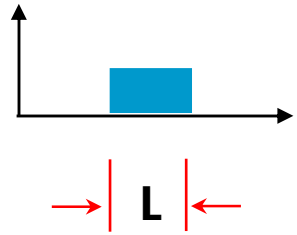
Why use microfluidics?

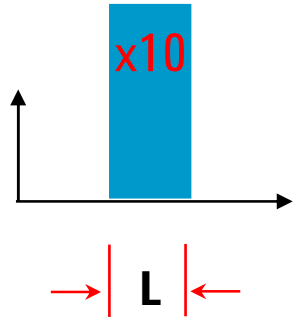
	Nano LC	Capillary LC	Micro LC	HPLC	Prep LC
Flow Rate (µL/min)	0.1	1 10	100	1,000	10,000 100,000
Column i.d. (mm)	0.05	0.2	1	2	4 50
Sensitivity gain	6400	400	16	4	1

Achieve Sensitivity by Reduction of ID (2r)



$$\text{Intensitiy} = f(c) = \frac{m}{V_{sp}}$$

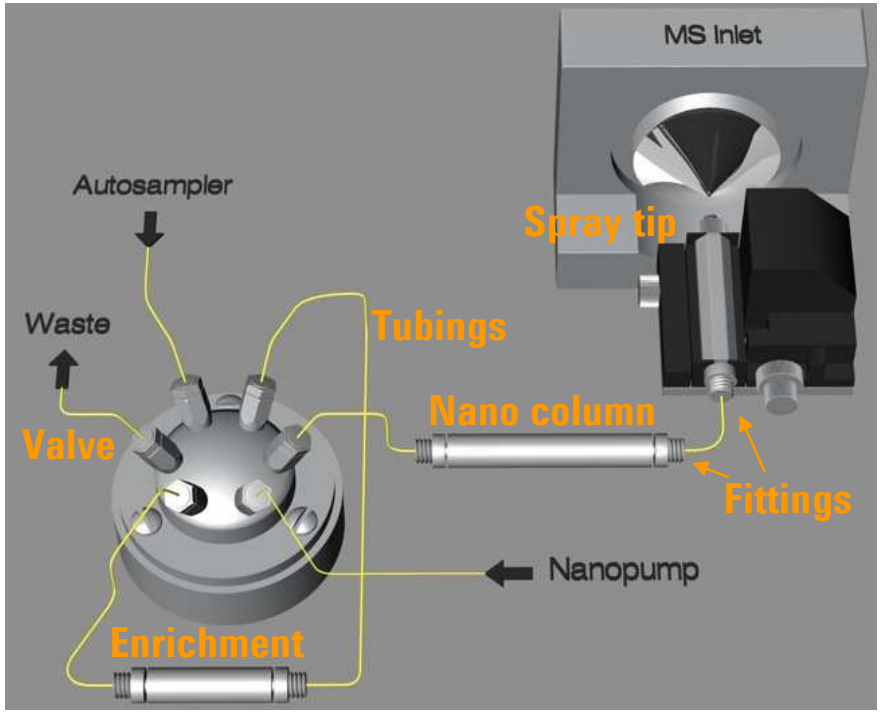




Nanospray LC/MS

Nanospray LC/MS combines both high resolution chromatography and high sensitivity MS detection and is used for ultra-trace analysis applications but...

Nanospray LC/MS is challenging to implement and maintain: multiple small capillary tubing connections, frequent clogging and leaks at the columns and nanospray tip and chromatographic degradation caused by tubing dead volume compromise reliability, ease of use, robustness and chromatographic performance.



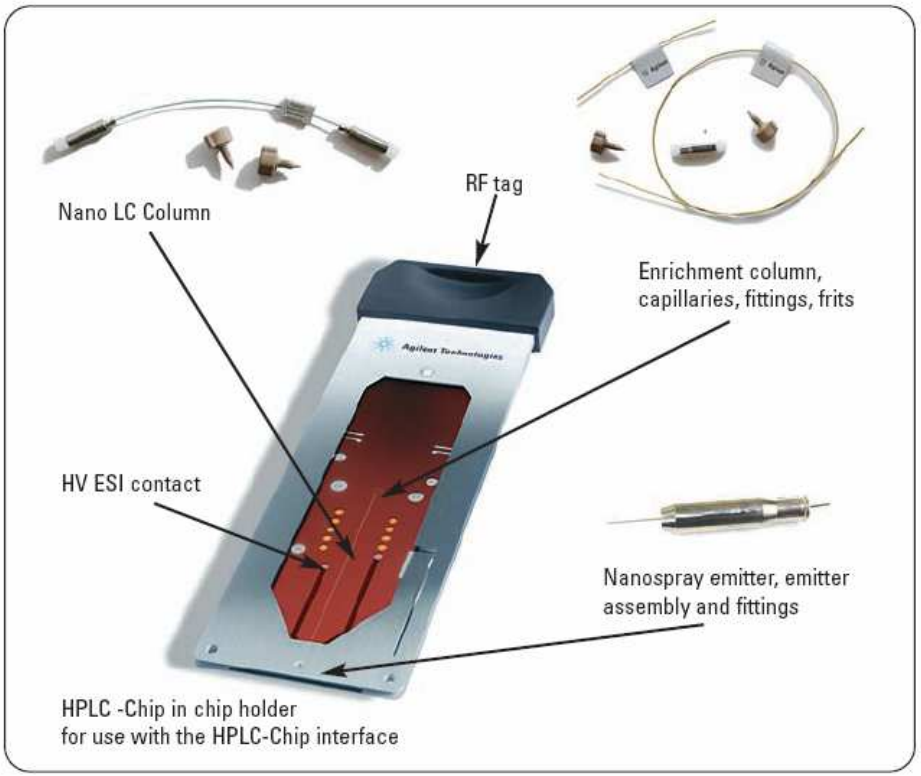
Why use Microfluidics for nanospray LC/MS?

Microfluidic chip devices are designed to be used at nano and pico flow rates and can integrate most functional components of a conventional nanocolumn LC/MS directly on to a chip.

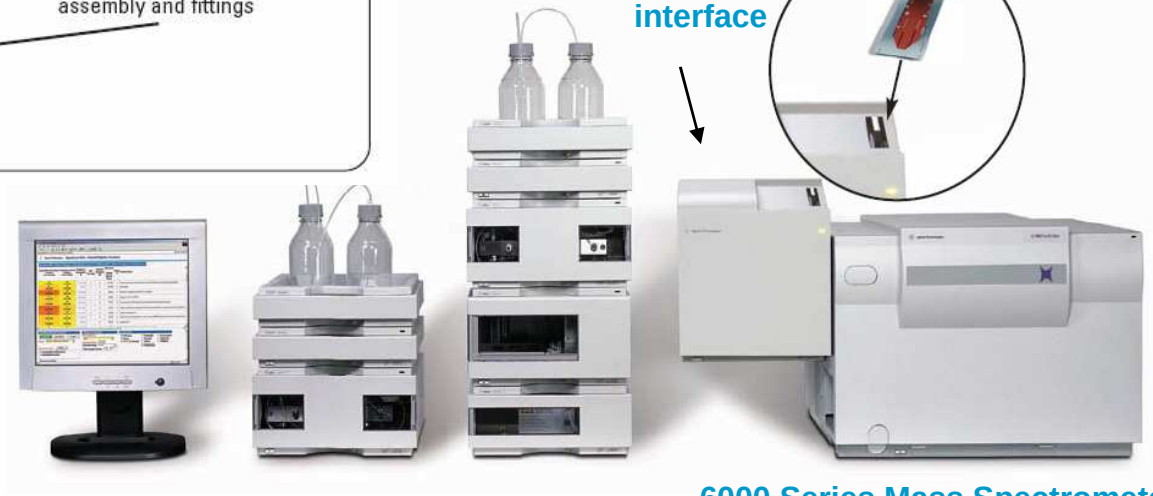
A microfluidic HPLC-Chip can integrate enrichment and analytical nanocolumns, nanospray emitter, fittings and connection capillaries directly on a reusable biocompatible polymer chip.

An automated HPLC-Chip interface ensures solvent and sample delivery to the chip, high pressure flow switching and automated chip loading and positioning in front of the MS inlet.

HPLC-Chip platform for nanospray LC/MS



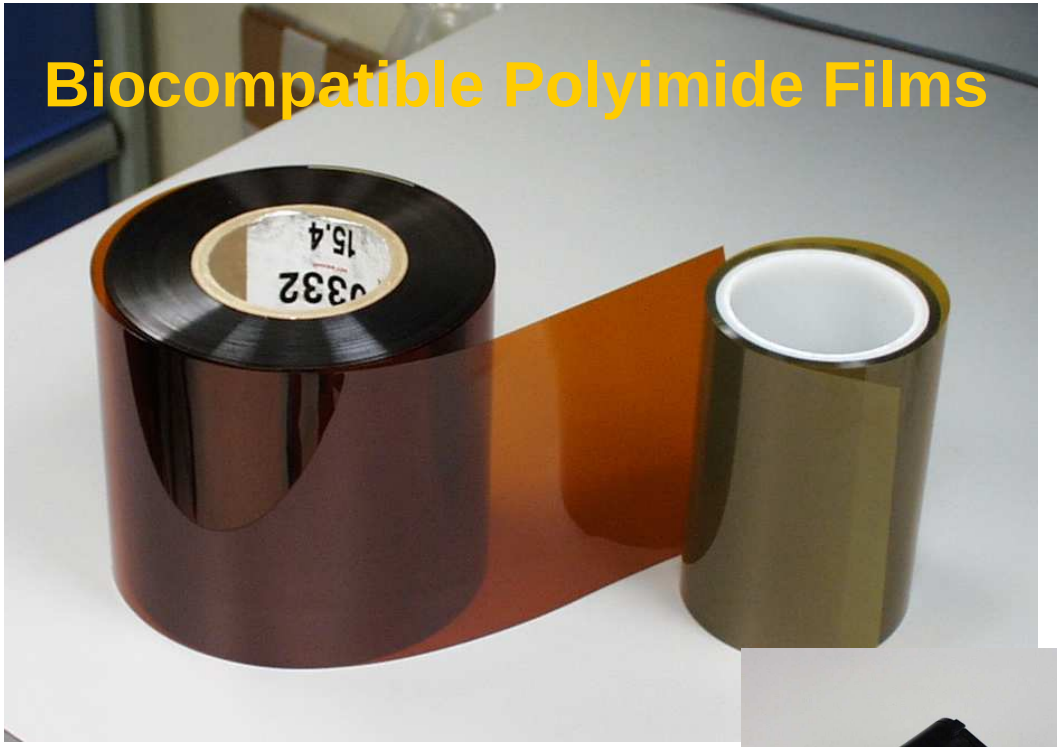
HPLC-Chip/MS interface



1200 NanoLC System

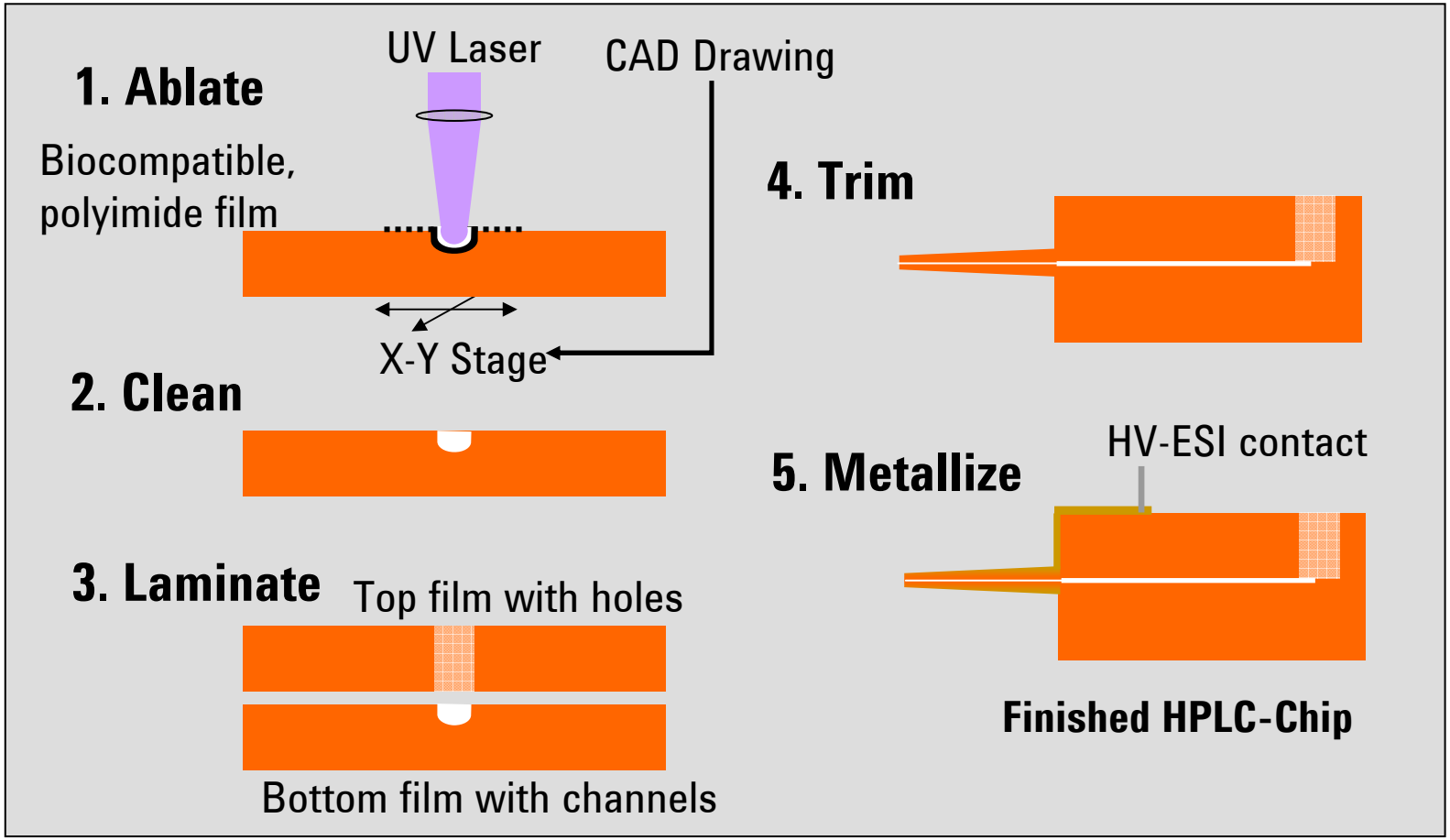
6000 Series Mass Spectrometer (Ion trap, SQ, QQQ, TOF, Q-TOF)

How Do You Make an HPLC-Chip?

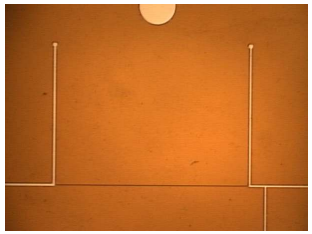
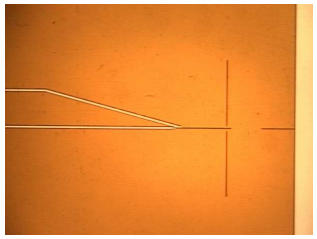


- Films 25 - 150 µm
- Special brand with high solvent resistance
- Multiple layers to make a HPLC-Chip

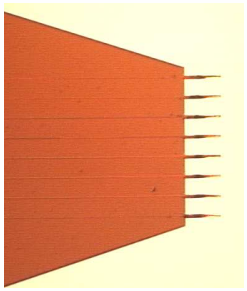
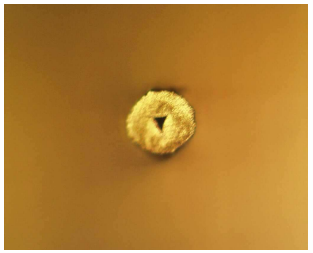
Rapid and High-Precision Fabrication of Polymer Microfluidics using Direct Write Laser Ablation



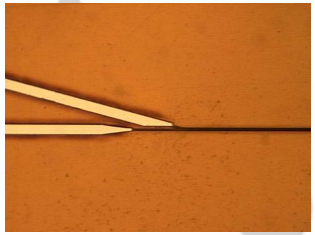
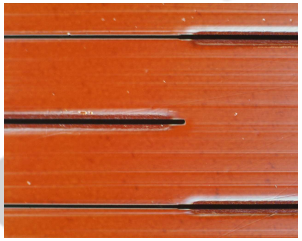
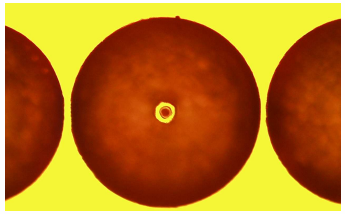
Laser Ablated Microfluidic Elements

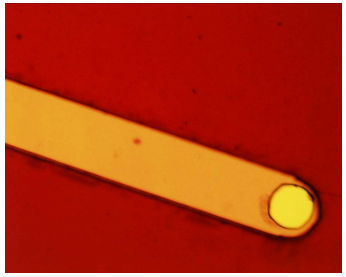
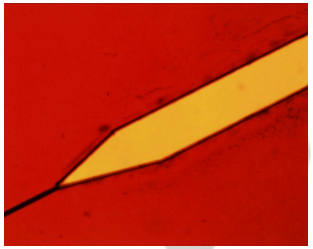
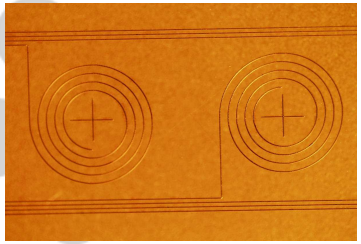
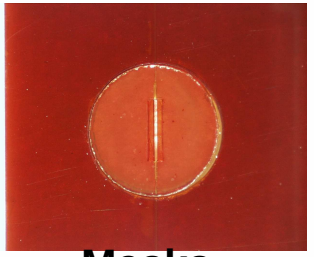
Channels

ES Tips

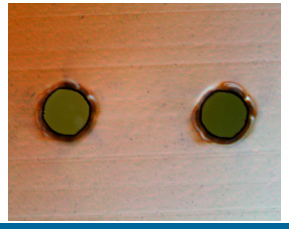
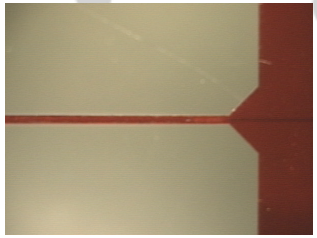
UV Window

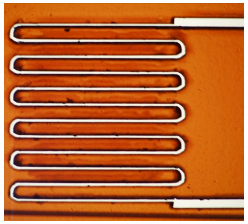





Holes

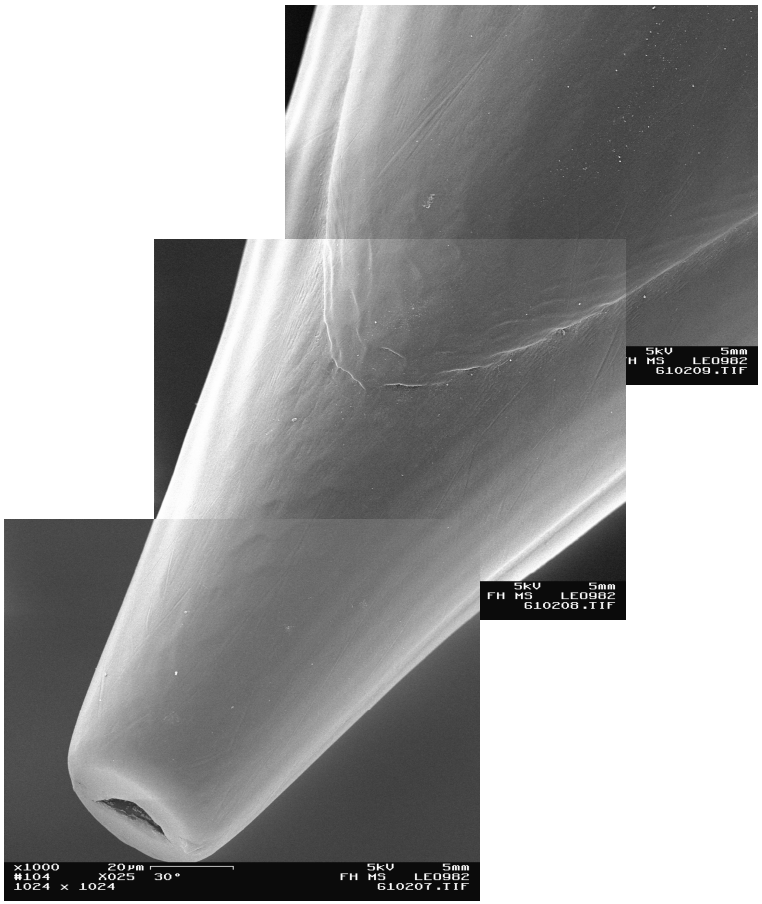
Sippers

Masks

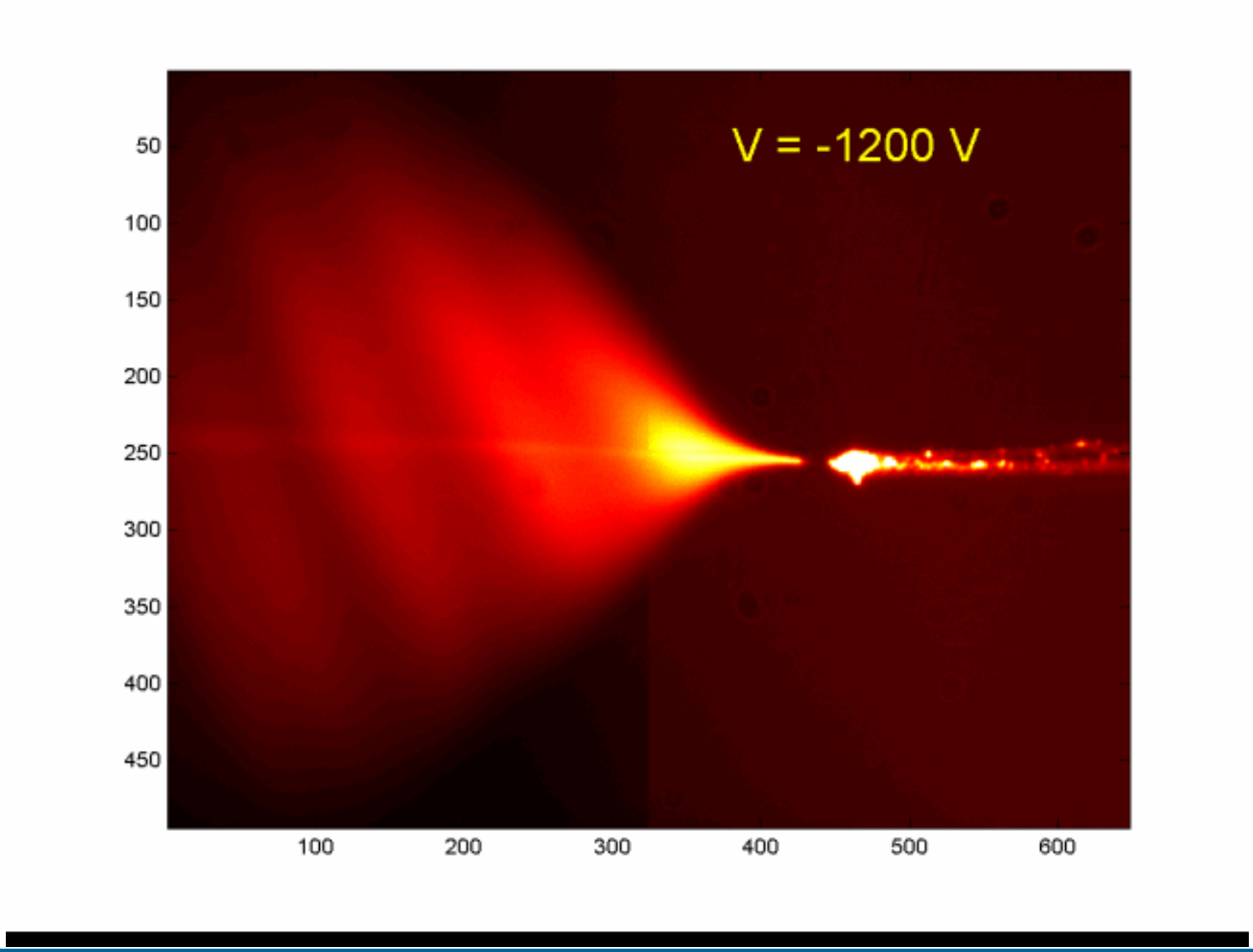
Laser Ablated Polyimide Electrospray Tips



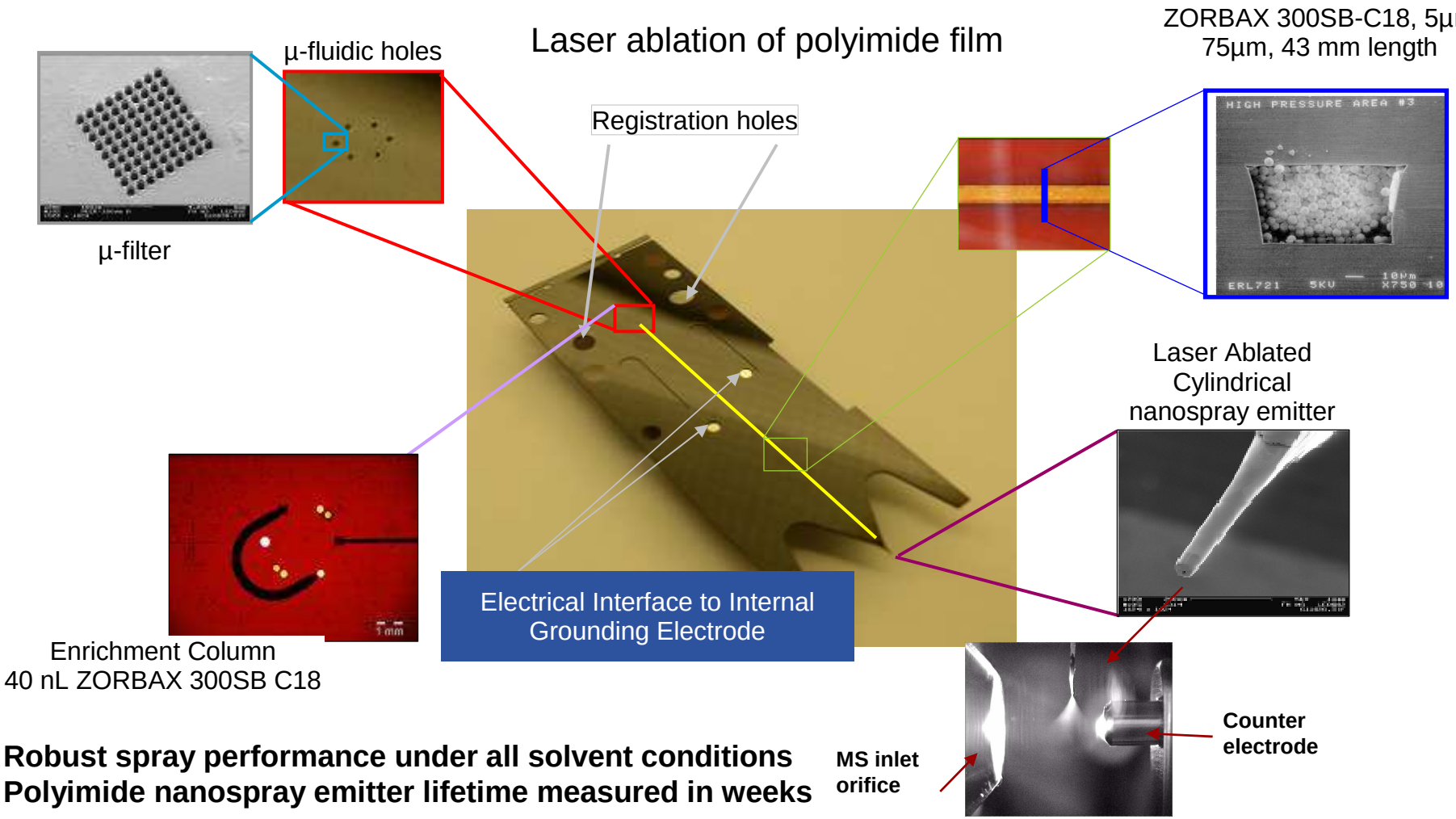
20 um

20 um

Visualization of Electrospray



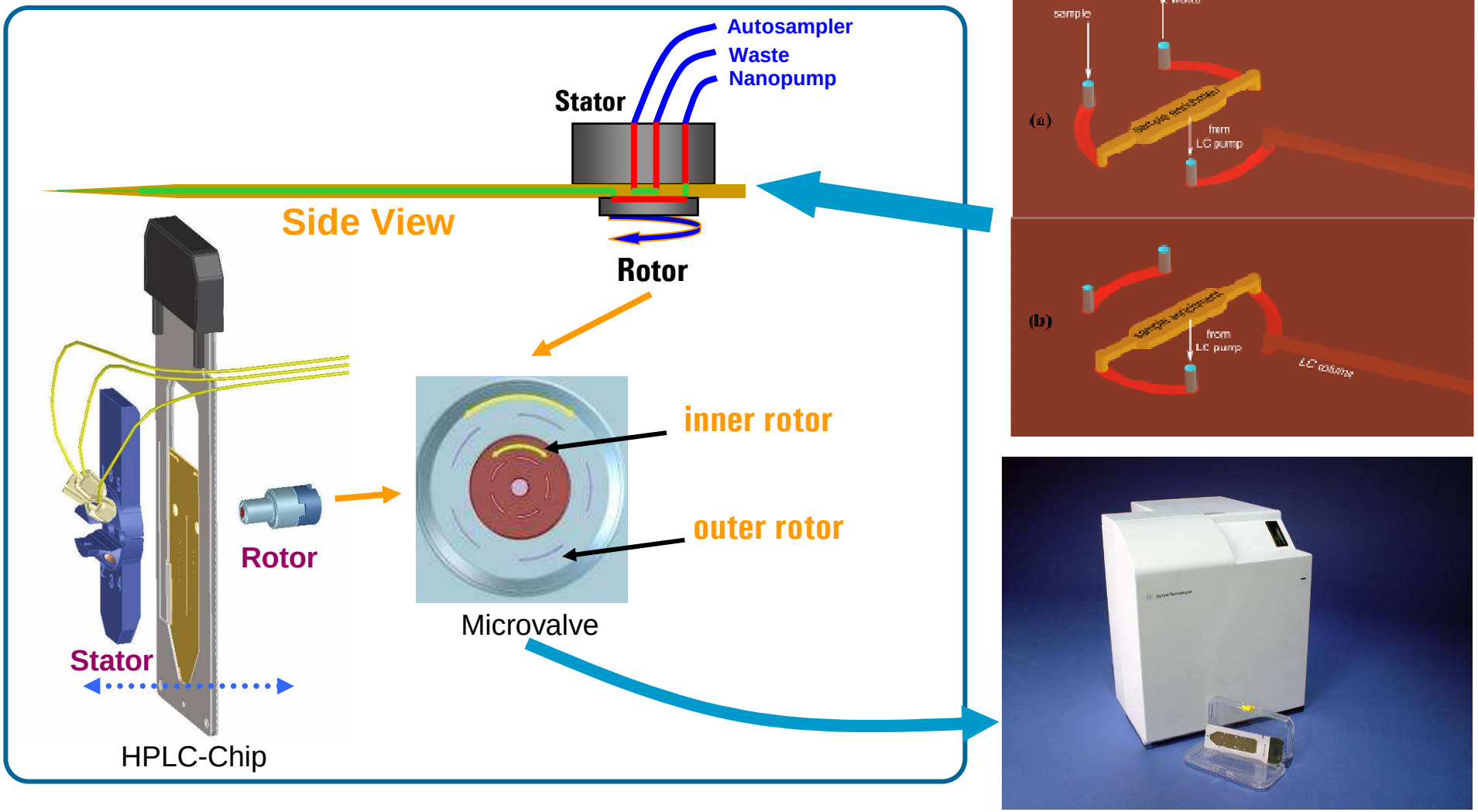
HPLC-Chip design features



Robust spray performance under all solvent conditions
Polyimide nanospray emitter lifetime measured in weeks

HPLC-Chip/MS system hardware components

- Putting it all together



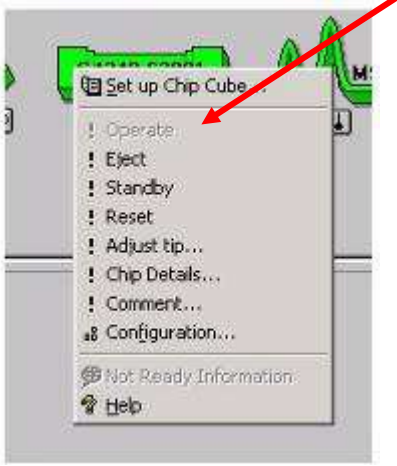
Putting it all together: how does it work?

