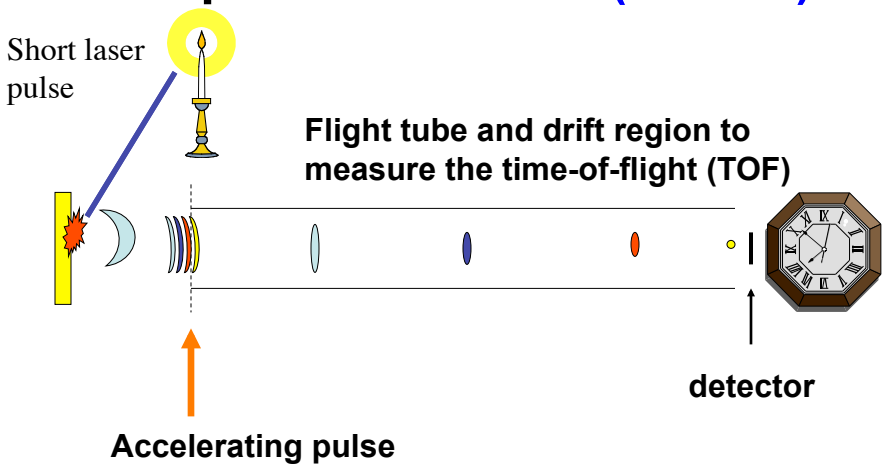
Application of mass spectrometry to the analysis and identification of peptides, proteins and other biological molecules

Stephen Barnes, PhD
4-7117
sbarnes@uab.edu

Overview

- **MALDI-TOF MS**
  - **Protein modifications**
  - **Peptide mass fingerprinting**
- **Electrospray MS**
  - **Analysis of intact proteins**
  - **Molecular weight calculations**
  - **Max Entropy for MW estimation**
- **Peptide analysis**
  - **Purity - ESI-MS is a revelation**
- **Integration of MS with LC and CE**
  - **Multidimensional LC of peptides**
- **Tandem MS**
  - **Identifying peptide amino acid sequences**

Matrix-Assisted Laser Desorption Ionization (MALDI)



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Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

- **Advantages of MALDI-TOF**
  - More tolerant to common buffers than ESI, but…
  - High degree of sensitivity, moderate mass accuracy, and mass resolution
  - High mass compounds, i.e. proteins, PEG…
- **Common Applications of MALDI-TOF**
  - Masses of large proteins and other compounds
  - Enzymatic digestion profiles of proteins to establish their identity
  - Peptide sequencing (TOF-TOF)
  - *In situ* protein/peptide imaging

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Factors from conventional experiments that impact MALDI-TOF analysis

- Tolerance of buffers/chemicals used in sample preparation
  - NaCl up to 150 mM
  - Urea up to 2-3 M (carbamoylation can occur!)
  - Guanidinium-HCl up to 2 M
- Tolerance of detergents
  - SDS up to 0.05%
- Staining Protocols
  - Whole proteins form adducts with Coomassie
  - Silver staining modifies selected peptides

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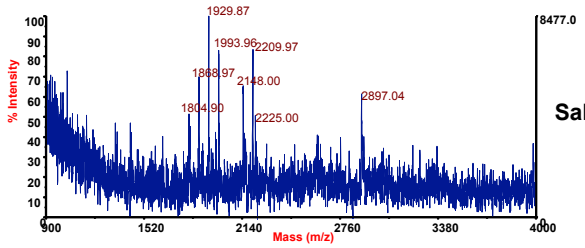
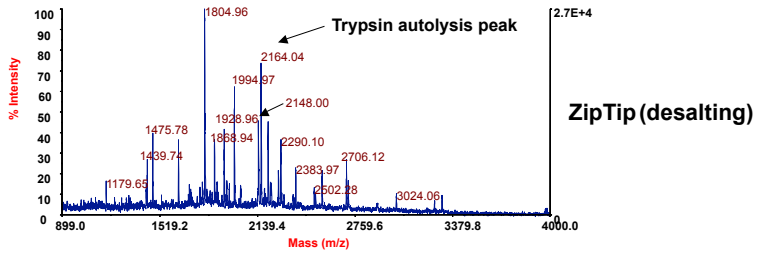
Benefit of removing salt from tryptic digest





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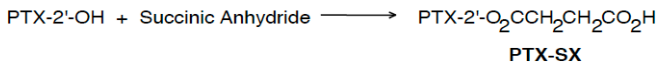
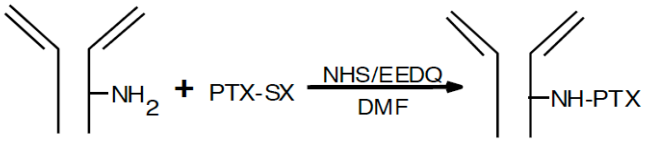
Chemically modifying an antibody to bind radioactive metal ions

Scheme 1



PTX-SX

Scheme 2



MAb **PTXMAb**

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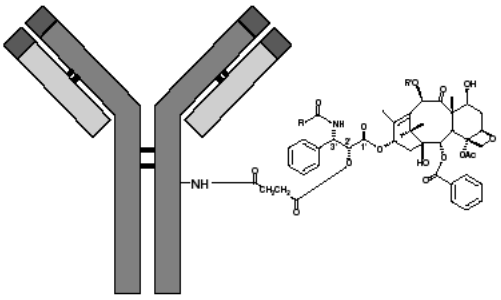
Structure of modified antibody



PTX-MAb

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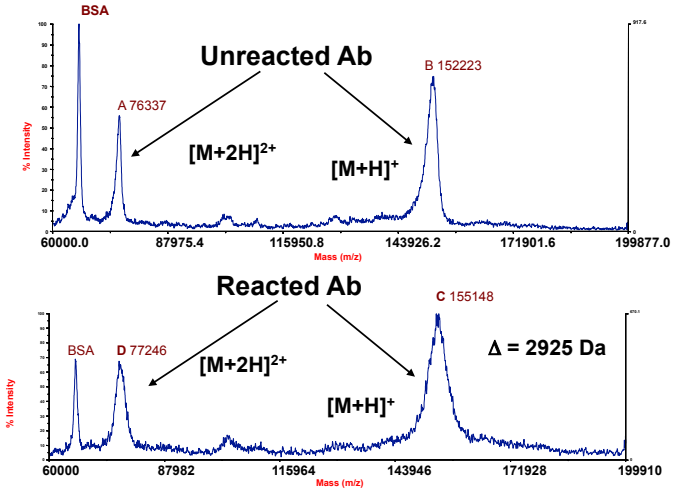
Modification of an antibody by MALDI-TOF



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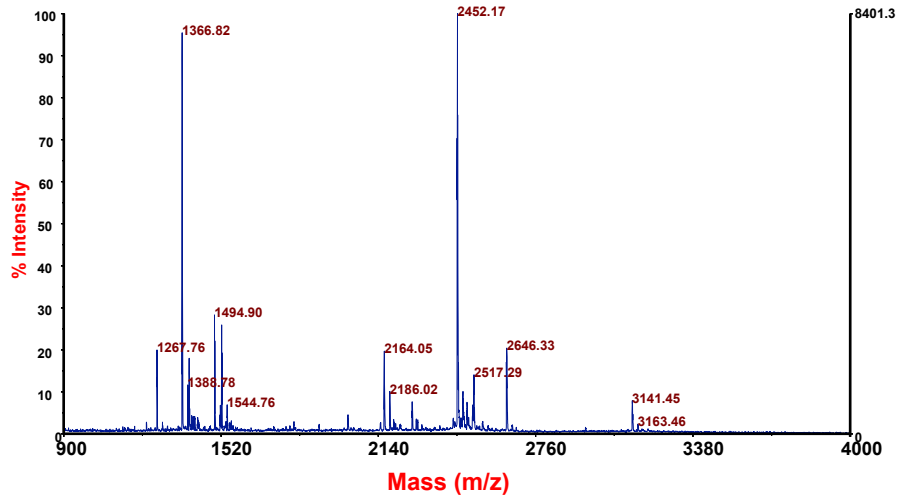
A MALDI-TOF mass spectrum of peptides



