CAPILLARY LC COLUMNS FOR WHOLE PROTEIN SEPARATION

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Introduction

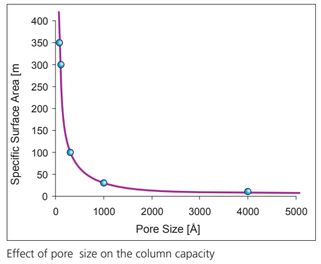
One major hurdle in proteomic research is the large difference in expression levels of different proteins. A small number of proteins make up the majority of the protein mass while proteins or peptides of scientific interest are usually only present in small quantities. In a typical MuDPIT experiment the protein extract of a sample is digested in total and then analyzed by multidimensional (orthogonal) LC-MS. As a consequence a high abundant protein is now spread over 20-30 high abundant peptide peaks.

An alternative to whole sample digest is the use of gel electrophoresis prior to the digest and LC separation. However, this approach requires the development of the electrophoretogram, in-gel digest, cutting of bands/spots and extraction to obtain a sample suitable for the LC-MS analysis. We introduce a capillary LC column tailored for the separation of proteins. The packing material is a 3µm C8 silica with 1000Å pore size. These columns allow the separation of proteins with minimized band-broadening due to restricted pore diffusion. The protein separation step adds another independent dimension to the 2D-LC fractionation of the digest in a similar fashion as the electrophoresis does. However, because the sample remains in solution, the process is more convenient and has the potential for automation. High abundant proteins are localized and will only affect the fraction which they elute in. Fractions can then be digested off-line or using an in-line enzymatic reactor and analyzed with more conventional 2D-LC-MS.

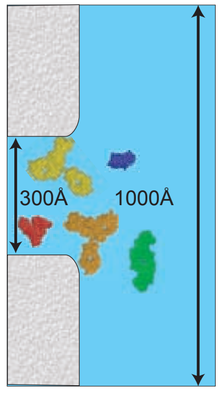
Importance of Pore Size for the Analysis of Large Molecules

At the point of injection the sample components bind to the first available surface. If the pore size is insufficient, larger proteins at the pore entrance can prevent proteins further down the pore system to elute or prevent the rest of the sample to gain access to the pore. This remains the case until the gradient has reached the stage where the blocking protein is desorbed.

Pore Size	Target Molecule
60-120Å	Small molecules; Pharmaceuticals
250-300Å	Peptides; Oligosachharrides
1000Å	Proteins
>2000Å	Protein complexes



Effect of pore size on the column capacity



The 5 most abundand proteins in serum
Serum Albumin
IgG
IgA
Transferrin
Antitrypsin

Analysis of Mouse Liver Microsomal Fraction Proteins

The Asia Oceania Human Proteome Organisation (AOHUPO) started an initiative to analyze the membrane associated part of the proteome. In the initial phase of the Membrane Proteomics Initiative (MPI) a protein standard was developed in the laboratories of Bill Jordan at the Victoria University in Wellington, NZ, and distributed to participating laboratories in order to develop and optimize a separation protocol which would be applicable to other proteomics samples. The sample was prepared from the large lobe of the liver of male C57BL/6J mice.

Membrane associated proteins form very important functions in cell recognition, disease markers and cell signalling and trafficking. They also form a particular challenging sample for RPLC separation due to their nature. Membrane proteins usually contain relative hydrophilic sections forming the intra or extracellular domains and very hydrophobic membrane spanning domains. However, because of their exposure to the cell surface they form interesting commercial targets for both diagnostic and therapeutic purposes.

Sample preparation:

1. Dilute the MPI rat liver microsomal membrane protein (11.3mg/mL) to 5mg/mL with Milli-Q. i.e. Aliquot 110mL Milli-Q to the bottom of Eppendorf tube (1.7mL) and 90 mL of liver microsomal protein is added to the tube and the solution is mixed by pipeting up-and-down several times.