- Easy to use HPLC-Chip system

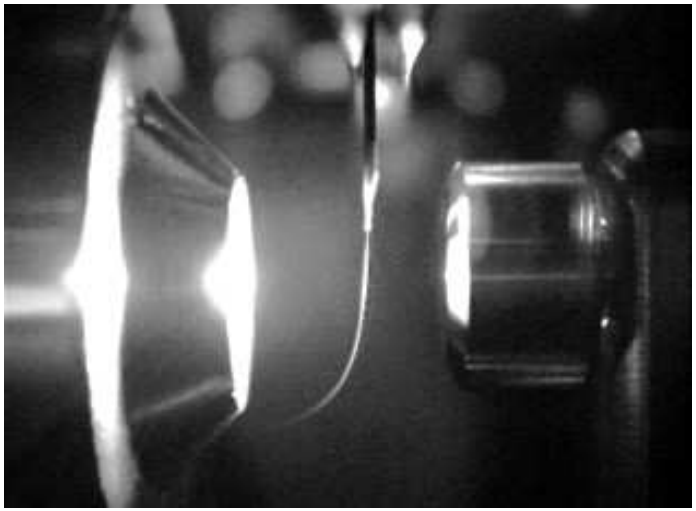
1 **Set up the nanoLC flow and gradient**

2 **Insert HPLC-Chip**

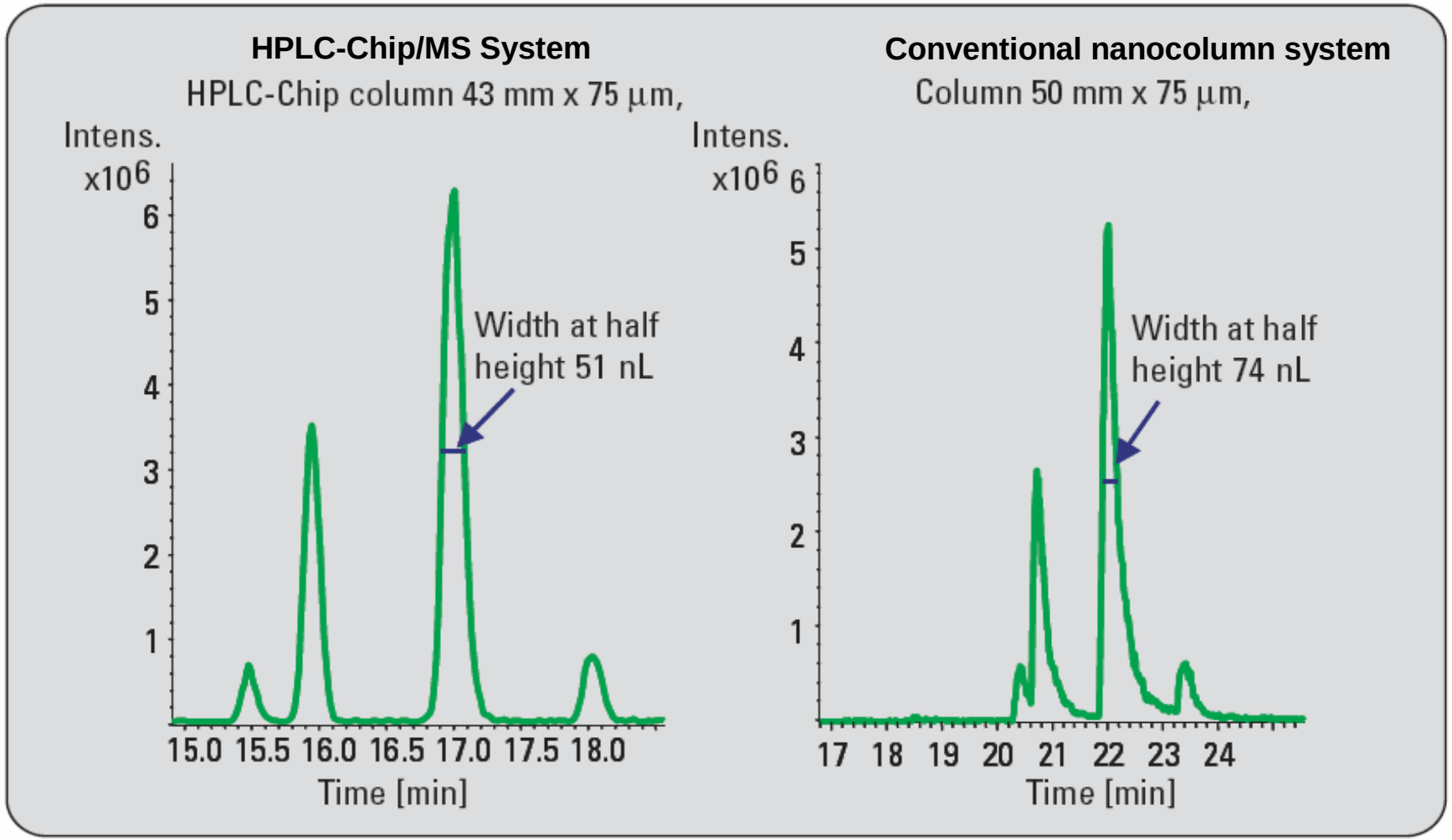
3 **Click on the Operate command in Chemstation**



4 **HPLC-Chip automatically moves into spray position**



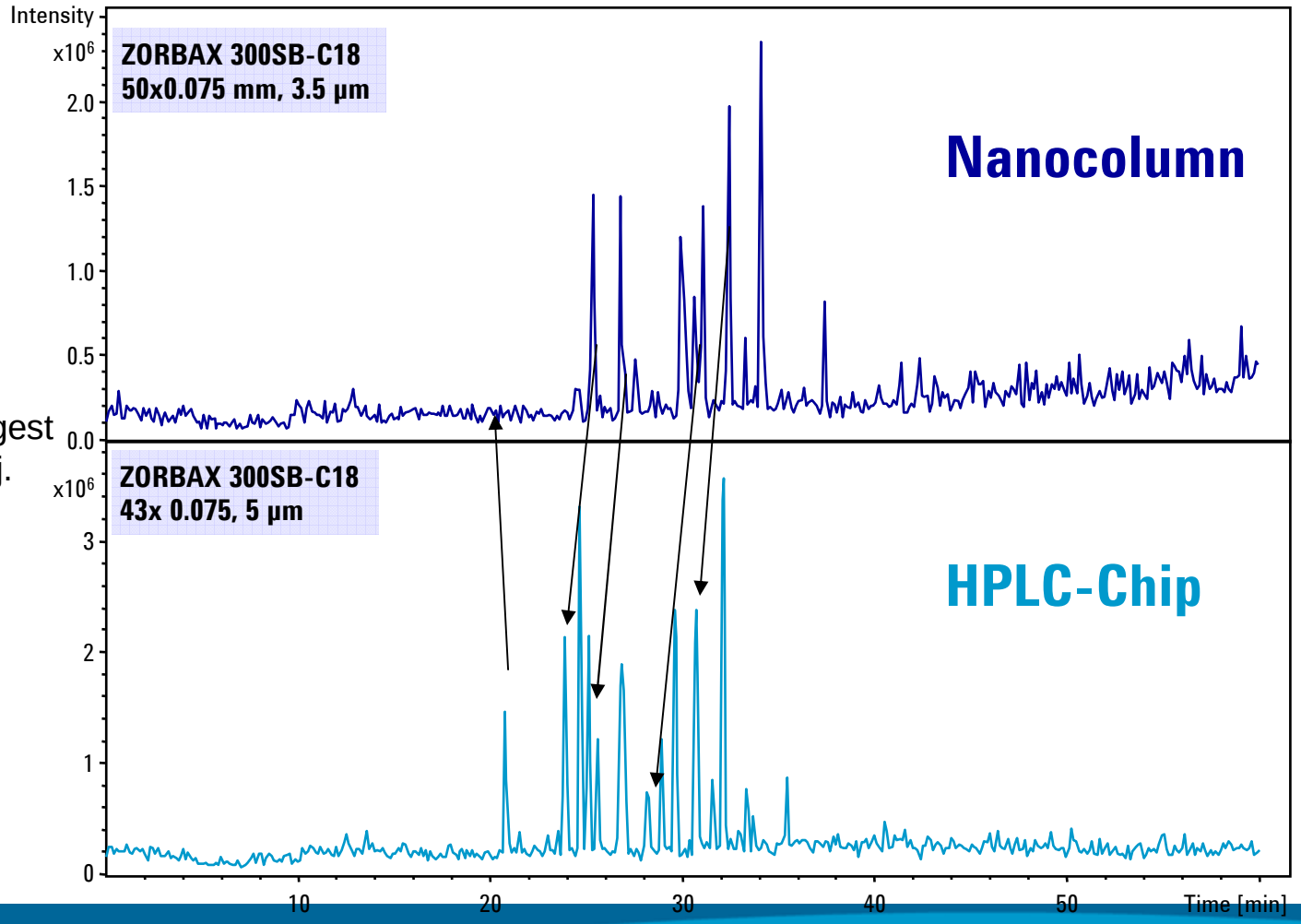
Chromatographic Performance



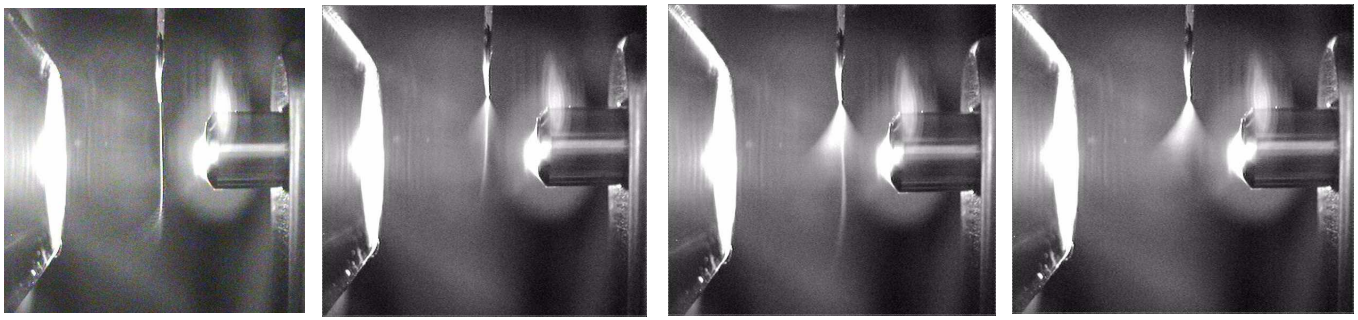
The integrated HPLC-Chip eliminates dispersion and band broadening and increases chromatographic performance and MS sensitivity.

Chromatographic Performance: HPLC-Chip Vs. Nanocolumn

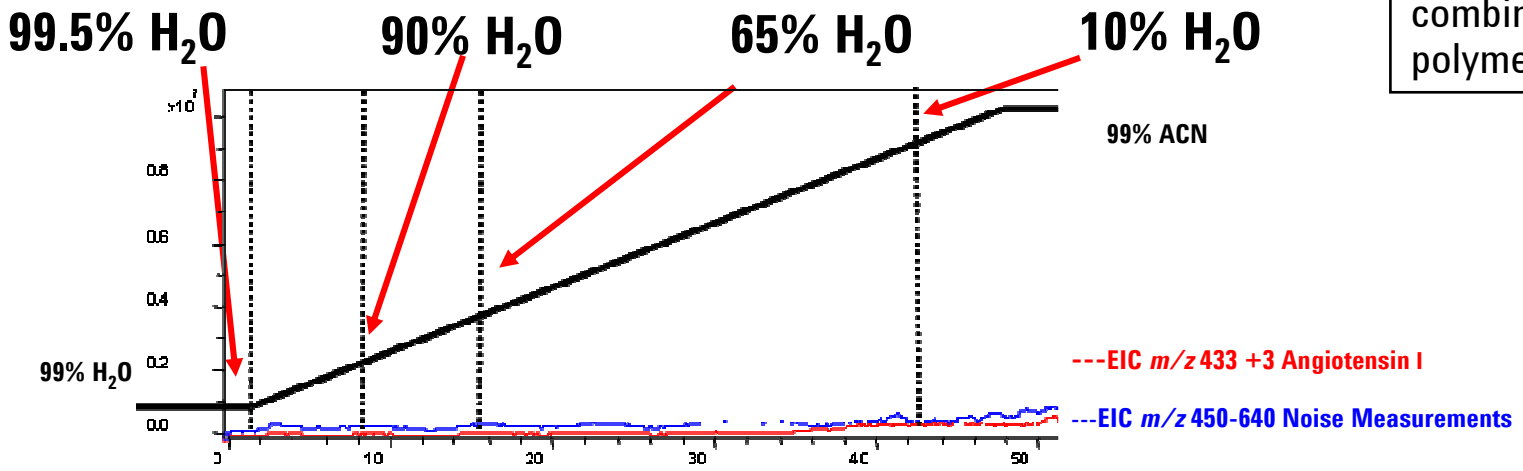
BSA tryptic digest
10 fmol, 1ul inj.



Nanospray performance



Use of the orthogonal dual electrode nanospray ion source shows constant signal-to-noise for all types of Taylor cone plumes when combined with the polymer chip

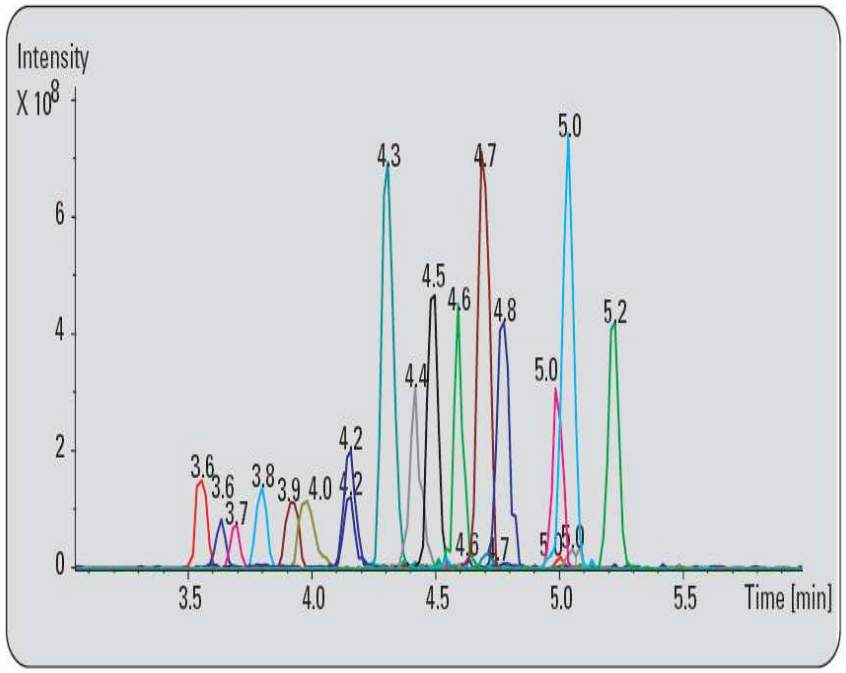


Flow: 320nL/min V: 2100volts
Angiotensin infused at constant flow rate over the 50 min gradient.

- **HPLC-Chip delivers robust spray performance for all solvent conditions.**
- **Does not require voltage change or optimization during a gradient.**
- **Polyimide nanospray emitter lifetime measured in weeks!**

Data courtesy of Paul Goodley, Agilent Technologies (USA)

Retention Time reproducibility



Extracted ion chromatograms for 17 peaks from a single analysis of BSA tryptic digest (50 fmol on-column). Rapid gradient method

	Average RT	SD	%RSD
EIC 487.8 ±All MS	3.618	0.014	0.40
EIC 752	3.788	0.011	0.29
EIC 740.6 ±All MS	5.018	0.010	0.20
EIC 874.4	3.968	0.012	0.31
EIC 653.6	4.289	0.012	0.28
EIC 511.7	3.681	0.012	0.31
EIC 722.7	3.547	0.012	0.35
EIC 778	4.143	0.010	0.23
EIC 526.3	4.399	0.015	0.34
EIC 547.5	4.472	0.011	0.25
EIC 746.7	5.196	0.011	0.20
EIC 519.1	4.142	0.011	0.26
EIC 508.2	4.972	0.011	0.23
EIC 582.4	4.679	0.011	0.23
EIC 461.9	3.905	0.012	0.30
EIC 474	4.759	0.011	0.22
EIC 628	4.584	0.010	0.22

RT reproducibility evaluated using 69 repeat injections
Worst case variation was 0.4% RSD, typical 0.25% RSD

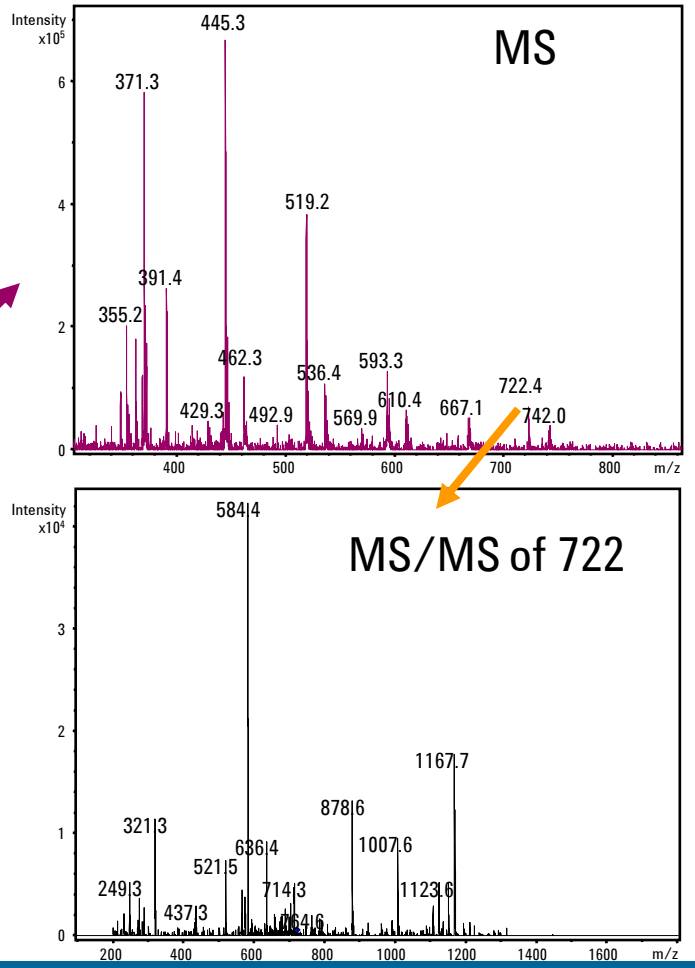
HPLC-Chip/MS

Application examples

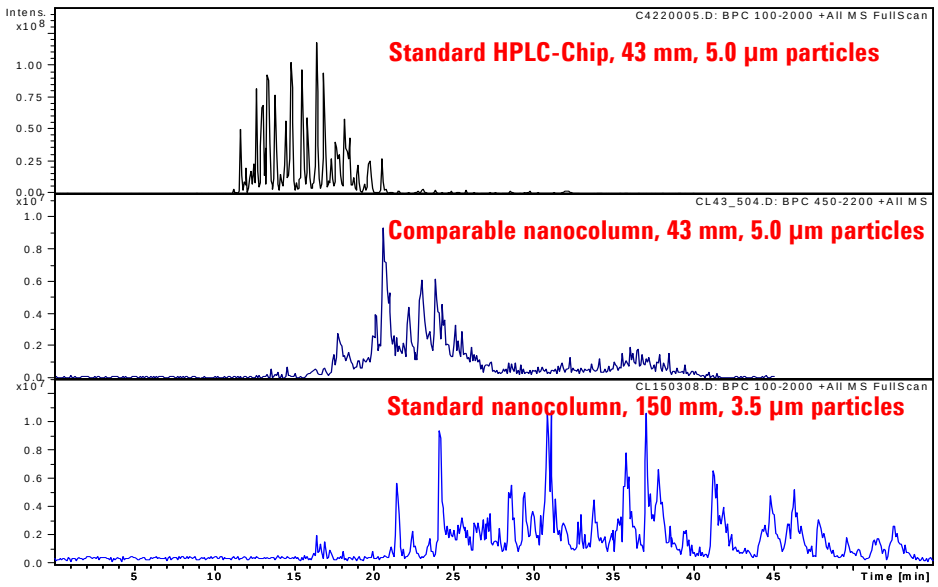
Sensitivity of the HPLC-Chip/MS System: Results for 100 amol HSA Digest

Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	6	6	83.98	12	1.40e+006	IPI00022434	Serum albumin

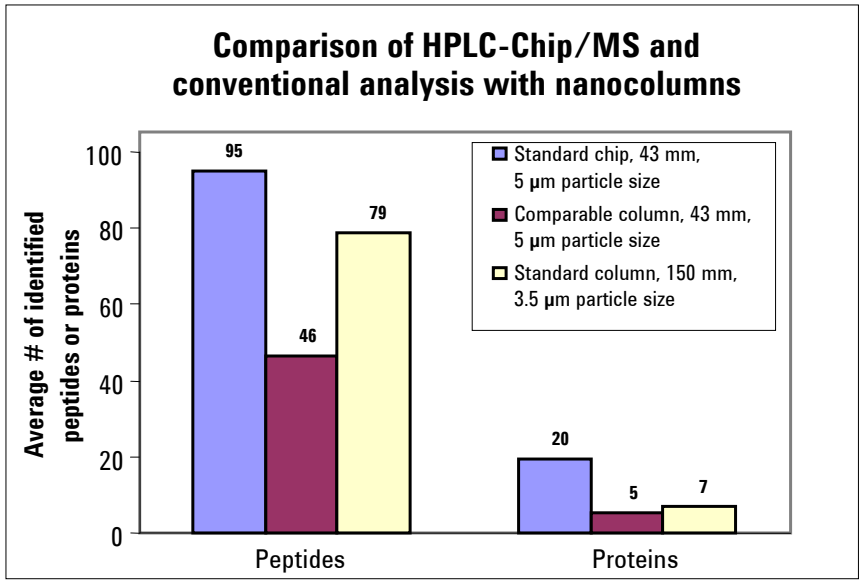
#	Sequence	m/z Measured (Da)	MH+ Matched (Da)	A100-1 Intensity
1	(K)FQNALLVR(Y)	480.97	960.5631	1.80e+006
2	(K)KVPQVSTPTLVEVSR(N)	547.47	1639.9383	1.50e+006
3	(K)LVNEVTEFAK(T)	575.46	1149.6156	1.93e+006
4	(K)QNCELFEQLGEYK(F)	829.49	1657.7532	1.04e+006
5	(R)RPCFSALEVDETYVPK(E)	637.96	1910.9322	1.02e+006
6	(K)YICENQDSISSK(L)	722.43	1443.6426	1.13e+006



Proteomics: Protein ID from yeast gel band tryptic digest.



Yeast proteome sample. The proteins from the yeast cell extract were separated by SDS-PAGE. Fifteen micrograms of protein was loaded in each lane of the gel. A single gel band was excised. Resulting peptides were isolated using the Agilent Protein In-Gel Tryptic Digestion Kit



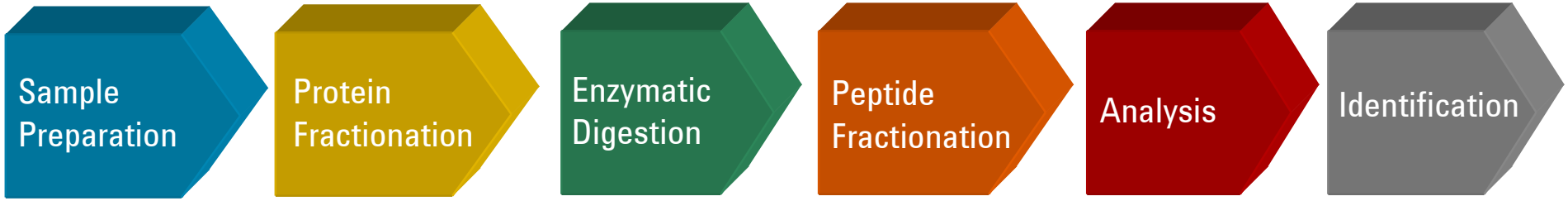
Average number (triplicate) of identified peptides and proteins (Spectrum Mill) from the yeast gel band using the HPLC-Chip/MS(43 mm) versus conventional LC/MS with nanocolumns (43 mm and 150 mm).

- **4X more proteins were identified with better sequence coverage for the tryptic digest of the yeast gel band with HPLC-Chip vs. comparable conventional nanocolumn.**
- **Better chromatographic performance and MS sensitivity than conventional nanocolumns.**

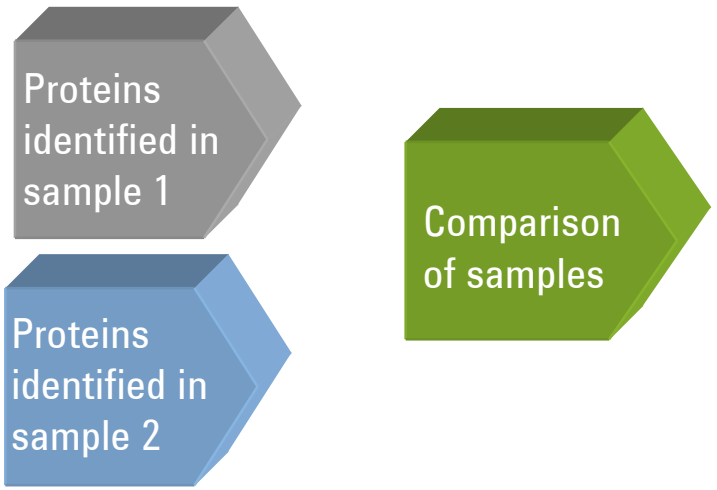
Data courtesy of Christine Miller and Martin Vollmer Agilent Technologies. (DE and USA)

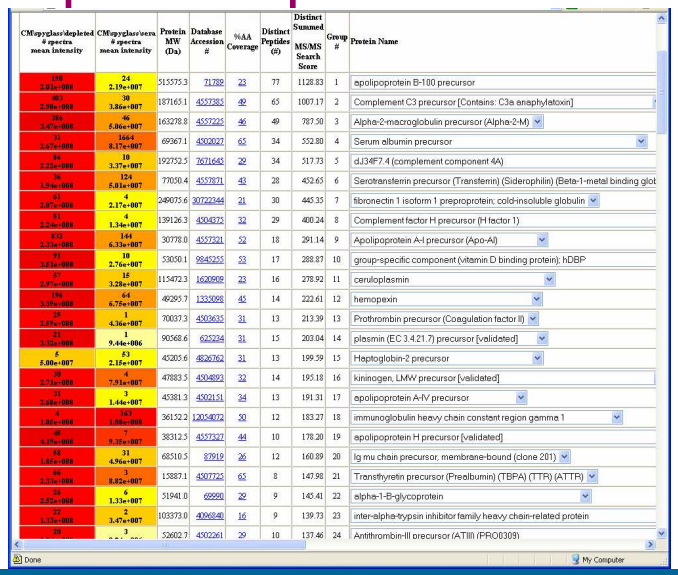
Protein Biomarker Workflow: Protein Identification Approach

Identify proteins in each sample



Compare identified proteins across multiple samples





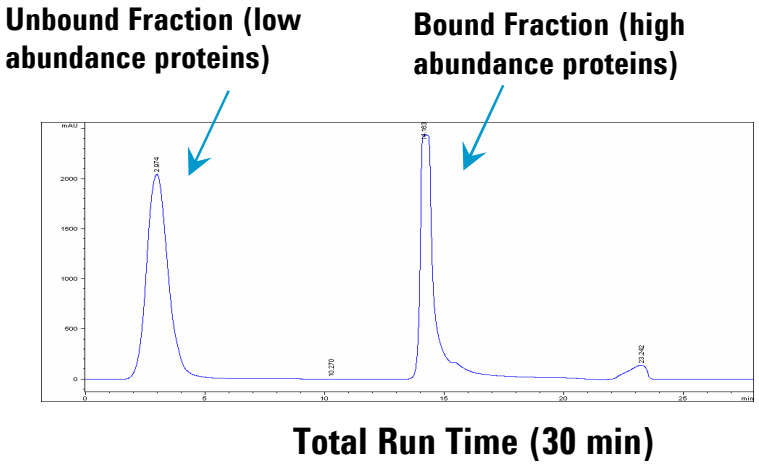
CM\spyglass\depleted # spectra mean intensity	CM\spyglass\sera # spectra mean intensity	Protein MW (Da)	Database Accession #	%AA Coverage	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	Group #	Protein Name
198 2.01e+008	24 2.19e+007	515375.3	71789	23	77	1128.83	1	apolipoprotein B-100 precursor
463 2.98e+008	30 3.86e+007	187165.1	4557385	49	65	1007.17	2	Complement C3 precursor [Contains: C3a anaphylatoxin]
345 3.47e+008	46 5.06e+007	163278.8	4557225	46	49	787.50	3	Alpha-2-macroglobulin precursor (Alpha-2-M)
31 2.47e+008	1664 8.17e+007	69367.1	4502027	65	34	552.80	4	Serum albumin precursor
86 2.22e+008	10 3.37e+007	192752.5	7671645	29	34	517.73	5	dJ34F7.4 (complement component 4A)
36 1.96e+008	124 5.01e+007	77050.4	4557871	43	28	452.65	6	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal binding glob
63 2.07e+008	4 2.17e+007	249075.6	30722344	21	30	445.35	7	fibronectin 1 isoform 1 preproprotein; cold-insoluble globulin
63 2.22e+008	4 1.34e+007	139126.3	4504375	32	29	400.24	8	Complement factor H precursor (H factor 1)
132 2.33e+008	164 6.33e+007	30778.0	4557321	52	18	291.14	9	Apolipoprotein A-I precursor (Apo-AI)
93 1.61e+008	10 2.76e+007	53050.1	9845255	53	17	288.87	10	group-specific component (vitamin D binding protein); hDBP
67 2.97e+008	15 3.28e+007	115472.3	1620909	23	16	278.92	11	ceruloplasmin
196 3.39e+008	64 6.75e+007	49295.7	1335098	45	14	222.61	12	hemopexin
28 2.87e+008	1 4.36e+007	70037.3	4503635	31	13	213.39	13	Prothrombin precursor (Coagulation factor II)
21 1.32e+008	1 9.44e-006	90568.6	625234	31	15	203.04	14	plasmin (EC 3.4.21.7) precursor [validated]
4 5.08e+007	63 2.15e+007	45205.6	4826762	31	13	199.59	15	Haptoglobin-2 precursor
30 2.71e+008	4 7.91e+007	47883.5	4504893	32	14	195.18	16	kininogen, LMW precursor [validated]
31 2.66e+008	3 1.44e+007	45381.3	4502151	34	13	191.31	17	apolipoprotein A-IV precursor
4 1.86e+008	143 1.08e+008	36152.2	12054072	50	12	183.27	18	immunoglobulin heavy chain constant region gamma 1
46 4.19e+008	7 9.35e+007	38312.5	4557327	44	10	178.20	19	apolipoprotein H precursor [validated]
59 1.85e+008	31 4.96e+007	68510.5	87919	26	12	160.89	20	Ig mu chain precursor, membrane-bound (clone 201)
66 2.13e+008	3 8.82e+007	15887.1	4507725	65	8	147.98	21	Transthyretin precursor (Prealbumin) (TBPA) (TTR) (ATTR)
25 2.52e+008	6 1.33e+007	51941.0	69590	29	9	145.41	22	alpha-1-B-glycoprotein
22 1.33e+008	2 3.47e+007	103373.0	4096840	16	9	139.73	23	inter-alpha-trypsin inhibitor family heavy chain-related protein
28	3	52602.7	4502261	29	10	137.46	24	Antithrombin-III precursor (ATIII) (PRO0309)

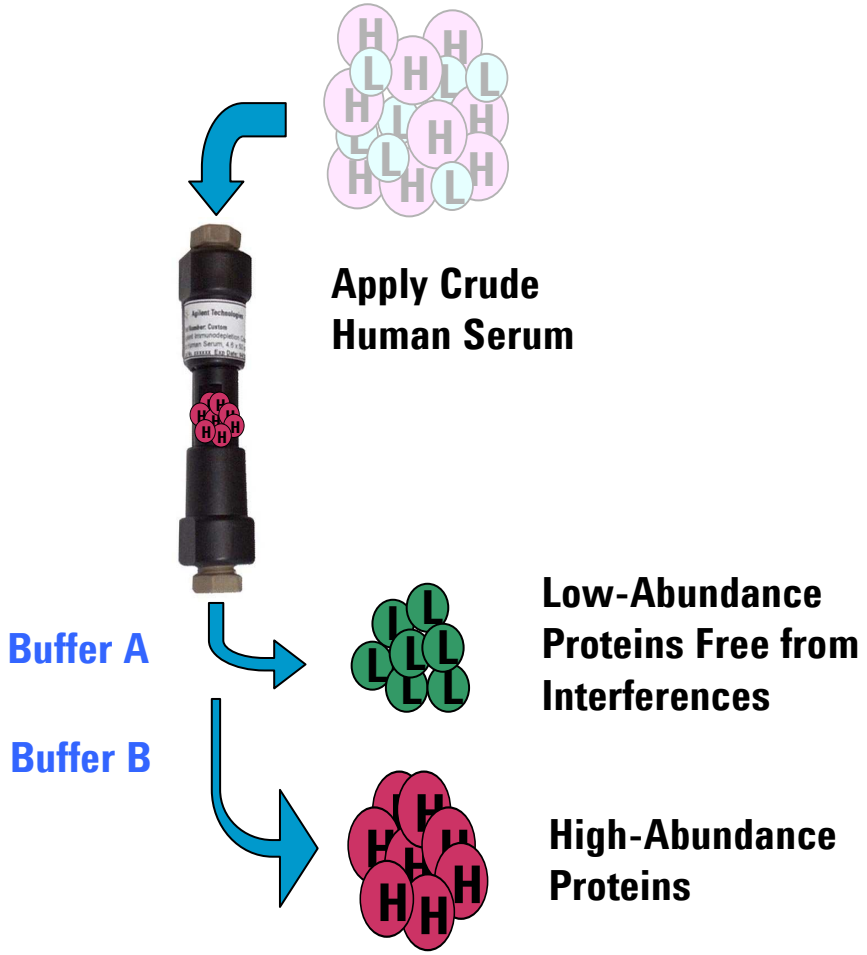
Sample Preparation For Biofluids
Blood, Plasma, Cerebral Spinal Fluid, Urine

Sample Preparation

A polyclonal antibody based system to rapidly deplete multiple high abundant proteins in serum/plasma/CSF.

- Launched in August 2003
- Individual Ab materials are mixed in selected percentages and packed into a column format
- Agilent continues to innovate and lead this market





Agilent Multiple Affinity Removal System: Where Are We? What is Next?

