Chromatography Lecture 4: LC, HPLC and IC

CU- Boulder
CHEM 5181
Mass Spectrometry & Chromatography

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CIRES and NOAA
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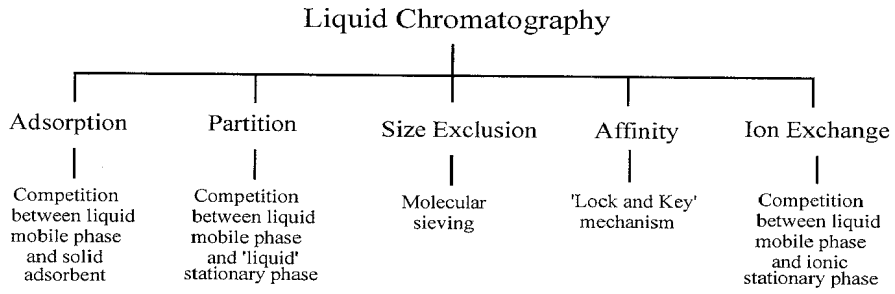
Classification of LC Techniques (Contemporary)



Historical Review

- LC used extensively since 1930s
  - Gravity feeding or peristaltic pumps (low P)
    - Limit on how small you can make the particles in packing
  - Low resolution
- Giddings, 1963: pointed the way to high efficiency
  - Following the model of GC
  - Smaller particles by 100 => high performance (HP)
- 1972: Majors, Kirkland demonstrate small particles as packings
- 1985: ESI starts to be applied to LC-MS
  - Boom for bioanalytical MS

HPLC

Advantages of HPLC

- Speed (minutes)
- High resolution
- Sensitivity (ng to fg)
- Reproducibility of +/- 1% (not so for LC)
- Accuracy
- Automation

Disadvantages of HPLC

- Cost
- Complexity
- Low sensitivity for some compounds
- Irreversibly adsorbed compounds not detected
- Coelution difficult to detect

Normal-Phase ('NP') Chromatography I

- Polar stationary phase
- Less polar mobile phase
- The more polar the analyte
  - the greater the retention
- Increasing polarity of mobile phase
  - Decreased retention
- Most commonly adsorption
  - Sometimes called 'adsorption chromatography'

Normal-Phase Chromatography II

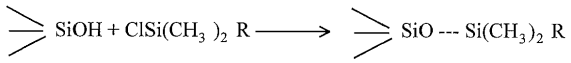
- SP: Silica
  - Available, low cost, very well characterized
  - Hydroxyl groups (-OH) on surface
- Problems:
  - lack of selectivity: compounds always eluted in the same order
  - Water adsorbed into the strongest sites

Table 2.1 General Order of Elution for Species Separated by Normal-Phase Chromatography

Saturated hydrocarbons < olefins < aromatic hydrocarbons ≅ organic halides < sulfides < ethers < nitro compounds < esters ≅ aldehydes ≅ ketones < alcohols ≅ amines < sulfones < amides < carboxylic acids

Reversed-Phase ('RP') Chromatography

- The most widely used mode of HPLC
- Separate molecules in solution on basis of their hydrophobicity (good word for Scrabble)
  - Non-polar stationary phase
  - Polar mobile phase
- In practice: non-polar functional group 'bonded' to silica
  - "bonded-phase chromatography"
- Sometimes polymeric (e.g. polystyrene) or solid (porous graphitic carbon) SP



Models of Interaction in RP Chromo

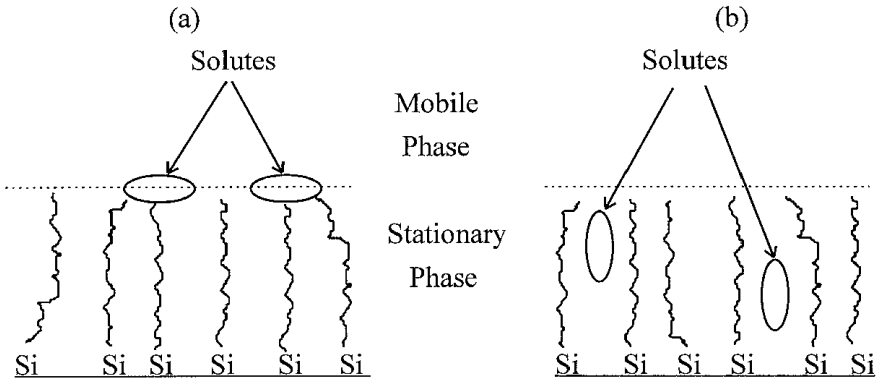


Figure 2.4 (a) Solvophobic and (b) partitioning models of solute retention.

More Details on RP Chromo

- Stationary phase
  - Functional group bonded to silica
  - This corresponds to a volume (and VanDeemter)
  - Alkyl groups $-CH_3$, $-C_4H_9$, $-C_8H_{17}$, $-C_{18}H_{37}$
    - Retention increases exponentially with chain length
- Mobile phases
  - Polar solvent (water) with addition of less-polar solvent (acetonitrile or methanol)

Gradient Elution I

- Gradient Elution
  - Progressive change of the mobile phase
  - Normally (but not always) to increased concentration of the organic solvent
  - Result: decreased retention
- Why is this important?
  - What concept from intro?

Gradient Elution II

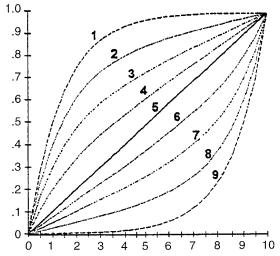


Figure 1.3 Gradient profiles. (Reprinted courtesy of Dionex Corporation, from the DX500 pump manual.)

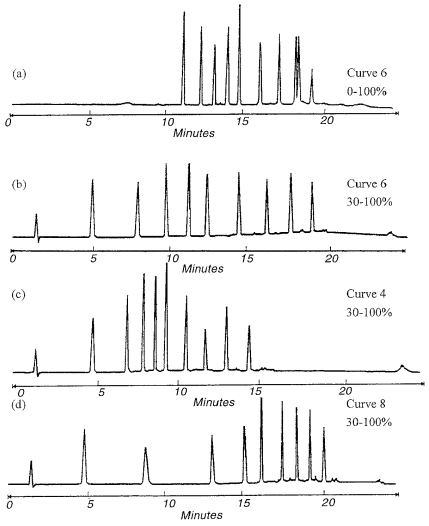
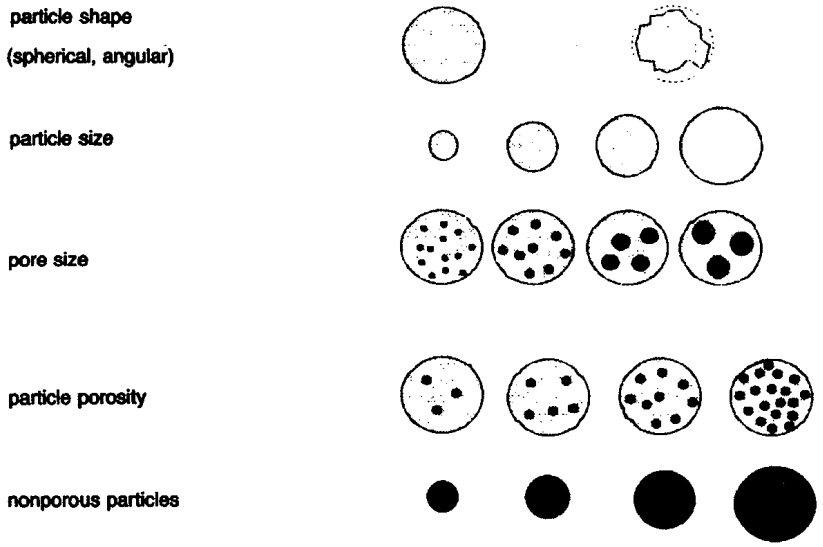


