Getting the sample from the matrix to the mass spectrometer

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Opening statement

- The analytes we are most interested in are a very minor part of the biological specimens in which they are contained
- The challenge is to remove the parts we don't want without losing any of those we plan to measure
- Sometimes the principal "contaminants" are in the same class as those to be measured, e.g., albumin in plasma/serum

Source for Reference

- **You are recommended to refer to an excellent 2007 review paper by Cañas and colleagues entitled,**

  "*Trends in sample preparation for classical and second generation proteomics*"

  published in the Journal of Chromatography A, vol. 1153, pp 235-258.

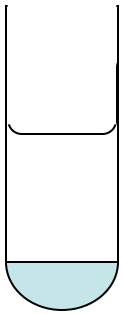
  It is available through Lister Hill Library

But first, drugs and their metabolites

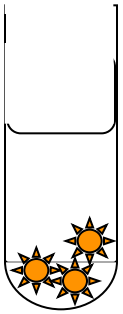
- In general, drugs are lipid soluble. If they are to be absorbed through the gastrointestinal wall, they have to be as hydrophobic as octanol
- Drugs are absorbed from the intestines at places where the pH varies from 1-2 (in the stomach) to neutral pH in the colon
- Most drug metabolites are more hydrophilic
  - Glucuronides, sulfates, amino acids
  - Acetates, methylation usually more hydrophobic

Recovery of drugs from samples

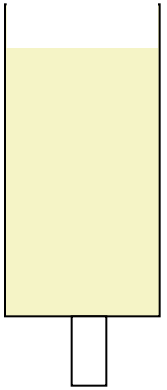
Partition across a non-miscible organic solvent and water



Protein precipitation



Solid-phase extraction



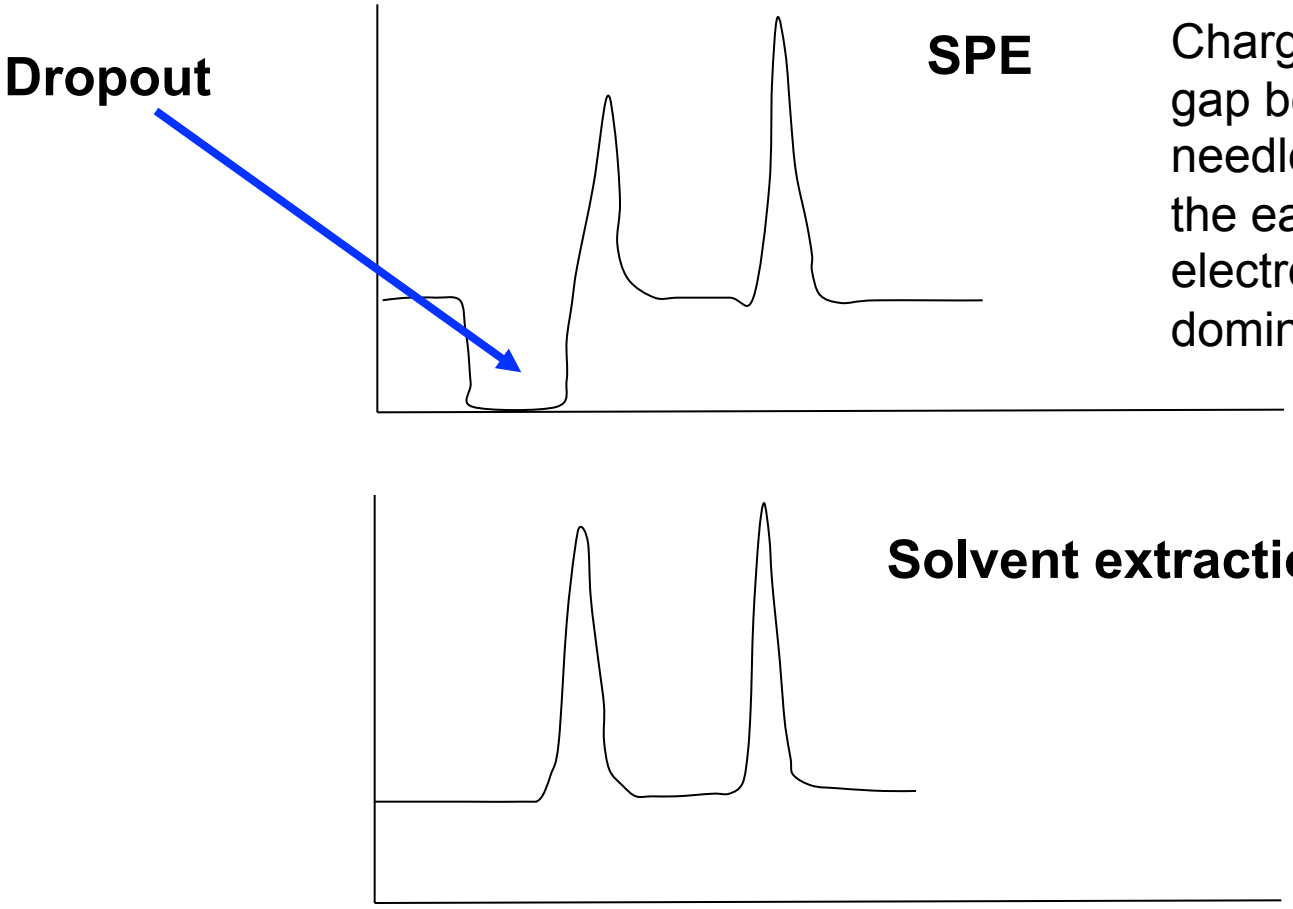
Solvent partition

- **Partition across a non-miscible organic solvent and water**
  - **Ethyl acetate, diethyl ether, dichloro(m)ethane, chloroform, heptane and hexane**
  - **Solubility in the organic phase depends on the pH**
    - **Organic acids should be acidified (formic acid, HCl)**
    - **Bases treated with ammonia**
    - **Some organic acids are treated with a hydrophobic base to create a hydrophobic salt**
  - **Highly reproducible, but slow**
  - **Generally free of electrolytes**
  - **Fats can be extract with hexane (protects HPLC column)**
- **Proteins and peptides are not recovered by solvent extraction**

Solid-phase extraction

- If the biological sample is a clear fluid, e.g., bile, plasma/serum, urine, then the analytes can be adsorbed to a chemically treated solid phase (as a cartridge or a ZipTip) – see later in class
- Silica chemically bonded with C_4, C_8 and C_{18} hydrocarbon chains forms a "reverse phase"
- Other modifications include for example cyano groups
- After washing with water or 5% acetonitrile to remove unbound materials, the bound substances are eluted with a water-miscible and volatile solvent, e.g., MeOH, acetonitrile, isopropanol
- The extract generally contains some electrolytes

Ion chromatograms - SPE vs solvent extraction



Charge transfer across the gap between the spraying needle and the orifice takes the easiest route - if electrolytes are present, they dominate the process

Protein precipitation

- This is carried out by the addition of a water-miscible solvent (methanol, ethanol)
- Typically 1 volume of the sample and 4 volumes of the solvent are mixed - the precipitated protein is removed by centrifugation
- Small volumes of the deproteinized supernatant can be analyzed directly if the concentrations of the drug are sufficiently high
- Otherwise, the solvent should be evaporated (under N_2)
- Ammonium hydroxide or formic acid can be added to displace the drug from the protein

