Protein Identification by CE-MS-TOF.

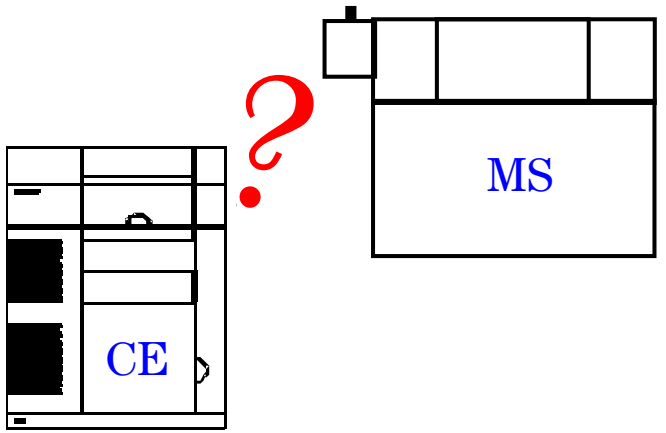
Martin Haex
Product Specialist Mass Spectrometry
Agilent Technologies.

Part 1: CE/MS requirements.

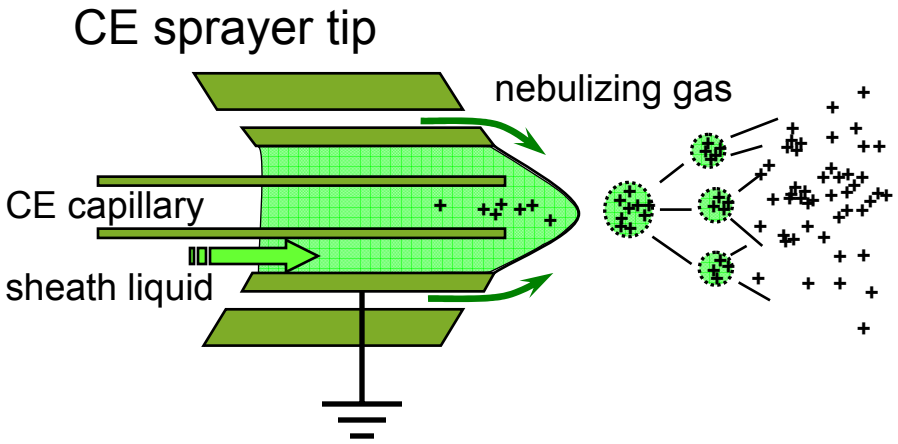
- Requirements for CE/MS interfacing
- Triple tube sprayer design
- Physical set up
  - capillary exits instrument
  - electrical connection
  - siphoning
  - sheath delivery methods
- Buffers for CE/MS
- Sheath liquid effects

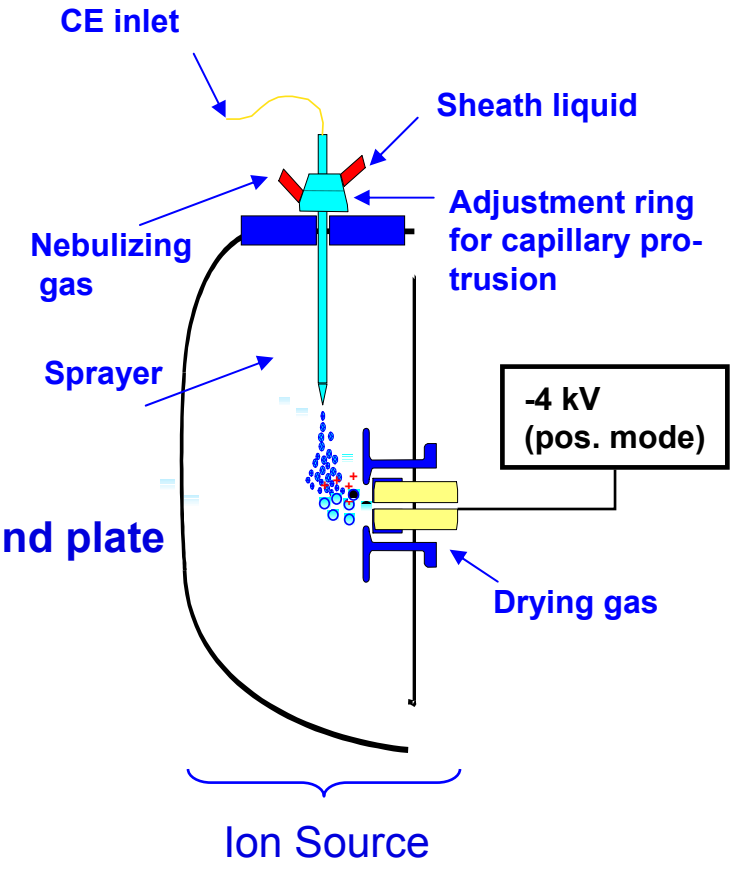
Checkpoints for On-Line CE-ESI-MS

- Capillary outside of CE instrument
- Capillary plugs in at MS
- HV on both instruments
- Physical setup
- Software control
- Compatibility of chemistry



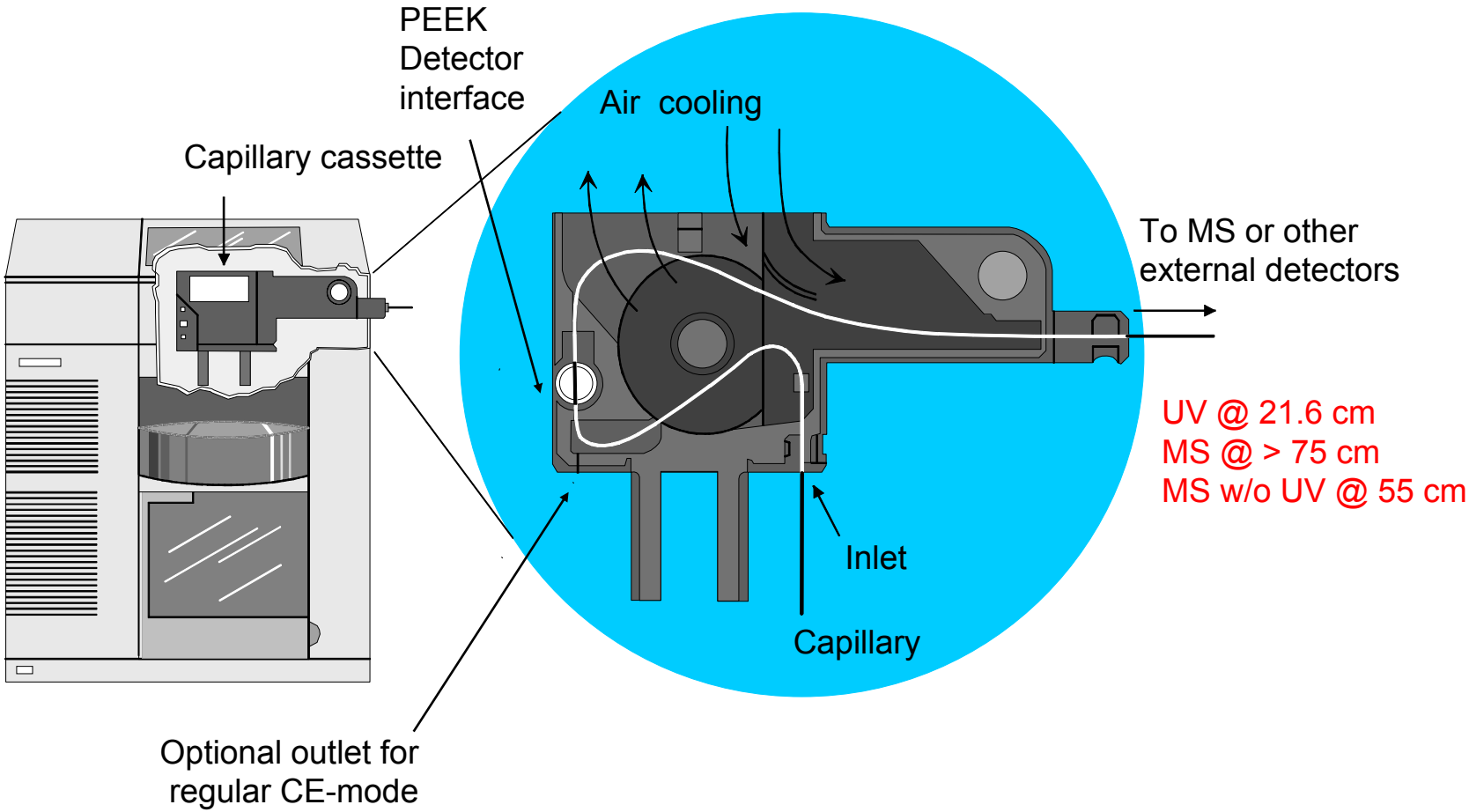
Orthogonal ESI Interface for CE/MS



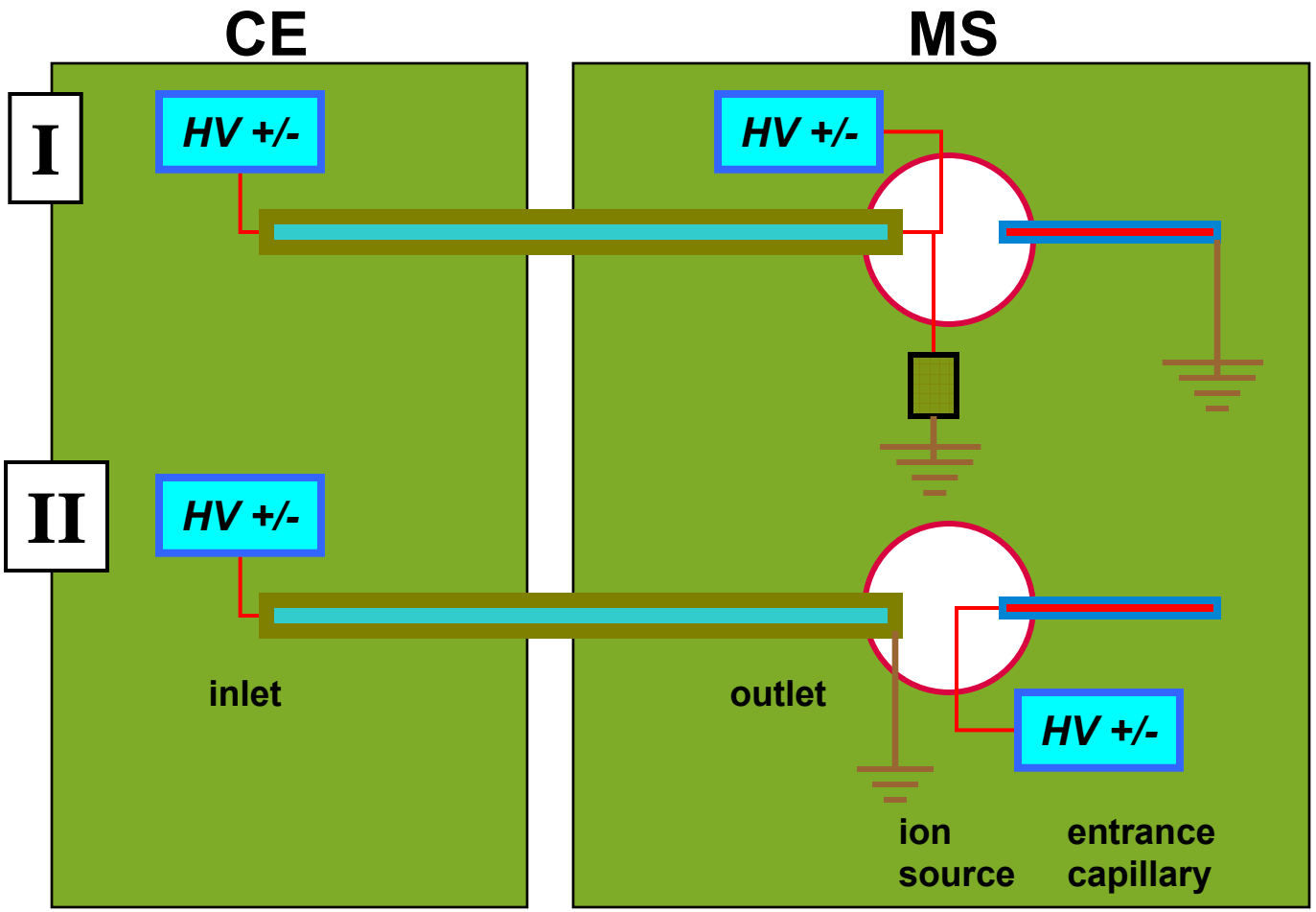


"Triple tube" or "co-axial sheath flow" design
Potential drop (ca. 4kV) between Spray needle and end plate
Sheath liquid provides electrical contact for CE.

