Reversed-Phase Preparative and Process Chromatography

A Powerful But Often Overlooked Method for Protein Purification

Reversed-phase chromatography on Vydac® 300Å pore-size adsorbents, first introduced in 1981, is a very useful high-resolution method for protein separations. The power of reversed-phase for protein chromatography is demonstrated, for example, by the separation of species-specific insulins, many of which differ by a single amino acid residue **(Figure 1)**, the separation of various apolipoproteins from human serum **(Figure 2)**, the separation of reduction products which differ mainly in conformation from native insulin **(Figure 3)**, and the separation of ribosomal proteins **(Figure 4)**.

Subtle differences in conformation can permit protein separation by reversed-phase chromatography because retention depends on the "hydrophobic footprint" of a protein molecule – the minority of hydrophobic amino acid residues that are actually accessible at the surface of the folded protein. Since sample loading occurs at low organic concentration, this can reflect subtle differences in native structure.

A rule of thumb for developing preparative and process purifications is that a method that succeeds in revealing an impurity will often be the best method for removing that impurity. The frequent use of reversed-phase for protein analysis would therefore argue for its utility in purifications. To be fair, chromatography on Vydac® reversed-phase adsorbents is already used in process purification of several FDA-approved bio-pharmaceuticals, and new reversed-phase purifications of protein products are reported regularly. However it also appears that reversed-phase methodology, while highly regarded as an analytical method and in spite of its prodigious resolving power, is often dismissed or overlooked as a candidate method for purification.

Species-Specific Insulins

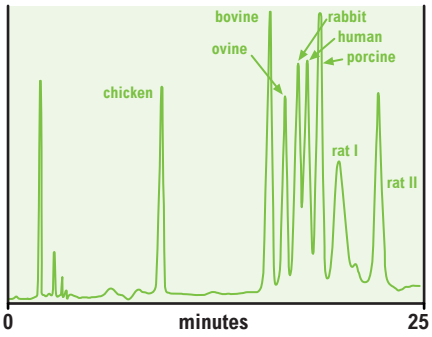

Figure 1: Separation of species-specific insulins, some of which differ by a single amino acid, demonstrates the ability of reversed-phase HPLC to separate very similar polypeptides. **Column:** Vydac® 214TP54 (C4, 300Å, 5µm, 4.6mm i.d. x 250mm). **Mobile phase:** Gradient from 27 to 30% ACN with 0.1% TFA over 25min. From J. Rivier and R. McClintock, J. Chrom. 268, 112-119 (1983).

Human Apolipoproteins

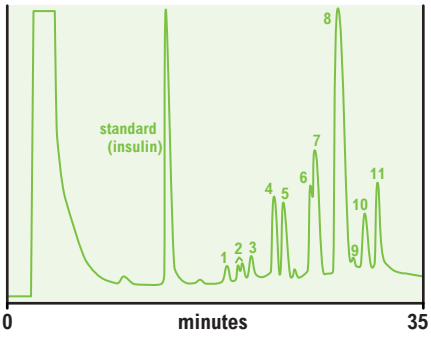

Figure 2: Separation of apolipoproteins isolated by gradient ultracentrifugation from human serum. **Column:** Vydac® 218TP54 (C18, 300Å, 5µm, 4.6mm i.d. x 250mm). **Flow rate:** 1.2mL/min. **Temperature:** 50°C. **Mobile phase: A =** 25% ACN/0.1% TFA in water. **B =** 58% ACN/0.1% TFA in water. Gradient from 0 to 100% B in 33 min. **Peaks:** 1. apoC-IIIa; 2. apoC-IIIb; 3. apoC-IIa; 4. apoC-IIIc; 5. apoC-I; 6. apoC-IIb; 7. apoA-Ia; 8. apoA-Ib; 9. apoA-IIc; 10. apoA-IIa; and 11. apoA-IIb. Reproduced with author's permission from Hughes, et. al., J. Lipid Res. 29, 363-376 (1988).