Handling proteins and peptides

- **Collecting and storing the sample**
  - **Use the same type of storage device for all the samples in a study**
  - **Some samples are sensitive to freezing**
    - **Mitochondria and other organelle fractions should be prepared using fresh tissue**
    - **Samples for analysis (fluids and purified organelle preparations) should be placed in divided tubes and only thawed one time to avoid the effects of multiple freeze-thaw cycles**
  - **Freeze fast (into liquid N_2)**
    - **Buffers such as sodium phosphate can selectively precipitate while ice and water are in equilibrium (down to -20^{o}C) - this can lead to a substantial change in pH**
    - **Similarly, melt fast rather than slowly**

Tissue disruption/cell lysis

- Manual and mechanical homogenization
  - Pestle and mortar, Dounce and Potter-Elvehjem homogenizers, Waring blender, Polytron
- Grinding with beads, sonication and freeze-thaw – plant cells
- Osmotic shock - or not! High pressure
- Bugbuster for bacteria
- Detergents
  - CHAPS, Triton X-100, cholate and deoxycholate, octylglucoside
- Protease inhibitors

Commercial kits for proteomics

- **InVitrogen**
  - http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Protein-Expression-and-Analysis/Protein-Sample-Preparation-and-Protein-Purification.html
- **Sigma-Aldrich**
  - http://www.sigmaaldrich.com/life-science/proteomics/recombinant-protein-expression/cell-lysis.html
- **Millipore**
  - http://www.millipore.com/immunodetection/id3/proteinextractionkits
- **ThermoFisher/Pierce**
  - http://www.piercenet.com/Proteomics/browse.cfm?fldID=FA97D803-6953-48E4-A7BD-6947D35FE83B
- **Promega**
  - http://www.promega.com/literature/Brochures/Proteomics/BR148.pdf

Preparing proteins for peptide mass fingerprinting

- The mass spectrometry procedure has to be preceded by high resolution protein chromatography steps
- Or, it can be a sample with a few intense bands of interest - e.g., a recombinantly expressed protein in bacteria lysate
- The samples are in a variety of buffers, even high salt and detergents, prior to SDS-PAGE electrophoresis - the salts migrate faster than the proteins

Preparing proteins for peptide mass fingerprinting (2)

- Since the protein is precipitated in the SDS-PAGE gel matrix, the electrolytes/salts and the SDS are largely removed by washing the gel pieces with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8
- Solvent in the gel is removed by evaporation and the gel piece is rehydrated in 25 mM NH_4HCO_3 buffer, pH 8 and trypsin added
- The resulting peptides are extracted with 50% aqueous acetonitrile containing 25 mM NH_4HCO_3 buffer, pH 8 - the extract is evaporated

Peptide mass fingerprinting

Eppendorf tube

destain

Speed-Vac

trypsin

1:20

Incubate

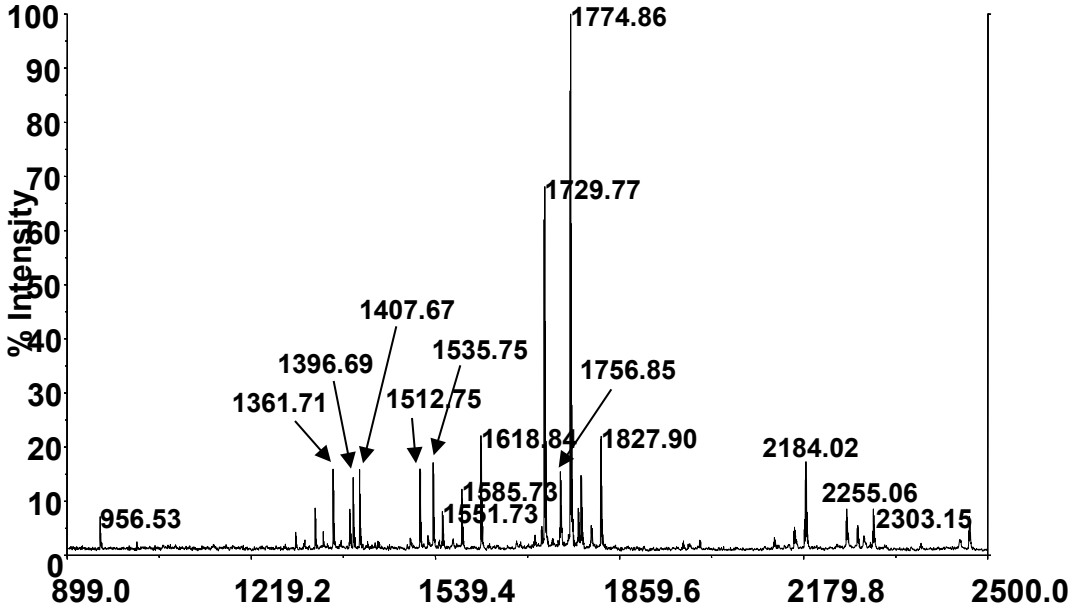
overnight

Water Bath 37°C

Peptide extraction

MALDI plate

Desalting Ziptip



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Preparing for 2D-electrophoresis

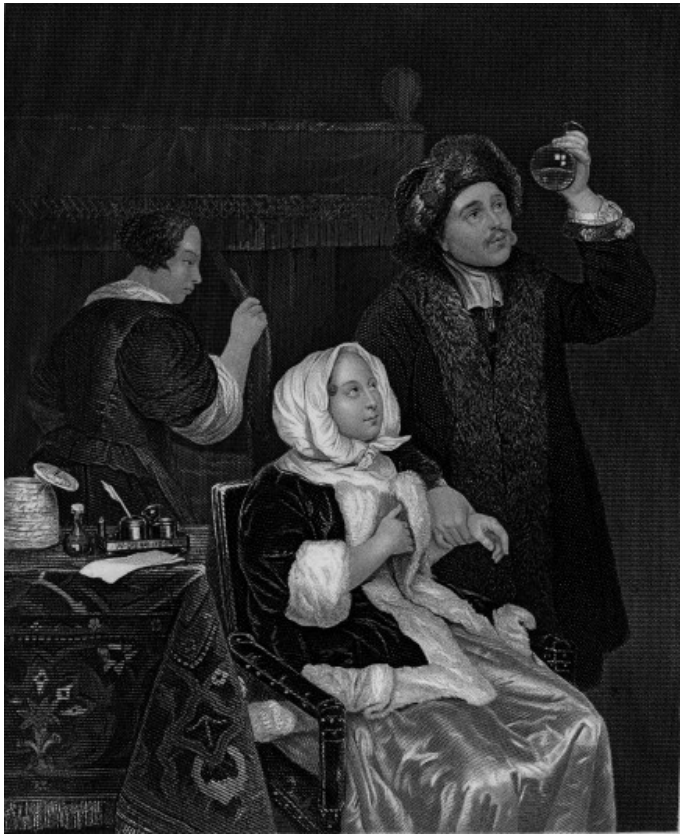
- **Extract tissue to recover proteins**
  - **Freeze tissue in liquid N_2 - grind to a powder**
  - **Extract into 7 M urea - 2 M thiourea - use high quality reagents**
  - **Need to remove salt and SDS (if used)**
- **Serum and plasma may require removal of common proteins**
- **Urine is by definition an aqueous solution**
  - **Proteins can be recovered by $CHCl_3$:MeOH precipitation (concentrates and desalts)**

Different types of 2D-electrophoresis

- **Isoelectric focusing/SDS-PAGE**
  - **This method is suited to proteins that are soluble in 7 M urea-2 M thiourea**
- **2D-blue native**
  - **Uses Coomassie Blue as the protein carrier**
- **2D-clear native**
  - **Uses deoxycholate as the protein carrier**
- **Both of the latter procedures are good for intrinsic membrane proteins (see Dr. Kim's talk)**
  - **The membrane proteins need to be solubilized with a mild non-ionic detergent**

