Analysis and Purification of Polypeptides by Reversed-Phase HPLC

Reversed-phase HPLC is a valuable tool for the analysis and purification of proteins and peptides. It is effective in separating peptide fragments from enzymatic digests, in the separation and purification of natural and synthetic peptides, and in purifying proteins as large as 100 kD for characterization.

Why is reversed-phase so widely used?

The answer is resolution. RP-HPLC is able to resolve very similar polypeptides, some of which differ by a single amino acid residue. The separation of insulin variants (below) is an example of the high resolution capabilities of reversed-phase HPLC. Some insulin variants differ by as little as a single amino acid. For instance, rabbit insulin has a threonine where human insulin has a serine—a difference of one methylene group in a single residue—and RP-HPLC is able to separate these two variants. In another instance, the purification of insulin-like growth factor (IGF), RP-HPLC separated native IGF from its oxidized methionine derivative—a difference of a single oxygen atom in a molecule of nearly 5800 MW.

The power and popularity of reversed-phase HPLC is in its high resolving power!

Insulin variants

The separation of insulin variants, some of which differ by a single amino acid, demonstrates the ability of reversed-phase HPLC to separate very similar polypeptides.

Conditions

Column: Vydac 214TP54 (C4, 5 µm, 4.6 mm i.d. x 250 mm).
Eluent: 27-30% ACN with 0.1% TFA over 25 min.
Separation conditions

Polypeptides are eluted from reversed-phase columns using aqueous mobile phases containing an ion pairing agent and an organic modifier. The most commonly used ion pairing agents include:

**ACIDS**
- Acetic
- Formic
- Perchloric
- Phosphoric
- Trifluoroacetic (TFA)
- Heptafluorobutyric (HFBA)

**BASES**
- Triethylamine (TEA)
- Tetramethylammonium (TMA)
- Tetrabutylammonium (TBA)
- Triethylammonium acetate (TEAA)

Trifluoroacetic acid is by far the most commonly used ion pairing agent because of its excellent separation capabilities, low UV absorbance, and high volatility for easy removal in peptide isolation. TFA is also very effective in solubilizing hydrophobic polypeptides.

HFBA is effective in the chromatography of basic proteins, and TEAA has shown unique selectivity for a variety of peptides.

The most commonly used organic modifiers are:
- acetonitrile (ACN)
- isopropanol (IPA)
- ethanol (EtOH)

Acetonitrile offers low viscosity, excellent UV absorption characteristics, and high volatility for easy removal. Isopropanol is used either alone or in combination with acetonitrile (1:2 to 2:1) to elute large or hydrophobic proteins. Ethanol is most often used in large-scale process applications.

Gradients in the organic modifier concentration are normally used to obtain sharp peaks and optimum selectivity. Initial organic concentrations range from 1-2% for hydrophilic peptides to 20-40% for large or hydrophobic proteins. While most peptides will elute in 70% organic modifier or less, large or hydrophobic proteins may require as high as 85% organic to elute.

The gradient slope (percent change in organic modifier per unit volume or time) is normally around 1-2% per minute, however very shallow gradients—as low as 0.05-0.2% per minute—are used to separate complex mixtures or very similar peptides.

Polypeptides are usually detected by UV absorption at wavelengths from 210 to 220 nm, where the peptide bond absorbs. Higher wavelengths such as 280 nm are sometimes used to monitor proteins with aromatic residues such as tryptophan.

References

Available FREE from Vydac:
The 2nd edition of The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC

The Handbook answers many questions commonly asked about the reversed-phase chromatography of proteins and peptides.
Keys to Well-Characterized Biotechnology Products

Recent changes in FDA rules simplify the regulatory treatment of “well-characterized biotechnology products”*. HPLC often plays a crucial role in characterizing biotechnology products as to purity, potency, and identity.

