обращеннофазовая хроматография высокого разрешения RP HPLC

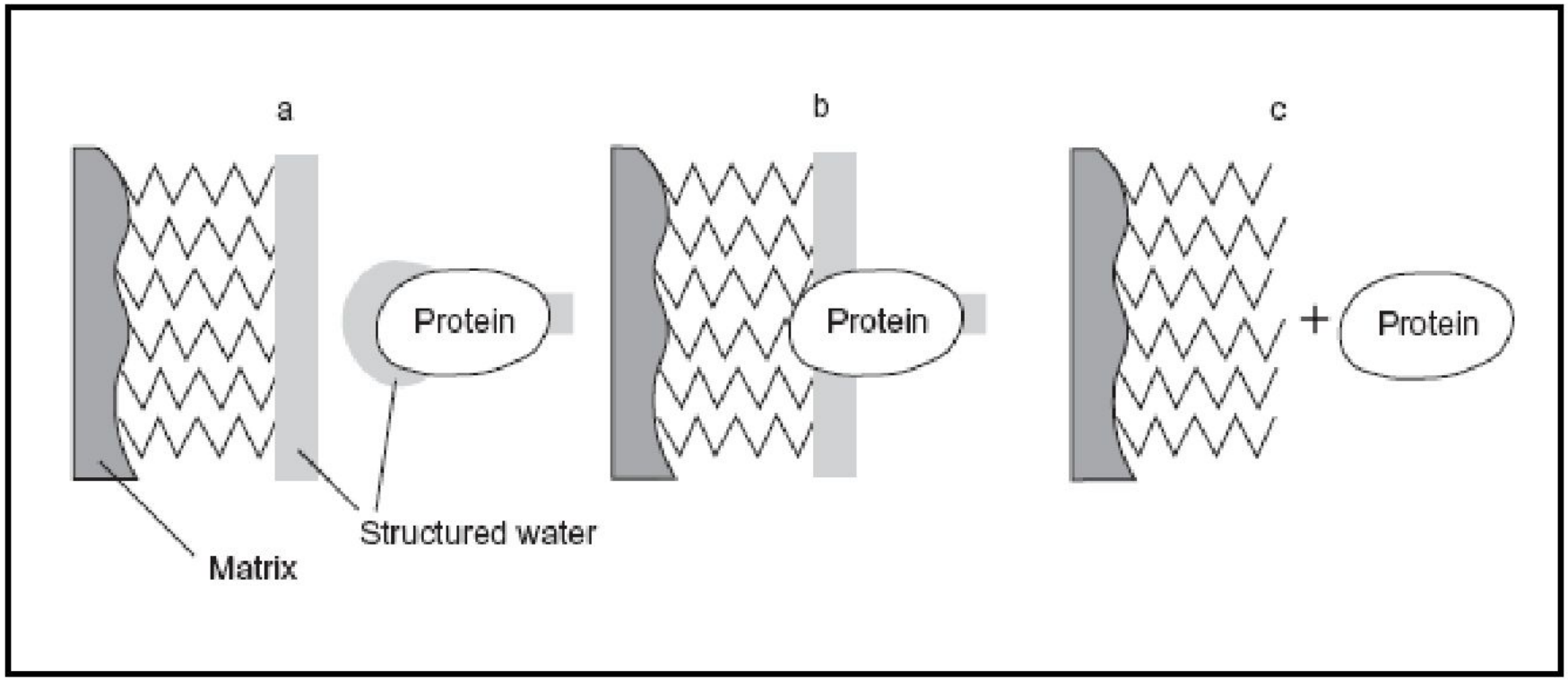
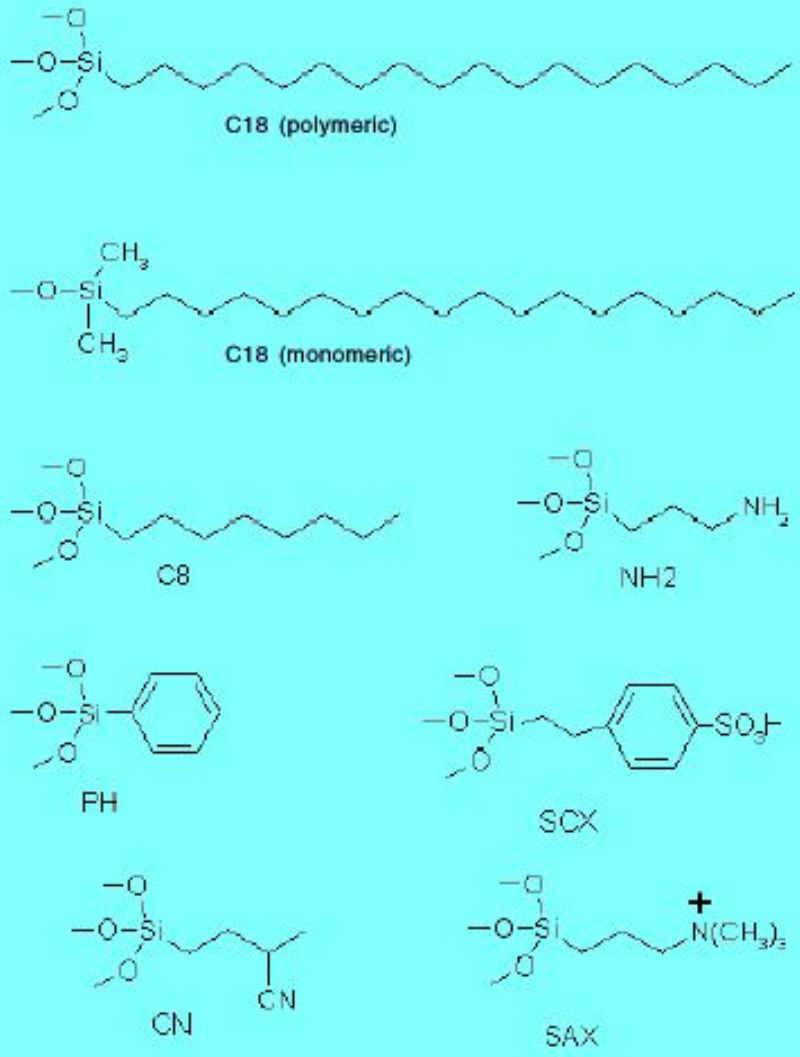
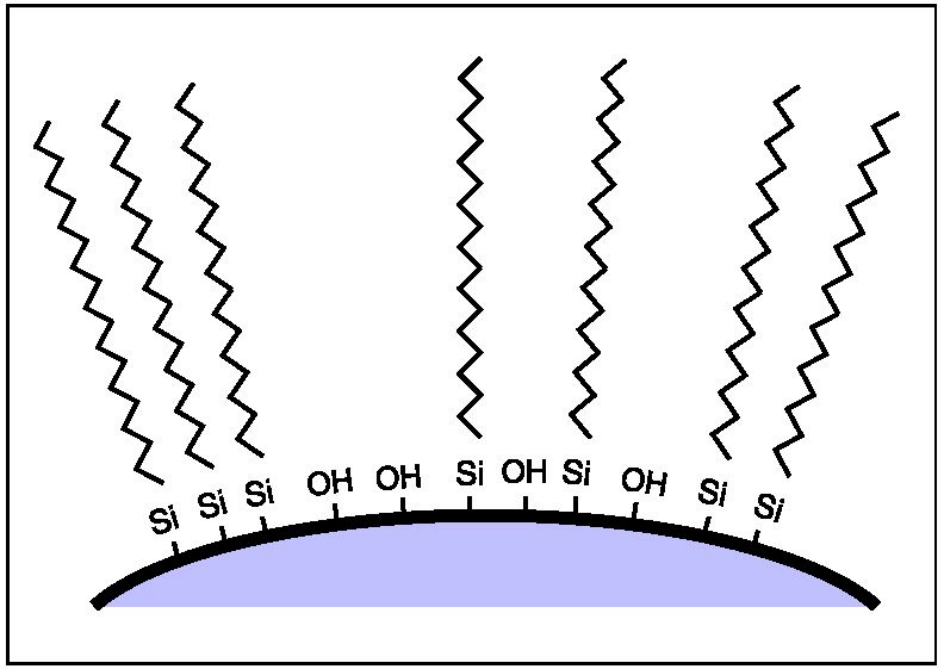


Fig. 1. Interaction of a solute with a typical reversed phase medium. Water adjacent to hydrophobic regions is postulated to be more highly ordered than the bulk water. Part of this 'structured' water is displaced when the hydrophobic regions interact leading to an increase in the overall entropy of the system.

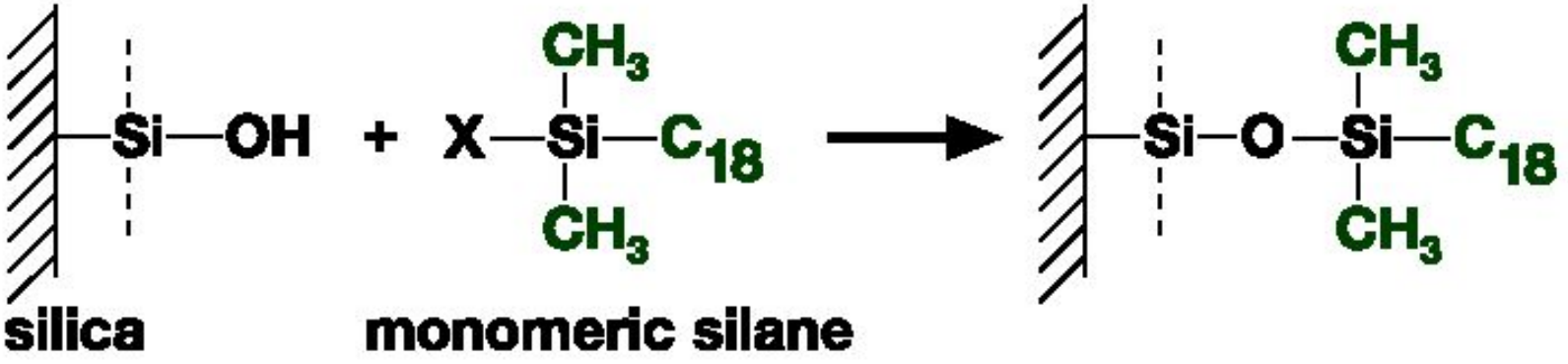
Structures of Common Silica-based Sorbents



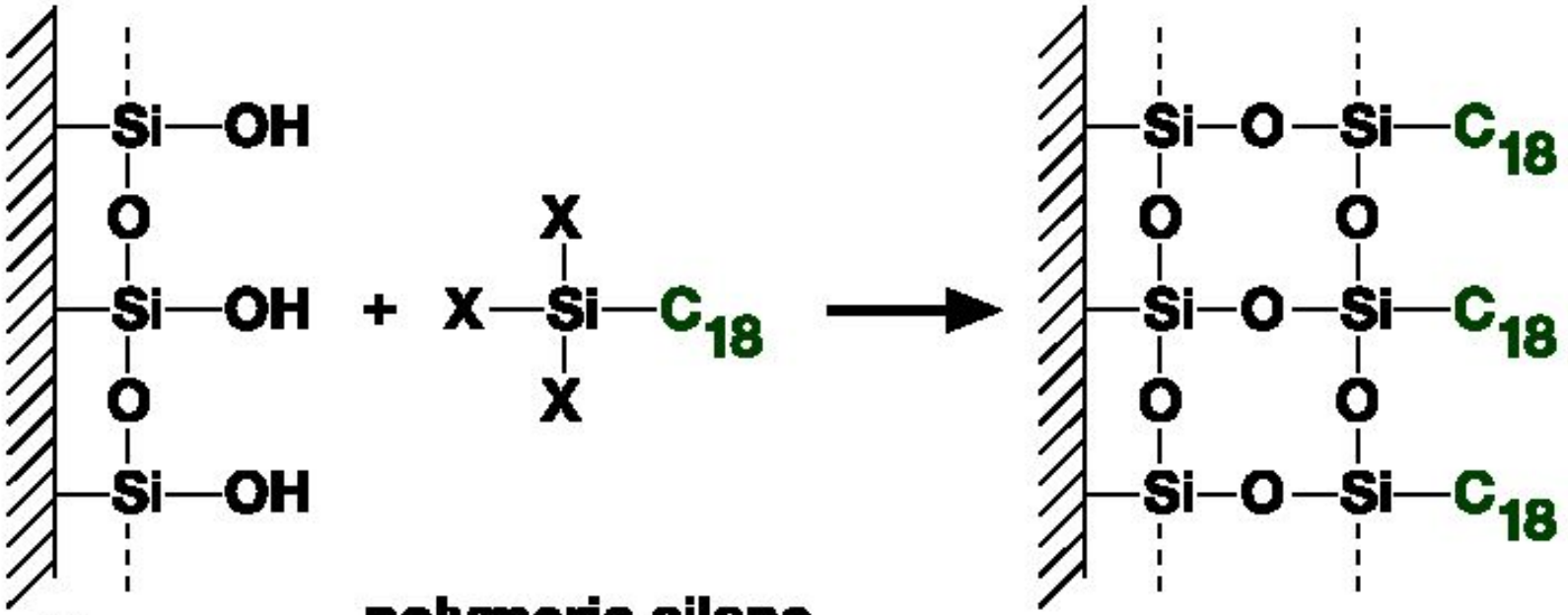
C18 Bonded Silica Containing Free Silanols