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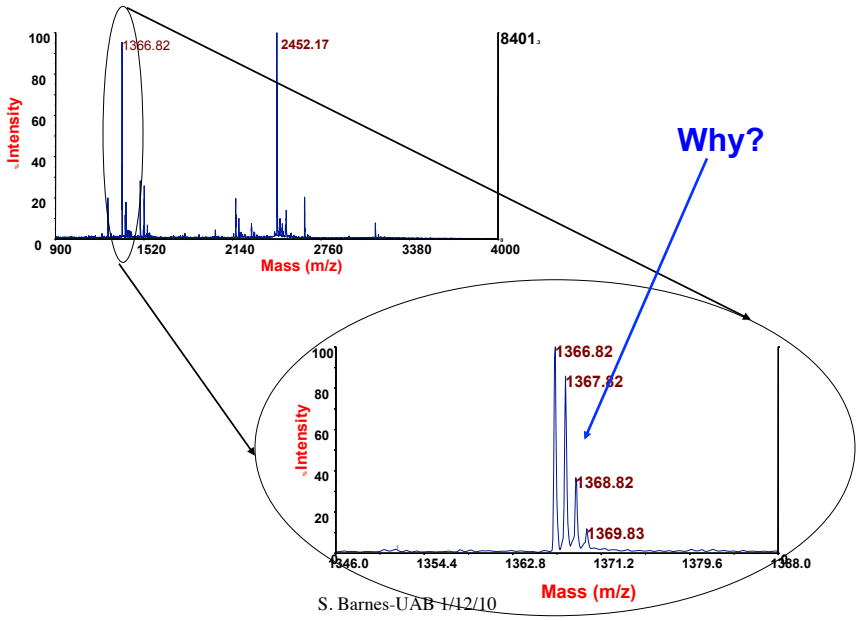
Isotope profile of an individual peptide ion

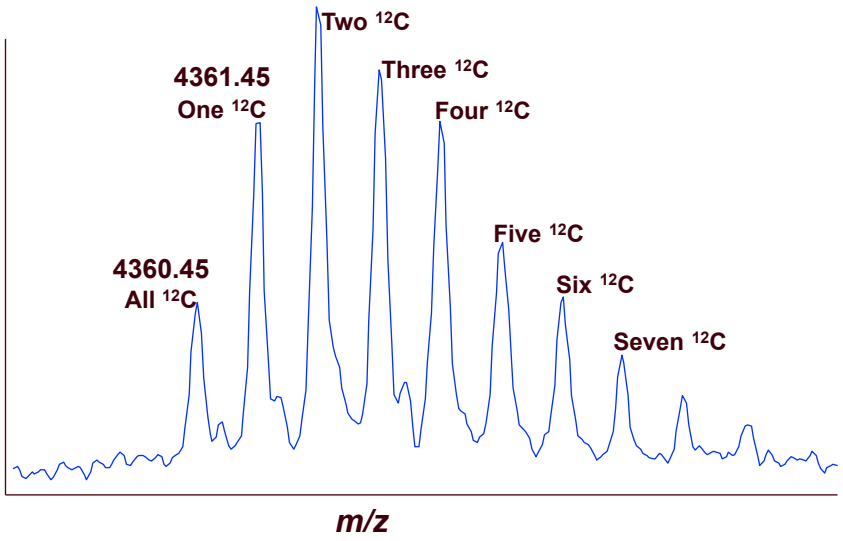


Stable isotopes of the most abundant elements found in peptides

Element	Mass	Abundance
H	1.0078	99.985%
	2.0141	0.015%
C	12.0000	99.89%*
	13.0034	1.11%*
N	14.0031	99.64%*
	15.0001	0.36%*
O	15.9949	99.76%*
	16.9991	0.04%*
	17.9992	0.20%*
S	31.9721	94.93%*
	32.9715	0.76%*
	33.9679	4.29%*
	35.9671	0.02%*

***Varies according to its source**

Isotope pattern for a larger peptide (207 C-atoms)



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How to represent the mass of compound?

- At high resolution where the isotopic peaks are fully resolved, then we can determine the ***monoisotopic mass*** for each one
- At low mass resolution (where the isotope peaks cannot be resolved) what is observed is the ***average mass***

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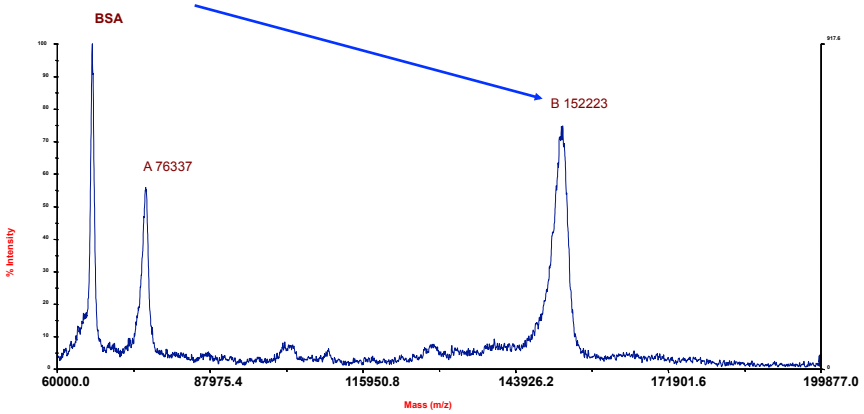
Average mass

For MALDI-TOF spectra above *m/z* 5000, it becomes difficult/ impossible to resolve the isotope profile. Instead, the average mass is calculated.



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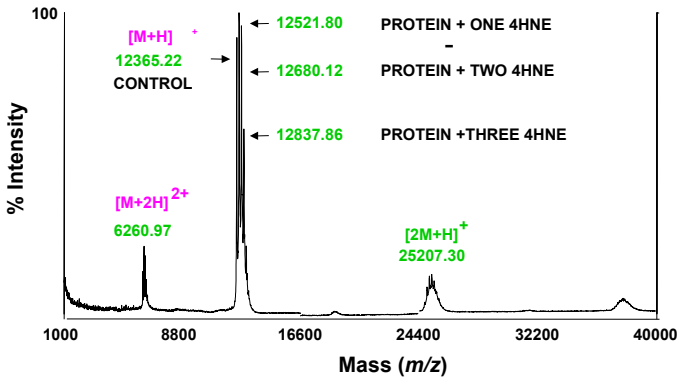
Modification of cytochrome C by the reactive aldehyde 4HNE



MALDI spectra usually contain only the **molecular ion $[M+H]^+$**. The mass accuracy is approximately ±1-2 Da. Hard to pick out the ^{12}C isotope peak. The 4HNE Michael adduct should be 156 Da.

