Application Note 20506

Analysis of Intact Proteins on a Thermo Scientific Accucore 150-C4 150 Å Pore Diameter HPLC Column

Joanna Freeke, Valeria Barattini, Thermo Fisher Scientific, Runcorn, Cheshire, UK

Key Words

Accucore C4, top down proteomics, proteins, fused core, superficially porous, 150 Å, ovalbumin, insulin, cytochrome c, lysozyme, myoglobin, carbonic anhydrase

Abstract

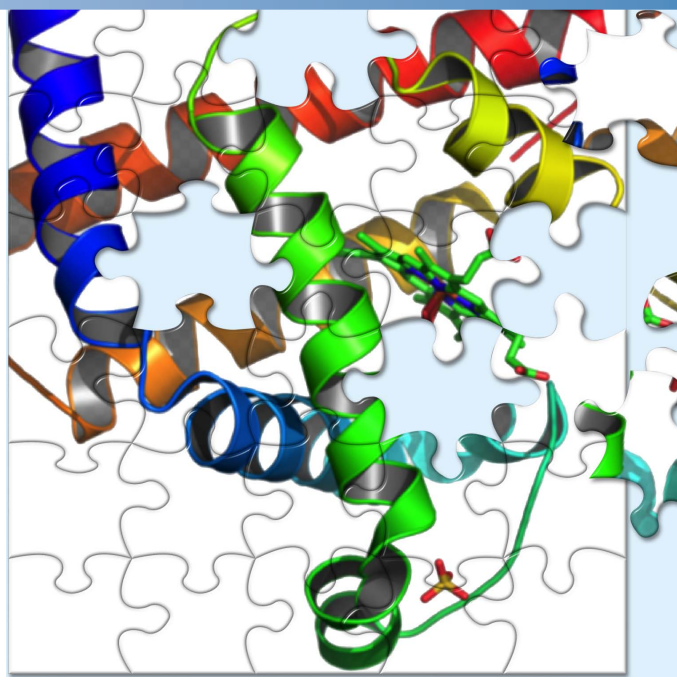
This application note demonstrates the analysis of intact proteins using a Thermo Scientific Accucore 150-C4 (150 Å pore diameter) HPLC column. The analysis of six proteins ranging in mass from 6 to 45 kDa is carried out in 15 minutes with pressures compatible with conventional HPLC instrumentation. Excellent loading capacity was observed for this column.

Introduction

Accucore™ HPLC columns use Core Enhanced Technology™ to facilitate fast and high efficiency separations. The 2.6 µm diameter particles have a solid core and a porous outer layer. The optimized phase bonding creates a series of high-coverage, robust phases. The tightly controlled 2.6 µm diameter of Accucore particles results in much lower backpressures than typically seen with sub 2 µm materials. For the analysis of large biomolecules the Accucore pore size has been further optimized and a C4 phase with reduced hydrophobic retention has been prepared. This 150 Å pore size enables the effective analysis of molecules unable to penetrate into smaller diameter pores, whilst the low hydrophobicity C4 phase results in protein separation by hydrophobicity.

Currently protein analysis is carried out either using the intact proteins or, after enzymatically digesting the proteins, on the peptide level. The latter approach leads to greatly increased sample complexity, which results in challenging data interpretation requiring deconvolution of the range of peptide fragments produced. Analysis of proteins at the intact level is preferred for the reduced sample complexity as well as the additional global protein information available. Developments in MS technology have enabled detection and analysis of intact proteins and this is being utilized in 'top-down' proteomics approaches.

These approaches rely heavily on separation of proteins prior to MS analysis. In this application note we demonstrate the excellent performance of an Accucore 150-C4 HPLC column (150 Å pore size) for the chromatographic separation of six intact proteins (6-45 kDa).



