N-terminal Edman Sequencing Sample Preparation

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http://www.arbf.org

Objective: Get **sample** onto the **instrument** in sufficient **purity**, **quantity** and **cleanliness** to allow successful **sequence analysis**.

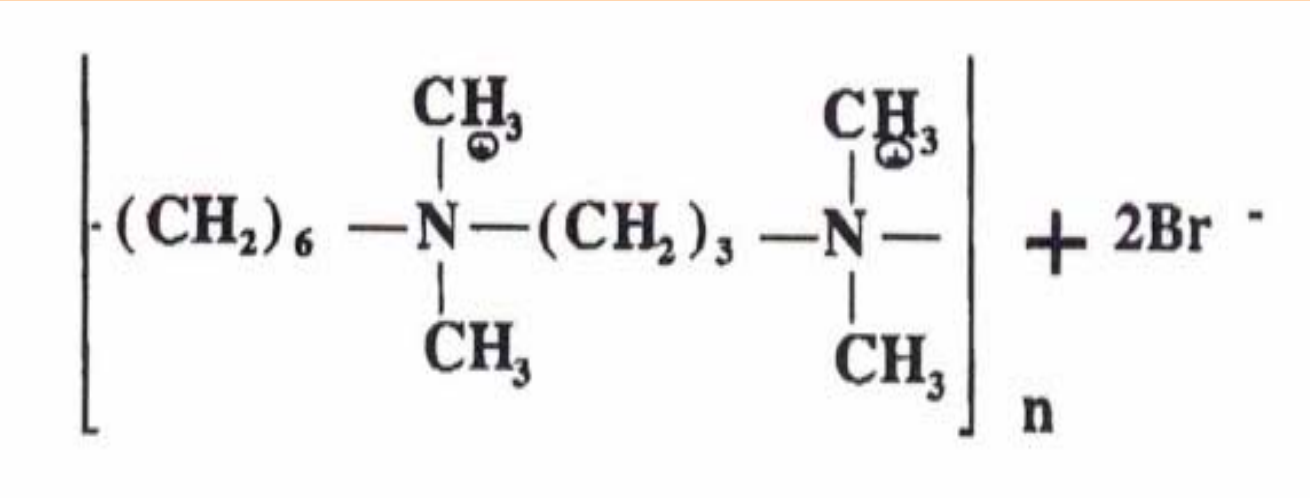
- **Sample** is the intact protein(s) or peptide(s) for analysis.
- **Instrument** defines the types of **supports** used for analysis.
- **Purity** describes the number of proteins or peptides in a sample; assess by various methods including SDS-PAGE gel, HPLC.
- **Quantity** can be determined by AAA, Bradford/Lowry/BCA assay, gel stain intensity, semi-quantitative HPLC.
- **Cleanliness** is a lack of common **interfering compounds** that could impede the analysis.

These five factors will define what types of sample preparation we must do for a successful analysis.

Supports for analysis

- Glass fiber filter, w/polybrene (quaternary polyamine) (ABI)
- PVDF membrane by electroblotting, filtration, adsorption, and covalent linkage.
- Bimodal reaction columns (RP/SAX) (HP)

Biobrene / Polybrene



Common Interferences

- TRIS and other amines cause large chemical artifacts.
- Buffering salts can interfere with the chemistry of the analysis.
- Detergents can affect sample washout from reaction cartridge and conversion flask dynamics. SDS can also precipitate in the instrument.
- Free amino acids contribute to high background in early cycles.

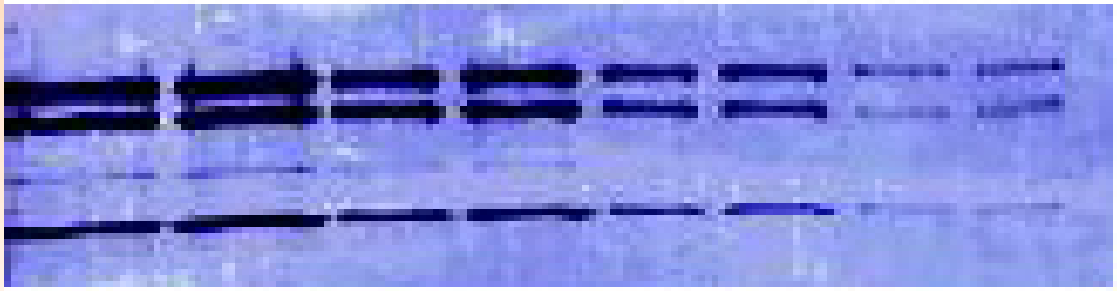
Quantitation basics

- **Limited by the absolute instrument sensitivity, about 1pmol loaded at ~50% IY for an average lab.**
- **Important to establish a reasonable level of quantitation for the researcher and the lab.**

- Rule: 1ug of 1kDa protein = 1 nmol

  - **100ug of 100kDa protein = 1,000 pmol**
  - **10ug of 100kDa protein = 100 pmol**
  - **1.0ug of 100kDa protein = 10 pmol**
  - **0.5ug of 250kDa protein = 2 pmol**

PVDF Stain Intensity by Amido Black



Common Sample Preparation Techniques for Soluble Samples

- **Direct Adsorption to PVDF**
- **Prosorb PVDF cartridge**
- **Reversed-phase (other media) packed micro pipettor tip devices**
- **Ultrafiltration membrane cartridge**
- **Precipitation methods**
- **1D / 2D gel electrophoresis to PVDF by electroblotting**
- **HPLC separation and collection**
- **Micro-spin type cartridges**
- **Bimodal reaction cartridges**
- **On-bead synthetic peptides**
- **Covalent bonding to PVDF membrane**

Direct absorption to PVDF (and Zitex) membrane

- Use for samples with high purity.
- Suitable for samples of reasonably high concentration.
- Allows removal of salts, buffers, free amino acids and some detergents.
- Sample remains bound to membrane for analysis (addition of polybrene is recommended).

Prosorb PVDF membrane

- Use for samples with high purity.
- Can be used with dilute samples.
- Allows extensive washing to remove most contaminants.
- Sample remains bound to membrane for analysis (addition of polybrene recommended).

