

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**

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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.

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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

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Recovery of drugs from samples

**Partition across
a non-miscible
organic solvent
and water**



**Protein
precipitation**



**Solid-phase
extraction**



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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
- **Proteins and peptides are not recovered by solvent extraction**

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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)
- Silica chemically bonded with C₄, C₈ and C₁₈ 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

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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 N₂)
- Ammonium hydroxide or formic acid can be added to displace the drug from the protein

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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 N₂)
 - Buffers such as sodium phosphate can selectively precipitate while ice and water are in equilibrium (down to -20°C) - this can lead to a substantial change in pH
 - Similarly, melt fast rather than slowly

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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**
- **Osmotic shock - or not!**
- **Bugbuster for bacteria**
- **Detergents**
 - CHAPS, Triton X-100, cholate and deoxycholate
- **Protease inhibitors**

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Preparing proteins for peptide mass fingerprinting (1)

- 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

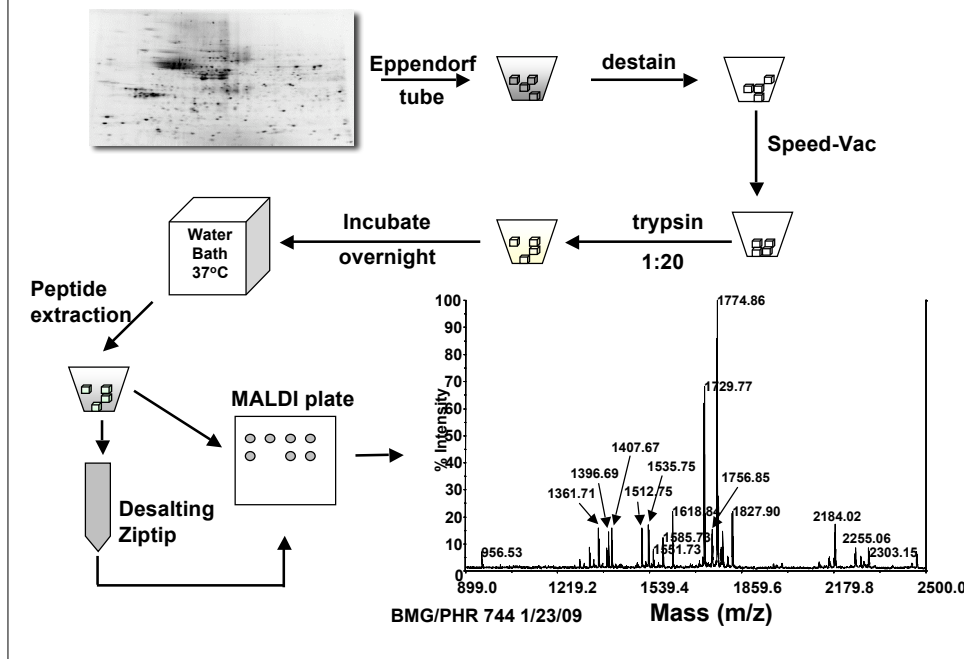
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Preparing proteins for peptide mass fingerprinting (2)

- Since the protein is precipitated in the 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

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Peptide mass fingerprinting



Preparing for 2D-electrophoresis

- **Extract tissue to recover proteins**
 - Freeze tissue in liquid N₂ - 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 CHCl₃:MeOH precipitation (concentrates and desalts)

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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

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Fluids that are full of common proteins

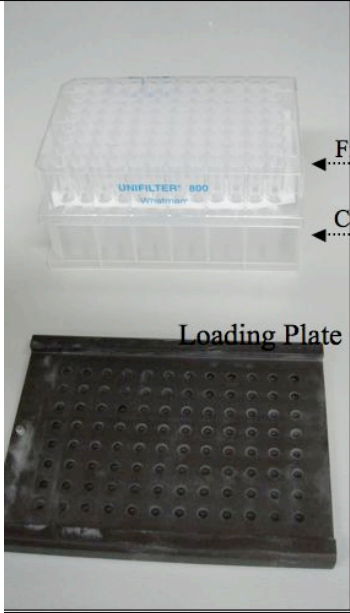
- **Serum, plasma and urine**
- **Passed over affinity columns that contain purified antibodies to the most abundant blood proteins**
 - E.g., albumin, transferrin, α 1-anti-trypsin
- **Columns optimized for the species under study (rat, human, mouse)**
- **The other proteins pass through the column and are therefore enriched**
 - There is a question of how reproducible and complete these procedures are