Chemistry of Monomeric and Polymeric Bonding



silica monomeric silane



silica polymeric silane

X = CH_3O, CH_3CH_2O, Cl

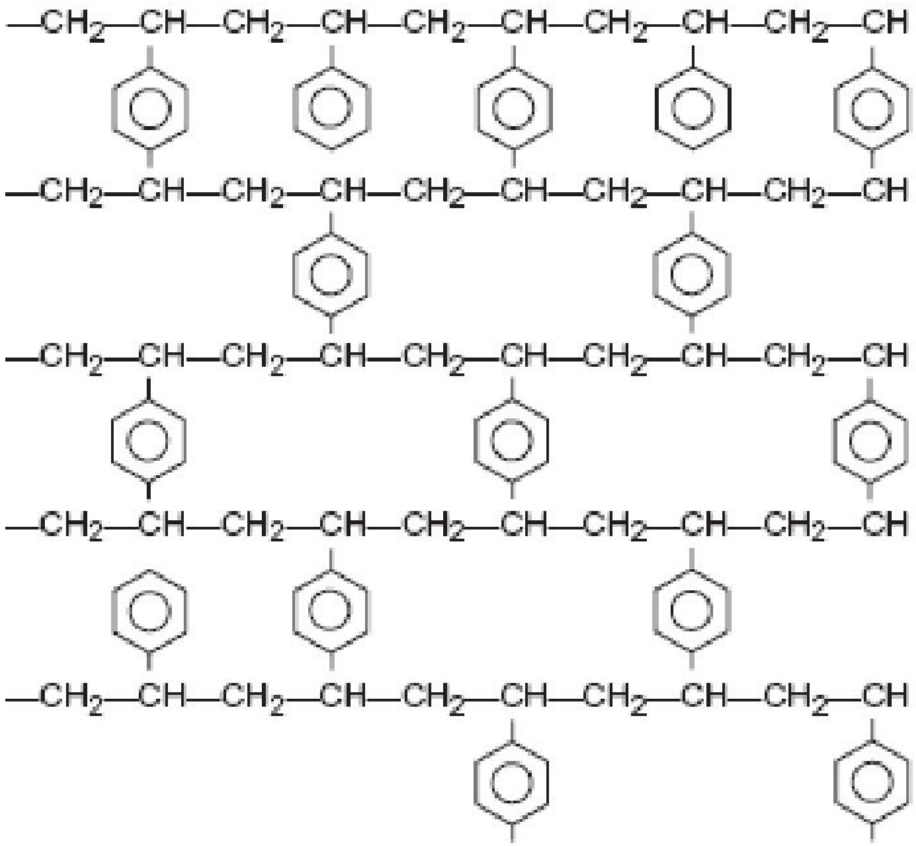
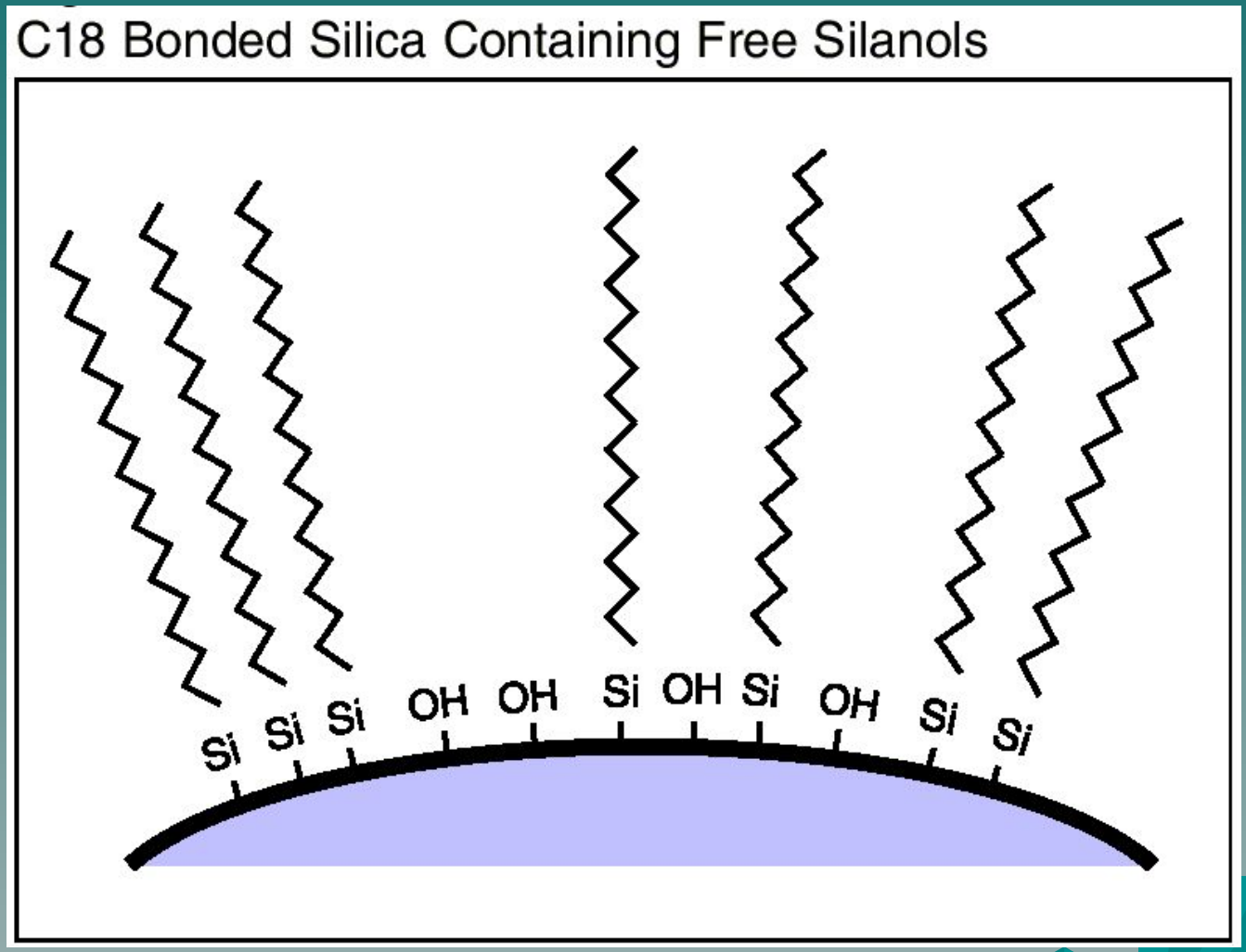
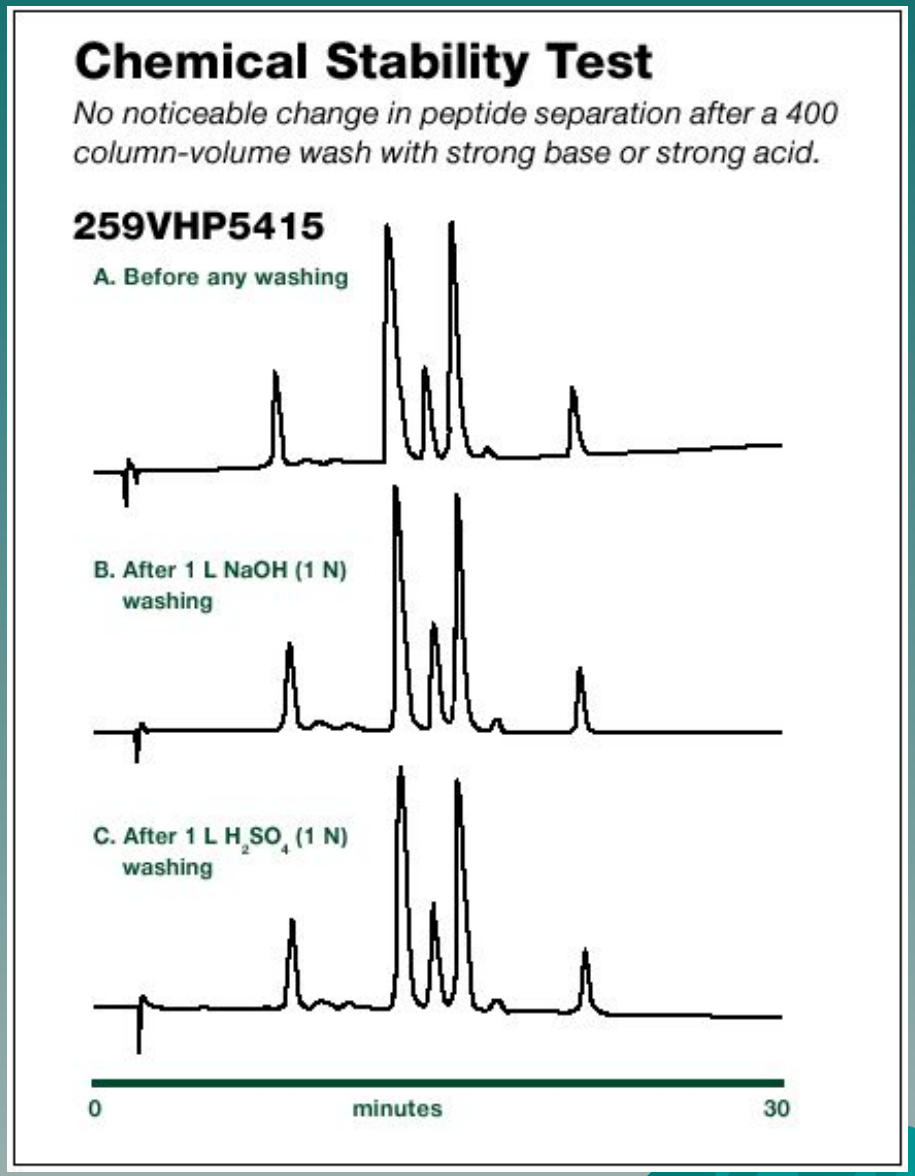


Fig. 4. Partial structure of a polystyrene-based reversed phase medium.