Keys to defining a “well-characterized biotechnology product” by HPLC are:

✓ **Selectivity**: To monitor biotechnology products for purity and identity: important impurities must be separated from the major product, and digest fragments with minor changes must be resolved from normal fragments. Resolving minor impurities such as deamidation products and oxidized methionine variants place the ultimate demands on HPLC column selectivity.

✓ **Stability**: Column selectivity must be constant over hundreds of injections to ensure robust and reliable assays. Only columns which are physically and chemically stable and which maintain selectivity over time are practical for monitoring purity, potency, or identity of “well-characterized biotechnology products”.

✓ **Reproducibility**: Column selectivity and sample resolution should remain the same when used columns are replaced with columns from a new batch. HPLC columns used to monitor purity, potency, or identity of “well-characterized biotechnology products” must be reproducible from batch to batch.

* The term “specified product” is favored for future use to eliminate the subjective aspect of “well characterized.”

**Selectivity**

Selectivity is the primary measure of column performance; a column must separate key impurities to be useful for an assay or purification.

Selectivity and resolution of peptides and proteins are very sensitive to column characteristics such as the silica matrix and bonding chemistry. Vydac reversed-phase HPLC columns for bioseparations are made with the 300 Å pore diameter silica that pioneered the separation of polypeptides nearly two decades ago and continues to be the standard against which all polypeptide separations are compared. Vydac’s unique polymeric bonding chemistry offers unrivaled selectivity for separating polypeptides.

Vydac reversed-phase columns have been used to separate all types of polypeptides including nearly identical insulins (see Ref. 3 on p. 11), interleukin muteins (see Ref. 4 on p. 11) and deamidated peptides (see figure below).

Vydac selectivity is illustrated in the figure below by the separation of two very similar digest peptide fragments where asparagine is converted to isoaspartate by deamidation.

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**Separation of tryptic digest fragments from normal and deamidated bovine somatotropin**

**Conditions:**
Column: Vydac 218TP54 (C18, 5 µm, 4.6 mm i.d. x 250 mm). 
Eluent: 0-15% ACN over 20 min., 15-2% ACN over 12 min., 21-48% ACN over 27 min., 48-75% ACN over 4 min.; in 0.1% aqueous TFA at 2.0 mL/min.

For details on how HPLC column selectivity can be optimized to best characterize your biotechnology product, request Vydac Technical Report:

**Column Selectivity: The Primary Key to Achieving High-Performance HPLC Separations of Proteins and Peptides**

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Stability

Column stability is the second measure of column performance. Stable columns result in constant column selectivity and sample resolution over hundreds of sample injections, thus ensuring robust and reliable assays for monitoring purity, potency, or identity of biotechnology products.

HPLC column stability in polypeptide separations requires that columns retain their selectivity even under the somewhat harsh conditions of peptide analysis – pH 2 or less with trifluoroacetic acid. Vydac reversed-phase HPLC columns have long been known for unusually long column lifetimes under these demanding conditions, often exceeding a thousand sample injections per column. The extended lifetime of Vydac columns is attributed to the exceptionally stable silica matrix and to unique, polymeric bonded phases.

To show how column stability results in reliable assays for proteins and peptides, Vydac tested a 214TP54 (C4, 5 µm, 4.6 mm i.d. x 250 mm) column under extreme conditions of pH and temperature. Over 1000 gradient runs produced no change in column selectivity; resolution between all of the peptides and proteins tested remained the same – a remarkable indication of long-term column stability (see figures at right).

