Chapter 28

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

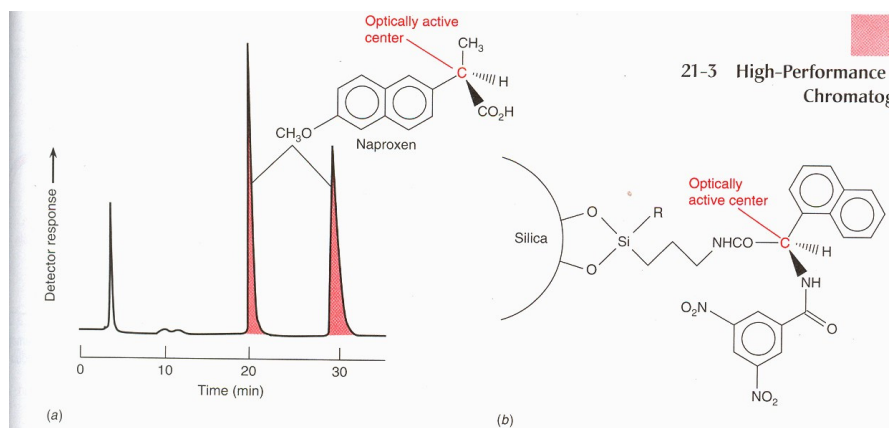NOTE: HPLC came about because <u>not all</u> compounds can be <u>vaporized</u> and analyzed on a GC

FIGURE 21-15 (a) HPLC separation of the two optical isomers (mirror image isomers) the drug Naproxen eluted with 0.05 M ammonium acetate in methanol. Naproxen is the [acti]ve ingredient of the anti-inflammatory drug Aleve. (b) Structure of the bonded stationary [pha]se. [Courtesy Phenomenex, Torrance, CA.]

Source: D.C. Harris, Exploring Chemical Analysis, 2nd ed. (2001)

Summary of Method

High Performance Liquid Chromatography (HPLC)

❖ An analytical separation technique that involves the high-pressure flow of a liquid through a column that contains the stationary phase.
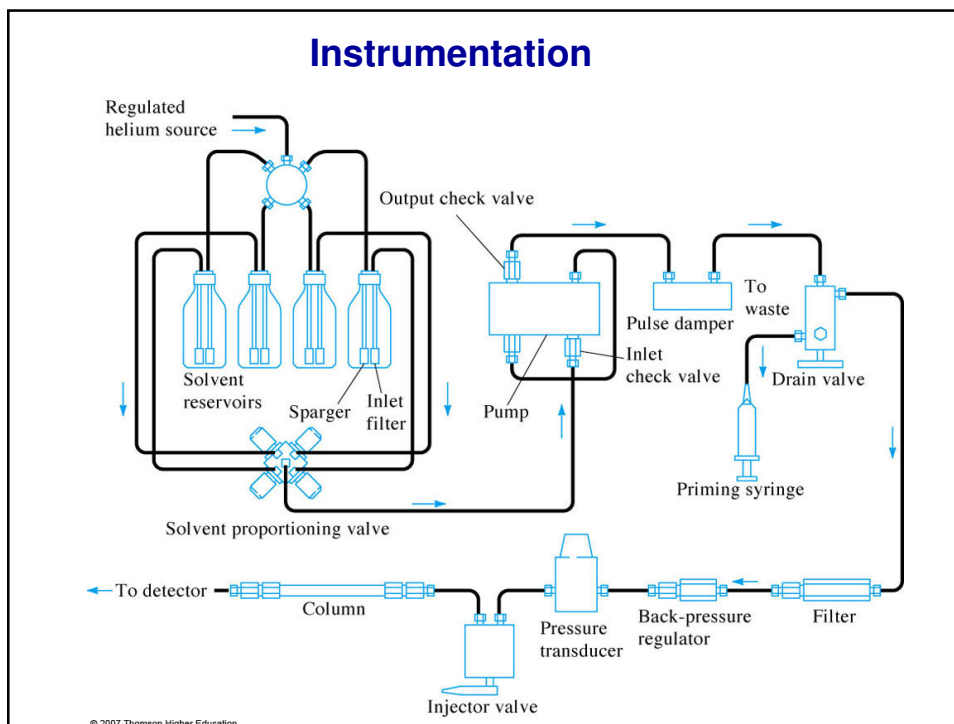
　　Mobile phase: Liquid

　　Stationary phase: Can be a solid (LSC) or a liquid (LLC)

❖ A mixture of compounds injected at one end of the column separates as the compounds pass through.