Схеме деструкции носителя



Анализ на химическую стабильность носителя и его качество



C18 Pyridine Peak Asymmetry

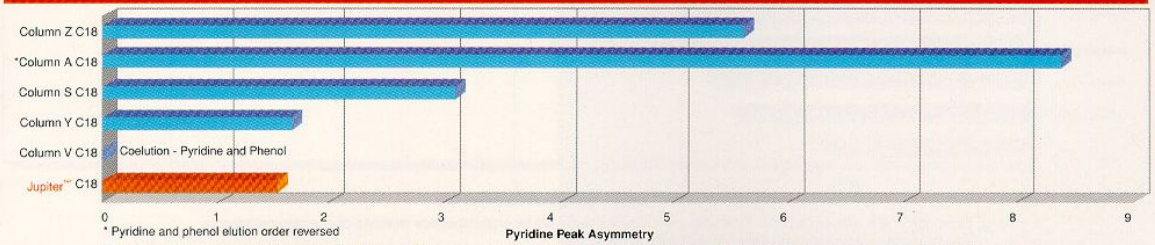
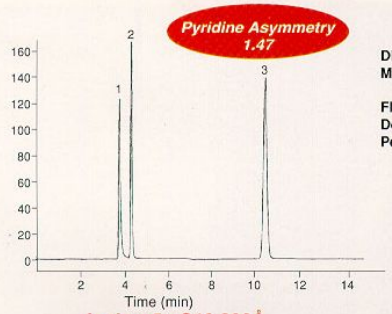


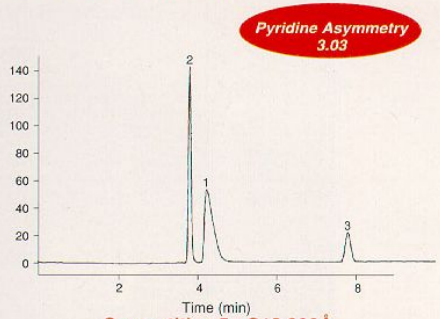
Figure 12. Comparison of 6 different 5µ C18 300Å wide pore columns designed for protein/peptide reversed-phase chromatography. Test measures degree of silanol activity on the surface of each silica. Jupiter 5µ C18 300Å material demonstrates the lowest silanol activity.



Jupiter 5µ C18 300Å

(expected elution order)

Dimensions: 250 x 4.6 mm
Mobile Phase: Water/Acetonitrile 50:50
Flow Rate: 1.0 mL/min
Detection: UV @ 254nm
Peaks: 1. Pyridine 2. Phenol 3. Toluene



Competitive 5µ C18 300Å

(elution order reversed)

C4 Pyridine Peak Asymmetry

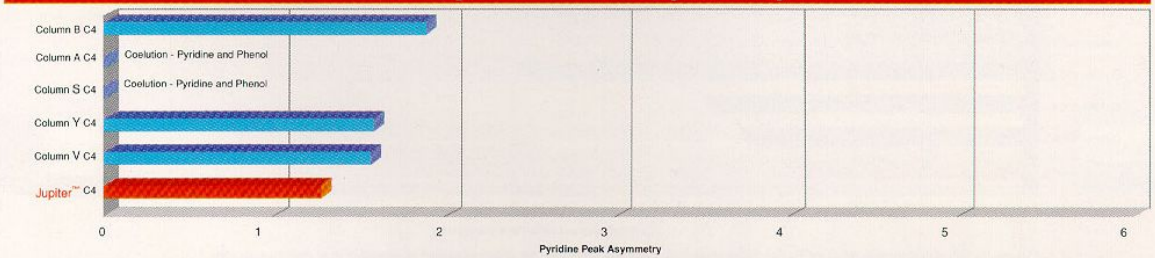
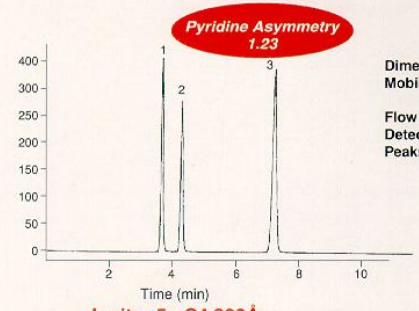
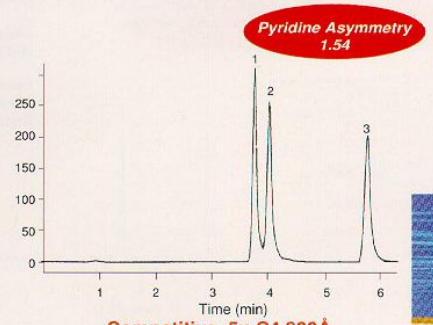


Figure 13. Comparison of 6 different 5µ C4 300Å wide pore columns designed for protein/peptide reversed-phase chromatography. Test measures degree of silanol activity on the surface of each silica. Jupiter 5µ C4 300Å material demonstrates the lowest silanol activity.



Jupiter 5µ C4 300Å

Dimensions: 250 x 4.6 mm
Mobile Phase: Water/Acetonitrile 50:50
Flow Rate: 1.0 mL/min
Detection: UV @ 254 nm
Peaks: 1. Pyridine 2. Phenol 3. Toluene



Competitive 5µ C4 300Å

Column Selection and Characteristics of Sample Molecule

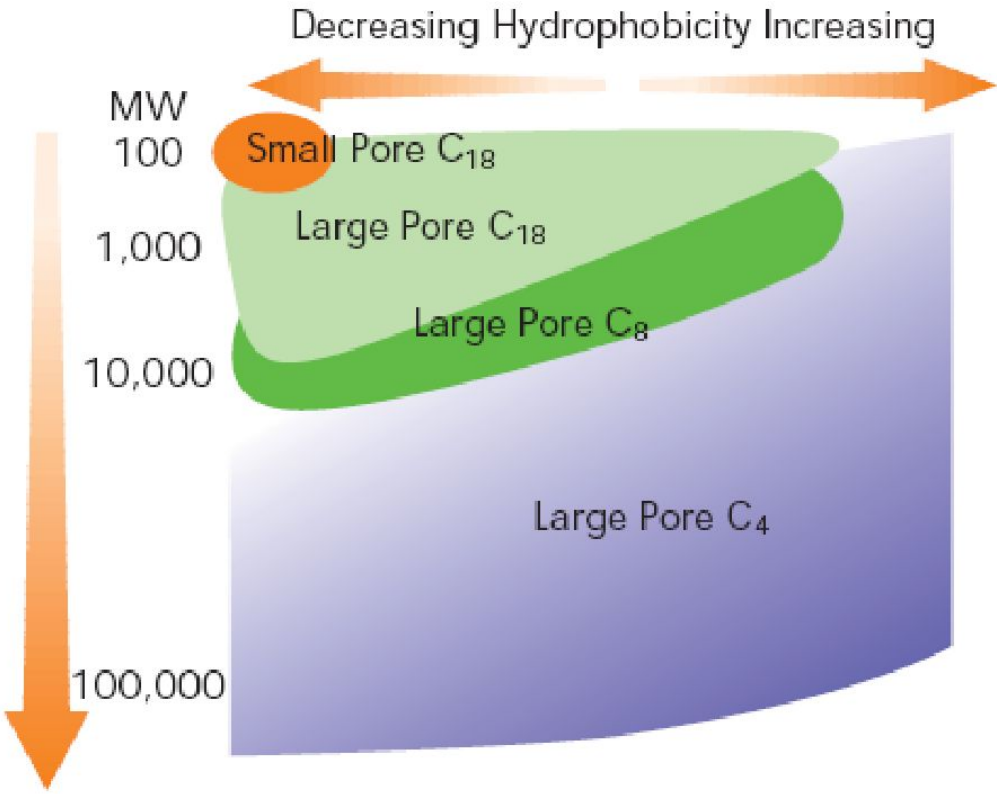


Figure 7. *This chart indicates the pore size and bonding recommended for various molecular weights and hydrophobicities.*

Peptide Separation on Different Reversed-Phase Columns

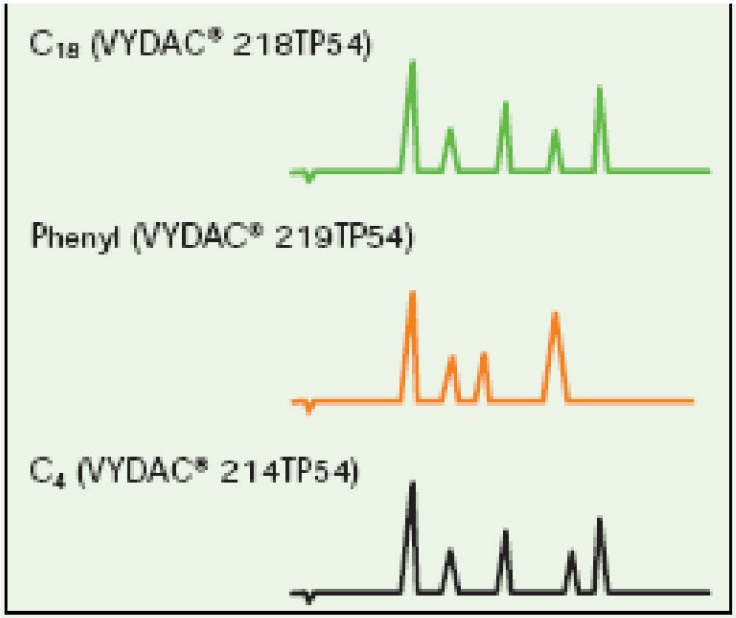


Figure 8. *Peptide separation on different reversed-phase columns* ***Columns:*** *VYDAC® 218TP54 (C_{18}); 214TP54 (C_4); 219TP54 (phenyl);* ***Eluent:*** *15–30 % ACN in 0.1% aqueous TFA over 30 minutes at 1.0 mL/min.* ***Sample:*** *1. oxytocin, 2. bradykinin, 3. angiotensin II, 4. neurotensin, 5. angiotensin I.*

Molecule Retention Versus Organic Modifier Concentration

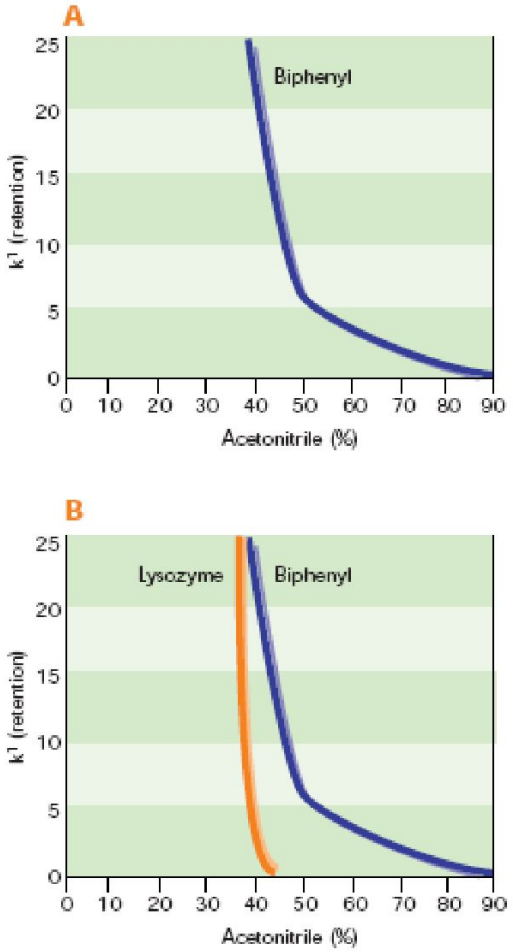


Figure 4. *A: The retention of small molecules such as biphenyl decreases gradually as the organic modifier concentration increases because they are retained by partitioning.* ***B:*** *The retention of polypeptides such as lysozyme changes suddenly and drastically as the organic modifier reaches the critical concentration needed to desorb the polypeptide, evidence of the adsorption/desorption model of polypeptide-reversed-phase surface interactions.*

