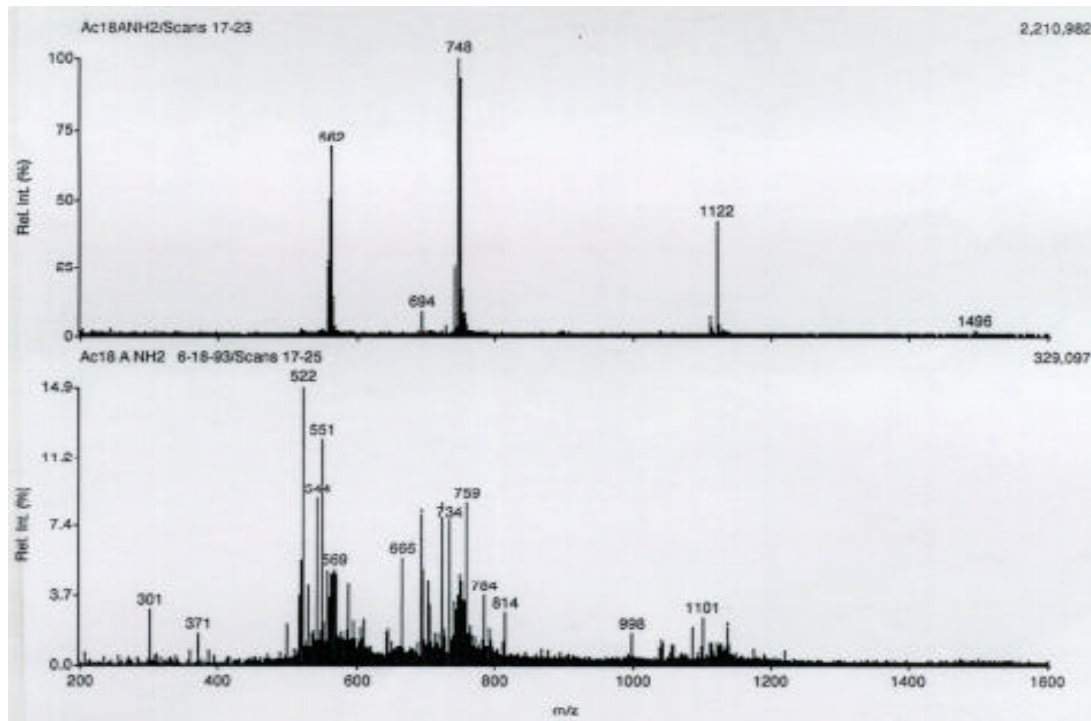


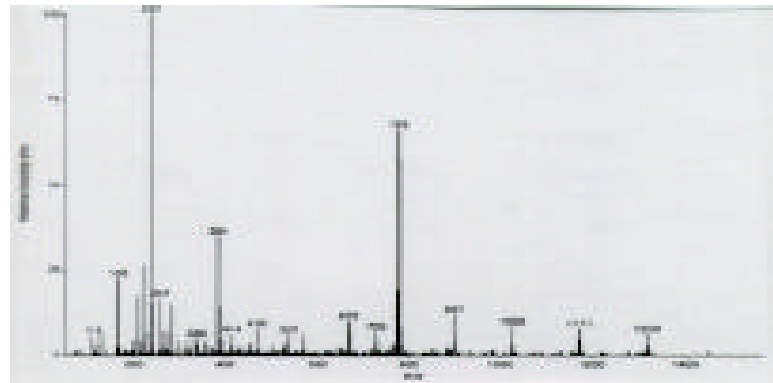
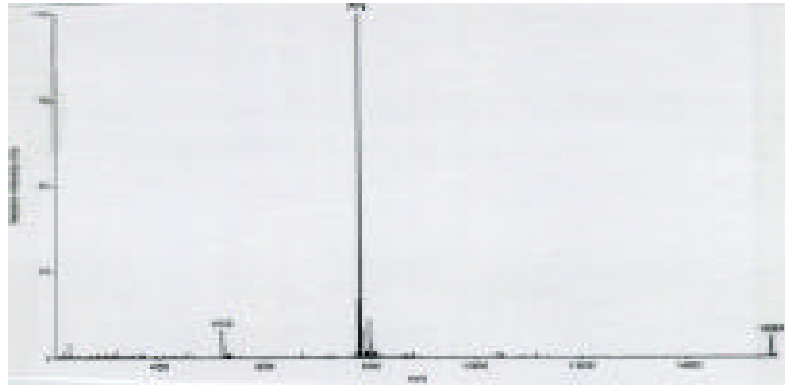
Applications of ESI-MS for peptides

