Intact Protein LC-MS: How to Overcome the Challenges?

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Overview

Purpose: Facilitate intact protein LC-MS without compromising performance.

Methods: Construct prototypes of integrated column and sprayer dedicated for intact protein separations and compare performance.

Results: Three prototypes have been directly compared and all showed good protein separations, each with their unique area of strength.

Introduction

Mass-spectrometry-based protein characterization finds two major applications in top-down proteomics and biopharma analysis of recombinant proteins, antibodies and other biotherapeutics. However, analysis of high-molecular-weight proteins can be challenging. The methodology chosen should minimize common problems in protein analysis such as degradation, poor recovery, broad elution profiles, poor ionization efficiency, ion sensitivity, and resolution. To address these performance challenges, we compared different prototype column-spray interfaces.

Methods

Samples

Column performance was evaluated beforehand by injecting 50 fmol of Bovine Serum Albumin (BSA) tryptic digest (data not shown). The test sample consisted of a mixture of seven proteins (Ribonuclease A, Cytochrome C, Lysozyme, Myoglobin, Carbonic Anhydrase, and BSA) with size-range 10–66 kDa. Each protein was also injected individually to determine artifacts from protein degradation which could lead to peaks that are not readily assigned by mass.

Liquid Chromatography

A Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system in nano (75 µm ID columns) and capillary (200 µm ID columns) modes with a cooled autosampler was used for all separations.

All direct-injection experiments were with mobile phase A water with 0.1% formic acid (FA) and mobile phase B with 80% ACN in water and 0.08% FA. Pre-concentration was performed for the monolithic column only, with water with 0.05% TFA as the loading solvent.

The chemistry selection was based on inherent attributes (Table 1). The solid core material was designed for protein separations. The fully-porous (referred to as wide-pore C18) material is traditionally used. The monolithic column presents excellent performance and robustness.

All conditions were kept as similar as possible to facilitate comparison. Gradient conditions are discussed with the results. The flow rates were 300 nL/min for the 75 µm ID columns and 2 µL/min for the 200 µm ID column, resulting in the same approximate linear flow velocity. The gradient was 5–65% at 15, 30, and 60 min. The injected sample amount was varied from 0.5, 2, 4, to 10 ng each protein on column. The temperature was set to 30, 45, and 60^{0}C.

TABLE 1. Overview of tested LC-MS column sprayer characteristics

Name	Column				Format		
	Technology	Chemistry	Particle	Pore Size	ID	Length	Sprayer ID
Accucore	Solid core	C4	2.6 µm	150 A	75 µm	15 cm	7 µm
PepMap	Fully porous	C18	5 µm	300 A	75 µm	15 cm	7 µm
PepSwift	Monolithic	PS-DVB	n.a.	n.a.	200 µm	25 cm	15 µm

Mass Spectrometry

All experiments were performed on a Thermo Scientific™ Velos Pro™ ion trap mass spectrometer with 1.9 kV spray voltage, and full MS-only recording from 400–2000 m/z. The S-Lens setting was 63%. All experiments were performed using the EASY-Spray source.

Data Analysis

Data was analyzed manually using the Qualbrowser feature in Thermo Scientific™ Xcalibur™ software revision 2.2. Peak detection was performed with the Genesis algorithm at 13.4 % peak height for gradient comparison and 50% peak height for loadability.

Results

Comparison of the Three Column Designs

A TIC (mass range 550–2000 to exclude background) is shown in Figure 1. The separation of the protein mixture for all three columns, with a 30-min gradient, is shown. Elution order was identical, however the peak width, peak shape and degree of separation vary. The myoglobin-BSA pair at the bottom of Figure 1 shows the best example of this.