Effect of Acetonitrile Concentration on Elution

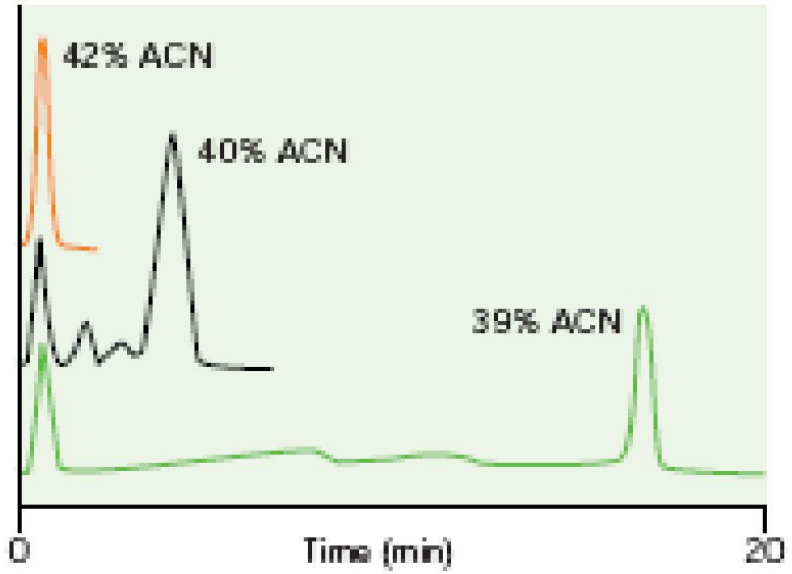
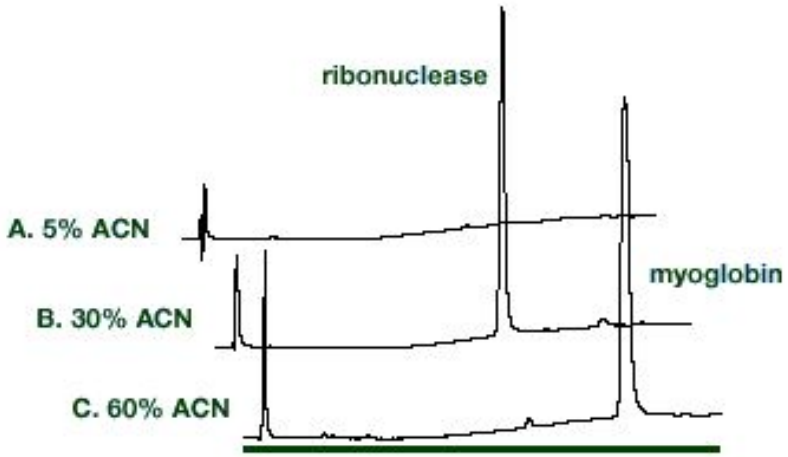


Figure 5. *At 39% ACN, the retention time of lysozyme is nearly 18 minutes. Increasing the ACN concentration to 40% reduces the retention time by more than half, to 7.6 minutes. Increasing the ACN concentration to 42% reduces the retention time of lysozyme again by more than half, to 3.1 minutes.* ***Column: VYDAC® 214TP54 Eluent:*** *ACN at 39, 40 and 42% in 0.1% aqueous TFA.*

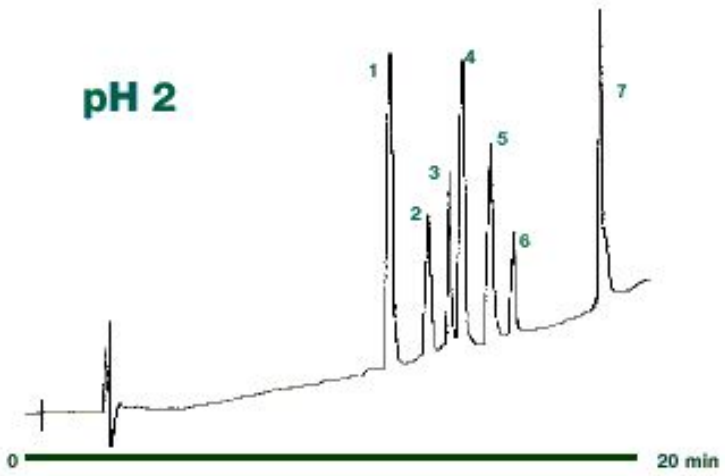
Example:

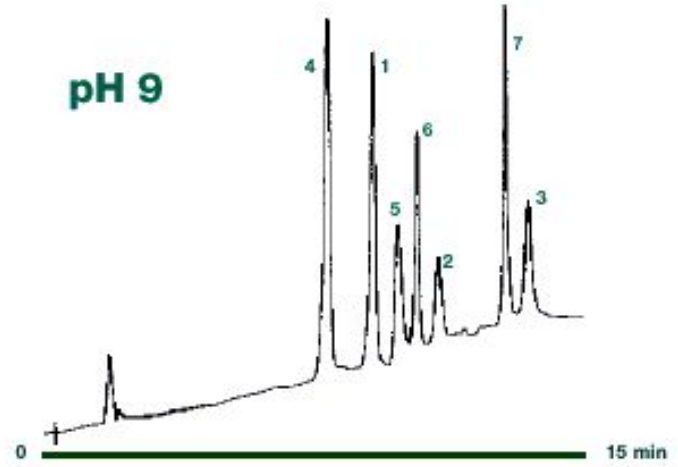
A 3-mL cartridge was conditioned with 1 mL of ACN followed by 1 mL of 5% ACN with 0.1% TFA and then loaded with 100 mg each of ribonuclease and myglobin in 30% ACN/0.1% TFA . The cartridge was washed with 1 mL of 5% ACN/0.1% TFA to remove weakly bound compounds, and then washed with 1 mL of 30% ACN/0.1% TFA to elute the ribonuclease. Myoglobin was eluted with 1 mL of 60% ACN/0.1% TFA. Protein was measured in each wash by HPLC. Analysis of the 5% ACN wash (Figure A) revealed only a small amount of ribonuclease. Most of the ribonuclease eluted in the 30% ACN wash (Figure B). The myoglobin eluted entirely in the 60% ACN wash (Figure C).



HPLC conditions:

Column: 214TP5405 (C_4, 5 µm, 4.6 mm i.d. x 50 mm). Gradient 15-70% ACN with 0.1% TFA in 10 min.





Effect of pH on Peptide Retention

The relative retention of peptides changes with pH, offering an additional dimension to reverse phase peptide separations.

Conditions

Column: Vydac 228TP104 (C_8, 10 µm, 4.6 mm i.d. x 250 mm).
Eluent: Left panel (pH 2) = 7-70% ACN / 0.5% TFA. Right panel (pH 9) = 7-70% ACN / 0.1% ammonium acetate.
Peaks: 1. oxytocin; 2. bradykinin; 3. angiotensin II; 4. eledoisin; 5. neurotensin; 6. angiotensin I; and 7. insulin.

Separation of a Tryptic Digest on Different Reversed-Phase Columns

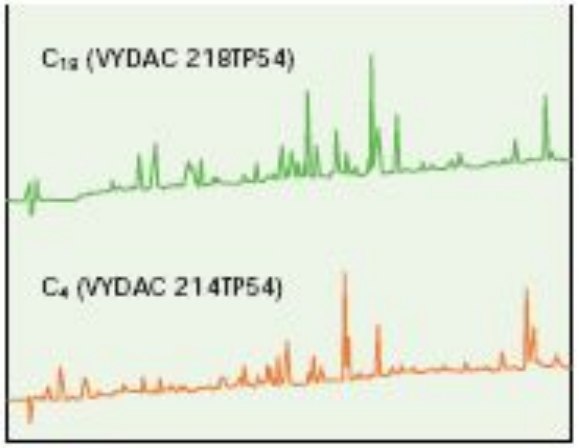
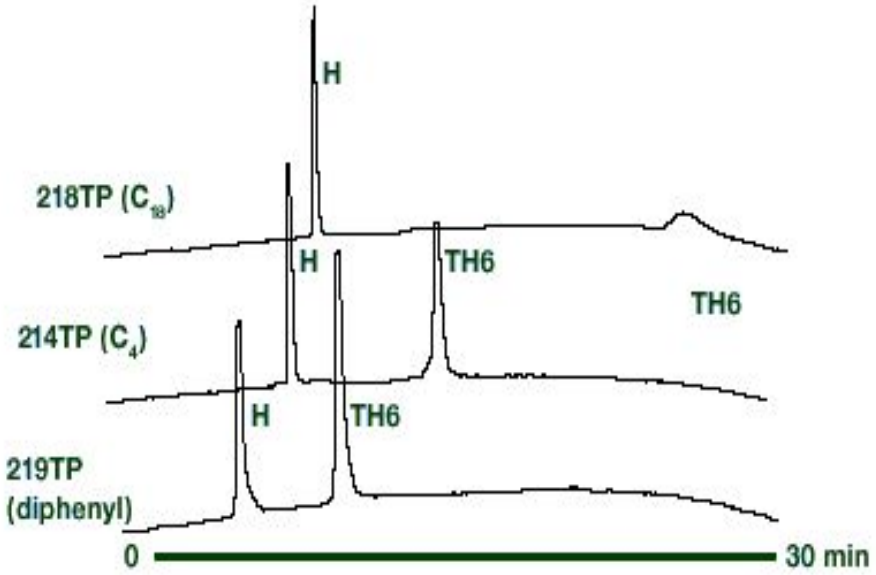


Figure 10. *Columns: VYDAC® 218TP54 (C_{18}); 214TP54 (C_4); Eluent: 0–30 % ACN in 0.1% aqueous TFA over 60 minutes at 1.0 mL/min. Sample: tryptic digest of β-lactoglobulin A.*

Comparison of C_{18}, C_4 and Diphenyl Polypeptide Reversed-Phase Columns



Conditions

Columns: Vydac 218TP (C_{18}), 214TP (C_4) and 219TP (diphenyl), 5 µm, 4.6 mm i.d. x 150 mm. Eluent: Gradient from 10-90% ACN with 0.1% TFA over 30 min. Sample: 18-residue helical peptide (H) and six-helix template assembled synthetic protein (TH_6).

From V. Steiner, M. Schar, K.O. Bornsen, and M. Mutter, *J. Chrom. 582* (1991).

Separation of Proteins on RP-HPLC Columns of Different Particle Size

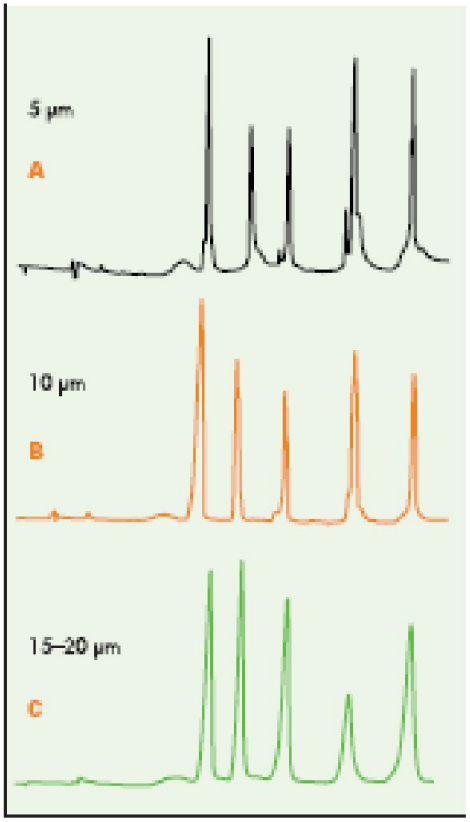


Figure 41. *Protein selectivity is the same on RP materials of different particle sizes. The only difference between materials of different particle sizes is that peak width increases and resolution decreases as particle size increases. Column materials: A. VYDAC® 214TP, 5 µm B. VYDAC® 214TP, 10 µm C. VYDAC® 214TP, 15–20 µm Mobile phase: 24–95 % ACN with 0.1% TFA over 30 min at 1.5 mL/min.*

Protein Loading Capacity of RP-HPLC Materials of Different Particle Size

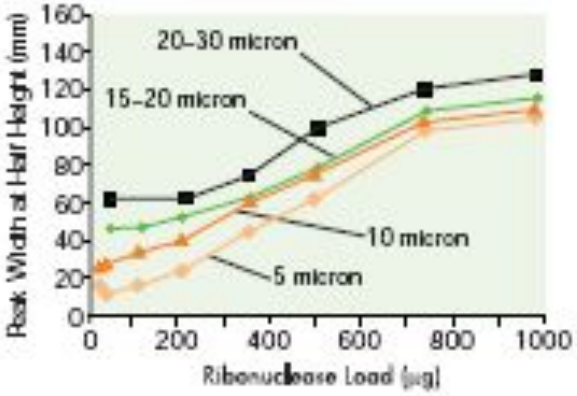


Figure 42. *Although peak widths are much narrower with small particle materials at low sample loads, there is little difference in peak widths at high loads, where the column is "overloaded". Column materials: VYDAC® 214TP, 5 µm; VYDAC® 214TP, 10 µm; VYDAC® 214TP, 15–20 µm; VYDAC® 214TP, 20–30 µm Eluent: 24–95 % ACN in 0.1% aqueous TFA over 30 min at 1.5 mL/min; Protein: ribonuclease.*