Focus on: Preparing samples from Biofluids for Proteomic Analysis

Biofluids: Serum, plasma, urine, Saliva, Broncheoalveolar lavage (BALF), Nipple Aspirate



Eknoyan, *American Journal of Kidney Diseases*, **49**, 2007, 865-872

Need to consider:

- *What are we interested in d*etermining?
  - molecular weight determination
  - identifying proteins
  - biomarker discovery
  - characterizing post-translational modifications (glycosylation, phosphorylation)

- Nature of sample – simple vs complex mixture

- Matrix (medium) of samples

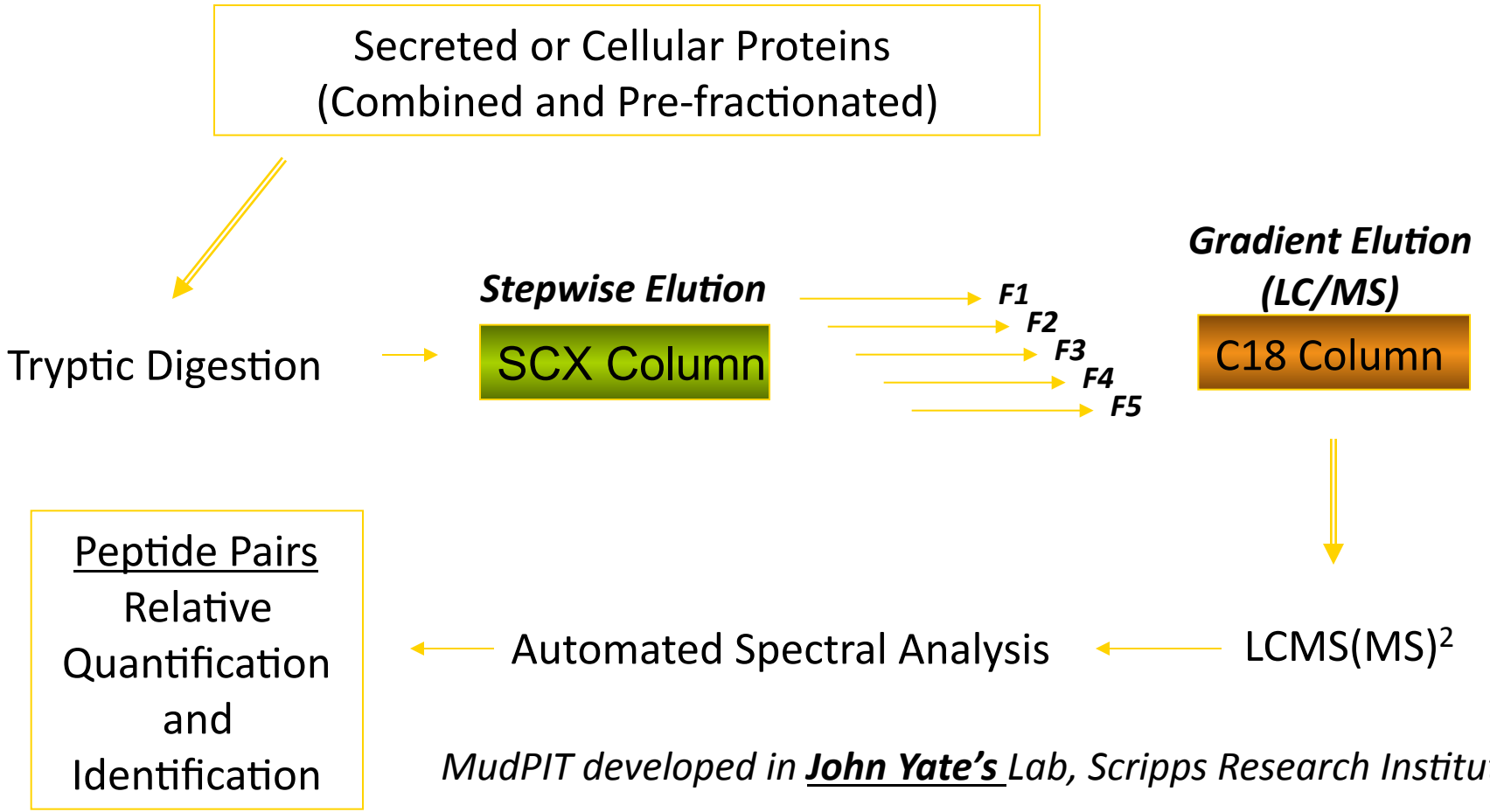
Biofluids – complex mixture and matrices

- How do we prepare samples for MS analysis?
  - assuming appropriate experimental design
  - proper collection and storage of samples

Depends …

(i) composition of samples,
(ii) compatibility with analytic method
(avoid molecules that interfere with ionization/detection by mass spectrometry)

Multidimensional Protein Identification Technologies (MuDPIT)



*MudPIT developed in **John Yate's** Lab, Scripps Research Institute*

Proteins/peptides of interest are of low abundance

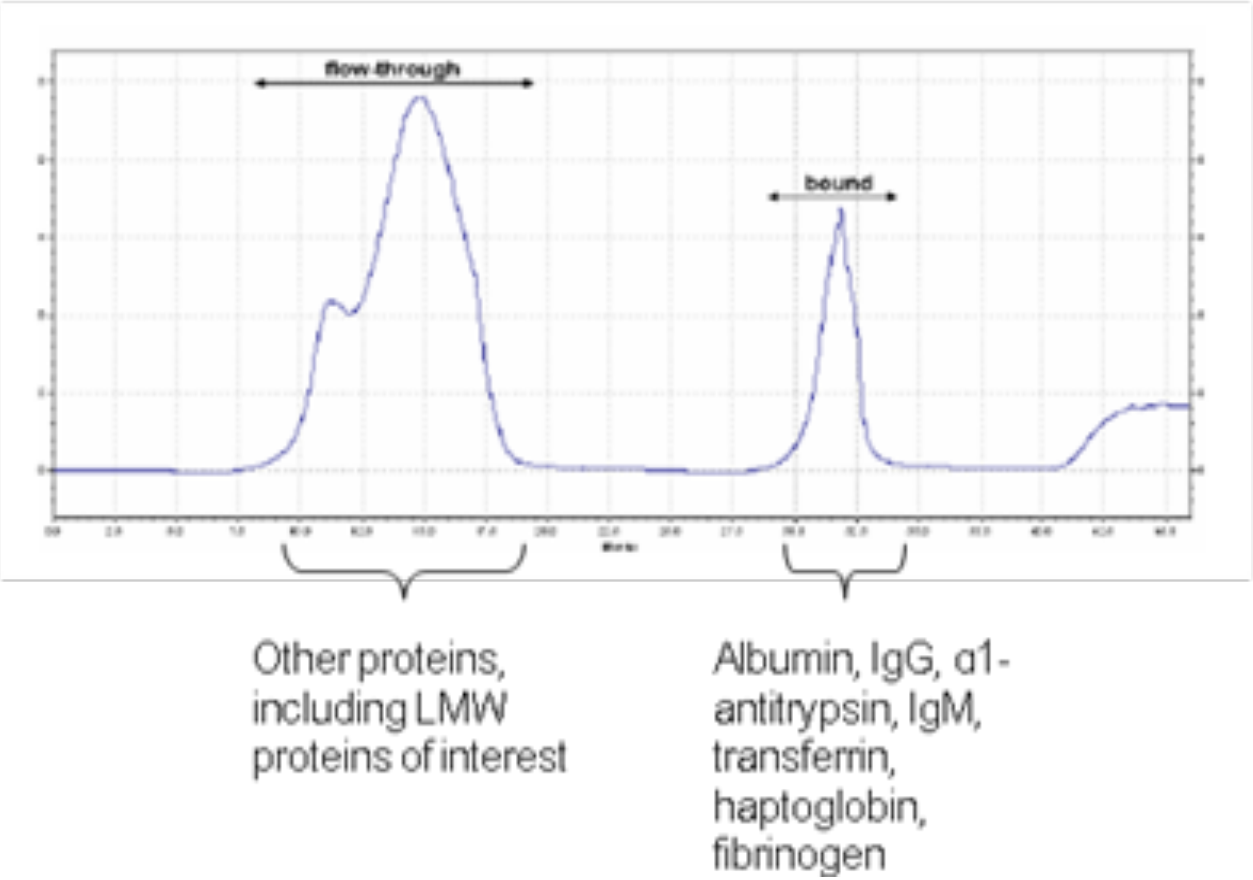
For e.g. in serum/plasma:

Large dynamic range mg/ml (serum albumin) to pg/ml (interleukins)
-difficult to detect and quantify low abundance proteins/peptides

Top 12 most abundant serum proteins:

- HSA (Human Serum Albumin)
- IgG
- Fibrinogen
- Transferrin
- IgA
- IgM
- Haptoglobin
- a2-Macroglobulin
- a1-Acid Glycoprotein
- a1-Antitrypsin
- Apo A-I
- Apo A-II

Depletion techniques to remove most abundant proteins



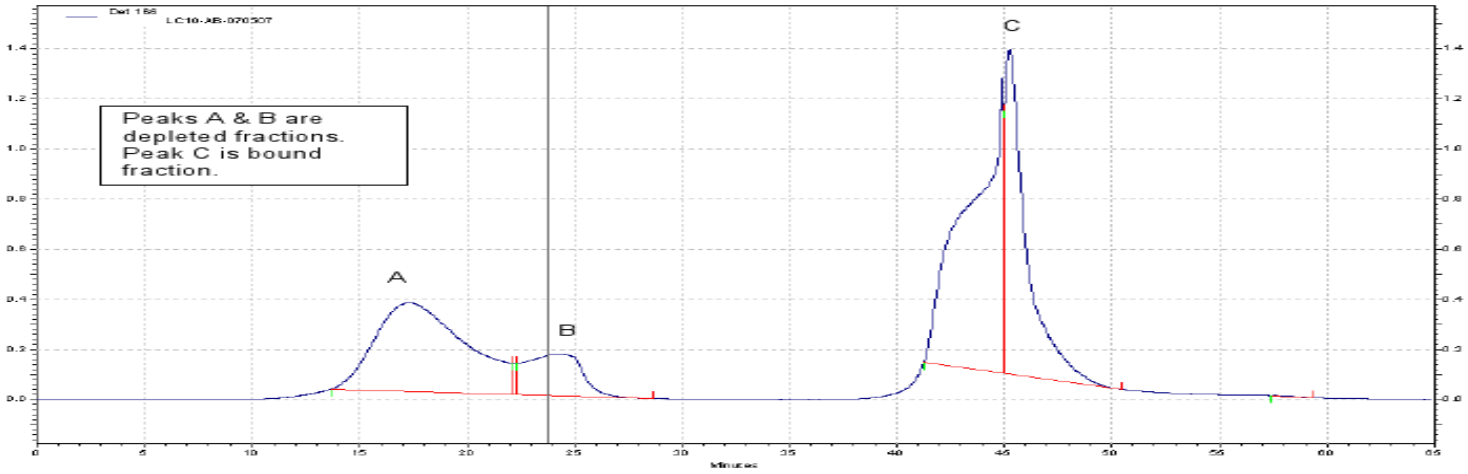
IgY R7 Immunoaffinity column

Table 1.
Timetable for IgY 14 LC2 column

Cycle	Time (minutes)	1× Dilution Buffer (%)	1× Stripping Buffer (%)	1× Neutralization Buffer (%)	Flow Rate (ml/minute)	Maximum Pressure (psi)
Injection						
Wash	0	100	0	0	0.2	350
Wash	17.01	100	0	0	1.5	350
Elution	22.01	0	100	0	1.5	350
Neutralization	36.01	0	0	100	1.5	350
Re-equilibration	42.01	100	0	0	1.5	350
Stop	50.00					

Method for 6.4 × 63.0 mm column is optimized for Beckman System Gold HPLC, Pump Module 1 Type: 118, Detector Model: 166.

Results
Figure 1.
Typical Depletion Chromatogram



Seppro IgY 14 LC-2 column

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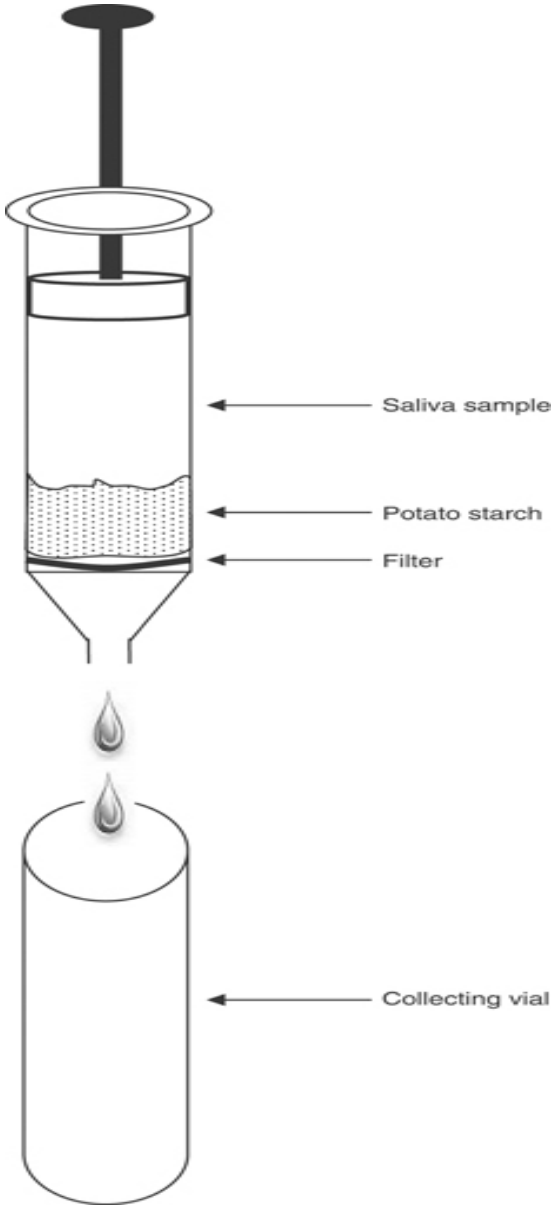

