PURITY ANALYSIS AND IDENTITY CONFIRMATION OF PROTEINS USING OPTIMIZED REVERSED PHASE CHROMATOGRAPHY LC/ EXACT MASS TOF-MS SYSTEMS

Waters

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INTRODUCTION

The separation of proteins with reversed chromatography has proven successful in identifying structural variants and modified proteins. The technique requires careful optimization of the chromatographic conditions because the differences that must be recognized are very small relative to the sizes of the molecules being separated. Certain preferences have become common for achieving the required resolution. These include the use of wide pore packing materials and shorter chain bonded phases. The gradients are usually shallow. It is becoming more common to substitute propanol for acetonitrile as the strong solvent. At the same time, temperatures are elevated, in some cases as high as 70—80°C. These parameters are primarily focused on achieving selectivity.

An equally important consideration is recovery and carryover. With protein samples, it is frequently observed that not all the injected protein reaches the detector. The components of the sample can be observed in repeat gradients without additional injections. With small molecules, such carryover often originates with unswept volumes of the injector. With proteins, however, the more common source is material that does not elute from the column because of slow chromatographic equilibria.

Efficient protein separations are also affected by the compromises required between detection with ESI/MS. The replacement of TFA with formic acid to achieve better sensitivity often degrades peak shape.

In these studies, the various chromatographic variables are compared .

METHODS

Samples:
- RNase A, cytochrome c, transferrin, hemoglobin, and enolase were purchased from Sigma Chemical Company, St. Louis
- Concentration = 0.1 µg/µL of each protein of mix in aqueous 0.1% formic acid
- Diluent = Aqueous 0.1% formic acid
- 10µL injections = 1 µg/protein
- RNase A oxidation mix = 1:1 untreated and 24 hour H_2O_2 treated 0.05mg/mL each in normal saline

Chromatography:
- Waters 2796 Alliance System
- BioSuite™ pPhenyl, 2.0 x 75 mm column
- Symmetry®300 C4, 2.1 x 100 mm column
- Flow rate 200 µL/min on BioSuite™ pPhenyl
- Flow rate 220 µL/min on Symmetry®300 C4
- Buffer A = Aqueous, 0.1% TFA (or 0.1% formic acid if noted)
- Buffer B = Acetonitrile, 0.1% TFA (or 0.1% formic acid if noted) with isopropanol (IPA) as noted
- Gradient range, 10-60% Buffer B
- Gradient time as noted
- Temperature 60°C unless noted

Mass Spectrometry:
- Waters Micromass LCT Premier
- Electrospray Ion Source 100° C
- Cone = 30 V
- Tunnel 1 = 90 V

RESULTS

Figure 1: Effect of Organic Solvent

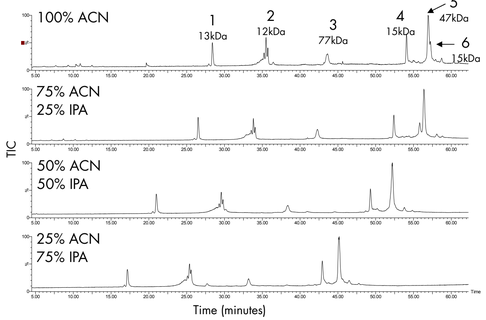

Figure 1: The standard protein mixture was separated on a Symmetry300 C4 column in the presence of varying proportions of acetonitrile and propanol. With increasing propanol all the proteins elute earlier. There is relatively little difference in peak shape or in selectivity. It is interesting to note the last pair of peaks. They resolve in 75% acetonitrile:25% isopropanol, but not at higher or lower proportions.

Figure 2: Effect of Temperature

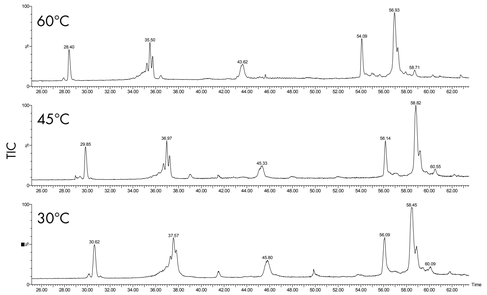

Figure 2: The standard protein mixture was separated on a Symmetry300 C4 column over a range of temperatures. At higher temperatures, all the proteins elute earlier. This effect is approximately equivalent to a 2% change in acetonitrile concentration over the range 30°C to 60°C. No changes in selectivity are associated with this change.

The effects of organic solvent and temperature may be more profound for other proteins, particularly larger and more hydrophobic ones. These variables may be important in eliminating memory effects.

Figure 3: Effect of Mobile Phase Modifier on Chromatography

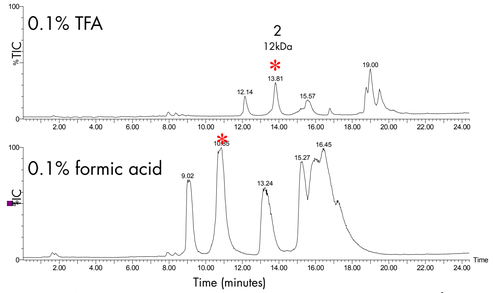

Figure 3: The protein standard was separated on a Symmetry C4, 300Å, in TFA and in Formic Acid. As expected, there was less retention with formic acid since there is no ion-pairing. The same phenomenon explains the enhanced sensitivity observed here as the TIC. Spectra were extracted from the marked (*) peaks.