- All synthetically prepared peptides should be analyzed by ESI-MS.
- Guarantees of purity based on observation of “a single peak by reversed-phase HPLC” and by “it gave the correct sequence when analyzed by Edman degradation” are hollow. This also applies to nucleotides (later).
- When we began examining synthetic peptides produced at UAB in 1993, we found that in many cases the blocking groups used during synthesis had not been removed and in extreme cases certain amino acids were missing from the peptide. This also applied to synthetic peptides produced off-campus by commercial suppliers.



The lower figure is the mass spectrum of a peptide as it was first sent to us. None of the ions corresponded to the supposed MW of the peptide. The material was a “pure” HPLC peak. After modification of the isolation procedure, the mass spectrum of some PURE peptide was obtained.

MS-MS analysis



- When peptide molecular ions are collided with neutral argon gas, daughter ion fragments are formed. For tryptic peptides, the principal molecular ion is $[M+2H]^{2+}$, and the two positive charges are at opposite ends of the molecule. This is the preferred ion for MS-MS experiments.
- The most common fragmentation occurs at the peptide bonds ($-\text{CO.NH}-$), producing C-terminal fragment ions (γ -ions) and N-terminal fragment ions (β -ions). The m/z value spacings between successive members of each series can be used to deduce the amino acid sequence.
- Amino acids of identical molecular weight (isoleucine and leucine) cannot be distinguished this way. However, loss of the R-group for these two amino acids is different (CH_2CH_3 for isoleucine and CH_3CHCH_3 for leucine) and w -ions may be observed.

MALDI-TOF analysis of proteins, peptides and other macromolecules

- Spectra usually contain only the **molecular ion** $[M+H]^+$. This is an advantage since it maximizes the signal, but is a disadvantage in that it gives no clues as to structure.
- Use of a reflector folds the energy distribution of ions in the drift region on itself and thereby increases mass resolution. Delayed extraction gives similar mass resolution in a non-reflector mode.
- If ion fragmentation occurs during the drift phase, the daughter ions have the same velocity as the parent molecular ion. Daughter ions formed during application of the extraction pulse simply increase the background noise.
- It may be possible to analyze proteins directly on a gel blot using an IR laser. Can scan a 2'' by 2'' blot from a 2D-electrophoresis experiment.

Structural experiments in a TOF-mass spectrometer

- Daughter fragment ions formed in the drift region are separated by the reflector. Suitable resolution only occurs over a limited range of m/z values. This can be overcome by recording individual spectra over a wide range of voltage settings (10-12) for the reflector. Alternatively, a curved applied voltage can be used to obtain the daughter ion spectrum in a single experiment.
- If we wait sufficiently long after the ions are initially generated, daughter ions can be formed before the extraction pulse is applied. This can give an MS-MS spectrum. However, there is no selection of the molecular ion (i.e., only pure compounds can be studied) and resolution is low due to premature drift before acceleration.
- The high mass resolution (5 ppm) offered by delayed extraction MALDI-TOF MS with internal standards can be used to distinguish between lysine and glutamine.