Amylase depletion in whole saliva

Electrophoresis Vol.29, 20 Pages: 4150-4157

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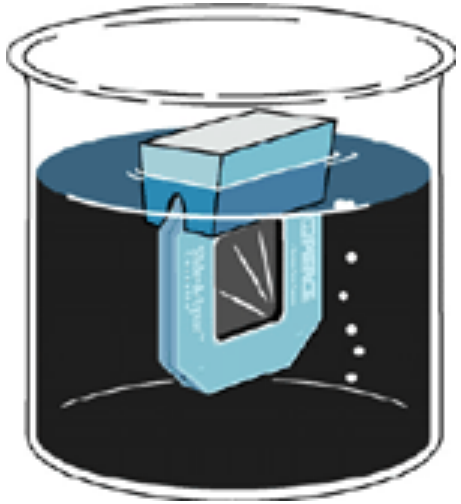
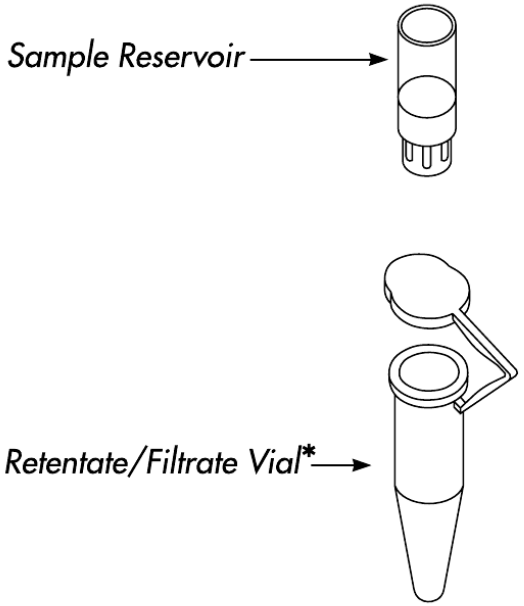
Desalting, concentration,buffer exchange



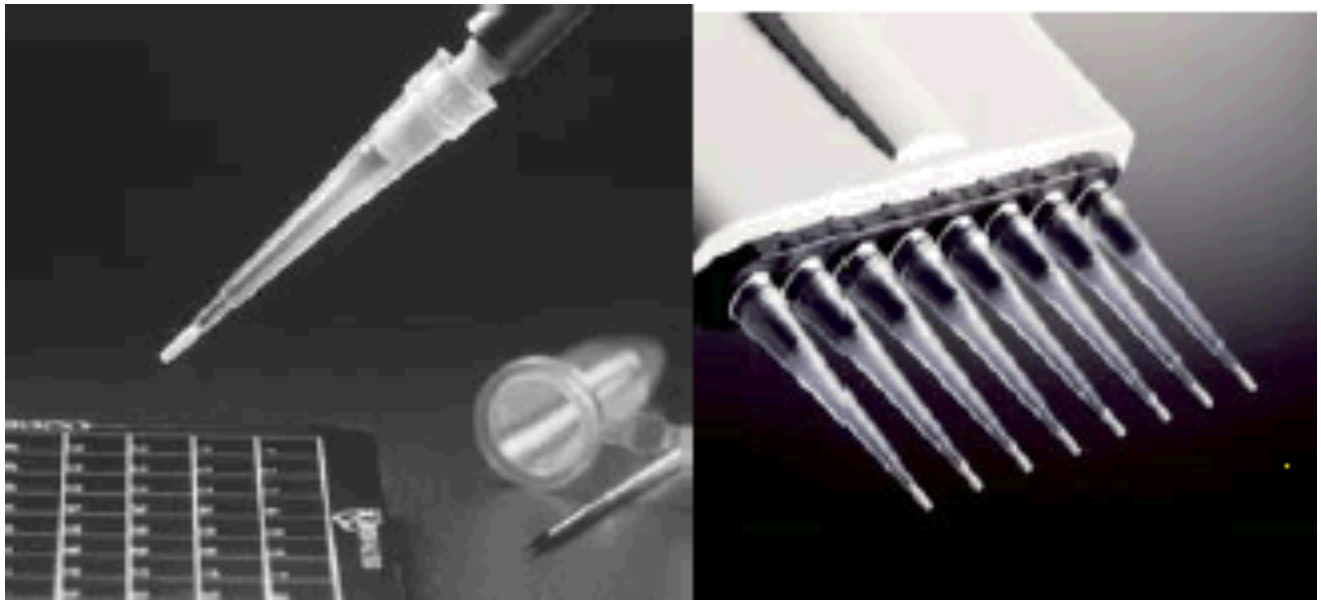
Slide-A-Lyzer™ Dialysis (Pierce)

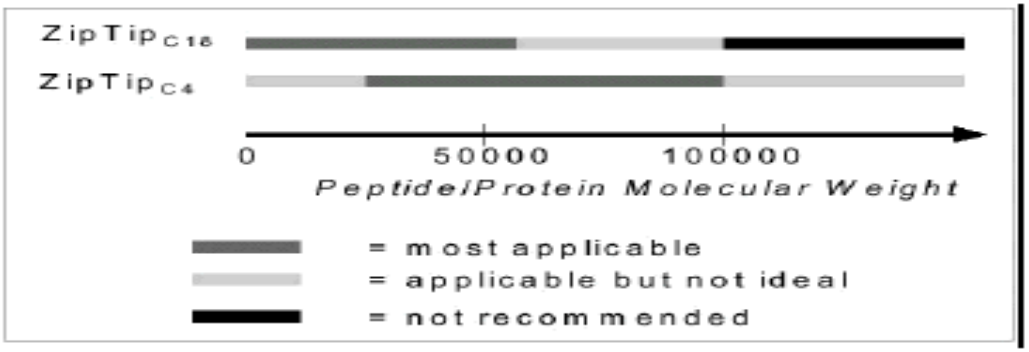
http://biotech.matcmadison.edu/resources/proteins/labManual/chapter_4/section4_3.htm



Microcon centrifugal device (Millipore)

http://www.millipore.com





http://www.millipore.com/techpublications/tech1/ps2342enus

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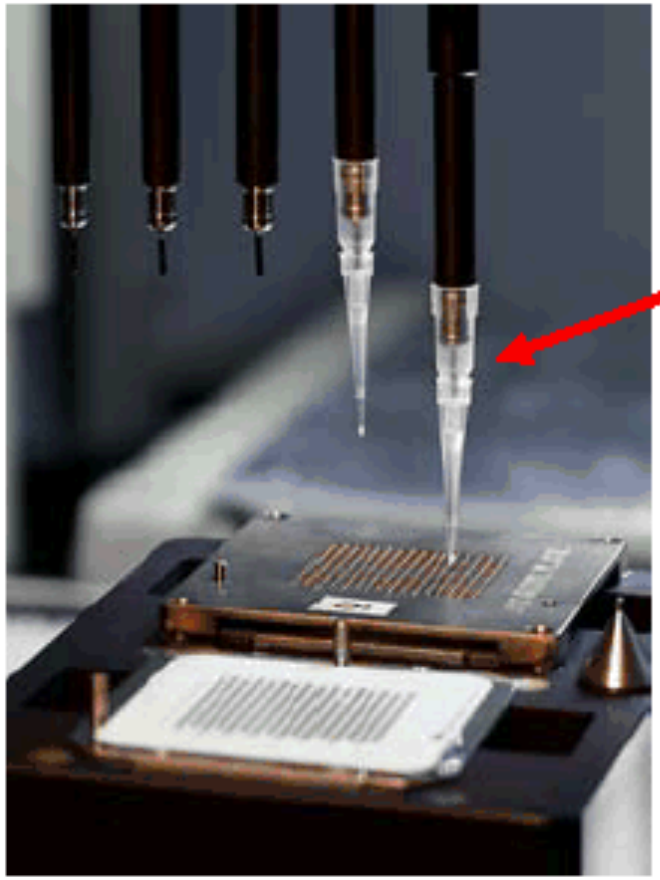


Micromass Mass*PREP*™ and PE MultiPROBE® compatibility. Integrated device containing an adapter collar press fit into a ZipTip$_{\mu\text{-}C18}$ containing a 200 nl bed of C18 allowing elution in minimal volumes.