❖ Separated compounds are detected <u>electronically</u> as they elute at the other end of the column.

Comparison with GC

Parameter	GC	HPLC
Basis of separation	<u>Interaction</u> of solutes with the s.p.; solute <u>vapor pressure</u>	<u>Interaction</u> of solutes with <u>both</u> the s.p. and m.p.
Analysis time	*Fast* (a few minutes for simple mixtures)	*Slower than GC* (several minutes for a simple mixture
Temperature for separation	Usually requires a *high temperature* (>40 ^{0}C)	Usually a *room temperature* technique
Applications	Separation of <u>volatile</u> and <u>thermally stable</u> compounds - cannot be used for high MW and highly polar compounds	Separation of a wider range of compounds -- <u>high MW</u>, <u>polar</u>, and <u>ionic</u> compounds

Instrumentation

Regulated
helium source →

Output check valve

Solvent
reservoirs — Sparger — Inlet filter

Solvent proportioning valve

Pulse damper

To waste

Inlet check valve

Pump

Drain valve

Priming syringe

← To detector

Column

Pressure transducer

Injector valve

Back-pressure regulator

Filter

Instrumentation (Cont.)

Major components

1) **Solvent** or **mobile phase**

➤ Usually a mixture of an organic solvent (Ex. methanol, IPA) and water

➤ Solvent polarity affects the separation process

➤ Sometimes buffered - keeps solutes in electrically neutral form
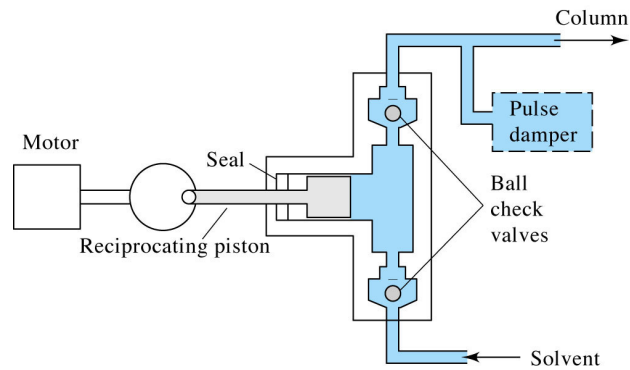
Mobile phase considerations

❖ Must be filtered (to prevent tiny solids from depositing at the column head) and degassed

➤ Bubbles could interfere with detection

➤ Degassing is done by **helium sparging**
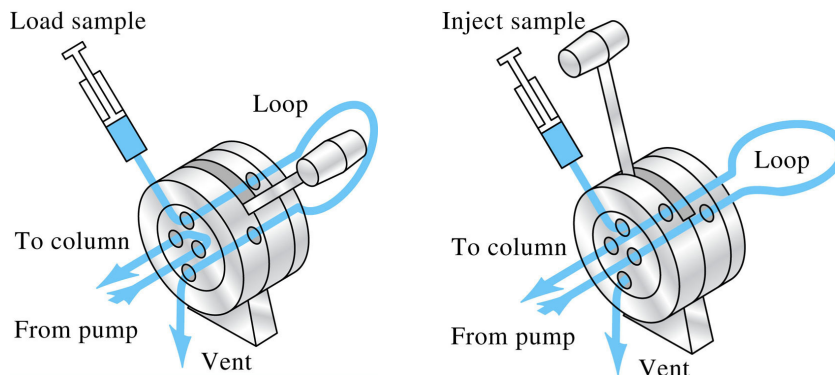
Instrumentation (Cont.)

2. **Pump**

➢ Role is to pump the solvent at a high pressure (usually from 1000 to 6000 psi) through the packed column

Column

Pulse
damper

Motor

Seal

Ball
check
valves

Reciprocating piston

Solvent

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Instrumentation (Cont.)

3. **Sample introduction system**

➢ Introduces the injected sample to the flowing mobile phase
➢ Usually a loop injector – see image below
➢ Automated injectors are common

Load sample

Loop

To column

From pump

Vent

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Inject sample

Loop

To column

From pump

Vent

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Instrumentation (Cont.)