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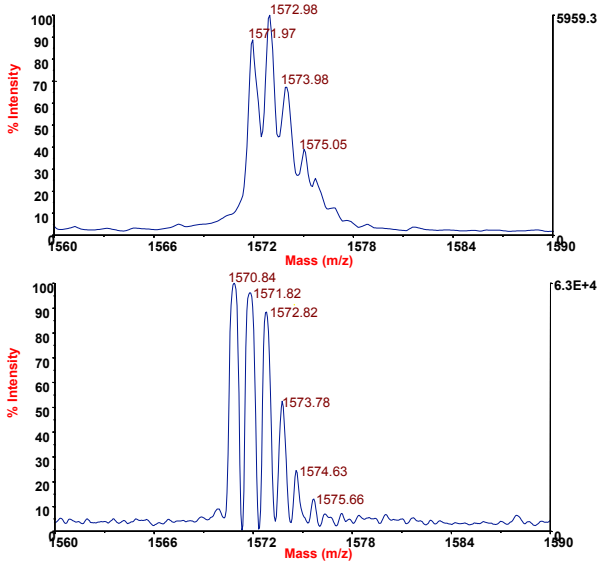
Increased resolution and sensitivity in reflector vs. linear mode



Linear Mode

Reflector Mode

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Peptide mass fingerprinting

- This method was developed because of the availability of predicted protein sequences from genome sequencing
- Proteins did not have to have been previously sequenced - only that the open reading frame in the gene is known - the rest is a virtual exercise in the hands of statisticians, bioinformaticists and computers
- However, remember the matching is only as good as the database content - this can change

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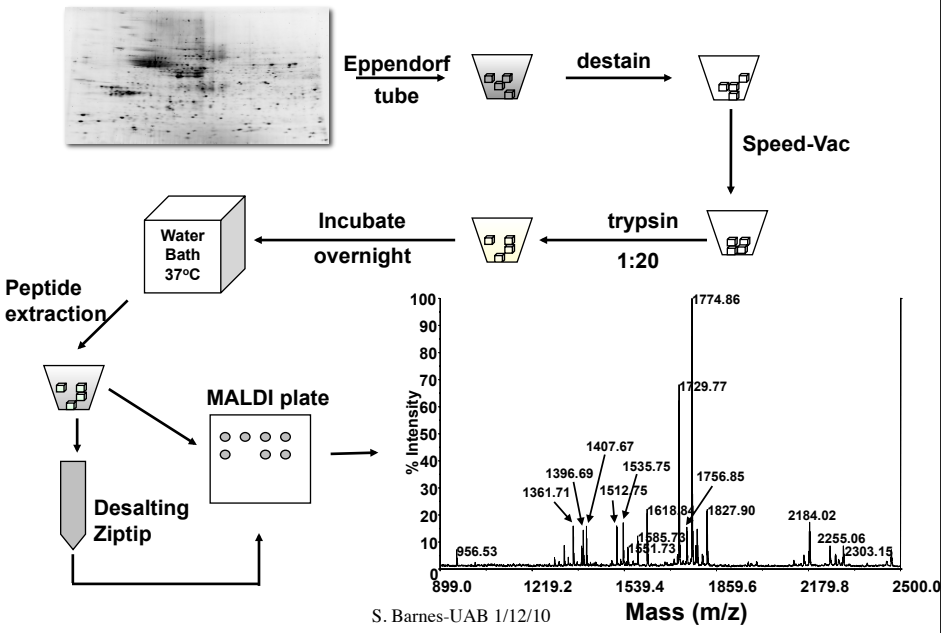
Protein analysis by MALDI 2010



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Proteolytic enzymes used to hydrolyze proteins

The choice of enzyme largely depends on the nature of the amino acid sequence and the specific issue that is being addressed

- **Trypsin - *cleaves at arginine and lysine residues***
- **Chymotrypsin - *cleaves hydrophobic residues***
- **Arg-C - *cleaves at arginine residues***
- **Glu-C - *cleaves at aspartate/glutamic acid residues***
- **Lys-C - *cleaves at lysine residues***
- **V8-protease - *cleaves at glutamic acid residues***
- **Pepsin - *cleaves randomly but consistently, at acid pH***

See http://www.abrf.org/JBT/1998/September98/sep98m_r.html

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Searching databases with peptide masses to identify proteins

Best site is at www.matrixscience.com

The program (MASCOT) can search the OWL or NCBI databases using a set of tryptic peptide masses, or the fragment ions (specified or unspecified) of peptides

Presents the expected set of tryptic peptides for each matched protein

Choice of peptidase

- **Analogous to DNA restriction enzymes**
- **Tryptic peptide fingerprinting may identify, not one, but several highly related protein candidates (e.g., actins)**
- **Inspection of the sequences may reveal that there is a difference at one residue that distinguishes between two candidates.**
- **If for instance it is a glutamate, then use of Glu-C or V8-protease may enable the two proteins to be correctly identified**
- **INSPECT sequences carefully**

Sequence of β-lactoglobulin

MKCLLLALAL TCGAQALIVT QTMKGLDIQK
VAGTWYSLAM AASDISLLDA QSAPLRVYVE
ELKPTPEGDL EILLQKWENG ECAQKKIIAE
KTKIPAVFKI DALNENKVLV LDTDYKKYLL
FCMENSAEPE QSLACQCLVR TPEVDDEALE
KFDKALKALP MHIRLSFNPT QLEEQCHI

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GLDIQK CLLLALALTCGAQALIVTQTMK VYVEELK

MK VAGTWYSLAMAASDISLLDAQSAPLR TK

ALK YLLFCMENSAEPEQSLACQCLVR K K

WENGECAQK PTPEGDLEILLQK VLVLDTDYK

FDK IPAVFK ALPMHIR IIAEK

TPEVDDEALEK IDALNENK LSFNPTQLEEQCHI

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Peptides from digestion with Glu-C

MKCLLLALALTCGAQALIVTQTMKGLD

IQKVAGTWYSLAMAASD ISLLD AQSAPLRVYVE

E LKPTPE GD LE ILLQKWE NGE CAQKKIIAE

KTKIPAVFKID ALNE NKVLVLD TD YKKYLLFCME

NSAE PE QSLACQCLVRTPE VD D E ALE KFD

KALKALPMHIRLSFNPTQLE E QCHI

Amino acid residue masses

Alanine	71.037	Leucine	113.084
Arginine	156.101	Lysine	128.094
Asparagine	114.043	Methionine	131.040
Aspartic acid	115.027	Phenylalanine	147.068
Cysteine	103.009	Proline	97.053
Glutamic acid	129.043	Serine	87.032
Glutamine	128.058	Threonine	101.048
Glycine	57.021	Tryptophan	186.079
Histidine	137.059	Tyrosine	163.063
Isoleucine	113.084	Valine	99.068

The m/z value of a peptide $[M+H]^+$ is the sum of the residue masses plus 18.015 for H_2O plus 1.008. So, what is it for ISLLD?

113.084 + 87.032 + 113.084 +113.084 + 115.027 + 18.015 + 1.008 = 560.334

Expected peptides from trypsin and Glu-C digestion of bovine β-lactoglobulin

837.4764	800.4876
916.4734	929.5455
1064.4466	1003.5605
1065.5827	1232.6634
1245.5845	1259.7722
1658.7843	1337.6632
2275.2586	1447.7032
2313.2588	1811.8996
2647.2023	2307.3006
2707.3760	2819.5265

Assumes all cuts are complete, there is no oxidation of Met residues, and Cys residues are unmodified

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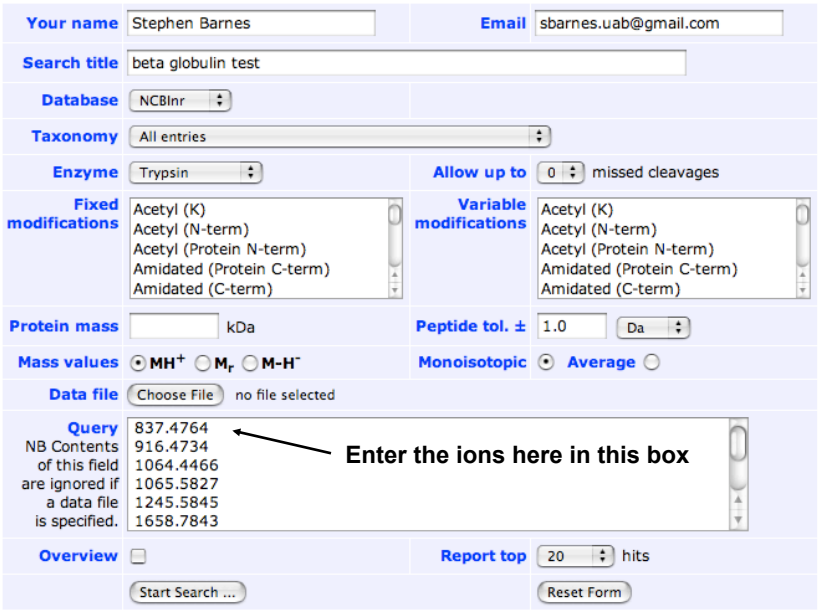
MASCOT Peptide Mass Fingerprint