Insulin and Reduction Products

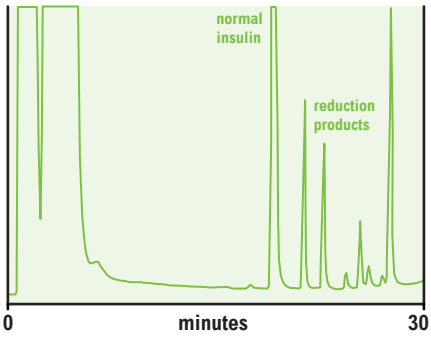

Figure 3: Separation of insulin and its partial reduction products. In a study of bridged peptides, disulfide bonds were reduced and the reduction products separated from normal insulin by C18 reversed-phase HPLC. **Column:** Vydac® 218TP54 (C18, 300Å, 5µm, 4.6mm i.d. x 250mm). **Flow rate:** 1.0mL/min. **Mobile phase: A =** 0.1% TFA. **B =** 0.092% TFA in 60:40 ACN:H2O. Gradient from 35 to 85% B in 25 min. Data from W. R. Gray, Prot. Sci. 2, 1732-1748 (1993).

Ribosomal Proteins

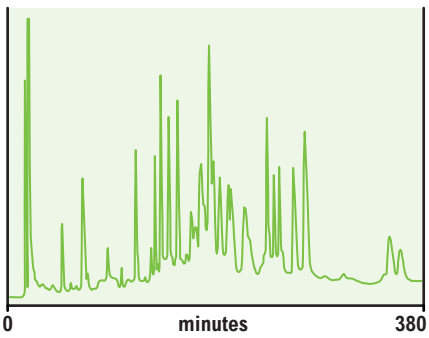

Figure 4: Separation of ribosomal proteins. **Column:** Vydac® 214TP54 (C4, 300Å, 5µm, 4.6mm i.d. x 250mm). **Mobile phase:** Gradient from 10 to 38% isopropanol with 0.1% TFA over 355min. From R.M. Kamp, A Rossenthoff, D. Kamp, and B. Wittman-Liebold, J. Chrom. 317, 181-192 (1984).

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Why is this?

We believe it reflects some apprehensions about reversed-phase that actually have simple answers.

Don't reversed-phase conditions denature proteins and destroy activity?

Although the organic solvents used in reversed-phase elution may disrupt tertiary structure, most proteins will refold and regain activity when returned to aqueous buffers. Some proteins, especially those with disulfide bridges that help maintain structure, will either not lose activity or refold very rapidly. For others, proper refolding is often favored by gradual removal of solvent at moderate temperatures. A little experimentation will usually discover the best conditions.

Use of organic solvents does provide some advantages. For example microbial growth and endotoxin contamination are less likely in organic-aqueous mixtures than in purely aqueous mobile phases. Reversed-phase chromatography can also provide an effective method for endotoxin removal.

Isn't reversed-phase chromatography too expensive for process-scale use?

Media for preparative and process reversed-phase are available in quantity at lower cost than adsorbents typically used to pack analytical HPLC columns. It is often possible to choose solvents for reversed-phase that are not more expensive than buffer components used in other forms of chromatography.

The separation power of reversed-phase as well as its utility in desalting, endotoxin removal, and ability to produce highly concentrated fractions often allows reversed-phase chromatography to replace multiple steps that are more expensive in a purification process.

Aren't organic solvents toxic? Don't they cause waste disposal problems?

Proper choice of solvents is the key. While the acetonitrile and methanol often used for analytical reversed-phase can be problematic, process protein separations can normally be performed with less expensive and nontoxic ethanol or isopropanol. Ethanol is a good choice because it is available in USP grade at reasonable cost, is familiar to regulatory agencies, and is environmentally innocuous. For ion pairing, acetic or formic acid can often replace the TFA commonly used in analytical separations.

In developing reversed-phase protein separations, whether analytical or preparative, it helps to have a partner that is experienced in this technology. As the leading provider of 300Å reversed-phase adsorbents for protein and peptide chromatography, Grace has more experience than any other supplier.

In addition, we provide a full range of Vydac® 300Å reversed-phase packed columns plus media for preparative and process applications. Vydac® preparative media consist of larger silica particle sizes – 10-15µm, 15-20µm, and 20-30µm – with the same 300Å pore size and bonded reversed-phase chemistries available in Vydac® columns for analytical protein and peptide HPLC. The availability of identical chemistries simplifies method development by allowing separations to be initially scouted on analytical-size columns, then scaled up, with appropriate adjustments in conditions of elution, to virtually any scale necessary for purification of larger quantities.

Grace has a history of supporting process reversed-phase customers. We have the capacity to supply Vydac® preparative media in 100-kg quantities with extensive regulatory support information for GMP applications. Our technical department can assist in developing separations, scaleup strategies, and providing Vydac® adsorbents in the quantities needed for your process.

To order call 1.800.255.8324 or contact your local Vydac® column distributor.

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