Reverse phase (or other media) packed micropipettor-tip devices

- Suitable for samples of medium to high purity.
- Can be used with dilute samples, binding to media can be a concern.
- Extensive desalting, detergent removal possible.
- Enrichment of specific species (i.e.. PO4), possible.
- Sample is eluted in high concentration in solution for analysis or purification.

Ultrafiltration membrane cartridge

- Can be used to remove unwanted low MW components, peptides and proteins from complex mixtures by MW cutoff selection.
- Can be used with dilute samples.
- Allows extensive washing, buffer exchange, removal of detergents.
- Sample is eluted in high concentration in solution for analysis or other chemistries.

Precipitation methods

- Acetone, chloroform/methanol, TCA
- Low to high purity samples.
- Used to concentrate dilute samples from solution, as little as 50ng total protein.
- Complete buffer exchange possible.
- Sample resolubilized in high concentration solution, ideal for loading onto SDS-PAGE gel.

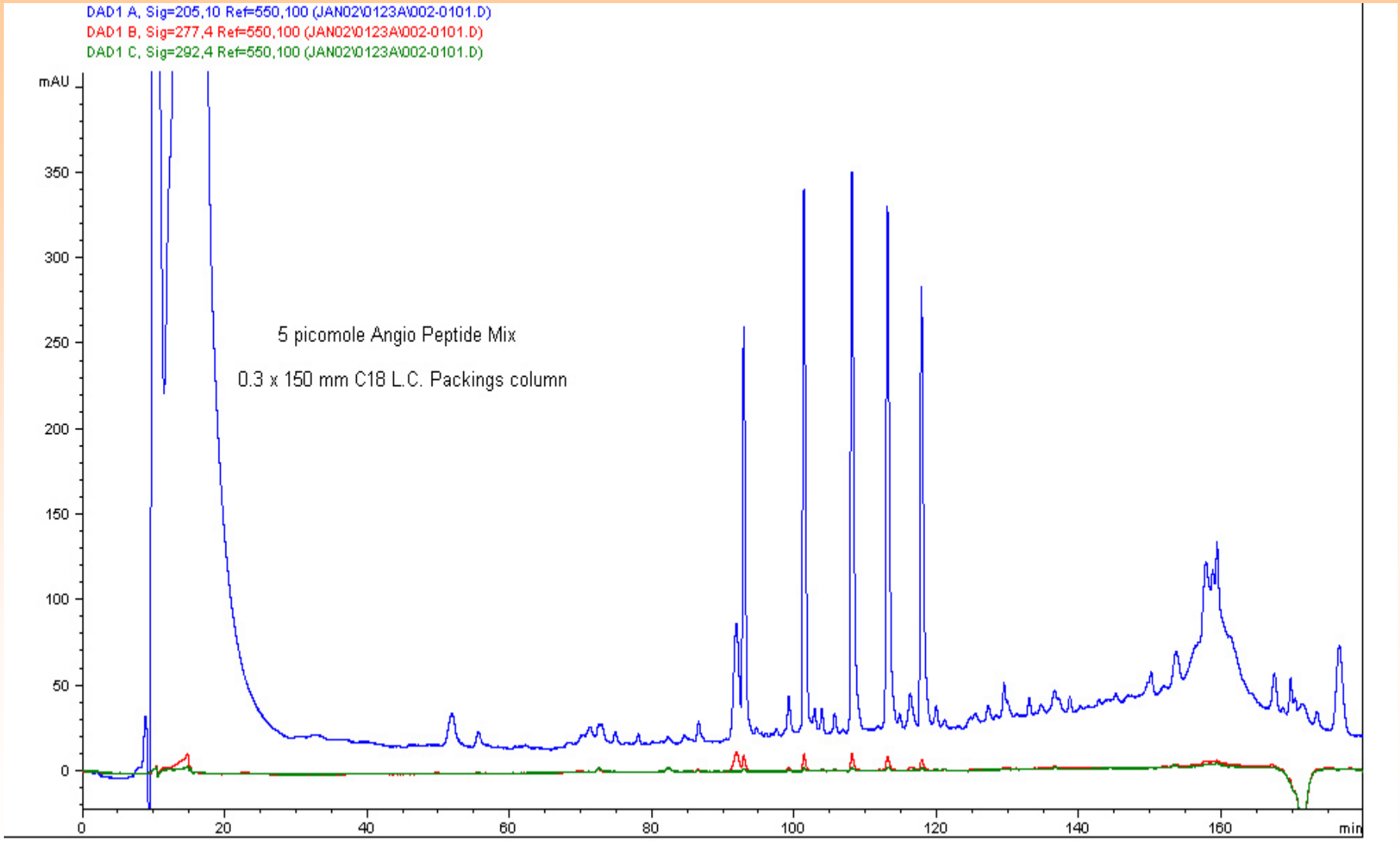
1D or 2D Gel Electrophoresis Electroblotted to PVDF

- Used for low (1D) to very complex mixtures (2D) of proteins or peptides.
- High sample concentration needed due to limiting gel load volumes. Sample solubility issues may arise.
- Sample is strongly bound to PVDF membrane in a highly concentration for analysis.

HPLC separation and collection

- Suitable for complex mixtures of proteins and peptides.
- High sample concentration needed, injection volumes can be limited by column and flow.
- Potential separation and concentration of individual protein and peptide species.
- Sample is collected in high concentration solution.

Capillary RP HPLC Separation of Peptides



Micro-spin cartridges for protein and peptide preparation

- Many new vendors, many chemistries and formats.
- Uses range from crude preparation of mixtures to final clean up of single analyte for analysis.
- Match sample with media for optimal results.
- Multistage processes in convenient formats.

Hewlett Packard Bimodal reaction columns

- Very convenient reverse phase sample loading, concentration and washing.
- On-column chemistries possible, easy clean up.