Experimental Details

Consumables	Part Number
Fisher Scientific HPLC grade water	W/0106/17
Fisher Scientific HPLC grade far UV acetonitrile	A/0627/17
Fisher Scientific HPLC grade trifluoroacetic acid	A116-50
Sigma-Aldrich® insulin, cytochrome c, lysozyme, myoglobin, carbonic anhydrase and ovalbumin	
Liquid handling hardware FinnPippette Kit 1	4700870
Vials and closures	MSCERT4000-34W

Thermo SCIENTIFIC

Separation Conditions			Part Number
Instrumentation:	Thermo Scientific Accela HPLC System		
Column:	Accucore 150-C4, 2.6 µm, 100 x 2.1 mm		16526-102130
Mobile phase A:	0.1 % TFA in 30:70 acetonitrile:water		
Mobile phase B:	0.1 % TFA in 98:2 acetonitrile:water		
Gradient:	Time (min)	%B	
	0	0	
	8	30	
	10	95	
	11	95	
	11.1	0	
	15	0	
Flow rate:	400 µL/min		
Backpressure at starting conditions:	185 bar		
Run time:	15 minutes		
Column temperature:	40 °C		
Injection details:	2 µL 10 pmol/µL solution except for loading capacity study		
Injection wash solvent:	Acetonitrile		
UV detector wavelength:	214 nm		

Solution	
Standard preparation:	A 1 mg/mL solution of each protein was prepared in water. These solutions were combined, with the resultant solution diluted to a final concentration of 10 pmol/µL.

Data Processing	
Software:	Thermo Scientific Xcalibur 2.0 SR2

Results

Under these conditions, six proteins covering the mass range of 6 to 45 kDa can be separated on an Accucore 150-C4 HPLC column in less than fifteen minutes with backpressures compatible with conventional HPLC equipment. The chromatography is shown in Figure 1 with all of the proteins eluting with sharp, symmetrical peaks and being baseline resolved, with the exception of an impurity from carbonic anhydrase which co-elutes with lysozyme. The identities of the proteins analyzed, their retention times, the percentage relative standard deviation (% RSD) in the retention time over six replicate injections, peak width and peak asymmetry are summarized in Table 1.

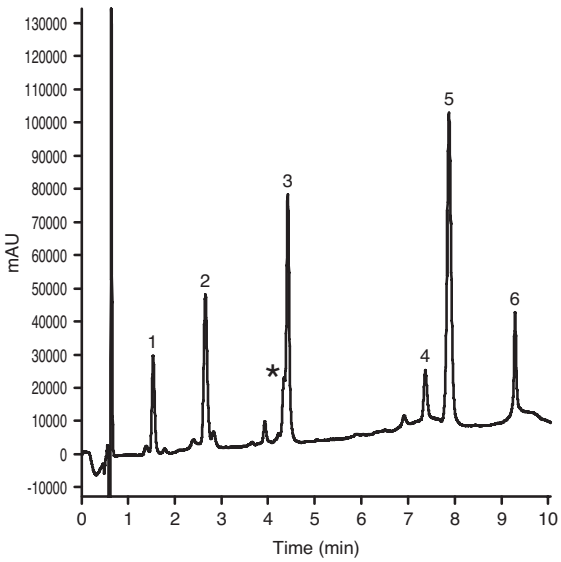


Figure 1: UV chromatogram for six proteins separated on an Accucore 150-C4, 100 x 2.1 mm HPLC column. The proteins are listed in Table 1, * indicates an impurity from the carbonic anhydrase sample.

	Protein	t_R minutes	% RSD	Peak width at half height / minutes	Asymmetry
1	Insulin (6 kDa)	1.54	0.67	0.06	1.20
2	Cytochrome C (12 kDa)	2.67	0.44	0.08	1.13
3	Lysozyme (14 kDa)	4.43	0.55	0.06	-
4	Myoglobin (18 kDa)	7.37	0.16	0.08	0.97
5	Carbonic anhydrase (30 kDa)	7.88	0.16	0.10	1.15
6	Ovalbumin (45 kDa)	9.30	0.06	0.05	1.14

Table 1: List of proteins analyzed, average retention time (t_R), percentage relative standard deviation (% RSD) in retention time over six replicate injections, peak width at half height and peak asymmetry

Loading capacity

With fused core materials there is a perception that the loading capacity of the material is much lower than that of a fully porous particle, however the peak shape for Accucore 150-C4 column is excellent for a wide range of sample loads. A large dynamic range is advantageous for proteomics applications as often only low picomolar concentrations are available, while for purification of overexpressed proteins there may be concentrations up to 1000 pmoles. A range of sample loads was compared on the Accucore 150-C4 column. A total protein load of 30-1200 pmoles was investigated. The chromatography is shown in Figure 2 and excellent peak shape was observed for all injections.