CE/MS Cassette Design



CE/MS Electrical Interfacing



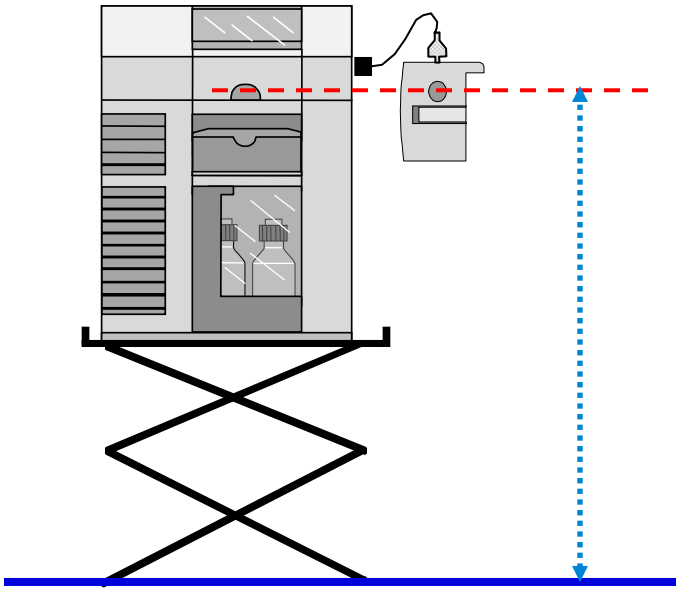
Needle at Voltage
- Field = 30kV - ES kV
- Ground cable and resistor sink required
- Polarity switching affects electrical field

Needle at Ground
- Field = 30kV

Siphoning Effects

Height adjustment to prevent siphoning effects

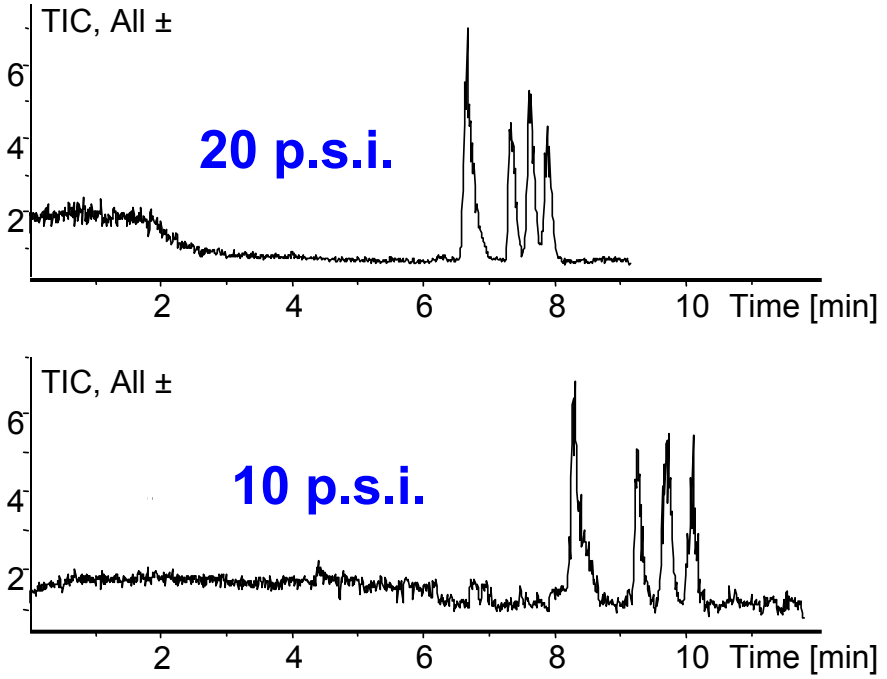
Height inlet vial liquid = Height outlet (sprayer tip)



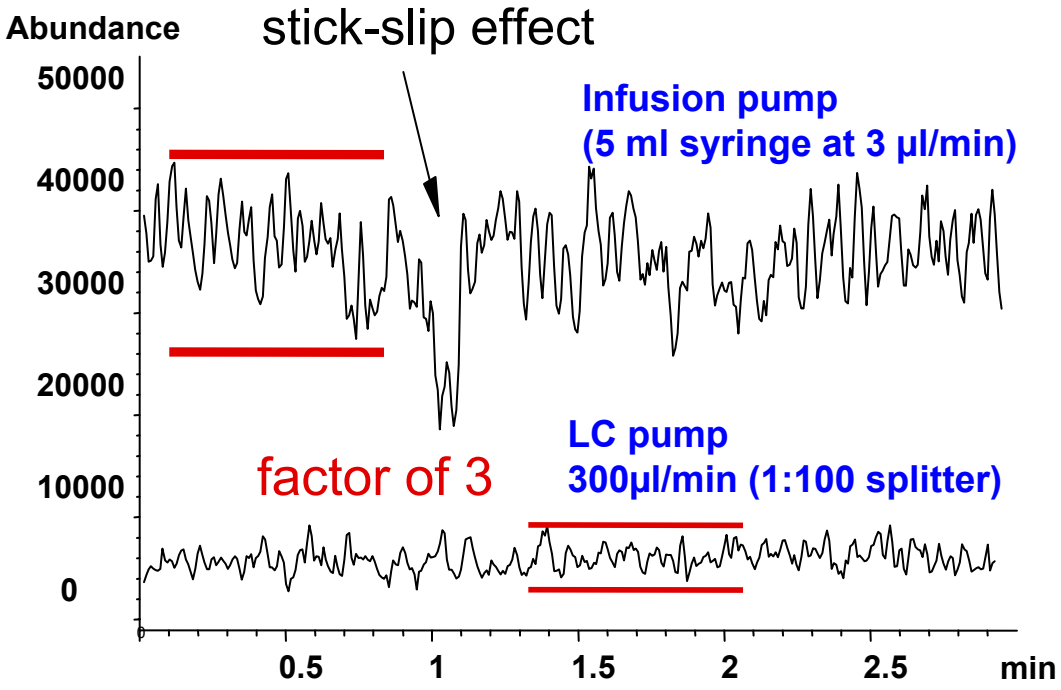
Degree of siphoning depends on capillary i.d. and length.
For 1.4cm liquid in a vial, the CE should be 1cm higher than the MS.

Due to Nebulising Gas Pressure

Capillary: 80 cm x 75µm id

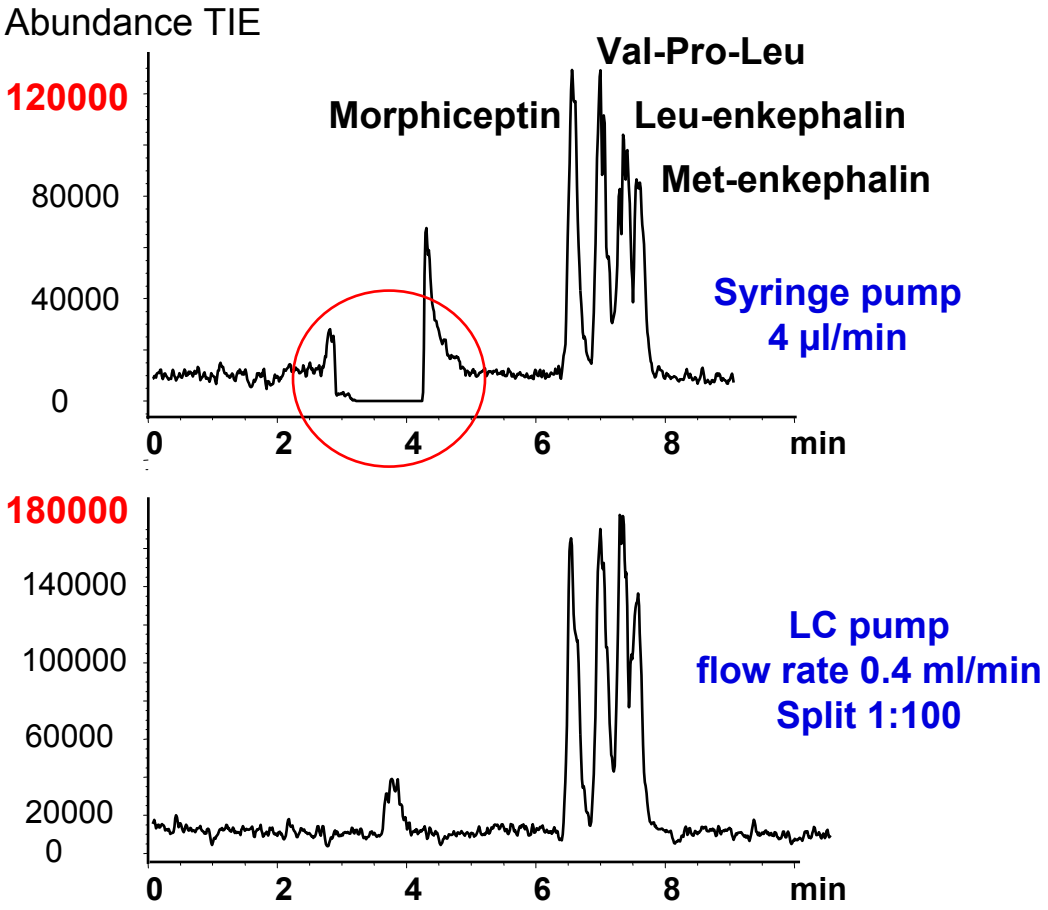


Sheath Liquid Delivery:
Flow Rates and Baseline Noise



pH	2.5	9.0
CE (µL/min)	0.042	0.212
Sheath Liquid (µL/min)	4.0	4.0
Dilution ratio	94	19

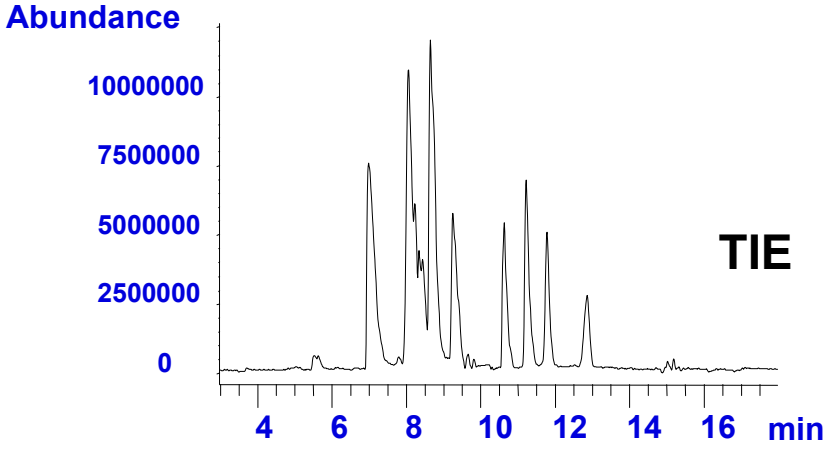
Sheath Liquid Addition - Abundance



Sample:	**0.25 mg/ml each**
Buffer:	**10 mM acetic acid**
Capillary:	**80 cm x 75µm id**
Injection:	**20 mbar x 2 s**
Voltage:	**25 kV**
Temp:	**25 °C**
DAD:	**signal 206/10 nm ref 450/80nm**
Sheath:	**0.5% HAc in 50% MeOH**
Neb. Gas:	**20psi**
Dry. Gas	**10 l/min, 150°C**
ES Volt:	**- 4kV**
MS:	**m/z 300-600**

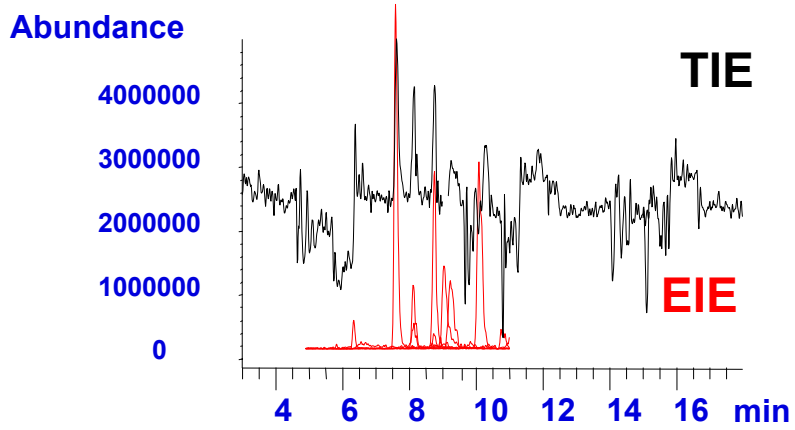
Volatile vs non-volatile buffers for CE/MS

10 mM acetic acid pH 3.4



Buffer:	20mM phosphate pH 2.5 or 10 mM acetic acid pH 3.4
Capillary:	75cm (22cm) x 50µm
Injection:	150mbar*s
Voltage:	27kV
Temp:	25°C
DAD:	206/10 nm ref 450/80nm
Sheath	0.5% HAc in 50% MeOH 4µl/min
Neb. Gas:	10psi
Dry. Gas:	10 l/min, 150°C
ES Volt:	- 4kV
MS:	m/z 350-650
Sample:	0.16mg/ml 10 peptide mix