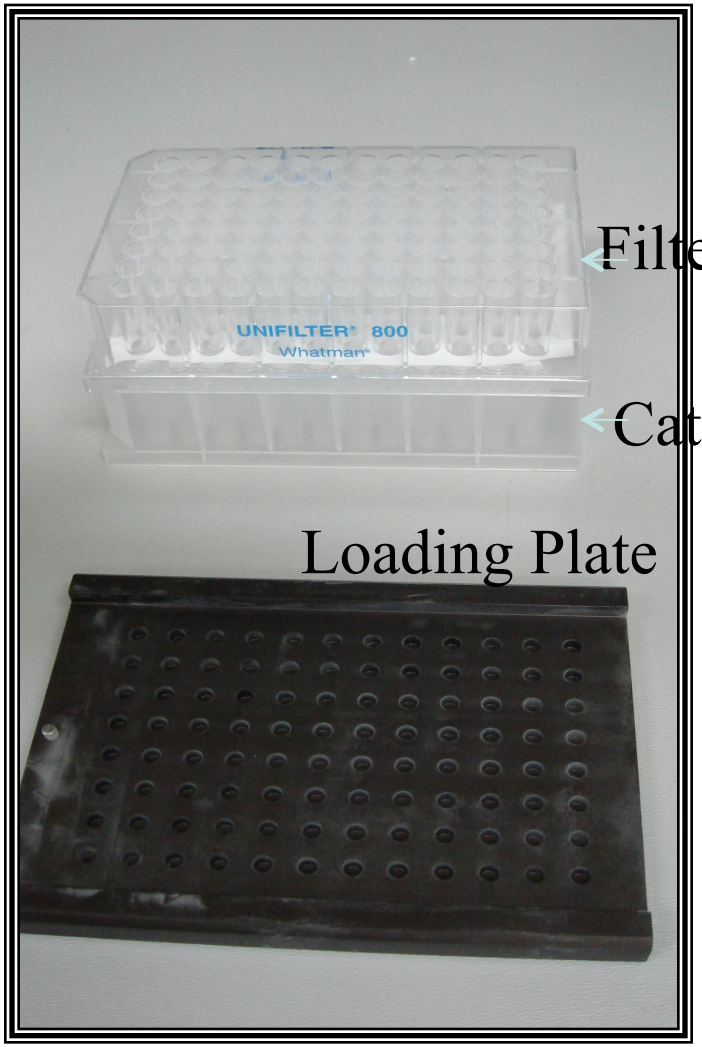
Additional Automation Protocols:

- ABI Symbiot™
- Bruker MAP™ II and MAP II/8
- Genomic Solution ProMS™
- Tecan Genesis

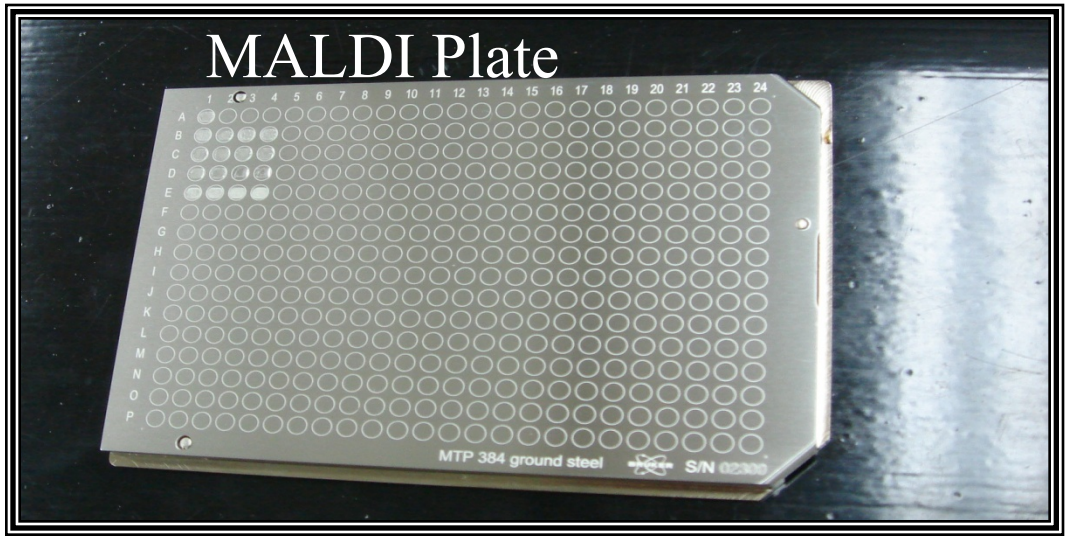
Automation reduces the labor involved with sample processing and target spotting.

http://www.millipore.com/techpublications/tech1/ps2342enus

C18 Packed Filter Plate



1) *Add C18 and Organic to activate*
2) *Remove Organic*
3) *Add Sample*
4) *Adsorb by Shaking*
5) *Centrifuge off Non-Binding Proteins*
6) *Elute With Organic*
7) *Spot to MALDI target Plate*



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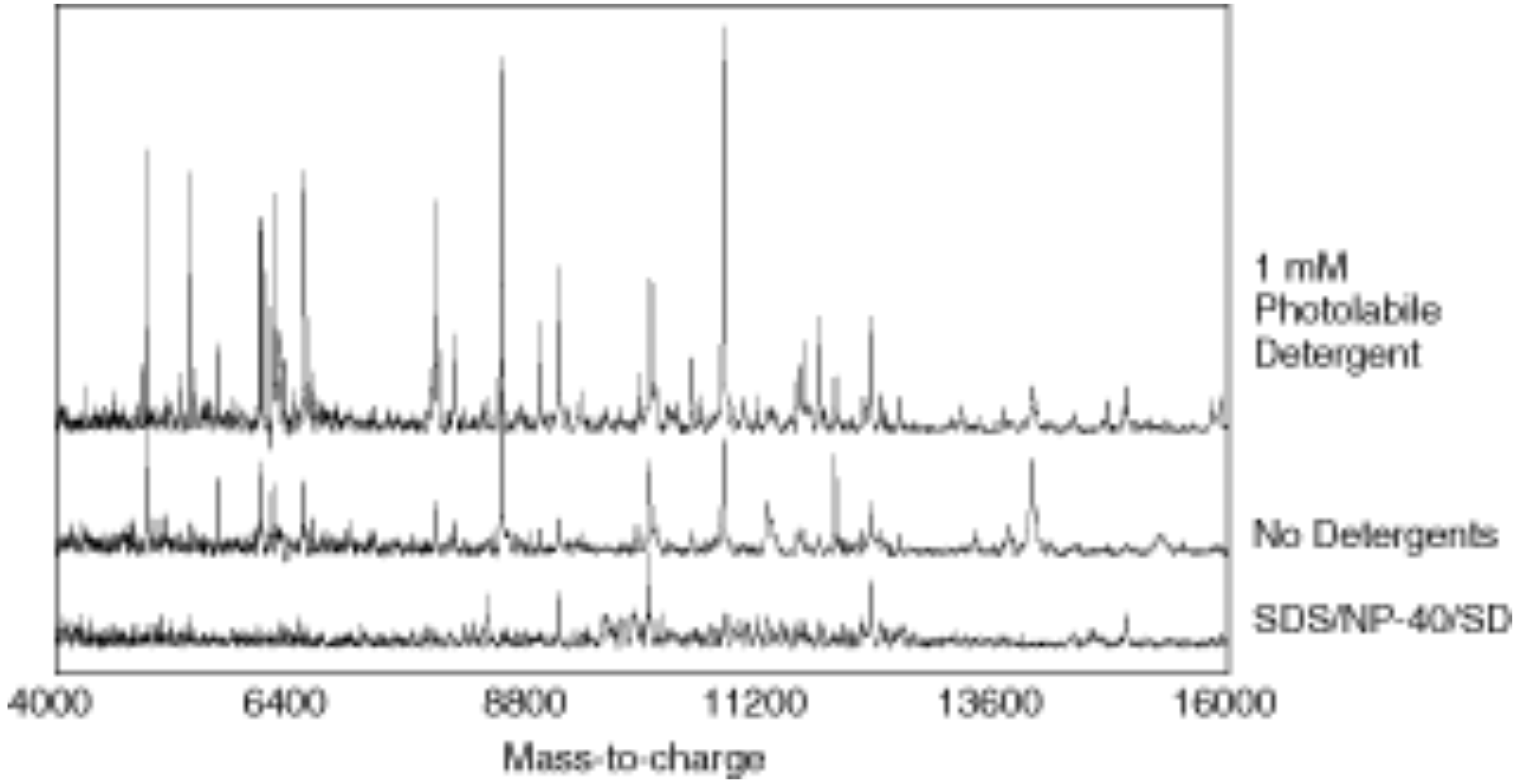
Effect of detergents – example of use of nonacid cleavable detergents designed for MS