Effect of Sample Load on Protein Peak Shape and Resolution

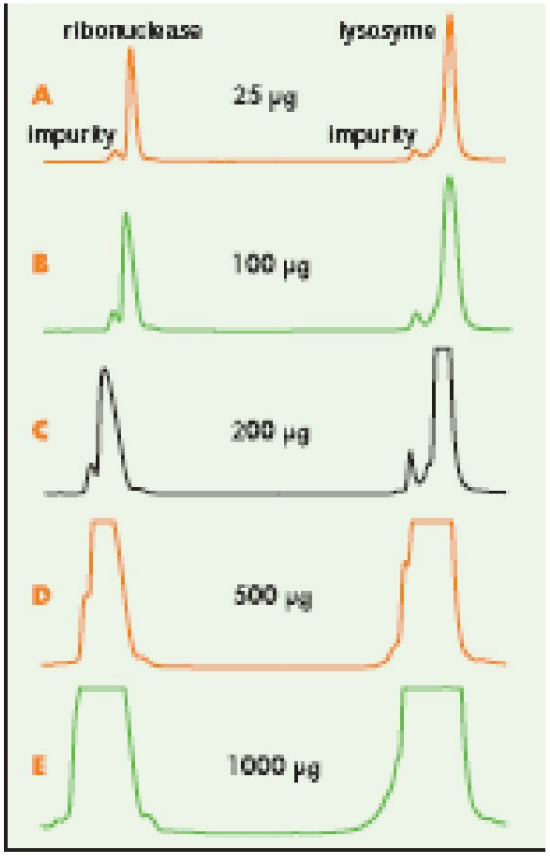


Figure 44. *A. 25 µg each protein B. 100 µg each protein C. 200 µg each protein D. 500 µg each protein E. 1000 µg each protein Column: VYDAC® 214TP54 (C_4, 5 µm, 4.6 x 250 mm) Eluent: 25–50% ACN in 0.1% TFA over 25 minutes at 1.5 mL/min. Sample: ribonuclease and lysozyme.*

Protein Loading Capacity

Protein	M.W.	Capacity (mg/g)
Lysozyme	13930	73
Ovalbumin	43500	56
BSA	67000	28

Frontal loading, 10 mg/mL protein solutions. Column: 259VHP5405, 4.6 mm i.d. x 50 mm. Conditions: Loading mobile phase = 80:20 H_2O:acetonitrile with 0.1% (v/v) TFA. Flow rate = 1 mL/min.

The Use of Low Concentrations of TFA for Peptide Separations

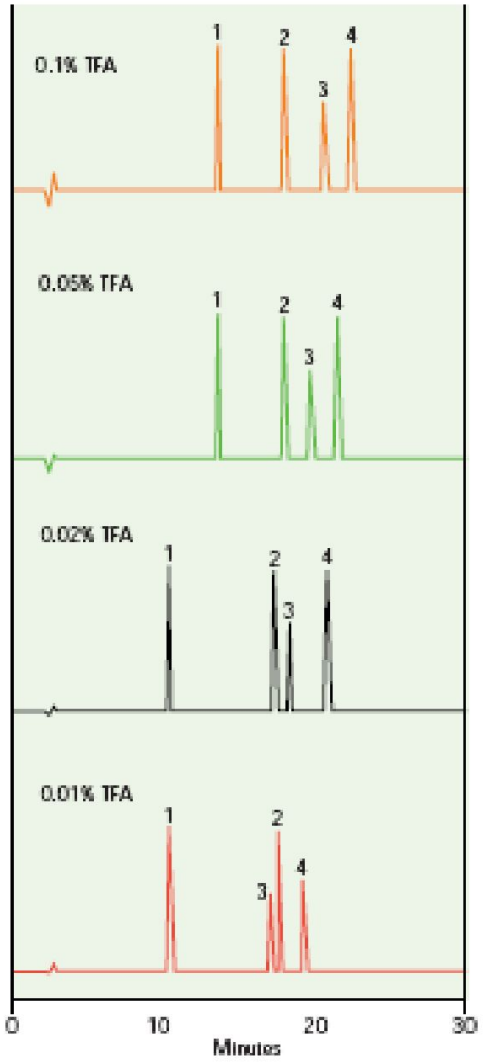


Figure 25. *Column: C_{18}, 5 µm, 4.6 x 250 mm (VYDAC® 218MS54). Flow rate: 1.5 mL/min. Eluent: Gradient from 5–19% ACN in aqueous 0.1% TFA. Sample: 1. neurotensin (1–8 frag) 2. oxytocin 3. angiotensin II 4. neurotensin.*

Comparison of TFA and Alternate Ion-Pairing Agents/Buffers for the Separation of Peptides

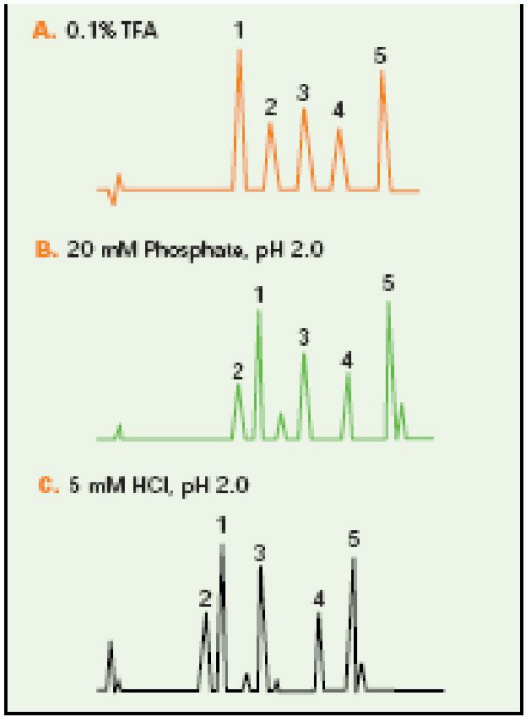


Figure 20. *Elution of five peptides using TFA (A), Phosphate (B) or HCl (C) as the buffer/ion-pairing agent. Column: VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm). Eluent: 15–30% ACN in 30 min at 1.0 mL/min; plus A. 0.1% TFA B. 20 mM phosphate, pH 2.0 C. 5 mM HCl, pH 2.0 Peptides: 1. oxytocin 2. bradykinin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

The Effect of TFA Concentration on Peptide Selectivity

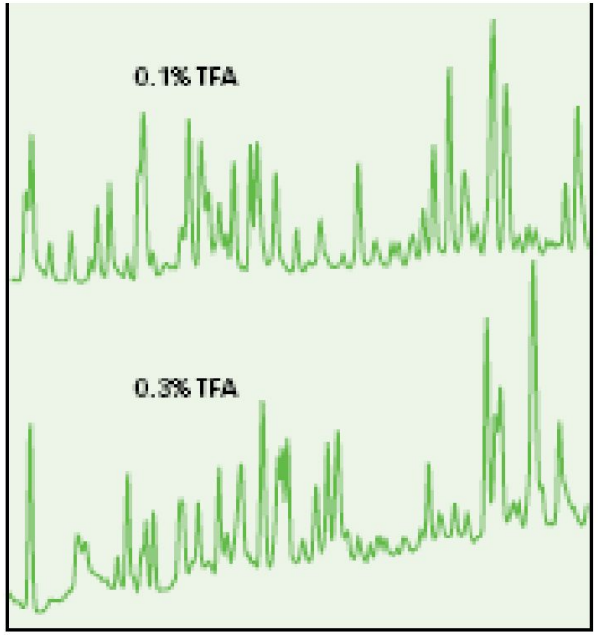


Figure 19. *Significant differences in the peptide separation pattern due to differences in TFA concentration are evident. Column: C_{18} (VYDAC® 218TP54). Flow rate: 1 mL/min. Eluent: Gradient from 0–50% ACN in aqueous TFA, concentration as indicated. Sample: Tryptic digest of apotransferrin. Note: Only part of the chromatogram is shown.*

The Effect of pH on Peptide Separations

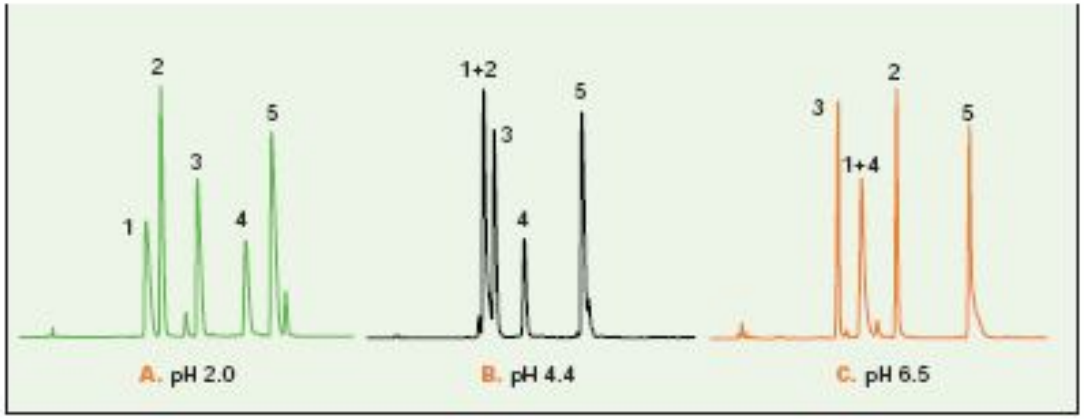


Figure 21. *Elution of five peptides at pH 2.0, 4.4 and 6.5 with phosphate as the buffer. **Column:** VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm). **Eluent:** 15–30% ACN in 30 min at 1.0 mL/min; plus A. 20 mM phosphate, pH 2.0 B. 20 mM phosphate, pH 4.4 C. 20 mM phosphate, pH 6.5 **Peptides:** 1. bradykinin 2. oxytocin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

Separation of Peptides on Synthetic Polymer (Polystyrene-Divinylbenzene) Column at Low and High pH

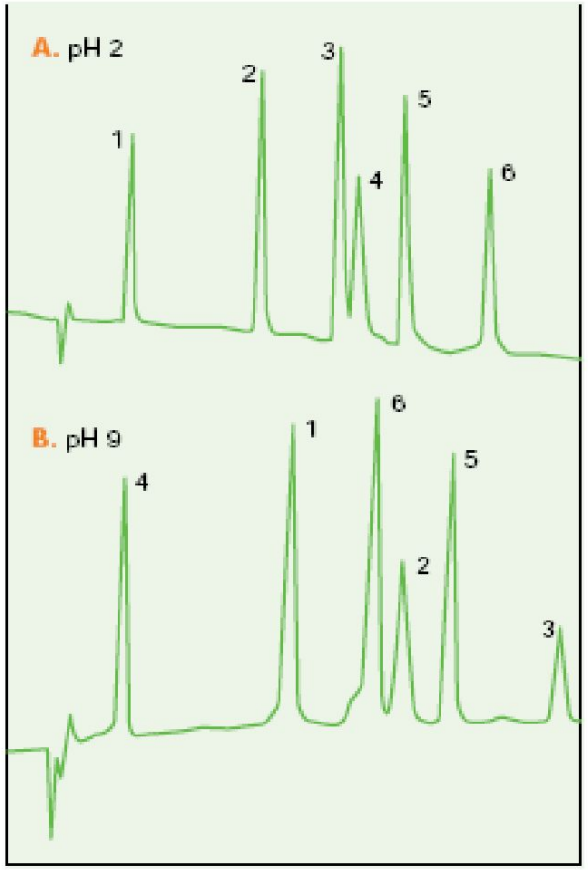


Figure 22. ***Column:** VYDAC® 259VHP5415 (PS-DVB, 5 µm, 4.6 x 150 mm) **Eluent:** 15–30% ACN over 15 min. with A. 0.1% TFA, pH 2. B. 15 mM NaOH, pH 9. **Flowrate:** 1.0 mL/min. **Peptides:** 1. oxytocin. 2. bradykinin. 3. neurotensin. 4. neurotensin 1-8. 5. angiotensin III. 6. val-4 angiotensin III.*