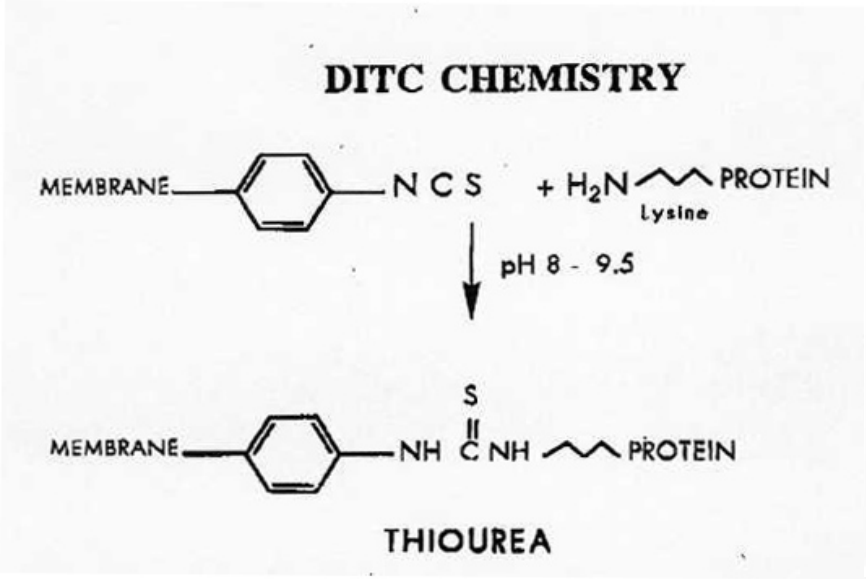
On-bead synthetic peptides

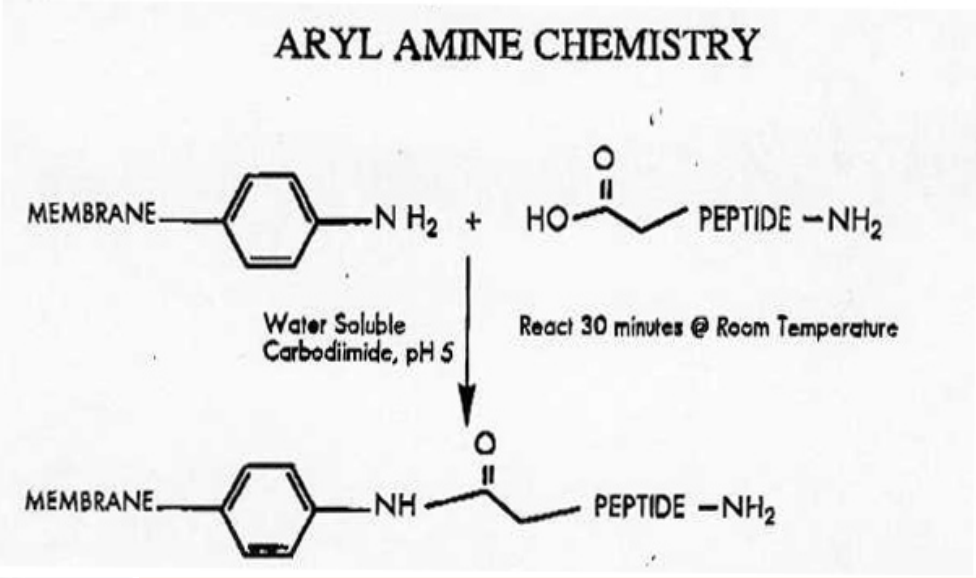
- Used to confirm synthesis efficiency and correctness.
- Single beads can contain nanomoles of peptide.
- In practice, cleavage from the resin is best.
- Handling of single beads is a difficult task to do reproducibly.

Covalent binding to Sequelon membrane

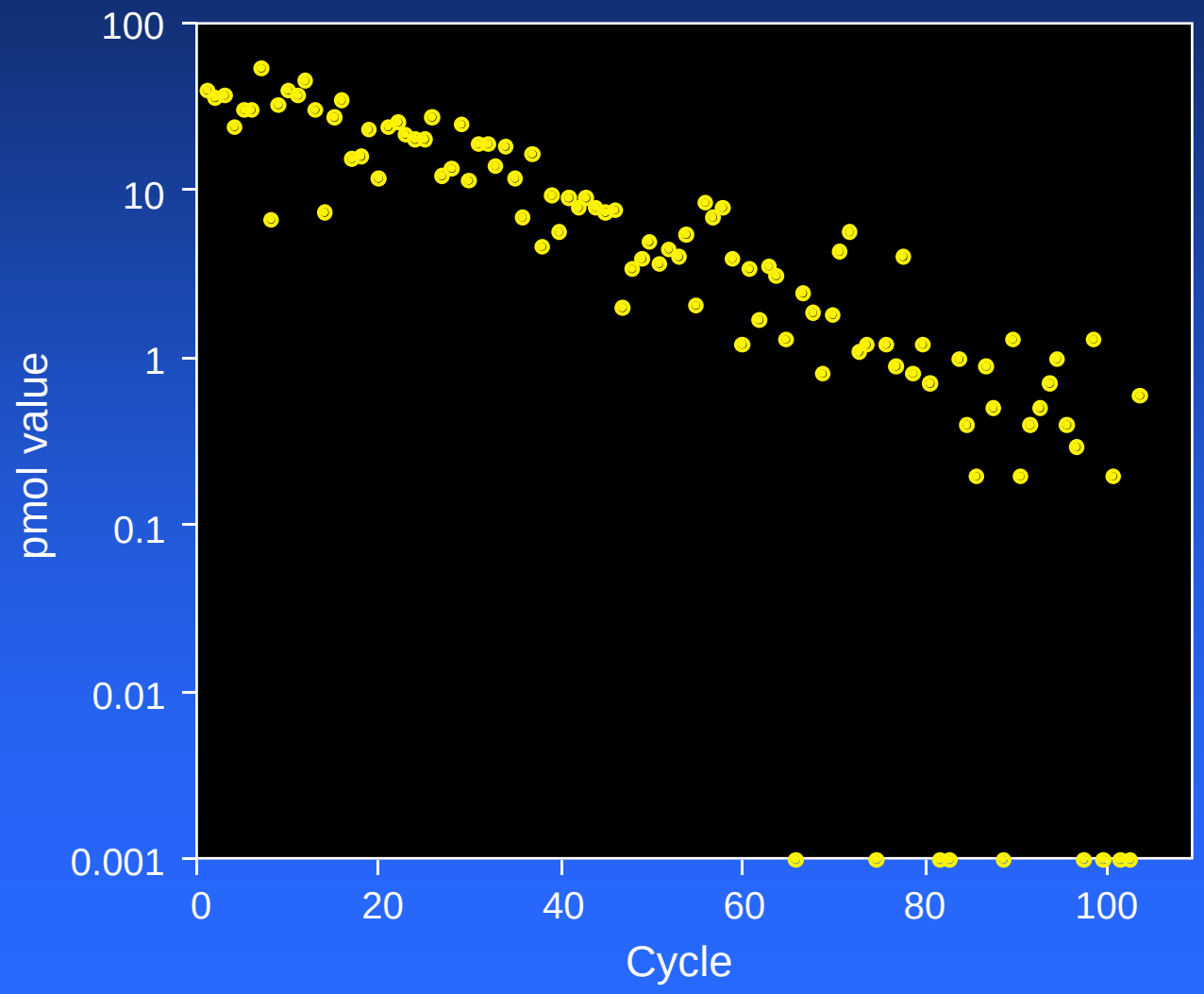
- Chemically bind a single protein or peptide to hydrophobic membrane for analysis.
- Allows efficient extraction of P32 labeled residues, extended sequencing of immobilized proteins, and other special chemistries to be applied.
- Detergents can inhibit efficient binding.

