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Intact Protein RP-HPLC Method Development Using Minimal Sample

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The ExpressLC-100™ with a ChromXP™ 3C4-300 column is used to develop an optimized RP-HPLC (reversed phase HPLC) method for a protein mixture using minimal sample. Fifteen chromatograms are generated using only 4 μL of protein solution.

Developing RP-HPLC separation methods for new compounds often requires the chromatographer to scan through a variety of conditions (columns, solvents, gradients, etc.) to determine how to best analyze the mixture. With a novel protein sample, the amount of material required for this process can be prohibitive, even at 10 μL per injection.

The ExpressLC-100 dramatically reduces the amount of sample required for this process for several reasons. As the instrument is optimized for use with capillary columns in the 0.3–0.5 mm i.d. range, typical injection volumes are only 100–300 nL per chromatogram.

Even more important for a method development exercise, the ExpressLC-100 uses time-sliced injections. That is, rather than filling then injecting the entire sample loop, the loop is filled and portions are injected sequentially. Here a 2 μL loop is filled using 4 μL of sample. From this 2 μL, 100 nL is used to generate a single chromatogram. Time slicing the loop this way, up to 15 chromatograms can be generated, allowing the chromatographer to investigate 15 different gradients while requiring only 4 μL of sample. The ~250 nL of sample used for a single chromatogram is nearly 50 × less than would be required for a single injection with a 4.6 mm column.

Experimental

Protein samples are prepared by dissolving a standard protein mix (Sigma-Aldrich, p/n H2899) in mobile phase A to a final concentration of 1 mg/mL.

RP-HPLC separations are performed at 30 °C on an ExpressLC-100 (Eksigent Technologies) instrument equipped with a CTC PAL autosampler and using a ChromXP 3C4-300 column (0.3 × 50 mm, dp = 3 μm, 300 Å pores, Eksigent Technologies) with the following conditions:

Mobile Phase A:	0.1% TFA/water
Mobile Phase B:	0.1% TFA/acetonitrile
Gradients:	A 10 → 50%, 4 min, 10 μL/min
	B 5 → 70%, 8 min, 5 μL/min
	C 10 → 50%, 7 min, 5 μL/min
	D 10 → 50%, 8 min, 5 μL/min
	E 20 → 50%, 8 min, 5 μL/min
Detection:	absorbance, 215 nm

Results and Conclusions

The top of Figure 1 shows the results for five separations of the protein mixture. Here the initial mobile phase composition,

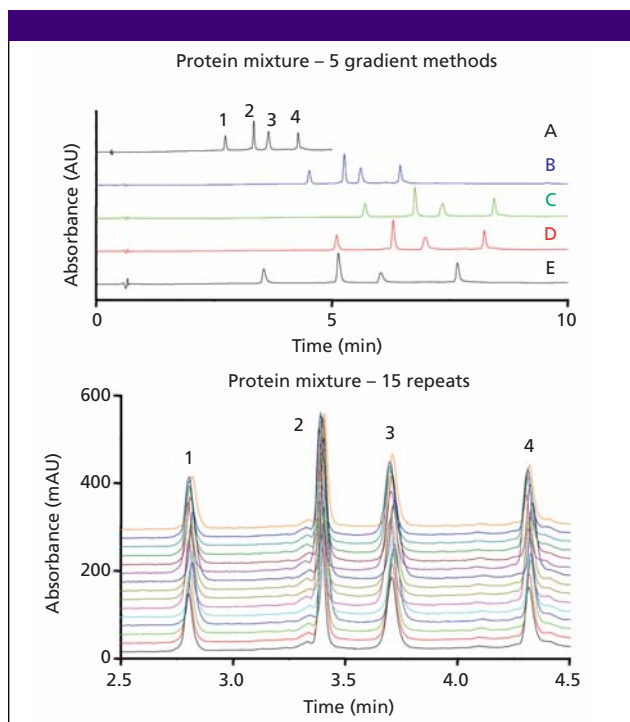


Figure 1: Top: 4 μL of intact protein mixture is analyzed by five different methods. Bottom: after identifying method A as the best procedure 15, 100 nL time sliced injections are performed and plotted. Peaks: 1 = ribonuclease A (14,000 Da); 2 = cytochrome C (12,000 Da); 3 = holo-transferrin (77,000 Da); 4 = apomyoglobin (17,000 Da).

gradient slope, and/or flow rate are varied. This shows the high quality chromatography possible with the ExpressLC-100. Peak widths (FWHM) range from 2–3 s, while base widths are between 3.5–5.0 s. System pressures varied during the run between 350–750 psi for the 5 μL/min separations and from 700–1500 for the 10 μL/min runs.

In the bottom panel of Figure 1, method A is used to show repeatability for the time-sliced technique. Here 15, 100 nL injections are plotted. The data yielded retention time RSDs of < 0.5% and area RSDs of < 5%.

Beyond the fifteenth injection quantitation degrades. As 100 nL time slices are pushed out of the loop the upstream end of the sample becomes diluted with mobile phase, ultimately limiting the total number of injections. The data in the figure show typical performance as 75% of the total sample loop may be used to produce repeatable results.

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