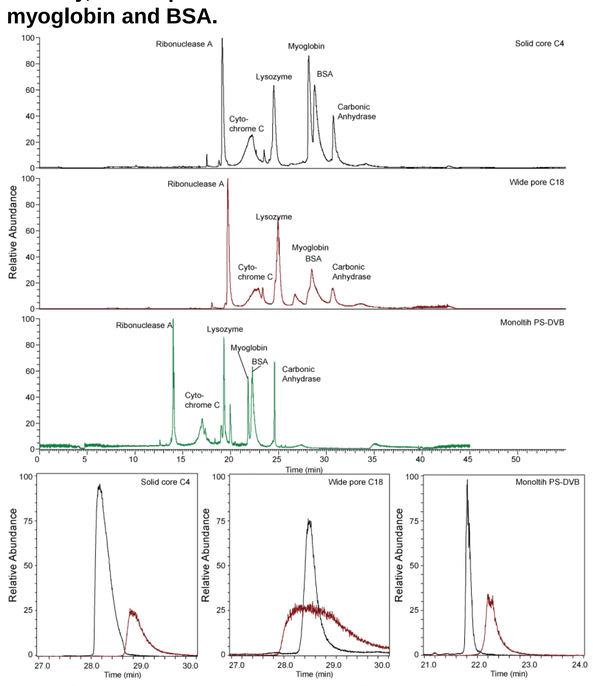
The noise for the monolithic column appeared higher. This was attributed to the 2 µL/min flow rate (vs. 300 nL/min for the other two columns). The dilution effects of wider ID did not significantly influence sensitivity because the total signal ion count was on the same order as for the 75 µm ID columns.

The selectivity difference, peak shape and degree of separation is shown at the bottom of Figure 1. Two extracted ion chromatogram traces (based on relevant peaks of the myoglobin and BSA charge envelope) were compared to show the degree of separation between these proteins. The BSA signal was enlarged two times for visibility. The C4 and monolith showed baseline separation, but the C18 column showed coelution, as well as a broad elution profile for the multiple species in the BSA sample.

Gradient Variation

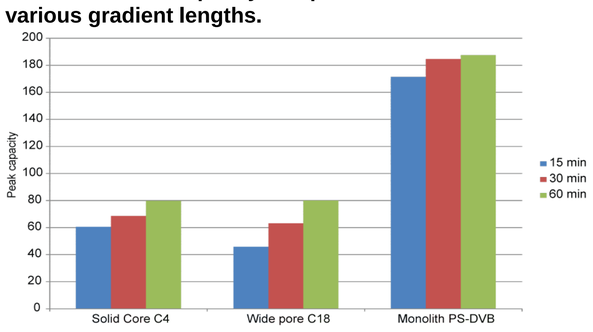
Different gradient lengths were used for the separation and to determine the peak capacity. The base peak chromatogram (BPC) signal of Ribonuclease A, Myoglobin, Lysozyme and Carbonic Anhydrase were used because these presented relatively clean charge envelopes without coelution of modified versions of these proteins. The underrepresentation in intensity for the larger species did not affect the peak width measurably, therefore the BPC can be used for data analysis.

FIGURE 1. Comparison the TICs for the three different columns. The same protein mixture, with the same gradient and linear flow velocity, was separated 5–65% B in 30 min at 60ºC on the EIC for myoglobin and BSA.



The columns compared here were different in many aspects: stationary phase structure, particle size and pore size. These differences lead to differences in the diffusion paths and affect peak widths. Large molecules will benefit most from the shorter diffusion pathways provided by the PS-DVB monoliths. The surface interaction on the monolithic structure provides the perfect backbone for short diffusion paths and narrow peaks. This is shown in Figure 2, where the monoliths exhibited the highest peak capacity. The lowest peak capacity was obtained for the fully porous 5 µm particles, which is explained by the longer diffusion paths for this type of stationary phase. Peak capacity was determined by dividing the gradient length by the average peak width at 13.4% for Ribonuclease A, Lysozyme, Myoglobin and Carbonic Anhydrase. These were chosen because they cover the complete elution window, and elute with a relatively "clean" charge envelope, avoiding interference from isoform elution.