Sequelon DITC & Aryl Amine Chemistries

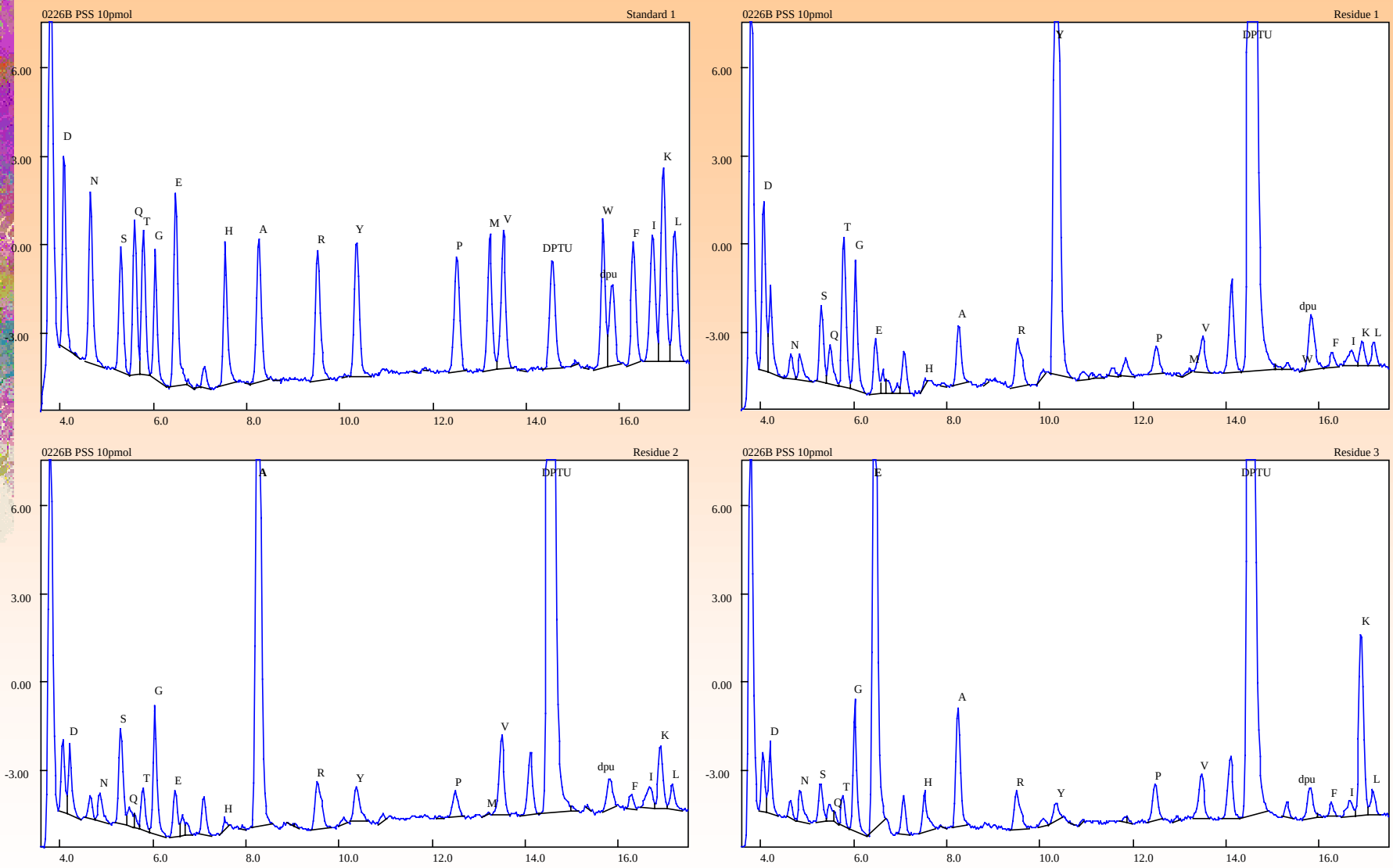




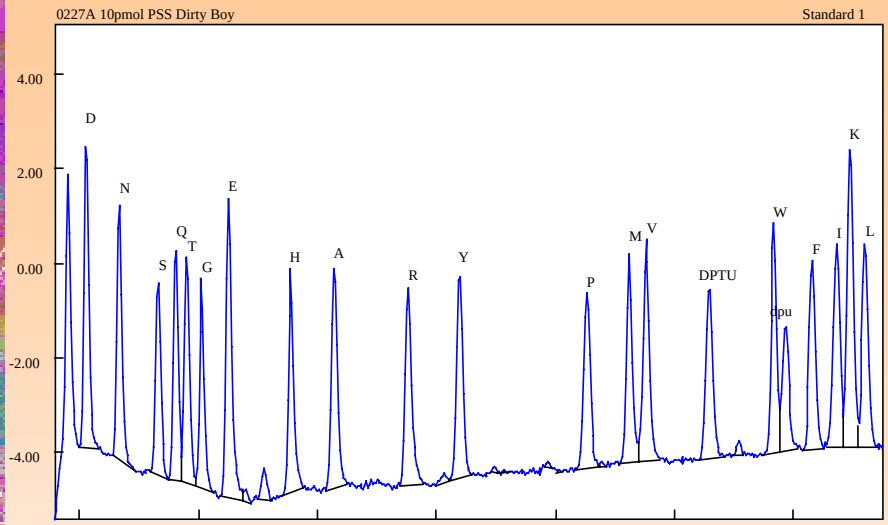
Solid Phase Sequencing of DITC Coupled Betalactogolublin