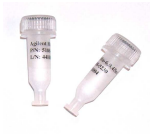
Sample Preparation

Capacity

Depth

2004

"Original" Top-6 Human Serum

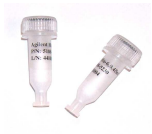


Spin Tube format

Mouse-3

2005

"High Capacity" Top-6 Human Serum



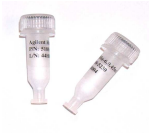
2006

"High Capacity" Top-7 Human Plasma

Spin Tube format

2007

"High Capacity" Level-II human



Multiple Affinity Removal System 7

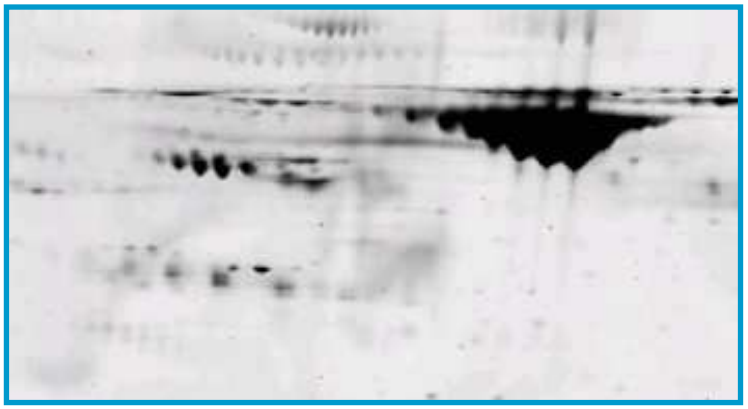
New depth with speed

Sample Preparation

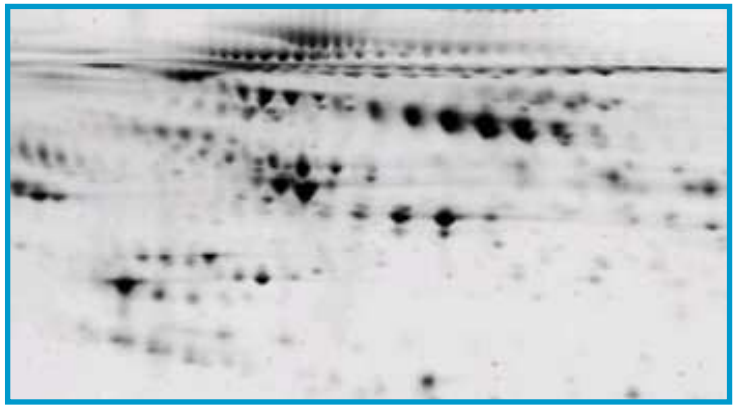
Orderable Now

Fully removes seven most abundant proteins in human plasma to unmask important otherwise undetectable proteins

- Highest reproducibility
- Lowest cost µL/sample
- Rapid methodology (30 minutes)
- Reusable for more than 200 experiment runs

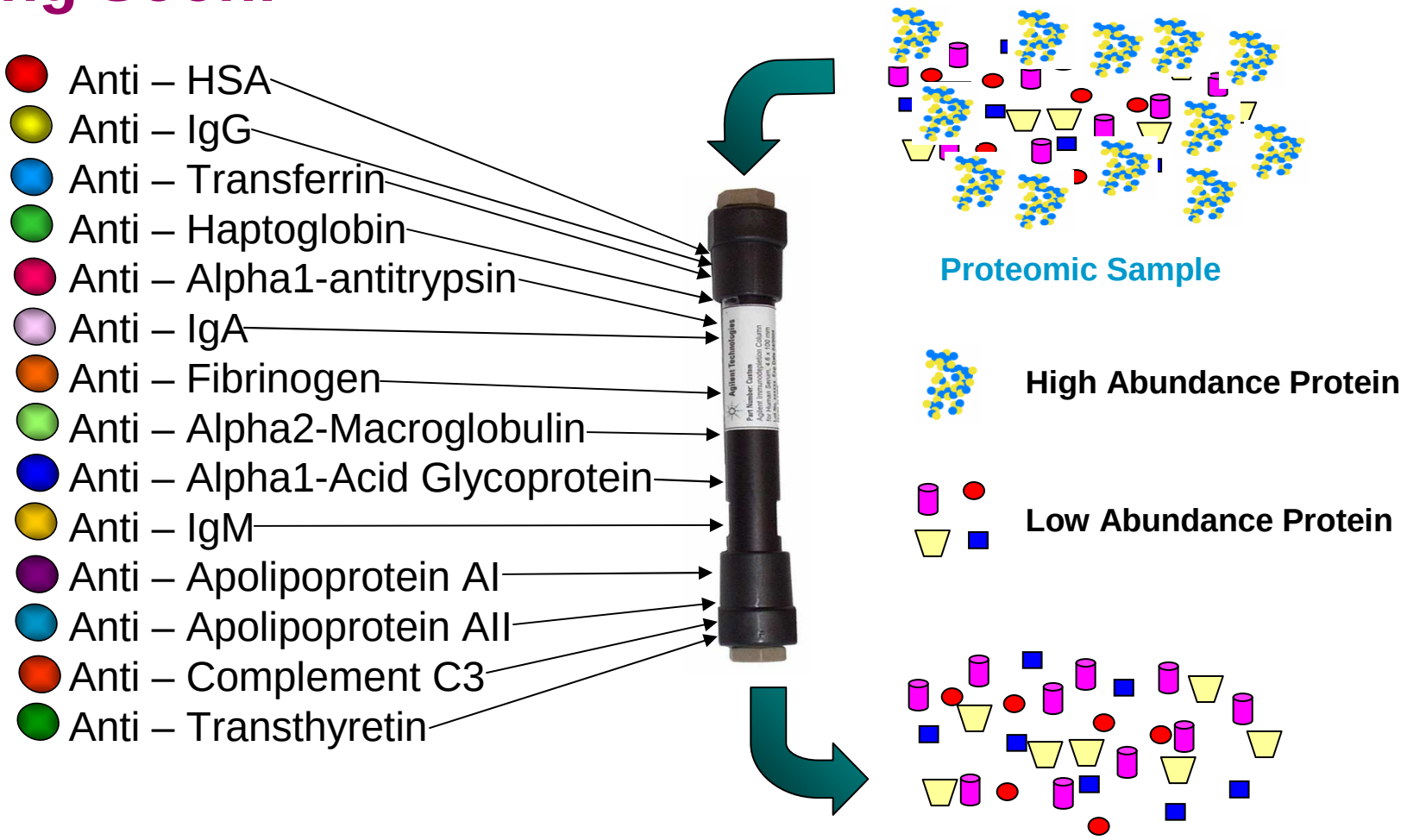


2DGE- Before



2DGE- After

Multiple Affinity Removal System 14
Coming Soon!



Removes 92-94% of total protein

pI-based Fractionation: OFFGEL Principle

- after rehydration the IPG gel seals tightly against the well frame
- the diluted sample is distributed across all wells in the strip
- after fractionation, the liquid fractions are recovered with a pipette

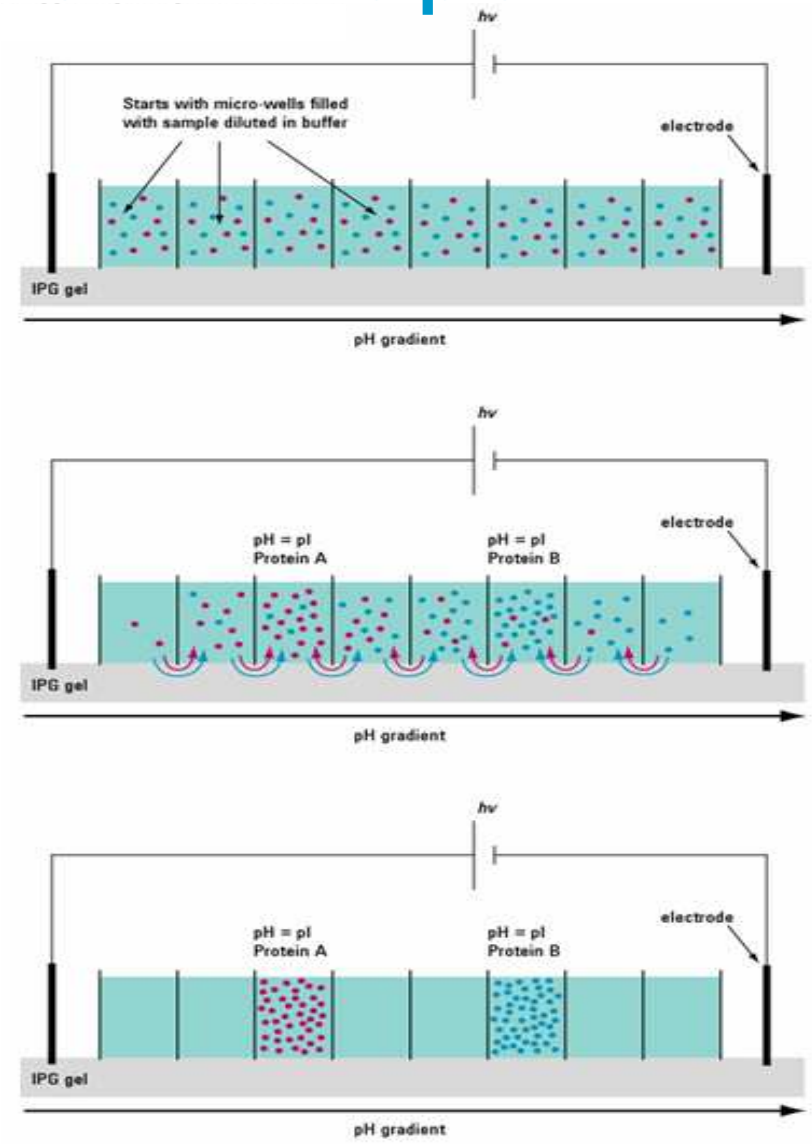
Number of fractions: 12 or 24

Fraction volume: 150 µL

Resolution: 0.1/0.6 pH

Loading capacity: 50 µg – 5 mg per sample

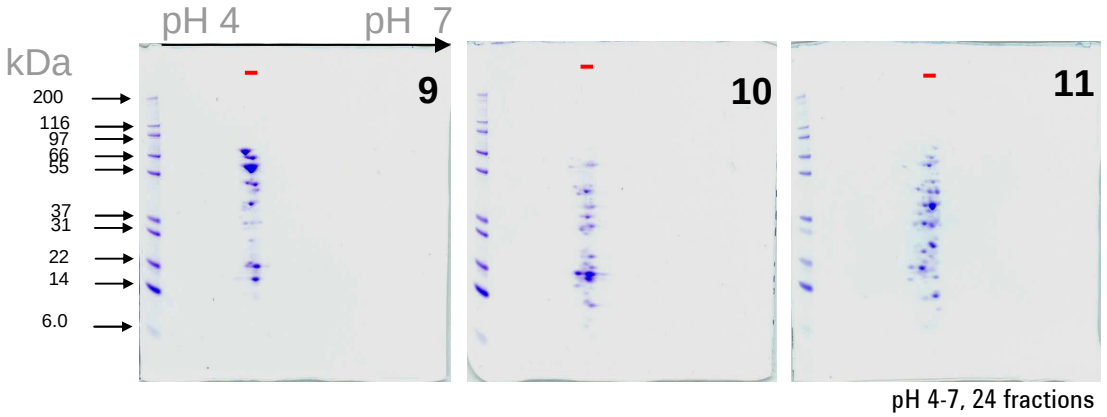
Fractionation time: 8 - 24 h



OFFGEL Protein Fractionation

High *pI* resolution

- theoretical resolution 0.1 pH

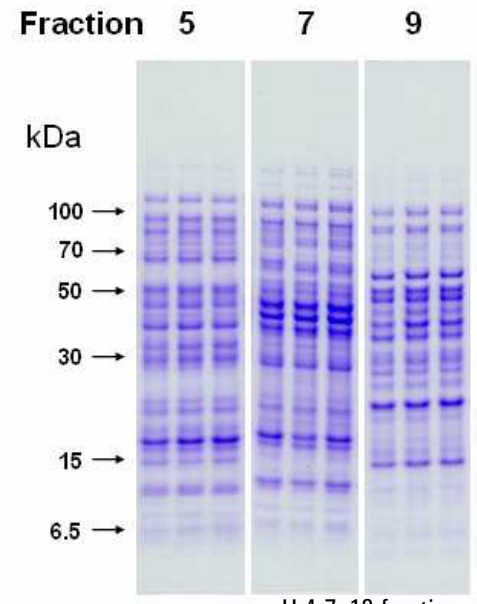


pH 4-7, 24 fractions

E.coli lysate

2D-Gel Electrophoresis

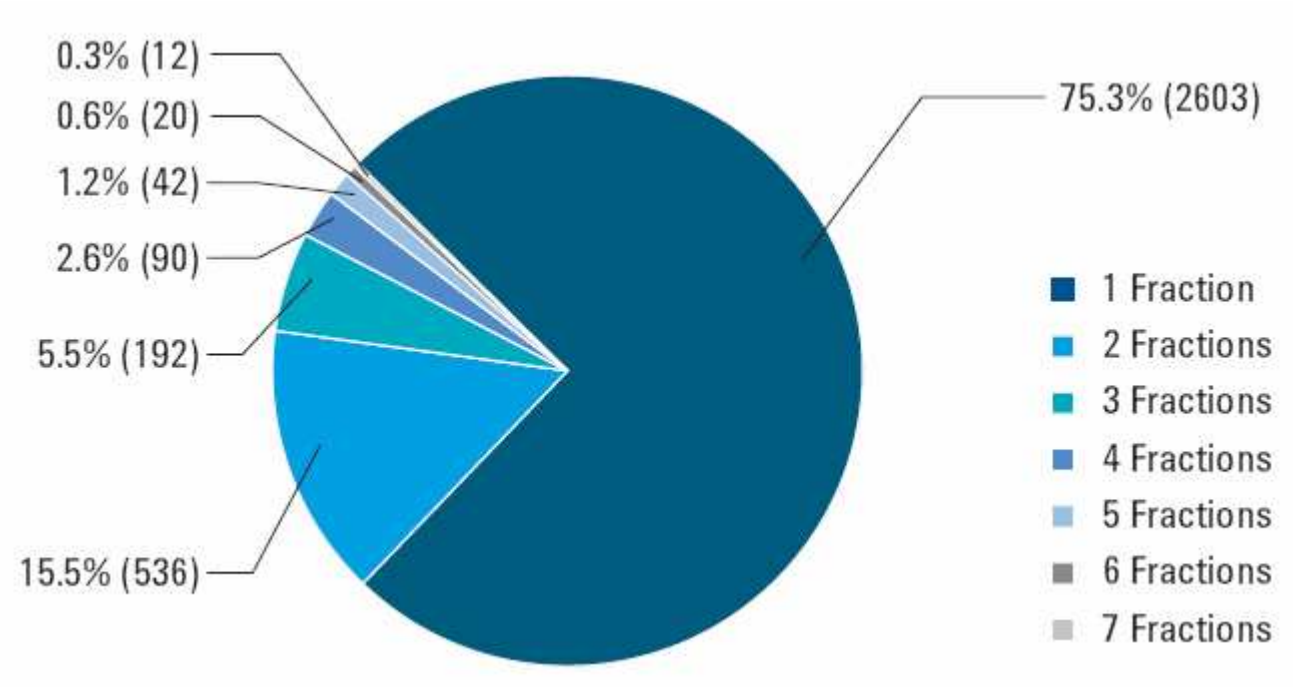
1D-Gel Electrophoresis



pH 4-7, 12 fractions

OFFGEL Peptide Fractionation

E.Coli Tryptic Digest



=> 90% of peptides are found in 1 or 2 fractions!

Number of OFFGEL fractions containing each individual peptide (absolute numbers of peptides in parenthesis)

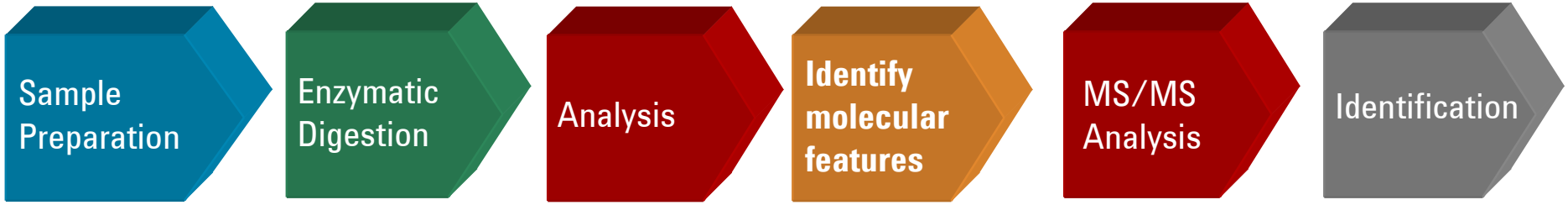
Mass Profiling- Find The Differences Between Samples

Molecular Feature: a discrete molecular entity defined by the combination of

- retention time, mass and response in an LC/MS analysis
- retention time, mass spectrum and response in a GC/MS analysis

- **Find real differences in sample sets using statistical analysis**
- **Reproducible measurements minimize the number of samples!**

Protein Biomarker Workflow: Protein Profiling Approach



Goal:
extract proteins from sample

Typical samples:
- Cells
- Conditioned media
- Tissue
- Body fluids

Techniques
- Laser capture microdissection
- Subcellular fractionation

Goal:
Clip proteins into smaller pieces (peptides) for subsequent analysis

Goal:
Obtain identifying information: accurate mass MS and retention time

Ionization:
- ESI

Mass analyzer:
- TOF
- QTOF
- FT MS

Goal:
Identify peptides that are up- or down-regulated

Software:
- "identify" features
- compare "features" between data sets

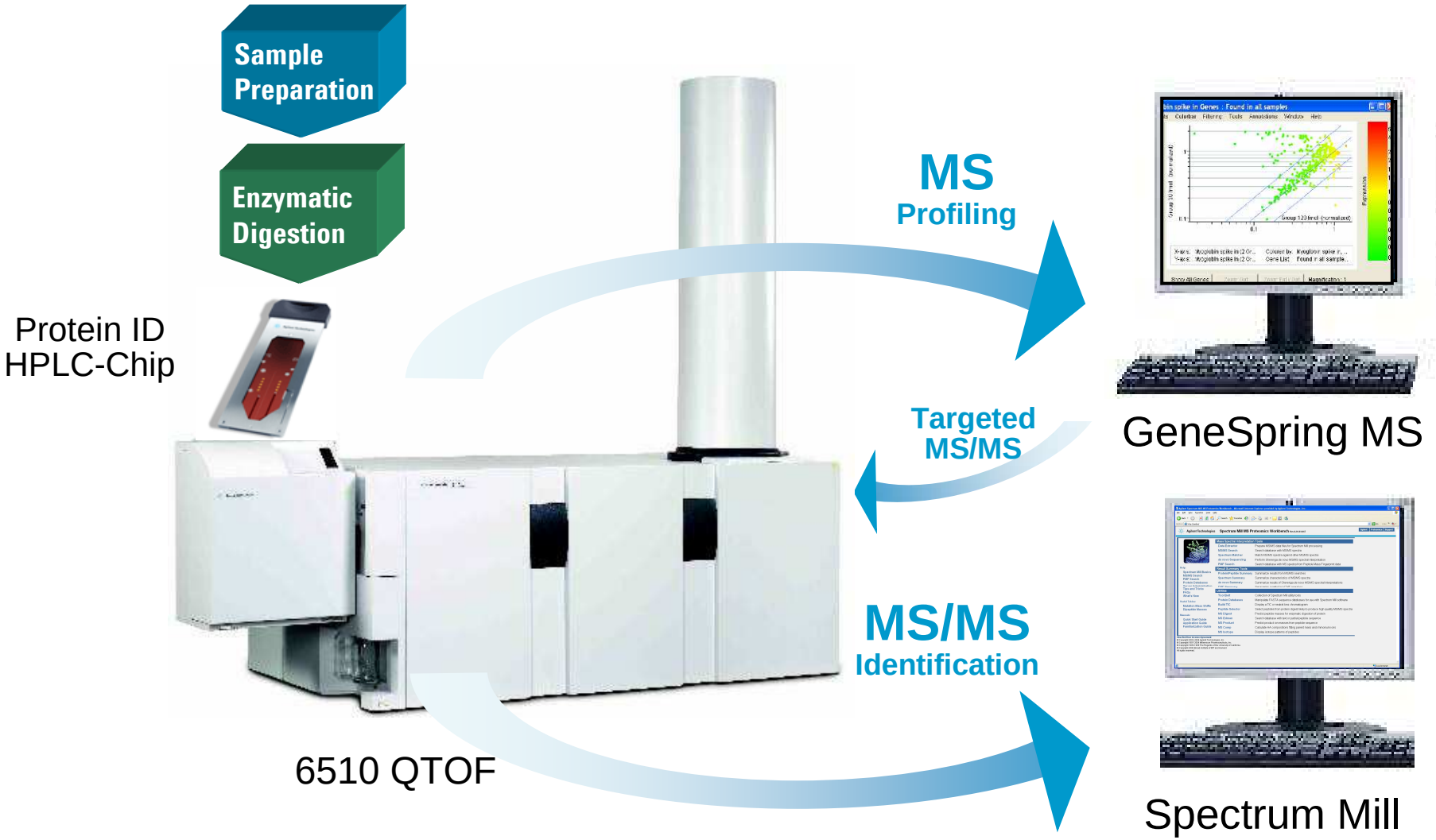
Goal:
Obtain **targeted** MS/MS on **features of interest only**

Ionization:
- ESI

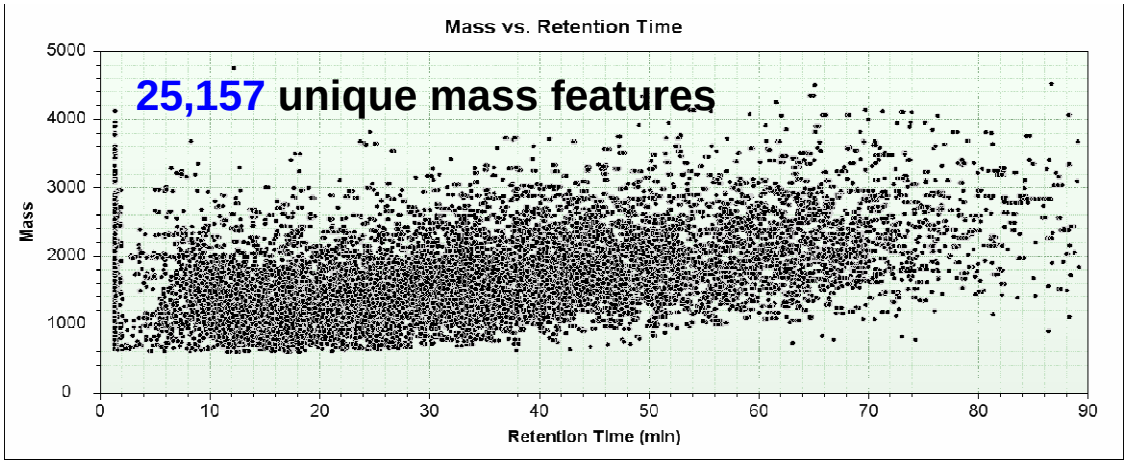
Mass analyzer:
- Ion trap
- QTOF
- TOF/TOF

Goal:
Identify peptides and thus proteins via database searching

MS Profiling And MS/MS Identification With The Agilent 6510 Q-TOF And HPLC-Chip



MassHunter Profiling Software

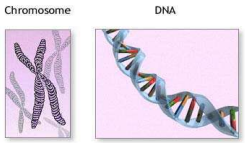


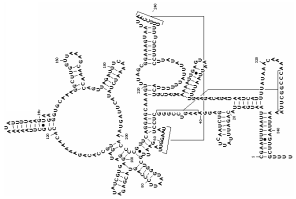
Mass Profiler

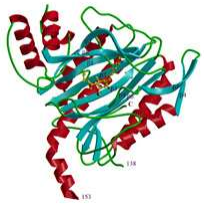
Importance of Informatics In Integrating Systems Biology

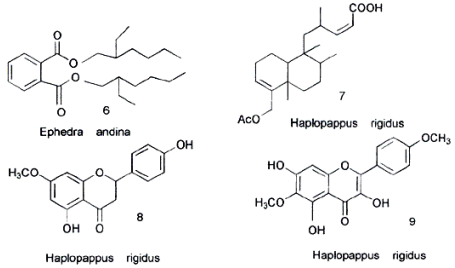
Genetics ➡ **Transcriptomics** ➡ **Proteomics** ➡ **Metabolomics**

Chrom. DNA/Genes | **mRNA** | **Proteins** | **Metabolites**





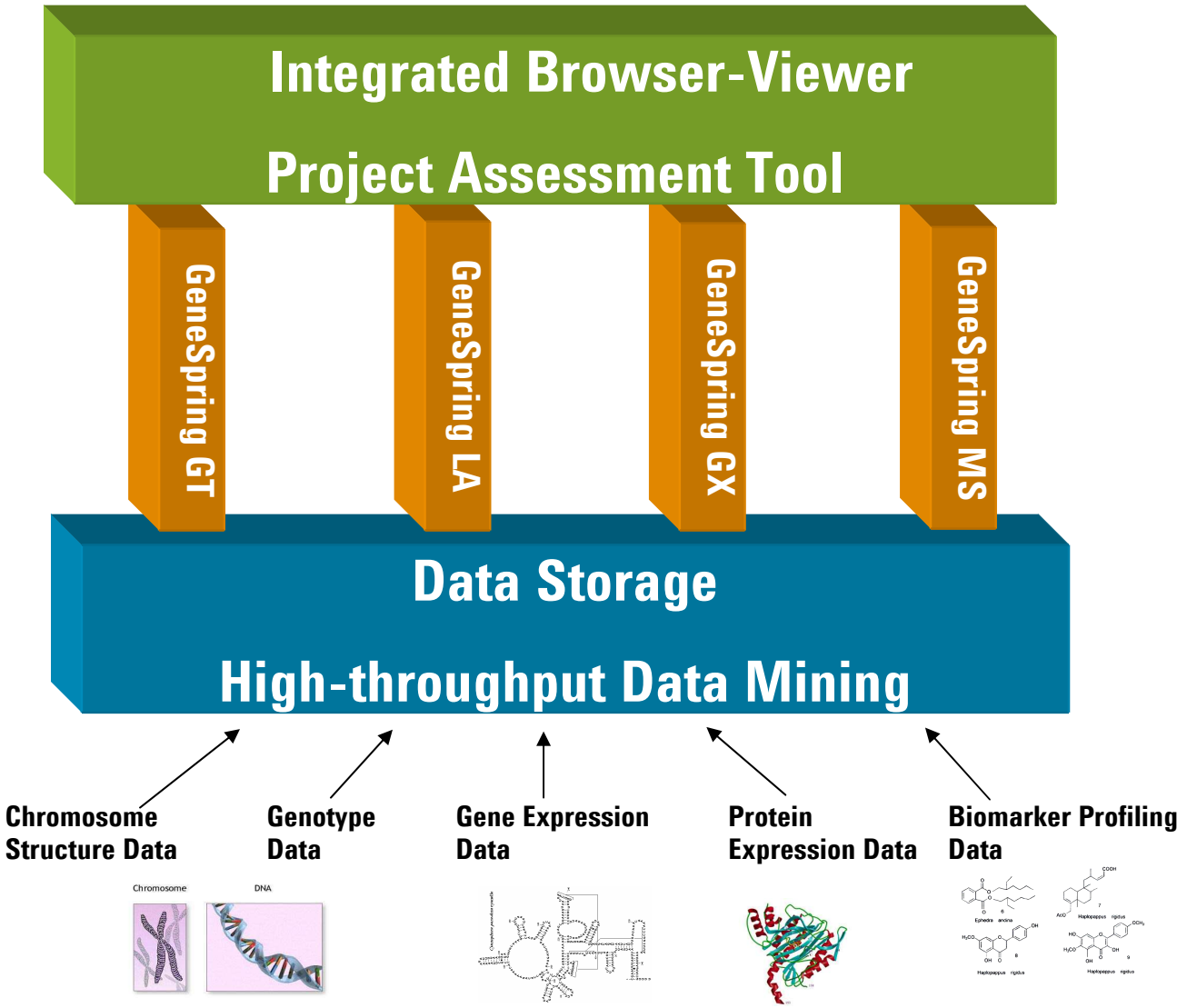




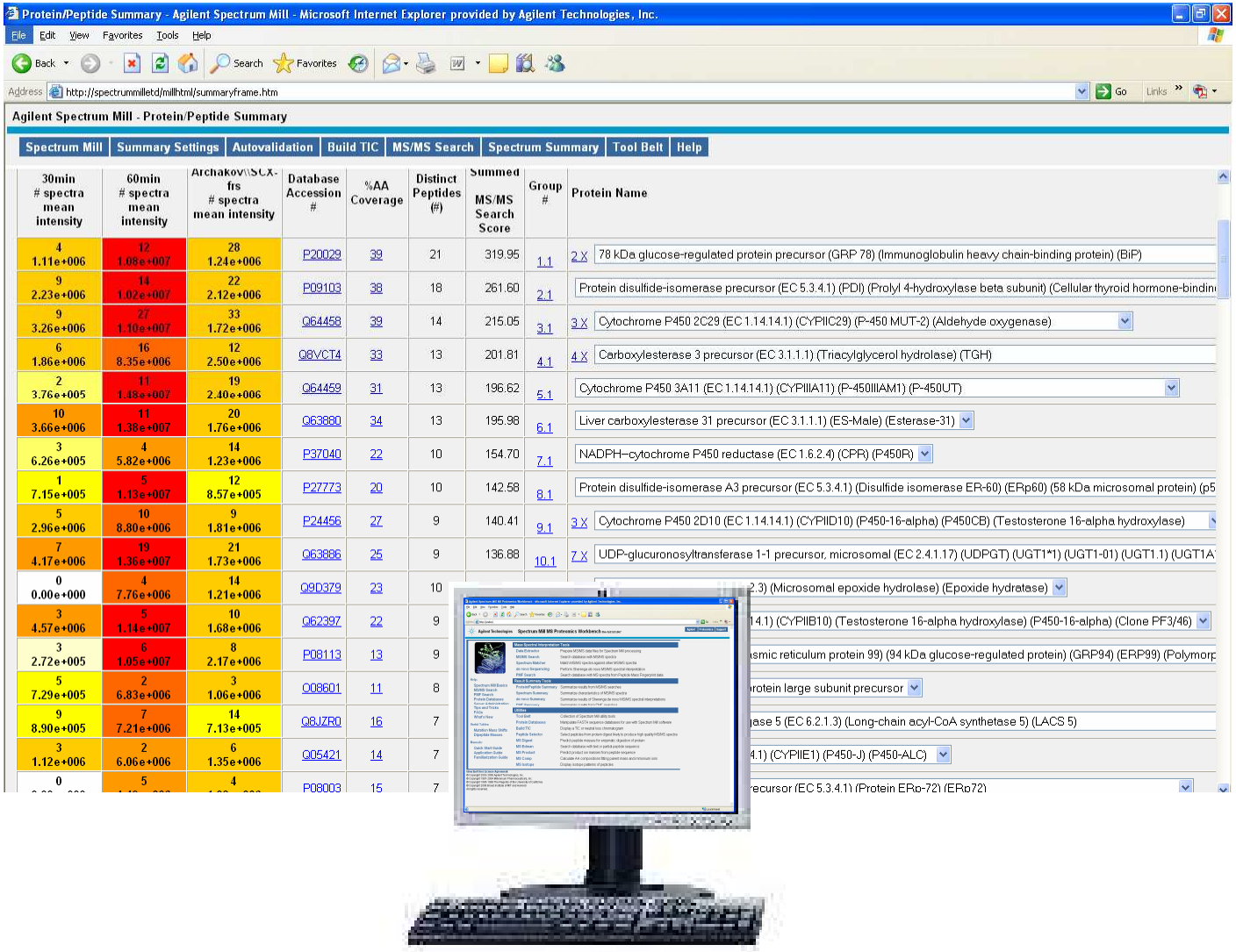
Informatics

Knowledge

GeneSpring Platform



Candidate Identification From Targeted MS/MS



Spectrum Mill

Protein Biomarker Studies: Simple Model Mixture

Three mixtures prepared which contained:

- 100 fmol/µL BSA digest and
- either 25, 50 or 100 fmol/µL serotransferrin digest

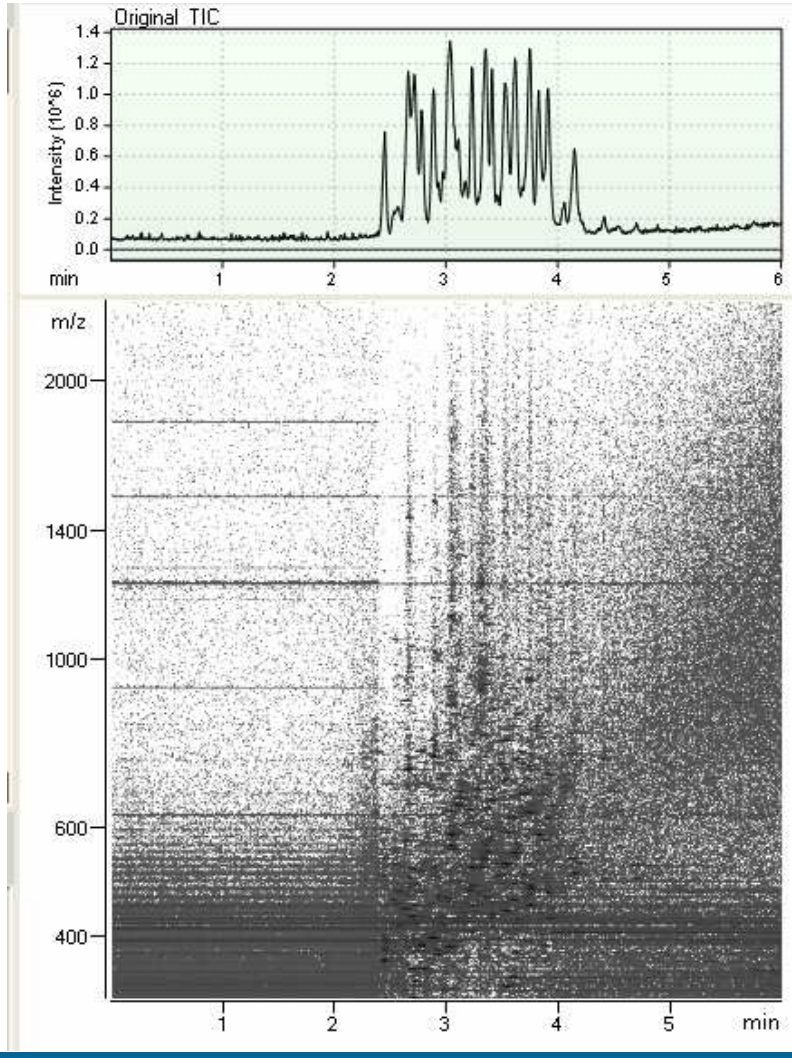
Five replicate injections (0.2 µL injection volume)