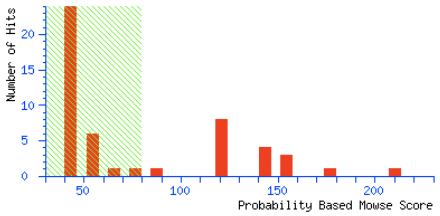


Mascot Search Results

User : Stephen Barnes
Email : sbarnes.uab@gmail.com
Search title : beta globulin test
Database : NCBInr 20061230 (4378862 sequences; 1508892933 residues)
Timestamp : 1 Jan 2007 at 02:17:51 GMT
Top Score : 210 for gi|87196497, lactoglobulin, beta [Bos taurus]

Probability Based Mowse Score

Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 79 are significant (p<0.05).



Protein Summary Report

Format As | Protein Summary | Help
Significance threshold p< 0.05 | Max. number of hits 20

Protein records provided by MASCOT search

	Accession	Mass	Score	Description
1.	gi|87196497	19870	210	lactoglobulin, beta [Bos taurus]
2.	gi|4388846	18269	179	Chain , Bovine Beta-Lactoglobulin Complexed With Palmitate, Lattice Z
3.	gi|223780	18165	152	lactoglobulin beta
4.	gi|72079	18255	152	beta-lactoglobulin - water buffalo
5.	gi|520	19908	150	beta-lactoglobulin [Bos taurus]
6.	gi|20178290	20010	148	Beta-lactoglobulin precursor (Beta-LG)
7.	gi|165839	19934	148	beta-lactoglobulin
8.	gi|2194088	18297	147	Chain A, Bovine Beta-Lactoglobulin, Lattice X
9.	gi|110612608	19891	144	beta-lactoglobulin [Bubalus bubalis]
10.	gi|162748	17156	126	beta-lactoglobulin
11.	gi|125912	19962	125	Beta-lactoglobulin precursor (Beta-LG)
12.	gi|7245834	18363	124	Chain A, Structural Changes Accompanying Ph-Induced Dissociation Of The
13.	gi|229460	18355	124	lactoglobulin beta
14.	gi|4388939	18355	124	Chain , Structural Basis Of The Tanford Transition Of Bovine Beta-Lacto
15.	gi|49259423	18339	124	Chain X, The Cys121ser Mutant Of Beta-Lactoglobulin
16.	gi|54037712	18139	120	Beta-lactoglobulin (Beta-LG)
17.	gi|57164367	19908	117	beta-lactoglobulin [Ovis aries]
18.	gi|90108547	18264	82	Chain A, Reindeer Beta-Lactoglobulin
19.	gi|71980384	20035	80	beta-lactoglobulin [Rangifer tarandus tarandus]
20.	gi|26352113	13020	60	unnamed protein product [Mus musculus]

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Comparison of observed and predicted tryptic peptides

gi|87196497 **Mass:** 19870 **Score:** 210 **Expect:** 4.4e-15 **Queries matched:** 10
lactoglobulin, beta [Bos taurus]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
837.4764	836.4691	836.4691	0.0001	158	164	0	K.ALPMHIR.L
916.4734	915.4661	915.4661	-0.0000	100	107	0	K.IDALNENK.V
1064.4466	1063.4393	1063.4393	0.0001	77	85	0	K.WENGECAQK.K
1065.5827	1064.5754	1064.5753	0.0001	108	116	0	K.VLVLDTDYK.K
1245.5845	1244.5772	1244.5772	0.0000	141	151	0	R.TPEVDDEALEK.F
1658.7843	1657.7770	1657.7770	0.0000	165	178	0	R.LSFNPTQLEEQCHI.-
2275.2586	2274.2513	2274.2513	0.0000	3	24	0	K.CLLLALALTCGAQALIVTQTMK.G
2313.2588	2312.2515	2312.2515	0.0001	57	76	0	R.VYVEELKPTPEGDLEILLQK.W
2647.2023	2646.1950	2646.1950	0.0001	118	140	0	K.YLLFCMENSAEPEQSLACQCLVR.T
2707.3760	2706.3687	2706.3686	0.0001	31	56	0	K.VAGTWYSLAMAASDISLLDAQSAPLR.V

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Search against SwissProt database

	Accession	Mass	Score	Description
1.	LACB_BOVIN	19870	271	Beta-lactoglobulin precursor (Beta-LG) (Allergen Bos d 5) - Bos taurus (Bovine)
2.	LACB_BUBBU	20010	197	Beta-lactoglobulin precursor (Beta-LG) - Bubalus bubalis (Domestic water buffalo)
3.	LACB_CAPHI	19962	135	Beta-lactoglobulin precursor (Beta-LG) - Capra hircus (Goat)
4.	LACB_OVIMU	18139	104	Beta-lactoglobulin (Beta-LG) - Ovis orientalis musimon (Mouflon)
5.	LACB_SHEEP	19908	102	Beta-lactoglobulin-1/B precursor (Beta-LG) - Ovis aries (Sheep)
6.	YKH5_YEAST	52055	41	Hypothetical 52.1 kDa protein in SMY1-MUD2 intergenic region - Saccharomyces cerevi
7.	TCPH_VIBCH	16247	40	Toxin coregulated pilus biosynthesis protein H (TCP pilus biosynthesis protein tcpH
8.	POLG_DEN22	54291	39	Genome polyprotein [Contains: Envelope protein E] (Fragment) - Dengue virus type 2
9.	PFPI_PYRKO	18404	38	Intracellular protease 1 (EC 3.2.-.-) (Intracellular protease I) - Pyrococcus kodak
10.	VIRB9_AGRT5	32181	38	Protein virB9 precursor - Agrobacterium tumefaciens (strain C58 / ATCC 33970)
11.	VPS71_YEAST	32001	36	Vacuolar protein sorting-associated protein 71 (SWR complex protein 6) - Saccharomy
12.	YHR7_YEAST	71812	34	TPR repeat-containing protein YHR117W - Saccharomyces cerevisiae (Baker's yeast)
13.	LGB_LOTJA	15745	34	Leghemoglobin - Lotus japonicus
14.	YH13_VACCV	13615	34	Hypothetical 13.6 kDa HindIII-C protein - Vaccinia virus (strain Western Reserve /
15.	MKT1_YEAST	94435	34	Protein MKT1 - Saccharomyces cerevisiae (Baker's yeast)
16.	H2A1_LILLO	12165	34	Histone H2A.1 (GcH2A) - Lilium longiflorum (Trumpet lily)
17.	RS12_BRUA2	13863	33	30S ribosomal protein S12 - Brucella abortus (strain 2308)
18.	RS12_BRUAB	13863	33	30S ribosomal protein S12 - Brucella abortus
19.	RS12_BRUME	13863	33	30S ribosomal protein S12 - Brucella melitensis
20.	RS12_BRUSU	13863	33	30S ribosomal protein S12 - Brucella suis

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Things to consider when doing peptide mass fingerprinting