4. **Column**

 ➢ A small metal tube (typically 5 to 30 cm long; 1-5 mm i.d.) that contains the stationary phase

 ➢ Role is to separate the components of a mixture
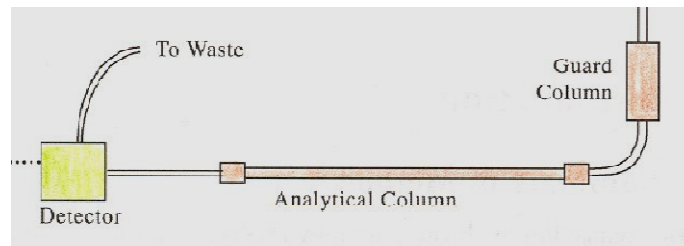
Column – Cont.

 ➢ Much shorter than columns used in GC --- Why?

 ❖ **Highly efficient separations** achieved in HPLC due to interactions of both m.p. and s.p. with the components of a mixture

 ➢ No need for long columns

 ➢ vs. GC, where only the s.p. interacts with components

Instrumentation (Cont.)

5. **Detector**

➢ Different design from those of GC detectors because the components are dissolved in a <u>liquid</u> m.p. (vs. gas in GC)

TABLE 28-1 Performance of HPLC Detectors

HPLC Detector	Commercially Available	Mass LOD* (typical)	Linear Range† (decades)
Absorbance	Yes	10 pg	3–4
Fluorescence	Yes	10 fg	5
Electrochemical	Yes	100 pg	4 5
Refractive index	Yes	1 ng	3
Conductivity	Yes	100 pg–1 ng	5
Mass spectrometry	Yes	<1 pg	5
FTIR	Yes	1 µg	3
Light scattering	Yes	1 µg	5
Optical activity	No	1 ng	4
Element selective	No	1 ng	4–5
Photoionization	No	<1 pg	4

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Detectors – *Cont.*

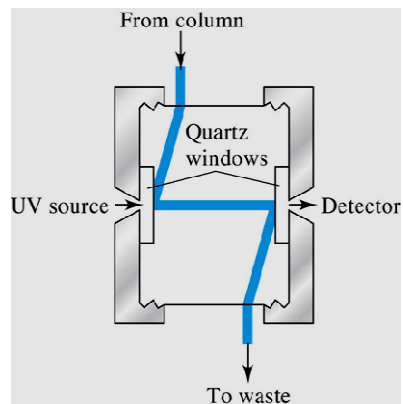**UV detectors** – most common

➢ Applications: Respond to substances that absorb light in the range 180 to 350 nm

  ❖ Most organics

    ➢ π systems (aromatics, alkenes, alkynes)

    ➢ Carbonyls

UV absorption cell for HPLC

➢ Z-shaped flow cell - > more time for UV light to pass th/

From column

Quartz windows

UV source → Detector

To waste

Mass spectrometric detectors – in LCMS

➢ Challenges: (1) Converting <u>liquid column effluent to gas</u>
(Recall: MS is a gaseous phase technique) and

(2) removing a lot of solvent before entering MS

Electrospray ionization*

❖ Use of nanoscale capillary LC – flow rates of μL/min

HPLC system	→	Ion source	→	Mass analyzer	→	Ion detector

Vacuum system

Data system
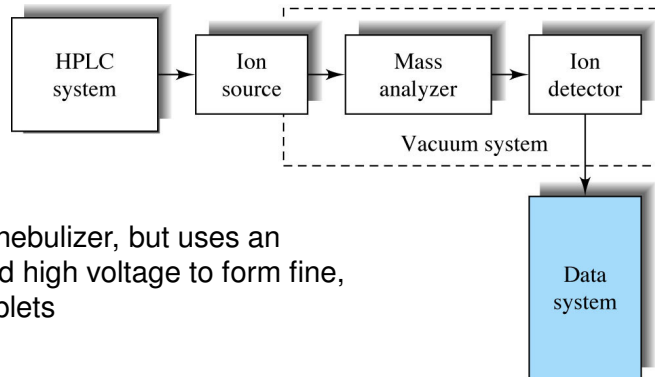
* Similar to a nebulizer, but uses an electrode and high voltage to form fine, charged droplets

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HPLC Column Selection

Dependent on the:

(1) type of mixture being separated, and

(2) type of interaction with the s.p.

Two **kinds of liquid chromatography** based on the **type of mixture** being separated

1) **Normal phase chromatography** utilizes a:

  ❖ **polar s.p**.
  ❖ nonpolar m.p.

➤ Has its origin in older LSC, which utilized <u>polar silica</u> or alumina s.p.