Rapid (9 min injection-to-injection) method used

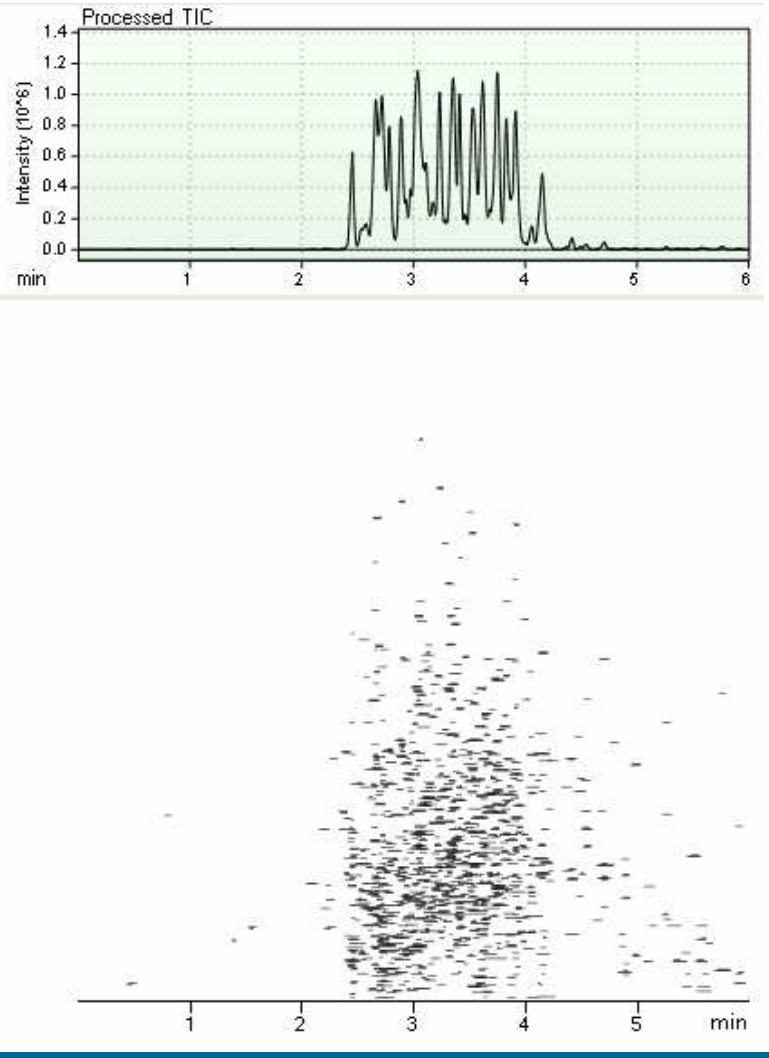
Optimization of acquisition rate also investigated

Simple Model Study: Molecular Feature Extraction

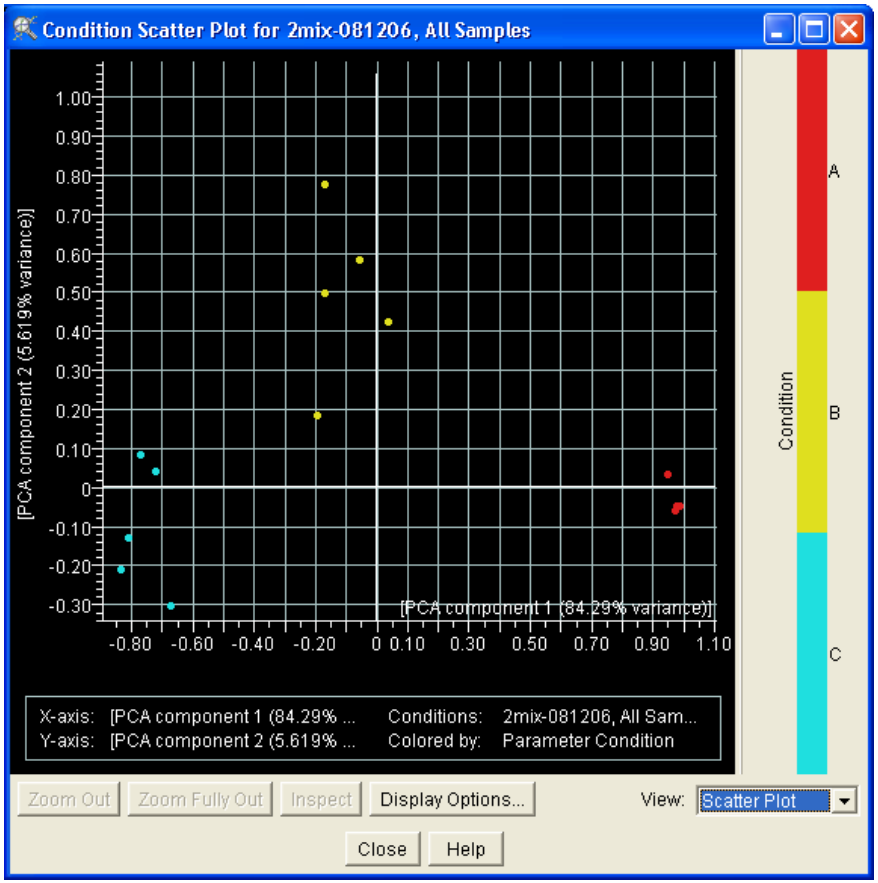
Raw Data



Extracted Features

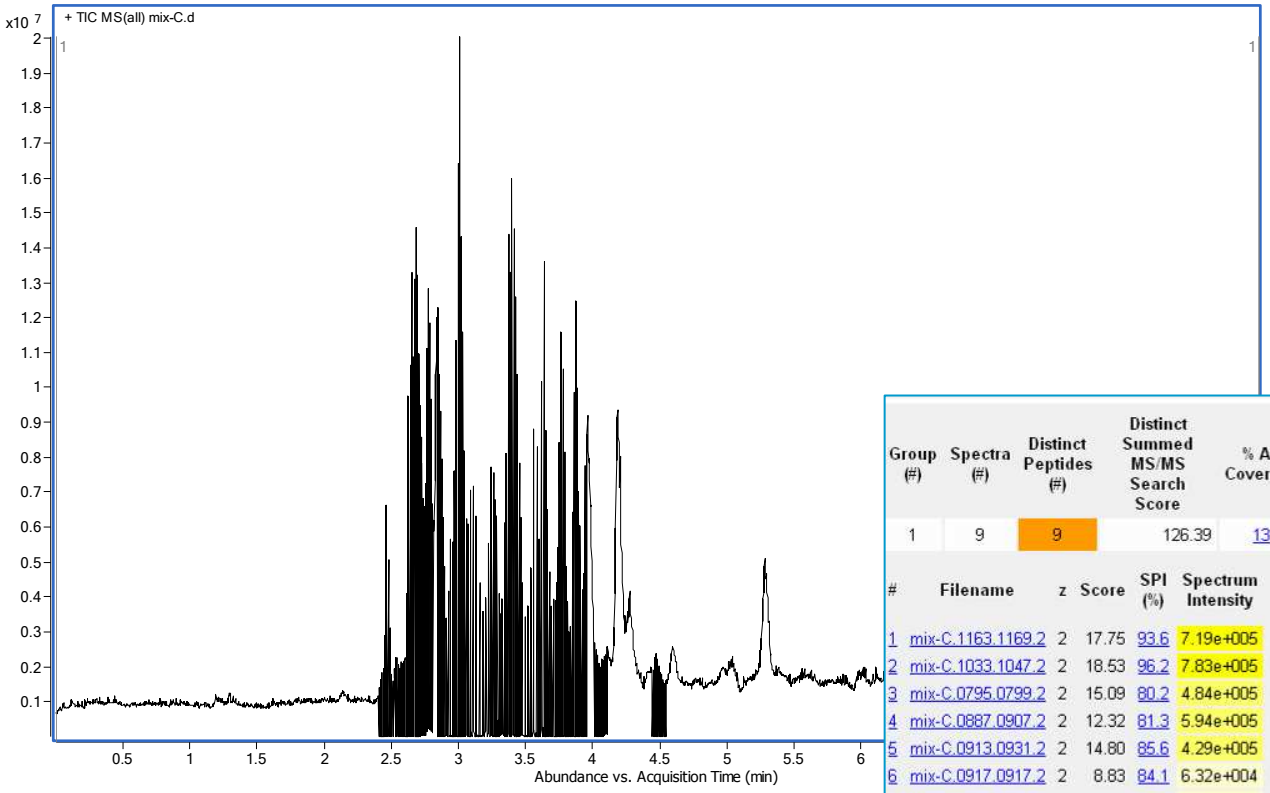


Simple Model Study: Finding The Differential Features in GeneSpring MS



- Filter data by relative frequency in replicates, fold change etc.
- Perform ANOVA to identify features that account for differences between sample groups
- PCA to visualize results shows clear differentiation between 3 sample groups
- Export list of m/z and retention time for targeted MS/MS

Simple Model Study: Protein Identification From Targeted MS/MS



Group (#)	Spectra (#)	Distinct Peptides (#)	Distinct Summed MS/MS Search Score	% AA Coverage	Mean Peptide Spectral Intensity	Database Accession #	Protein Name
1	9	9	126.39	13	5.96e+005	Q29443	Serotransferrin precursor (Transferrin)

#	Filename	z	Score	SPI (%)	Spectrum Intensity	Sequence	RT (min)	m/z Measured (Da)	MH+ Matched (Da)
1	mix-C.1163.1169.2	2	17.75	93.6	7.19e+005	(K) CGLVPVLAENYK (S)	4.08	681.8701	1362.709
2	mix-C.1033.1047.2	2	18.53	96.2	7.83e+005	(K) GYLAVAVVK (K)	3.60	460.2938	919.561
3	mix-C.0795.0799.2	2	15.09	80.2	4.84e+005	(K) CLASIAK (K)	2.72	381.7190	762.418
4	mix-C.0887.0907.2	2	12.32	81.3	5.94e+005	(K) DSADGFLK (I)	3.06	426.7164	852.410
5	mix-C.0913.0931.2	2	14.80	85.6	4.29e+005	(K) ELPDPQESIQR (A)	3.16	656.3430	1311.654
6	mix-C.0917.0917.2	2	8.83	84.1	6.32e+004	(K) DQTVIQNTDGNNNEAWAK (N)	3.17	1009.4843	2017.921
7	mix-C.0956.0962.2	2	12.94	85.4	2.83e+005	(K) NYELLCGDNTR (K)	3.31	677.8183	1354.606
8	mix-C.0965.0973.2	2	9.89	68.0	1.63e+006	(K) ENFEVLCK (D)	3.35	519.7610	1038.493
9	mix-C.1021.1028.2	2	16.24	97.7	3.78e+005	(K) TSDANINWNNLK (D)	3.55	695.3555	1389.676

Protein Biomarker Studies: Complex Model Mixture

Five mixtures prepared which contained:

- Tryptic digest of an *E. coli* lysate (complex background)
- Tryptic digests of bovine serotransferrin and BSA at specified levels

Each mixture was prepared 10 times

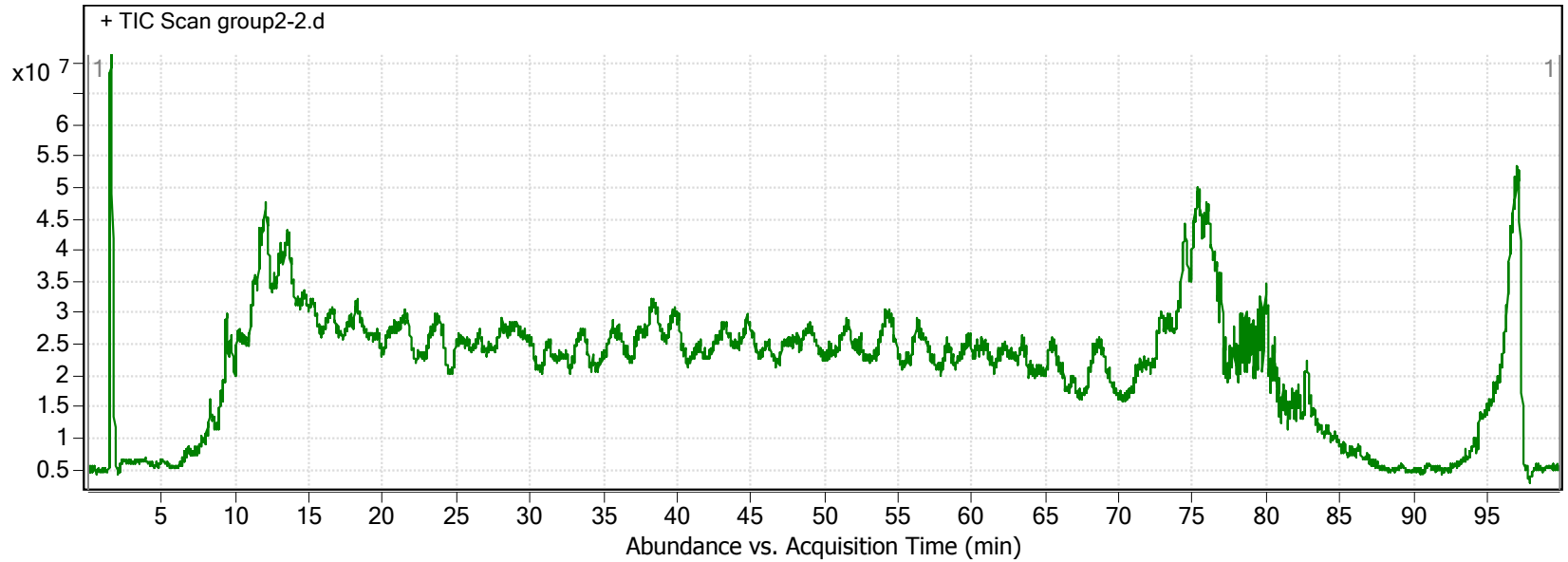
One injection of each sample (1 µL injection volume)

Long (100 min injection-to-injection) method used

Complex Model Study: Spiked *E. coli* Lysate

Sample	*E. coli* lysate (ng total protein)	BSA (fmol)	Serotransferrin (fmol)
1	400	25	200
2	400	50	100
3	400	100	50
4	400	200	25
5	400	400	5

Complex Model Study: Spiked *E. coli* Lysate Total Ion Chromatogram

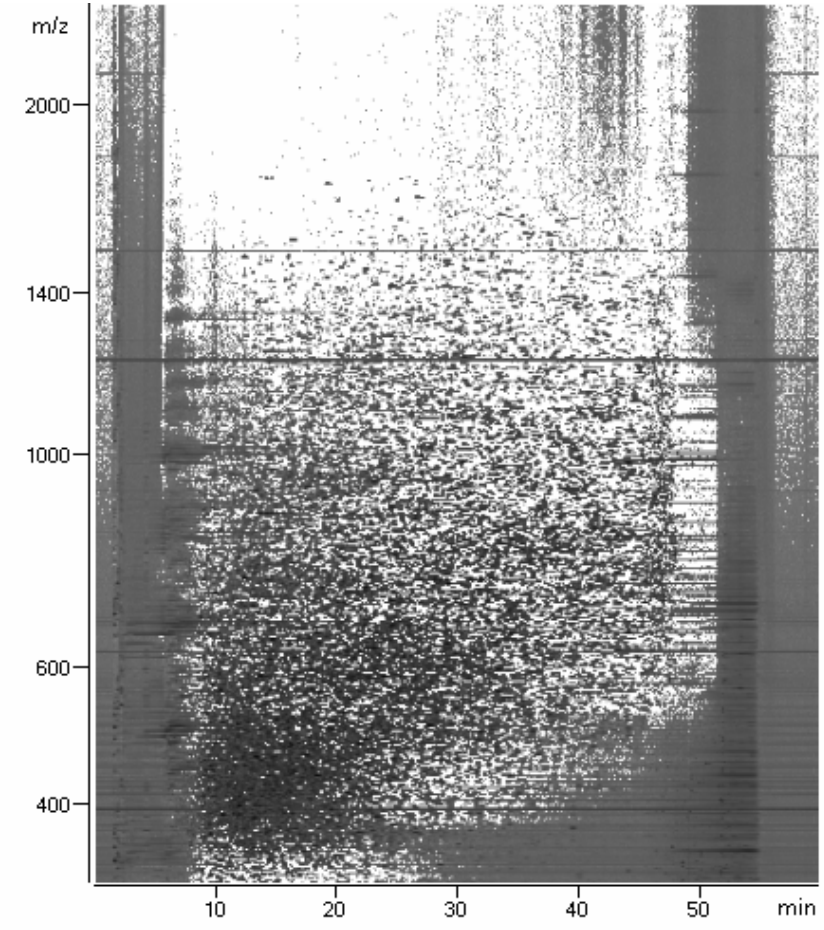


Digest of *E. coli* lysate creates a complex background of thousands of peptides

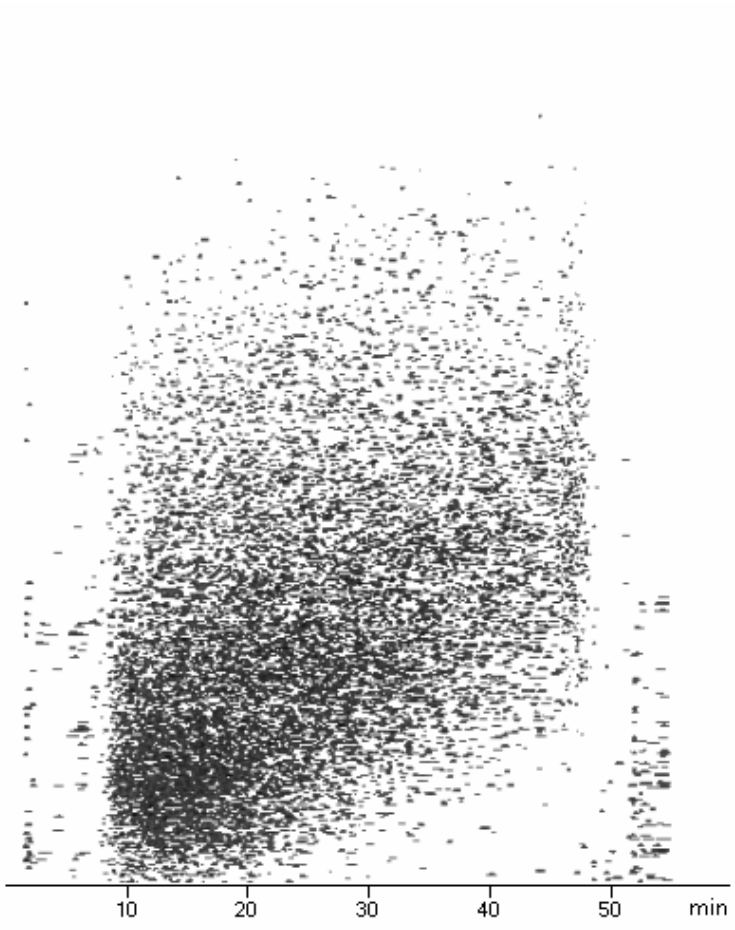
Profiling software must be able to find the bovine peptides that were spiked into the *E. coli* mixture

Complex Model Study: Molecular Feature Extraction

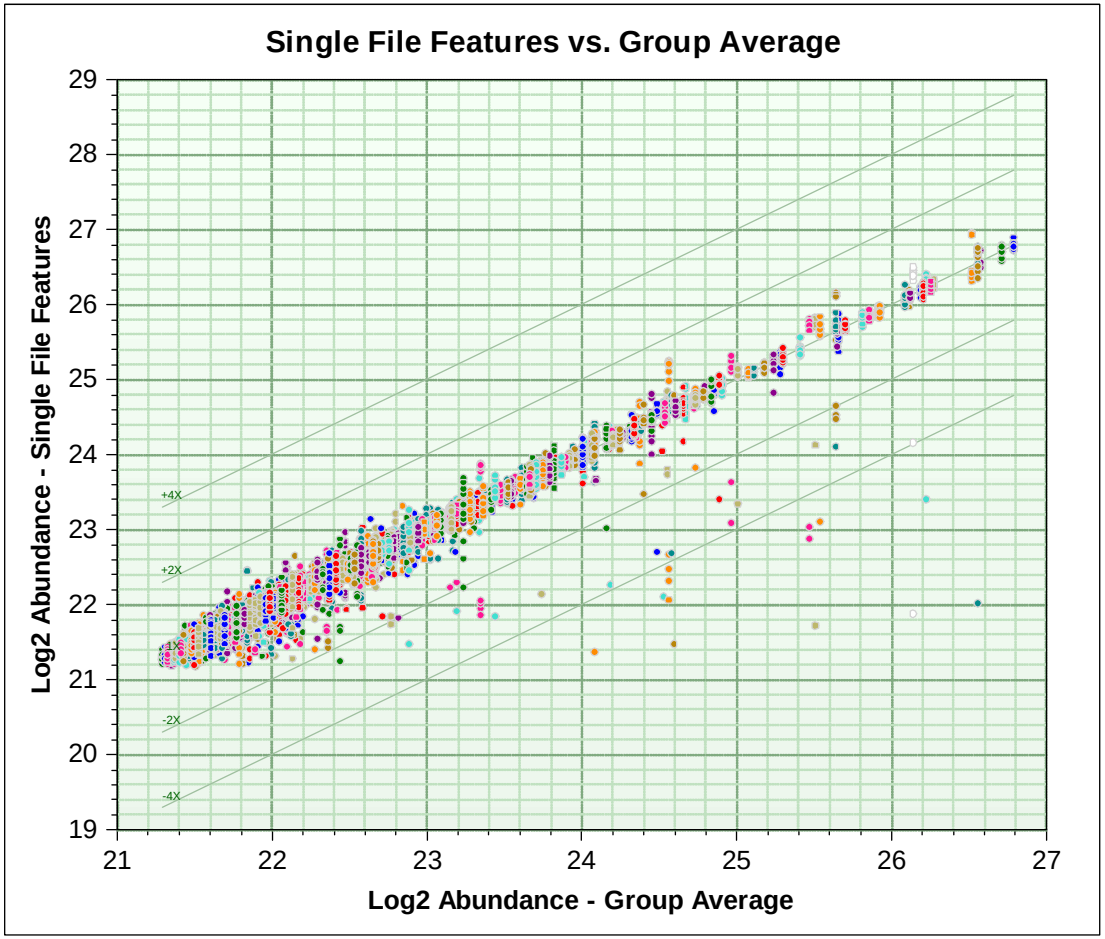
Raw Data



Extracted Features



Complex Model Study: Reproducibility With Technical Replicates



Color-coding by molecular feature

Total of 762 features shown

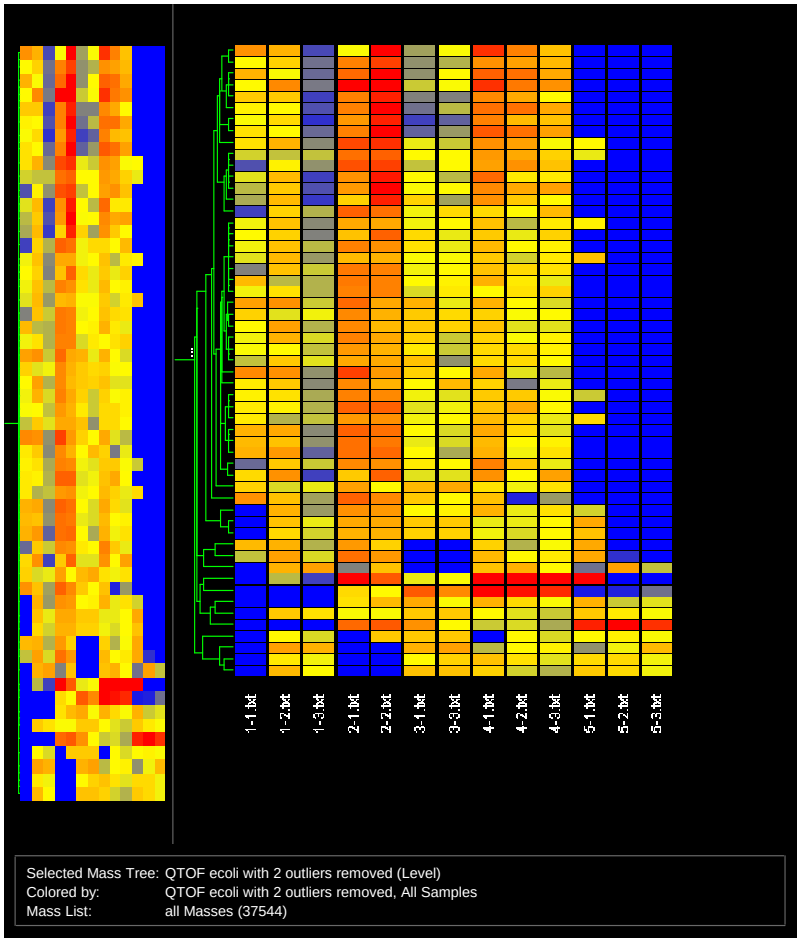
Average SD is

0.0519 min for RT

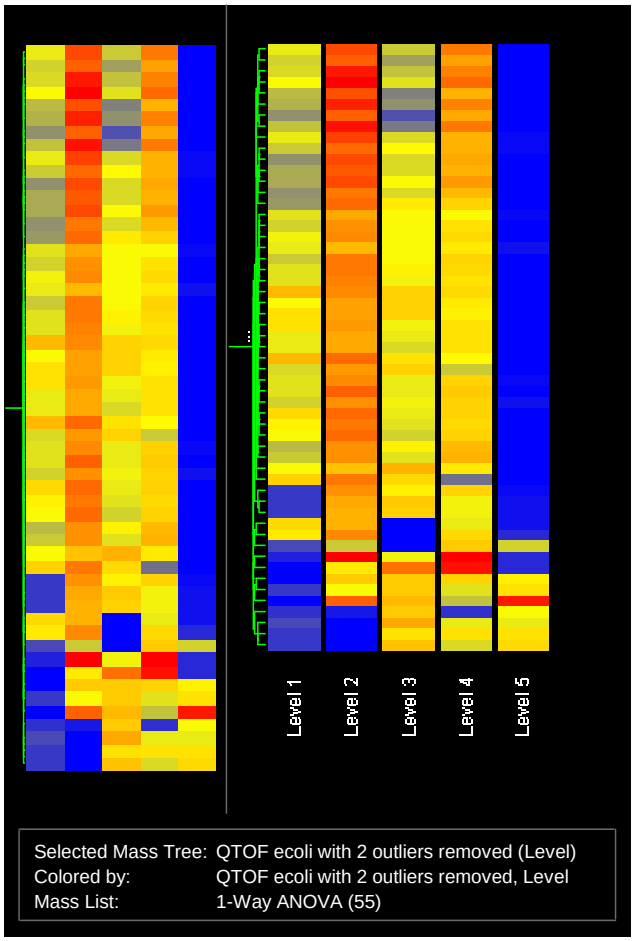
1.6 mDa for mass

Complex Model Study: Clustering of Features That Are Significantly Different (ANOVA)

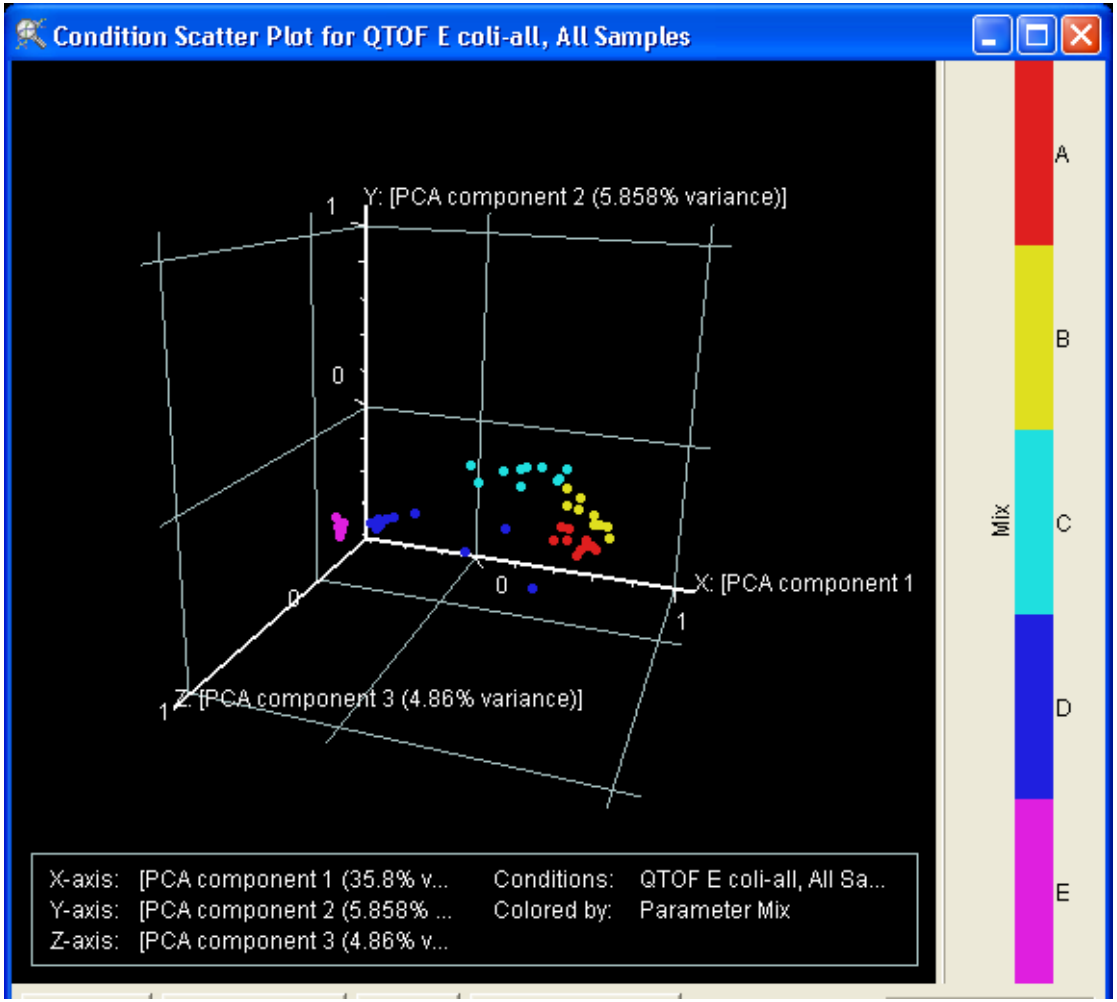
Cluster by sample



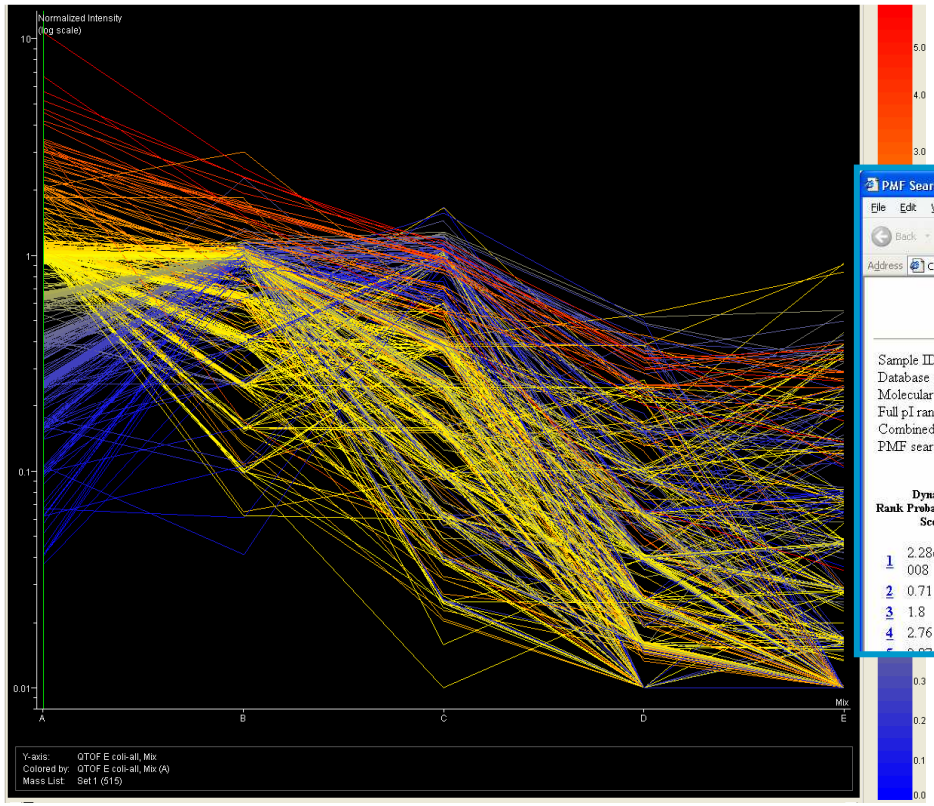
Cluster by level



Complex Model Study: Finding The Differential Features in GeneSpring MS



Complex Model Study: K-Means Clustering of Differential Features And PMF Search



PMF Search Results - Agilent Spectrum Mill - Microsoft Internet Explorer

File Edit View Favorites Tools Help

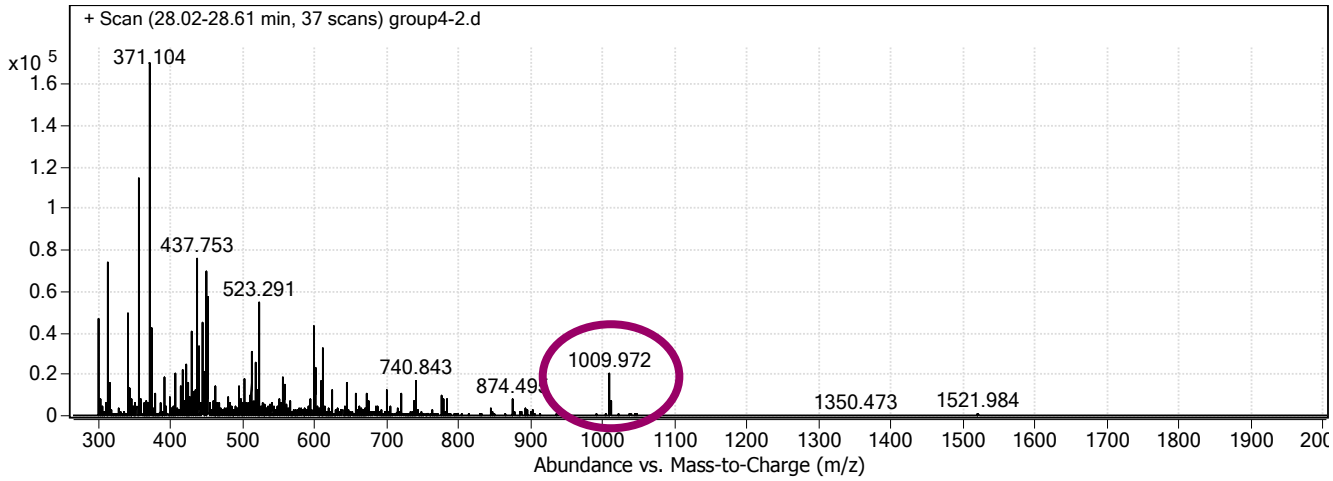
Address C:\Temp_wb141656049511564744548 61\SMResult1156474907526.html

PMF Search Results

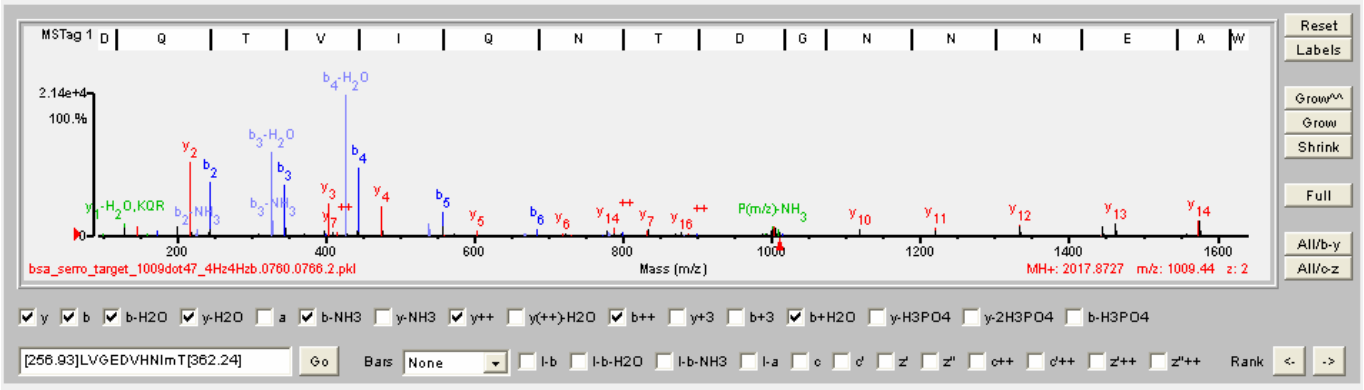
Sample ID (comment): **Enter_Comment**
Database searched: **SwissProt.mammals** Parameters used in Search
Molecular weight search (**1000 - 100000 Da**) selects **31701** entries.
Full pI range: **35824** entries.
Combined molecular weight and pI searches select **31701** entries.
PMF search selects **25** entries.