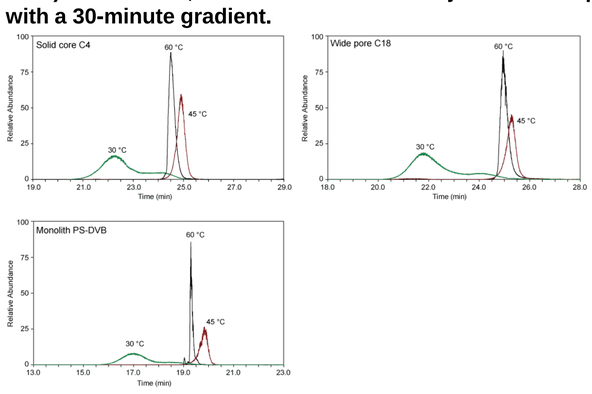
FIGURE 2. Peak capacity comparison for the tested columns at various gradient lengths.



Temperature Effect

Temperature can have a strong effect on the recovery and separation efficiency of proteins. Shown in Figure 3, this effect was most visible for Lysozyme, which at the three different temperatures had a completely different retention time, elution profile and peak height. Interestingly, the behavior was similar for all three of the RP stationary phases, which could be explained by temperature-dependent conformational changes of Lysozyme.

FIGURE 3. Lysozyme elution at three temperatures (30, 45, and 60ºC). In all cases, the same amount was injected and separated with a 30-minute gradient.

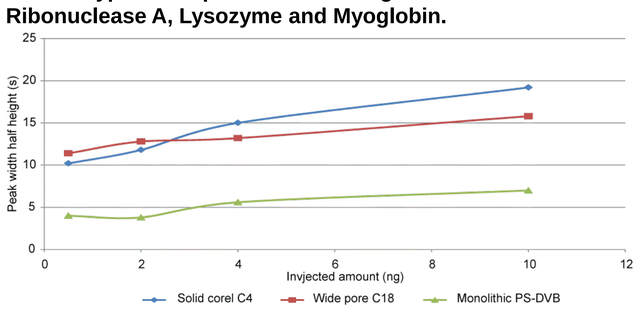


Sensitivity and Protein Loading Capacity (Loadability)

The base concentration was 4 ng for each protein injected. The sensitivity was evaluated by injecting 2 (2 ng) and 8 (0.5 ng) times less. Protein loadability was evaluated by injecting 2.5 times more (10 ng). Figure 4 shows the trend for peak width half height (PWHH) for the three proteins. In all runs, good signal-to-noise ratio resulted in higher sensitivity.

The increase in PWHH is a measure of loadability, where the strongest increase represents the lowest loadability. The monolithic column is wider in ID, giving it better loadability by default. The solid-core particles have lower surface area compared to the fully-porous particles and hence a lower loadability was expected.

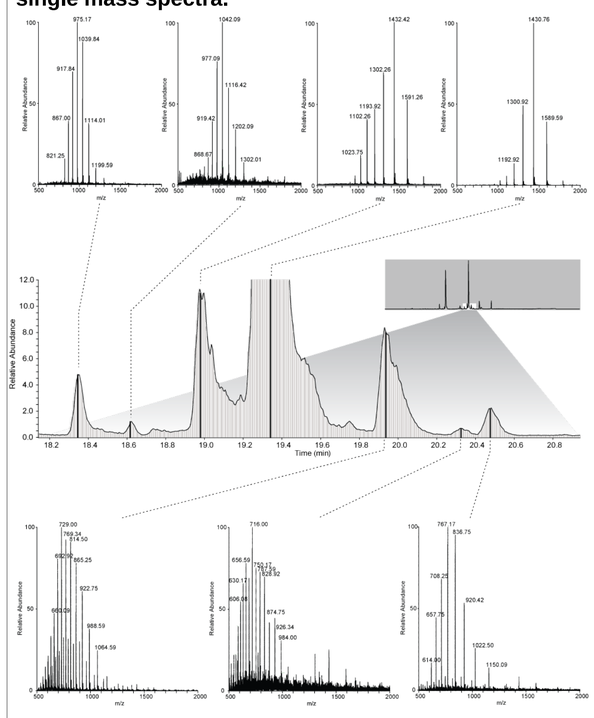
FIGURE 4. PWHH plot for different injected amounts for each column type. Each point is the average of the PWHH of Ribonuclease A, Lysozyme and Myoglobin.