- Proteins can be oxidized both biologically (real data) and during the workup
- Treat the protein or the peptide digest with a reagent that reacts with Cys sulhydryl groups - e.g., iodoacetamide, iodoacetic acid, N-ethylmaleimide or 4-vinylpyridine. Cysteines may also have reacted with acrylamide in the gel.
- Set the options in the fixed or variable modification boxes before searching
- Allow for at least one missed cleavage - trypsin does not cut when Lys or Arg are followed by a Pro residue

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Other web sites for peptide analysis

- http://prowl.rockefeller.edu/
  - Choose ProFound
- http://prospector.ucsf.edu/
  - Choose MS-fit

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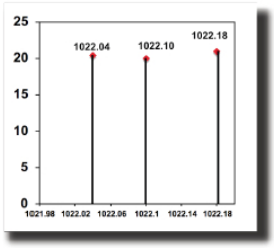
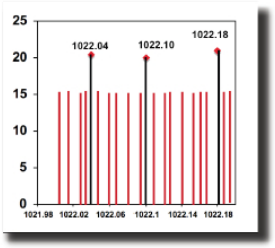
Further information on identified protein

- **Take the protein identifier number:**
  - **For bovine β-lactoglobulin it is gi|520**
  - **Go to http://www.ncbi.nlm.nih.gov**
  - **Under protein, paste in the gi number**
  - **A link to the protein will appear**
  - **Click on Blink - this is similar to BLAST, but better**
  - **Scroll down the list and select 1CJ5**
  - **Click on image of protein structure**
  - **To view a 3D-image of the protein, first download Cn3D from the NCBI site or RasMol**
    - **Bring a picture of beta-lactoglobulin to the next class**

Blurring of protein space

- **Identification using MALDI-TOF with MASCOT depends on:**
  - Number of peptides recognized as being part of the protein
  - The mass accuracy of the peptides that are recognized
  - Pre-2000, an accuracy of better than 0.05 Da in a 1000 Da peptide (i.e., 50 ppm) was sufficient to distinguish the unknown protein from the other proteins in the databases at that time
  - Now, the protein information space has become more dense and MALDI-TOF is no longer adequate
  - Previously identified proteins may not be correct





Electrospray ionization

- ESI-MS is very sensitive to the presence of electrolyte species -
  - these ionize more easily than solutes and may also form adducts with solutes
- In ESI-MS, multiple charge states are possible
  - These lead to more accurate MWs
- This is a softer ionization than MALDI where the UV laser at 337 nm alters the chemistry of modifications such as Tyr-NO_2 and Cys-SNO

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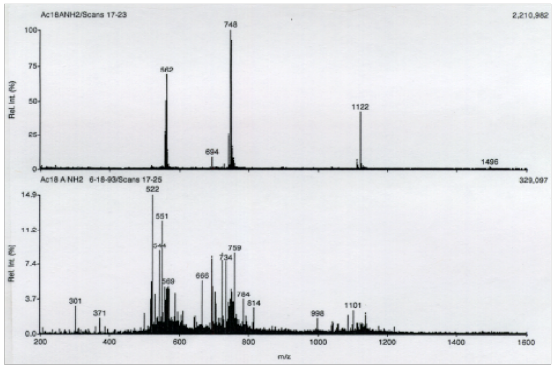
ESI-MS and purity of peptides



Guarantees of purity based on observation of "a single peak by reverse-phase HPLC" and by "it gave the correct sequence when analyzed by Edman degradation" are hollow. The lower spectrum was of a "pure" HPLC peak. The method of purification was amended and the upper spectrum was obtained

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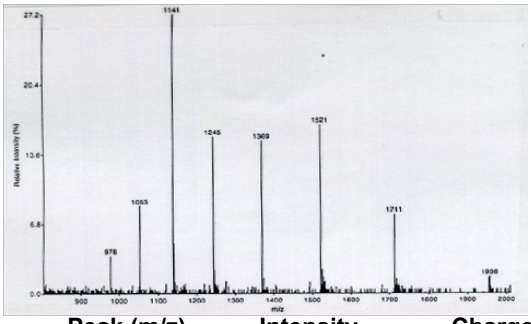
Ionizing proteins and peptides

- $^{+}H_3NCHR_1CO(NHCHR_nCO)_nNHCHR_2COOH$ is the ion that's found in dilute acid solution
- If there are internal basic residues, then the ions will be of the form $[M+nH]^{n+}$, where n = 1, 2, etc.
- A tryptic peptide will have a N-terminal amino group and an amino group from Arg or Lys
  - If the peptide has a mol. wt. of 1000 Da, then the singly charged ion will have a *m/z* of 1001, whereas the doubly charged ion has a *m/z* of 501

ESI mass spectrum of ribonuclease



Cumulative MW estimate = 13,680.29

SD = 2.94

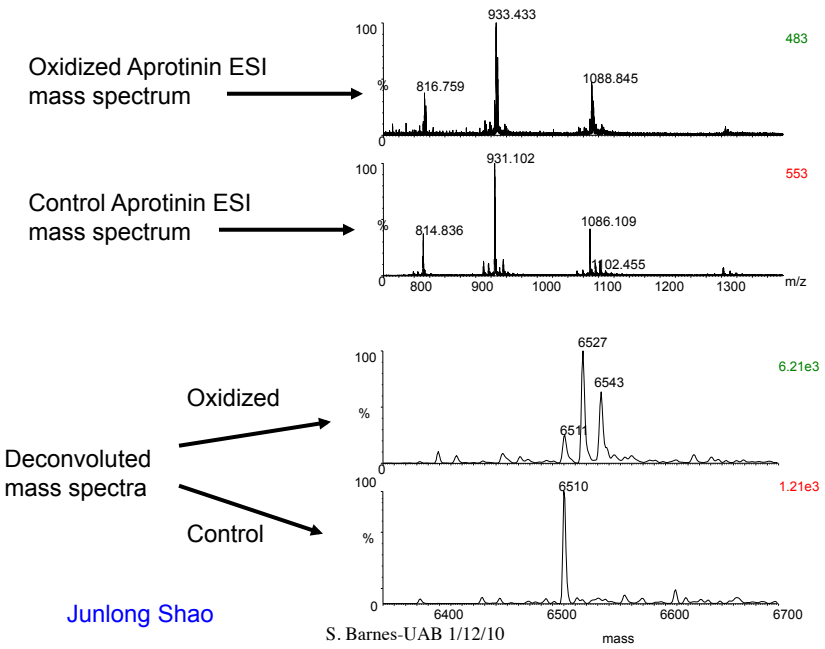
Peak (m/z)	Intensity	Charge (est.)	Mol. Wt. (Est.)
978.00	7,778	14.00000	13,677.89
1,053.00	18,532	13.02656	13,675.90
1,141.00	59,087	11.95446	13,679.91
1,245.00	33,275	10.96146	13,683.91
1,369.00	32,390	10.03219	13,679.92
1,521.00	35,668	8.99995	13,679.93
1,711.00	16,624	7.99996	13,679.94
1,956.00	3,333	6.97955	13,684.94