➤ Uses **normal phase columns**

   ➤ Made of <u>polar</u> <u>stationary phase</u> bonded to a solid support

Called ***bonded phase s.p.***

Application: Separation of <u>polar</u> <u>compounds</u> (amino acids and peptides, alcohols, carboxylic acids)

Examples of **polar bonded (stationary) phases**:

Bonded polar phases		Bonded nonpolar phases	
$R = (CH_2)_3NH_2$	Amino	$R = (CH_2)_{17}CH_3$	Octadecyl
$R = (CH_2)_3C \equiv N$	Cyano	$R = (CH_2)_7CH_3$	Octyl
$R = (CH_2)_2OCH_2CH(OH)CH_2OH$	Diol	$R = (CH_2)_3C_6H_5$	Phenyl

Examples of **nonpolar** or **low polarity mobile phases**

➤ Hydrocarbons, such as **hexane** (C_6H_{14}) and **toluene** (C_7H_8)

➤ Chloroform, $CHCl_3$

2) **Reverse phase chromatography** - utilizes a:

❖ **nonpolar s.p.** and
❖ **polar m.p.**

➢ Uses **reverse phase columns** = contains ***nonpolar bonded phase s.p.***

Application: Separation of nonpolar compounds

Bonded polar phases		Bonded nonpolar phases	
$R = (CH_2)_3NH_2$	Amino	$R = (CH_2)_{17}CH_3$	Octadecyl
$R = (CH_2)_3C \equiv N$	Cyano	$R = (CH_2)_7CH_3$	Octyl
$R = (CH_2)_2OCH_2CH(OH)CH_2OH$	Diol	$R = (CH_2)_3C_6H_5$	Phenyl

Octyl = C_8 *Octadecyl* = C_{18}

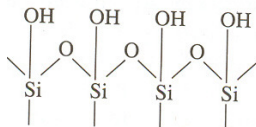**Reverse phase chromatography** (Cont.)

Example of **nonpolar bonded (stationary) phases**:
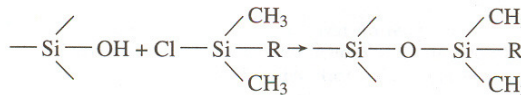➢ Hydrocarbons (with C_8 and C_{18}; most common)

Example of **polar mobile phases**
➢ A mixture of water and an organic solvent (methanol, acetonitrile)

Note: Bonded phases are usually attached to a silica or silica-based solid support

Silica w/ reactive
silanol (Si-OH) groups

Replacement of silanol group w/ R
groups to produce **bonded phases**

Selection of Mobile Phase

Eluent strength = ability of the m.p. to elute a solute from the column

➢ Increased by *approaching the polarity of the s.p.* (i.e. by competing w/ the s.p. for the solute)

❖ Results to better elution of components at a shorter analysis time

Q. What changes can be done to increase the eluent strength in HPLC?

Normal phase chromatography

↑ eluent strength by ↑ polarity of the m.p.

In practice: Add a miscible, <u>more polar</u> solvent

Reverse phase chromatography

↑ eluent strength by ↓ polarity of the m.p.

In practice: Add a miscible, <u>less polar</u> solvent

Increasing eluent strength (Cont.)

Drill: Arrange the following solvents in the order of <u>increasing polarity</u>.

❖ Acetonitrile; hexane; water; methanol; isopropyl alcohol

Answer:

Hexane < Acetonitrile < Isopropyl alcohol < Methanol < Water

Increasing eluent strength (Cont.)

➢ Normal phase chromatography => Increase proportion of water or methanol, or

➢ decrease amount of hexane (becomes more polar)

➢ Reverse phase chromatography => Decrease proportion of water or methanol

Effect of other parameters on retention of solutes

1. **Column length effect**

    $\uparrow$ length, $\uparrow t_R$

    $\uparrow$ analysis time

2. **Polarity of the s.p.**

    ❖ Polar solutes are more soluble (more retained) in polar s.p.

