Affinity Trap Columns and In-Line Enzymatic Reactors

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Introduction

Routine biochemical analysis often deals with very complex samples. Such samples not only contain a large number of components but their concentrations vary greatly.

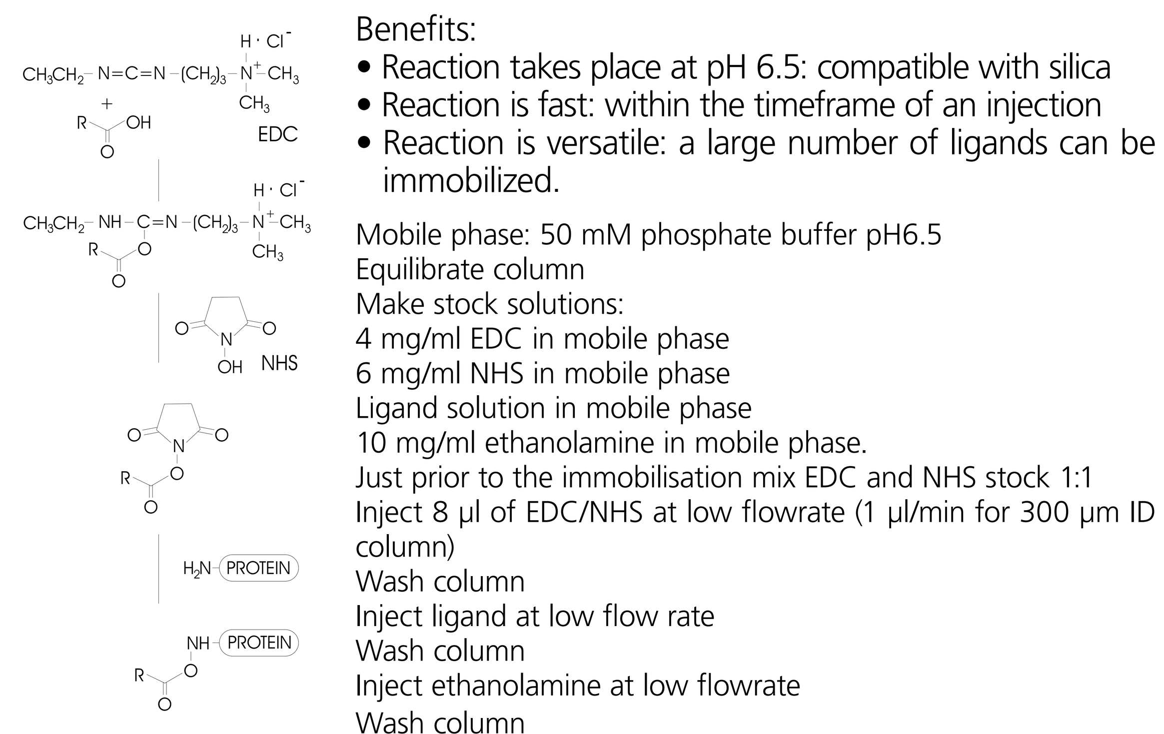
In proteomic research for example the total protein content is commonly digested with trypsin and the resulting peptide mixture is analysed by LC-LC/MS-MS. Such a sample not only contains tens of thousands of peptides but their concentration levels span several orders of magnitude. Of all these peptides only a small fraction is of scientific interest. An efficient way of sample preparation can reduce the complexity of the analytical task. In one possible approach, affinity chromatography, the highly specific interaction of naturally occurring molecule pairs (or their mimics) are utilized to selectively bind one partner from a complex matrix, while the remainder is washed off.

SGE has developed two affinity trap columns. The first one is an immobilized metal affinity column (IMAC). When loaded with Fe3+, Ga3+ or Zr4+ this column shows a high affinity for phosphorylated peptides.

The second column is a generic affinity column. The column is packed with a 2000 Å pore size silica coated with crosslinked carboxy-methylated dextran. Using a very fast activation chemistry these columns can be modified with any ligand contain a primary amine group by injecting the reagents with an auto sampler. Depending on the ligand chosen by the analyst these columns can then be used as an affinity trap column or as an in-line enzymatic reactor.

Examples are given for all the described applications to demonstrate the use of these columns.

Immobilization Chemistry



Benefits:
- Reaction takes place at pH 6.5: compatible with silica
- Reaction is fast: within the timeframe of an injection
- Reaction is versatile: a large number of ligands can be immobilized.