Rank	Dynamic Probability Score	Static Probability Score	# (%) Masses Matched	Mass Error Mean (Std Dev) (ppm)	Protein Coverage	Protein MW (Da)/pI	Species	Accession #	Protein Name
1	2.28e-008	3.16e-007	32/515 (6%)	3.6 (6.6)	49%	77753.7/6.75	BOVIN	Q29443	Serotransferrin precursor (Transferrin) (Siderophilin) (Beta-1-metal-binding globulin)
2	0.71	0.71	27/515 (5%)	-2.5 (10.9)	40%	75755.5/5.34	MOUSE	P14824	Annexin A6 (Annexin VI) (Lipocortin VI) (P68) (P70) (Protein III) (Chromobindin-20) (67 kDa calel
3	1.8	1.8	13/515 (2%)	-2.8 (11.4)	30%	46512.7/6.54	MOUSE	Q91WL8	WW domain-containing oxidoreductase (EC 1.1.1.-)
4	2.76	2.76	14/515 (2%)	-2.8 (10.9)	28%	41450.6/10.65	HUMAN	O75683	Surfeit locus protein 6

Complex Model Study: Mass Spectra From a Targeted Peptide



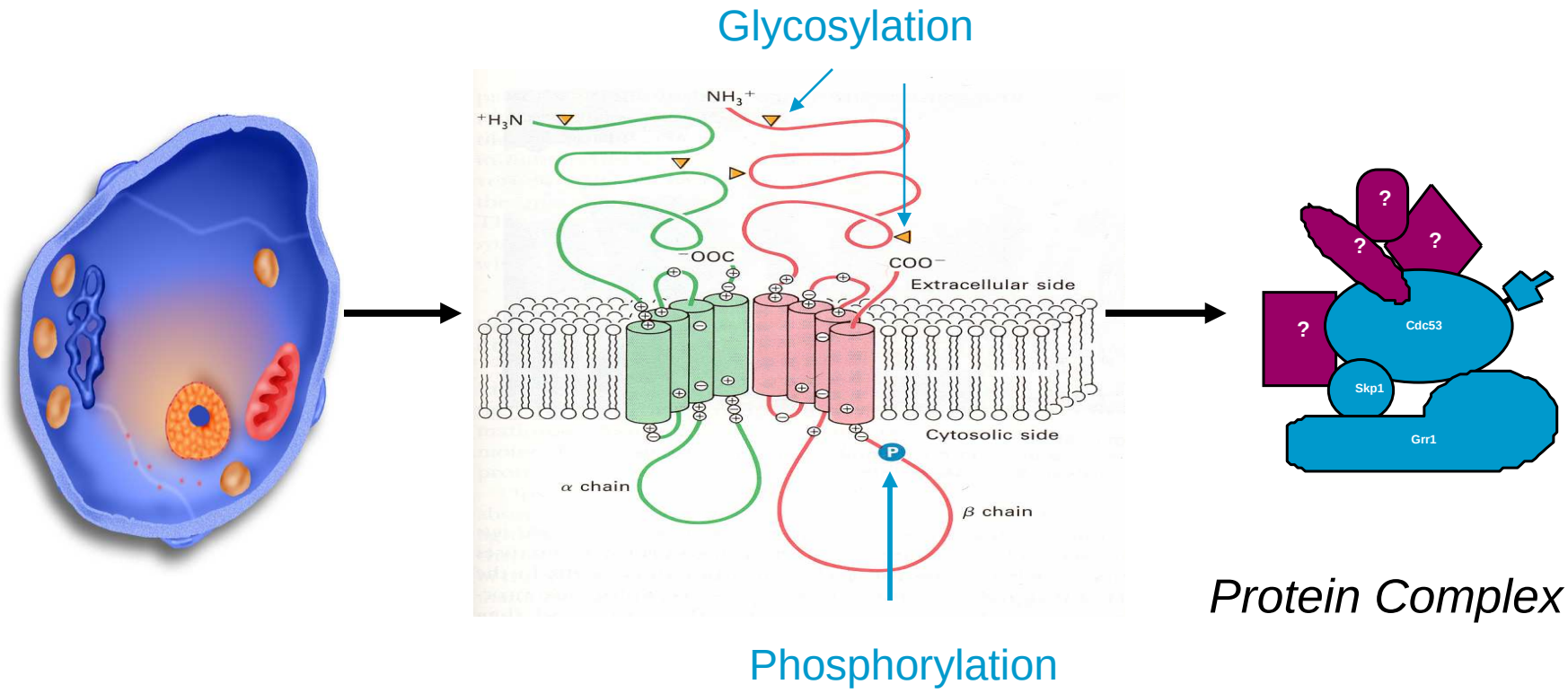
Apex mass spectrum for a targeted species



Fragment ion assignments for targeted peptide (from transferrin)

Post-translational Modifications (PTMs)

•Are chemical structures added to proteins AFTER they are translated
•*Control* or *regulate* the protein's structure or activity, such as ability to form a complex or interact with other proteins



Most Common Post-translational Modifications

Table A.1C.4 Mass Changes Due to Some Post-Translational Modifications of Peptides and Proteins[a]

Modification[b]	Monoisotopic mass change	Average mass change
Common modifications		
Pyroglutamic acid formation from Gln	−17.0265	−17.0306
Disulfide bond (cystine) formation	−2.0157	−2.0159
C-terminal amide formation from Gly	−0.9840	−0.9847
Deamidation of Asn and Gln	−0.9840	−0.9847
Methylation	14.0157	14.0269
Hydroxylation	15.9949	15.9994
Oxidation of Met	15.9949	15.9994
Proteolysis of a single peptide bond	18.0106	18.0153
Formylation	27.9949	28.0104
Acetylation	42.0106	42.0373
Carboxylation of Asp and Glu	43.9898	44.0098
Phosphorylation	79.9663	79.9799
Sulfation	79.9568	80.0642
Cysteinylation	119.0041	119.1442
Glycosylation with pentoses (Ara, Rib, Xyl)	132.0423	132.1161
Glycosylation with deoxyhexoses (Fuc, Rha)	146.0579	146.1430
Glycosylation with hexosamines (GalN, GlcN)	161.0688	161.1577
Glycosylation with hexoses (Fru, Gal, Glc, Man)	162.0528	162.1424
Modification with lipoic acid (amide bond to lysine)	188.0330	188.3147
Glycosylation with *N*-acetylhexosamines (GalNAc, GlcNAc)	203.0794	203.1950
Farnesylation	204.1878	204.3556
Myristoylation	210.1984	210.3598

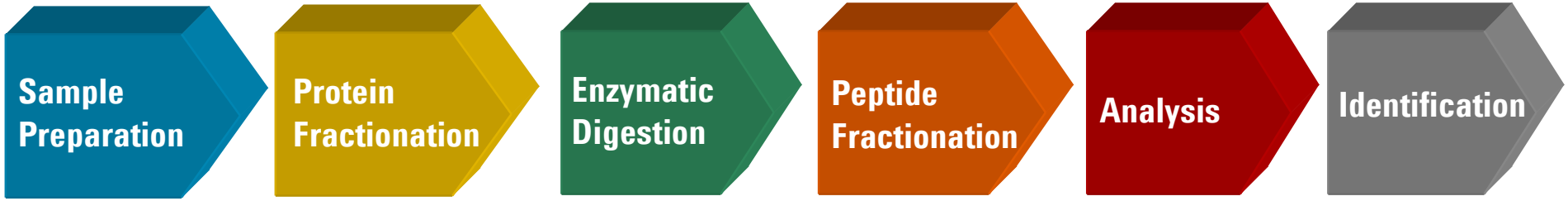
PTMs Are Dynamic In An Organism

The number of phospho groups on a phosphoprotein, or the number and type of glycans on a glycoprotein, change in response to changes in the environment

- Phosphate (sugar) concentration
- Enzyme concentration
- Cofactor concentration
- Nutrients present
- State (healthy or diseased, etc.)

In turn, the phospho- or glycoprotein interacts with other proteins (or enzymes, receptors…), triggering other actions

PTM Identification Workflow



Goal:
Extract phosphoproteins from sample

Typical samples:
- Cells
- Conditioned media
- Tissue
- Body fluids

Techniques:
- Laser capture microdissection
- Subcellular fractionation

Goal:
Reduce sample complexity

Techniques:
- **Immunodepletion**
- Affinity purification

- SDS-PAGE (1D)
- 2D gel
- **OFFGEL**

- **mRP**
- **RPLC**
- **Ion exchange**
- **Size exclusion**

Goal:
Clip proteins into smaller pieces (peptides) for subsequent analysis

Goal:
Reduce sample complexity

Techniques:
- **OFFGEL**
- **RPLC**
- **Ion exchange**
- IMAC trapping
- TiO2 enrichment
- Chemical methods

Goal:
Obtain MS/MS information

Ionization:
- LC/ESI
- LC/MALDI

Mass analyzer:
- **Ion trap**
- **Ion trap with ETD**
- **QTOF**
- TOF/TOF
- FT MS

Goal:
Identify peptides and thus proteins via database searching

Software:
- **SpectrumMill**
- Mascot

Global Proteomic Profiling of Phosphopeptides Using HPLC-Chip Electron Transfer Dissociation Ion Trap Mass Spectrometer

- Identified 1435 unique phosphorylation sites from 500 proteins
- ETD identified 60% more phosphopeptides than CID, with an average of 40% more fragment ions
- Discovered 15 novel phosphorylation motifs

PNAS

Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry

Henrik Molina*†, David M. Horn‡, Ning Tang‡, Suresh Mathivanan*§, and Akhilesh Pandey*¶

*McKusick-Nathans Institute for Genetic Medicine and Departments of Biological Chemistry, Pathology, and Oncology, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; †Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense 5230, Denmark; ‡Agilent Technologies, Santa Clara, CA 95052; and §Institute of Bioinformatics, International Tech Park, Bangalore 560 066, India

Communicated by Paul Talalay, Johns Hopkins University School of Medicine, Baltimore, MD, December 25, 2006 (received for review November 1, 2006)

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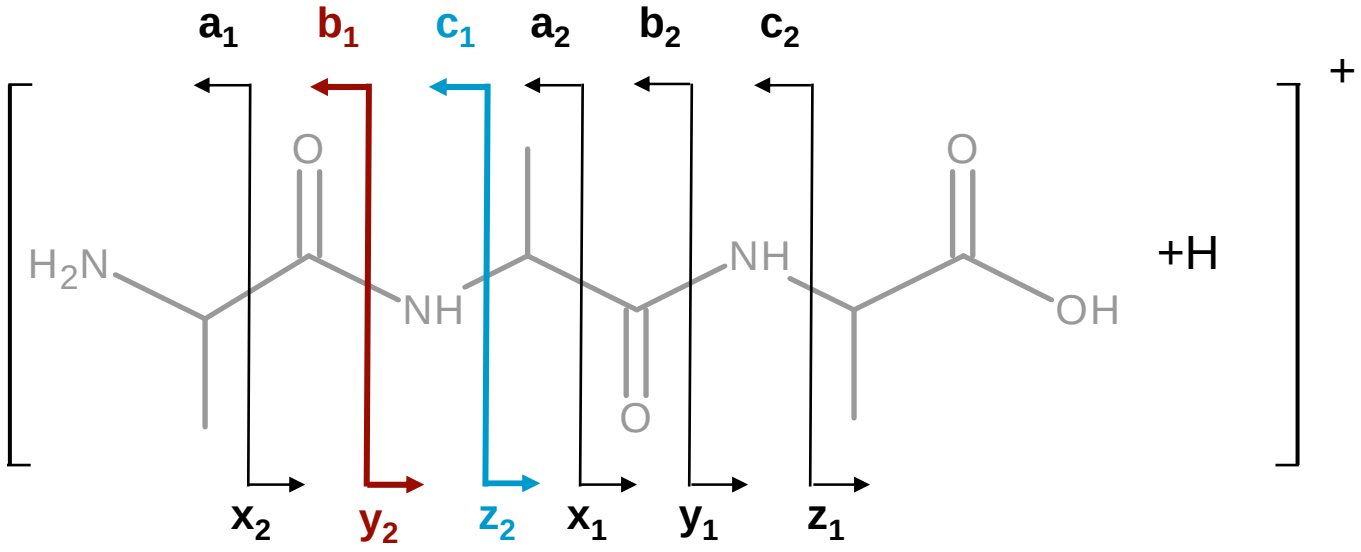
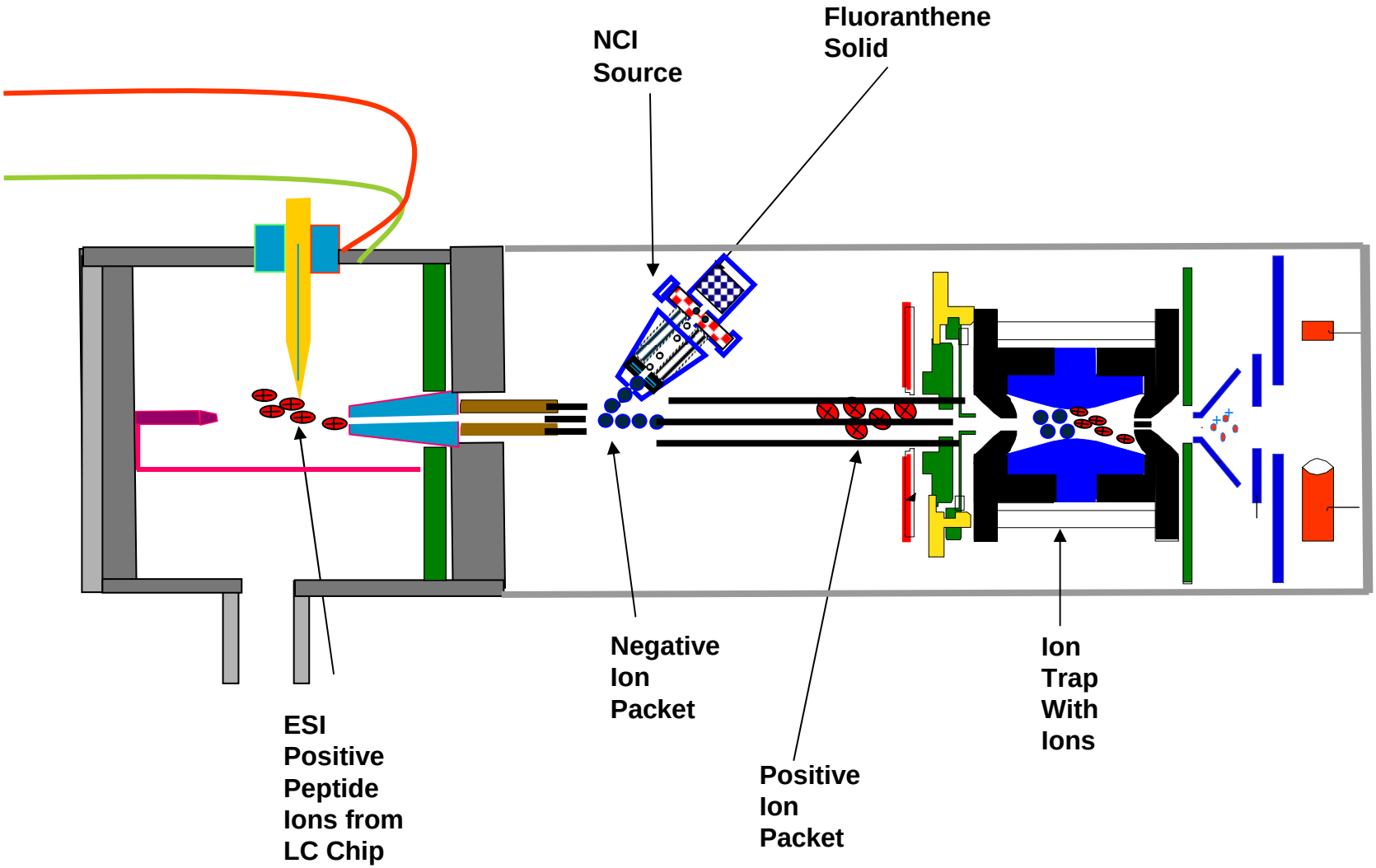
ETD Fragmentation

- Produces c and z fragment ions, while CID produces b and y fragment ions
- **Preserves post-translational modifications**
- ETD fragmentation is random and less sequence dependent



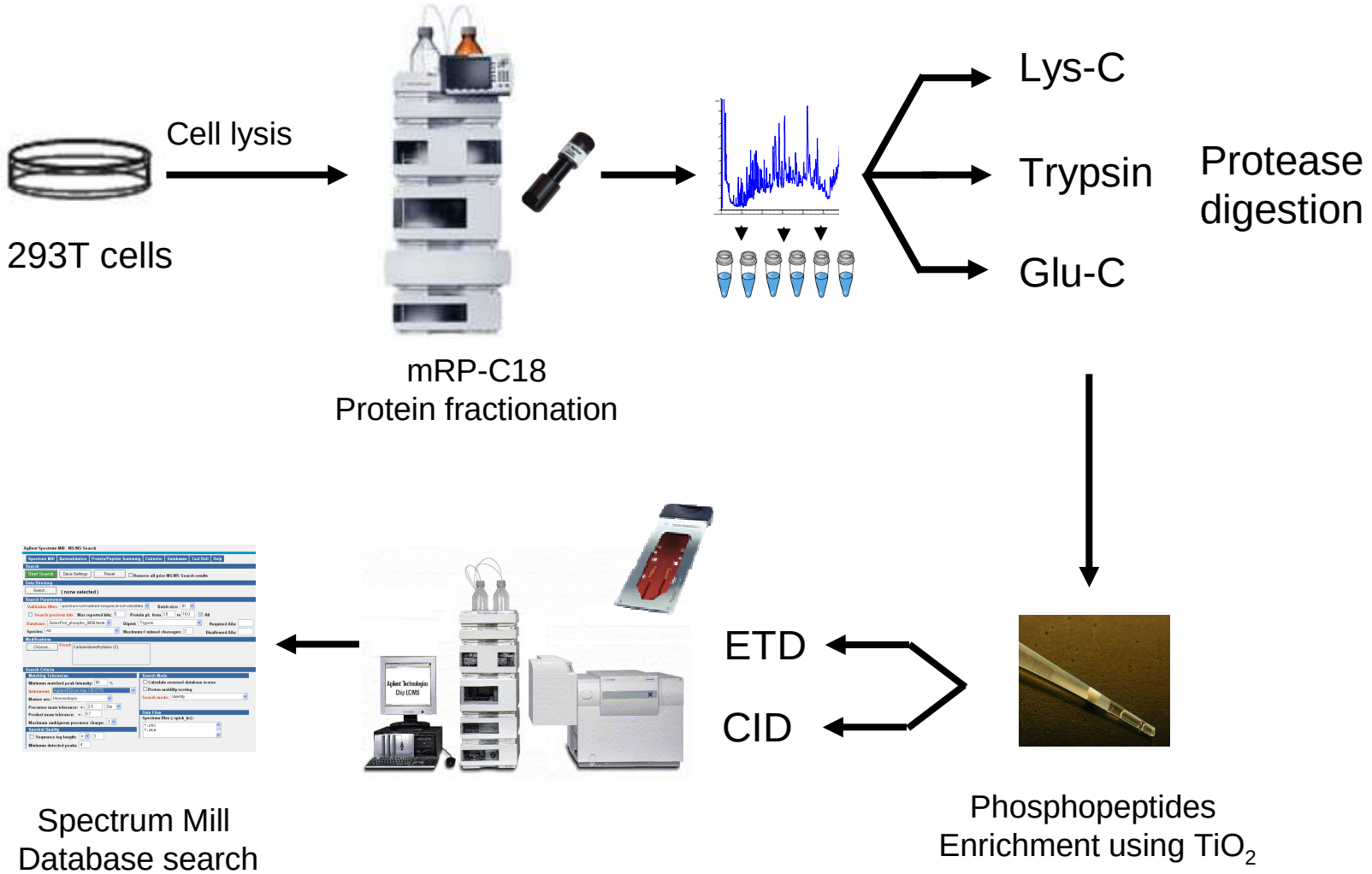
Instrument Schematic For ETD Ion Trap



ETD Advantages:

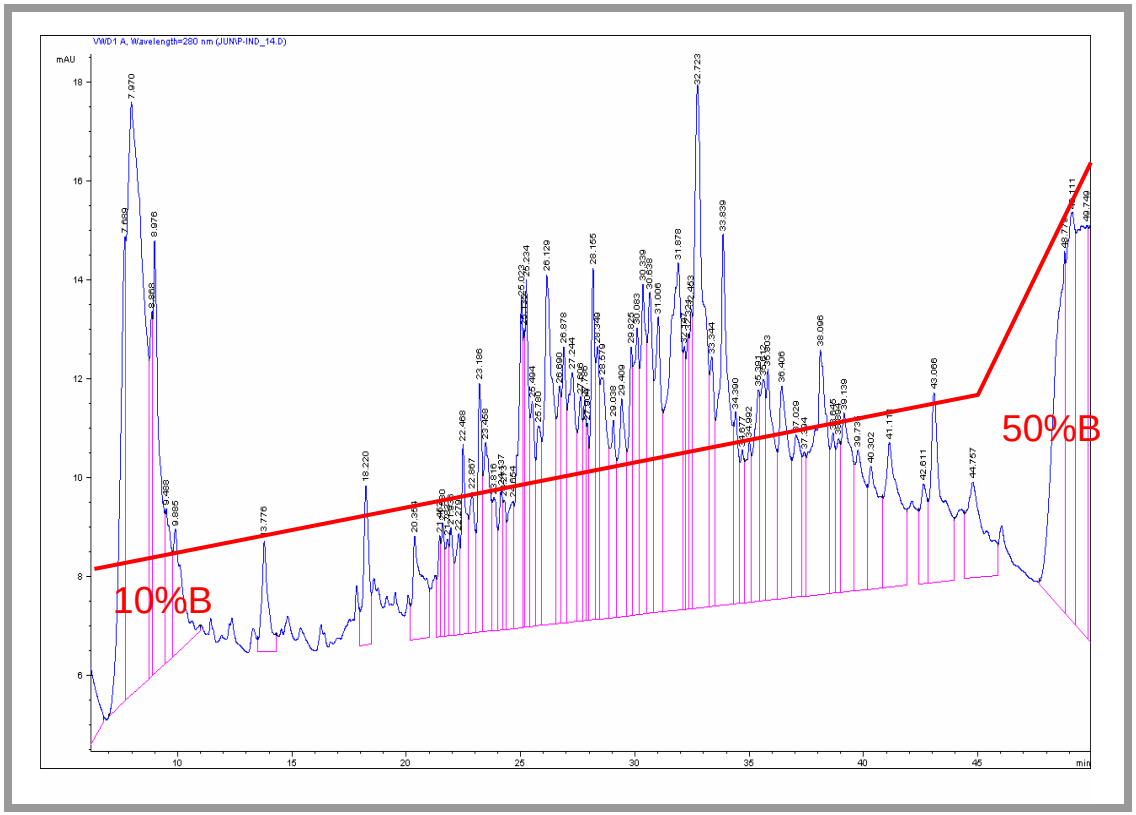
- Complementary fragmentation to CID by providing additional c and z ions
- Soft fragmentation process which maintains intact phospho-residues allows phosphorylation site determination
- Multiple MS(n) fragmentation methods with the combination of CID and ETD enrich information content
- Provides a new gas phase ion reactor for studying gas phase ion-ion chemistry reactions in real time

Experimental Design



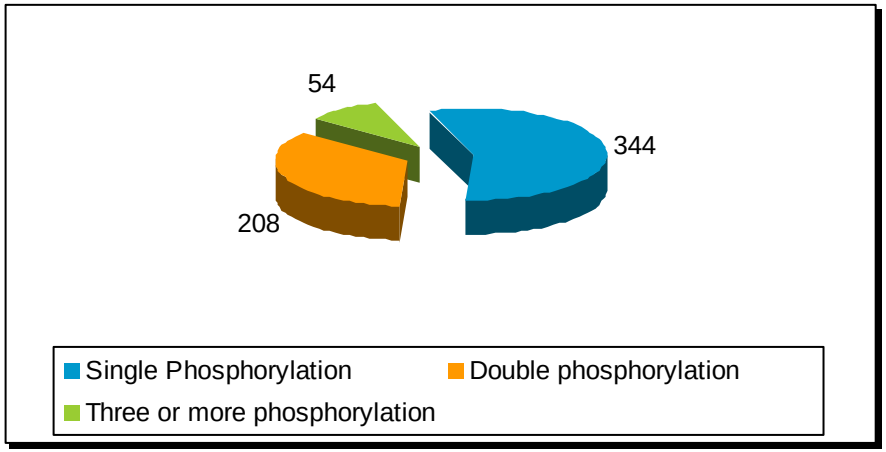
Fractionation of Intact Proteins Using mRP Column

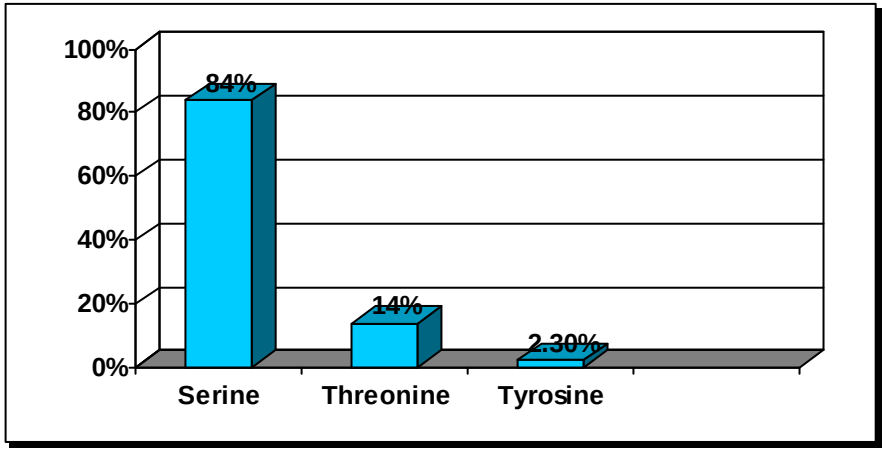
- Protein recovery > 98%
- Achieve high resolution
- Highly reproducible



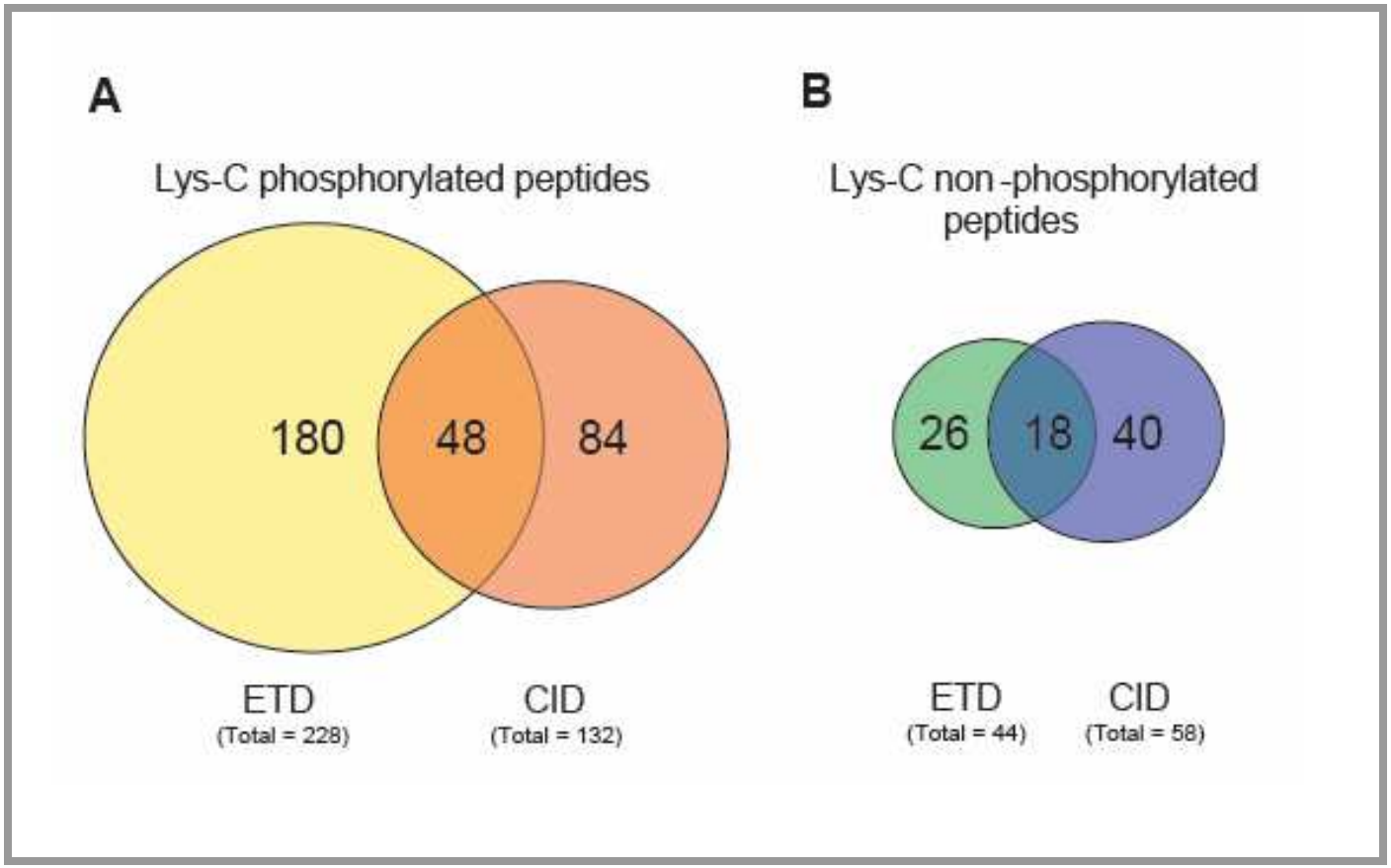
Detailed Analysis of Phosphorylation Sites Identified Using ETD From the Lys-C digests

- 676 unique peptides were identified, derived from 287 proteins
- 606 (90%) were phosphorylated
- 344 singly phosphorylated, 208 doubly phosphorylated and 54 peptides contain three or more phosphorylation residues
- 84% serine phosphorylation, 14% threonine phosphorylation and 2.3% tyrosine phosphorylation



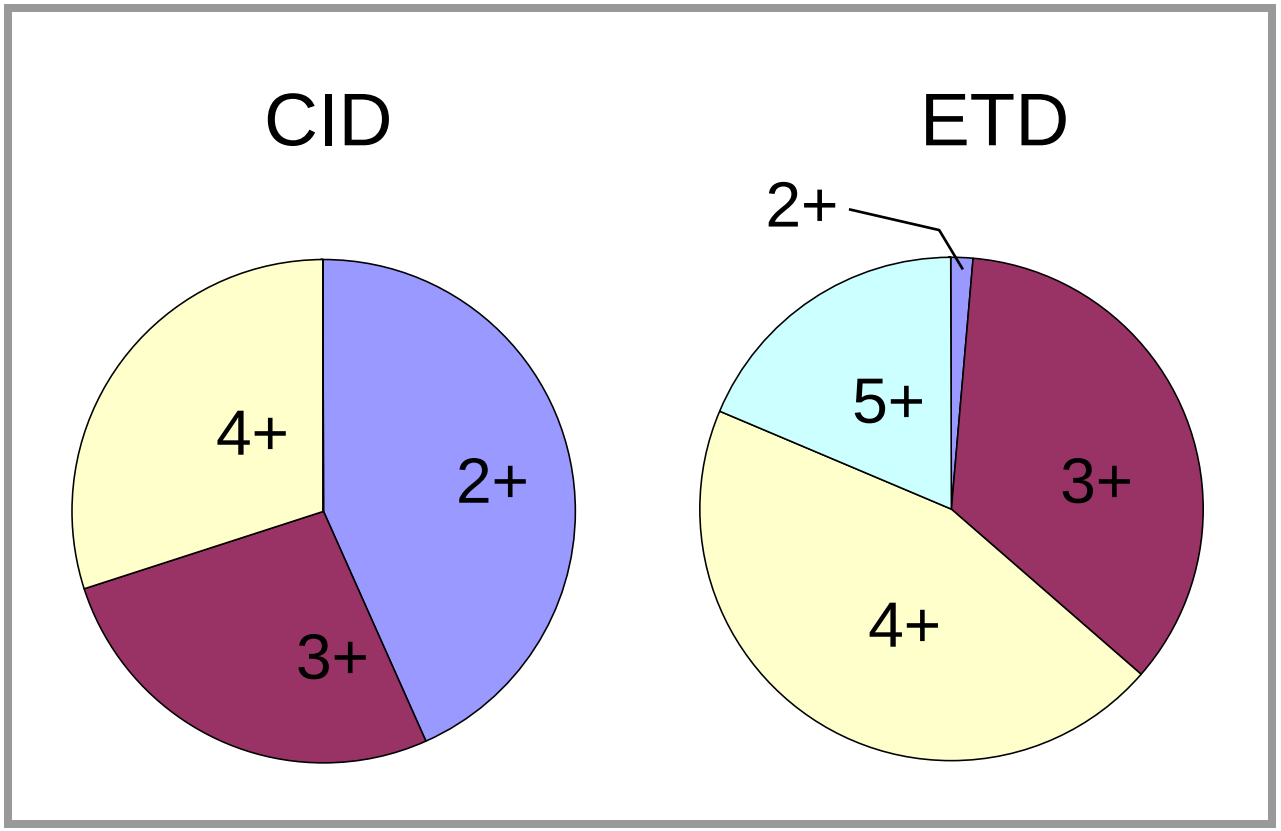


Phosphopeptides Identified by ETD and CID From LysC digest



ETD resulted ~40% more back-bone fragment ions transforming into a 23% better sequence coverage over CID

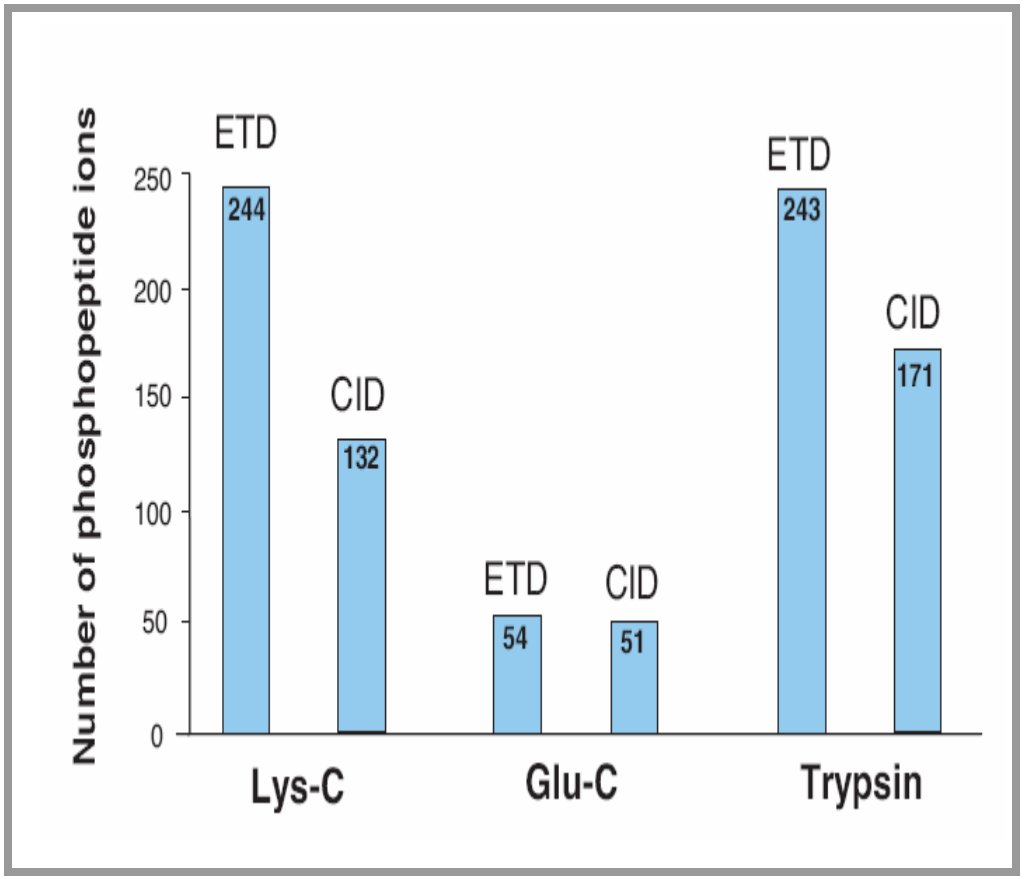
Charge State Distribution of the Identified Phosphopeptides



ETD identified more phosphopeptides with higher charge states, while CID identified more doubly charged precursors.