Calculation of molecular weights and ion states

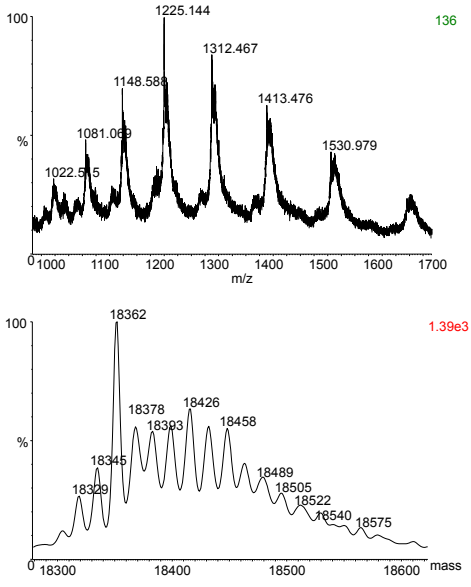
- For two ions in a series for a peptide of molecular weight M, the lower *m/z* value (x) will be for the n+1 ion state and the larger *m/z* value (y) will be for the n+ ion state.
  - (1) $(M+n)/n = y$
  - (2) $(M+n+1)/(n+1) = x$
- Hence
  - (3) $M+n = ny$ and $M = ny-n$
  - (4) $M+n+1 = (n+1)x$ and $M = (n+1)x-(n+1)$
- Hence
  - $ny-n = (n+1)x - (n+1)$
  - $ny-n-xn+n = x-1$
  - $n(y-x) = x-1$
  - $n = (x-1)/(y-x)$
- The value of n can then be substituted in equation (1) to obtain the molecular weight of the peptide

Deconvolution of MS data

- When several proteins are present, then their multiply charged ion clusters overlap
- Can this be overcome? - yes, use the MaxEntropy program provided by Micromass



Deconvolution of oxidized forms of β-lactoglobulin



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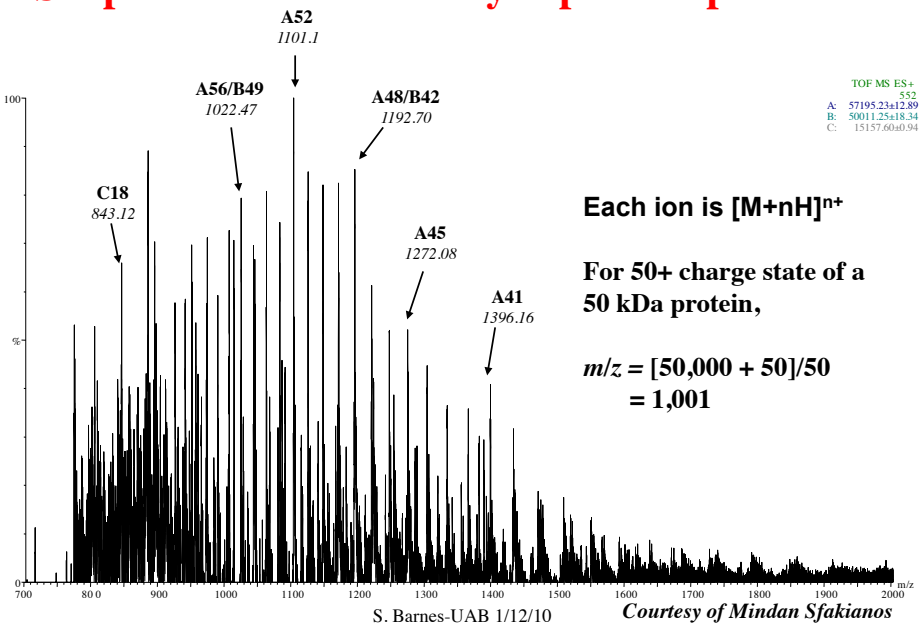
Junlong Shao

ESI spectrum of bacterially expressed protein



Each ion is $[M+nH]^{n+}$

For 50+ charge state of a 50 kDa protein,

***m/z* = [50,000 + 50]/50**
= 1,001

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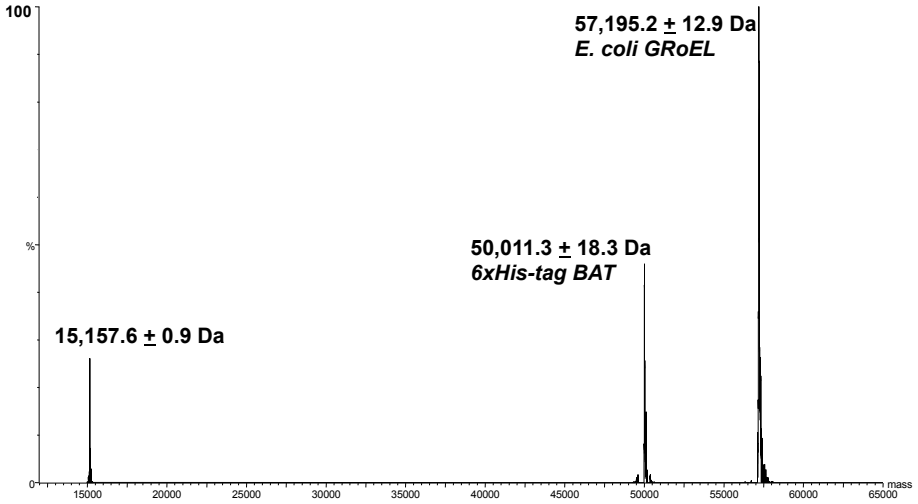
Courtesy of Mindan Sfakianos

MaxEnt deconvolution of MWs



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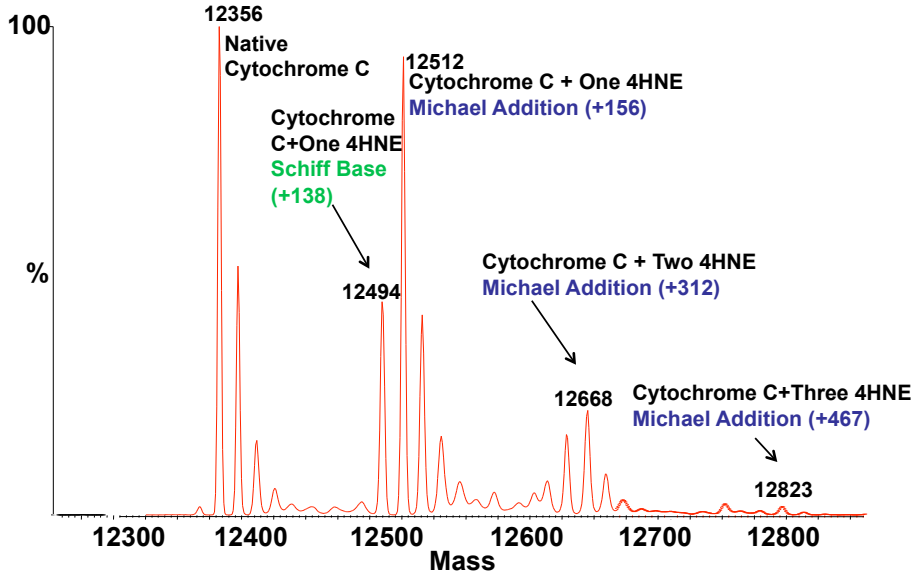
Courtesy of Mindan Sfakianos

ESI-MS of 4HNE-Modified Cytochrome C



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Summary of determining MW by ESI

- The multiple charge states of a protein allow:
  - Mol Wt of large proteins to be estimated
  - It's a super SDS-PAGE gel
- Important to remember that the protein sample must be free of salt
  - Typically, a sample is cleaned up on a short reverse-phase column prior to electrospray
  - Alternative, use ammonium acetate as buffer

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Studying high molecular weight complexes by ESI

- Most instrument ESI interfaces have a limited *m/z* range - up to 3,000
- In protein complexes water, and hence H+ ions, is "squeezed" out, thereby substantially increasing observed *m/z* values
- Interfaces that pass ions with *m/z* values above 10,000 have been designed