Mobile phase: 50 mM phosphate buffer pH6.5
Equilibrate column
Make stock solutions:
4 mg/ml EDC in mobile phase
6 mg/ml NHS in mobile phase
Ligand solution in mobile phase
10 mg/ml ethanolamine in mobile phase.
Just prior to the immobilisation mix EDC and NHS stock 1:1
Inject 8 µl of EDC/NHS at low flowrate (1 µl/min for 300 µm ID column)
Wash column
Inject ligand at low flow rate
Wash column
Inject ethanolamine at low flowrate
Wash column

IDA-IMAC trap colums for the enrichment of phosphorylated peptides

Proteins can be phosphorylated by adding a phosphate group to a serine residue

- Activation or deactivation of enzymes
- Trans-membrane transportation
- Signal trafficking

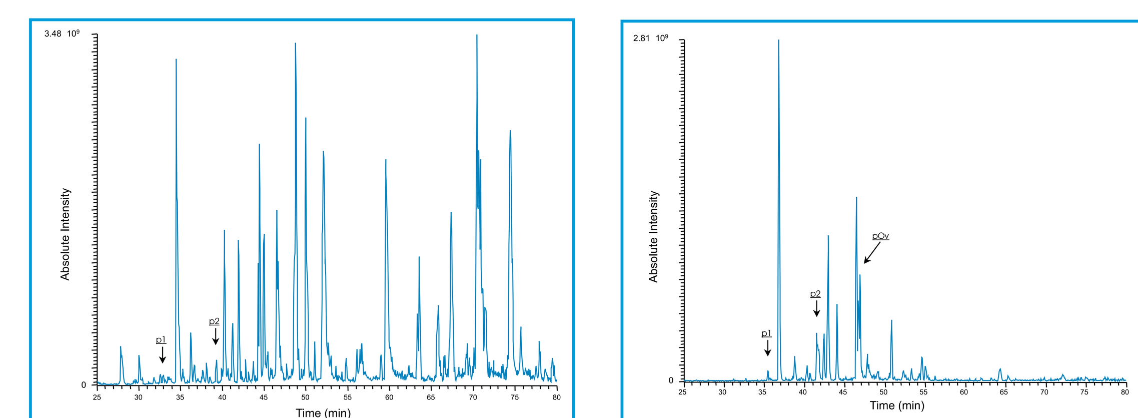
The affinity of phosphate to certain metal ions can be utilized to enrich phosphorylated proteins or peptides.

Column Specifications:
Length: 5 or 10 mm
ID: 150 or 300 µm
Connection tubing: 2 x 70 mm; 25 or 50 µm ID; 1/32" OD
Tubing material: PEEK coated fused silica
Packing: imino diacetic acid (IDA) modified silica;
3 µm particles, 300 Å pores
Suitable metal ions: Fe(III), Ga(III) or Zr(IV)

IMAC phosphopeptide enrichment procedure

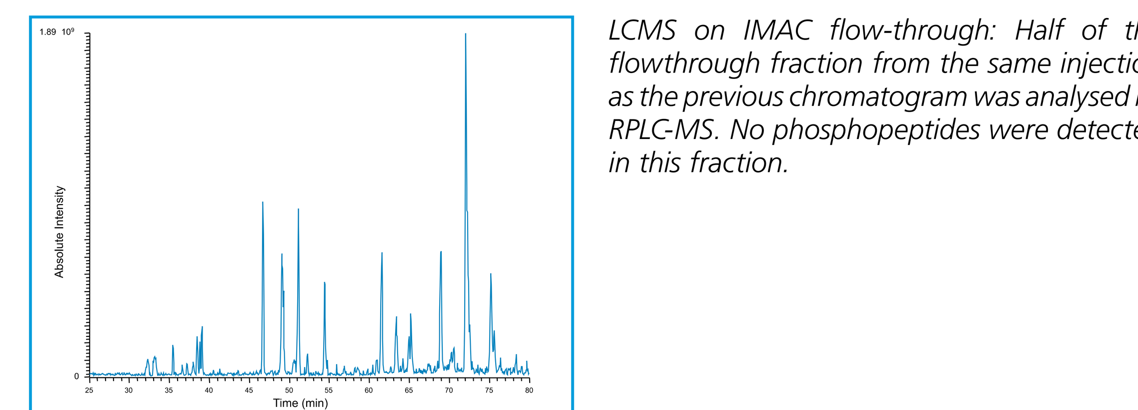
Step	Solution	Volume	Comment
1	50 mM EDTA, 1M NaCl, pH 8.5	25 µl	Stripping column
2	HPLC grade H_2O	10 µl	
3	0.1 % HAc	25 µl	
4	100 mM $FeCl_3$	50 µl	Cation charging
5	0.1 % HAc	25 µl	
6	Sample	variable	Flow-through collected
7	0.1 % HAc	25 µl	Wash fraction
8	25 % Acetonitrile, 1 % HAc, 100 mM NaCl	25 µl	Wash fraction
9	0.1 % HAc	25 µl	Wash fraction
10	50 mM K_2HPO_4	20 µl	Eluted material collected

Figure 1: Procedure for the enrichment of phosphorylated peptides prior to RPLC-MS analysis. Bound, but not phosphorylated peptides are likely acidic and could be suppressed by methylation of the peptides.



