The effect of pH on peptide separations (Figure 2)

Peptide separations are often sensitive to eluent pH, as illustrated by the elution of five common peptides. All the peptides elute earlier at pH 4.4 than at pH 2.0, and relative retention changes. Bradykinin and oxytocin are well separated at pH 2.0, but co-elute at pH 4.4. Peptide retention at pH 6.5 is greater than at pH 4.4. However elution order is drastically different. Angiotensin II, which elutes third at pH 2.0 - 4.4, now elutes first. Neurotensin elutes before oxytocin, and bradykinin and neurotensin co-elute at pH 6.5.

Conclusion

pH can have a dramatic effect on peptide elution and can be a useful tool in optimizing separations. Evaluating a peptide assay with three different buffers and three different pH's provides a strong foundation for a robust peptide assay.

Optimizing selectivity: other factors

Temperature

Reproducible retention times require temperature control. Temperature may affect the polypeptide structure, the reversed-phase surface or both.

- Effect of temperature on polypeptide structure: Temperature may denature or change the tertiary structure of polypeptides. As a consequence, interaction with the reversed-phase surface will change and resolution may be improved or degraded.
- Effect of temperature on the reversed-phase surface: C18 polymeric reversed-phase layers undergo a transition from a wax-like state to a liquid-like state in the range of 30° - 60° C. This phase "melting" affects the interaction of polypeptides with the reversed-phase surface and may change resolution.
- Recommendation: Column temperature should be controlled in a reversed-phase polypeptide assay. Although in most cases the effect of temperature on a polypeptide separation may be small, critical separations may be affected by small changes in temperature. Controlling temperature is the best way to ensure a robust assay. Temperature is usually controlled at a few (~5) degrees above ambient, although higher temperatures may occasionally be useful and should be investigated.

System hardware

Two aspects of a system most affect the robustness of an assay:

- The volume from the gradient mixer to the top of the column. This "dead volume" determines the time that the column is eluted isocratically at the initial solvent concentration. HPLC systems with different dead volumes will give different results with the same sample, column and mobile phase. The effect of dead volume can be determined prior to validation by putting hold steps of different duration at the initial solvent concentration before the start of the gradient and looking for changes in resolution. In general, low-pressure-mixing systems have larger "dead volume" than high-pressure-mixing systems. Small bore columns (< 4 mm ID) are particularly sensitive to dead volume.
- The ability of the system to generate reproducible gradients at the flow rate and solvent concentrations selected. All HPLC systems do not produce identical, reproducible gradients. Gradients produced by low-pressure-mixing systems depend on electronically actuated valves, and high-pressure-mixing systems depend on check valves, all subject to wear and contamination.
- Recommendation: Test all the HPLC systems that may be used in performing the assay prior to finalizing the procedure. In the written procedure, retain the option of adjusting the initial gradient hold time to normalize system hardware.

Column stability

Robust polypeptide assays require HPLC columns to be stable over long periods of use (long column lifetime).

Column stability - meaning that selectivity and resolution are unchanged over hundreds of sample injections - ensures robust and reliable assays for monitoring purity, potency or identity of biotechnology products.

A robust peptide assay requires that columns retain their selectivity even under the somewhat harsh conditions of peptide analysis - pH 2 or below with trifluoroacetic acid or other buffers.

Column stability test procedure (Figure 3)

1037 repeat gradients were run on a Vydac 214TP54 (300Å, C4, 5 micron, 4.6 x 250 mm) column. 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 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) - ensured 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) was run every 50 - 100 gradients under the test conditions.

Column Stability

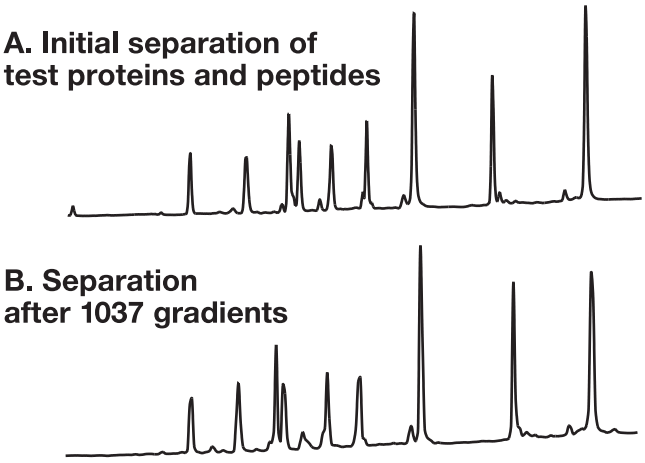

Figure 3. Study of column stability under extreme test conditions.