20 mM phosphate pH 2.5

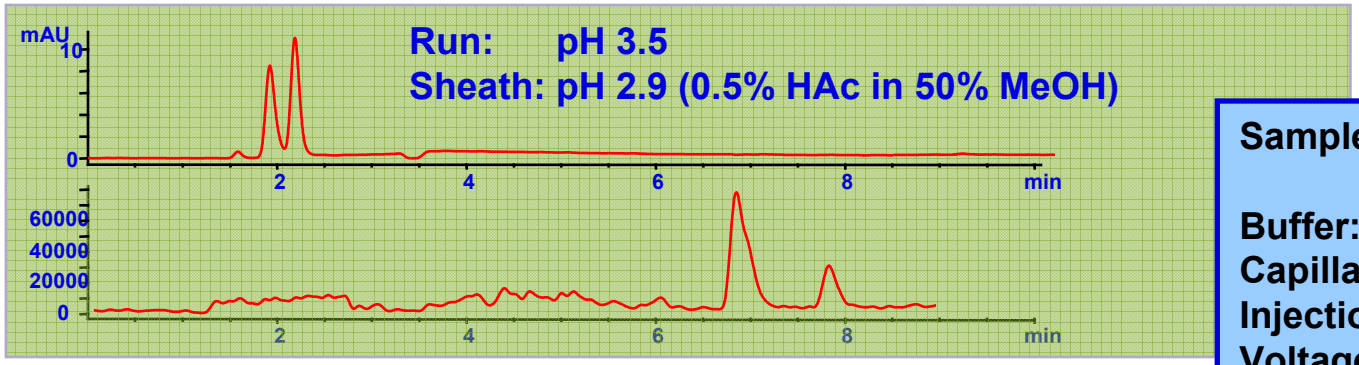


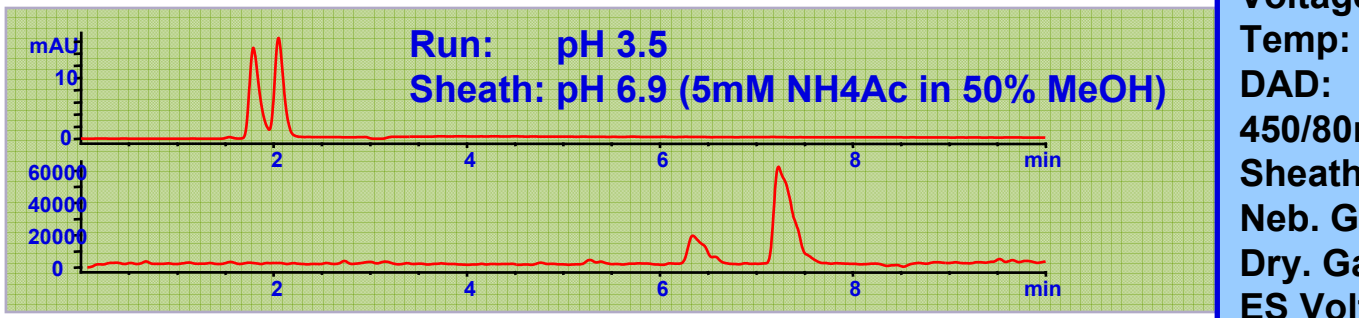
Buffers for CE/MS

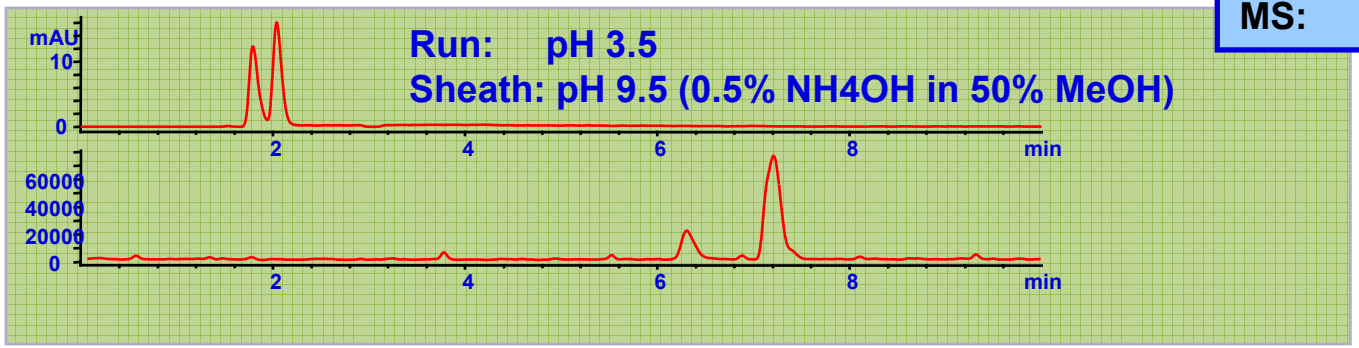
Buffers

- Acetic Acid
- Formic Acid
- Ammonium formate
- Ammonium acetate
- TRIS <20mM
- Phosphate <20mM
- Borate <10mM
- Chiral Buffers
- Try Dilute CE buffers

Effects of Varying Sheath Liquid pH
Angiotensin I and II migration times.

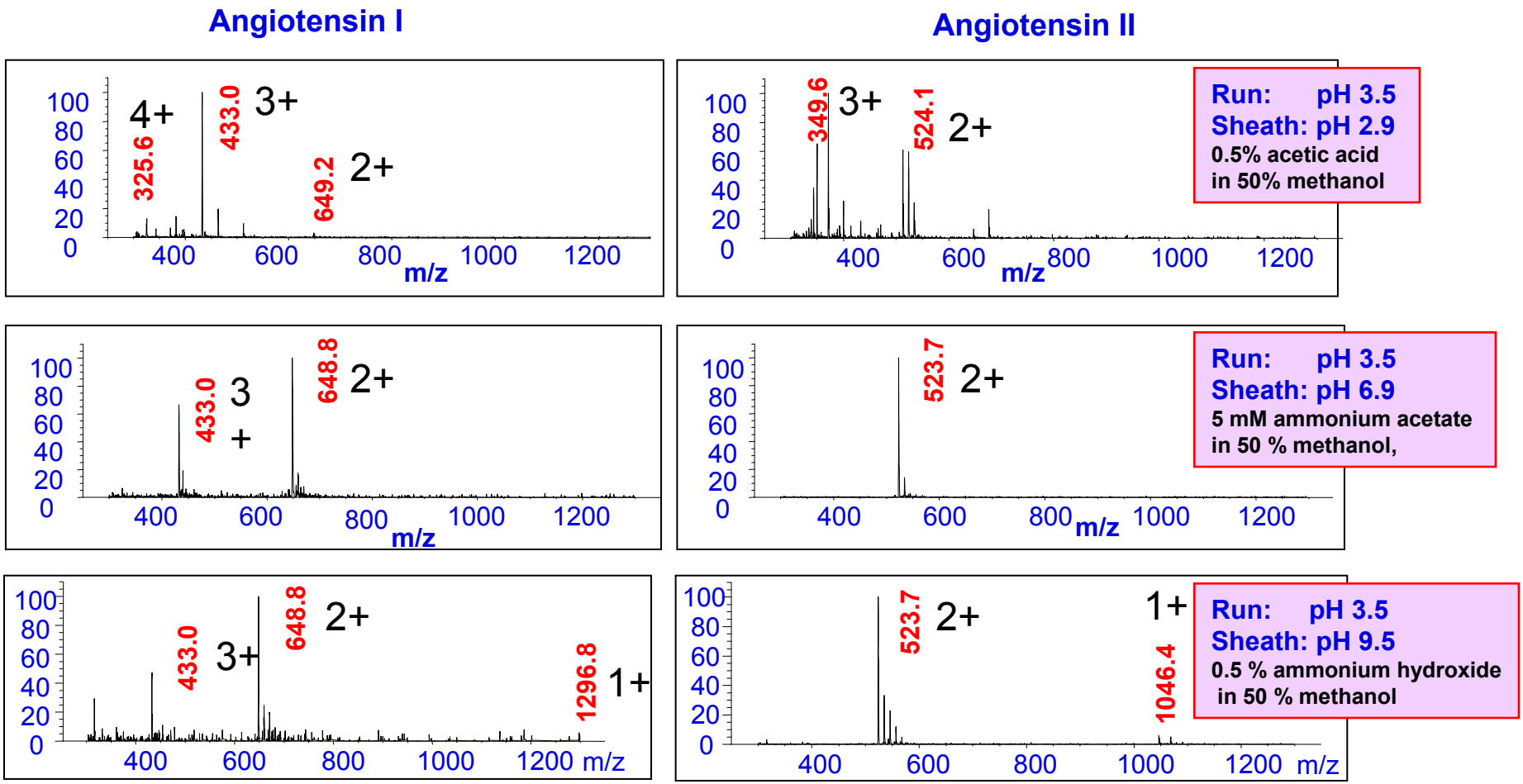






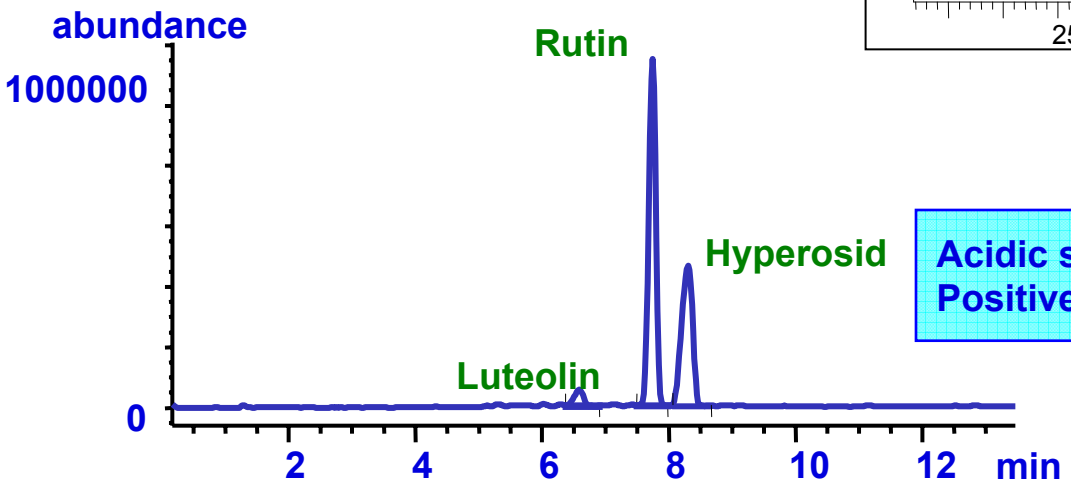
Sample:	**0.05mg/ml peptide**
Buffer:	**10mM HAc pH 3.5**
Capillary:	**75cm (22cm) x 50µm**
Injection:	**300mbar*s**
Voltage:	**27kV**
Temp:	**25°C**
DAD:	**206/10 nm ref 450/80nm**
Sheath	**4µl/min**
Neb. Gas:	**10psi**
Dry. Gas:	**10 l/min, 150°C**
ES Volt:	**- 4kV**
MS:	**m/z 300-1300**

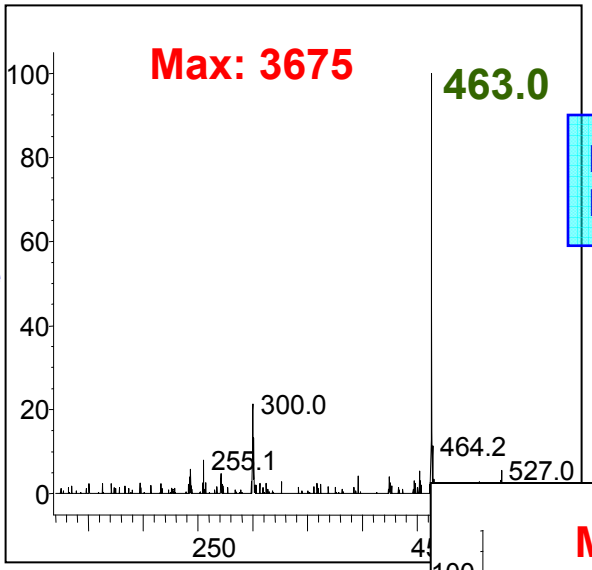
Influence of Sheath Liquid :
Ionisation State of Angiotensin I and II (Mass Spectra)



Influence of Sheath Liquid
Ion polarity, optimisation of signal

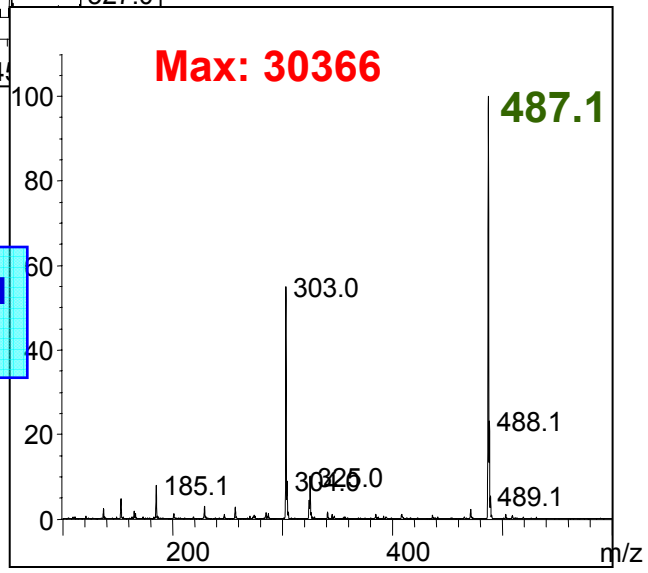
- Capillary — 75 cm tot (22cm to UV) x 75 µm ID
- Buffer — 5 mM Borate pH 9.3
- Voltage — 30 KV
- Temperature — 25° C
- Detection — 275 nm
- Injection — 10s @ 50 mbar
- Sheath Flow — 4 µl/min 1:1 MeOH / 1% Formic Acid
  or 1:1 IPA / 0.5% Ammonium Hydroxide
- API-ESI — Positive or Negative Ion, Full Scan Data
- Fragmentor — 150 V
- Dry. Gas Flow — 5 l/min Nitrogen
- Dry. Gas Temp — 150° C
- Nebulizer — 10 psi