J. Mass. Spectrom. 2005:40, 1319-1326

Design your protocols carefully:
- to avoid detergents, salts etc that interfere with ionization/detection
- to avoid introducing contaminants – such as keratin from skin, hair

Use chemistry of your samples to design effective clean-up, fractionation techniques

Use simplest possible procedure that allow desired result (sample recovery, throughput)

Reasons to purify proteins

- To characterize them
  - e.g., for their enzyme activity, chaperone function
- To determine structure
  - How they assemble in complexes
- To make antibodies
  - For immunoaffinity purification
- Their pharmaceutical properties

Pre-fractionation of proteins

- Can be based on molecular weight
  - Dialysis, centrifugation through membranes of different porosity
- Differential centrifugation
  - Nuclei, mitochondria, plasma membranes, microsomes, cytosol
- Gradient centrifugation (sucrose, Percoll)
  - lysosomes, peroxisomes, autophagosomes

Purification fundamentals

- How much do we need and should it be pure?
- Do we want it in the native form?
  - Many of the recombinant proteins are misfolded
- How are we going to measure it during the purification steps?
  - Enzyme or other biochemical assay
  - Using an antibody
  - Using SDS-PAGE gel

Where do we get the protein from?

- **Used to be from tissue or body fluids**
  - **Some proteins are from animal parts obtained at slaughter houses**
  - **These can include liver, brain, kidneys**
  - **Some proteins are richly expressed in one of these tissues, e.g., tau in brain**
- **Since 1980, recombinant expression in bacteria and other systems have provided replenishable and unlimited amounts**
  - **But the proteins still have to be purified**

Limitations of recombinant expression

- **Poor folding in bacteria**
  - **Large amount of proteins in inclusion bodies**
  - **Possible to coincidentally overexpress chaperone proteins**
- **Truncation**
  - **Chemical and enzymatic, deamidation**
- **Most of the posttranslational mechanisms in eukaryotic systems do not occur in bacteria**
  - **Phosphorylation on a serine can be simulated by mutating the serine to aspartate**

Assists using molecular biology approaches

- It is possible to add tags that assist in recovering the protein
  - 6xHis, HAT, maltose-binding protein, glutathione S-transferase
  - Biotinylation site
- But from a structural/functional point of view, is that a disadvantage?
  - Alterations in protein structure caused by the tag
  - Back to protein purification

What to consider

- Choice of biological source
- How to maximize the tissue recovery
- How to monitor the protein
- How to develop a purification strategy
- Techniques to be used
- How to integrate the techniques

Harvesting the protein

Remember that what ever you do from this point on, you're likely to have remnants of any buffer component in your final purified sample

- **Avoid detergents!!!!!!**
- **The reality is that proteins that will be studied by mass spec have to be water soluble or soluble in a detergent that isn't a polymer**
- **Grind tissue in liquid nitrogen to minimize degradation**
- **Use BugBuster™ for bacteria**

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 in order to see the complex ions

Carol Robinson, FRS - Oxford group

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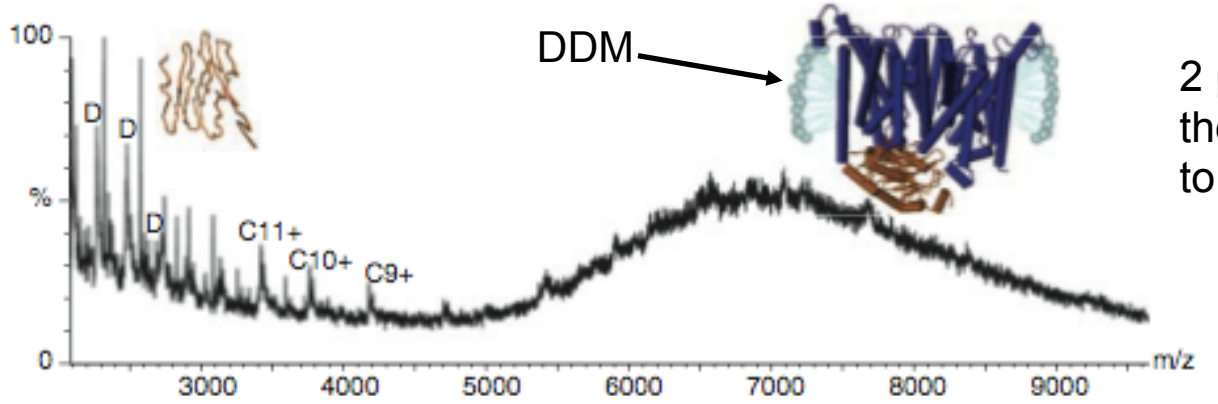
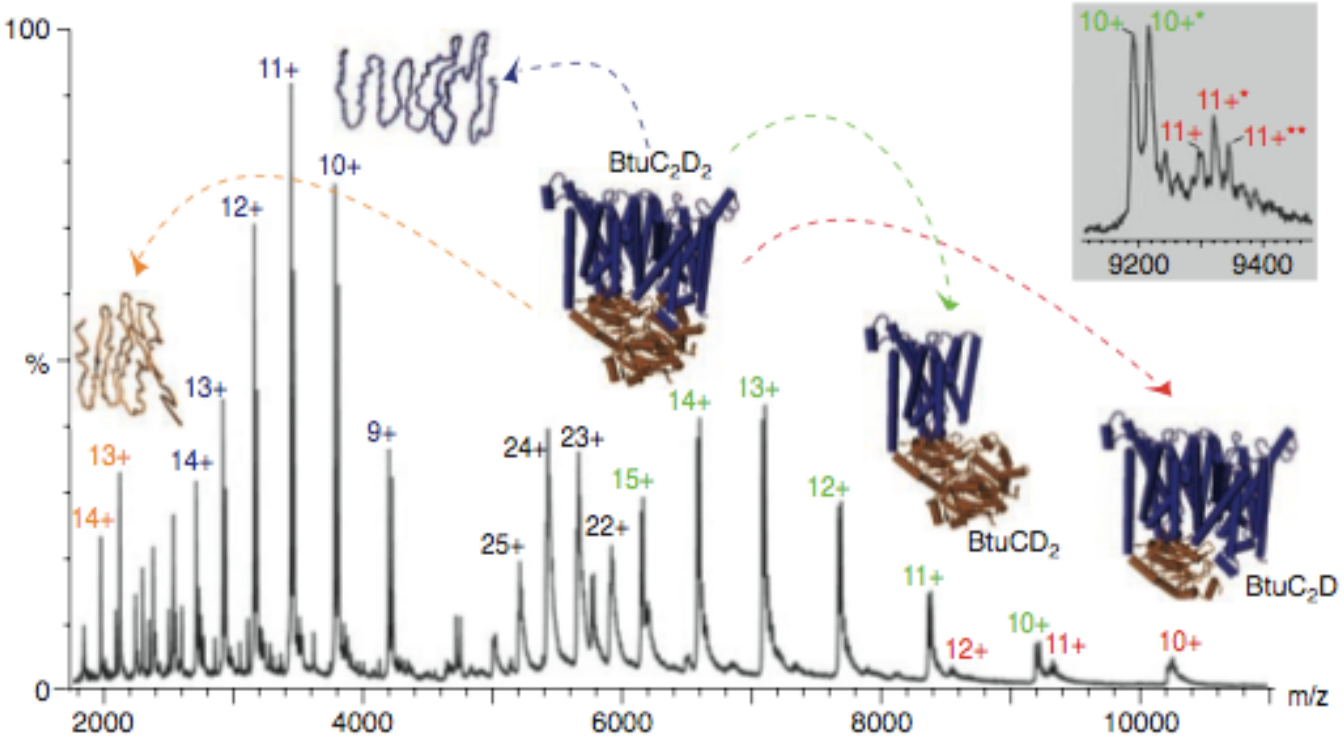
Effect of increasing voltage on $BtuC_2D_2$ membrane complexes observed in nanoMS