Microanalysis and identification of proteins

- Rapid identification of proteins by mass spectrometry has become a hot topic.
- The protein complement of a cell represents an important biological parameter in the new approaches to cell biology, pathology and physiology
- Proteins are isolated as follows:
 - by conventional chromatographic methods
 - by affinity methods (to substrate or binding protein)
 - 2D-SDS-PAGE procedures (for whole cell lysates)
- The peptides arising from proteolysis represent a recognizable fingerprint of the protein
- Identification of 50% of the tryptic peptides of the protein are sufficient to identify the protein if its amino acid or nucleotide sequence is already known.
- Confirmation of identity is made by carrying out MS-MS analysis of individual peptides.

Hydrolysis of proteins for mass spectrometry

- For proteins isolated in large amounts such as recombinant proteins, treat the protein with dithiothreitol and an alkylating agent (e.g., iodoacetamide) to protect cysteine groups and react with trypsin in an ammonium bicarbonate buffer. Evaporate the ammonium bicarbonate. The peptides are most easily analyzed by MALDI-TOF-MS.
- To avoid the problem of interfering tryptic peptides, load the protein samples onto a solid-phase trypsin column. As above, first protect the cysteine groups by alkylation. The protein may be denatured with urea for full hydrolysis, or left untreated so that only the tryptic sites in the folded protein are hydrolyzed.
- Proteins on 2D-gels (IEF/SDS-PAGE) may be analyzed in the gel or following transfer to PVDF membranes. A major advance in this method has been the introduction of immobilized pH gradients. The proteins are first located with a Cu^{2+} reagent or with Ponceau S. Newer reagents include Sypro Ruby and Cy3 and Cy5 fluorescent dyes.
- Gel pieces are dehydrated and then rehydrated in the presence of trypsin and ammonium bicarbonate buffer. After overnight incubation, the gel pieces or the membrane pieces are extracted with 50% aqueous acetonitrile several times. The eluates are combined, concentrated and analyzed by MALDI-TOF MS.

Separation of peptides prior to MS and MS-MS analysis

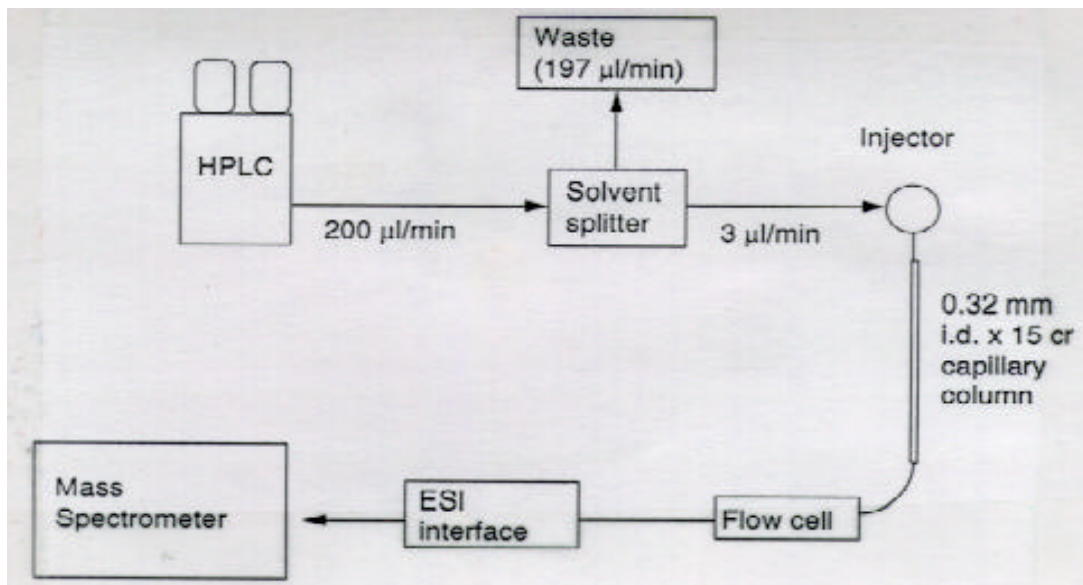
- Even “pure” peptides usually contain many contaminants
- Peptide mixtures typically arise from the enzymatic digestion of proteins

If the number of peaks in a spectrum of mixed components is not too complex, using nanoelectrospray it is possible to carry out multiple MS-MS experiments over a 30 min period and not require chromatographic separation of each component.