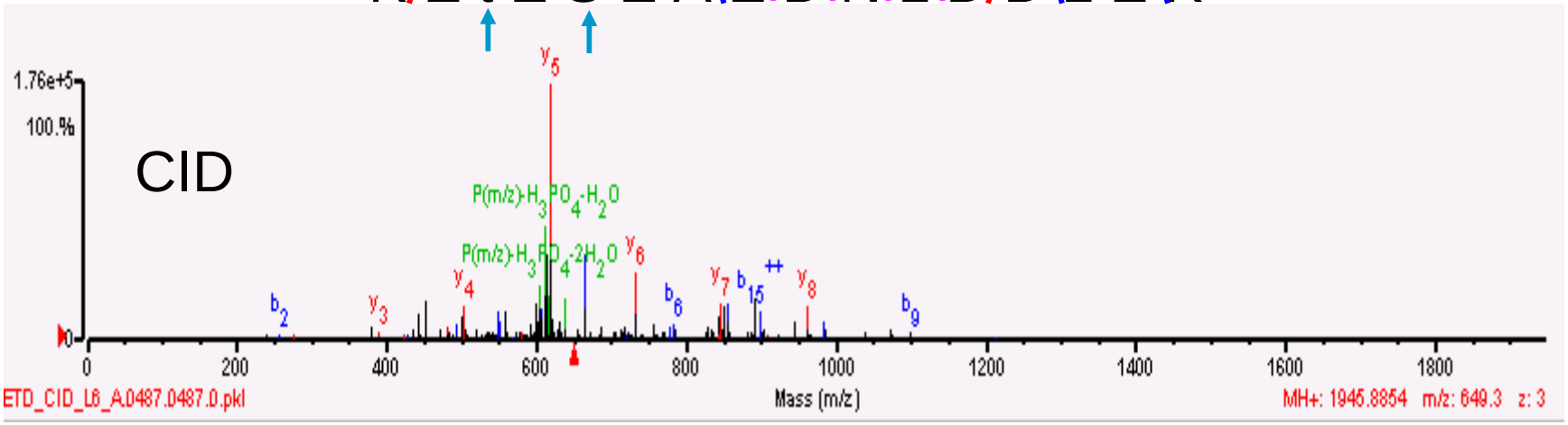
Phosphopeptides Identified by Three Proteolytic Digestion and CID/ETD

- ETD identified 60% more phosphopeptides than CID
- Trypsin and Lys-C identified similar number of phosphopeptides using ETD
- Glu-C identified fewer phosphopeptides than Trypsin and Lys-C

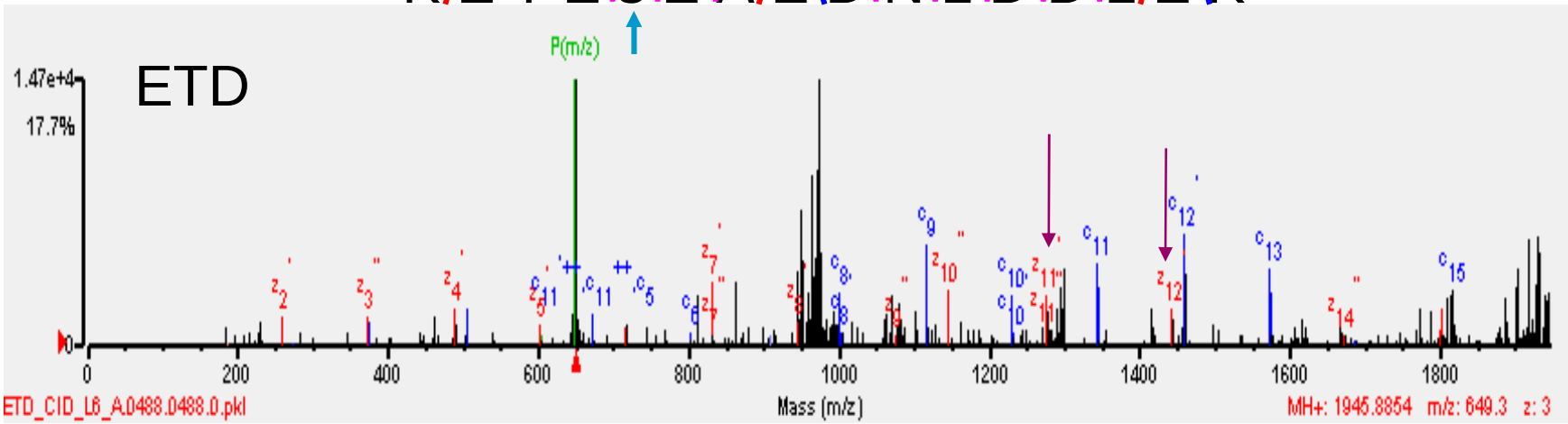


Correct Phosphorylation Location Determined by ETD

K/E t E S E A\E|D|N|L|D/D\L E\K

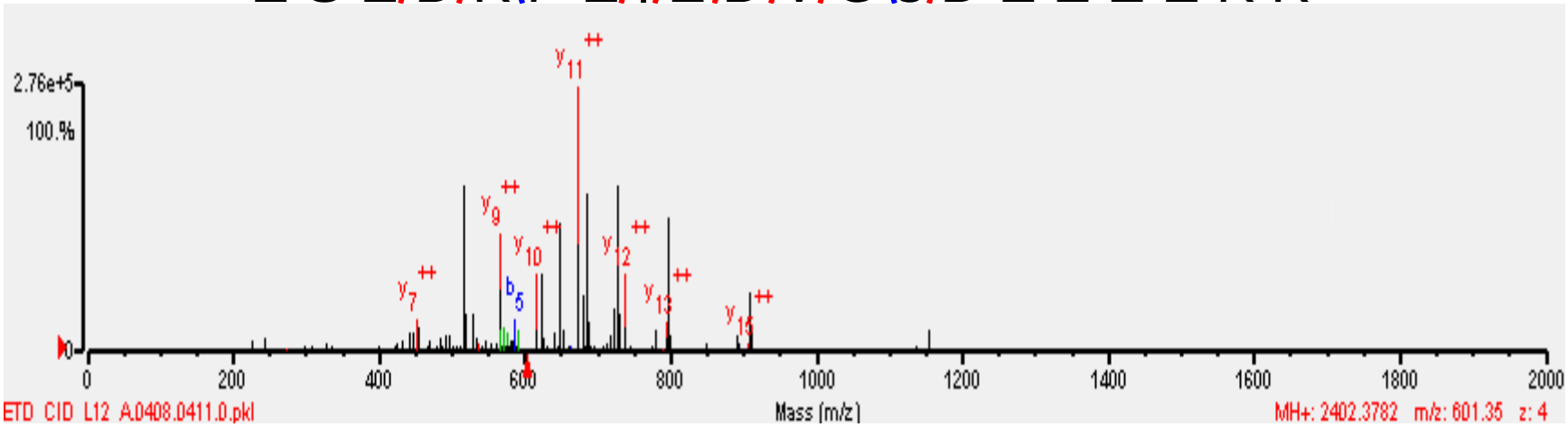


K/E T E|s|E|A/E\D|N|L|D|D|L/E\K



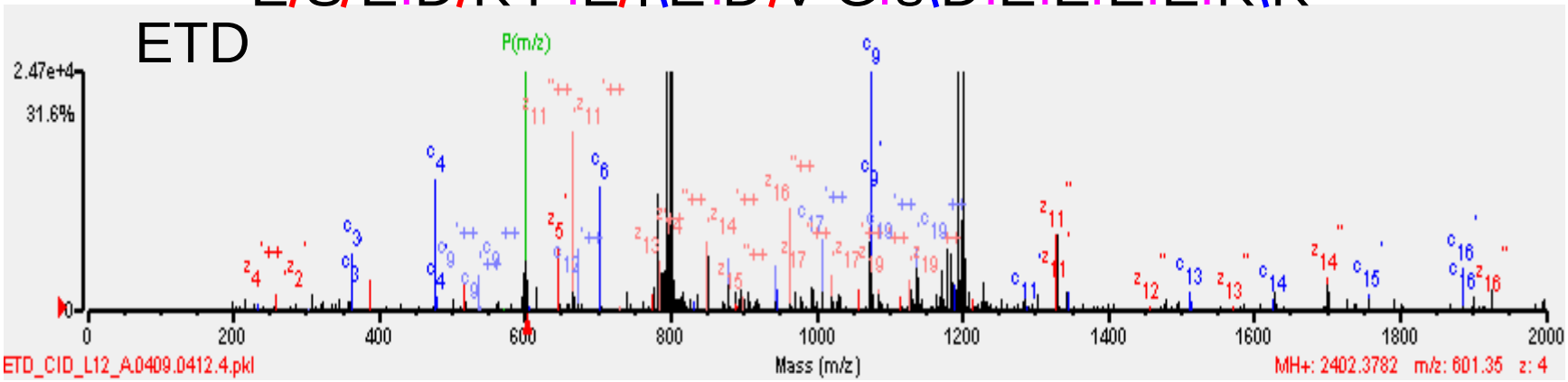
ETD Provided More Fragment Ions And Higher Sequence Coverage

E S E/D/K\P E/I/E/D/V/G\s/D E E E E K K



E/S/E|D/K P|E/I\E|D/V G|s\D|E|E|E|E|K\K

ETD



Discovery of New Phosphorylation Motifs

- 80% of identified phosphorylation sites were novel
- Identified 294 of 368 reported phosphorylation sites from 103 separated publications
- Of the 1141 novel phosphorylation sites, 85% matched known motifs
- 15 new motifs were identified by hierarchical clustering

	Novel motif	Occurrence in phosphopeptides
1	pS[E/D]X[E/D][E/D]	55
2	pSPXXXP	31
3	pSPXXXT	27
4	DXXXp[S/T]P	14
5	GGpS	13
6	p[S/T]PPP	12
7	QXp[S/T]P	12
8	PSp[S/T]P	11
9	PPXp[S/T]P	9
10	PPp[S/T]P	9
11	EXSXp[S/T]P	9
12	PXpSPX[R/K]	8
13	PpSXL	7
14	PLp[S/T]P	6
15	TpTP	5

Summary

- A complete solution for phosphoproteome analysis has been demonstrated
- 1435 unique phosphorylation sites from 500 proteins has been identified from human embryonic kidney cells
- ETD identified 60% more phosphopeptides than CID, with an average of 40% more fragment ions
- Discovered 15 novel phosphorylation motifs

Glycan Profiling

The majority of recombinant protein drugs used for treatment of disease are glycoproteins, parts of glycoproteins, or contain glycoproteins (examples: recombinant antibodies, vaccines)

The glycan portion of the product varies dramatically depending on the cell line used, fermentation conditions, and other production parameters, leading to batch-to-batch variations

The product quality and efficacy (e.g., circulation lifetime) varies dramatically depending on the identity and amount of the glycans present

One way of monitoring the quality of recombinant protein drugs is to profile the glycans, verifying that the product quality has not change appreciably

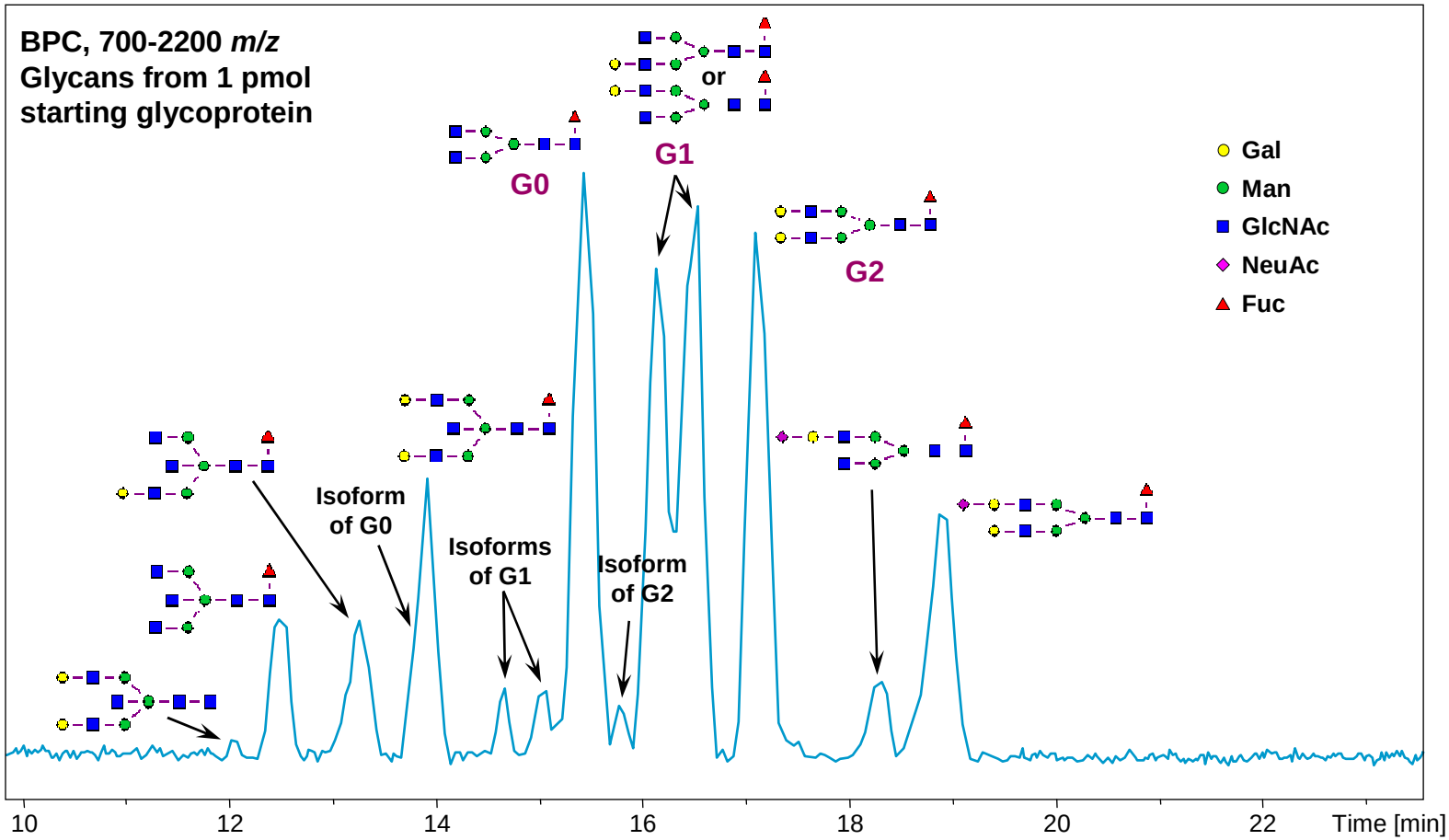
Some Product Names You May Recognize…

Product	Used to treat	Manufacturer
Activase®, **TNKase®** (t-PA, tissue plasminogen activator)	Heart attack (> 1 million US cases/ year), stroke (700,000 US cases/ year), pulmonary embolism (600,000 US cases/ year)	Genentech
Aranesp® (darbepoetin alfa)	Kidney failure, anemia	Amgen
Avastin® (monoclonal antibody)	Colorectal cancer	Genentech
Enbrel® (dimeric fusion protein)	Inflammation (e.g., rheumatoid arthritis)	Amgen
EPOGEN® (epoetin alfa)	Kidney failure, anemia	Amgen
Erbitux® (monoclonal antibody)	Colorectal cancer	ImClone Systems
Gardasil® (genetically engineered vaccine)	HPV infection leading to cervical cancer (10,000 US cases/ year)	Merck
Herceptin® (monoclonal antibody)	HER2-positive breast cancer	Genentech
Pediarix® (genetically engineered vaccine)	Diptheria, pertussis, tetanus, hepatitis B, and polio in infants	GlaxoSmithKline
Raptiva® (monoclonal antibody)	Plaque psoriasis	Xoma, Genentech
Rituxan® (monoclonal antibody)	Non-Hodgkin's lymphoma	Genentech

All of these products

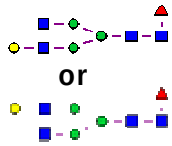
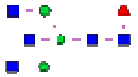
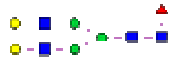
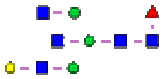
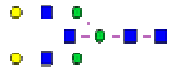
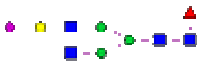
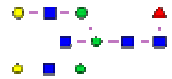
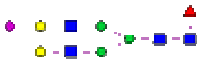
- are ***glycoproteins*** or contain glycoproteins produced by recombinant techniques
- have been approved by the FDA (commercially available) or are in the final stages of approval
- need to be checked for ***stability*** during their product development and phase 1 trials
- need to be checked for ***consistency*** during product development and phase 1 trials
- need ongoing QA/QC checks during production, especially of the glycan portion, to ensure
  - batch-to-batch uniformity of the product
  - consistent performance
  - they are ***safe*** for human consumption

Human Serum IgG N-Linked Glycan Alditols



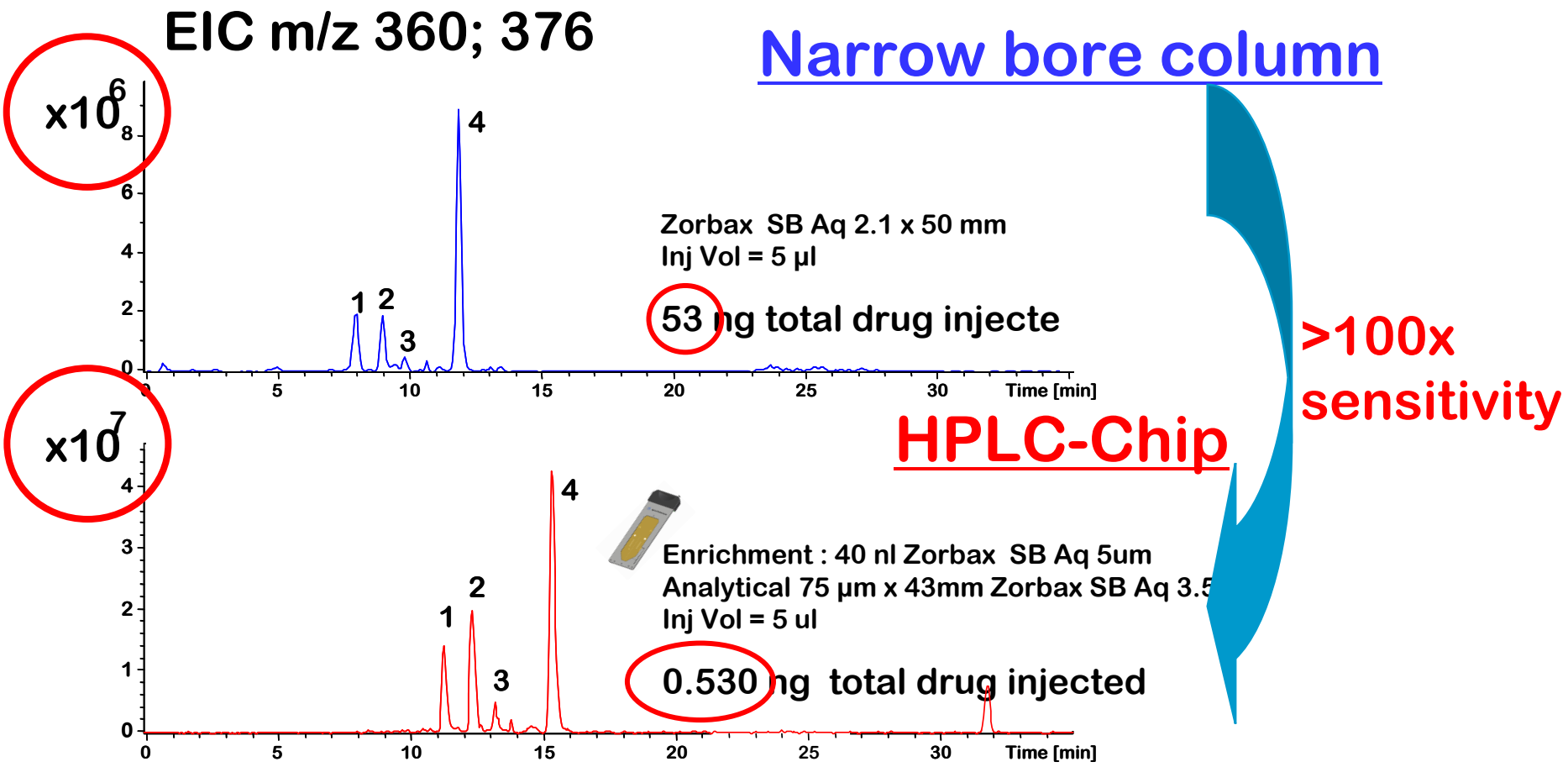
Assuming a GlycoSuite database search segment score of *1*, *2*, or *4*; species *Homo sapiens*, tissue *hemic system* or *immune system;* and attached protein is *IgG*, then these identifications are the only results that pass all criteria

Human Serum IgG N-Linked Glycan Distribution

Glycan (including isoforms)	Structure	*m/z* value of alditol $[M+2H]^{2+}$	peak height in averaged spectrum	relative percent
G0		733.4	46886	14.3
G1	 or	814.4	103172	31.4
G0 + bisecting GlcNAc		835.2	30969	9.4
G2		895.4	35483	10.8
G1 + bisecting GlcNAc		916.0	25222	7.7
G2 + bisecting GlcNAc - fuc		924.0	7623	2.3
G1 + NeuAc		959.9	16101	4.9
G2 + bisecting GlcNAc		997.0	24573	7.5
G2 + NeuAc		1041.0	38886	11.8

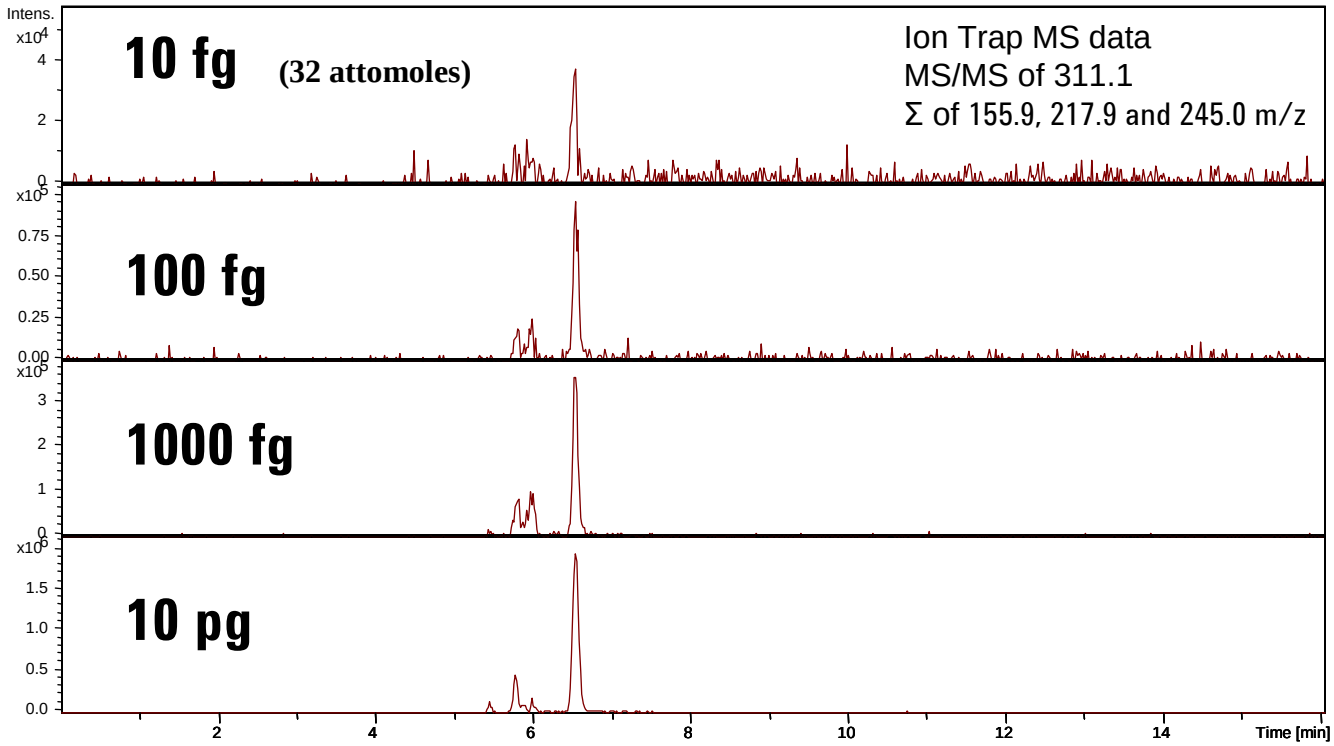
Some inaccuracy in the relative amount is to be expected because of variations in response, charge state distribution, and adduct formation among the various analytes

Metabolite study: 30µM Buspirone Incubation

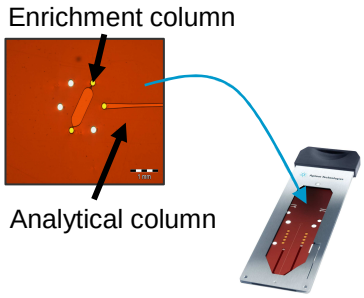


- Peaks 1 to 3 $[M+H]^+ = 376$ N,N-desethyl hydroxybuspirone
- Peaks 4 $[M+H]^+ = 360$ N,N-desethyl buspirone

Pharma: HPLC-Chip sensitivity, sulfadimethoxine in serum, 1 uL injection



Spiked plasma sample was centrifuged in a 3 kDa MW cutoff spin tubes. 1uL is injected on to the enrichment column of the HPLC-Chip.



Area RSD's of 2.6% (10 pg) to 10 % (10 fg) are obtained for 1 uL injection (without ISTD use).

Estimated LOD: 10 fg

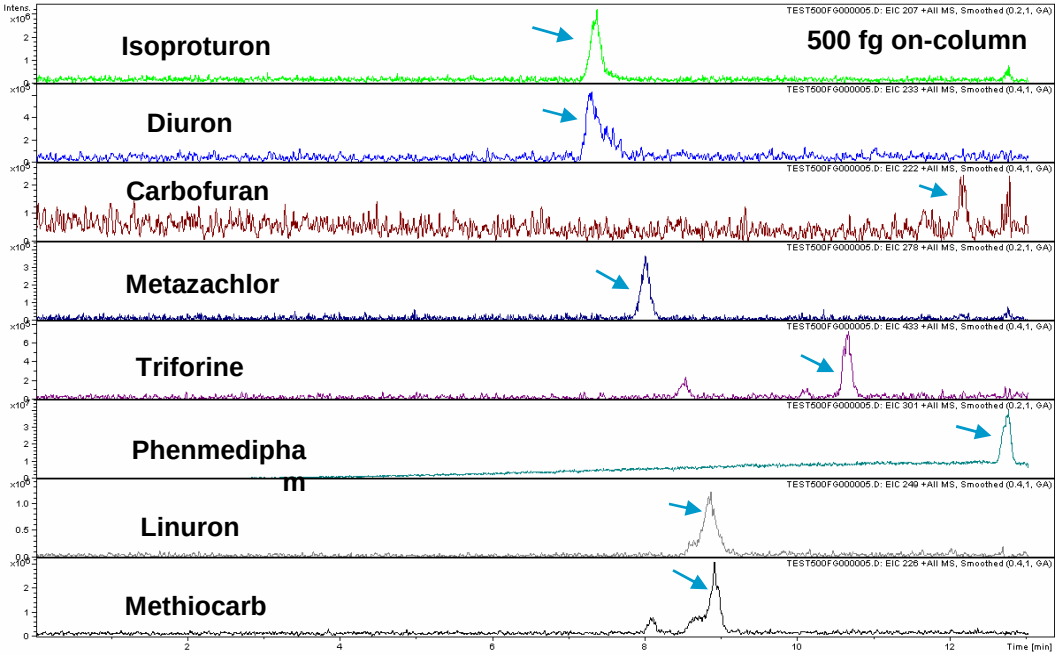
- **Automated sample enrichment at physiological concentration**
- **Small sample size: 1 µL**
- **125 X better sensitivity that best reported QQQ data (1.25 pg/µL in milk)**

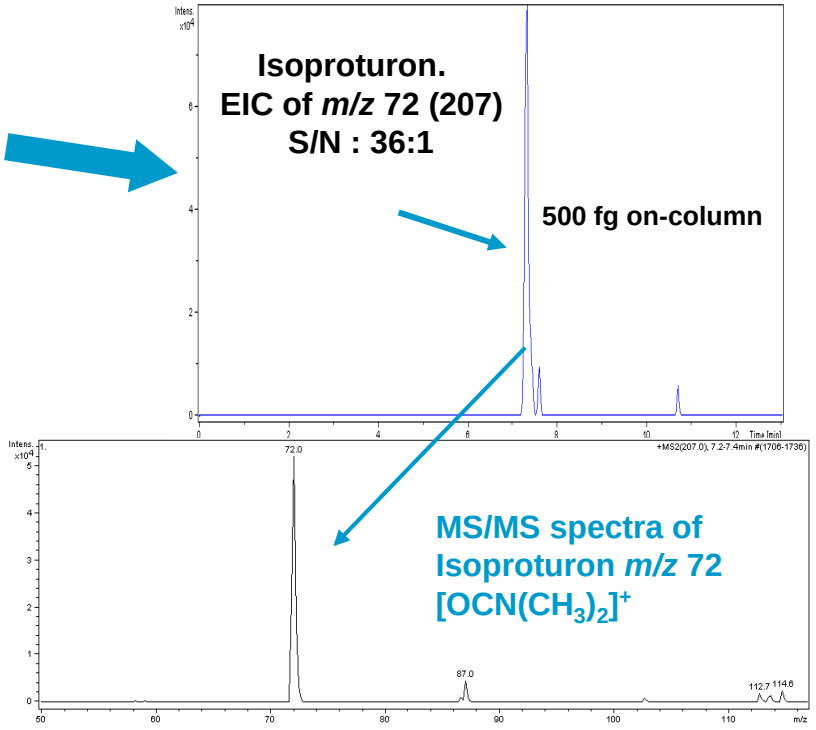
Environmental: Residual herbicides in drinking water with automated sample pre-concentration.

- **EU drinking water regulations require monitoring for neutral and acidic herbicides at the ppt level.**
- **Current procedures involving collecting from 1 to 5 liters of sample and then concentrating the herbicides using SPE prior to analysis.**
- **Detection using conventional 4.6 mm ID columns with LC/MS**
  - **Water sample spiked with herbicides at 1 pg/µL level.**
  - **Direct injection of 0.5 µL of water with automated sample pre-concentration on HPLC-Chip (500 fg on-column).**
  - **Detection on 6340 Ion Trap MS**

Environmental: Residual herbicides in drinking water with automated sample pre-concentration.

Agilent 6340 Ion Trap MS with HPLC-Chip





- **Sensitivity enhancement of nanospray LC/MS allows detection at levels that meet EU drinking water regs (< 100 ppt) without SPE pre-concentration (reduced sample handling/preparation cost).**
- **HPLC-Chip enables routine, reliable use of nanospray LC/MS.**

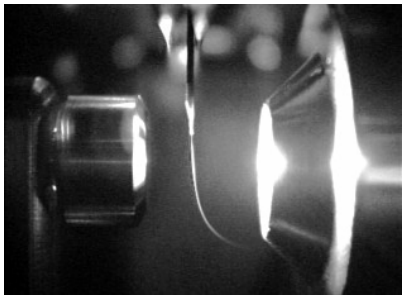
Michael Zumwalt and Hongfeng Yin
Agilent Technologies, Inc.