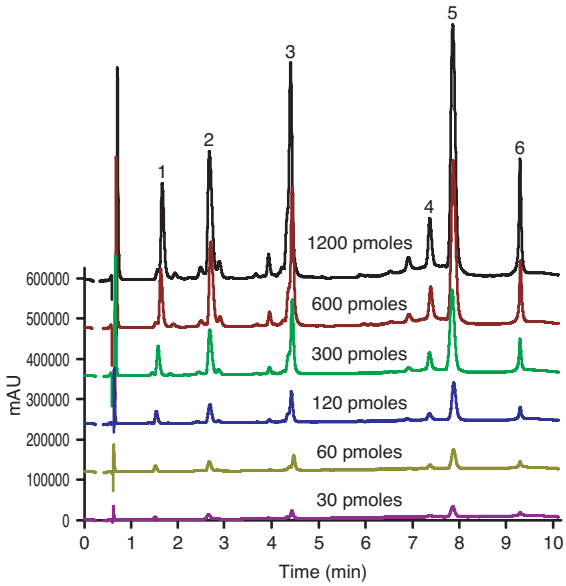


Figure 2: UV chromatograms showing the result of different loads of six protein mix. The total protein picomolar load on the column is reported

Peak capacity

For many analysts investigating a range of unknown proteins, the number of peaks that can be resolved within a gradient run is an important parameter and this is defined as the peak capacity. For the six protein mix with a 10 minute gradient the peak capacity was calculated to be 67 using the following formula [1]:

$$n_c = 1 + \left(\frac{t_g}{W}\right)$$

Where n_c = Peak capacity

t_g = gradient time

W = average peak width 10% height

The effect of sample loading on peak capacity was investigated (Figure 3).

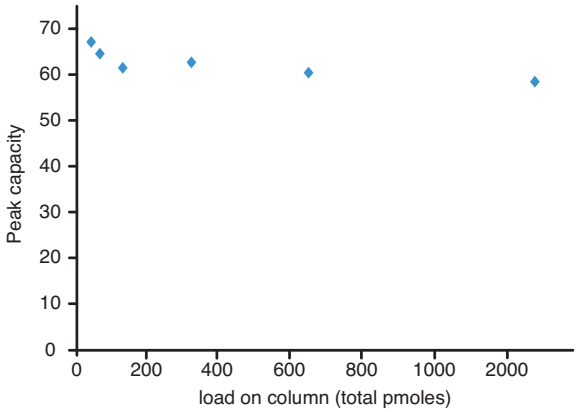


Figure 3: Peak capacity variation with picomolar load of total protein on column

Conclusion

- Accucore 150-C4 columns show excellent separation of six test proteins of differing mass (6-45 kDa) within 15 minutes
- Good peak shape is observed for all proteins across a range of concentrations (30-1200 pmol total load on column)
- High peak capacity is shown on a ten minute gradient separation
- The backpressure is compatible with use on a conventional HPLC system

References

[1] Wang X., Peak Capacity Optimization of Peptide Separations in Reversed-Phase Gradient Elution Chromatography (2006), 78 (10), 3406-3416

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USA and Canada +1 800 332 3331
France +33 (0)1 60 92 48 34
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China +86 21 68654588 +86 10 84193588
+86 20 83145199 800 810 5118
India +91 22 6742 9494 +91 27 1766 2352
Australia 1 300 735 292 (free call domestic)
New Zealand 0800 933 966 (free call domestic)
All Other Enquiries +44 (0) 1928 534 050

Technical Support
North America +1 800 332 3331
Outside North America +44 (0) 1928 534 440

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