Figure 1.4 Gradient elution of nine alkylphenones showing the effect of different gradient profiles on the peak resolution: (a) linear, 0–100% acetonitrile (ACN), (b) linear, 30–100% ACN, (c) convex, 30–100% ACN, and (d) concave, 30–100% ACN. Curve numbers refer to profiles of Figure 1.3. (Adapted from Ref. 23 with permission.)

The SP, MP, and VanDeemter

Physical Parameters of Silica



Van Deemter Model of Band Broadening

- H: plate height
- $\bar{u}$: average linear velocity

Reminder

$$\bar{u} = \frac{L}{t_M}$$

$$H = A + \frac{B}{\bar{u}} + C_S \bar{u} + C_M \bar{u}$$

- H: as small as possible

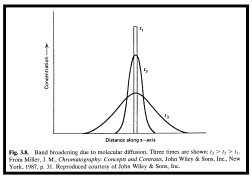
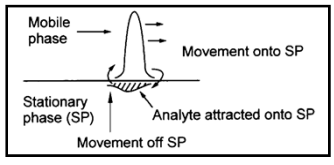
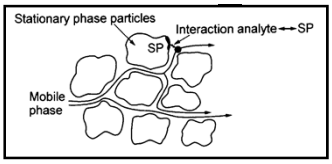


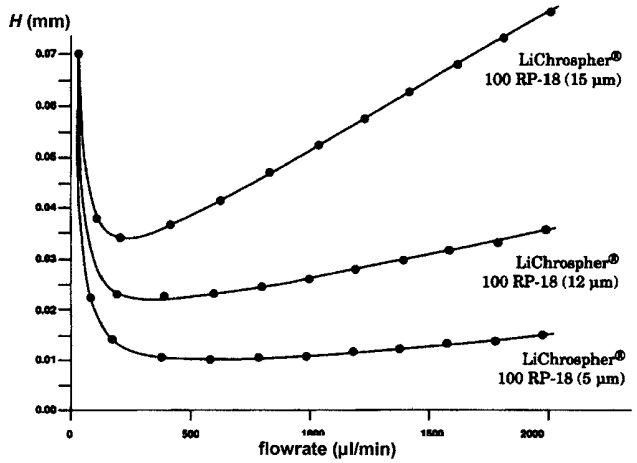
Fig. 3.8. Band broadening due to molecular diffusion. Three times are shown: $t_3 > t_2 > t_1$. From Miller, J. M., *Chromatography: Concepts and Contrasts*, John Wiley & Sons, Inc., New York, 1987, p. 31. Reproduced courtesy of John Wiley & Sons, Inc.





Effect of Particle Diameter on Plate Height

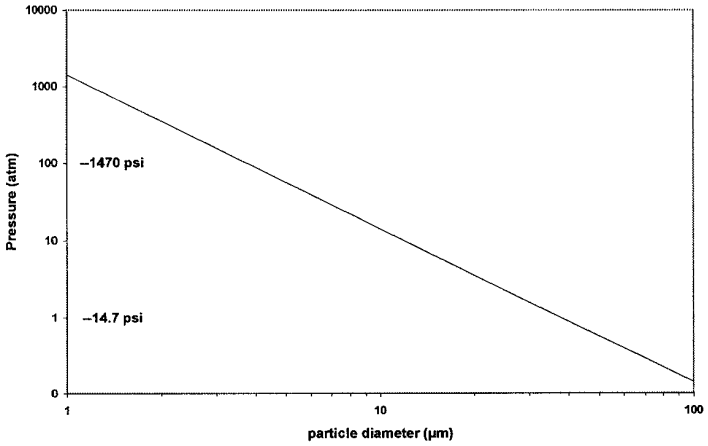
Figure 5.4 Effect of Particle Diameter on Plate Height



Column: as indicated, 250 x 4mm ID. Mobile phase: acetonitrile/water (75/25). Sample: anthracene. Reprinted from ref. 3 with permission of EM Separations.

Dependence of ΔP on Particle Size

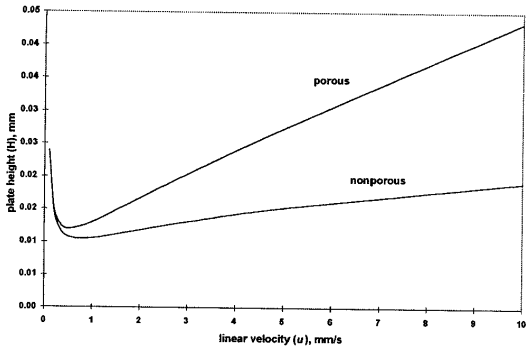
Figure 5.6 Dependence of Pressure on Particle Diameter



Graph of Eq. 5.2. L = 5cm; F = 1ml/min; η = 0.4cp; d_c = 0.46cm.

Effect of Porosity on Plate Height

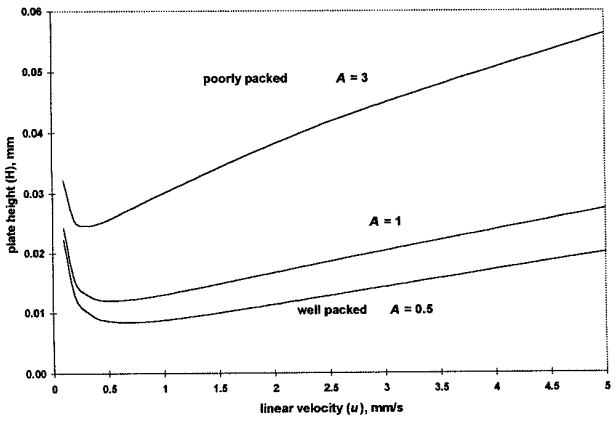
Figure 2.10 Effect of Porosity on Bandspreading



Graph of Eq. 2.17 using the constants of Eq. 2.27 for a 5μm packing and small molecule solute; $A = 1$ and $B = 2$.