Results and conclusion (Figure 3)

The chromatographic separation of six peptides and three proteins was unchanged even after 1037 gradient runs. The column did not degrade, and selectivity and resolution remained unchanged under the extreme conditions of pH (1.5) and temperature (60° C) used.

Lot-to-lot reproducibility

Robust polypeptide assays require HPLC columns to be reproducible in selectivity from lot to lot over years.

Reproducible HPLC columns are important in the development of robust assays of biotechnology-derived therapeutic products.

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

Column reproducibility test procedure (Figure 4)

The separation of peptide fragments from an enzymatic digest is a very effective test of batch-to-batch reproducibility of reversed-phase columns. A tryptic digest of b-lactoglobulin A was chromatographed on five Vydac 218TP54 (C18, 5 micron, 4.6 x 250 mm) columns from the following 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.

Analytical conditions: 0 - 30% acetonitrile with 0.1% TFA over 60 minutes at 1.0 mL/min. at 30° C.

Column Reproducibility

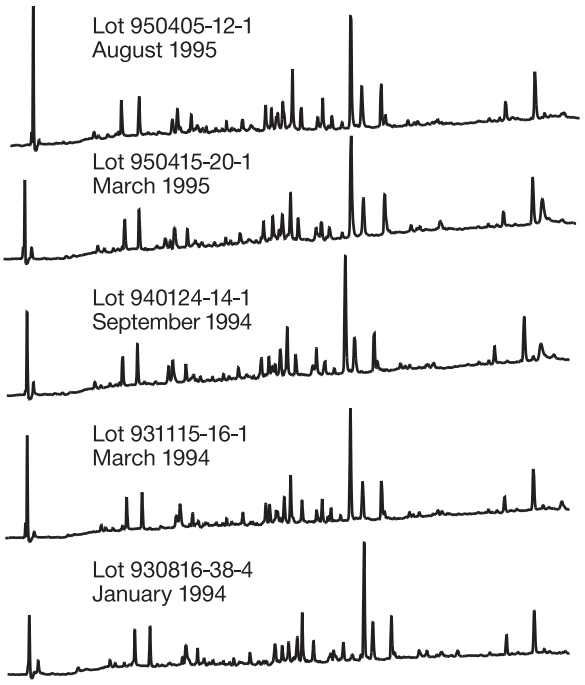

Figure 4. Tryptic digest maps of b-lactoglabulin A on 218TP54 (300•, C18) columns from five lots produced over a period of nearly two years.

Results and conclusion (Figure 4)

Tryptic digest maps from five lots of C18 reversed-phase materials produced over nearly two years were nearly identical, verifying lot-to-lot reproducibility over the time period tested.

Validation of multiple lots of packing materials ensures long-term assay robustness.

Once elution conditions have been optimized and a candidate column is chosen, columns from three different lots of material should be tested. Silica-based reversed-phase columns have three potential sources of variation:

- silica production lot
- sizing fraction
- bonding lot

The validation of a robust polypeptide assay should include the testing of material from three different lots of silica production, as well as different sizing and bonding.

Vydac lot number description

The lot number of the silica, the sizing fraction and the bonding is included in Vydac lot numbers.

This simplifies the selection of three different lots of silica for the validation of a robust peptide assay by ensuring that the three columns used do, in fact, come from three different silica manufacturing lots.

Example:

950613-5-2

year, month, & day of silica manufacture

sizing fraction

bonding lot

ORDERING INFORMATION:

Cat. No.	Description
218TP54	Column, Octadecyl (C18), 5mm, 300Å, 4.6mm ID x 250mm L
214TP54	Column, Butyl (C4), 5mm, 300Å, 4.6mm ID x 250mm

To place an order, call **The Nest Group 800-347-6378** your local Vydac distributor.

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