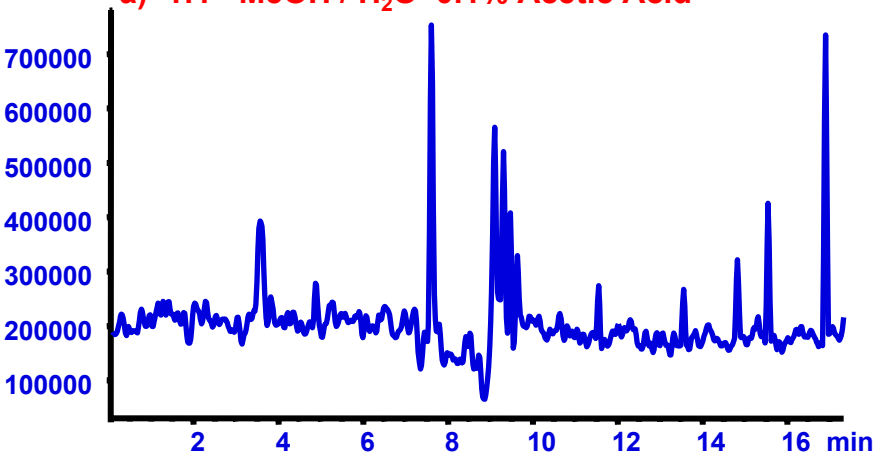
Basic sheath liquid
Negative ion mode

Acidic sheath liquid
Positive ion mode

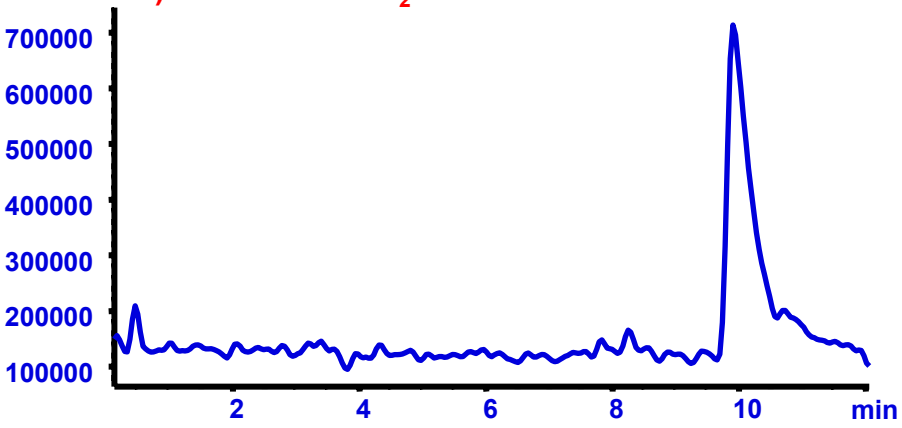


Influence of Sheath Liquid on MS Signal

a) 1:1 MeOH / H_2O 0.1% Acetic Acid



b) 1:1 MeOH / H_2O 1% Formic Acid

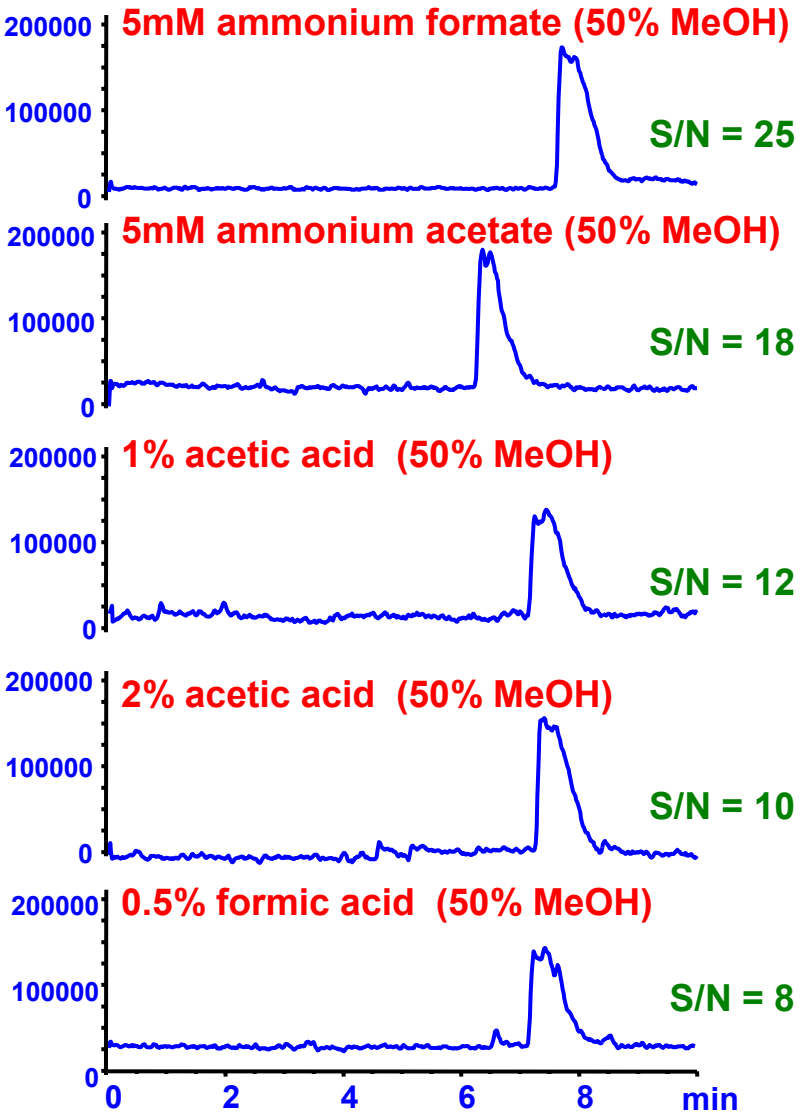


Capillary: 75 cm tot. l., 50 µm ID

Sample: 22kDa protein

Buffer	50 mM Am.Acetate pH 6.8
Voltage	30 KV
Temp	30° C
Detection	220 nm
Injection	50 mbar 10 sec
Sheath Flow	5 µl/min
	a) 1:1 MeOH / H2O 0.1% Acetic Acid
	b) 1:1 MeOH / H2O 1% Formic Acid
API-ESI	Positive Ion, Scan 1000 - 2100 amu
Fragmentor	Variable V
Dry. Gas Flow	4 l/min Nitrogen
Dry. Gas	Temp 150° C
Nebulizer	25 psi

Effects of Sheath Liquid Composition S/N



Buffer: 100mM formic acid

Sample: tetrandrine and fangchinoline

Capillary 50µm i.d. x 76cm (UV at 22cm)

Voltage: 27kV

Temperature: 20°

Injection: 200mbar*s

Detection: UV 200nm

Sheath: see caption

Flow rate: 5µl/min

Neb Gas: N_2, 10p.s.i.

Dry Gas: N_2, 250°C

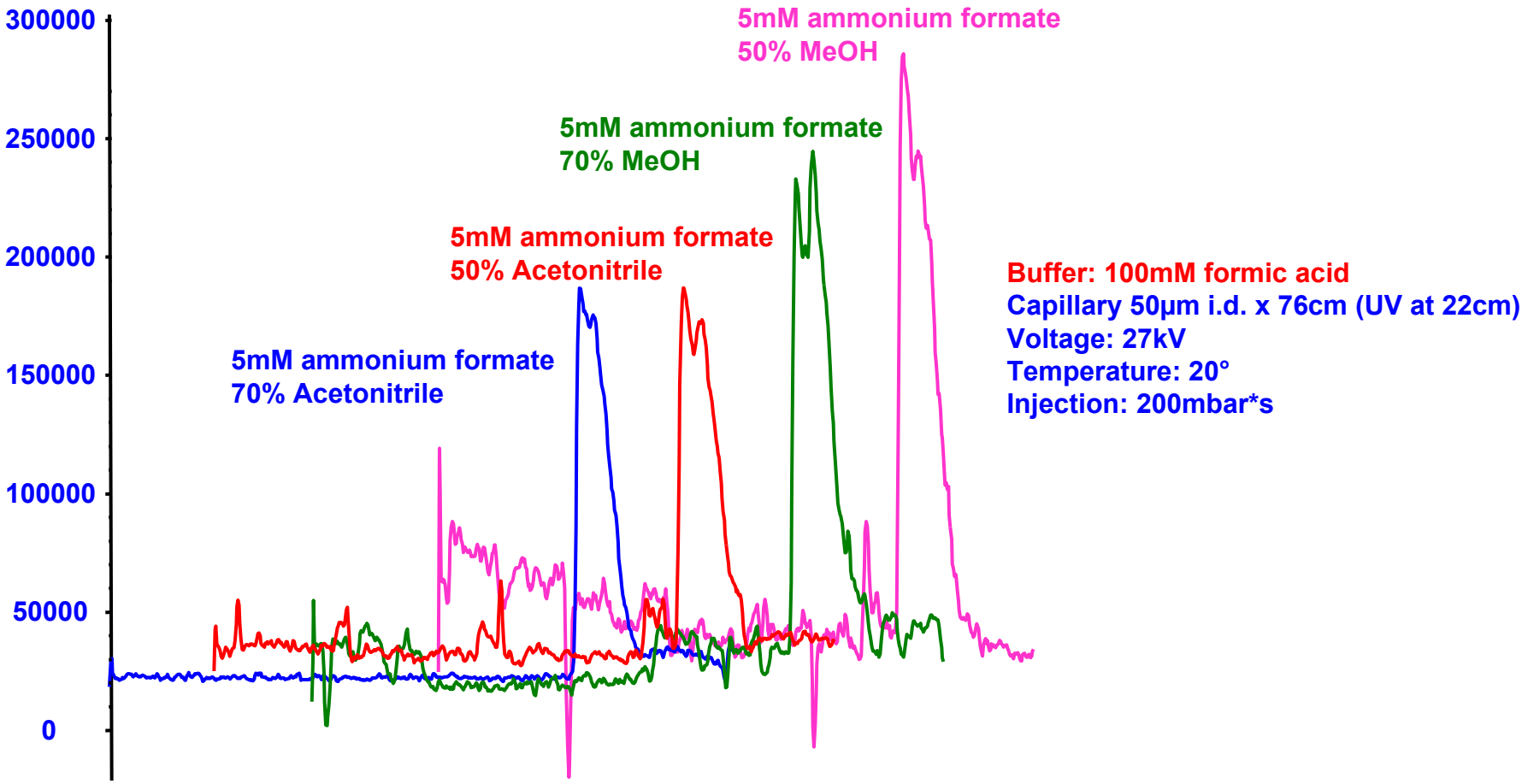
Acquisition: Positive Ion Mode

Vcap: - 4.0 kV

Frag: 70V

Scan: 300m/z to 650m/z

Effects of Sheath Liquid Organic Modifer on s/n

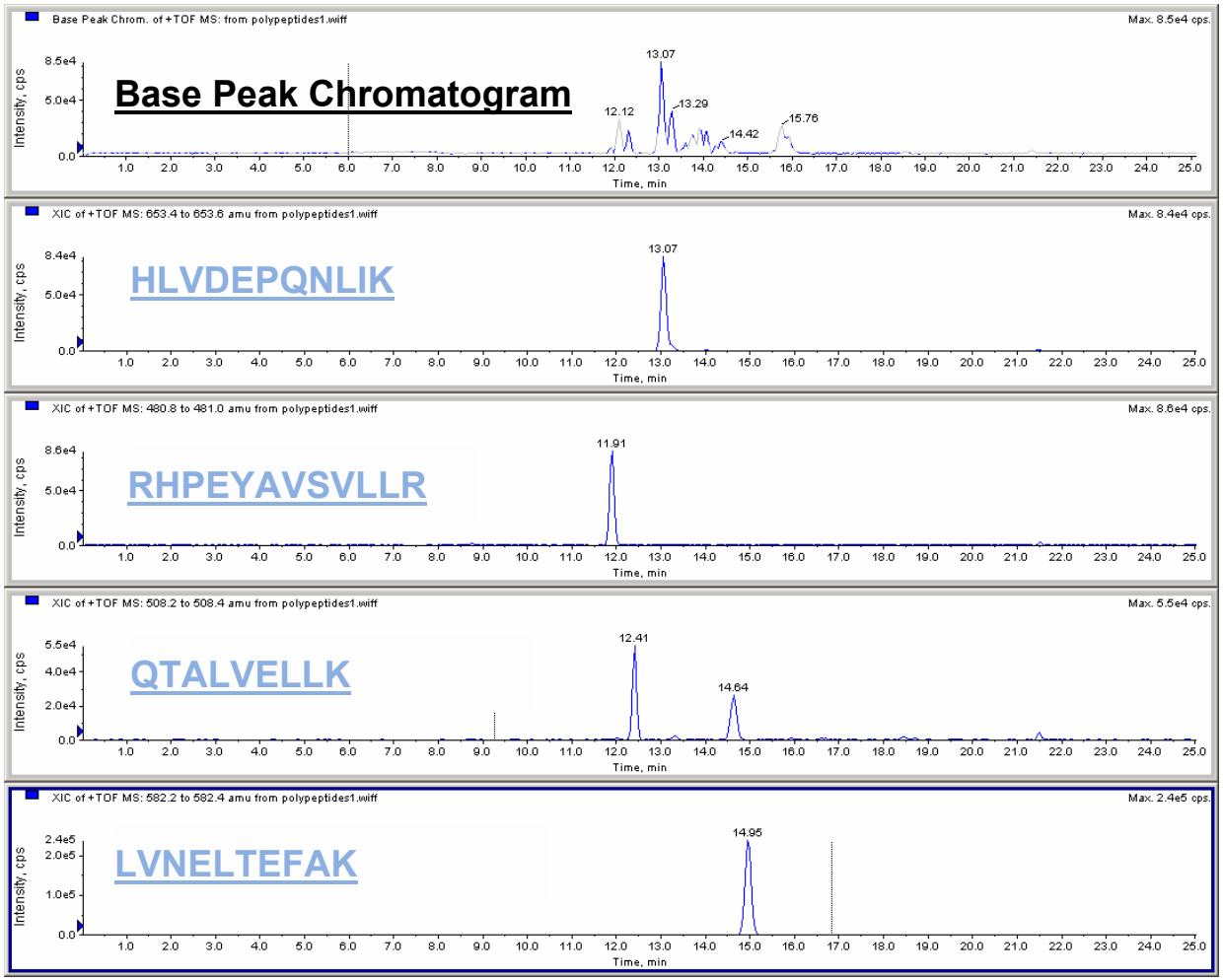


Part 2: CE-TOF Set-up

Agilent TOF:	**External calibration; Auto Tune; Vcap: 3900 V**
Agilent CE:	**Fused silica capillary: 90 cm x 50 µm**
	Buffer: ACN, i-Prop, HCOOH
	Sheath Flow: 2 µl/min
	Injection: 50 mbar x 60, 75, 150, 300 sec
Samples run:	**BSA digest (1 pmol/µl)**
	BSA (0.05 pmol/µl)
	Polypeptide Standard
	Complex tryptic digest from Bacteria

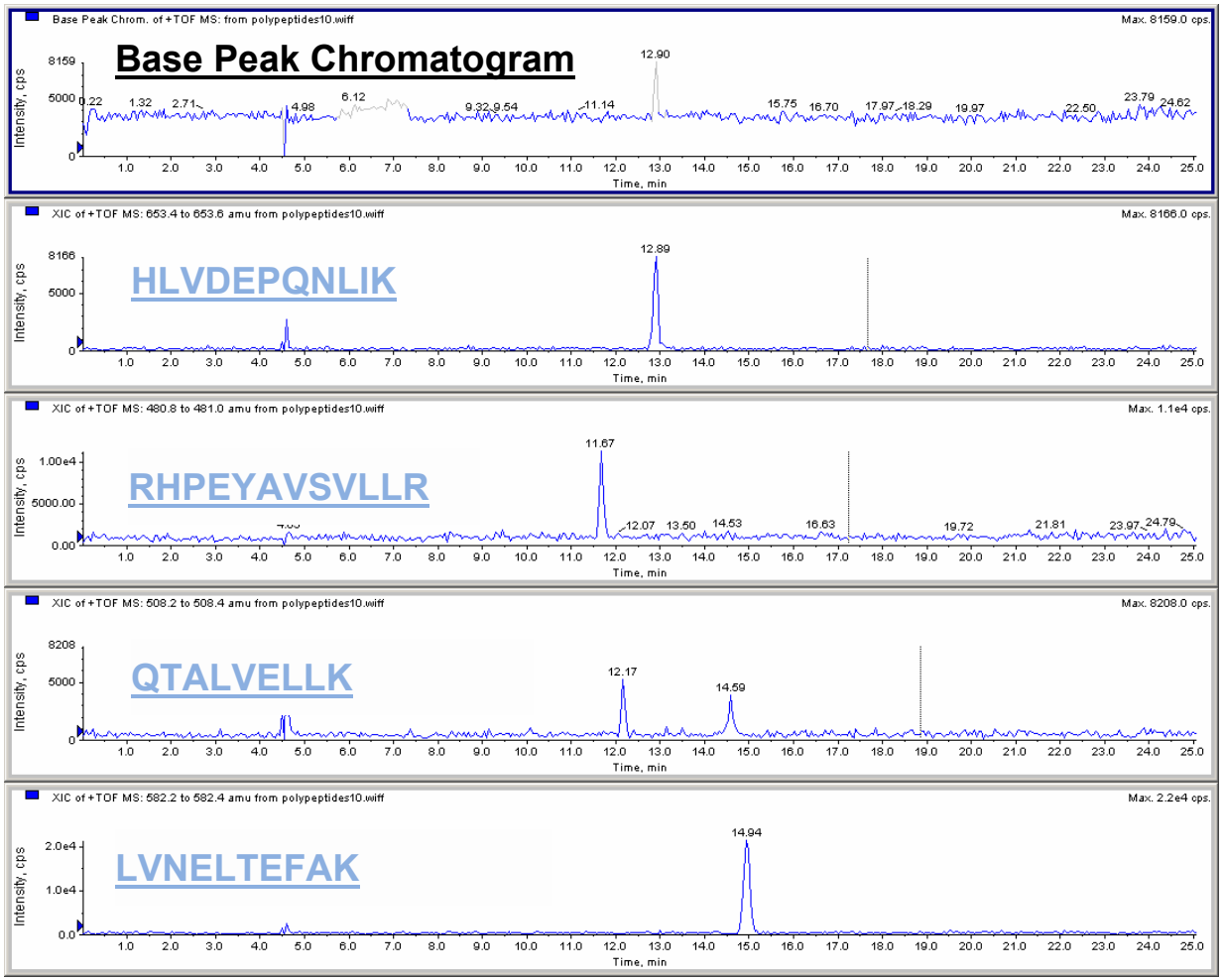
CE-TOF of BSA Digest

1 pmol/μl (50mbar * 75 sec ~ 88 fmol)