LC-MS of spiked ovalbumin tryptic digest: LC-MS chromatogram of 25 pmol ovalbumin digest containing 5 pmol of standard phosphopeptides. Peaks corresponding to spiked phosphopeptides are indicated as p1 and p2.

LCMS of IMAC enriched sample: The bound fraction of the IMAC column (see protocol on the left) was analysed by RPLC-MS. The spiked phosphopeptides are marked p1 and p2 while pOv is a phosphopeptide from the ovalbumin digest.



LCMS on IMAC flow-through: Half of the flowthrough fraction from the same injection as the previous chromatogram was analysed by RPLC-MS. No phosphopeptides were detected in this fraction.

In-line Enzymatic Reactor: Immobilized Trypsin

In-line tryptic digest has some advantages over traditional methods:
- only small sample amounts are needed since the total digested sample is eluted onto the analytical column
- no autodigest of trypsin since trypsin is covalently attached to the support surface
- when installed in-line with two switching valves digest and consecutive analysis can be automated.

Experimetal conditions for trypsin derivatised CMD columns

- Trypsin from porcine pancreas (EC 3.4.21.4) was immobilized onto a 150 mm x 300 µm ID CMD column.
- Column was equilibrated with a 50mM NaHCO solution
- 5 µl Bovine serum albumin solution (10mg/ml in mobile phase) was injected onto the column and the flow was stopped.
- The column was kept at 37 °C for two hours
- The digest was eluted and collected in a 100 µl sample vial
- The digest was then analysed by RP-HPLC

Reversed phase analysis of protein digest

Column: ProteCol-C18 100 mm x 300 µm ID
Packing: 3 µm particle size; 300 Å pore size; ODS
Part Number: 250137
HPLC: Agilent 1100 CapLC with multi wavelength UV detector and 80 nl flow cell
Mobile phase A: 0.1 % TFA in water
Mobile phase B: 0.1 % TFA in 80 % acetonitrile/water
Gradient: 0 to 60 min: 0 to 100 % B linear
60 to 70 min: 100 % B
70 to 80 min: 100 to 0 % B
80 to 100 min : 0 % B
Flow rate: 4.0 µl/min
Injection volume: 8 µl
Temperature: ambient

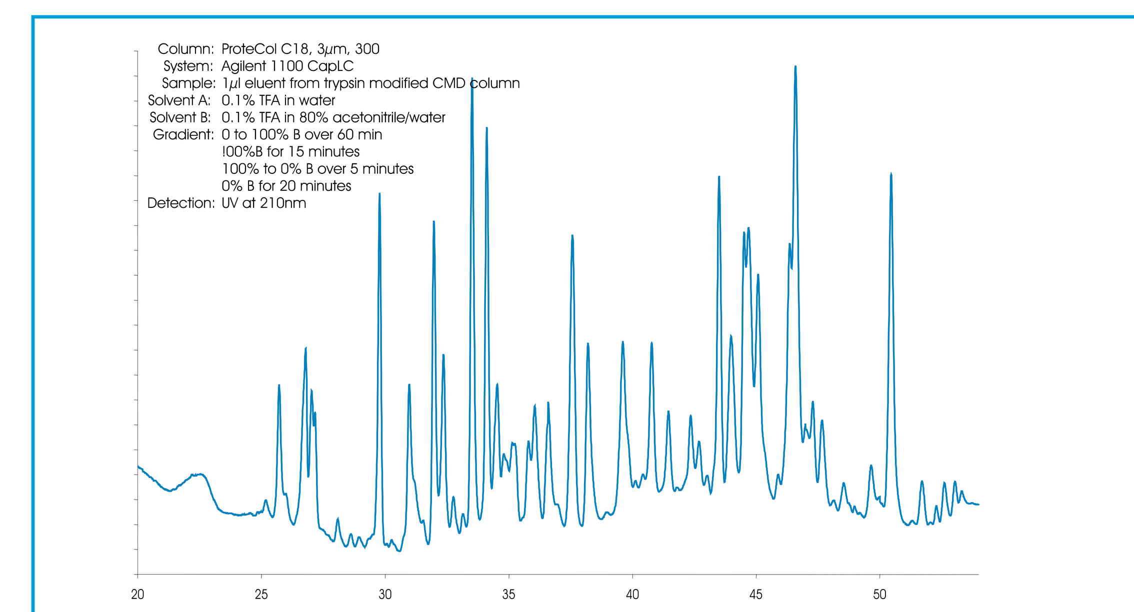


Figure 1: Reverse phase analysis of the tryptic digest of bovine serum albumin produced by on-column digestion with trypsin modified ProteCol-CMD column.