Effect of Eddy Diffusion on Plate Height

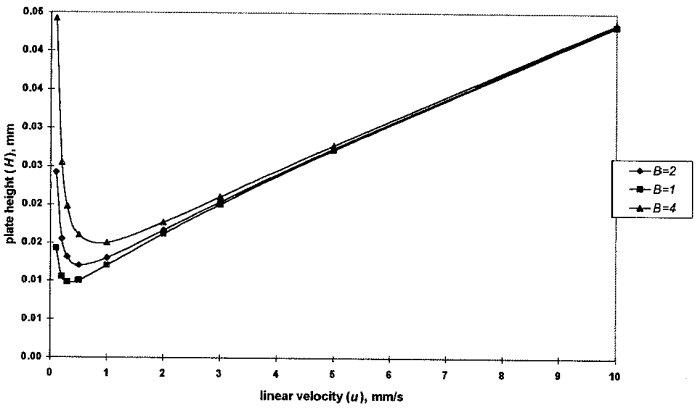
Figure 2.13 Effect of Eddy Diffusion and Flow Variations on Bandspreading



Graph of Eq. 2.17 using the constants of Eq. 2.27 for 5μm particles and a small molecule solute. $B = 2$ and $C = 0.1$.

Effect of Axial Diffusion on P. Height

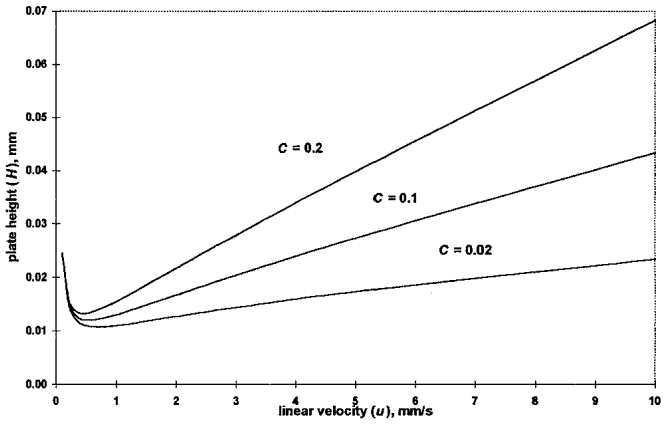
Figure 2.14 Effect of Axial Diffusion on Bandspreading



Graph of Eq. 2.17 using the constants of Eq. 2.27 for 5µm particles and a small molecule solute. $A = 1$ and $C = 0.1$.

Effect of Mass Transfer on P. Height

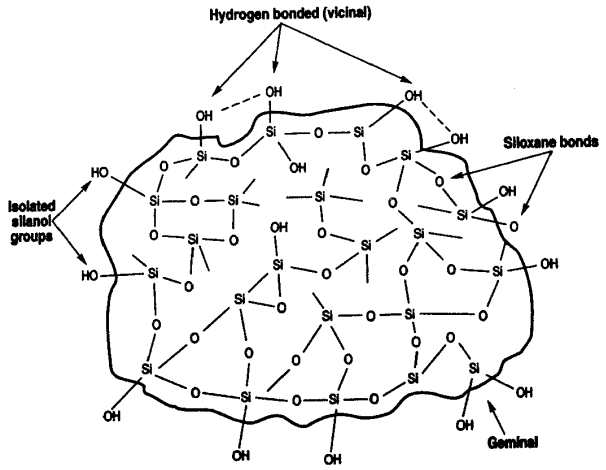
Figure 2.15 Effect of Mass Transfer on Bandspreading



Graph of Eq. 2.17 using the constants of Eq. 2.27 for 5µm particles and a small molecule solute. $A = 1$ and $B = 2$.

Structure of Silica

Figure 5.8 Structure of Silica



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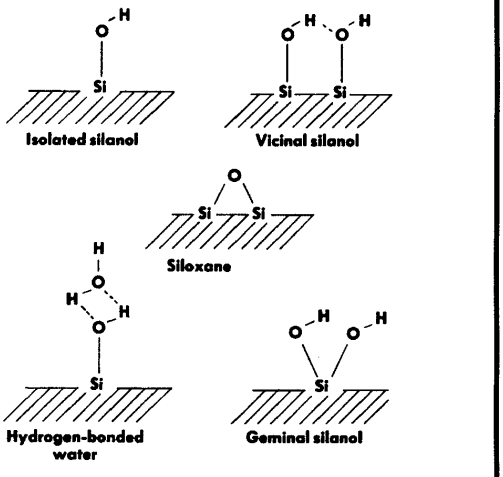
Silica Surface Groups

Figure 5.9 Silica Surface Groups



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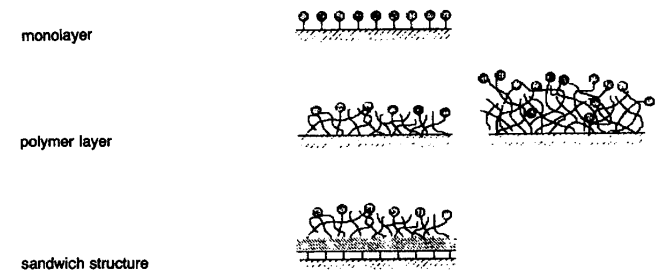
Bonded Phase to Silica

Figure 5.10 Bonded Phases



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Sources of Silica-Based Supports

Table 5.4 Selected Sources of Silica-based Supports

MANUFACTURER	ASSOCIATED OR MERGED COMPANIES
Alltech	Exmere Ltd.
Bio-Rad	
Beckman	
E. Merck	
Hewlett Packard	Rockland Technology
Higgins Analytical	
Keystone	
Macherey Nagel	
MICRA Scientific	SynChrom
Perkin-Elmer	Applied Biosystems, Perseptive Biosystems
Poly LC	
Separations Group	
Supelco	
Toso Haas	
Thermo Quest	Hypersil
Waters	Phase Separations, YMC

Limitations of Silica

Figure 5.12 Solubility of Silica with pH

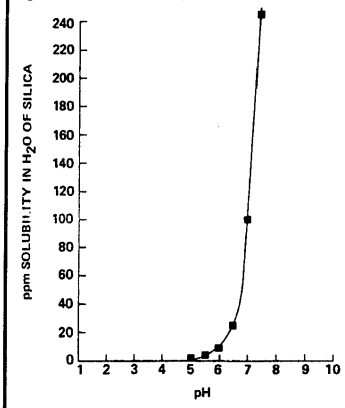

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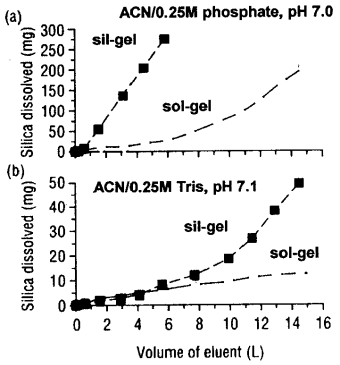
Figure 5.13 Relative Rate of Dissolution of Silicas



Columns: 150 x 4.6mm ID; ACN/buffer (20/80); temperature: 60°C; flowrate: 1ml/min. Sol-gel is double endcapped and sil-gel is single endcapped. Reprinted from *LCGC* with kind permission (14).