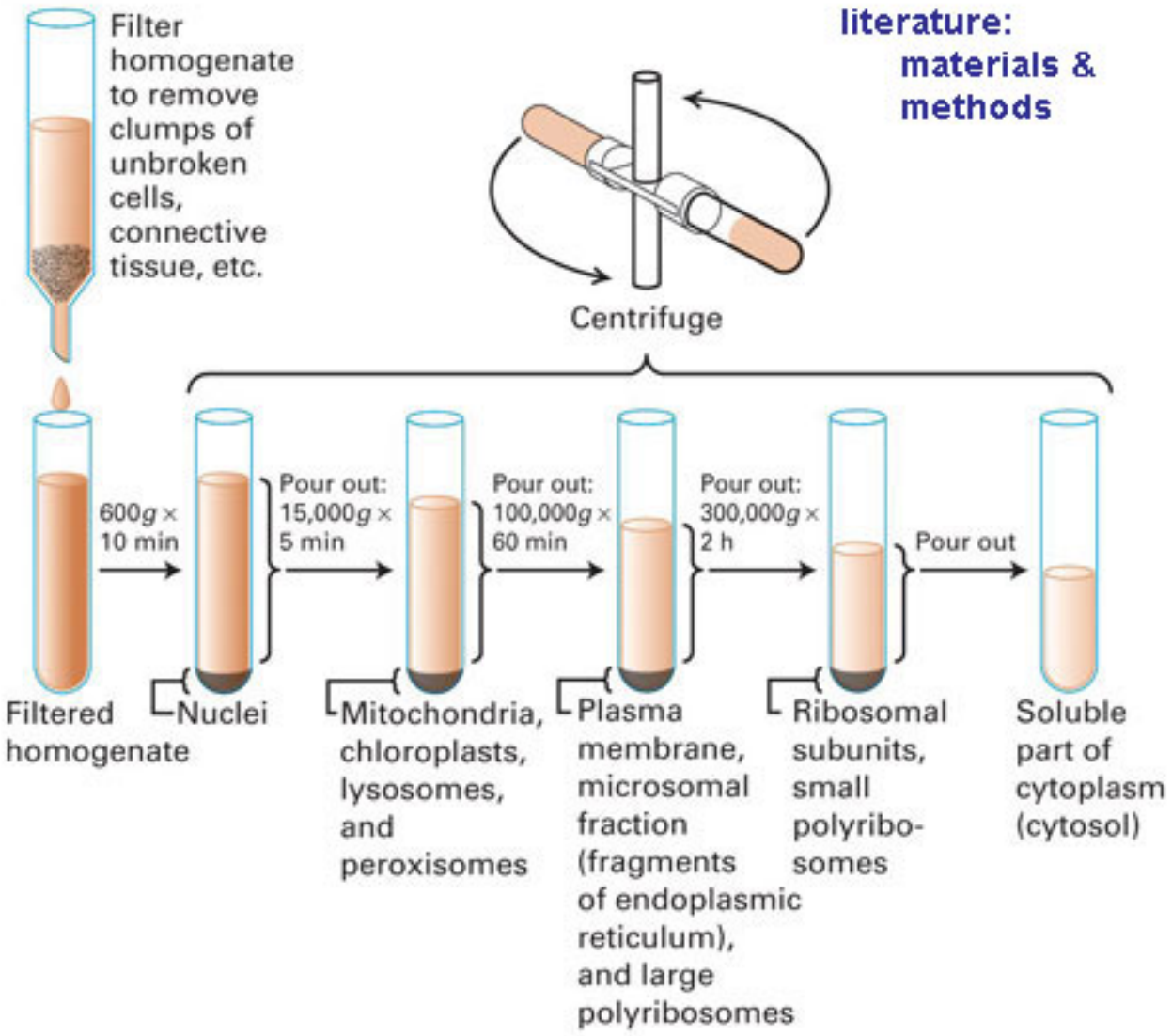
2 μl of 5 μM solution of the complex subjected to nanoelectrospray



For tissues choose the correct compartment

- Homogenize the tissue in an isotonic buffer
- Separate by differential centrifugation and with sucrose density gradients
  - Nuclear fraction (x800*g* pellet)
  - Lysosomes/plasma membrane (x10,000*g* pellet)
  - Mitochondria (20-35,000x*g* pellet)
  - Peroxisomes (OptiPrep gradient)
  - Endoplasmic reticulum (100,000x*g* pellet)
  - Cytosol (100,000x*g* supernatant)
- For bacteria, the cytosol or the inclusion bodies

literature:
materials &
methods



http://www.bio.miami.edu/~cmallery/255/255tech/mcb5.36.fuge.jpg

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Review on proteome research on organelles

Yan, W., Aebersold, R., Raines, E.W.

Evolution of organelle-associated protein profiling. *Journal of Proteomics*, 2009, 72:4-11.

http://www.ncbi.nlm.nih.gov/pubmed/19110081?itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_RVDocSum&ordinalpos=2

Not available electronically through the Lister Hill Library

Principles of protein purification

- **Proteins should remain at high concentrations throughout the work up**
  - **Proteins stick to surfaces (*sic*, ELISA assays)**
  - **Early stages can use large surface areas, but miniaturize the system as the purification proceeds**
- **Consider the chemical and physical properties of the protein**
- **Most proteins benefit from being kept cold**

Characteristics of proteins that can be exploited

- Solubility in different solvents
- Balance of charged amino acids (Asp and Glu versus Arg and Lys)
- Molecular weight
- Thermal stability
- Specific binding regions
- Availability of immunoaffinity reagents

Purification techniques

- $(NH_4)_2SO_4$ precipitation
- Ion exchange (anion and cation)
- Chromatofocusing (isoelectric point)
- Hydroxyapatite
- Hydrophobic interaction chromatography
- Reverse-phase chromatography
- Small molecule affinity chromatography
- Immunoaffinity chromatography
- Gel filtration