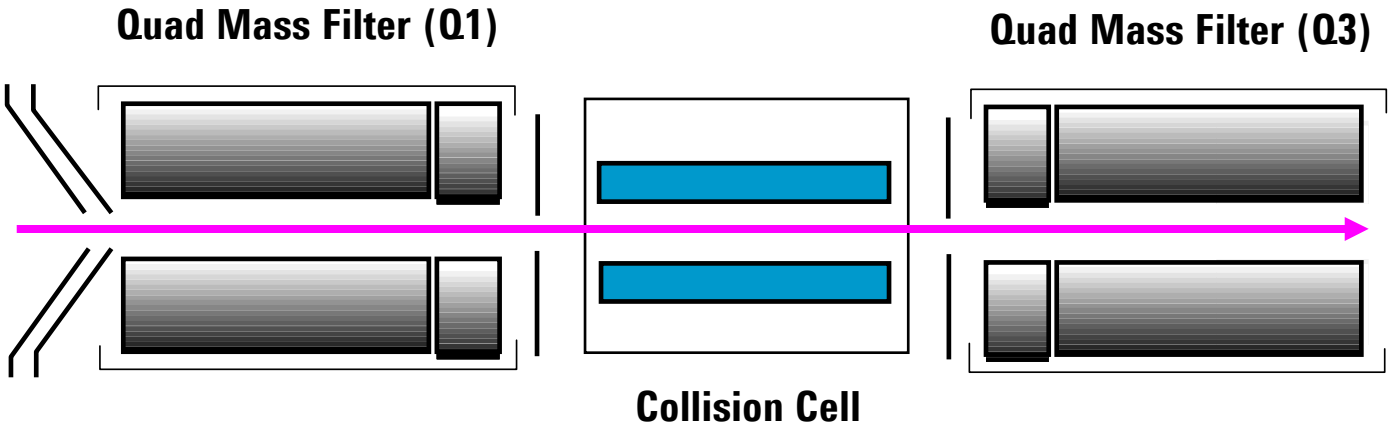
Diana Wilkins and Shannon McOmber
Center for Human Toxicology, Univ. Utah

ASMS 2007

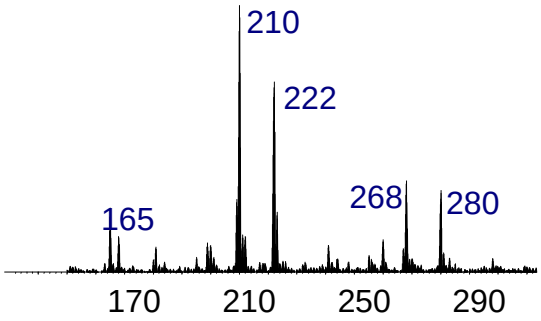
MS Solution: Sensitivity and confirmation

QQQ - MRM (Multiple Reaction Monitoring)

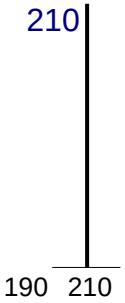




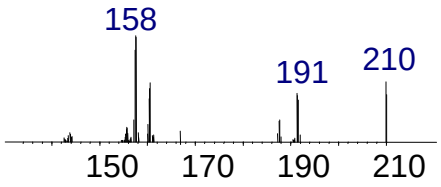
Spectrum with <u>background ions</u> (from ESI)



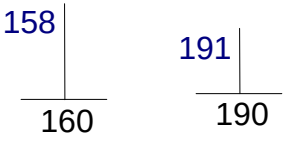
Q1 lets **only** target ion 210 pass through



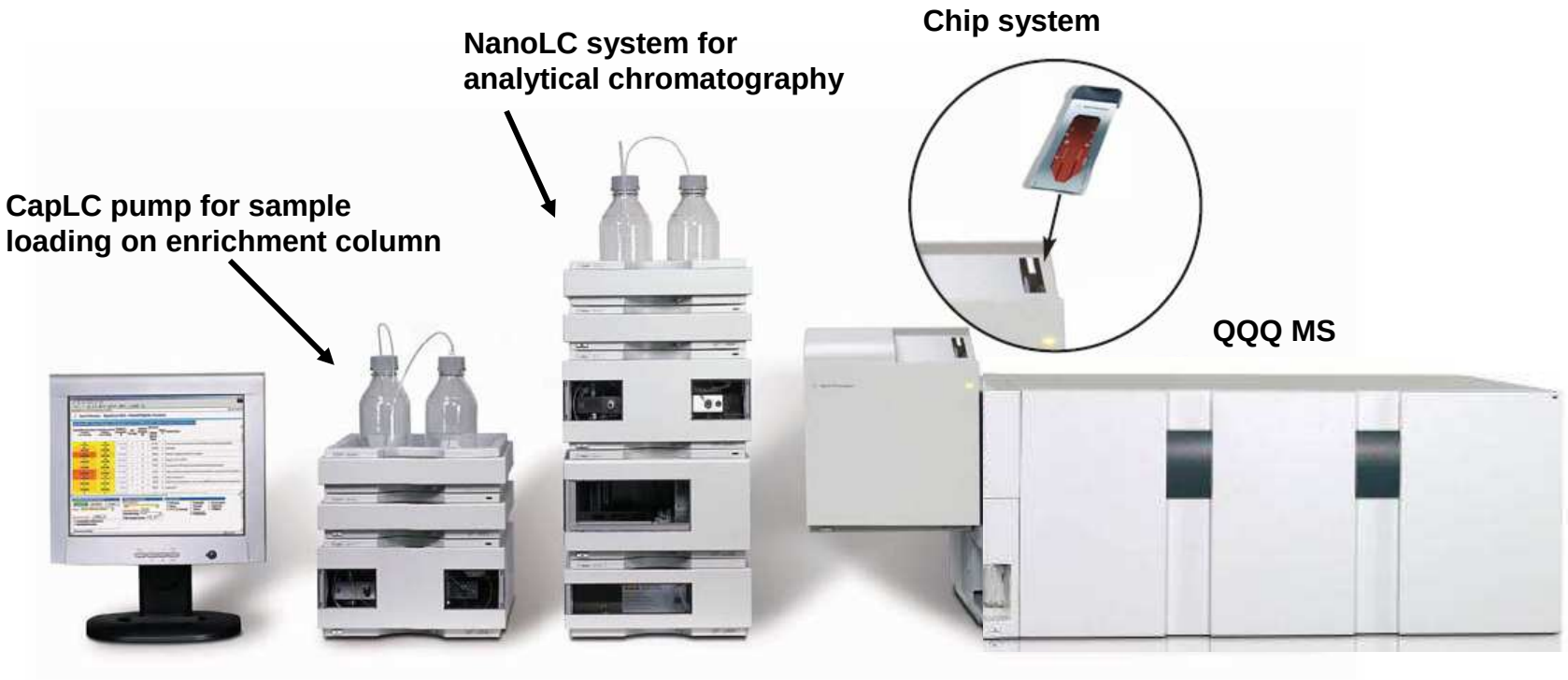
Collision cell breaks ion 210 apart



Q3 monitors **only** characteristic fragments 158 and 191 from ion 210 for quant and qual.



HPLC-Chip/QQQ Technology – system configuration



Analysis of cTHC using HPLC-Chip/QQQ

LC conditions

- 1200 Series NanoLC
  - solvent A: 0.1% formic acid in water
  - solvent B: 0.1% formic acid in 95/10 acetonitrile/water
  - flow: 300 nL/min
  - Analysis of **standards**
  - isocratic: 30% B
  - Stop 5 min

- 1200 Series CapLC (2nd pump for sample loading on enrichment column)
  - 1 µL inj. vol. (**standards**) 2 µL inj. vol. (**extracts**)
  - 30:70 water/acetonitrile
  - flow: 3 µL/min
  - 2 min load time

- HPLC-Chip/MS
  - Analytical column: 75 µm x 43 mm, 300 Å, 3.5 µm, C18

Analysis of cTHC using HPLC-Chip/QQQ – MS conditions

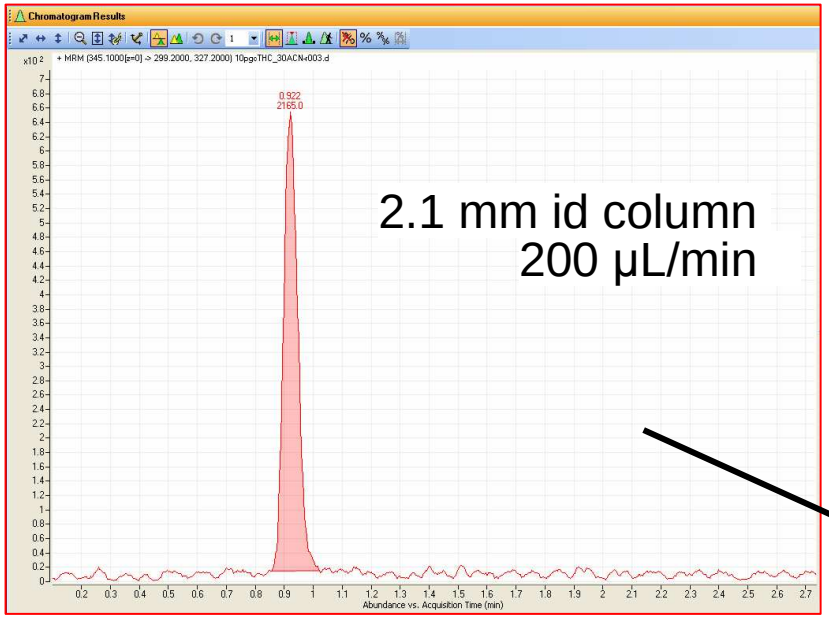
❑ Agilent G6410A QQQ Mass Spectrometer

Source (positive nanoelectrospray)
Capillary: 1800 V
Dry gas: 6 L/min
Gas temp.: 350 C

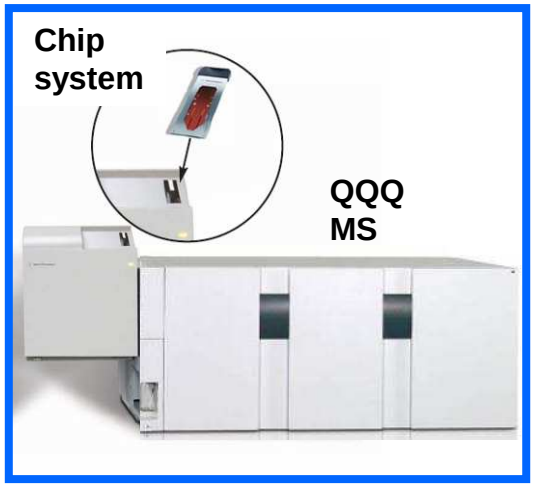
Analyzer
Monitored MRM transitions:

Cmpd	Transition	Dwell (msec)	Frag (V)	CE (V)
cTHC	345.1 > 299.2 (quant)	100	140	18
	345.1 > 327.2 (qual)			
d3-cTHC	348.1 > 302.2 (quant)			
	348.1 > 330.2 (qual)			

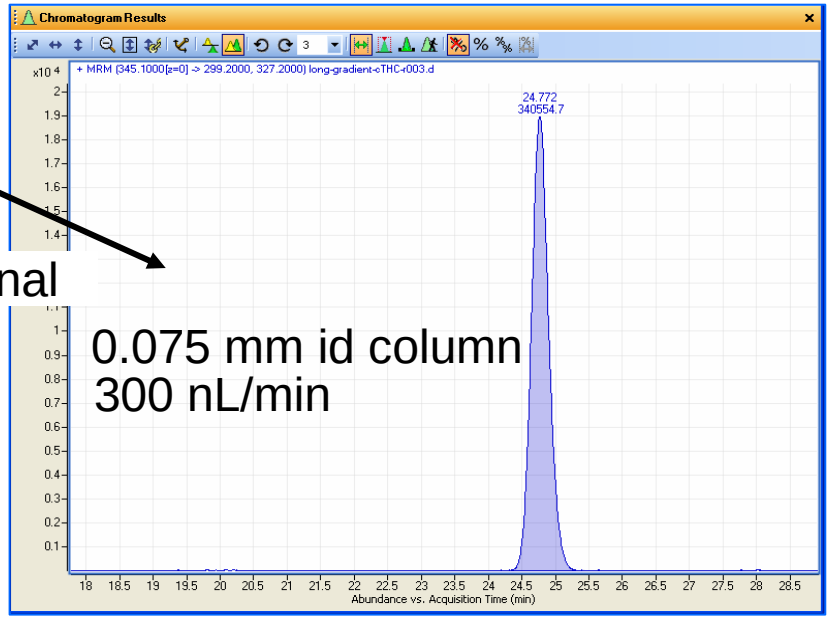
Increase in sensitivity over standard HPLC



2.1 mm id column
200 µL/min



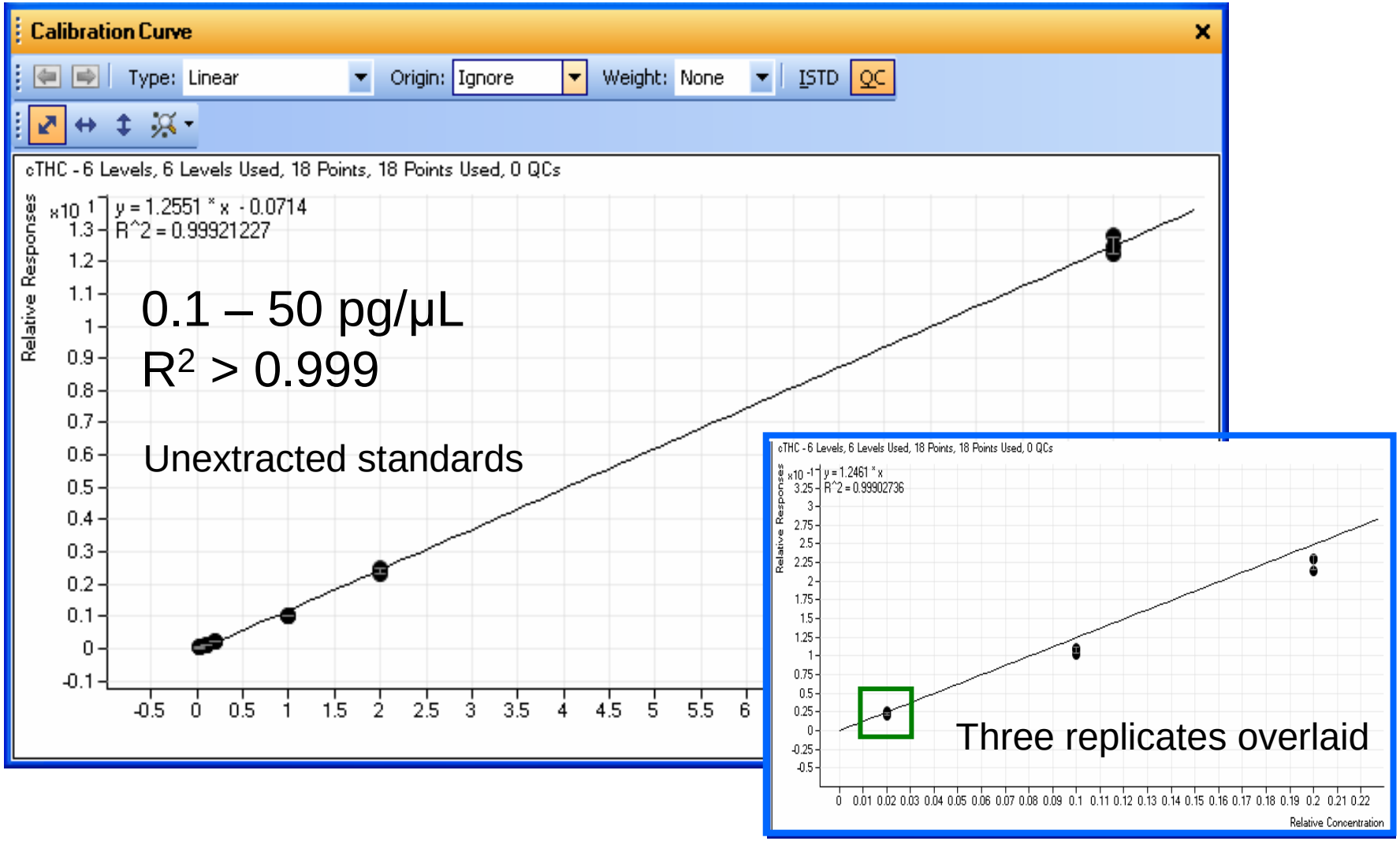
30 x increase in signal



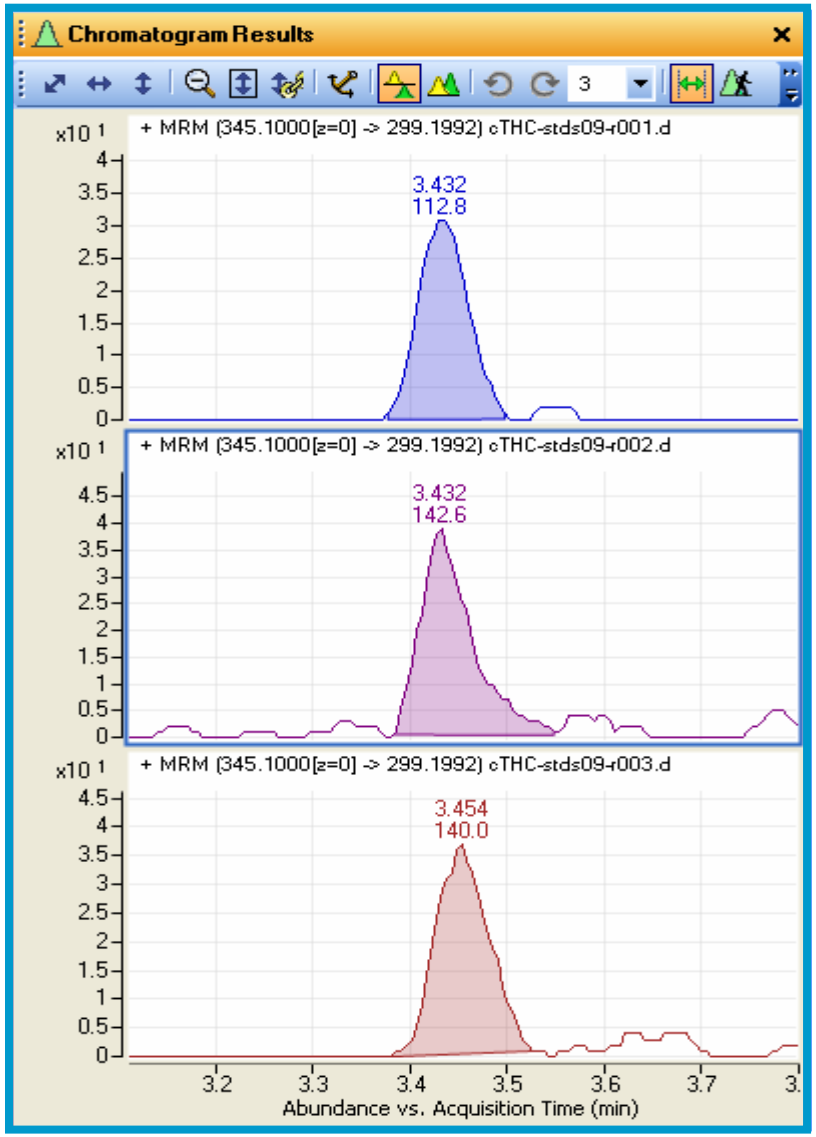
0.075 mm id column
300 nL/min

Injection of cTHC standard

Linearity and reproducibility of cTHC standard



Limit of quantitation in the low femtograms level



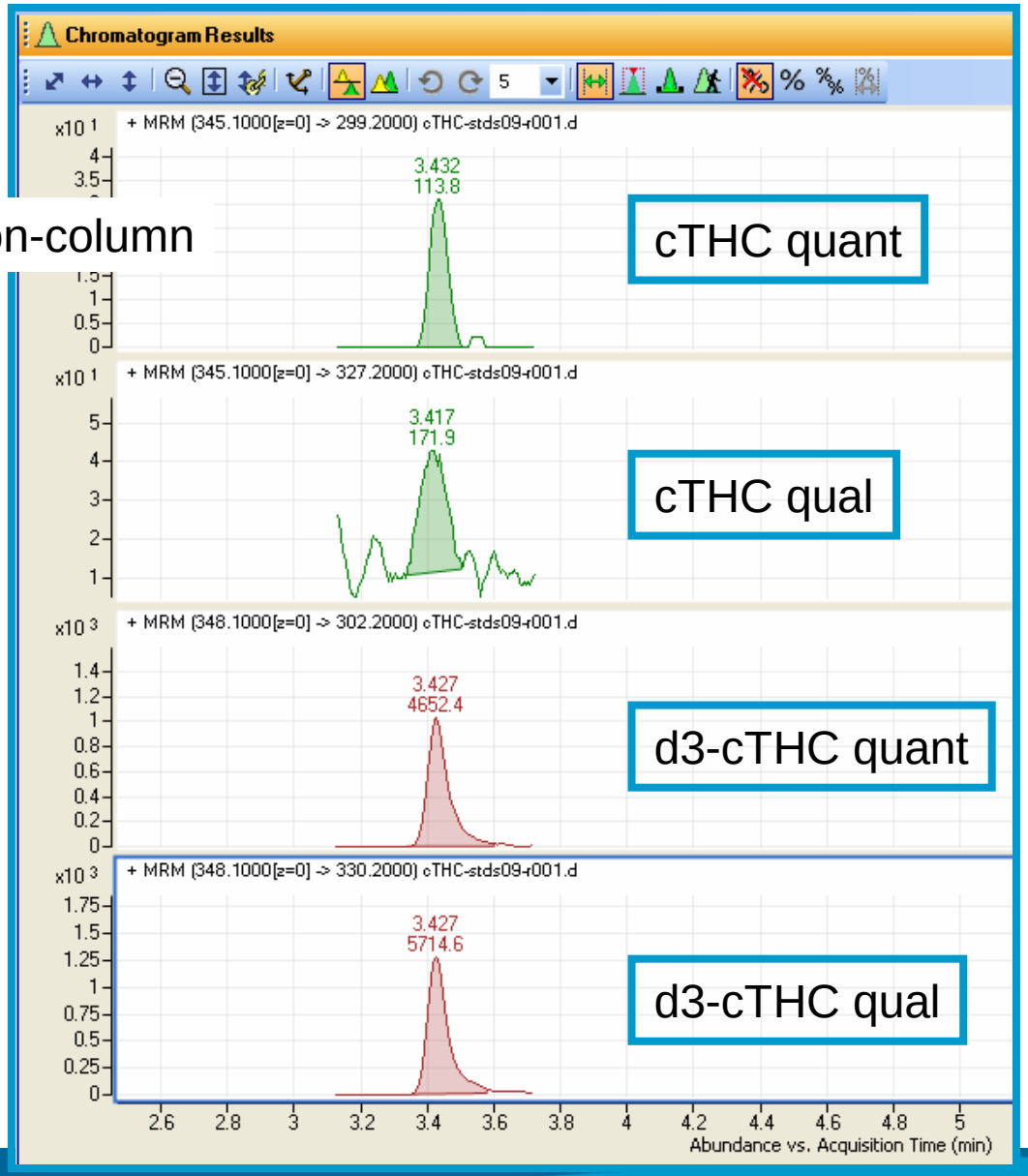
Replicate injections of
20 fg on-column

Area RSD = 12.5%

Average relative difference between calculated and expected concentrations is only 7%

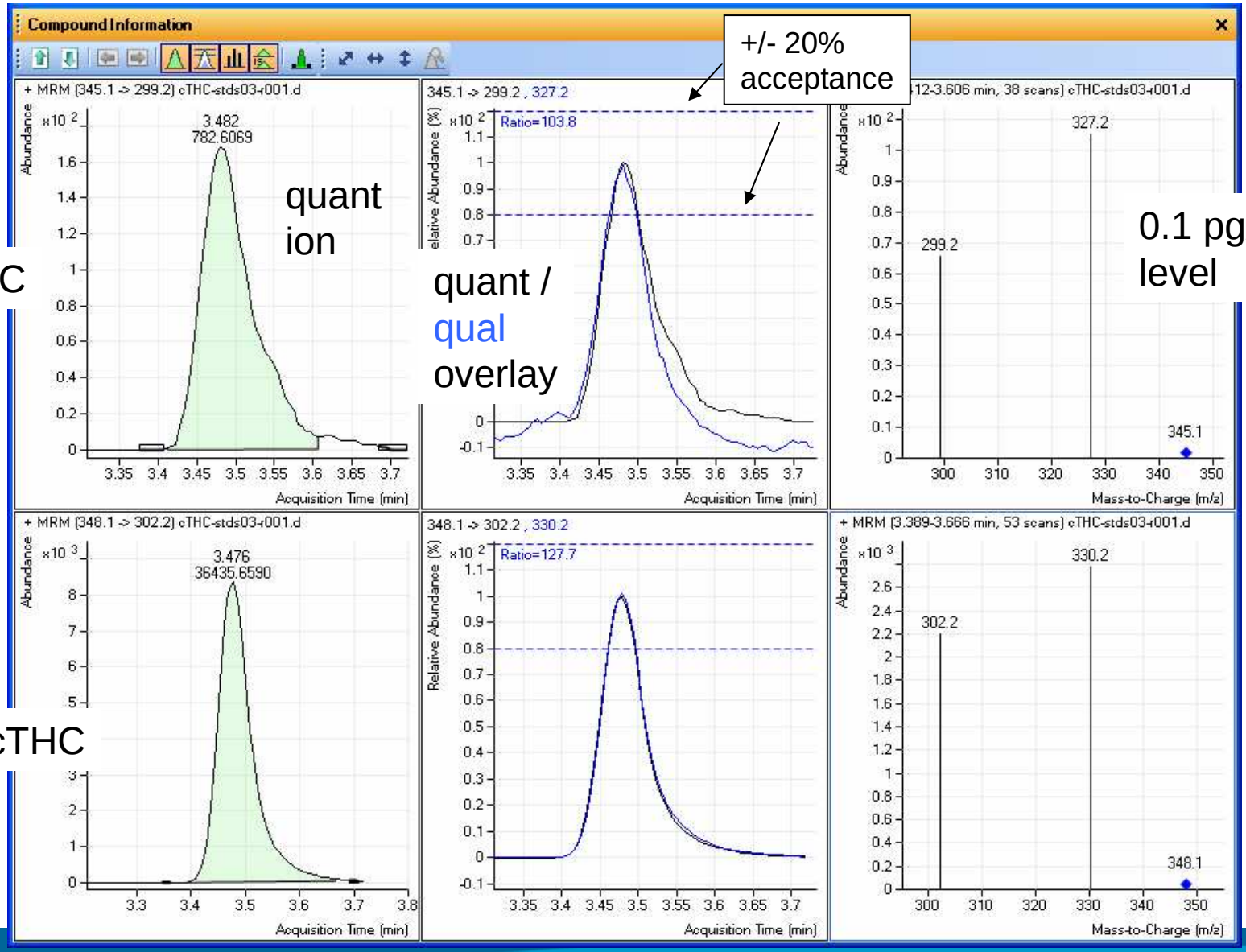
Results of standards – all transitions at lowest level

20 fg on-column

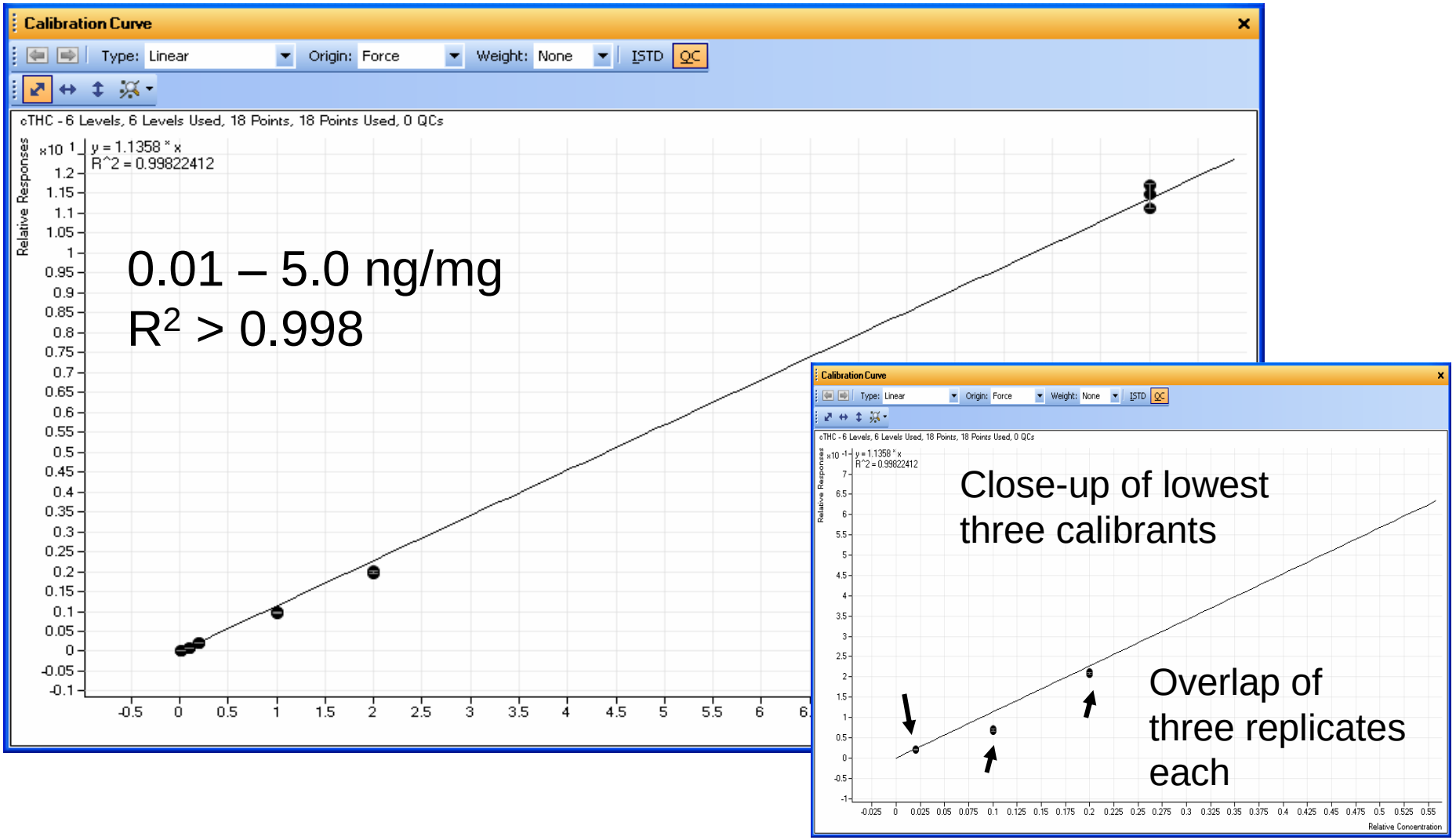


½ amount normally injected on-column by GC/MS/MS or GC/GC/MS

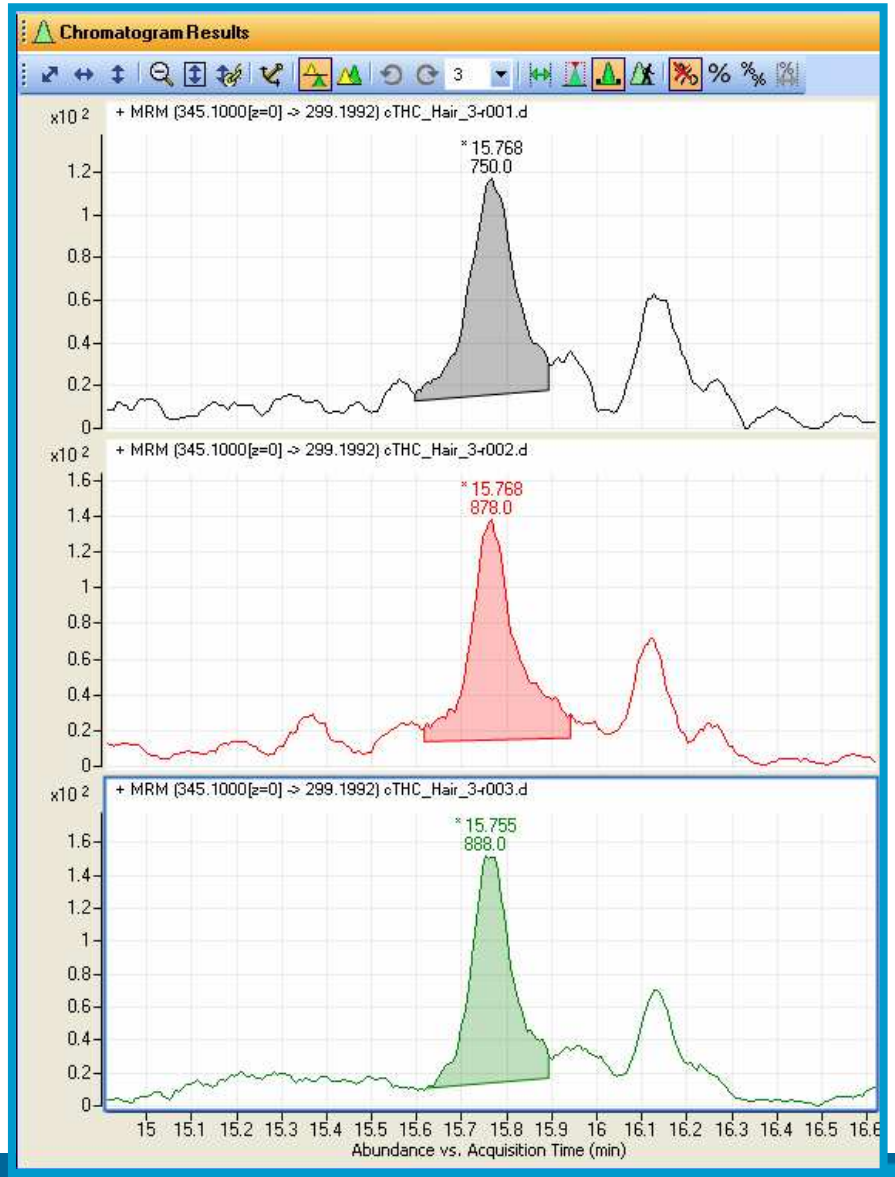
Results of standards – Compound Information



Linearity of cTHC in hair – >2 orders of magnitude



Limit of quantitation for cTHC in hair



400 fg on-column spiked blank

Corresponds to 0.50 pg/mg in hair with 100% extraction recovery

Area RSD = 9%

Summary

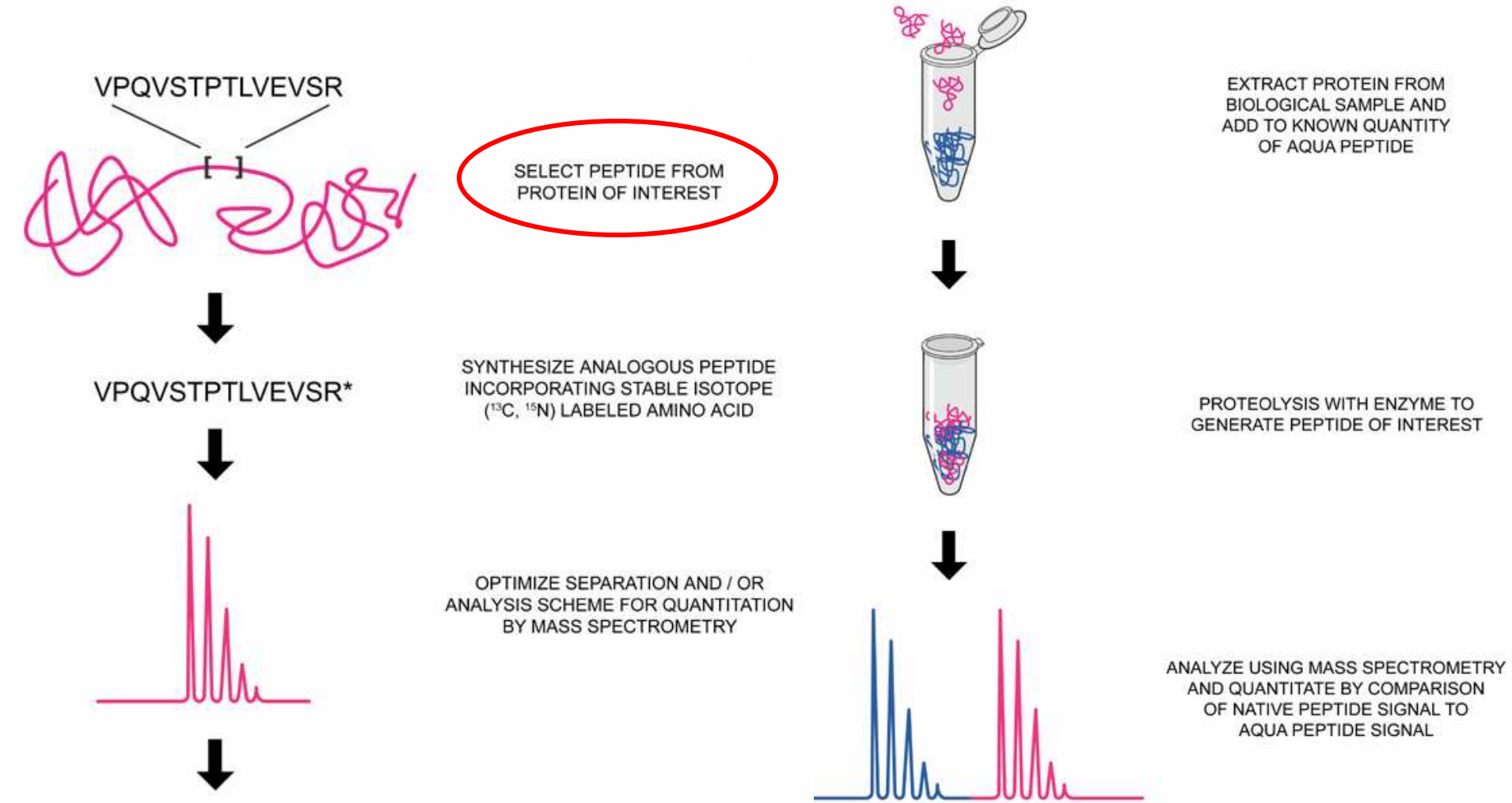
- HPLC-Chip increases sensitivity by factor of 30 over standard LC flow
- Excellent sensitivity with standards, 20 fg on-column easily seen
- Excellent reproducibility at each level and $R^2 > 0.999$
- Good linearity of sample calibration curve, $R^2 > 0.998$, with excellent reproducibility at each level
- LOQ of 0.01 ng/mg in hair with RSD = 6%
- 400 fg matrix spike on-column (0.5 pg/mg) RSD of 9% shows sensitivity of LCMS using HPLC-Chip is 10x level achievable by GCMS without the need for derivatization. Note that this assumes 100% sample extract recovery. Results can be improved with better sample extraction.

