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Evaluation of Reversed Phase Columns for Protein Separation using a single LC/MS - LC/UV Methodology

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Introduction

HPLC separations on reversed phase columns are an important part of characterizing complex protein samples from basic research and pharmaceuticals. Recent advances in mass spectrometry have allowed for rapid characterization of protein and peptide samples separated buy HPLC, greatly facilitating the identification of target proteins and contaminants. Thus, there is a need for good separation techniques using MS compatible methodology for the separation of proteins by HPLC. We present her the evaluation of peak capacity and recovery in protein separations along with LC/MS.

Our findings resulted in the determination that both Delta-Pak® and Symmetry® 300 are the preferred columns on which

Experimental

Separation Method

Various columns 300Å 5mm 4.6X50mm
Column heated to 60°C
A: 0.1% Formic acid in Water
B: 0.1% Formic acid in Acetonitrile
Gradient from 5% B to 70% B in 15 min at 60°C; 0.5 mL/min, split flow to ~0.2mL/min diverted to ZQ

Waters® Alliance® HPLC system

2690 or 2790

Waters® 996 PDA

Detection at 280 nm

Waters ZQ™ Mass Detector

Source = ESI (+)
Capillary (kV) = 3.3
Cone (V) = 22
Temperature (°C)
Source = 150
Desolvation = 350
Gas flow (L/Hr)
Cone = 50
Desolvation = 500
Scan Mode m/z = 500-1500

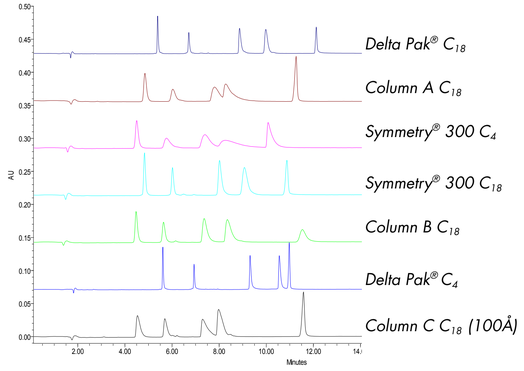
Proteins

RNase A
Cytochrome C
Horse Myoglobin
Carbonic Anhydrase

Protein LC/MS - LC/UV Protein Separation Configuaration

Allianance® (LC), ZQ® 4000 (MS), 996 PDA (UV), and Delta-Pak® C_{18} (column)

An Overlay of Protein Separations on Various Reversed Phase Columns viewed at 280nm



The peak capacity of each column tested as a function of each protein and an average of all four proteins.

Protein	Symmetry® 300 C_{18}	Symmetry® 300 C_4	Column A	Delta-Pak® C_{18}	Delta-Pak® C_4	Column B	Column C
RNase A	170	114	119	225	255	136	84
Cytochrome C	152	54	76	182	216	115	100
Myoglobin	103	43	53	111	137	74	56
Carbonic Anhydrase	66	20	40	78	107	57	59
Heme	134	71	127	161	103	55	134
Average (except Heme)	122	58	72	148	166	96	75

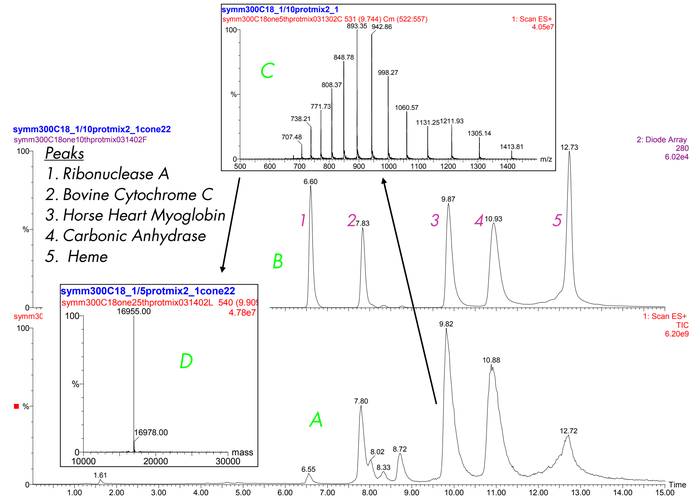
Peak Capacity = Time of Analysis (min)/ [S peak widths @ 50% height (min)/ number of peaks]

The recovery of each column tested as a function of each protein and an average of all four proteins. Four injections were run for each sample with three blank injections between each protein injection.

Protein	Symmetry® 300 C_{18}	Symmetry® 300 C_4	Column A	Delta-Pak® C_{18}	Delta-Pak® C_4	Column B	Column C
RNase A	92.1	93.9	83.9	100	93.0	86.6	94.9
Cytochrome C	101.0	98.3	98.0	85	90.0	84.9	103.0
Myoglobin	78.9	94.1	77.5	81	89.0	77.6	76.9
Carbonic Anhydrase	97.4	90.6	88.0	95	94.0	88.3	78.8
Heme	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Average (except Heme)	92.3	94.2	86.9	90.3	91.5	84.3	88.4

The UV, MS, and MaxEnt1 Data for the separation of RNase A, Bovine cytochrome C, Horse Heart Myoglobin, and Carbonic Anhydrase

A) Total Ion Chromatography of the Separation of a four protein mixture on Delta-Pak® C_{18} column.
B) The corresponding 280nm UV Chromatograph recorded concurrently with the MS TIC Chromatography.
C) The horse heart myoglobin protein charge envelope from the combined ion spectrum over the peak.
D) The MaxEnt1 process spectrum of horse heart myoglobin



Conclusions

This method is an excellent general method for separating proteins for UV/MS detection. Proteins of various sizes are separated and ionized well, giving good UV and MS signals.

The system described is a robust system with excellent separation and analysis capabilities for proteins.

Delta-Pak® and Symmetry® columns are outstanding for protein LC-MS. Excellent peak shape/capacity. No protein carryover. Recovery is excellent.

RNase A does not ionize well but we were still able to get a good MS signal with this method.

While contaminating protein degradation products are not readily detected by UV, they are readily detected by using mass spectrometry.