Two Main Types of Silica

Table 5.3 Silica Characteristics by Type

Type Designation	Sil-gel Type A	Sol-gel Type B
synthesis	gelling soluble silicates	aggregating silica sols
surface area	high	moderate
porosity	high	moderate
pore walls	variable	thick
silanols	isolated or unbonded	associated or bonded
pH stability	moderate	good
purity	may contain metal impurities	high
solubility	metals may increase stability	moderate

From ref. 14 -16.

Size Exclusion and Affinity Chromatography

Size Exclusion Chromatography

- Used to separate molecules of different "size" (MW)
  - Porous solute with pores of a given (average) size
  - MW differences of 10% are enough for small molecules
  - Macromolecules: x 2 in MW
- Two types
  - Gel filtration (GFC)
    - Aqueous MP, hydrophilic SP, biomolecules
  - Gel permeation (GPC)
    - Organic MP, hydrophobic SP, polymers

Size Exclusion Chromatography Example

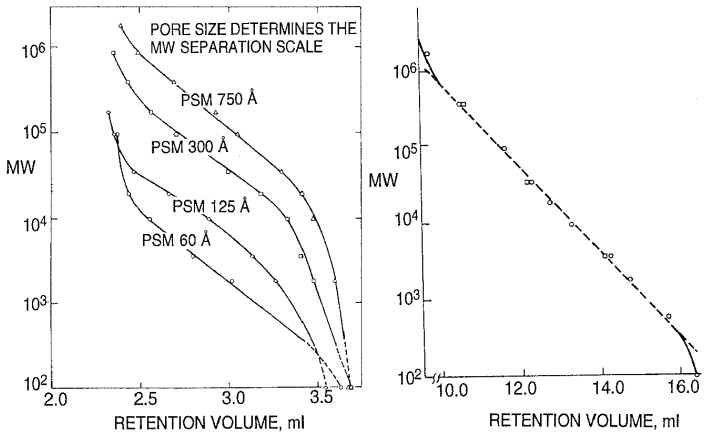
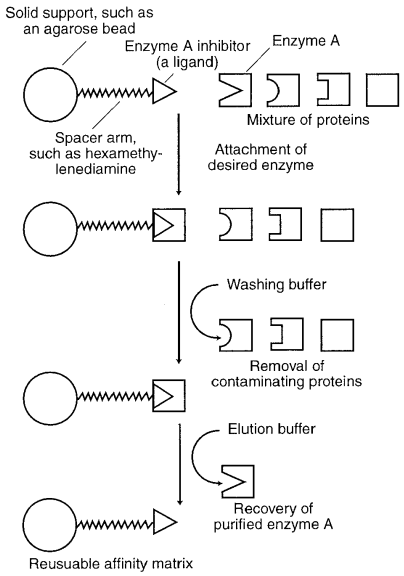


Figure 2.14 Molecular weight calibration curves for porous silica microsphere columns. Chromatography conditions: column, 10 × 0.78 cm; mobile phase, tetrahydrofuran, 22°C; flow rate, 2.5 ml/min; detection, UV absorbance at 254 nm; sample, 25 μl solutions of polystyrene standards. (a) Four individual columns showing four different calibration ranges; (b) four columns in series with two distinct pore sizes (60, 60, 750, 750Å), providing a single calibration curve with a broader molecular weight range than the individual columns. (Adapted from Ref. 54 with permission.)

Affinity Chromatography



SPs and MPs in Affinity Chromatography

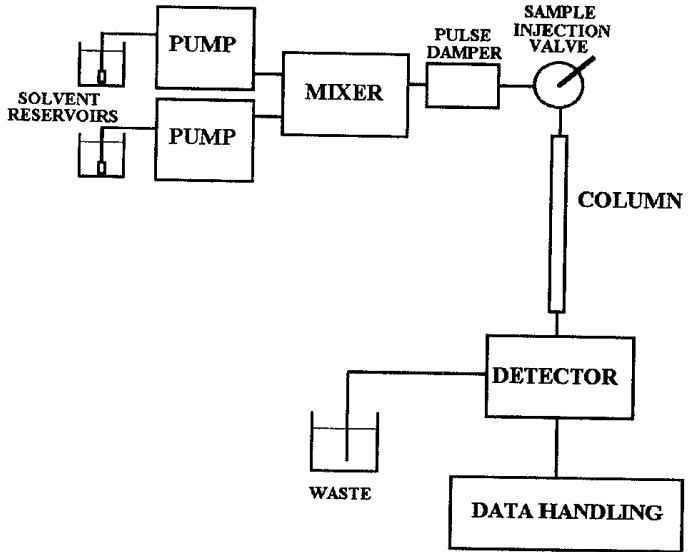
Table 2.7 Solvents and Packings Used in Size-Exclusion Chromatography[a]

Polymer types	Typical solvent system	Typical column packings[b]	Supplier
Proteins, polypeptides	Aqueous buffers	Zorbax Bio Series GF	Du Pont
Biopolymers, viruses, DNA, RNA	Aqueous buffers	TSK-G-PW	Toya Soda
		Superose	Pharmacia
Cellulose derivatives, polyvinyl alcohol, polysaccharides	Aqueous buffer, salts	TSK-G-SW	Toya Soda
Many polar noncrystalline synthetic polymers, some crystalline polymers, small molecules	Tetrahydrofuran	Ultra-Styragel	Waters
Nonpolar, noncrystalline synthetic polymers, hydrocarbon polymers, low molecular weight polymers	Toluene (benzene[c] or chloroform[c]	LiChrospher Si	Merck
Polar crystalline polymers (e.g., polyamides and polyesters)	*m*-Cresol (hot) or hexafluoroisopropanol (cold)	PL gel	Polymer Labs
Nonpolar crystalline polymers (e.g., polyethylene and stereoregular polyhydrocarbons)	1,2,4-Trichlorobenzene (hot) or 1,2-dichlorobenzene (hot)	Zorbax PSM (bimodal)	Du Pont