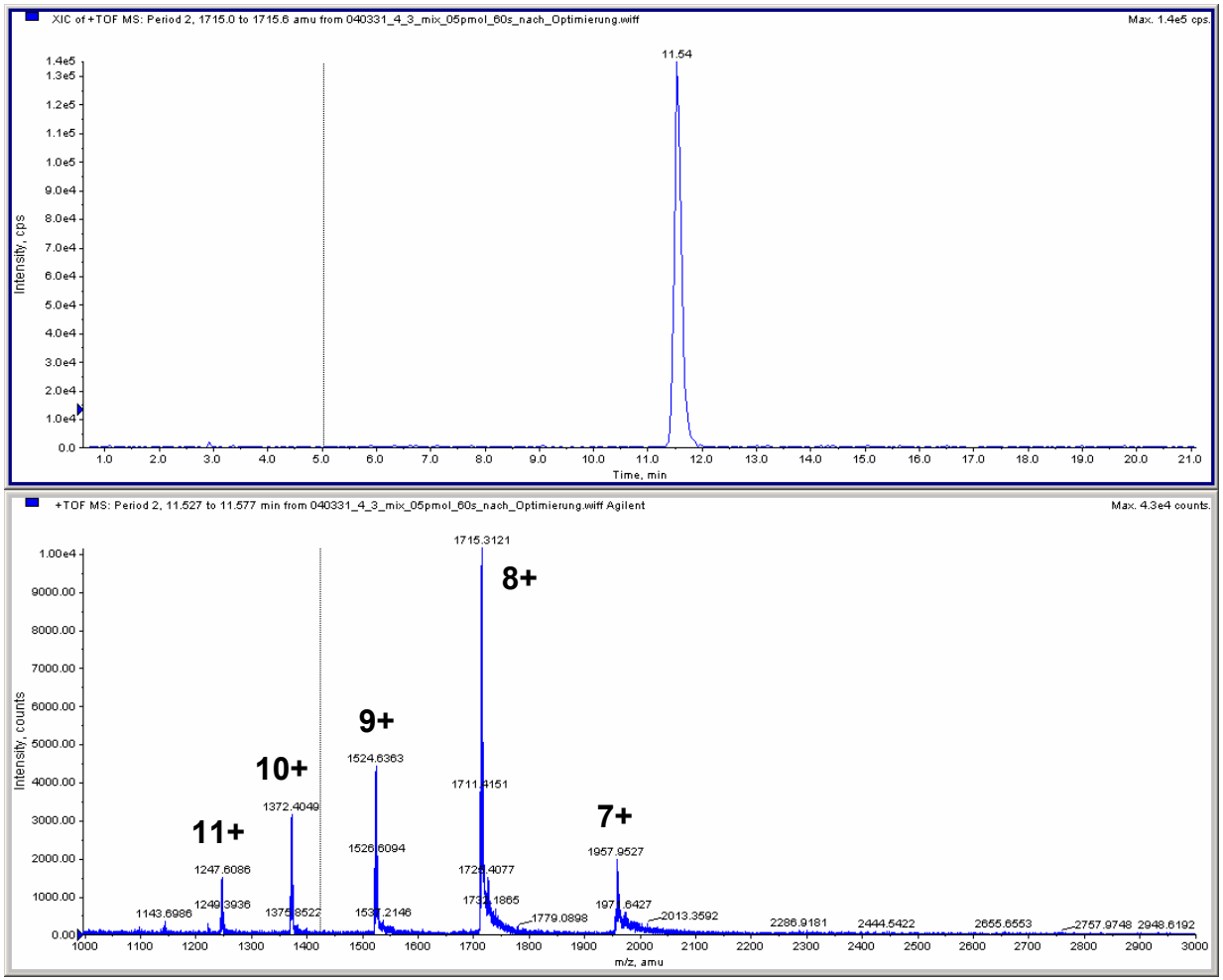
CE-TOF of BSA Digest

0.05 pmol/μl (50mbar * 75 sec ~ 4.4 fmol)



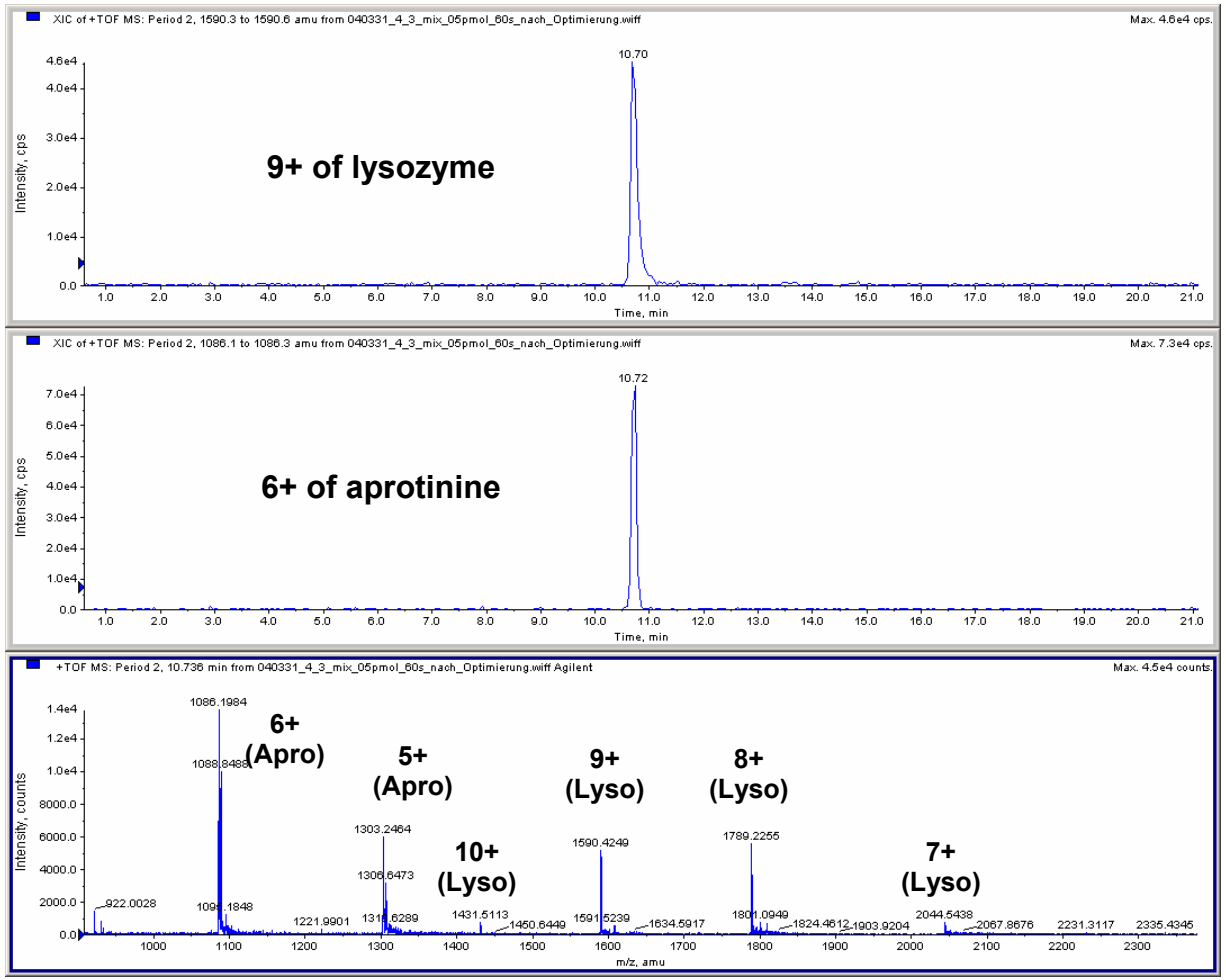
CE-TOF of Ribonuclease (13.6 kDa)

0.5 pmol/μl (50mbar * 60 sec ~ 35 fmol)



Lysozyme (14.3 kDa); Aprotinin (6.5 kDa)

0.5 pmol/μl (50mbar * 60 sec ~ 35 fmol)



Part 3: Molecular Profiling Toolkit

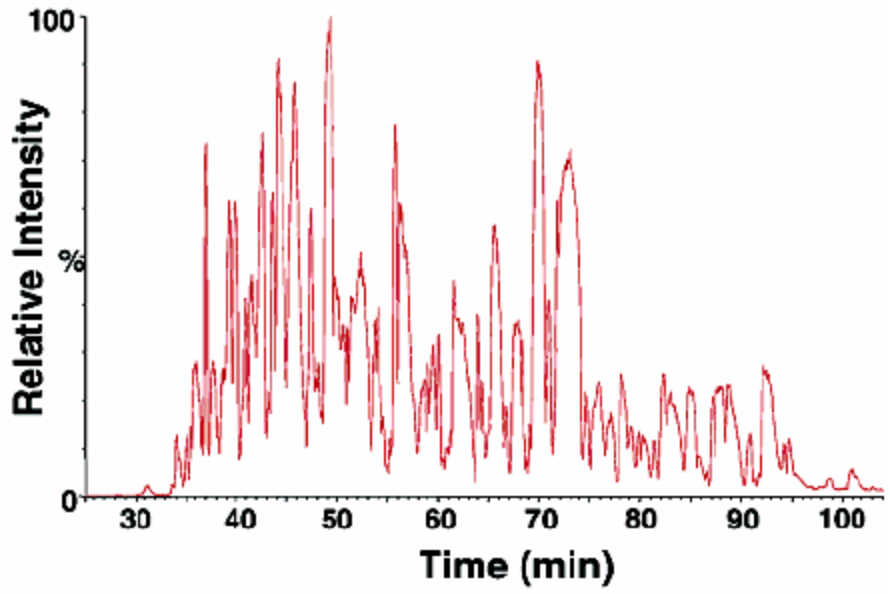
Mass Hunter/Mass Profiler: New Software Tools for Molecular Profiling with High-Performance LC or CE/TOF MS

Molecular Profiling

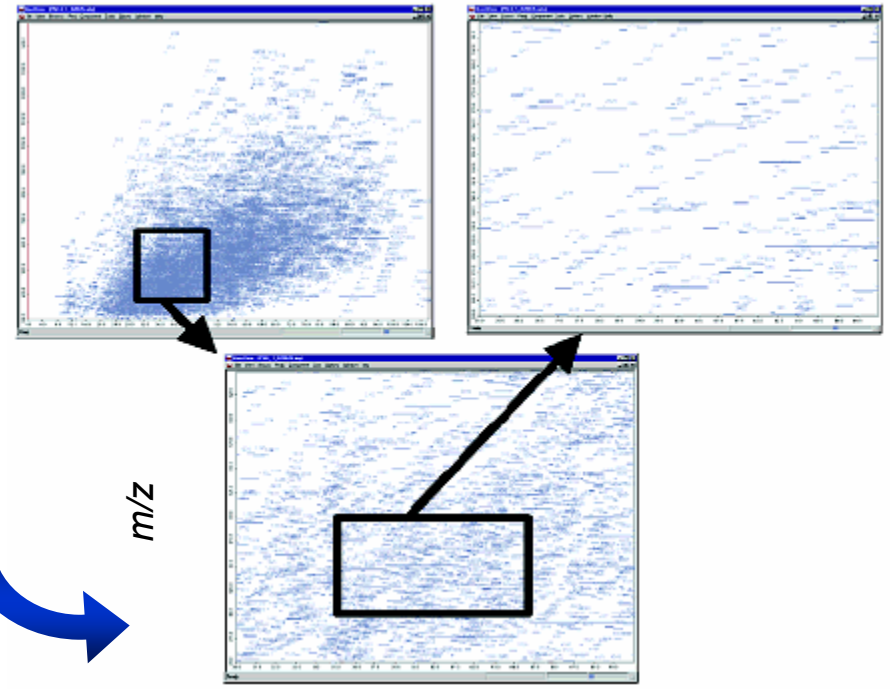
Comparative expression analysis for proteomics, metabolomics or other ‘omics technologies

- Measurement of potentially relevant molecular features from one or more LC or CE/MS analyses
- Differential expression analysis to reveal features that separate one sample set from another
- Selection of differential features for subsequent MS/MS identification
- Database searching and/or manual spectral interpretation remains an option at any point in the data reduction process

Expression Profiling of Proteins and Metabolites by LC or CE/ESI-TOF



BPC of digested human serum proteome.