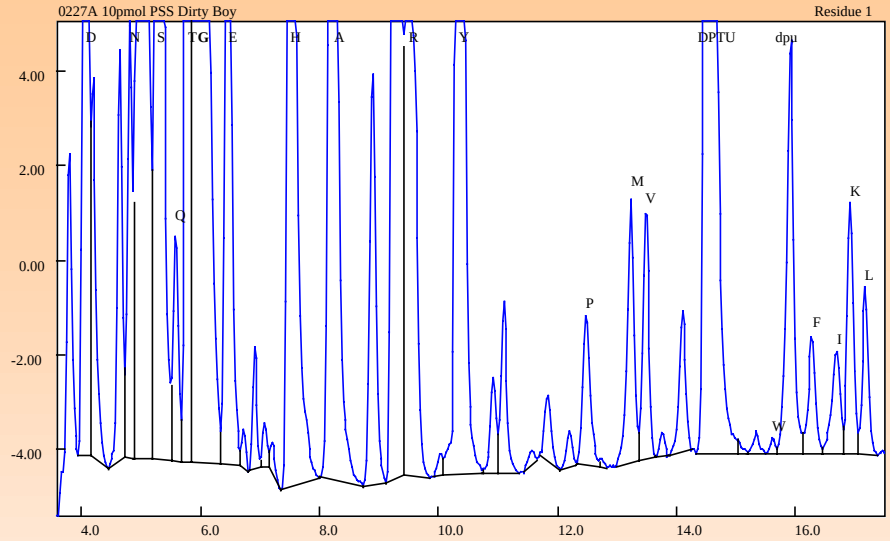


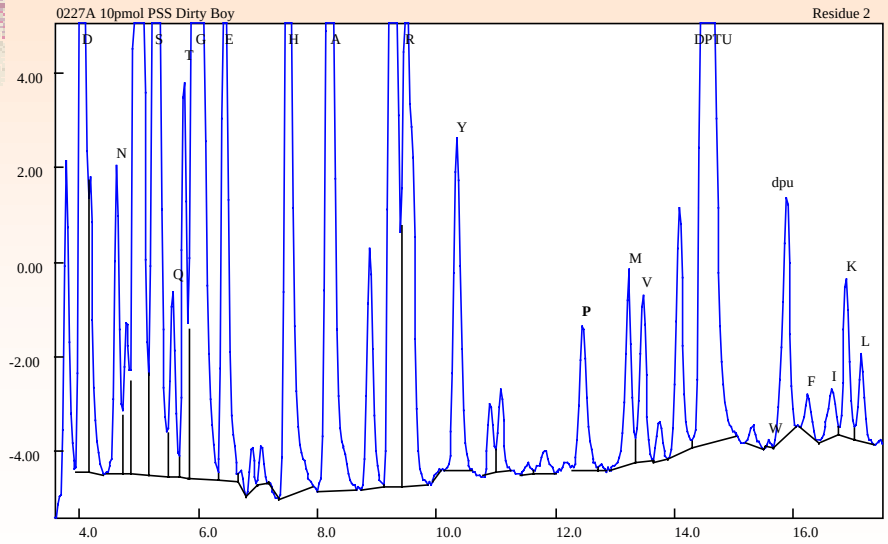
Peptide Standard: 10pmol (Tyr, Ala, Glu)