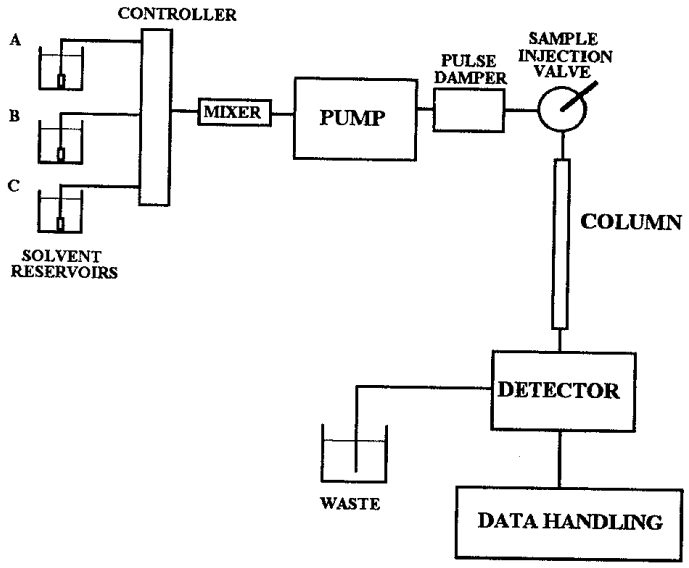
[a] Reprinted from Ref. 52 with permission.
[b] These examples merely illustrate commercially available packings. Each packing often can be used for many polymer types.
[c] Suspected carcinogen.

HPLC Instrumentation

HPLC with High P mixing



HPLC with Low P Mixing



P1: Syringe Pump + Control

Figure 3.2 Syringe Pump

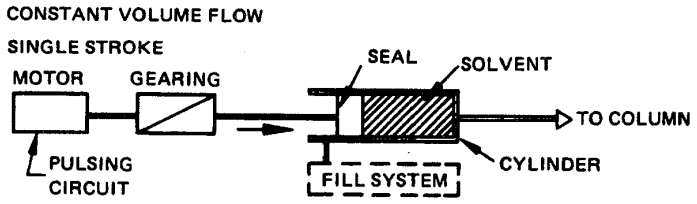


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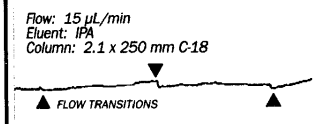
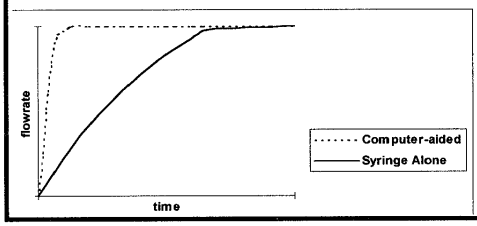
Figure 3.3 Pump Transitions



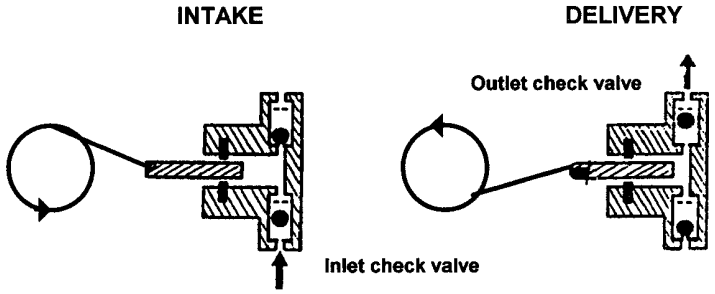
MicroPro® reciprocating system. Reprinted from ref. 3 with permission of Eldex.

Figure 3.4 Constant Flowrate in Syringe Pumps



P2: Single Piston Pump

Figure 3.6 Operation of Single Piston Pump



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P3: Dual Piston Pumps

Figure 3.7 Flow Variance of Dual Piston Pumps

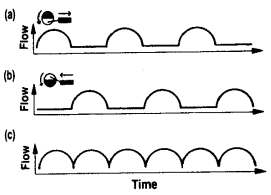

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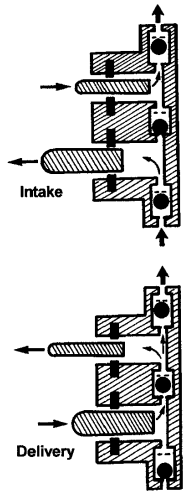
Figure 3.8 Tandem-Piston Pump



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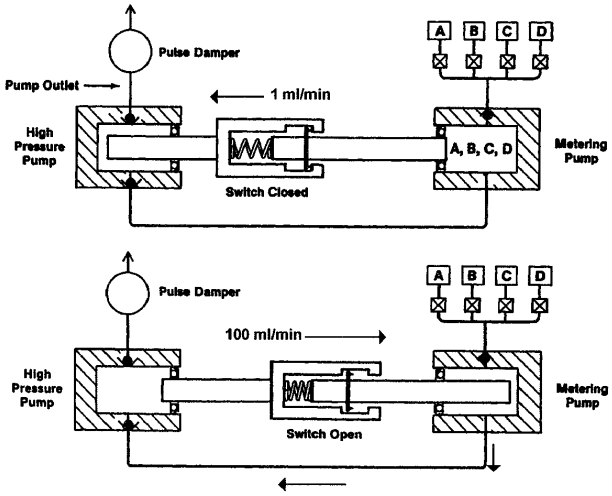
P4: Fast-Fill Single Piston Pump

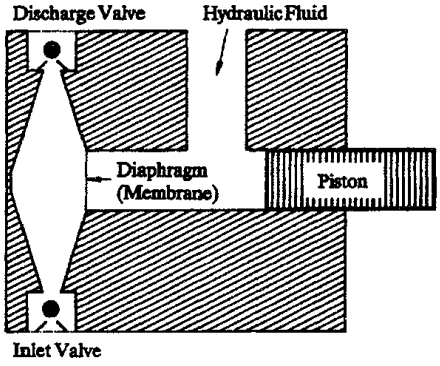
Figure 3.9 Fast-Fill Single Piston Pump



Perkin-Elmer Series 200 pump. Reprinted from ref. 7 with permission of Perkin-Elmer.

P5: Diaphragm Pump

Figure 3.10 Diaphragm Pump



Reprinted from ref. 10 with permission of LDC Analytical which merged with Spectra-Physics Analytical in 1993 to become Thermo Separation Products.