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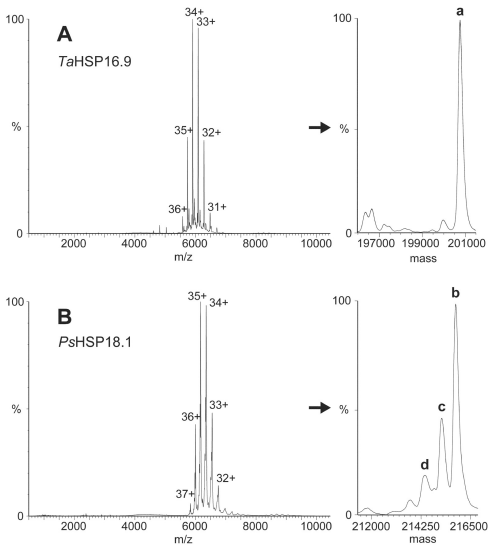
nanoESI-MS of HMW complexes of small heat shock proteins



Note the large *m/z* values (6,000-7,000) for the observed ions

The ESI data were deconvoluted to reveal the distribution of the masses of the complexes

Sobbott et al., J Biol Chem 277:38921

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Studying intact membrane protein complexes by gas-phase mass spectrometry (top-down analysis)

- Electrosprayed in neutral detergent
  - Dodecyl maltoside (DDM)
- See Barrera et al., Science 321: 243-246, 2008
- Carried out on Waters Qtof II with modification of the ESI interface
- Requires high voltage (Δ200 V) to be applied across the interface and collision cell

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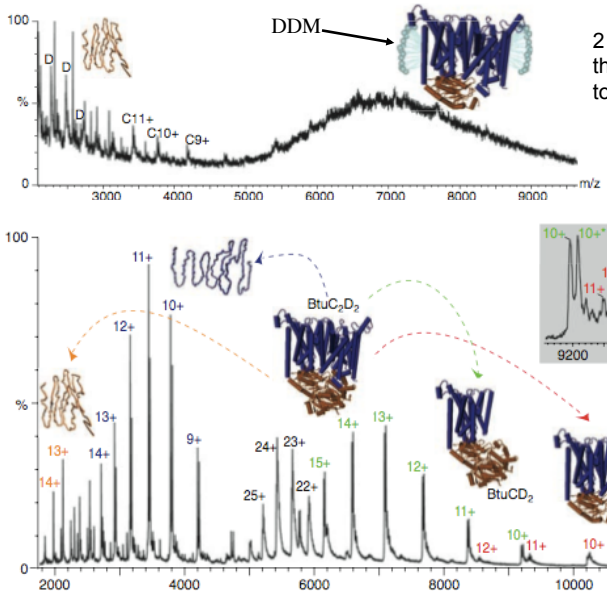
Effect of increasing voltage on $BtuC_2D_2$ membrane complexes observed in nanoMS



2 μl of 5 μM solution of the complex subjected to nanoelectrospray

Use of FT-MS in ESI of proteins

- The very high resolving power of FT-MS enables a direct measure of charge state of an individual ion since each peptide or polypeptide will have several/many isotope peaks
- The distance in Da between successive isotope peaks of a multiply charged ion is the reciprocal of the number of charges

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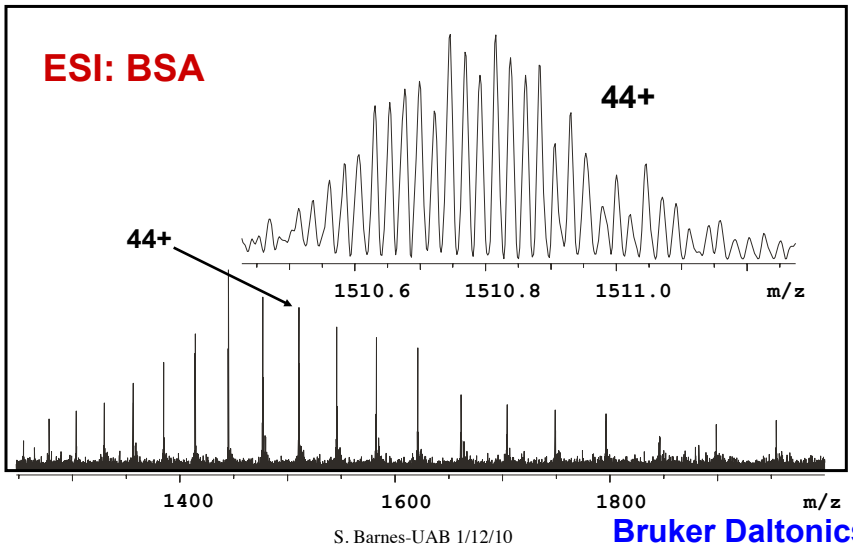
Bovine Serum Albumin (66 kDa)
4.7 T Actively Shielded Magnet



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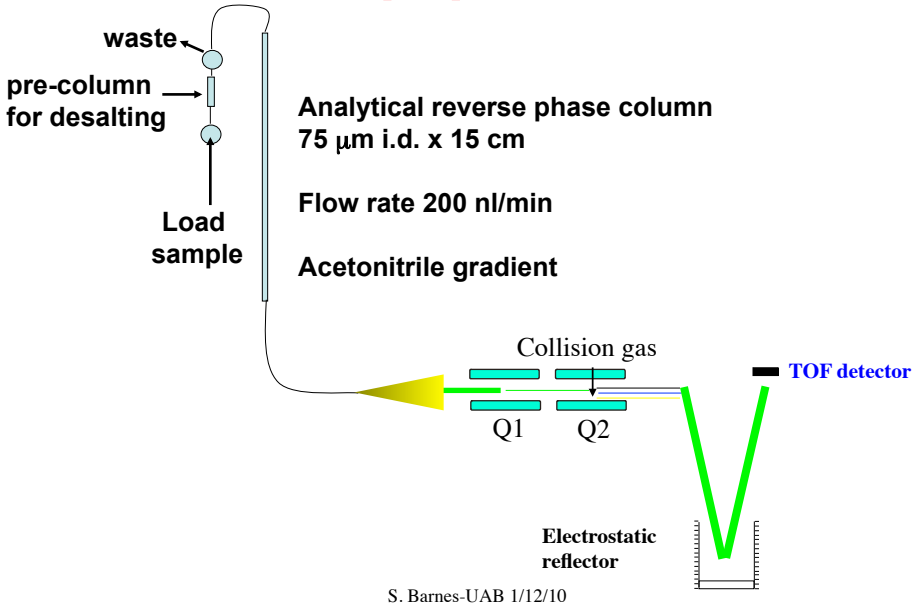
Bruker Daltonics

LC-MS of peptide mixtures



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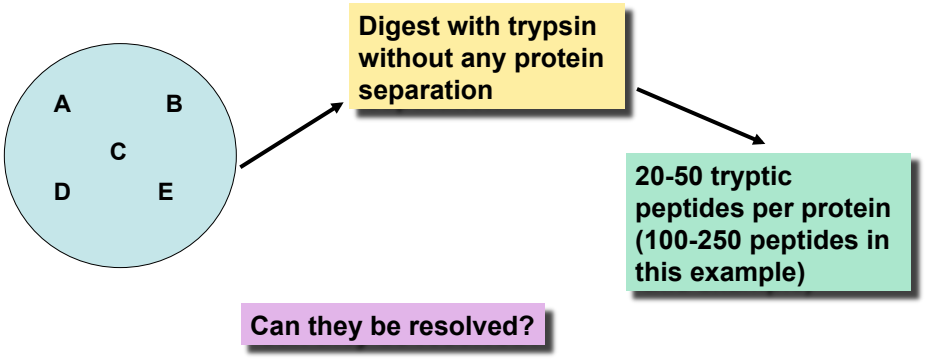
The MUDPIT approach