For all other circumstances, preliminary chromatographic separation is required (it is assumed for now that the protein has been purified to homogeneity).

- **WATCH OUT FOR KERATINS**
- Reversed-phase HPLC
- Capillary electrophoresis

Reversed-phase HPLC



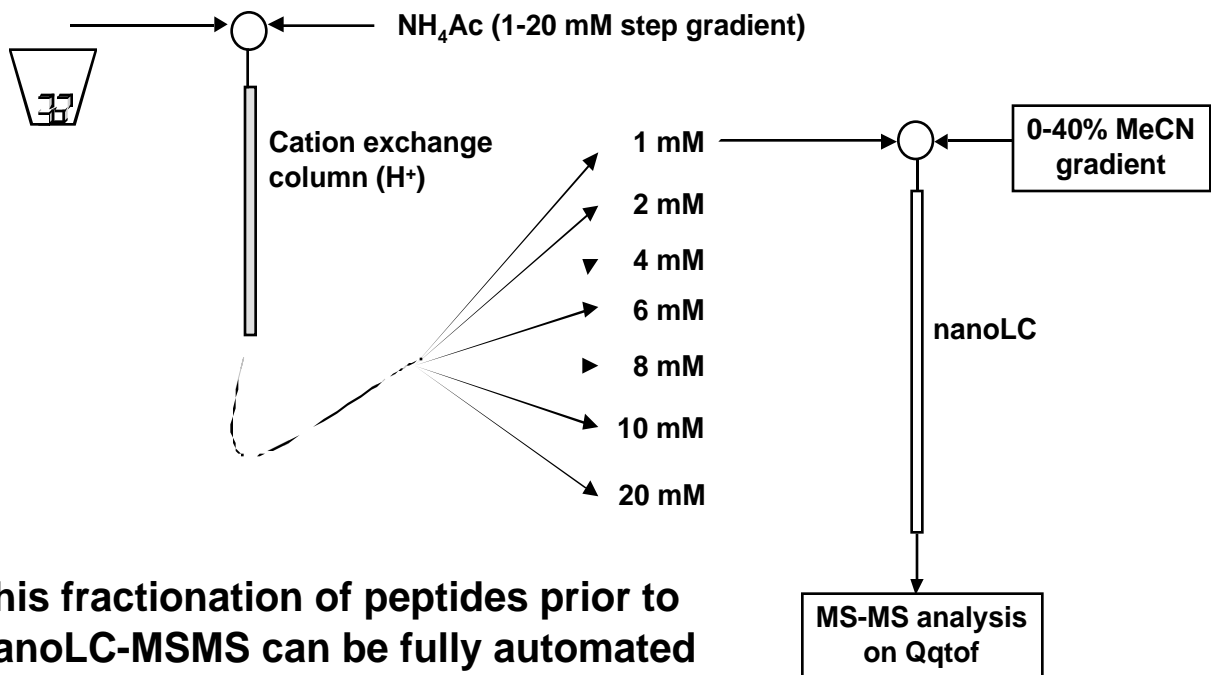
- Peptide peaks can be collected manually by chromatographing the peptide mixture over conventionally sized, reversed-phase columns (1.0 to 4.6 mm i.d.). Each fraction is then analyzed by ESI-MS or MALDI-TOF MS (better).
- Since the sensitivity of ESI is inversely proportional to the flow rate (slower is better), for a given mass of peptide, narrow capillary columns (0.075 to 0.32 mm i.d.) give much better sensitivity than microbore (1.0 mm i.d.) or narrow bore (2.1 mm i.d.) columns.
- The increase in sensitivity is approximately proportional to the inverse of the ratio of the cross sectional areas of the columns. Therefore, a 0.32 mm i.d. capillary column gives an approximately 10-fold increase in sensitivity over a microbore column and nearly a 50-fold increase over a narrow bore column.
- Low flow rates achieved using regular HPLC pumps followed by a flow splitter PRIOR to mixing with the sample stream.
- Newer methods involve smaller reversed-phase particles (1.5 µm), higher pressures and high flow rates. A typical analysis can be reduced to 35 sec a run (as opposed to 20-30 min under current conditions). To acquire data at these speeds, TOF analyzers are attached to ESI interfaces.