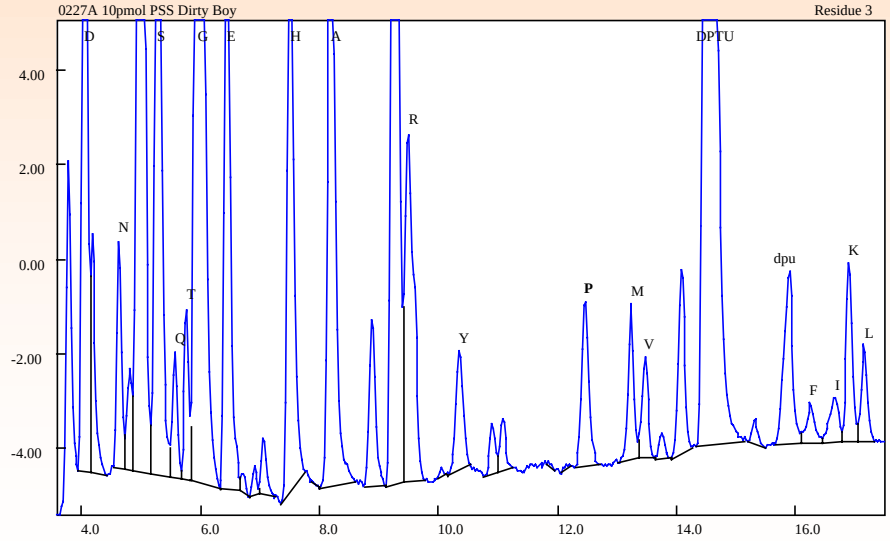


Dirty Peptide Run: No Sequence Data

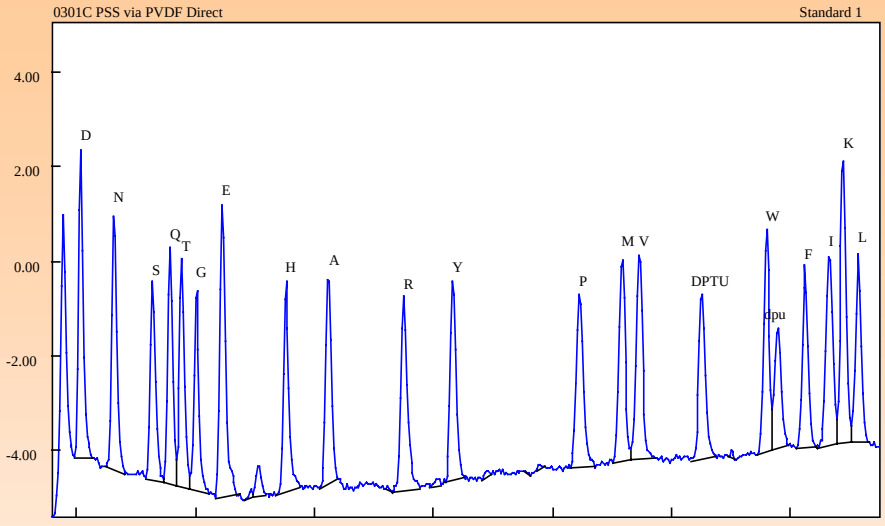


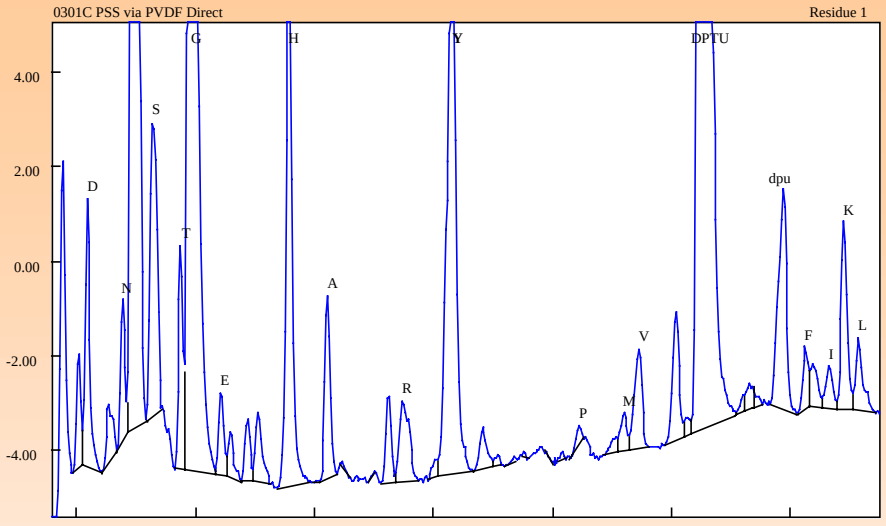


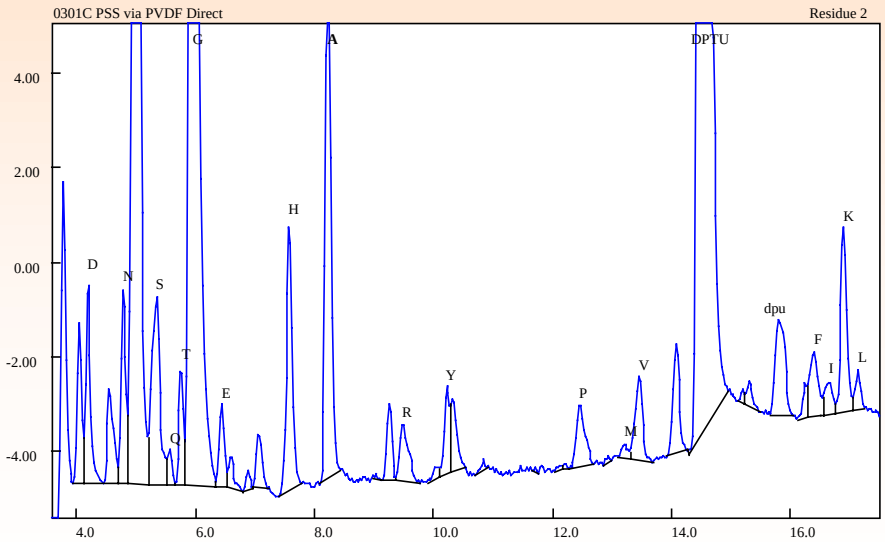


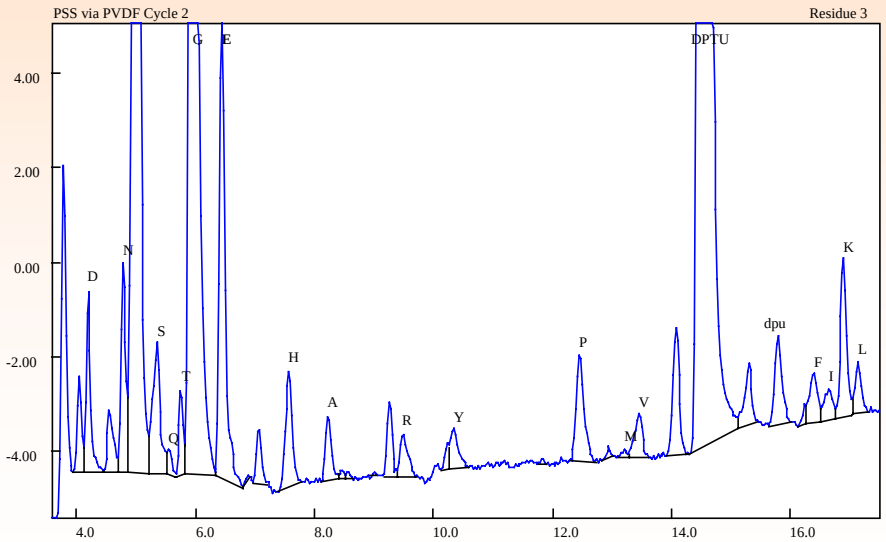


Direct Adsorption to PVDF Membrane