Sample: Angiotensin II (0.25 mg/ml), Angiotensin III (0.25 mg/ml)
Column: RESOURCE RPC 3 ml (i.d. 6.4 mm, length 100 mm).
Eluent A: (A) 0.1% TFA in water, pH 2
(B) NaOH 10 mM in water, pH 12
Eluent B: A) 0.1% TFA, 60% acetonitrile in water
(B) NaOH 10 mM, 60% acetonitrile in water
Gradient: 10-65% B in 10 min
Flow: 2 ml/min

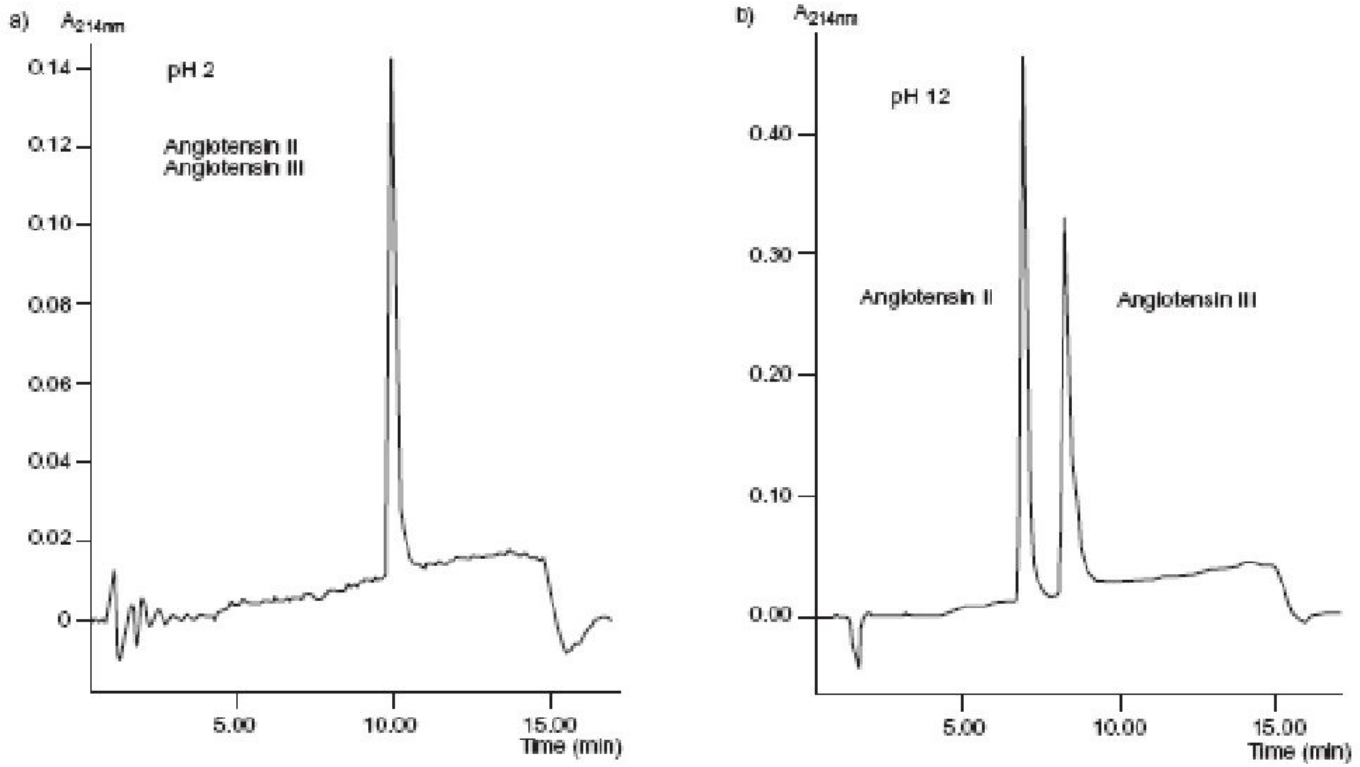


Fig. 30. Separation of angiotensin II and angiotensin III at a) pH 2 and b) pH 12. The selectivity is changed significantly by changing the pH. (Work by Amersham Pharmacia Biotech AB, Uppsala, Sweden.)

The Effect of Temperature on the Separation of Peptide Fragments

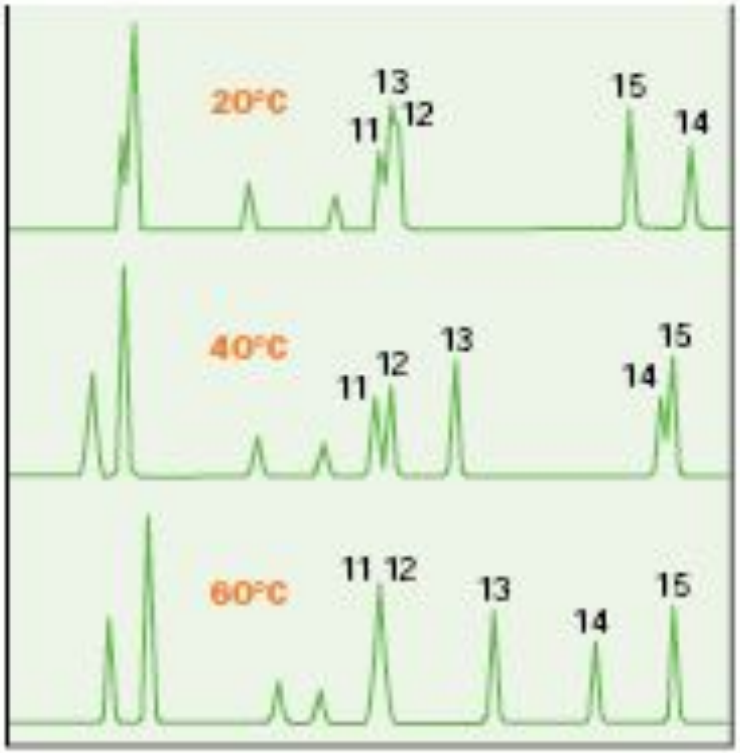


Figure 23. *Column: C_{18}, 4.6 x 150 mm. Flow rate: 1 mL/min. Eluent: Gradient from 0–60% ACN in aqueous .1% TFA in 60 min. Temperature: As indicated. Sample: Tryptic digest of human growth hormone. Data from Reference 39.*

Table 6. Solvents used in reversed phase chromatography.

Solvent	Boiling point (°C)	UV cut-off* (nm)	Viscosity (cP at 20 °C)	Comments
Acetonitrile	82	190	0.36	More powerful denaturant than alcohols. Toxic.
Ethanol	78	210	1.20	
Methanol	65	205	0.60	
1-propanol (n-propanol)	98	210	2.26	Viscous
2-propanol (iso-propanol)	82	210	2.30	Viscous
Water	100	<190	1.00	

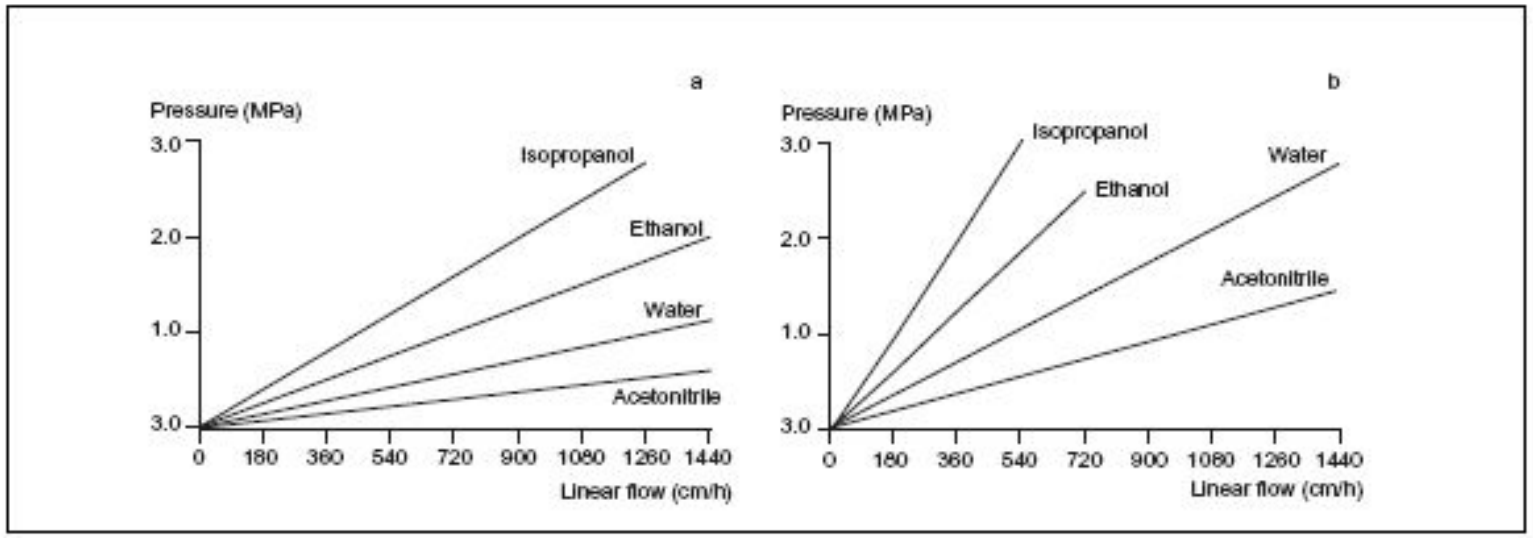


Fig. 25a. Pressure:flow curves for (a) RESOURCE RPC, 1 ml and (b) RESOURCE RPC, 3 ml with various organic solvents and water. (Work by Amersham Pharmacia Biotech AB, Lillestrøm, Norway.)

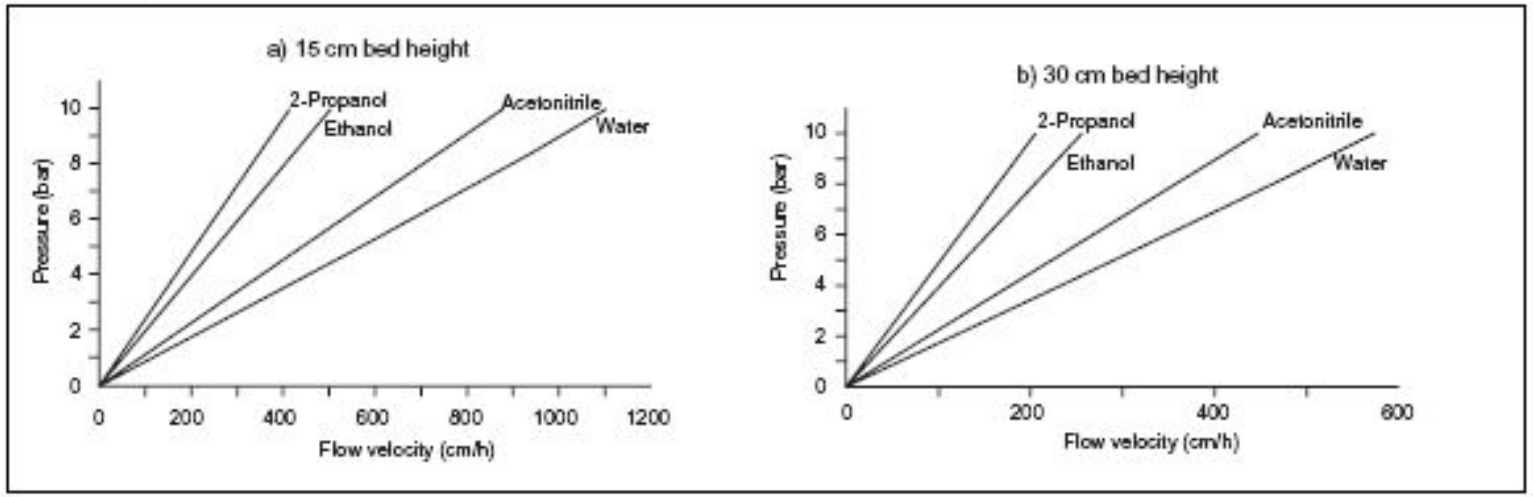
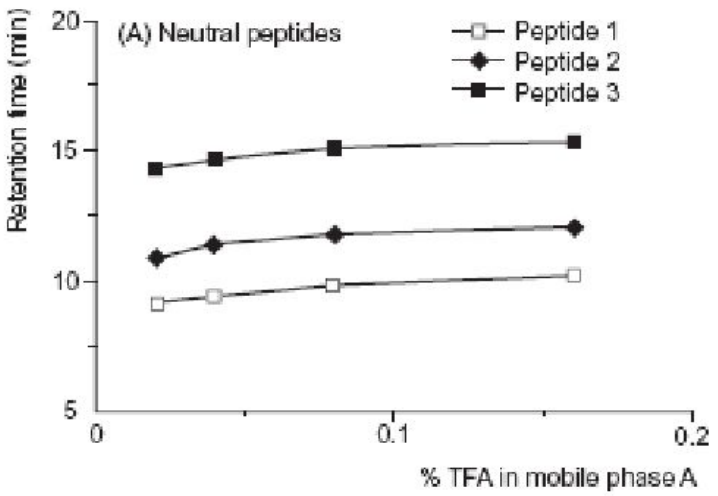