MUDPIT analysis

- A new approach to the analysis of proteins is to hydrolyze them all to tryptic peptides using the Multi Dimensional Protein Identification Technology.

Washburn MP. Wolters D. Yates JR 3rd. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nature Biotechnology.19:242-247, 2001

Fractionating tryptic peptides *Preliminary separation by ion-exchange*



This fractionation of peptides prior to nanoLC-MSMS can be fully automated

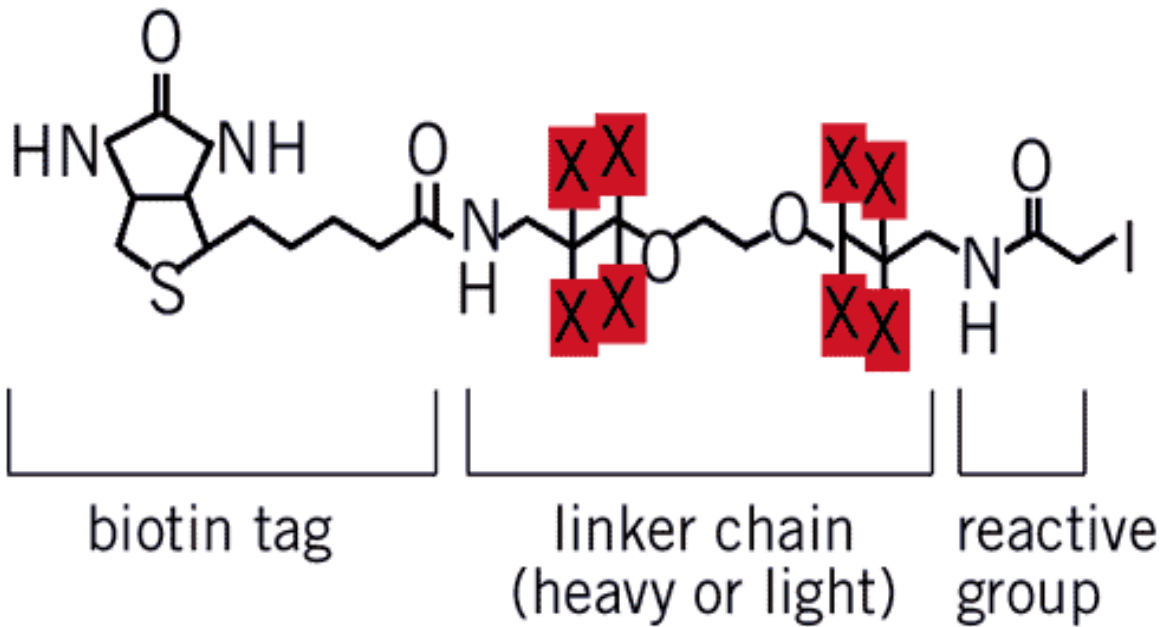
Affinity isolation

- Isotope Coded Affinity Reagent for quantitating protein expression

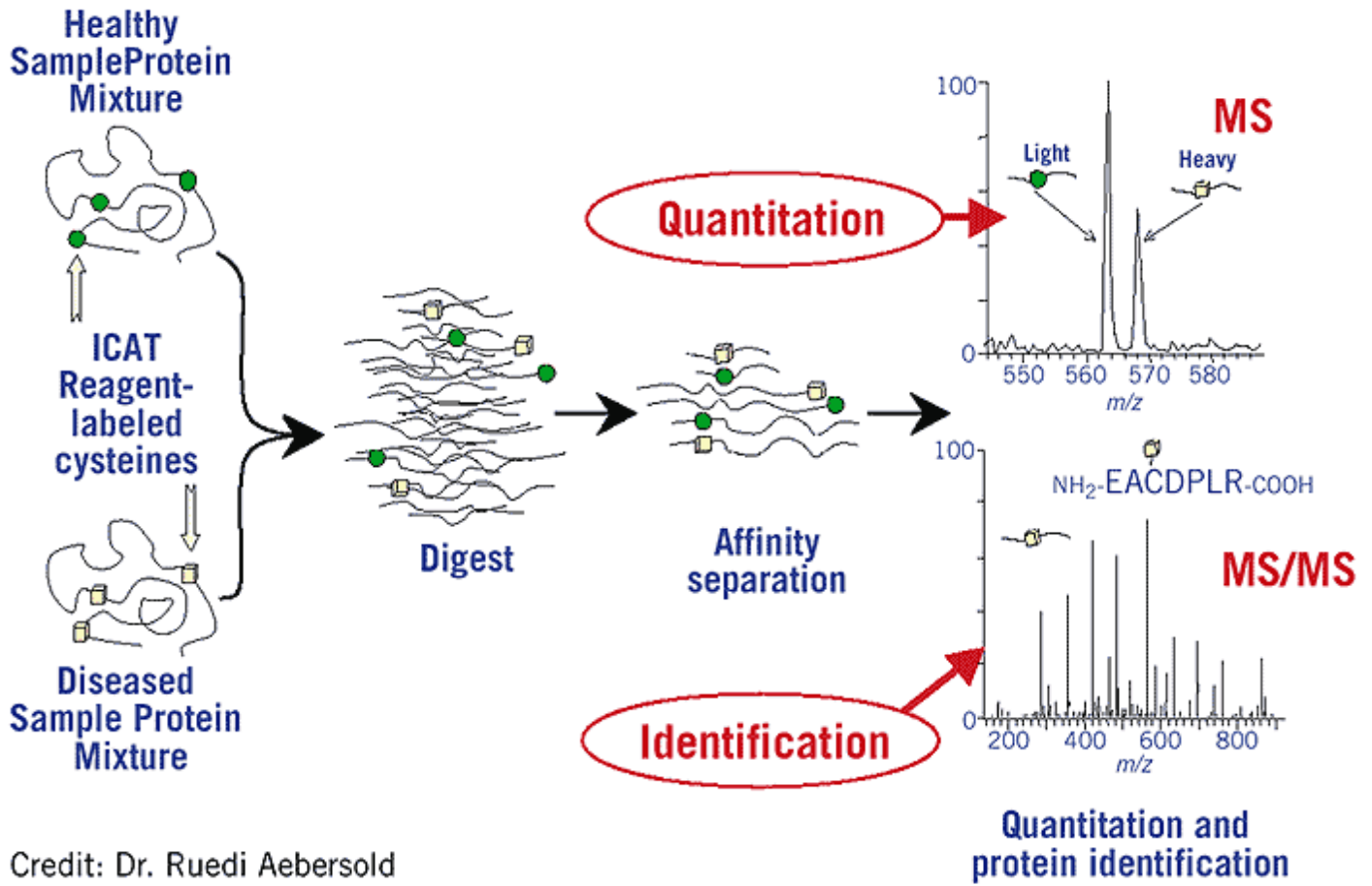
Isotope-Coded Affinity Tags

heavy reagent: D8-ICAT Reagent (X=deuterium)

light reagent: D0-ICAT Reagent (X=hydrogen)