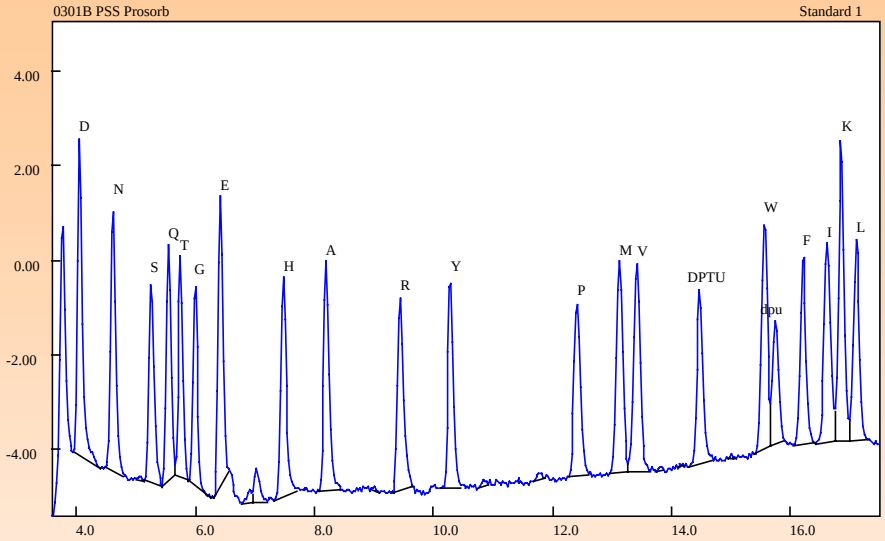


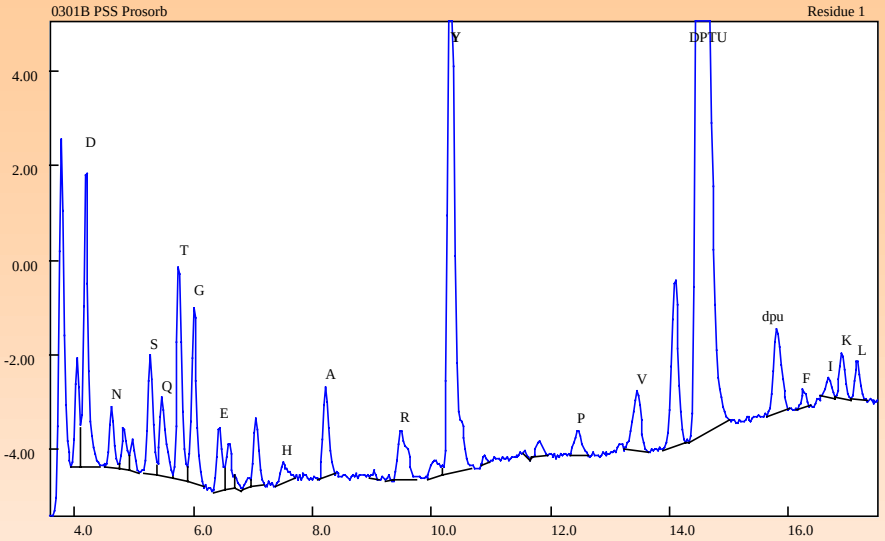


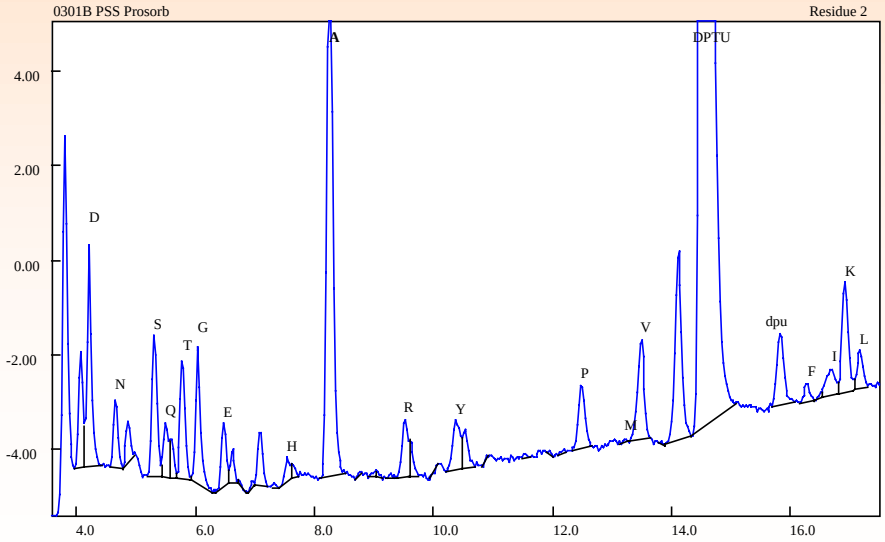


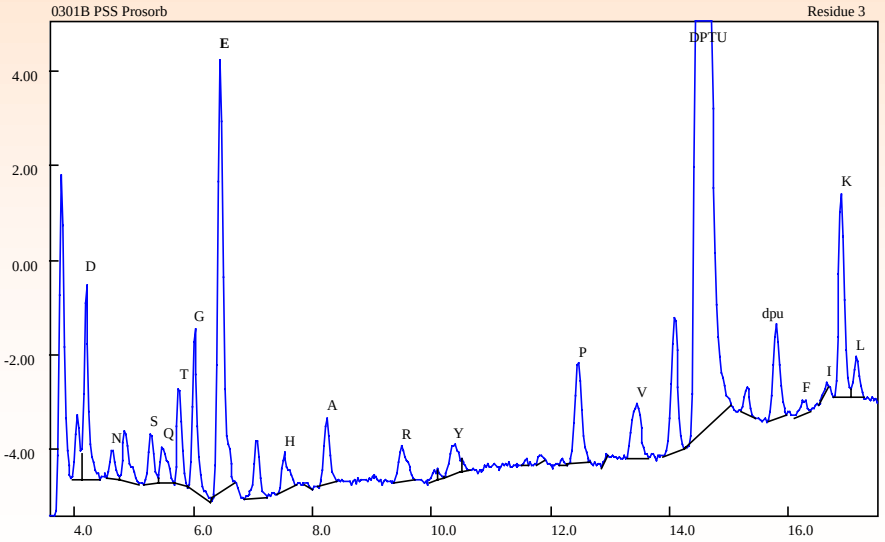


Prosorb PVDF Filter Cartridge

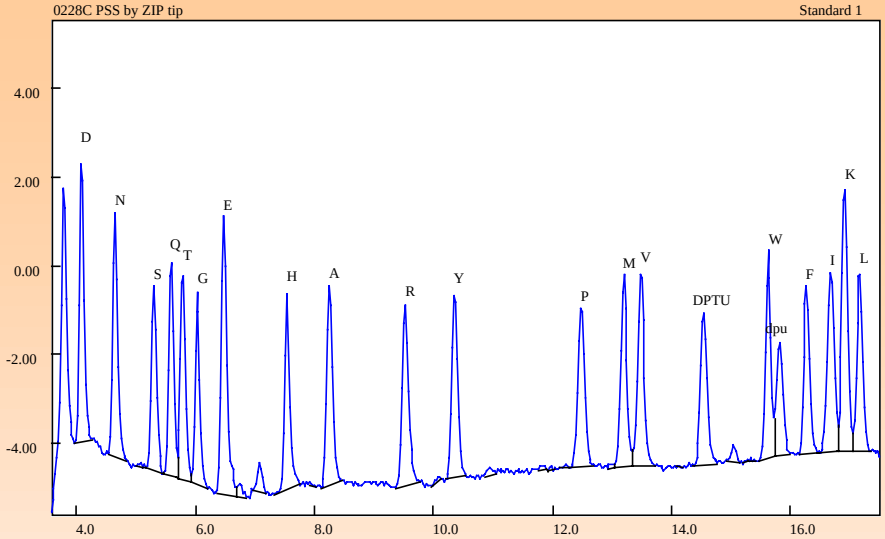


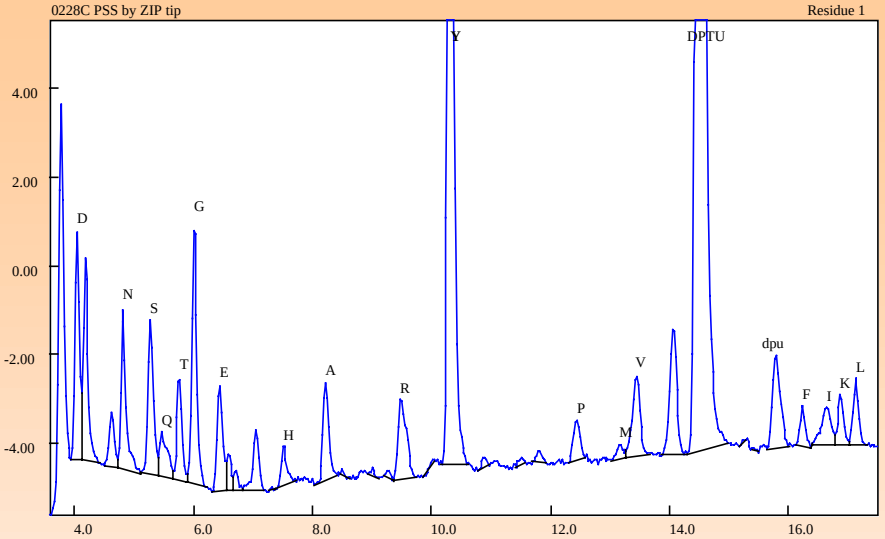


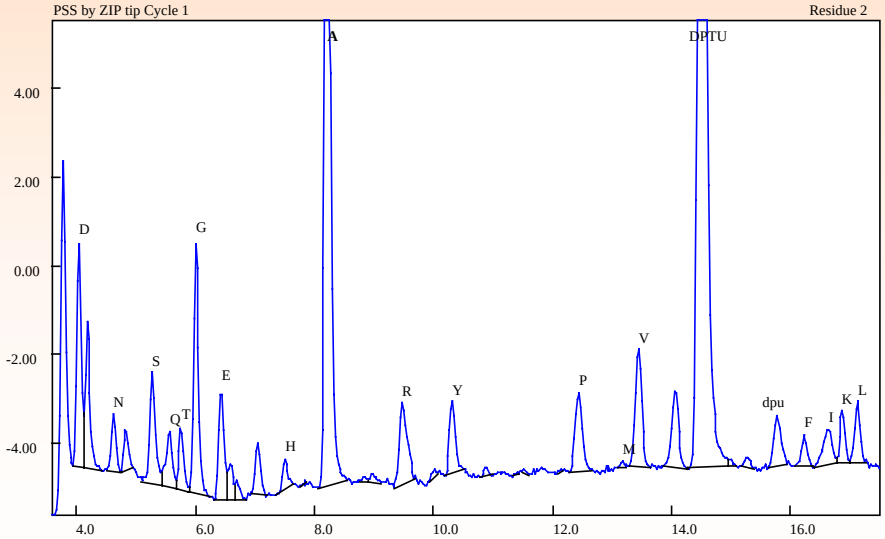