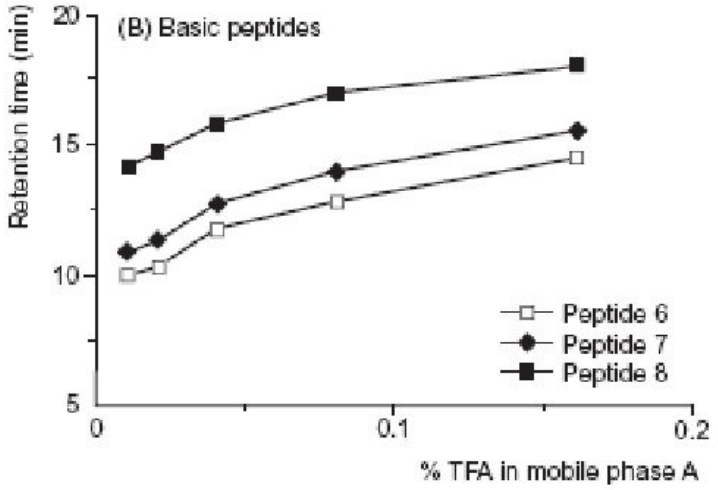
Fig. 25b. Pressure/flow characteristics of SOURCE 30RPC in various organic solvents and water at room temperature. The pressure/flow velocity data were determined in a FineLINE column with a) 15 cm and b) 30 cm bed height.

Table 7. Examples of mobile phases for use at different pH.

Buffer	Approximate pH	Comments
Hydrochloric acid	2 - 3	
Phosphoric acid	2 - 3	Non-volatile.
Trifluoroacetic acid (TFA)	2 - 3	
Triethylammonium phosphate (TEAP)	6	Non-volatile.
Ammonium acetate	6 - 7	
Sodium hydroxide	12	Non-volatile. Only with stationary phases based on organic polymers.



Sample: Standard peptides. See Table 8.
Sample volume: 20 ml
Column: Organic polymer-based matrix (i.d. 5 mm, length 200 mm).
Eluent A: TFA in water. TFA concentrations as in diagrams.
Eluent B: TFA, 95% acetonitrile in water. TFA concentrations as in diagrams.
Gradient: 15-100% B at 1% B/min
Flow: 1 ml/min



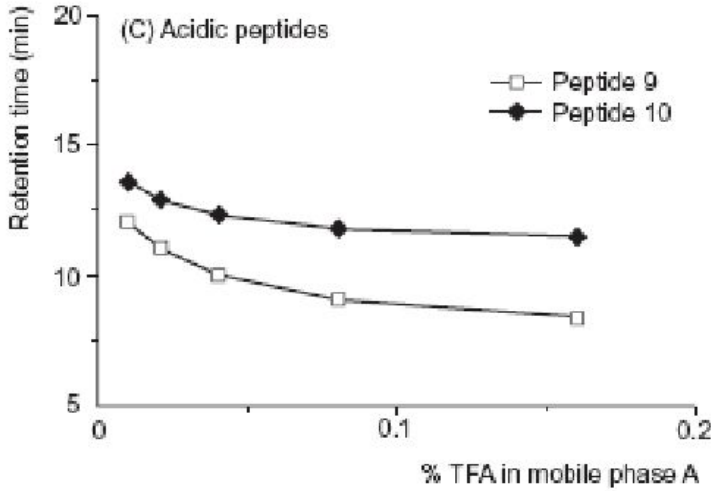


Fig. 31. Variation of retention time with concentration of the anionic ion pairing agent (TFA) for (A) neutral, (B) basic and (C) acidic peptides. (Work by Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA.)

Ион-парные агенты используемые в обратнофазовой хроматографии

Table 9. Ion pairing agents.

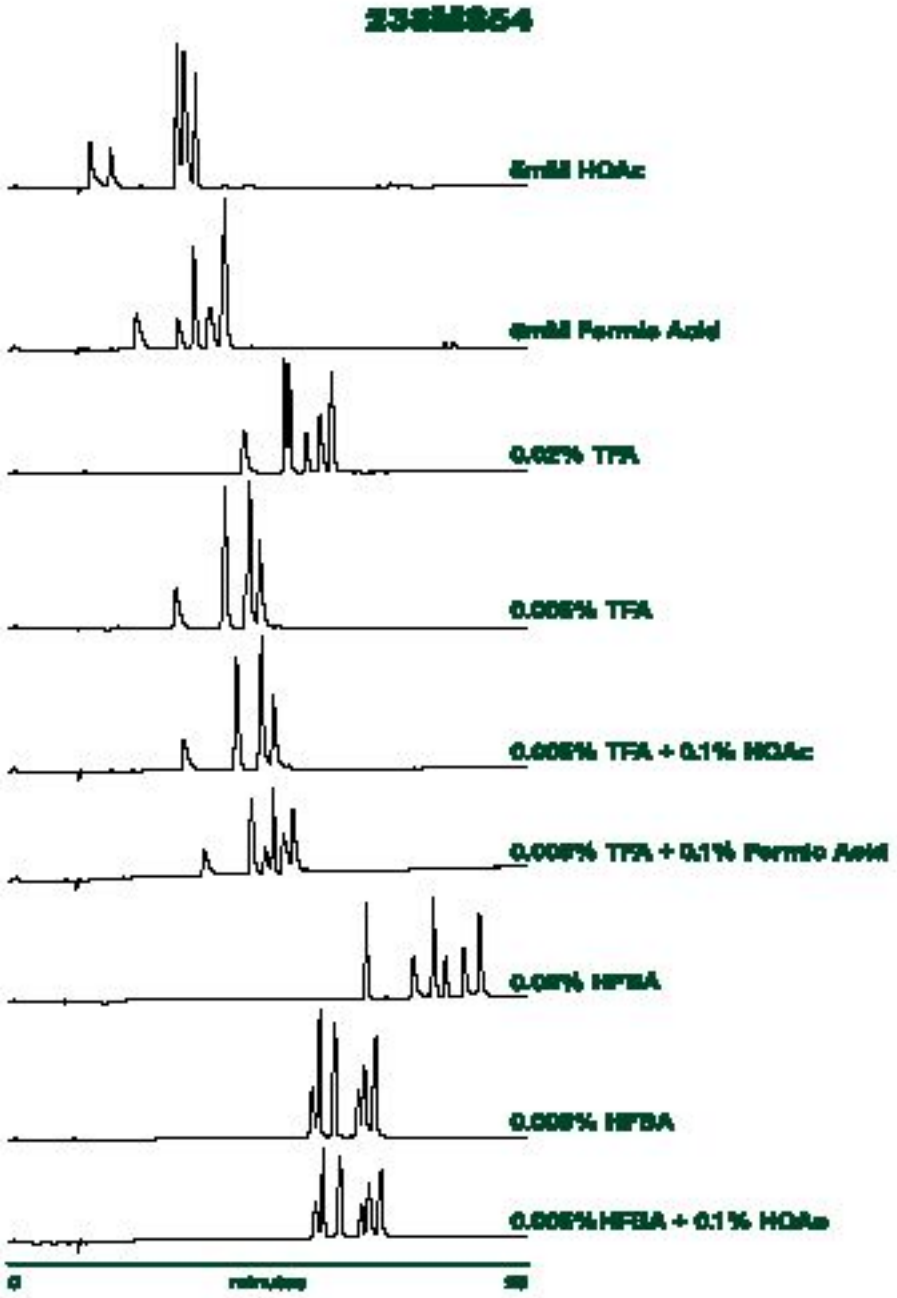
Ion pairing agent	Formula of pairing ion	Comments
Anionic		
Trifluoroacetic acid (TFA)	CF_3COO^-	Low UV-absorbance. Volatile, low pH,
Pentafluoroproprionic acid (PFPA)	$CF_3CF_2COO^-$	More hydrophobic than TFA. Volatile, low pH
Heptafluorobutyric acid (HFBA)	$CF_3CF_2CF_2COO^-$	More hydrophobic than TFA. Volatile, low pH
Ammonium acetate	CH_3COO^-	
Phosphoric acid	$H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}	Less hydrophobic than TFA.
Cationic		
Tetramethylammonium chloride	$+N(CH_3)_4$	
Tetrabutylammonium chloride	$+N(C_4H_9)_4$	
Triethylamine	$NH^+(C_2H_5)_3$	

Peptides on LC/MS C18

A mixture of six peptides was separated on a monomeric-C_{18} LC/MS column with a variety of ion-pair reagents.

Conditions

Column: Vydac 238MS54 monomeric C_{18}, 5 µm, 300 Å, 4.6 mm i.d. x 250 mm. Flow: 1.0 mL/min. Detection: 220 nm. Gradient: Linear, 10% to 40% ACN over 30 minutes for all chromatograms. Mobile phase modifiers as indicated (w/v). Sample components: oxytocin, bradykinin, angiotensin II, eledoisin-related peptide, neurotensin, and angiotensin I.



Rapid Separation of Proteins Using Short (50 mm) Column

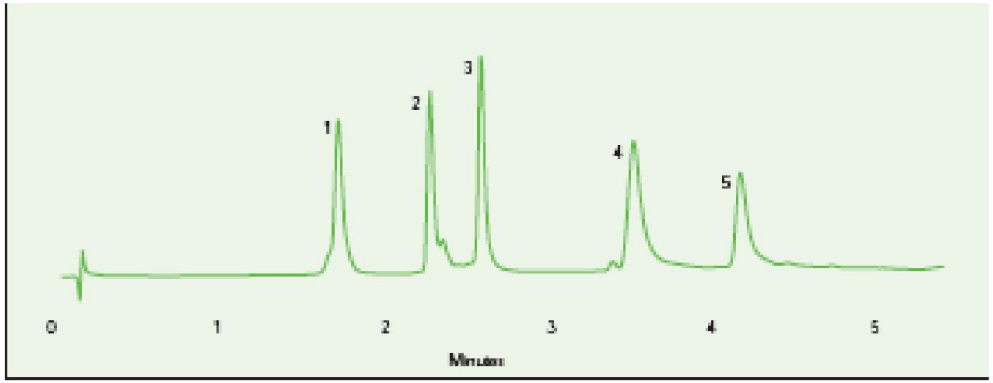


Figure 24. *Column: C_{18}, 3 µm 4.6 x 50 mm (VYDAC® 238TP3405). Flow rate: 4.0 ml/min. Eluent: Gradient from 20–45% ACN in aqueous 0.1% TFA in 4 min. Sample: protein standards. (1) ribonuclease, (2) insulin, (3) cytochrome c, (4) BSA and (5) myoglobin.*

Comparison of high performance reversed-phase and high performance ion-exchange chromatography in the separation of five peptides

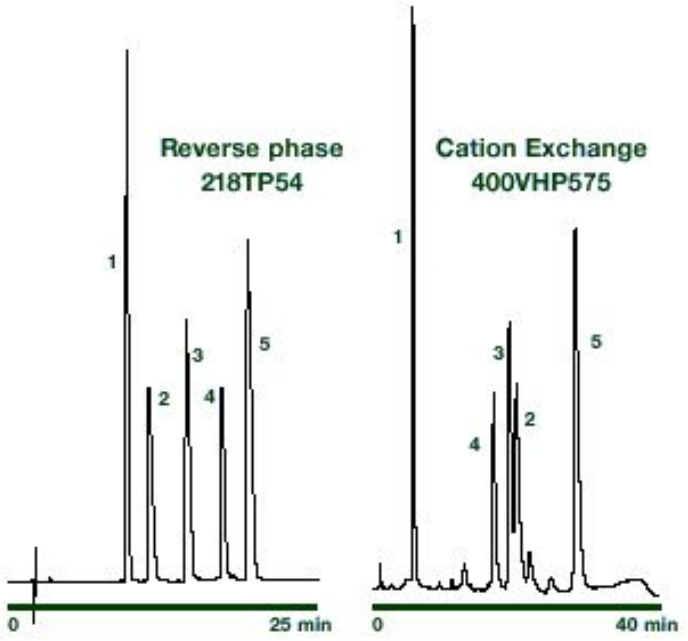
Comparison of the separation of several peptides by reversed-phase and cation-exchange HPLC illustrates the complementary selectivity of the two techniques. On the cation-exchange column, singly-charged oxytocin elutes early, followed by the three doubly-charged peptides—neurotensin, angiotensin II, and bradykinin. Angiotensin I with four charges elutes last. On reversed phase the peptides elute in the order of oxytocin, bradykinin, angiotensin II, neurotensin, and angiotensin I.