Glycopeptide Enrichment Using Immobilized Concanavalin A

Concanavalin A is a lectin isolated from canavalis ensiformis (Jack Bean) and has a selective binding affinity for terminal a-D-mannosyl and a-D-glucosyl groups. It can be used in affinity chromatography to selectively bind glycosylated proteins and peptides.

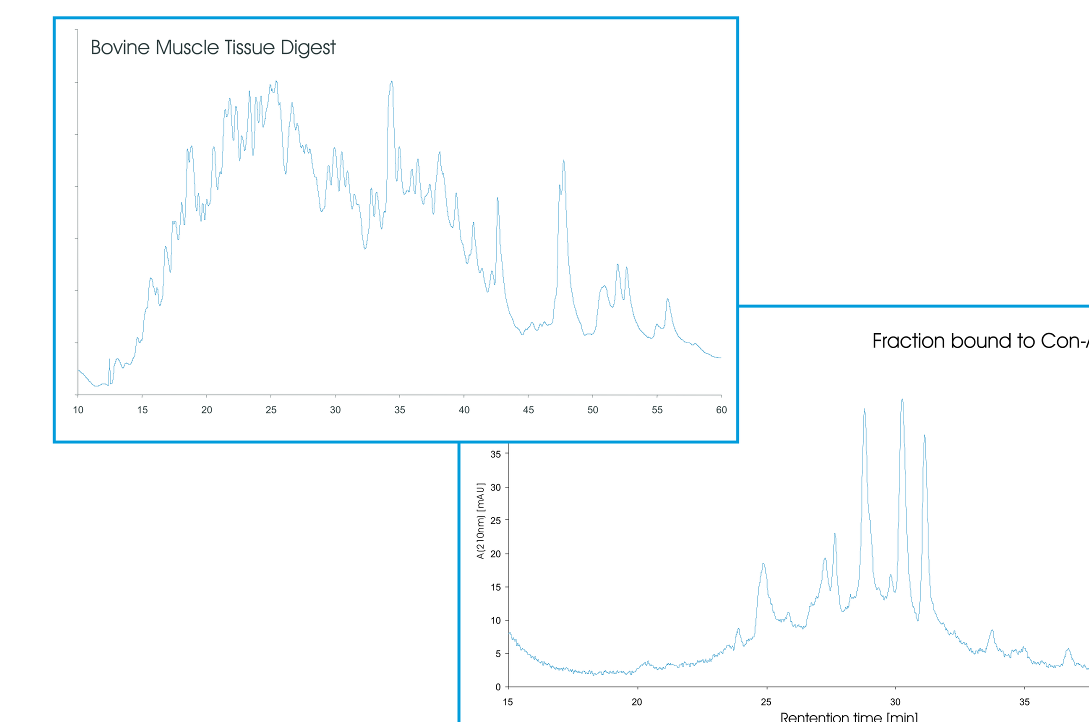
Bovine muscle tissue protein digest

- Freeze-dry tissue sample
- Dry and grind again
- Precipitate with TFA
- Add trypsin (protein to enzyme 20:1)
- Keep at 37 °C over night
- Filter with 0.45 µm filter
- Grind and extract fat with iso-octane
- Extract proteins with water
- Re-dissolve with 50 mM NaHCO solution

Experimetal conditions for Concanavalin A derivatised CMD columns

- The derivatisation with Con-A was performed as described at pH 5.5 (dimeric form of Con-A).
- The column was equilibrated with 0.1% TFA in water
- The peptide mix from the protein digest was loaded onto the Con-A/CMD column.
- The non-retained portion of the peptide mix was washed off.
- The bound fraction was eluted with methyl-a-D-mannopyranoside.

Con-A forms a number of oligomers depending on pH. The active dimeric form is stable at pH 5.6 and below. Between pH 5.8 and 7 Con-A forms tetramers; above pH 7 it forms higher aggregates.
Chromatographic conditions for the reversed phase analysis of the total protein digest and the eluate off the Con-A/CMD column were the same as for the analysis of the BSA tryptic digest.



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