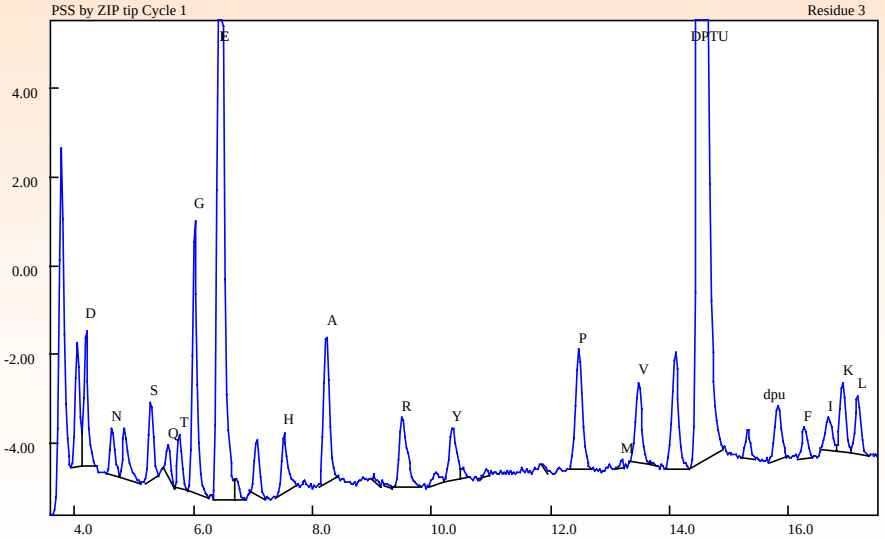


C18 Zip Tip Clean Up









OK, so what if we get **no data**?

- Insufficient quantities.
- Highly heterogeneous sample leading to uninterpretable results.
- A **blocked n-terminus**, leading into Bill Henzel's part of this presentation.