3. **Mobile phase flow rate**

    $\uparrow$ m.p. flow rate, $\downarrow t_R$ = $\downarrow$ analysis time
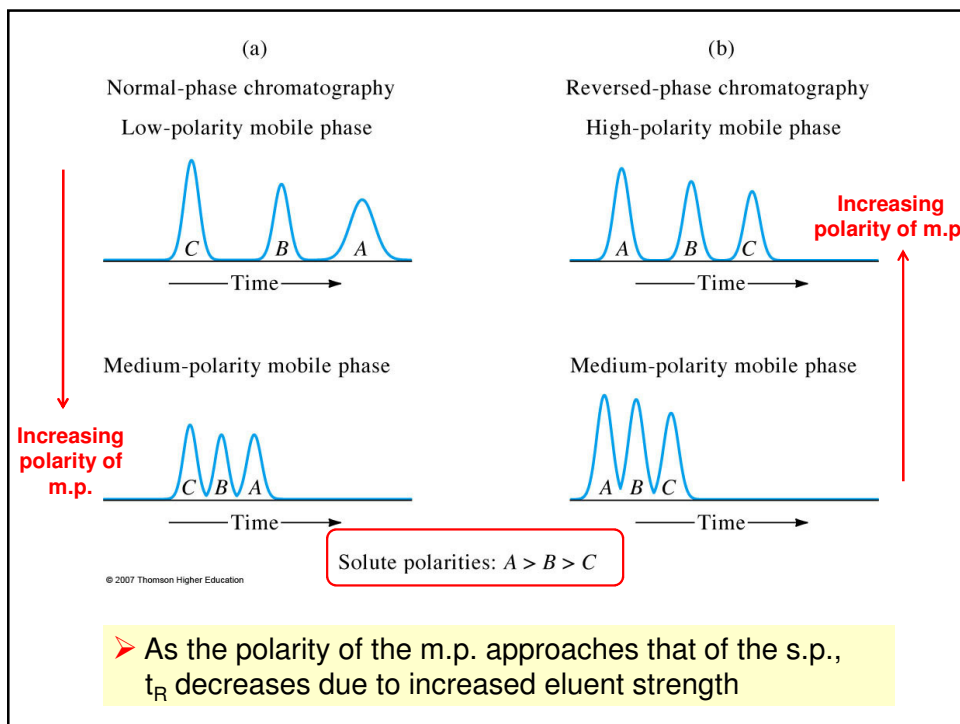

Effect of other parameters …

Q. How would you decrease t_R of strongly retained solutes?

    ❖ Increase m.p. flow rate, and/or

    ❖ Increase eluent strength, and/or

    ❖ Use a shorter column => last resort

4. **Polarity of mobile phase**

    ➢ Polar m.p. elutes polar components faster; Nonpolar m.p. elutes nonpolar components faster

(a)
Normal-phase chromatography
Low-polarity mobile phase

Medium-polarity mobile phase

(b)
Reversed-phase chromatography
High-polarity mobile phase

Increasing polarity of m.p.

Medium-polarity mobile phase

Increasing polarity of m.p.

Solute polarities: $A > B > C$

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➢ As the polarity of the m.p. approaches that of the s.p., t_R decreases due to increased eluent strength

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Effect of other parameters on retention of solutes (Cont.)

5. **Nature of bonded s.p.**
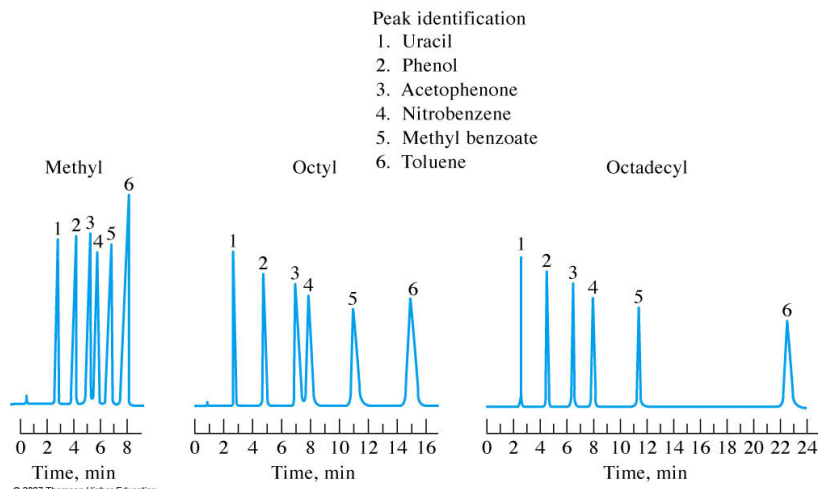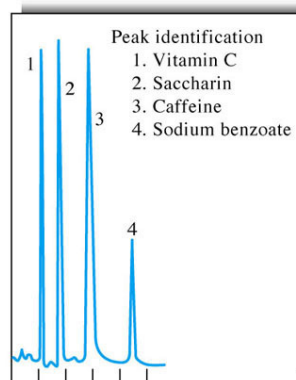
➢ Retention increases as chain length increases

Peak identification
1. Uracil
2. Phenol
3. Acetophenone
4. Nitrobenzene
5. Methyl benzoate
6. Toluene