2-D display of the LC or CE/ESI-TOF spectra, each plotted element is a separate molecular ion (component)

High resolution, high mass accuracy TOF permits superior reproducibility, sensitivity, and specificity for low abundance molecules

Analytical Chemistry, Vol. 75, No. 18, September 15, 2003

Agilent's Molecular Profiling Toolkit – Enabling Effective and Efficient Molecular Profiling and Differential Analysis

A multi-stage informatics system - starts with raw mass spectral data and ends with the discovery of statistically significant, differentially expressed molecular features

- Stage 1 – Extracting molecular features from individual LC or CEMS analyses (*Mass Hunter)*
- Stage 2 – Profiling of molecular features from closely related ("like") sample sets i.e., feature alignment within sample sets (*Mass Profiler)*

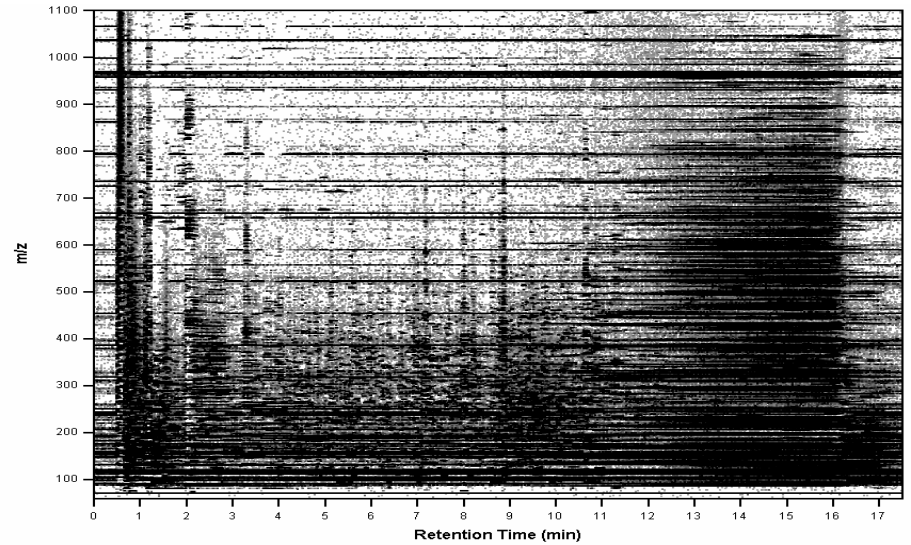
Mass Hunter: Molecular Feature Extraction

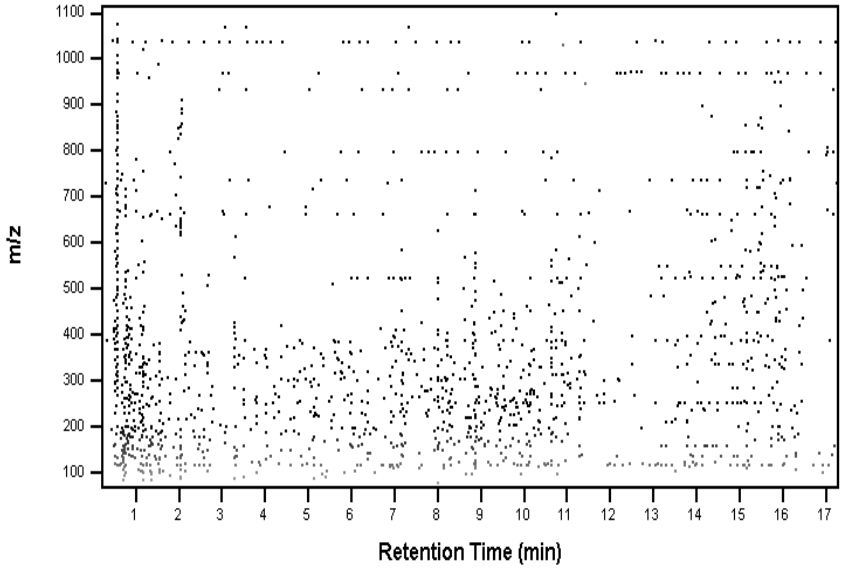
Molecular feature: a discrete molecular entity defined by combination of retention time, mass and response in an LC or CE/MS analysis.

Mass Hunter operates on raw mass spectral data generating lists of chemically qualified molecular features (eliminates interferences and reduces data complexity)

- Persistent chemical background is removed
- Co-eluting interferences are resolved
- Isotopic cluster recognized and grouped
- Charge state assignments and molecular adducts are recognized
- 2D/3D Data visualization
- Chemical identification (ppm, isotope matching)
- Feature lists storable in space-efficient binary format, and saved as text files.

Mass Hunter – Data Mining / Profiling – Step 1





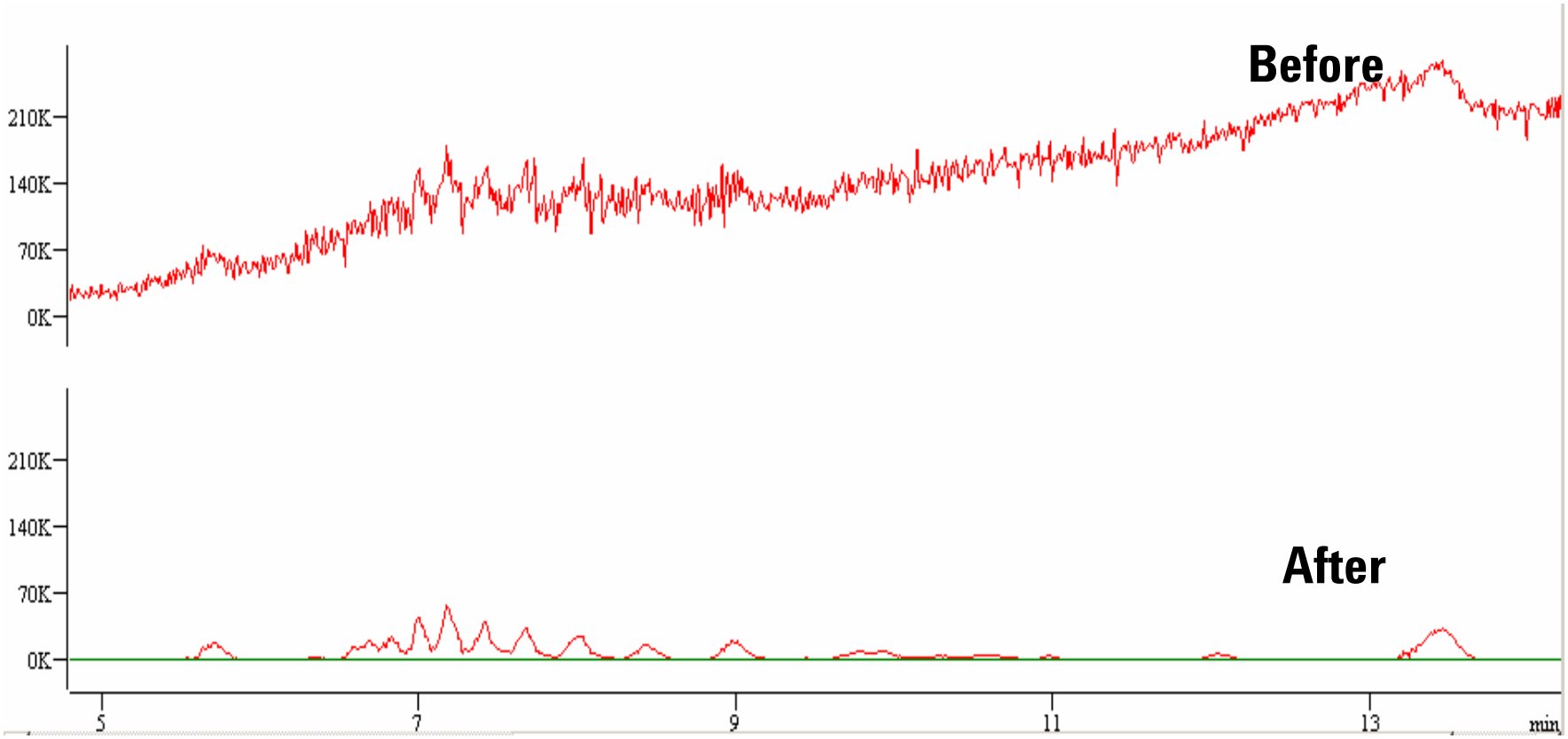
20 Million Data Points => 1500 Molecular Features

- Uses specificity in both Retention time and mass dimensions
- Removes persistent chemical noise and co-eluting interferences
- Performs charge state assignment, deisotoping, and adduct determination

Extracts all relevant chemical information for subsequent profiling

Mass Hunter Graphics

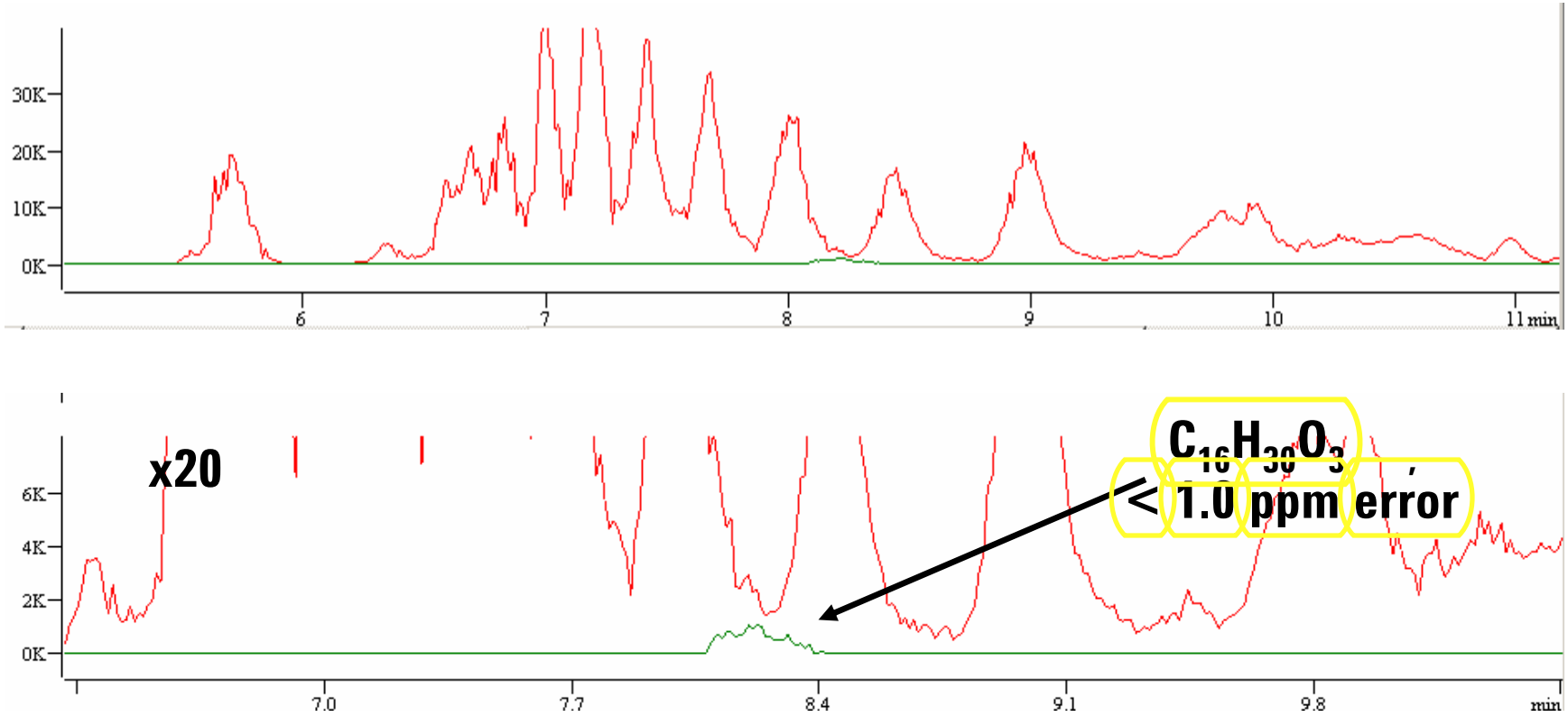
Mitochondria $CHCl_3$ Extract Human Lymphocyte Cells



Extract from the Medical College of Wisconsin

Wide Dynamic Range TIC/EIC *m/z* 270.2181

Oxidative addition to Palmitoleic Acid $C_{16}H_{30}O_2$



Extract from the Medical College of Wisconsin

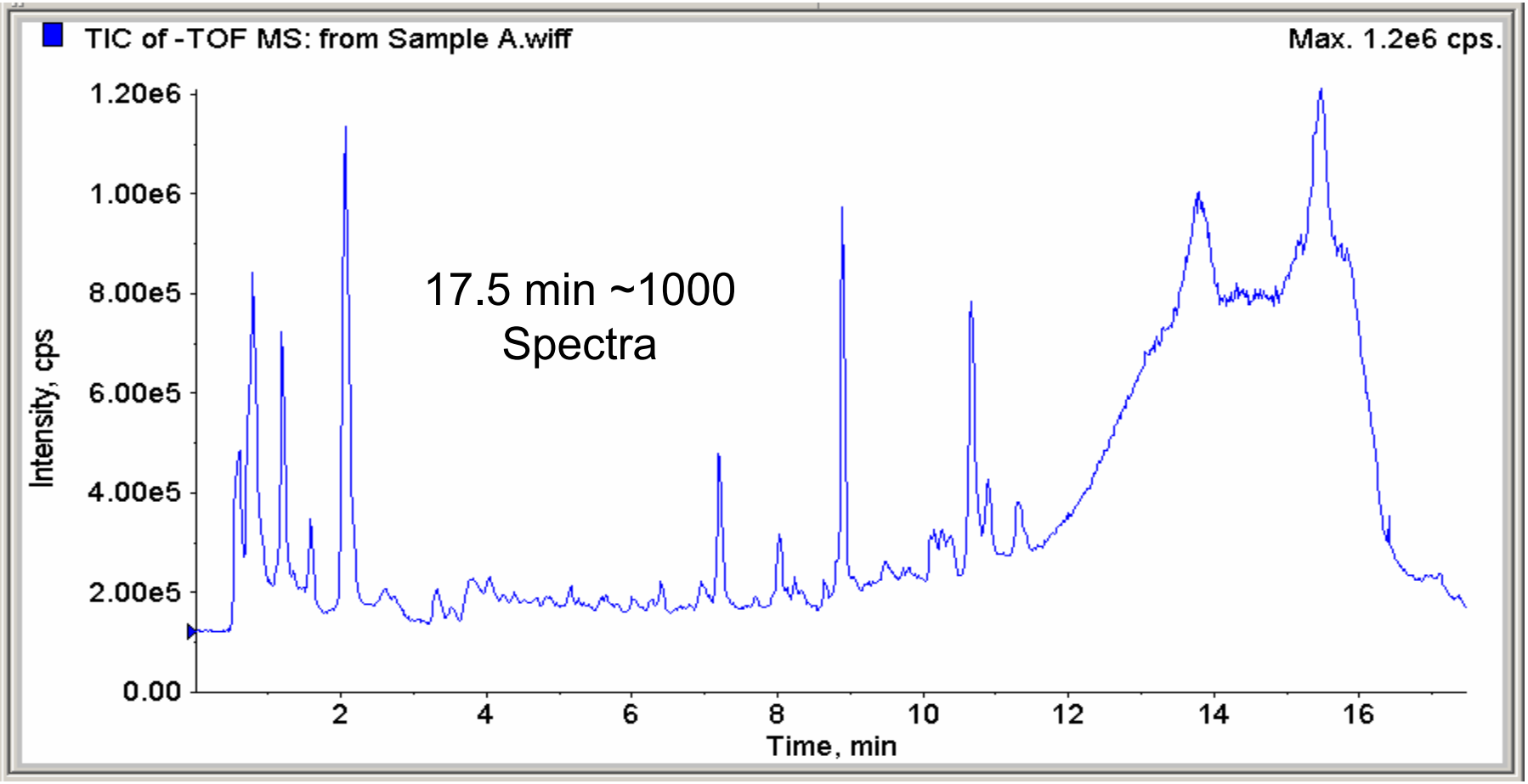
Accurate Mass Accuracy Across Mass Range