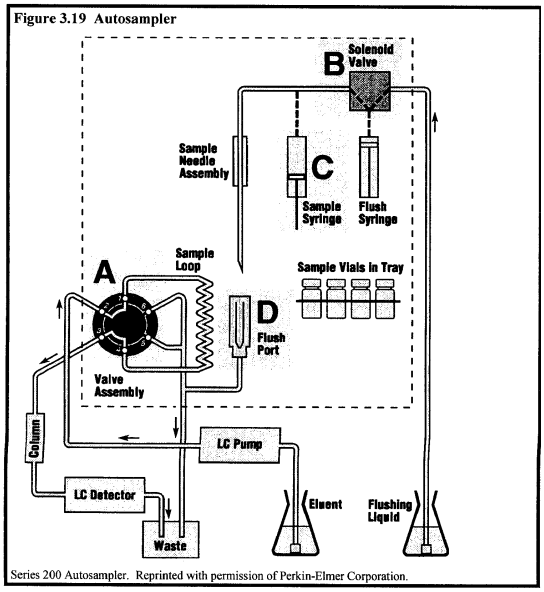
Pumps Used in Commercial Systems

Table 3.1 Selected HPLC Systems

		Syringe	Single Piston		Dual Piston
			Diaphragm	Fast-Fill	
Beckman	System Gold®			X	
Eldex	MicroPro®	X			
Gilson	Model 305			X	
Hewlett Packard	Model 1100 binary				X
ISCO	µLC-500®	X			
Hewlett Packard	Model 1090		X		
ISCO	Model 2350			X	
Perkin-Elmer	series 200			X	
ThermoSeparations	Spectra Vision®				X
Varian	LC Star®, 9000			X	
Waters	Model 515				X
Waters	Model 626			X	

HPLC Autosampler

Figure 3.19 Autosampler



Series 200 Autosampler. Reprinted with permission of Perkin-Elmer Corporation.

Appendix

Common Problems in HPLC

Table 3.5 Common Problems with HPLC Systems

Problem	Preventative Measures
Dirty check valves	filter mobile phase flush salts from pumps
Plugged filters (inlet, in-line)	filter mobile phase
Deteriorated pump seal	flush salts from pumps flush behind seal use appropriate seal for mobile phase replace at timely intervals, e.g. every 6 months
Inadequate mixing	use dynamic mixer if solvents not readily miscible
Pump pulsing	use pulse damper
Air bubbles in check valve	degas mobile phase
Broken or scored piston	flush salts from pumps
Blockage in analytical column	use guard column
Leaks	use correct fittings/ferrules
Pump failure	lubricate and maintain as recommended
Injector leaking	flush salts (18)

HPLC Validation Procedures

Table 3.6 Equipment Validation Procedures

Solvent Delivery	
flow accuracy	test flow rate volumetrically with back pressure
flow precision	reproducibity of retention time or peak area for 5 injections
Sampling Device	
precision	reproducibility of peak areas for 5 consecutive injections
accuracy	linearity of peak area vs. concentration or volume
Absorbance Detector	
linearity	absorbance vs. concentration
wavelength accuracy	measurement of wavelengths for maximum and minimum absorbance of a standard compound
noise	measure in mAU
Refractive Index Detector	
linearity	RIU vs. concentration
accuracy	calculation from linearity data of *dn/dv, a* calibration constant relating the change in RIU to that in voltage
noise	measure in RIU
drift	measure change in RIU for at least one hour
Temperature Control	
accuracy	measure temperature of eluent
reproducibility	measure temperature of eluent at 5 different times
Gradient Formation and Mixing	
accuracy	evaluate linear gradient as outlined in Section IV.C

Intro to Ion Chromatography (IC)

- HPLC
  - Normally neutral samples
  - Ionic samples
    - Inorganic ions
    - Ionized or ionizable organic molecules
- IC
  - More complicated than neutral HPLC
  - Some special problems
  - But more control (‘handles’) available
    - Easier to achieve successful separation

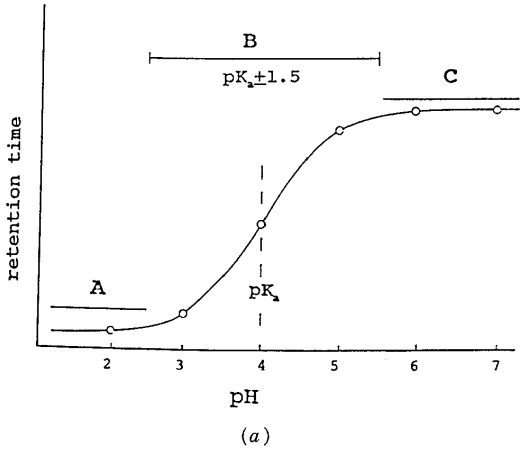
HPLC Approaches to Ionic Samples?

- Reversed-Phase IC (MP is more polar)
- Ion-Pair C
- Ion-Exchange C
  - For inorganic ions
- Try in that order, RPC is easiest, if it does not work move down the list

Definition of Ionic Solute

- Capable of acidic or basic behavior
  - H^+ donor or acceptor
  - In the usual pH range
    - 2 < pH < 8 for silica-based columns
      - Why?
    - 1 < pH < 14 for pH stable columns
  - Best to work in region where extent of dissociation is a strong function of pH
    - Why?

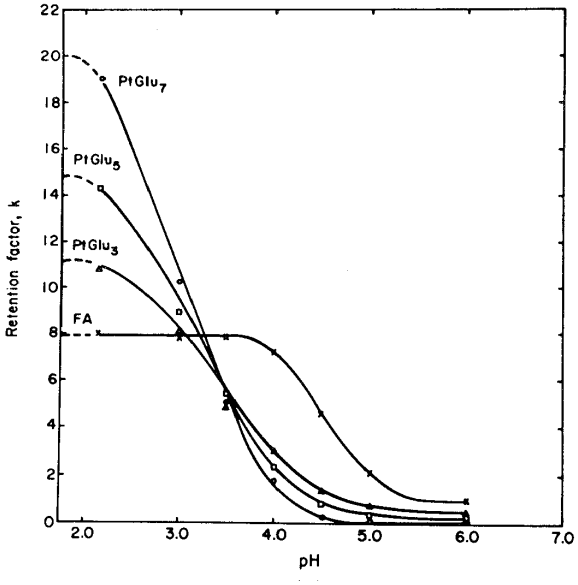
Retention time vs. pK_a



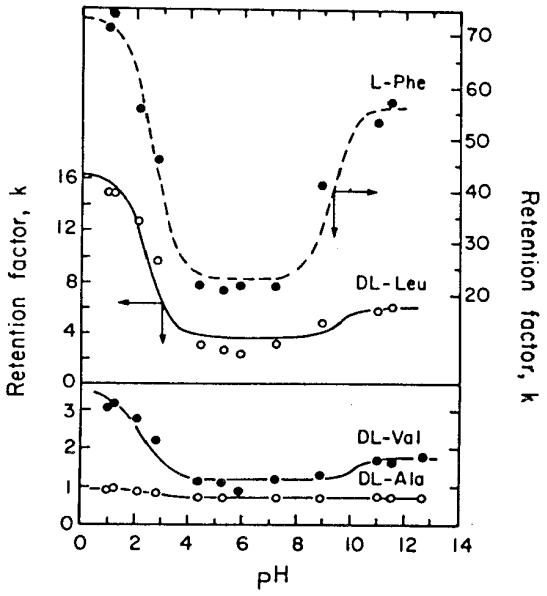
(*a*)

FIGURE 7.2 Retention and buffer capacity as a function of pK_a and pH. (*a*) Idealized dependence of retention on pH for a basic compound with pK_a = 4.0; (*b*) deterioration of peak shape as mobile-phase buffer capacity decreases; 3,5-Dimethylaniline solute (pK_a = 3.8); 25 × 0.46-cm cyano column, 25% MeOH–buffer (25 m*M* potassium phosphate), 1 mL/min, 35°C. (Reprinted with permission from Ref. 3.)