2. Aliquot 100 mL of the diluted protein solution to the bottom of a separated Eppendorf tube (1.7mL). So, each tube will have 100 mL protein solution.

3. Dry samples in a centrifugal vacuum concentrator (Speed-Vac).

4. Add 200 mL of 80% formic acid/Milli-Q to each dried sample and sonicate in a water bath for 30 seconds.

5. Dry samples in a centrifugal vacuum concentrator (Speed-Vac).

6. Add 500 mL of 80% formic acid/Milli-Q to each dried sample and sonicate in a water bath for 30 seconds or until a clear solution results.

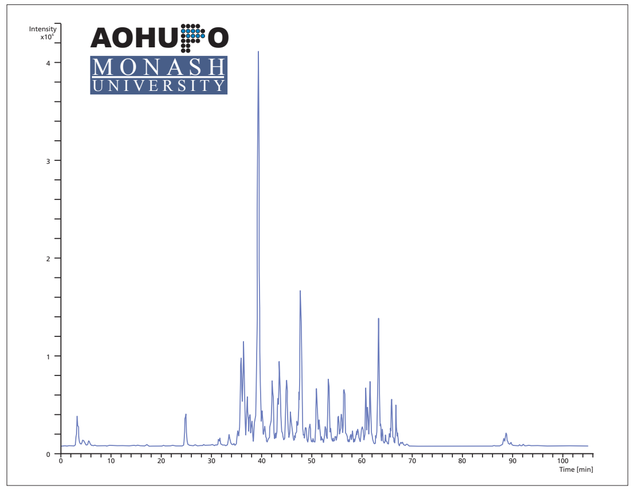
7. Centrifuge the sample in a bench-top centrifuge at 13,000rpm for 5min.

Note: The final sample protein concentration would be approximately 1.0 mg/mL in 80% formic acid/Milli-Q.

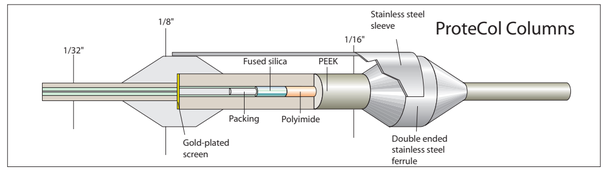
Chromatographic conditions

System: Agilent 1100 CapLC with Agilent MSD-iontrap MS
Column: ProteCol-C8 3µm; 1000Å 150mm x 300µm ID
Sample: 3µL AOHUPO-MPI standard
Flow rate: 5.0 µL/min
Temperature: 80°C
Mobile Phase A: 0.1% formic acid in water
Mobile Phase B: 0.09% formic acid in acetonitrile

Gradient profile: Time	%B
0	20
2	20
42	50
52	80
57	100
72	100
75	20
105	20



ProteCol Column Design



Standard Column Length: 50, 100 or 150mm
Frit design: deactivated gold-plated woven stainless steel mesh 140µm thickness
Standard IDs: 75, 150, 300 or 530 µm
Integrated connection tubing: PEEKsil (PEEK coated fused silica) 25 or 50µm ID
1/32" OD; inlet 200mm outlet 100mm
Columns are encapsulated in a stainless steel tube for added protection
Capillary ends are precision cut and polished to allow true zero-volume butt connections

Advantages of LC Protein Separation

Many protocols for the analysis of proteomic samples include an electrophoresis separation of the intact proteins. The separation is then followed by a staining process to make the proteins visible, an extraction of the protein by cutting out individual spots on the gel, a protease digestion and finally a LC-MS/MS analysis of the digest.

The process is time consuming and has the drawback that proteins with a concentration lower than the staining is able to visualize will not be excised and therefore will not be analyzed. Separating the proteins on a dedicated protein column has various advantages:

- Sample remains in solution - no gel cutting
- High abundant proteins form one interfering peak rather than >30 interfering digest peaks
- Protein and digest retention behavior is virtually independent - additional dimension of separation
- Can be combined with in-line protease digest and 2D-LC-MS/MS

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