Protein Quantitation with HPLC-Chip / MS and Stable Isotope Labeled Peptides

Christine Miller and **Hongfeng Yin**
Agilent Technologies, Inc.

ASMS 2007

Protein Quantitation Strategy

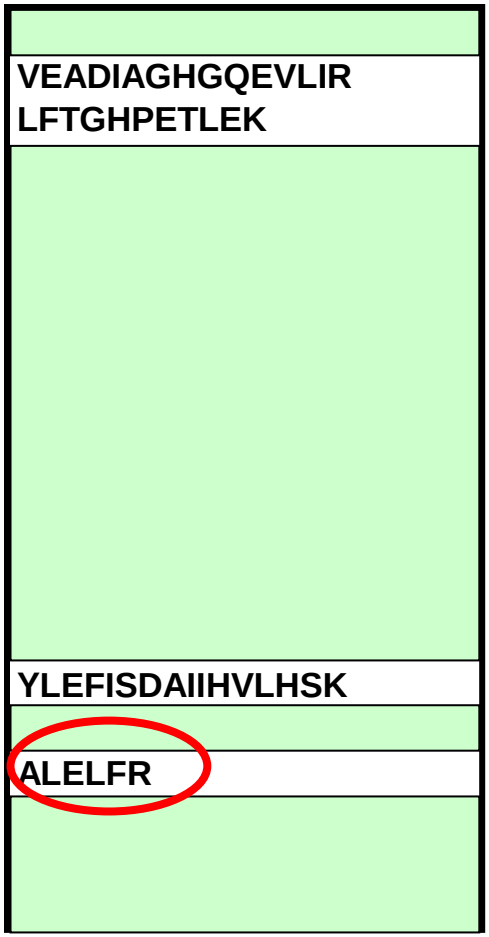


Information is kindly provided by Jon Gingrich of Sigma-Aldrich Co.

SIGMA-ALDRICH

Protein-AQUA

Peptide Selection (myoglobin)



1-16	
17- 31	
32- 42	
43- 45	
46- 47	
48- 50	
51- 56	
57- 62	
63- 63	
64- 77	
78- 78	
79- 79	
80- 96	
97- 98	
99-102	
103-118	
119-133	
134-139	
140-145	
146-147	
148-153	

Chemically reactive residues

(Cys = C, Met = M, Trp = W)

Peptides adjacent to multiple cleavage site

Chemically unstable residues (Asp-Gly = D-G; Asn-Gly = N-G; N-term Glu = E; N-term Asn = N)

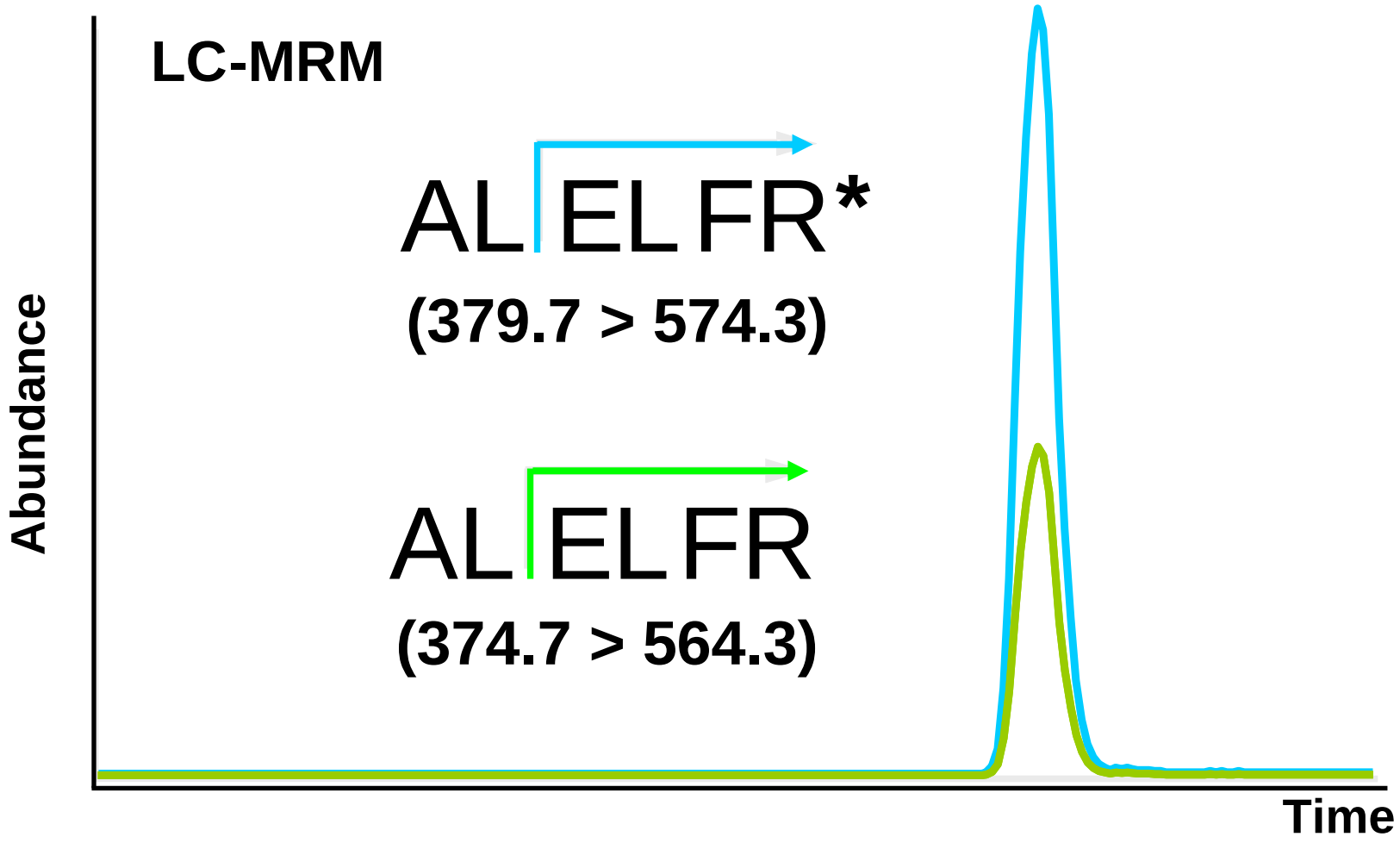
Eliminate "LC-incompatible" peptides

BLAST remaining sequences

AQUA peptide

Information is kindly provided by Jon Gingrich of Sigma-Aldrich Co.

MRM Transitions for Peptide ALELFR



Quantitation of Myoglobin Digest Spiked in Digested Human Serum

calibration level	Myoglobin final concentration	Myoglobin added amount	Internal standard	Serum	water
1	0.4fmol/µL	2µL*20fmol/µL	10µL	2µL	86µL
2	1fmol/µL	5µL*20fmol/µL	10µL	2µL	83µL
3	2fmol/µL	10µL*20fmol/µL	10µL	2µL	78µL
4	5fmol/µL	25µL*20fmol/µL	10µL	2µL	63µL
5	10fmol/µL	50µL*20fmol/µL	10µL	2µL	38µL
6	20fmol/µL	13.5µL stock	10µL	2µL	74.5µL
7	50fmol/µL	33.5µL stock	10µL	2µL	54.5µL
8	100fmol/µL	67µL stock	10µL	2µL	21µL

Myoglobin Stock Concentration is 150fmol/µL

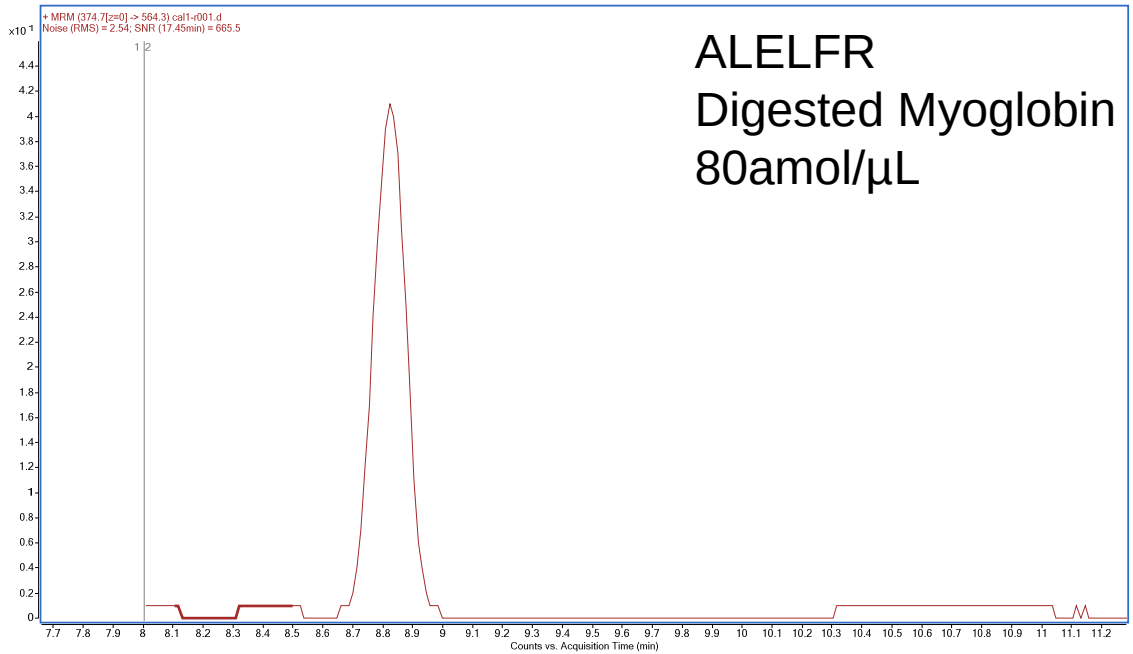
Internal Standard Stock Concentration is 10fmol/µLALELFR* and 100fmol/µL LFTGHPETLEK*

Final Internal Standard Concentration is 1fmol/µLALELFR* and 10fmol/µL LFTGHPETLEK*

Serum Concentration is 12mg/mL. Final concentration is 240ng/µL

Aqua peptides were kindly provided by Jeffrey Porter and Jon Gingrich of Sigma-Aldrich Co.

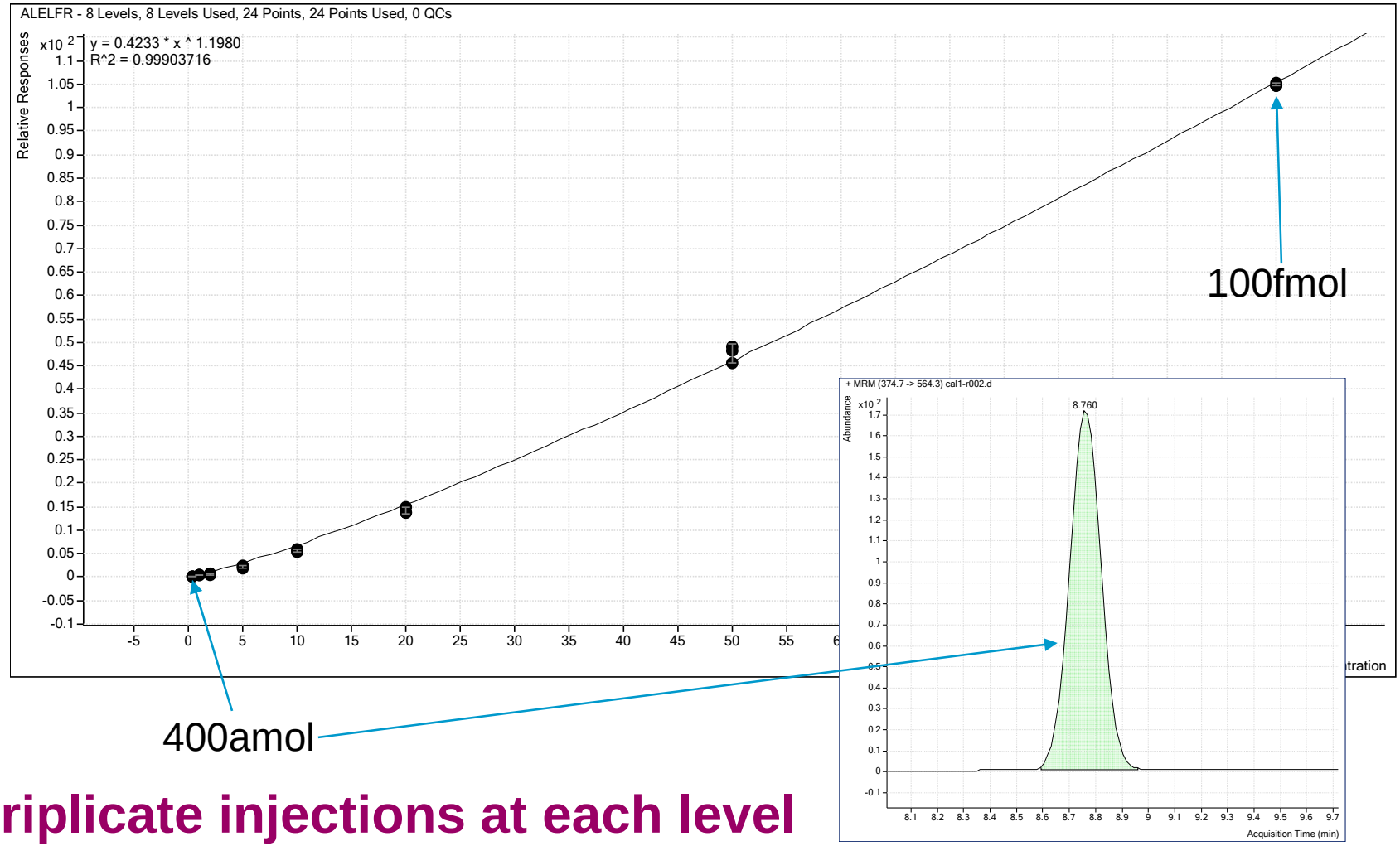
Detection Sensitivity of the Native Myoglobin Peptide with HPLC-Chip/QQQ



HPLC Chip: 43mm Zorbax SB C18
nanoPump: 400nL/min, A. water+0.1%FA, B. 95%ACN 0.1%FA
0 min 5%B, 10min 30%B, 12min 80%B
CapPump: 3µL/min, 2%ACN water
Sample: 0.4fmol/µL * 0.2µL injection, sample is in 240ng/µL digested serum.

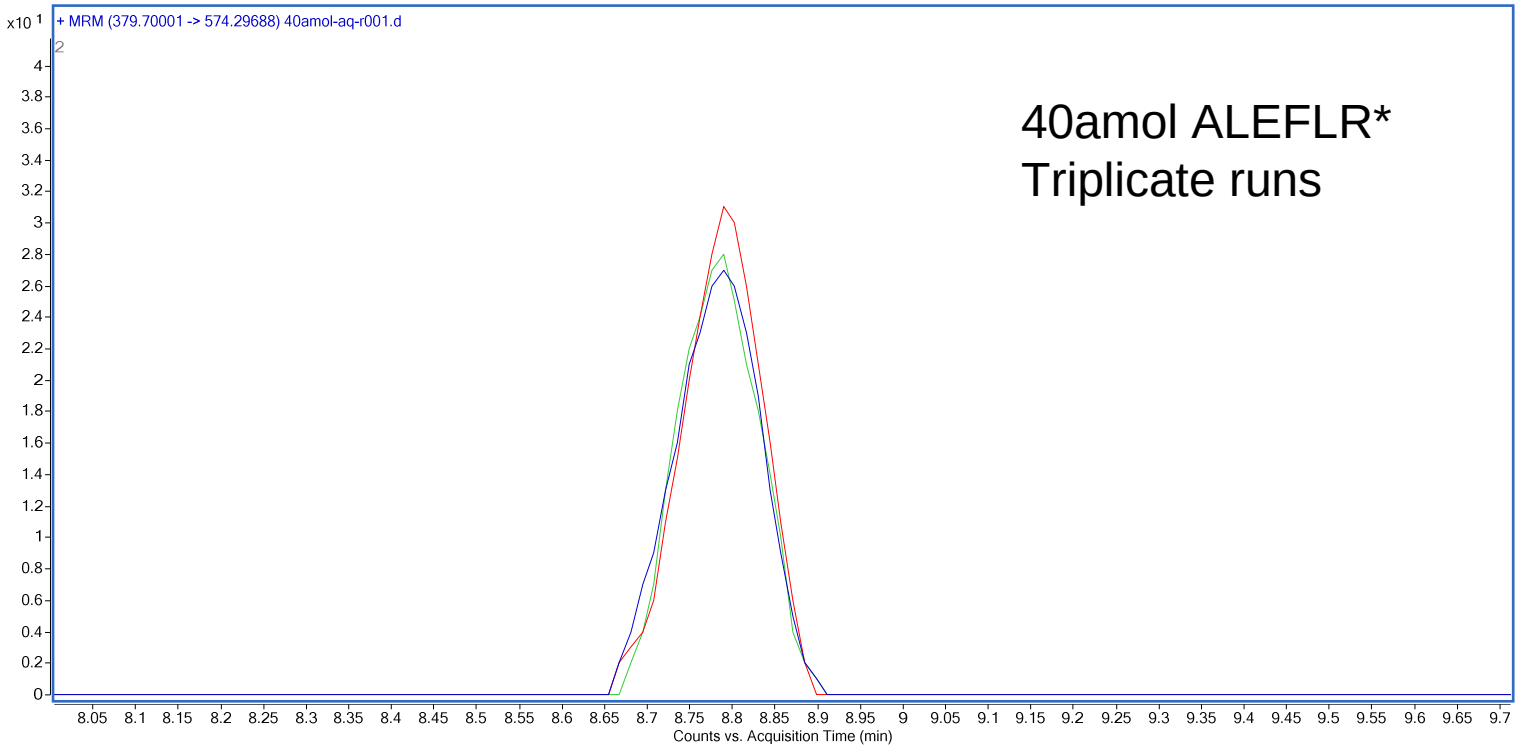
Peptide	precursor	fragment	quant / qual
LFTGHPETLEK	636.3	716.4	quant
LFTGHPETLEK	636.3	1011.6	qual
LFTGHPETLEK*	640.3	724.4	quant
LFTGHPETLEK*	640.3	1019.9	qual
ALELFR	374.7	564.3	quant
ALELFR	374.7	435.3	qual
ALELFR*	379.7	574.3	quant
ALELFR*	379.7	445.3	qual

Calibration Curve for ALELFR Using ALELFR* as Internal Standard



Triplicate injections at each level

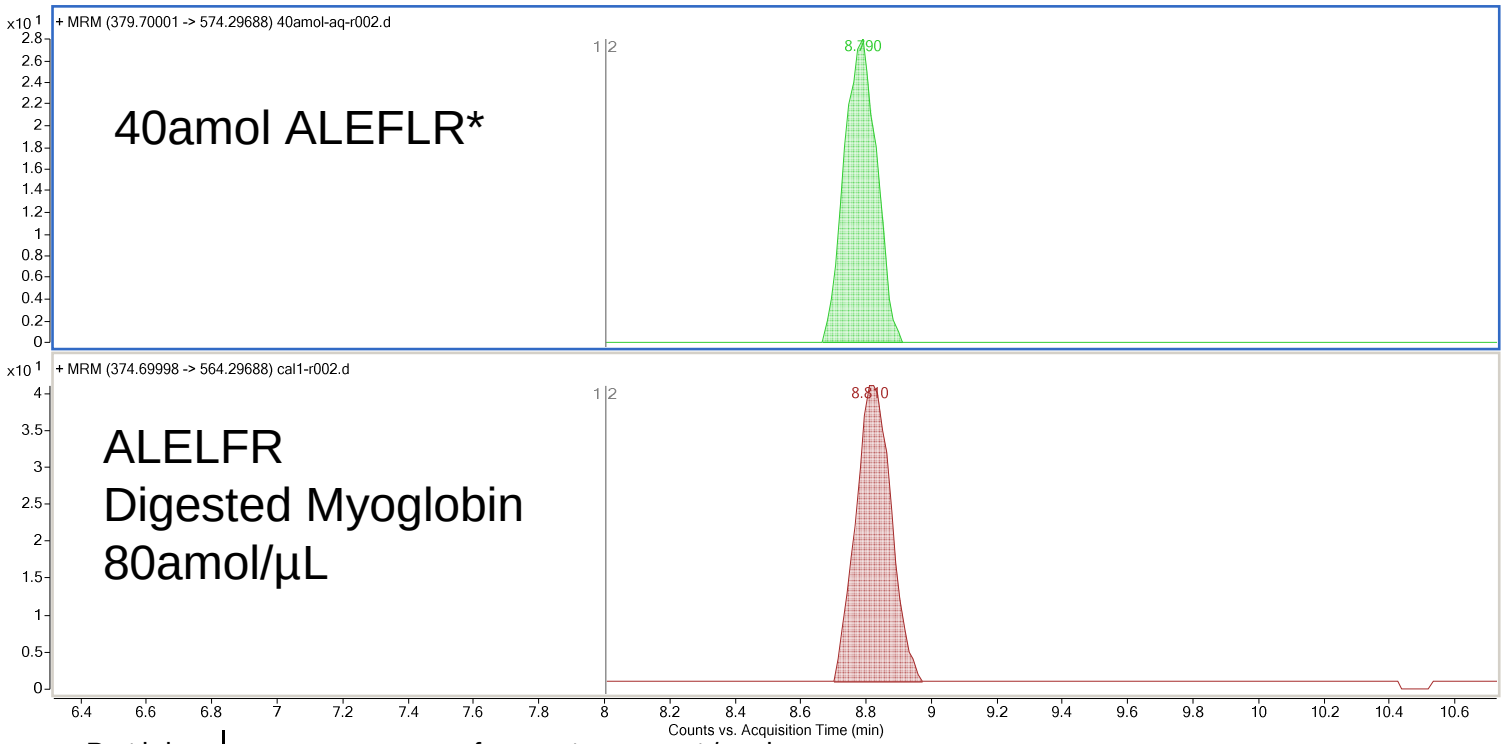
Detection Sensitivity with HPLC-Chip/QQQ



HPLC Chip: 43mm Zorbax SB C18
nanoPump: 400nL/min, A. water+0.1%FA, B. 95%ACN 0.1%FA
0 min 5%B, 10min 30%B, 12min 80%B
CapPump: 3µL/min, 2%ACN water
Sample: 0.04fmol/µL * 1µL injection, sample is in 240ng/µL digested serum

Peptide	precursor	fragment	quant / qual
LFTGHPETLEK	636.3	716.4	quant
LFTGHPETLEK	636.3	1011.6	qual
LFTGHPETLEK*	640.3	724.4	quant
LFTGHPETLEK*	640.3	1019.9	qual
ALELFR	374.7	564.3	quant
ALELFR	374.7	435.3	qual
ALELFR*	379.7	574.3	quant
ALELFR*	379.7	445.3	qual

Quantitation with Internal Standard



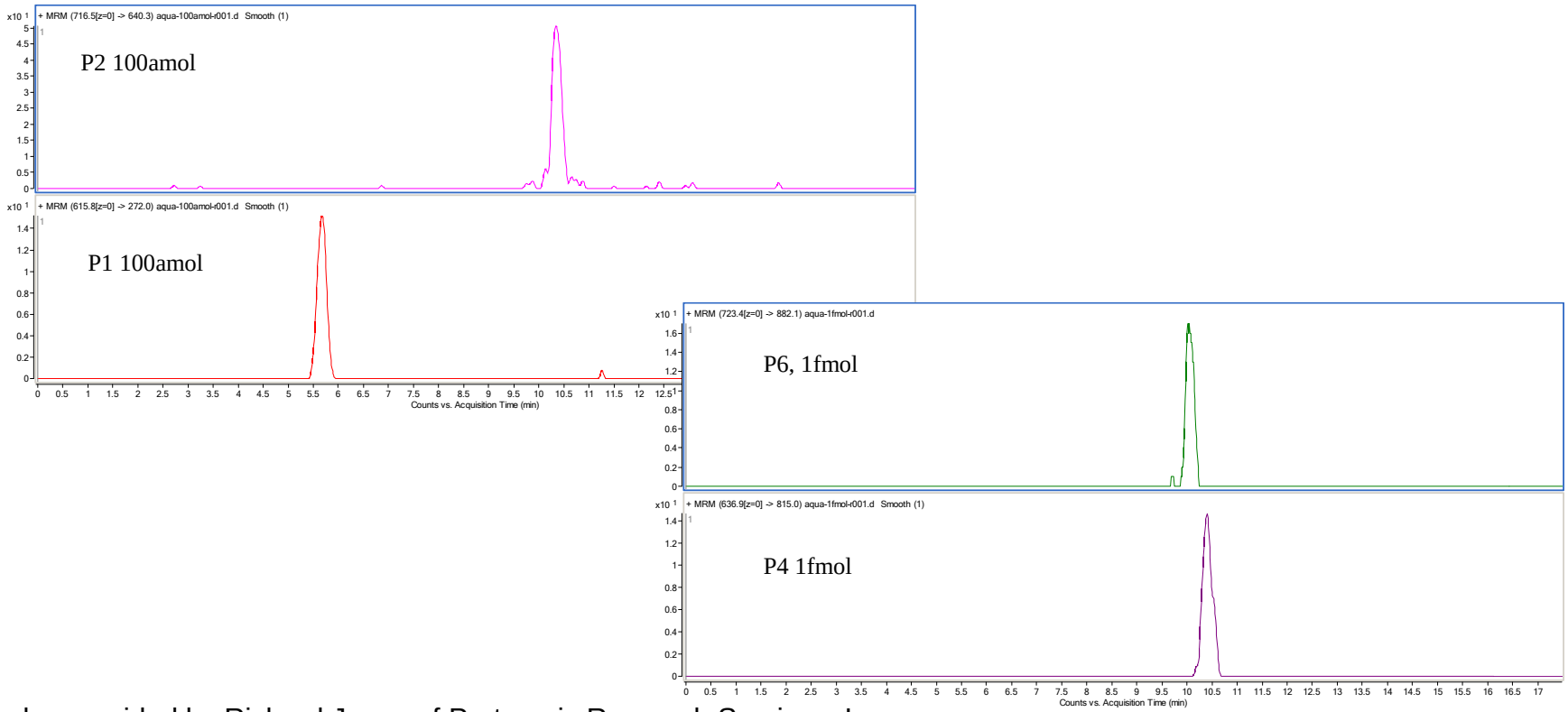
Peptide	precursor	fragment	quant / qual
LFTGHPETLEK	636.3	716.4	quant
LFTGHPETLEK	636.3	1011.6	qual
LFTGHPETLEK*	640.3	724.4	quant
LFTGHPETLEK*	640.3	1019.9	qual
ALELFR	374.7	564.3	quant
ALELFR	374.7	435.3	qual
ALELFR*	379.7	574.3	quant
ALELFR*	379.7	445.3	qual

The ALELFR peak from 80amol digested Myoglobin is calculated to be 63 amol using the 40amol ALEFLR* Aqua peptide. One reason for it could be that the digestion efficiency is 80%.

Serum Protein Quantitation using Aqua Peptides

Aqua Peptides from PRS proteomics

Peptide	sequence	Aqua peptide	fragment	native peptide	native peptide fragment
P1	ESYSGVTL*DPR	615.8++	272 (13eV)	612.3 ++	601.3 (y5 ion singly charged) and 272 (y2)
P2	DIPTNSPELEETLTHTITK*	716.5+++	640.3 (9eV)	713.7+++	637.6 (y17 triply charged)
P4	TNLESILSYPK*	636.9++	815 (17eV)	632.9 ++	807.5 (y7 singly charged)
P6	SLDFTELDVAAEK*	723.4++	882.1 (18eV)	719.4 ++	874.5 (y ion singly charged)



Samples provided by Richard Jones of Proteomic Research Services, Inc.

Conclusions:

- High sensitivity peptide analysis using HPLC-Chip/QTOF and HPLC-Chip/QQQ MS
- High efficiency chromatography separation with HPLC-Chip
- Good retention time and MS detection reproducibility
- Excellent quantitation when using stable isotope labeled peptides as internal standard
- Sample loading capacity is at least 300ng total protein digests on a standard short analytical column/40nL enrichment HPLC-Chip

HPLC-Chip portfolio

- **MS Calibration & Diagnostic chip**: Easy infusion of MS tuning mix, Calibration of the Chip Cube valve
- **Protein ID Chip#1**: 43 mm column, peptide separation. Low/medium complexity tryptic digest mixtures. Dilute samples 100-400 ng on-column
- **Protein ID Chip # 2:** 150 mm column, peptide separation, medium/high complexity tryptic digest mixtures. Concentrated samples 500 ng to 1 µg on column.
- **Graphitized Carbon Chip:** Glycan (oligosaccharides) separations, 43 mm column. Highly polar compounds normally not retained on C-18 material. Structurally related compounds such as geometric isomers and diastereoisomers
- **Small molecule Chip:** Metabolite ID, DMPK, dilute samples. Small molecules retained and well characterized by SB-C18. 43 mm column
- **Infusion Chip:** direct infusion or automated flow injection of the sample directly in to the mass spectrometer at nanoflow rates for the collection of MS and MS/MS data.
- **SPQ Chips:** high capacity enrichment column, or intact protein separation chip using C-8 material
- **Custom Chip:** Custom packing, enrichment and/or analytical column.

HPLC-Chip Interface: Part of the Agilent LC/MS platform.

6300 Series Ion Trap MS

6500 Series QTOF MS

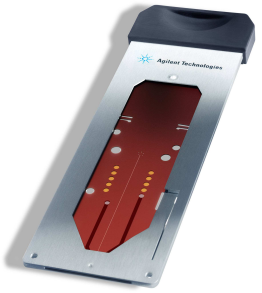
6100 MSD: Fall 2007

6200 Series TOF

1200 Series Nano-LC

6400 Series QQQ: June 2007

HPLC-Chip/MS: Conclusions



HPLC-Chip/MS Features:

- Increased productivity: easy to use, fast system setup and optimization, integrated workflows.
- Increased uptime: robust, reliable, plug and play operation designed for reliability and supportability
- Enhanced performance: improved MS/MS data quality, high sensitivity and high resolution chromatographic separations.
- Flexibility: optimized application and system performance simply by changing HPLC-Chip.

Customer feedback:

"The Chip changed my life"
"I have been able to triple the throughput in my lab!"
"Once you use the chip, you don't want to go back to needles!"
"My technician can now run nano-LC/MS!"
"So reliable, I now run nanoLC sequences over the weekend!"
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Thank you very much for your attention!