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Reproducible recovery of peptides

- Besides the familiar proteins, biological fluids contains both small proteins and fragments of larger proteins in the mass range from 2-20 kDa
- Peptides bind to reverse-phase cartridges and are more easily eluted with 50% aqueous acetonitrile, leaving the larger proteins (albumin) behind
- More automatable as well as reproducible

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The diagram shows a laboratory setup for peptide recovery. At the top is a white 96-well plate labeled 'UNFILTER 800'. Below it is a white 'Catch plate' with a grid of wells. At the bottom is a black 'Loading Plate' with a grid of wells. To the right is a 'MALDI Plate' with a grid of wells. Arrows point from the labels to the corresponding components.

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

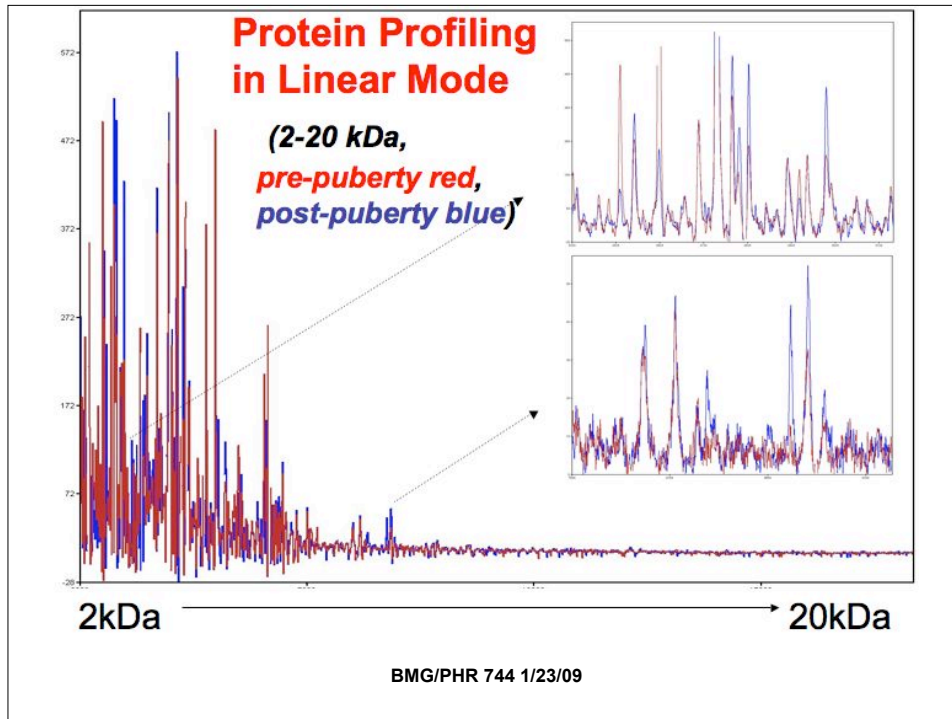
Filter plate

Catch plate

Loading Plate

MALDI Plate

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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**

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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

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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

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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

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Limitations of recombinant expression

- **Poor folding in bacteria**
 - Large amount of proteins in inclusion bodies
 - Possible to coincidentally overexpress chaperone proteins
- **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

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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

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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**

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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
- Grind tissue in liquid nitrogen to minimize degradation
- Use BugBuster™ for bacteria

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For tissues choose the correct compartment

- Homogenize the tissue in an isotonic buffer
- Separate by differential centrifugation and with sucrose density gradients
 - Nuclear fraction (x800g pellet)
 - Lysosomes/plasma membrane (x10,000g pellet)
 - Mitochondria (20-35,000xg pellet)
 - Peroxisomes (OptiPrep gradient)
 - Endoplasmic reticulum (100,000xg pellet)
 - Cytosol (100,000xg supernatant)
- For bacteria, the cytosol or the inclusion bodies

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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*, 2008, in press.

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

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Principles of protein purification

- Proteins should remain at high concentrations
 - 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

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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**

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Purification techniques

- **(NH₄)₂SO₄ precipitation**
- **Ion exchange (anion and cation)**
- **Chromatofocusing (isoelectric point)**
- **Hydroxyapatite**
- **Hydrophobic interaction chromatography**
- **Reverse-phase chromatography**
- **Small molecule affinity chromatography**
- **Immunoaffinity chromatography**
- **Gel filtration**

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