Reversed-phase

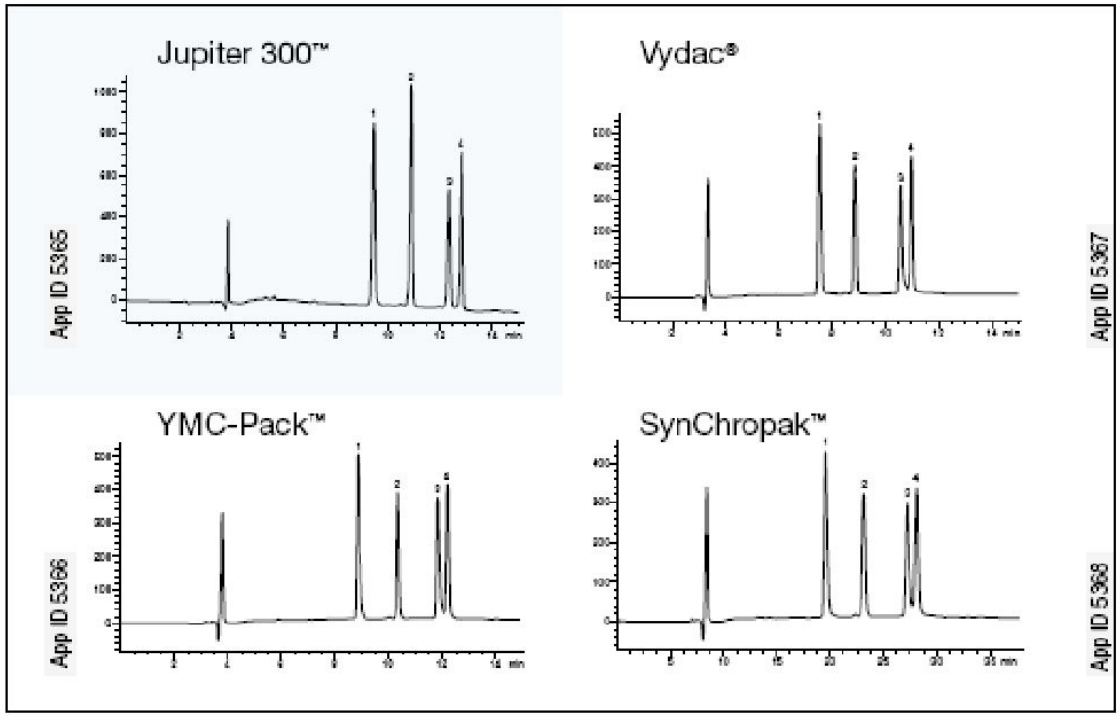
Column: Vydac 218TP54 (C_{18}, 5 µm, 4.6 mm i.d. x 250 mm).
Eluent: 15-30% ACN in 0.1% TFA over 30 min. at 1.0 mL/min.

Strong cation exchange

Column: Vydac 400VHP575 (cation exchange, 5 µm, 7.5 mm i.d. x 50 mm).
Eluent: 10 mM phosphate, pH 2.7/25% ACN; gradient 0-0.1 M NaCl in 20 min. at 1.0 mL/min. Sample: 1. oxytocin; 2. bradykinin; 3. angiotensin II; 4. neurotensin; and 5. angiotensin I.



Chromatographic Comparisons of 5µ, C4, 300Å 250 x 4.6mm Columns*,**



Peptide Hormones**

Conditions for all columns:

Dimensions: 250 x 4.6mm
Mobile Phase: A: 0.1% TFA in Water
B: 0.1% TFA in Acetonitrile/Water (90:10)
Gradient: a) A/B (90:10) to A/B (74:26) in 8 min (2% B/min)
b) A/B (74:26) to A/B (70:30) in 6 min (0.57% B/min)
Flow Rate: 1.0 mL/min
Detection: UV@214nm
Sample:

1. [Arg_8]-Vasotocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2)
2. [Arg_8]-Vasopressin (Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-NH_2)
3. Isotocin (Cys-Tyr-Ile-Ser-Asn-Cys-Pro-Ile-Gly-NH_2)
4. Oxytocin (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)

Comparison of High Performance Reversed-Phase and High Performance Ion-Exchange Chromatography in the Separation of Peptides

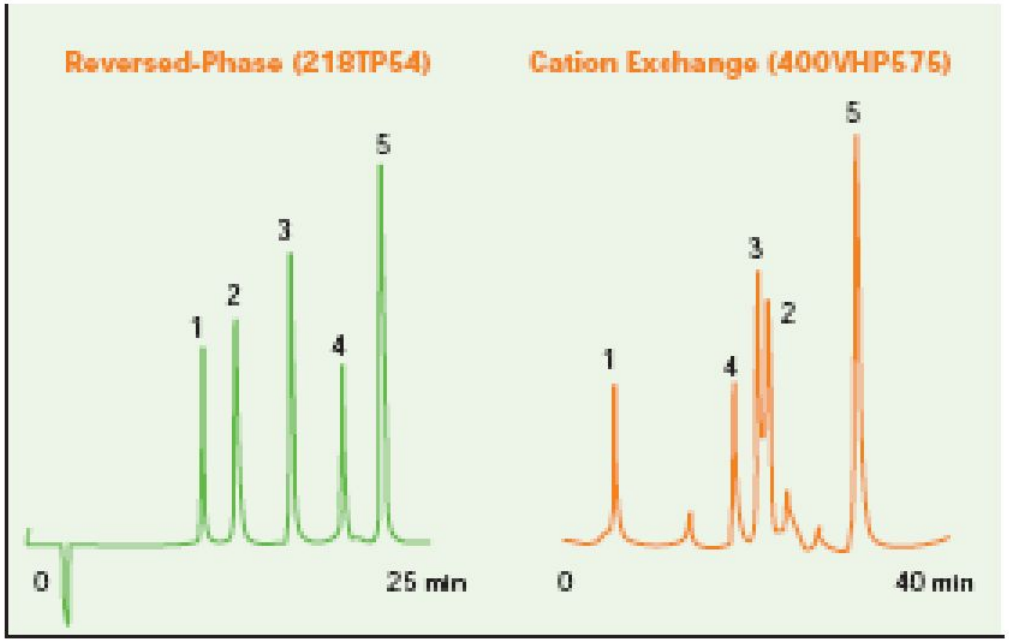
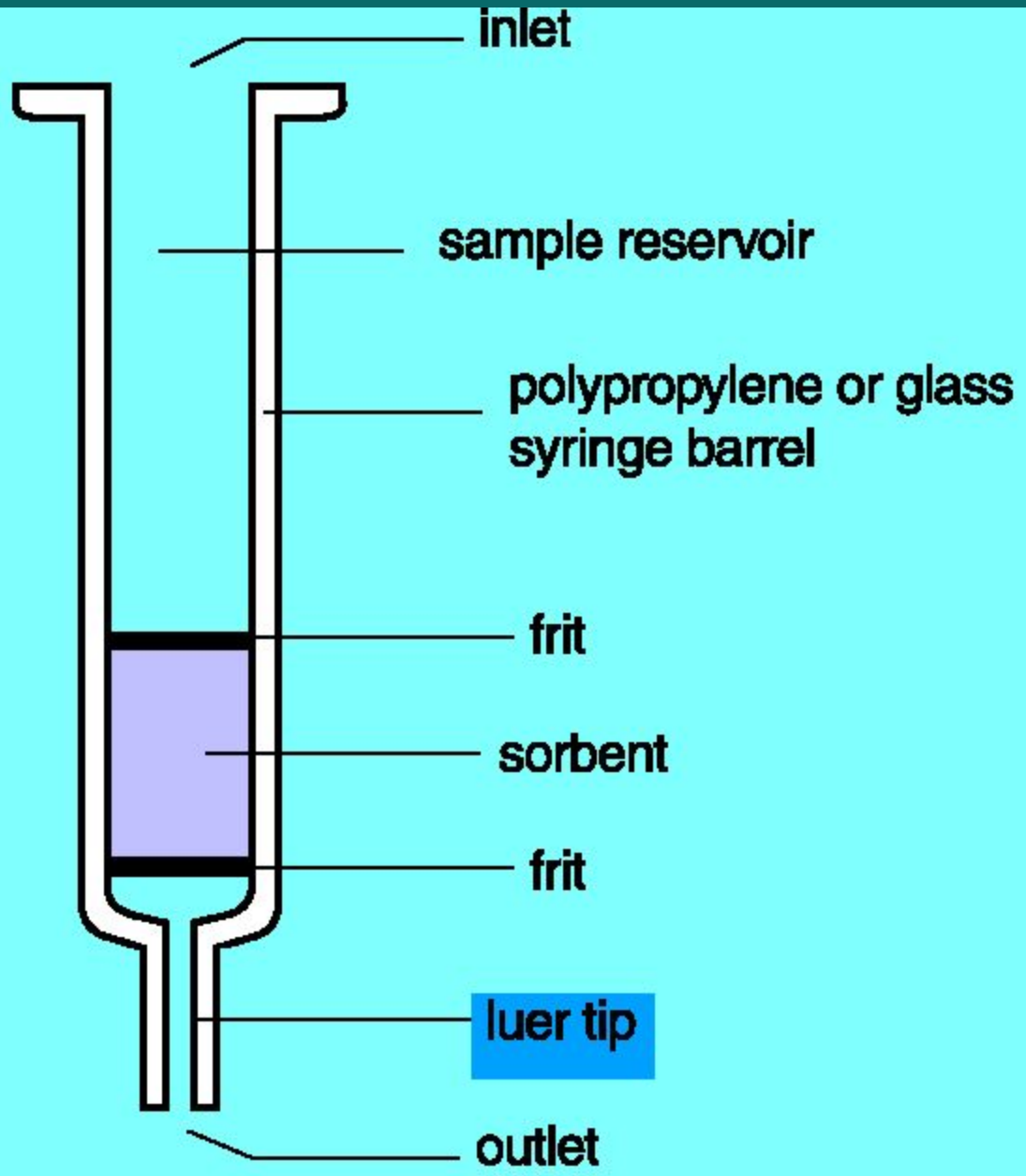


Figure D-1. *Reversed-Phase Column: VYDAC® 218TP54, C_{18}, 5 µm, 4.6 x 250 mm Eluent: 15–30% ACN in 0.1% TFA over 30 minutes at 1.0 mL/min Strong Cation Exchange Column: VYDAC® 400VHP575, Cation exchange, 5 µm, 7.5 x 50 mm Eluent: 10 mM phosphate, pH 2.7/25% ACN; gradient from 0-0.1 M NaCl in 20 min at 1.0 mL/min Sample: 1. oxytocin 2. bradykinin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

Figure 1.
Typical SPE
Column



Easy-to-use BioSelect™ SPE cartridges

Step 1
Prepare cartridge by washing with strong solvent followed by weak solvent.

Step 2
Load sample onto cartridge

Step 3
Rinse with weak solvent to elute weakly bound contami-nants

Step 4
Rinse with medium strength solvent to remove product of interest