Compounds with Several Acid Groups



Compounds with Acid + Base Func. Groups



pK_a values for Functional Groups

TABLE 7.2 pK_a Values for Acidic or Basic Functional Groups

	pK_a			
	Acid		Base	
Group	Aliph[a]	Arom[b]	Aliph[a]	Arom[b]
Sulfonic acid, $-SO_3H$	1	1		
Amino acid, $-C(NH_2)-COOH$	2–4		9–12	
Carboxylic acid, $-COOH$	4–5	4–5		
Thiol, $-SH$	10–11	6–7		
Purine		2–4		9
Phenol, $-OH$		10–12		
Pyrazine			1	
Sulfoxide, $-SO$			1–2	
Thiazole			1–3	
Amine, $-NH_2$, $-NR_2$, pyridine			8–11	5
Imidazole				7
Piperazine			10	

Source: Ref. 13.

[a] Aliph, aliphatic substituent (e.g., acetic acid for $-COOH$).

[b] Arom, aromatic substituent (e.g., benzoic acid for $-COOH$).

Effect of pH on Separation

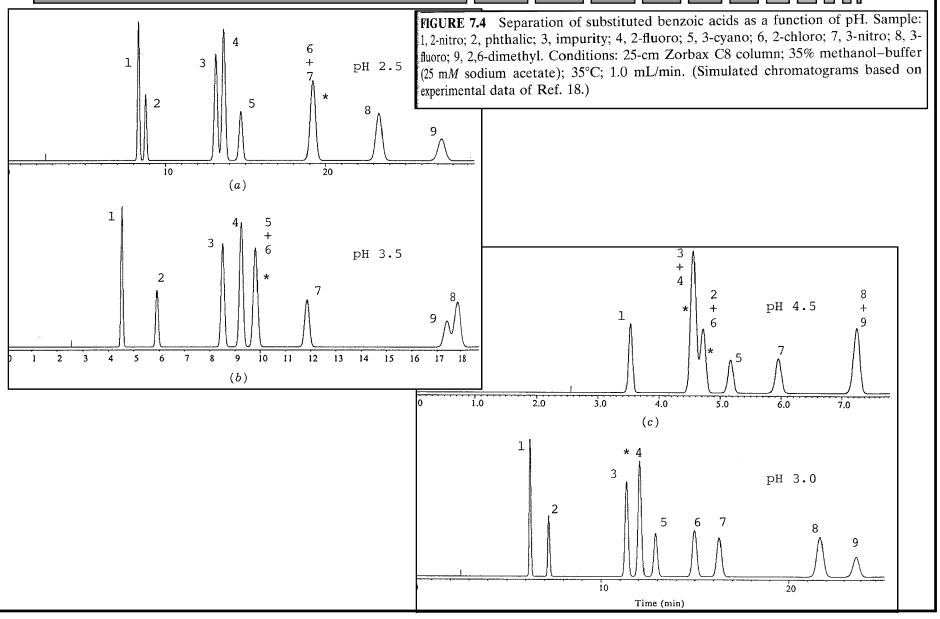


FIGURE 7.4 Separation of substituted benzoic acids as a function of pH. Sample: 1, 2-nitro; 2, phthalic; 3, impurity; 4, 2-fluoro; 5, 3-cyano; 6, 2-chloro; 7, 3-nitro; 8, 3-fluoro; 9, 2,6-dimethyl. Conditions: 25-cm Zorbax C8 column; 35% methanol–buffer (25 m*M* sodium acetate); 35°C; 1.0 mL/min. (Simulated chromatograms based on experimental data of Ref. 18.)

pH Buffers for RPC

TABLE 7.1 Buffers for Use in HPLC Separation

Buffer	pK_a	Buffer Range[a]	UV Cutoff[b]
Trifluoracetic acid	>>2	1.5–2.5	210 nm (0.1%)
Phosphoric acid/mono- or di-K phosphate	2.1	< 3.1	< 200 nm (0.1%)
	7.2	6.2–8.2	
	12.3	11.3–13.3	< 200 nm (10 m*M*)
Citric acid/tri-K citrate	3.1		
	4.7	2.1–6.4	230 nm (10 m*M*)
	5.4		
Formic acid/K-formate	3.8	2.8–4.8	210 (10 m*M*)
Acetic acid/K-acetate	4.8	3.8–5.8	210 nm (10 m*M*)
Mono-/di-K carbonate	6.4	5.4–7.4[c]	< 200 nm (10 m*M*)
	10.3	9.3–11.3	< 200 nm (10 m*M*)
Bis-tris propane[e] · HCl/Bis-tris propane	6.8	5.8–7.8	215 nm (10 m*M*)
	9.0	8.0–10.0	225 nm (10 m*M*)
Tris[d] · HCl/tris	8.3	7.3–9.3	205 nm (10 m*M*)
Ammonium chloride/ammonia	9.2	8.2–10.2	200 nm (10 m*M*)
1-Methylpiperidine · HCl/1-Methylpiperidine	10.1	9.1–11.1	215 nm (10 m*M*)
Triethylamine · HCl/triethylamine	11.0	10.0–12.0	< 200 nm (10 m*M*)

[a] pH range allowed with this buffer (conservative estimate).
[b] Absorbance <0.5 A; from Ref. 7.
[c] Requires addition of an acid (e.g., acetic or phosphoric).
[d] Tris(hydroxymethyl)aminomethane.
[e] 1,3-bis [Tris(hydroxymethyl)methylamino] propane.

Ion-Pair Chromatography

- Similar column + mobile phase as RP-IC
- IPC
  - Addition of an ion-pair reagent to the mobile phase ('matched' to the sample)
  - More complicated, try RPC first
  - IPC applied to RPC separations that need improvement
- Ion-pair reagent
  - E.g. C_6-SO_3^- on C_8 or C_{18} RPC packing
  - Na^+ in mobile phase attracted to SP
  - Analyte ions can substitute Na^+