Quantitating protein expression



Credit: Dr. Ruedi Aebersold
Institute for Systems Biology, Seattle, WA

Other tagged peptide methods

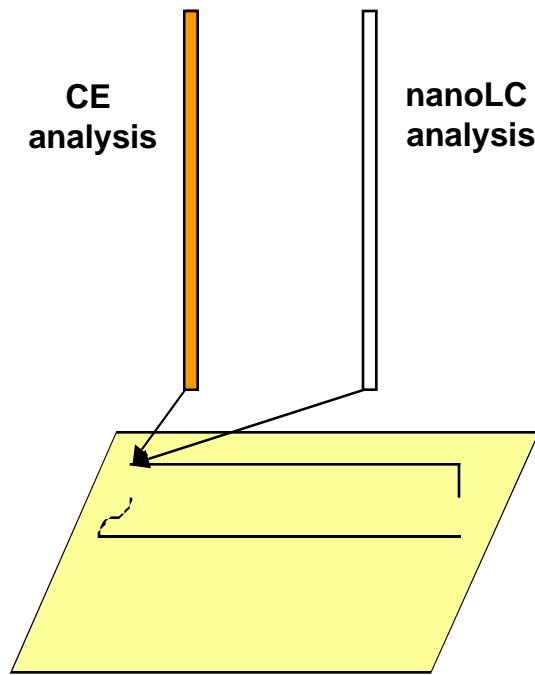
Affinity columns containing antibodies against specific epitopes

- anti-phosphotyrosine
- specific proteins if the intent is to recover protein complexes (mini-subproteomes)

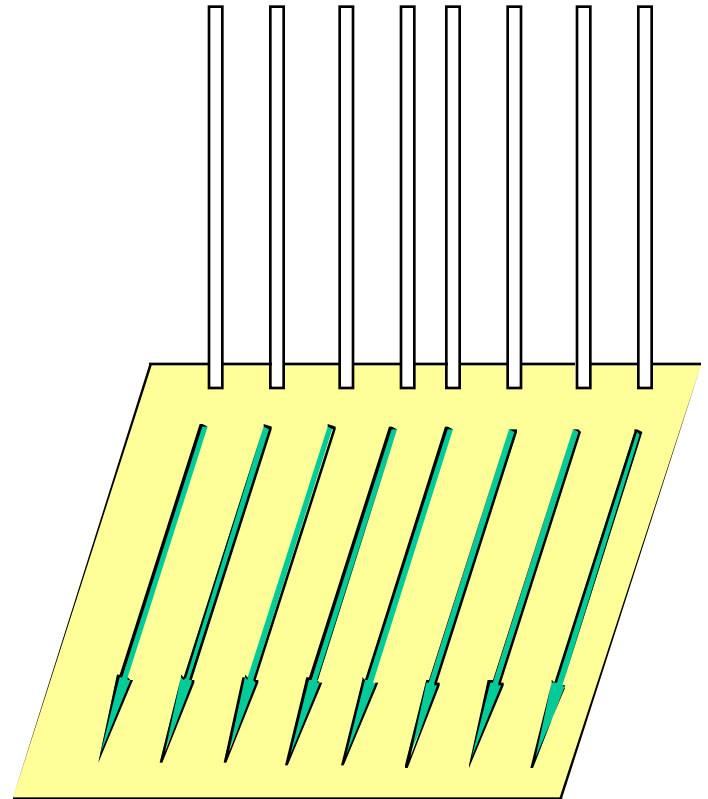
Subproteomes may be recovered using baits on recombinant proteins

- Flag
- GSH

MALDI capture of CE and nanoLC



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

Capillary electrophoresis

- CE and mass spectrometry is a marriage made in heaven. It has proved much harder to implement.
- The low flow rates of CE (10-20 nl/min) are suitable for ESI-MS. However, existing commercial interfaces are constructed such a make up flow is required. This dilutes the capillary eluate and thereby loses the sensitivity offered by the high resolving power of CE.
- In the past year, details on CE-MS interfaces which operate at 10-20 nl/min have been published.
- The optimum solvents for the separation of peptides by CE (e.g., phosphate buffers) conflict with the formation of ions by ESI.