MUlti-Dimensional Protein Identification Technology



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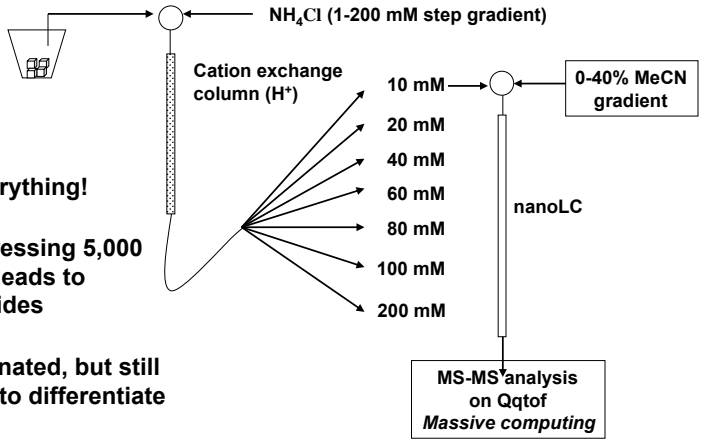
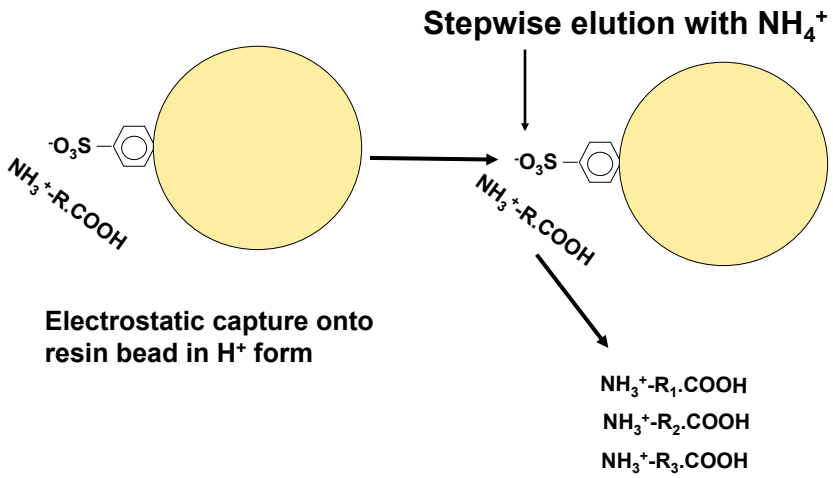
MUDPIT - MUlti-Dimensional Protein Identification Technology



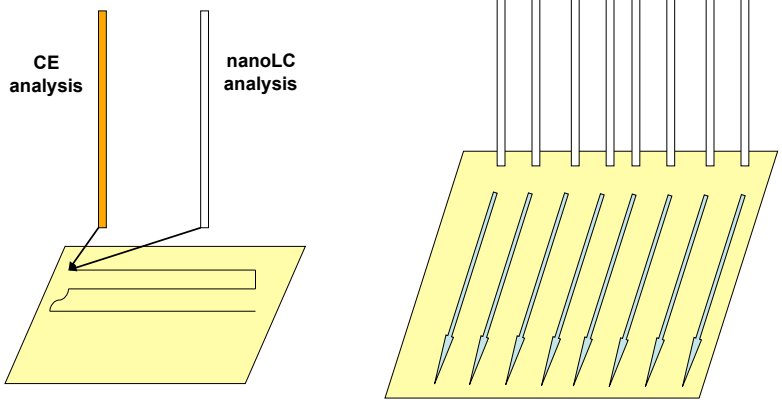
- Hydrolyze everything!
- For a cell expressing 5,000 proteins, this leads to >100,000 peptides
- Can be fractionated, but still 10,000-20,000 to differentiate
- Enormous bioinformatics problem

John Yates

Cation exchange of peptides



Connecting CE and LC to MALDI analysis



Creates 20 mm wide tracks that can be scanned by MALDI laser for MS analysis

Parallel capture of effluents of 8 nanoLC separations on Mylar - can be scanned simultaneously by fast laser

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Pros/Cons of laying down LC or EC separations on matrix plate

- Allows off-line analysis both in real time and then in a retrospective mode
- MALDI-TOF analysis is very fast
- Can also do TOF-TOF MS-MS analysis
- BUT what happens chemically on the acidic environment on the surface of the plate during storage?
- Also, can the laser beam cause chemical changes?

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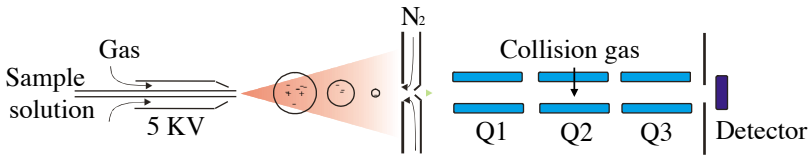
Sequencing of peptides

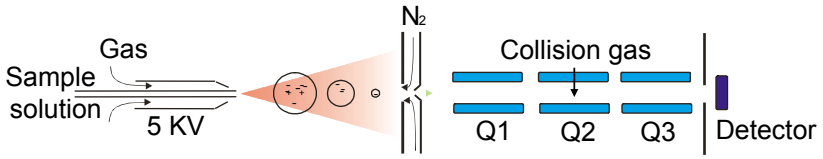
- Using tandem mass spectrometry in a triple quadrupole, Q-tof, or ion trap instrument, the parent ion is first selected in the first quadrupole
- The parent ion is collided with argon gas and it breaks into fragments (daughter ions)
- By identifying the daughter ions, the peptide amino acid sequence is inferred

Tandem mass spectrometry on a triple quadrupole instrument

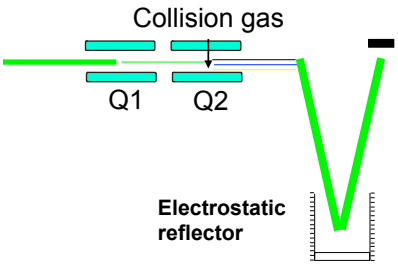


- Daughter ion spectra
- Parent ion spectra
- Multiple reaction ion scanning

Triple quad versus Q-tof and sensitivity



The quadrupole analyzer (Q3) is slow and insensitive - it's a filter - thus throws away large amounts of data



TOF detector collects all ions generated and yields fmol rather than pmol sensitivity

Also gives far greater mass accuracy - from 1000 ppm on the triple quad to <20 ppm on the Q-tof

Crucially important for automated interpretation of MS-MS spectra to yield amino acid sequence

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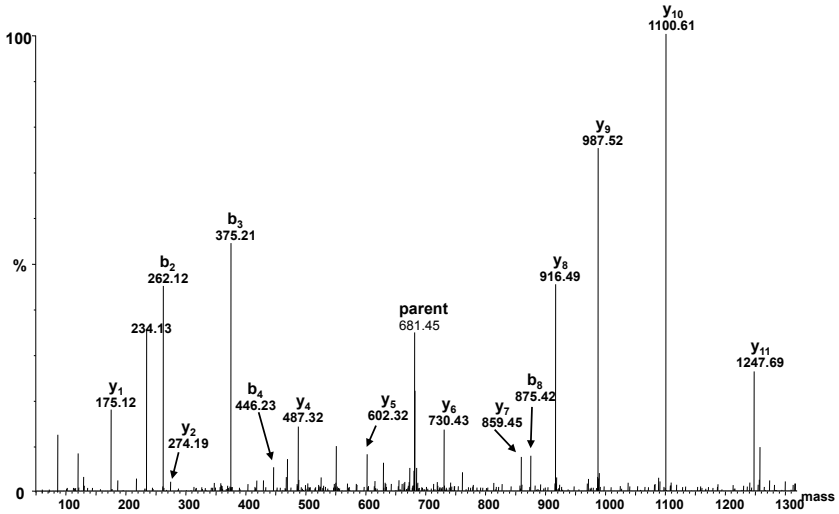
Fragmentation of a peptide



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