Concept of Ion-Pair Chromatography

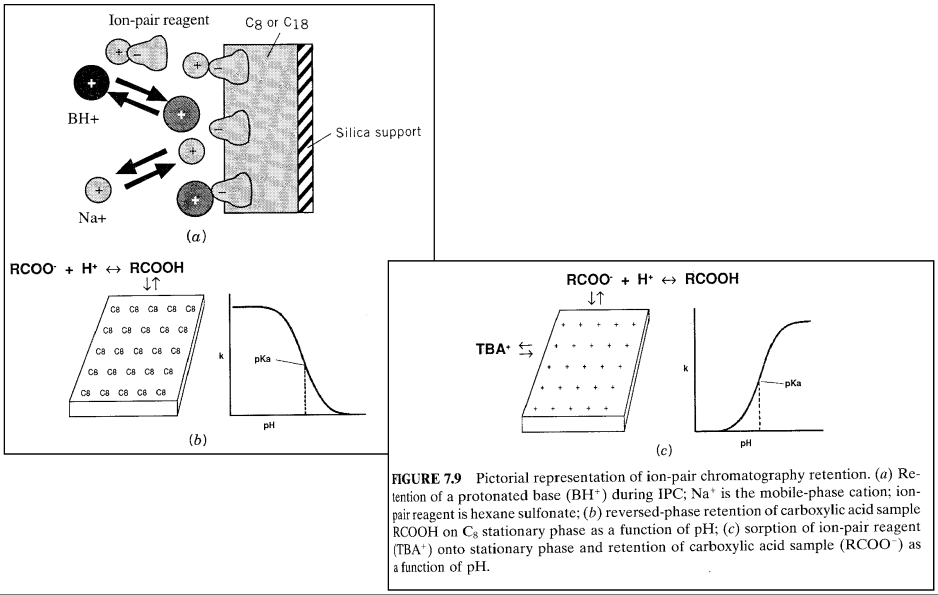


FIGURE 7.9 Pictorial representation of ion-pair chromatography retention. (*a*) Retention of a protonated base (BH^+) during IPC; Na^+ is the mobile-phase cation; ion-pair reagent is hexane sulfonate; (*b*) reversed-phase retention of carboxylic acid sample RCOOH on C_8 stationary phase as a function of pH; (*c*) sorption of ion-pair reagent (TBA^+) onto stationary phase and retention of carboxylic acid sample ($RCOO^-$) as a function of pH.

IPC Optimization

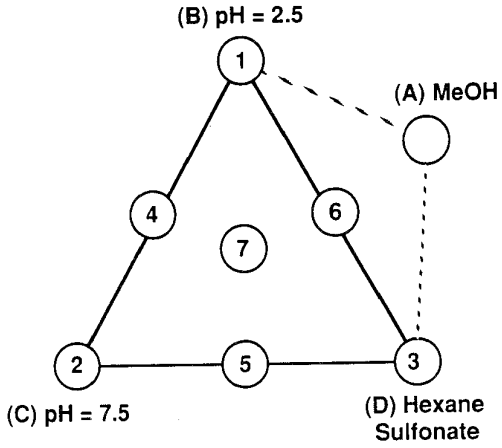
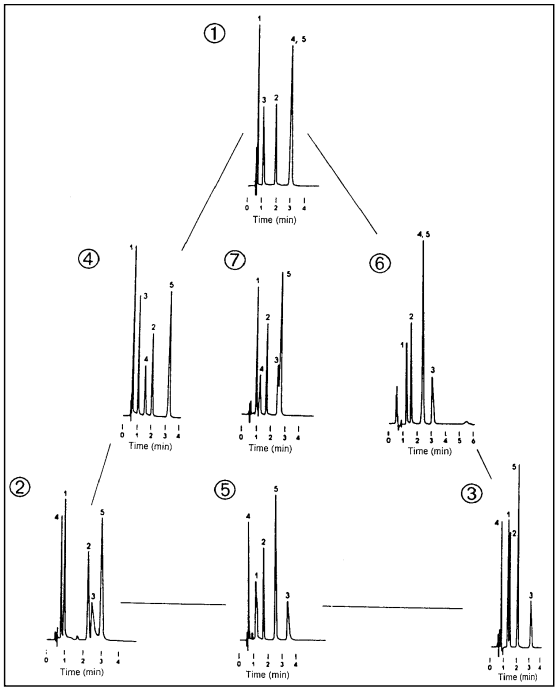


FIGURE 7.14 Experimental design for rapid optimization of retention range and selectivity in ion-pair HPLC. Simultaneous variation of mobile-phase pH and ion-pair reagent concentration. (Reprinted with permission from Ref. 43.)

IPC Optimization Example I



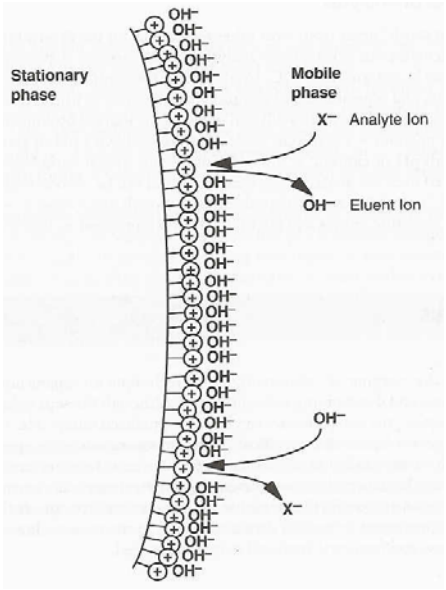
IPC Optimization Example II

FIGURE 7.15 Application of optimization scheme of Fig. 7.14 for the separation of a cold–cough remedy. Sample: a mixture of five compounds: 1, phenylephrine; 2, glycerol guaicolate; 3, pseudoephedrine; 4, sodium benzoate; 5, methylparaben. Conditions: 15 × 0.46-cm Zorbax C8 column, with mobile phases as follows:

	Vol % Solvent in Mobile Phases 1 to 7						
Solvent	1	2	3	4	5	6	7
A: methanol	30	27	34	29	30	32	30
B: pH 2.5 buffer	70	0	0	35	0	35	23
C: pH 7.5 buffer	0	73	0	36	36	0	24
D: 200 m*M* hexane sulfonate	0	0	66	0	33	33	22

(Reprinted with permission from Ref. 43.)

Concept of Ion-Exchange C



Synthesis of Ion Exchange Resins

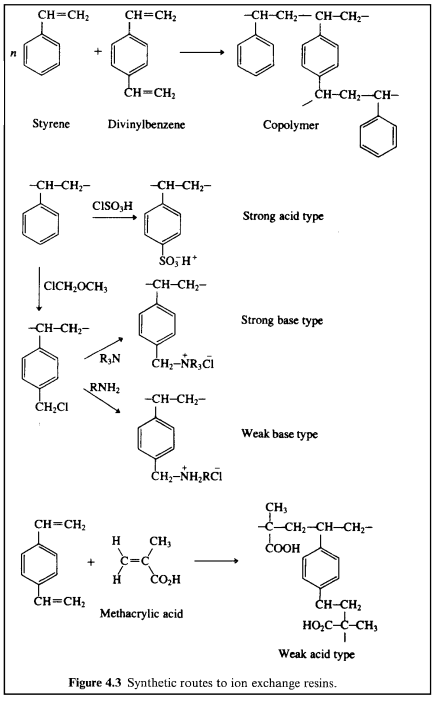


Figure 4.3 Synthetic routes to ion exchange resins.