Figure 4: Effect of Mobile Phase Modifier on Mass Spectra

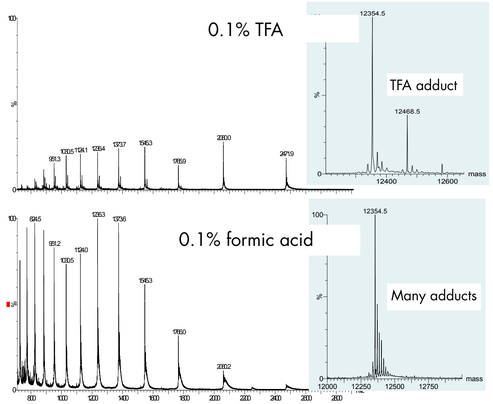

Figure 4: Mass spectra were extracted from the starred peaks in Figure 3. There are important differences between the spectra obtained in the two mobile phases. With formic acid as the modifier the total intensity is greater in the TIC and in the spectrum ion intensities. The charge-state intensity distributions are centered at different *m/z* values. The formic acid solution favored higher charge states, low *m/z*, while the TFA solution favored lower charge states, high *m/z*. Also, the TFA forms adducts with the protein as shown in the MW spectrum (inset). Oddly, there are low MW adducts suggesting solvent.

Figure 5: Analysis of Oxidized Proteins

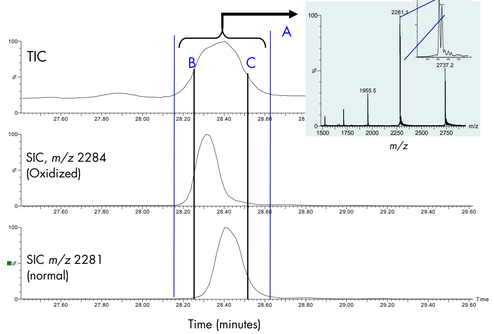

Figure 6: A 1:1 mixture of oxidized RNase A and untreated RNase A was separated on a Symmetry300 C4 column. The protein forms eluted from 28.15—28.63 minutes. When the summed mass spectra over this peak are examined (Top), a pair of closely spaced *m/z* species are observed. Single mass chromatograms were extracted for the two most intense signals, 2281 and 2284. These SIC's correspond to native and oxidized RNase, and the chromatograms show partial separations of these species.

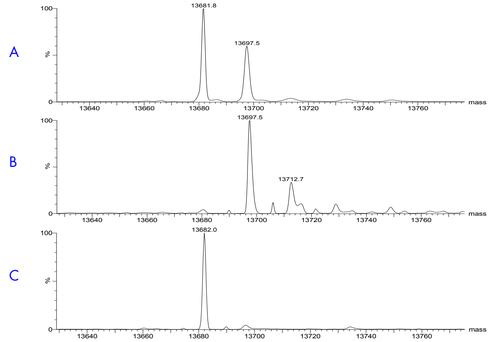

Figure 7: RNase A peak of mixed oxidized and untreated protein eluted from the Symmetry C4 column. The molecular weights were deconvoluted from spectra over the entire peak, A, an early eluting spectrum, B, and a later eluting spectrum, C (See figure 6). Spectra B and C are only 9 seconds apart, but they show no coelution. The spectra were deconvoluted with MaxEnt1™ and yield the correct molecular weights separated by 16 Daltons. These results demonstrate that the singly oxidized protein elutes prior to the untreated protein, and that chromatographic separations can improve spectral quality for intact proteins.

Figure 7: Measuring Carryover / Memory Effects

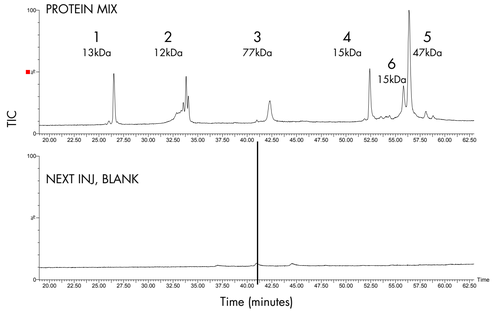

Figure 9: Reversed phase separations of proteins are often compromised by carryover. This observation may be more accurately described as a memory effect when the eluted material originates from sample that did not elute from the column, rather than from the injector. With the columns and conditions used in these experiments, injection of diluent buffer as a blank shows neither carryover nor memory effects.

Figure 8: High Sensitivity for Carryover Measurement

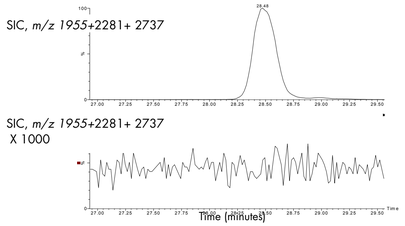

Figure 10: The highest sensitivity assessment of memory effects is achieved using ESI-MS. In this example, an extracted mass chromatogram was constructed for the three most intense ions of the protein eluting near 28.5min. No protein can be detected in the subsequent blank.

CONCLUSIONS

- Common chromatographic modifications have small impacts on the selectivity of protein separations.
- The substitution of formic acid for TFA can increase sensitivity in ESI/MS with some sacrifices in chromatography.
- Greater complexity of adduct formation is observed in mass spectra with formic acid than with TFA.
- Small chemical differences between proteins can be resolved.

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