Retention Time	Compound	Neutral Formula	Measured m/z	Calculated Mass [M+H]+	ppm Error
11.901	C18:0	C18H36O2	283.2646	283.2642	1.22
11.075	C18:1	C18H34O2	281.2487	281.2486	0.3406
10.447	C18:2	C18H32O2	279.2332	279.2329	0.8806
10.879	C16:0	C16H32O2	255.2327	255.2329	-0.996
10.2	C16:1	C16H30O2	253.2181	253.2172	3.14
9.831	C14:0	C14H28O2	227.202	227.2016	1.52
9.05	C14:1	C14H26O2	225.1867	225.186	3.09
13.068	C20:0	C20H40O2	311.2953	311.2955	-0.817
8.005	C16:1 (O)	C16H29O3	269.2127	269.2122	1.79
8.38	C18:1 (O)	C18H33O3	297.2438	297.2435	0.946

Mass Profiler step 2

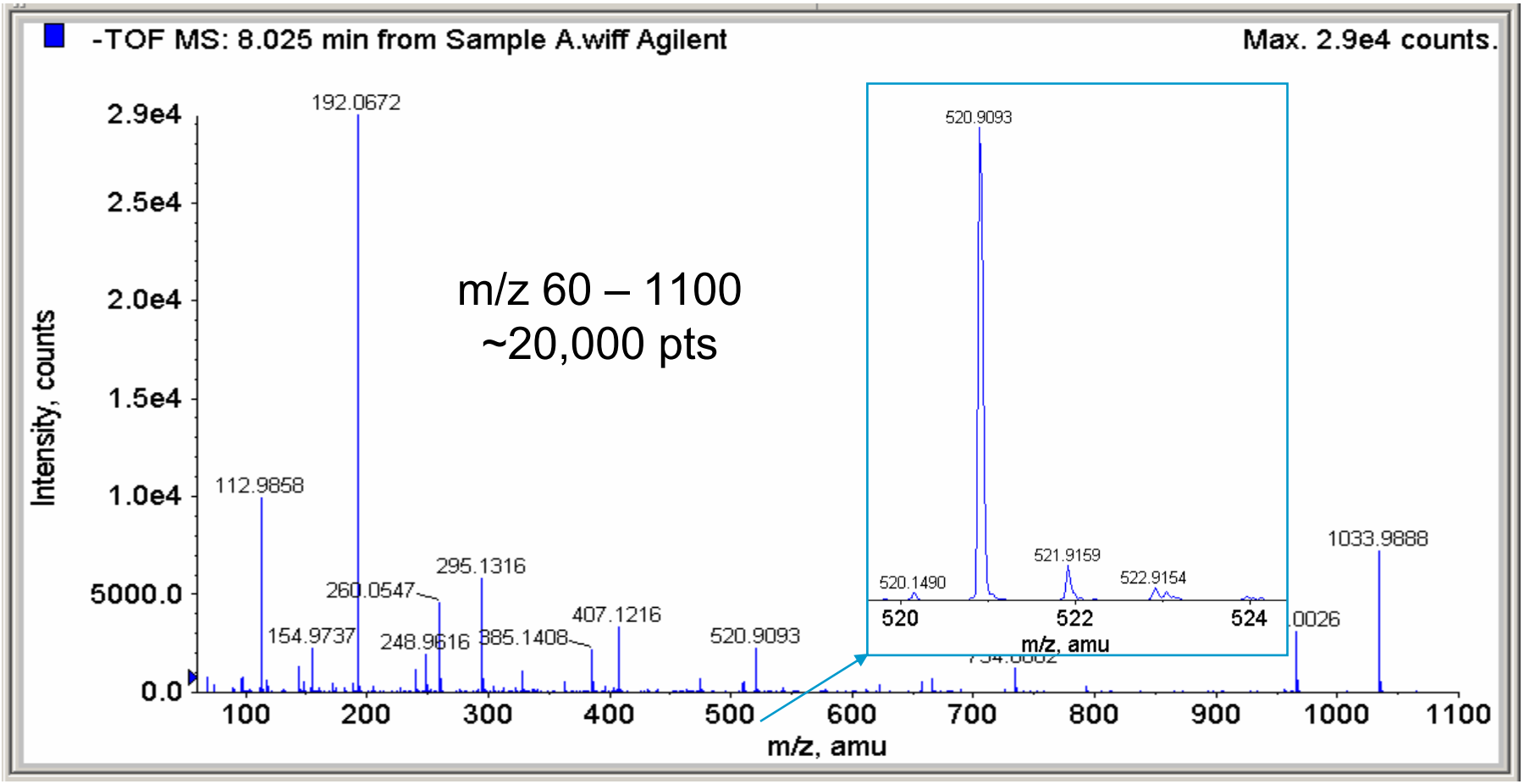
- Enables loading of a large number of molecular feature lists
  - Statistical assessment of feature occurrence and variation in retention time, mass and abundance across composite (one group) molecular feature set
  - Statistical comparison of two different groups of molecular feature sets
    - Multiple Visualization and identification of statistically significant molecular feature differences between the groups
  - Feature Details view provides for facile review of chemical details of putative differential features, and comparison of these details across the total groups of samples
  - Export a Molecular Profile table in text format
    - allow user-generated reports with programs such as Excel
    - transfer of differential feature lists to database search and/or other identification environments or statistical analysis packages (GeneSpring MS)

Total Ion Chromatogram of TOF Analysis of Sample A (Control Rat Urine)



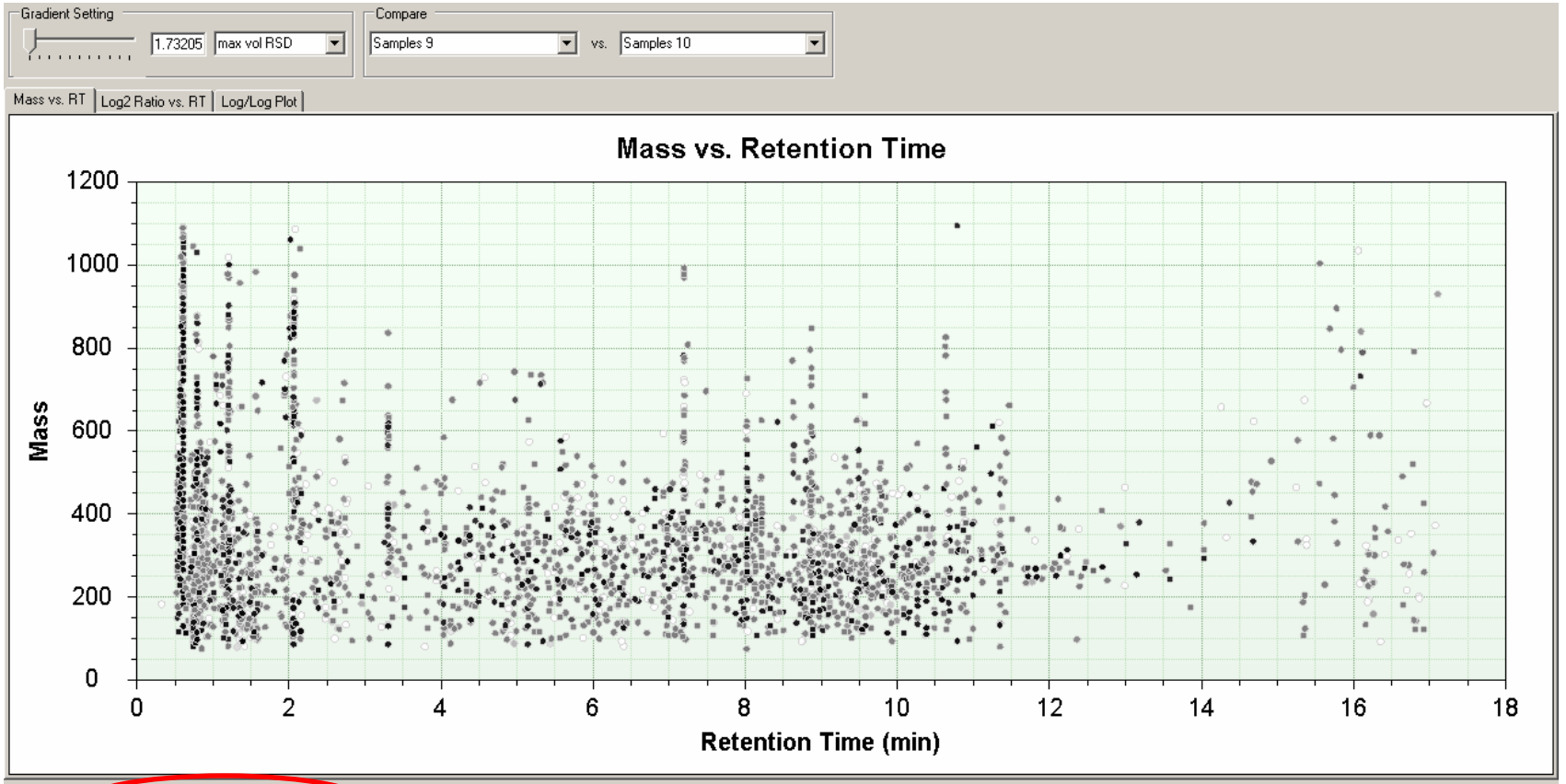
Data Courtesy of Mike Reily and Laura Egnash Pfizer Ann Arbor

One of Approximately 1000 High Resolution Spectra from a Single TOF/MS Analysis



Differential Biomarker Expression in Urine

All observed features in 3 replicates of treated/untreated rat



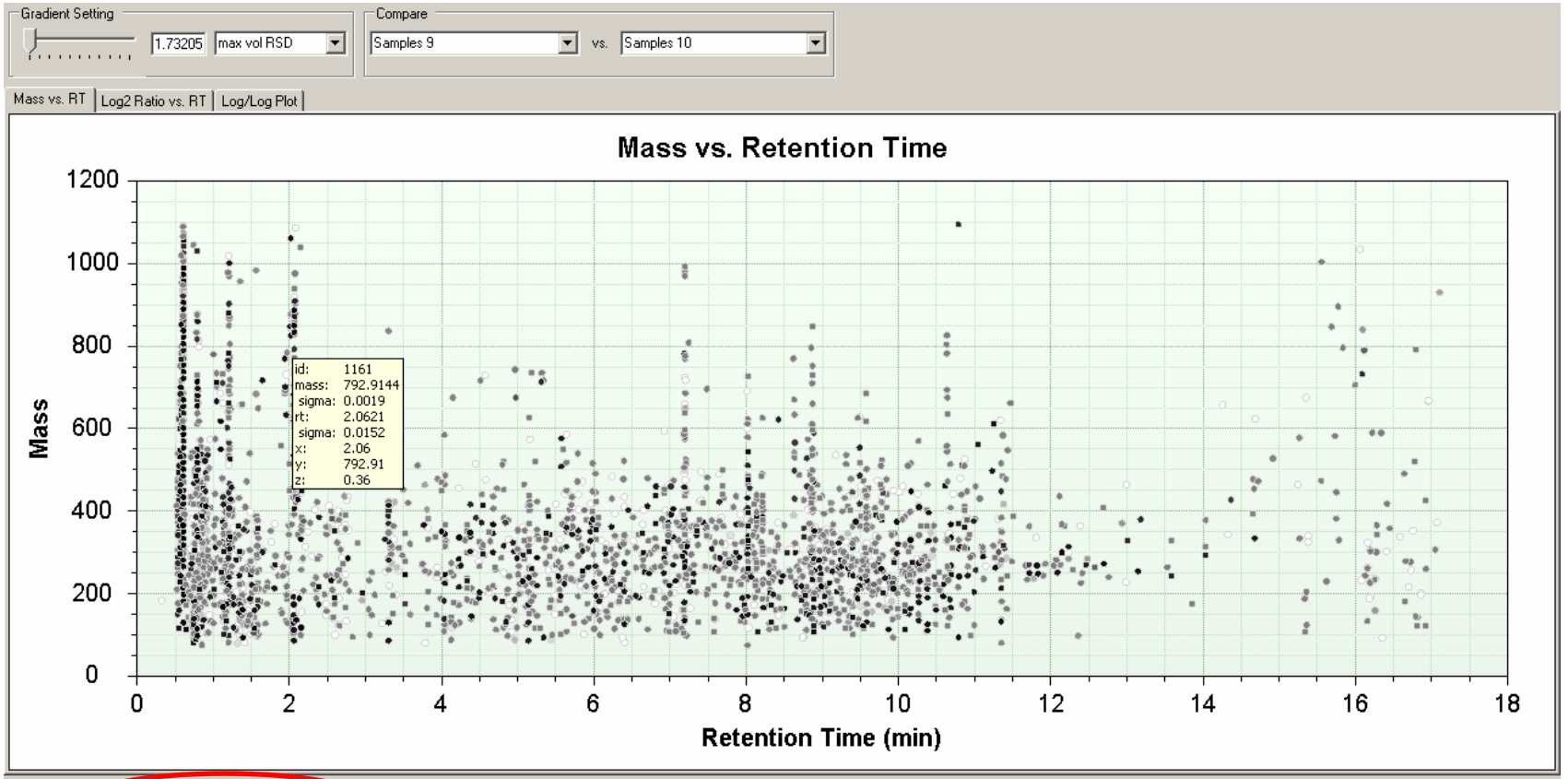
Export

18375 Differential Features

	Feature Summary								Samples 10(3)					Samples 9(3)					Comparison				
	ID	RT	sgm RT	mass	Sgm mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	Dlt RT	Dlt mass	lg2(V1/V2)	|lg2(V1/V2)|	Diff. Score
1	1	15.688	0.121	846.9617	0.0017	0.0	1.52	3			0.0		0	15.688	846.9617	0.1	0.84	3			-16.00	16.00	89.3
2	2	16.662	0.133	278.8313	0.0010	0.0	1.10	3			0.0		0	16.662	278.8313	0.0	0.10	3			-16.00	16.00	100.0
3	3	8.865	0.018	610.1441	0.0014	0.1	1.11	3			0.0		0	8.865	610.1441	0.1	0.12	3			-16.00	16.00	100.0
4	4	9.751	0.025	284.0701	0.0008	0.0	1.10	3			0.0		0	9.751	284.0701	0.1	0.09	3			-16.00	16.00	100.0
5	5	9.467	0.026	626.1490	0.0017	0.0	1.14	3			0.0		0	9.467	626.1490	0.1	0.24	3			-16.00	16.00	99.8