Methyl

Octyl

Octadecyl

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TABLE 28-3 Typical Applications of Partition Chromatography

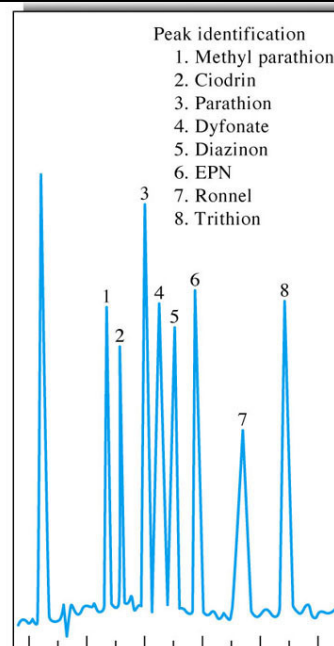| Field	Typical Mixtures
Pharmaceuticals	Antibiotics, sedatives, steroids, analgesics
Biochemical	Amino acids, proteins, carbohydrates, lipids
Food products	Artificial sweeteners, antioxidants, aflatoxins, additives
Industrial chemicals	Condensed aromatics, surfactants, propellants, dyes
Pollutants	Pesticides, herbicides, phenols, polychlorinated biphenyls
Forensic science	Drugs, poisons, blood alcohol, narcotics
Clinical chemistry	Bile acids, drug metabolites, urine extracts, estrogens

Challenge: *What kind of samples would contain these compounds?*

Peak identification
1. Methyl parathion
2. Ciodrin
3. Parathion
4. Dyfonate
5. Diazinon
6. EPN
7. Ronnel
8. Trithion

Peak identification
1. Vitamin C
2. Saccharin
3. Caffeine
4. Sodium benzoate

(a) Time, min

(b) Time, min

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Solvent composition in HPLC

ISOCRATIC vs. GRADIENT ELUTION

➢ Analogous to *isothermal vs. programmed* temperature in GC in terms of outcome

Isocratic elution

❖ Uses a *constant solvent composition* throughout the separation process

❖ Ideal for the separation of simple mixtures

Gradient elution

❖ Solvent *composition is changed* in time (by increasing eluent strength)

❖ Applies to the separation of more complex mixtures

Isocratic *vs.* Gradient Elution of chlorinated benzenes on a C_{18} column

Isocratic elution

➢ Poor resolution of early-eluting solutes
➢ Extremely long t_R of late-eluting solutes

(a) Gradient elution

Peak identity

1. Benzene
2. Monochlorobenzene
3. Orthodichlorobenzene
4. 1,2,3-trichlorobenzene
5. 1,3,5-trichlorobenzene
6. 1,2,4-trichlorobenzene
7. 1,2,3,4-tetrachlorobenzene
8. 1,2,4,5-tetrachlorobenzene
9. Pentachlorobenzene
10. Hexachlorobenzene
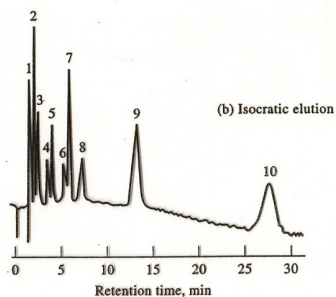
(b) Isocratic elution

Retention time, min

Figure 28-5 Improvement in separation efficiency by gradient elution. Column: 1 m × 2.1 mm i.d., precision-bore stainless; packing: 1% Permaphase® ODS. Sample: 5 μL of chlorinated benzenes in isopropanol. Detector: UV photometer (254 nm). Conditions: temperature, 60°C, pressure, 1200 psi. *(From J. J. Kirkland,* Modern Practice of Liquid Chromatography, *p. 88. New York: Interscience, 1971. Reprinted by permission of John Wiley & Sons, Inc.)*
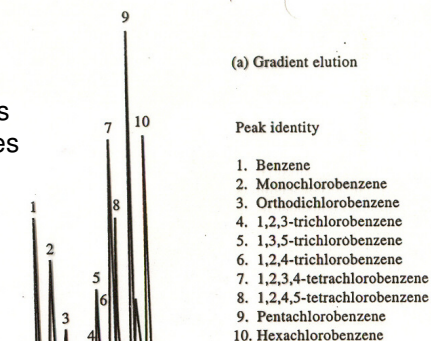
Gradient elution

➢ Improved separation within a much shorter time

Source: Skoog, Holler and Nieman, Principles of Instrumental Analysis." 5th ed., Harcourt Brace,1998.