Column stability test procedure:
To provide an extreme test of column stability, over 1000 repeat gradients were run on a Vydac 214TP54 (C4, 5 µm, 4.6 mm i.d. x 250 mm) column under extreme conditions of pH and temperature. Gradients were run from 0-100% B over 80 minutes at 1.0 mL/min at a temperature of 60°C. The starting eluent (A) contained 0.5% TFA (pH 1.5) in water. The final eluent (B) contained 0.45% TFA in acetonitrile. The unusually high concentration of TFA (0.5%) and unusually high temperature (60°C) ensure an extreme test of column stability. A test mixture consisting of six peptides (oxytocin, bradykinin, angiotensin II, eledoisin-related peptide, neurotensin, and angiotensin I) and three proteins (ribonuclease, insulin, and lysozyme) were run every 50-100 gradients under the test conditions. Results: The chromatographic separation of the peptides and proteins is nearly identical after 1037 gradient runs to that obtained during the initial separation. The column did not degrade, and selectivity and resolution remained unchanged over 1037 gradient runs under the extreme conditions of pH and temperature used.
Keys to Well Characterized Biotechnology Products

Reproducibility

Batch-to-batch column reproducibility is the third measure of column performance. Reproducible HPLC columns are essential in the development of robust assays of biotechnology products.

Column selectivity and sample resolution should remain the same when used columns are replaced with columns from a new batch. Columns which retain selectivity and separation characteristics from batch to batch reduce hassle and ensure reliable assays over the lifetime of the biotechnology product.

Batch-to-batch column reproducibility depends on absolute control over the manufacture of the silica matrix and the bonding chemistry. VyDAC manufactures HPLC silica in its own facility and maintains absolute control over the production process through in-process quality control and Statistical Process Control measurements. These ensure highly reproducible, high quality silica particles.

VyDAC uses very sensitive tests to monitor and control carbon loading on reversed-phase materials; tests which surpass conventional methods of measuring carbon loading such as carbon microcombustion. One such test used to monitor the carbon load of polymerically bonded C\textsubscript{18} is based on a test developed by the U.S. NIST (National Institute for Standards and Technology), involving the isocratic separation of three polyaromatic hydrocarbons and the calculation of a resolution factor, $\alpha$, between two of these (see figure). VyDAC is able to precisely control the carbon load of polymerically bonded C\textsubscript{18} through this highly sensitive test.

VyDAC Application Note #9703, “Developing a Robust Reversed-Phase Method for Analysis of Polypeptides”, discusses the importance of reversed-phase HPLC in research, production, and quality control of polypeptide therapeutics as well as its pivotal role in developing robust assays for well-characterized biotechnology-derived therapeutics. The publication emphasizes the need for column stability and reproducibility and describes methods for optimizing analyses by selection of ion-pairing agents, buffers, and eluant pH.

VyDAC Application Note #9803, “Optimizing Peptide Purifications”, emphasizes the importance of trial separations varying adsorbent type, pH, and buffer ions in optimizing HPLC methods for routine purification of specific peptides.
Highly sensitive tests enable precise control over reversed-phase carbon load and result in unparalleled batch-to-batch column reproducibility for Vydac columns used in the analysis and purification of proteins and peptides. Batch-to-batch column reproducibility is evidenced by the separation of peptide fragments from the tryptic digest of β-lactoglobulin A. This digest was chromatographed on Vydac 218TP54 columns (C₁₈, 5 µm, 4.6 mm i.d. x 250 mm) from several lots of materials produced over a period of more than a year (see figure). Digest maps performed on these columns were nearly identical—evidence of Vydac's exceptional batch-to-batch column reproducibility.

**Column reproducibility test procedure:**
The separation of peptide fragments from an enzymatic digest is a very effective test of batch-to-batch reproducibility of reverse phase columns. A tryptic digest of β-lactoglobulin A was chromatographed on Vydac 218TP54 (C₁₈, 5 µm, 4.6 mm i.d. x 250 mm) columns from the following five lots: lot 930816-38-4 produced in January 1994; lot 931115-16-1 produced in March 1994; lot 940124-14-1 produced in September 1994; lot 940415-20-1 produced in March 1995; and lot 950405-12-1 produced in August 1995. Conditions were: 0-30% acetonitrile with 0.1% TFA over 60 minutes at 1.0 mL/min. and 30°C. **Conclusion:** Vydac 218TP54 columns show remarkable lot-to-lot reproducibility.