Differential Biomarker Expression in Urine

Common features present in all replicate injections



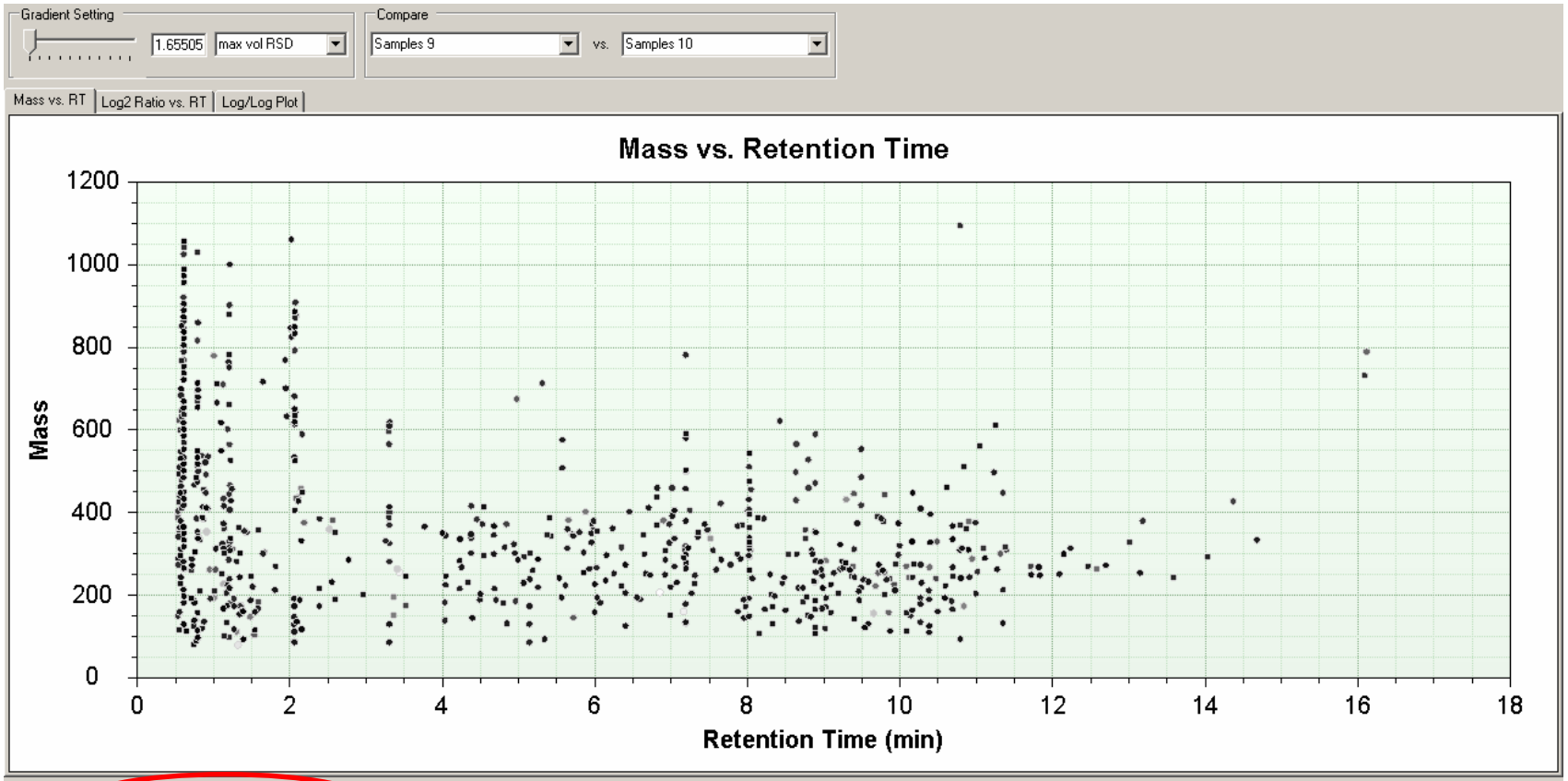
Export

2430 Differential Features

	Feature Summary								Samples 10(3)					Samples 9(3)					Comparison				
	ID	RT	sgm RT	mass	Sgm mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	Dlt RT	Dlt mass	lg2(V1/V2)	llg2(V1/V2)l	Diff. Score
316	316	7.700	0.019	364.0778	0.0009	0.4	0.95	5	7.716	364.0782	0.1	0.87	2	7.689	364.0776	0.7	0.11	3	-0.027	-0.0006	-3.66	3.66	100.0
317	317	9.056	0.108	356.1115	0.0006	0.1	0.97	4	9.216	356.1121	0.0	1.73	1	9.003	356.1113	0.1	0.17	3	-0.213	-0.0009	-3.66	3.66	99.8
318	318	8.882	0.020	107.0488	0.0007	0.1	0.94	5	8.898	107.0488	0.0	0.88	2	8.871	107.0488	0.1	0.12	3	-0.027	0.0000	-3.60	3.60	100.0
319	319	1.206	0.009	1018.9789	0.0031	0.1	1.10	4	1.200	1018.9836	0.0	1.73	1	1.208	1018.9773	0.2	0.49	3	0.008	-0.0063	-3.56	3.56	96.3
320	320	9.702	0.032	340.1179	0.0010	0.1	0.94	4	9.740	340.1191	0.0	1.73	1	9.689	340.1175	0.2	0.05	3	-0.051	-0.0016	-3.56	3.56	99.9

Differential Biomarker Expression in Urine

Common features present in both samples and all replicates



Export

646 Differential Features

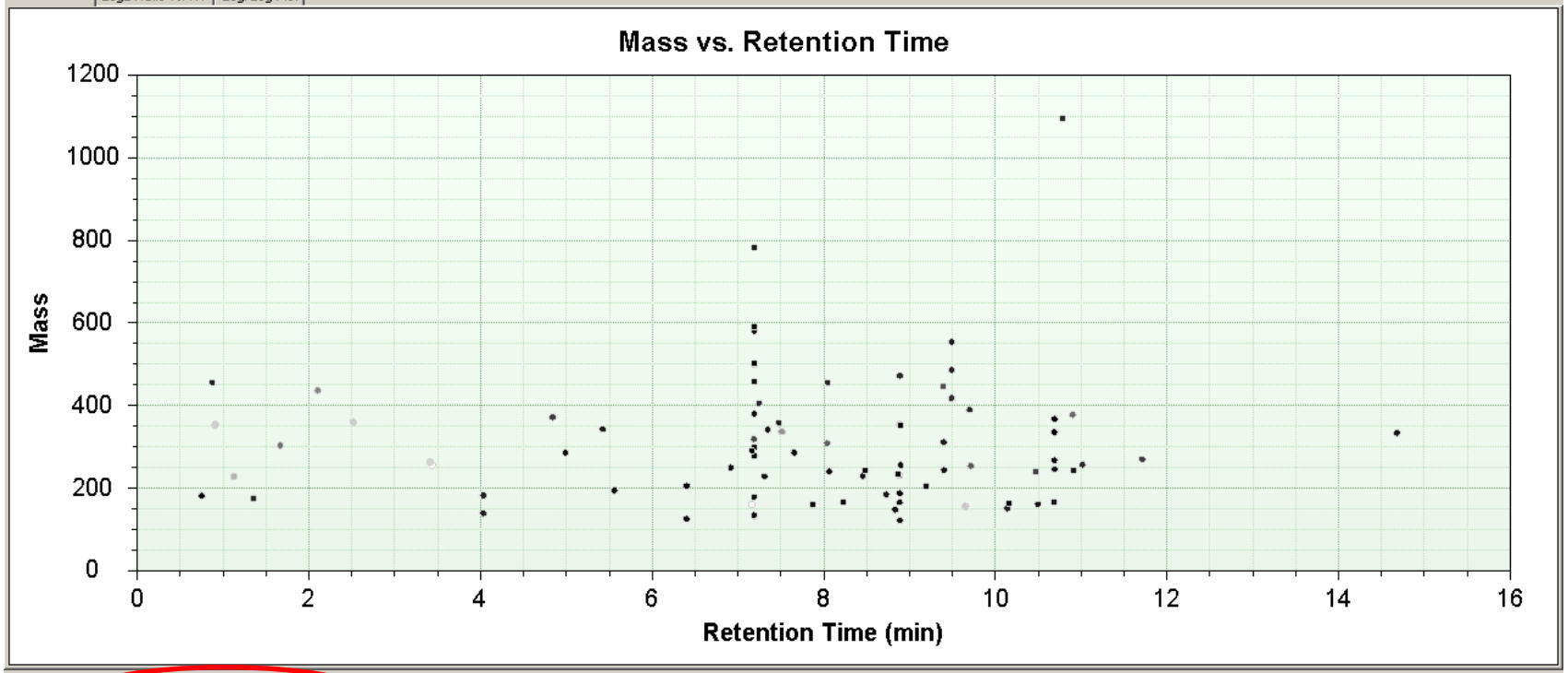
	Feature Summary								Samples 10(3)					Samples 9(3)					Comparison				
	ID	RT	sgm RT	mass	Sgm mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	Dlt RT	Dlt mass	lg2(V1/V2)	llg2(V1/V2)l	Diff. Score
1	1	7.475	0.012	359.0626	0.0006	1.2	1.12	6	7.482	359.0624	2.4	0.23	3	7.468	359.0628	0.0	0.24	3	-0.014	0.0004	7.24	7.24	99.8
2	2	7.511	0.101	337.0796	0.0012	3.3	1.61	6	7.558	337.0801	6.6	0.96	3	7.463	337.0790	0.1	0.13	3	-0.095	-0.0011	6.69	6.69	85.3
3	3	8.480	0.181	243.0199	0.0004	2.4	1.09	6	8.315	243.0201	4.7	0.16	3	8.645	243.0198	0.0	0.06	3	0.330	-0.0003	6.63	6.63	100.0
4	4	11.014	0.058	257.0715	0.0010	0.9	1.04	6	11.052	257.0723	1.7	0.17	3	10.977	257.0707	0.1	0.32	3	-0.075	-0.0016	4.88	4.88	99.9
5	5	7.189	0.010	591.0960	0.0013	0.4	1.01	6	7.194	591.0953	0.8	0.14	3	7.185	591.0967	0.0	0.05	3	-0.009	0.0014	4.44	4.44	100.0

Differential Biomarker Expression in Urine

Differentially expressed features (2x up or down regulated)

Gradient Setting: 1.59000 max vol RSD | Compare: Samples 9 vs. Samples 10

Mass vs. RT | Log2 Ratio vs. RT | Log/Log Plot



Export | 84 Differential Features

	Feature Summary								Samples 10(3)					Samples 9(3)					Comparison				
	ID	RT	sgm RT	mass	Sgm mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	RT	mass	vol	vol RSD	N	Dlt RT	Dlt mass	lg2(V1/V2)	llg2(V1/V2)l	Diff. Score
50	50	7.653	0.015	286.1783	0.0007	0.2	0.52	6	7.663	286.1781	0.1	0.15	3	7.644	286.1785	0.3	0.06	3	-0.019	0.0005	-1.45	1.45	100.0
51	51	8.867	0.018	234.0513	0.0003	2.4	0.51	6	8.878	234.0513	1.3	0.11	3	8.856	234.0513	3.5	0.02	3	-0.022	0.0000	-1.45	1.45	100.0
52	52	9.652	0.081	156.1156	0.0005	0.3	0.67	6	9.720	156.1157	0.4	0.12	3	9.584	156.1154	0.2	1.28	3	-0.135	-0.0002	1.39	1.39	90.0
53	53	4.031	0.021	183.0537	0.0002	0.5	0.55	6	4.038	183.0537	0.7	0.26	3	4.024	183.0537	0.3	0.15	3	-0.014	0.0000	1.39	1.39	98.4
54	54	10.899	0.034	378.1655	0.0013	0.1	0.79	6	10.923	378.1662	0.1	0.70	3	10.875	378.1648	0.0	0.08	3	-0.048	-0.0013	1.34	1.34	79.1

Summary:

CE: High separation power.

TOF MS: High mass accuracy < 2 ppm.
High speed, up to 40 datapoints/sec.
High sensitivity.
High resolution.

Molecular Profiling Toolkit: Software tools for data evaluation.