MS Detection of Proteins and their Isoforms

The goal of resolving analytes prior to MS analysis, is to obtain more information. For LC-MS analysis of proteins, this often identifies lower abundance species such as isoforms and modifications. For the chromatograms shown in Figure 1, six main peaks could easily be identified. However, zooming in revealed many smaller peaks. Figure 5 shows the charge envelopes for the "zoom in" at 18–21 min for the monolithic separation in Figure 1.

FIGURE 5. Zoom-in of 4 ng protein mix separation on monolithic column with a 30-min gradient at 60ºC. The charge envelopes are single mass spectra.



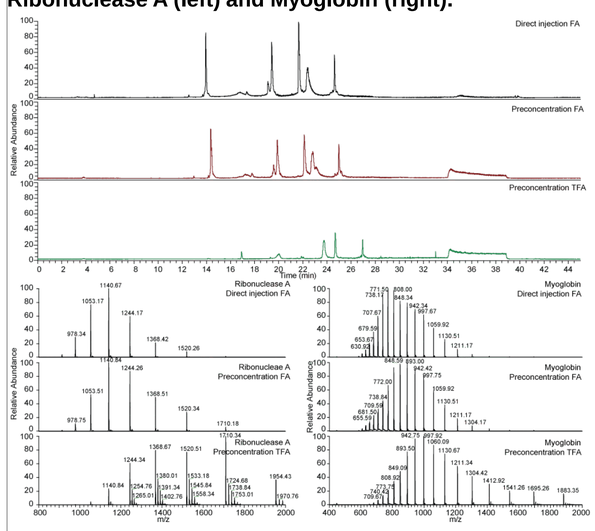
Influence of Trap Column and Ion-Pairing Agent

All previous data was collected by direct-injection. However, pre-concentration is preferred when larger sample injection volumes are required. In addition, the effect of using TFA compared to FA in eluents is summarized in Figure 6.

The use of a trap column did not impact the separation performance significantly. PWHH increased slightly by 1–1.5 seconds, which was expected due to the added volume. While the use of TFA as an ion pairing agent changes the separation selectivity, no significant improvements were observed with PWHH remaining the same. However, signal intensity was strongly influenced.

The effect on the MS spectra was also significant. TFA forms more adducts and shifts the charge envelope to higher m/z (lower charges). These two effects distribute the signal of a protein over more m/z values, all of which will have lower intensity. Hence, the use of FA is recommended for analysis of intact protein by LC-MS.

FIGURE 6. Comparison of different setups, direct injection and pre-concentration with FA as eluents and pre-concentration with TFA in the eluents. Top shows TIC on the same intensity scale. Bottom shows the effect on the charge envelope for Ribonuclease A (left) and Myoglobin (right).



Conclusion

- Three integrated sprayer and emitters were used to successfully separate a semi-complex protein mix.
- Both short and long gradients showed excellent peak capacity, but long gradients should be adapted to the sample complexity.
- Higher temperatures are recommended for increased separation performance and protein recovery, but possible effects on the confirmation should be considered.
- The high separation efficiency resulting from the integrated design allowed detection of low abundance fragments and modified forms.
- Direct-injection and pre-concentration techniques produced no significant differences in results.
- The use of TFA will decrease signal intensity, which cannot be compensated by improving the separation. Therefore FA is recommended.

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