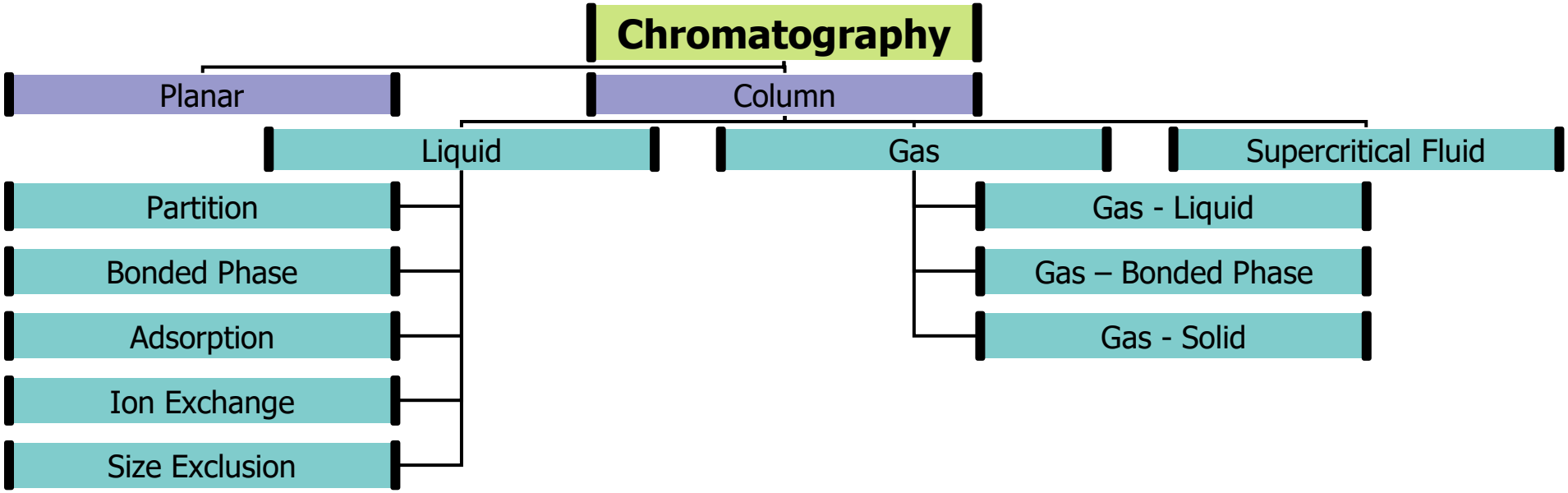
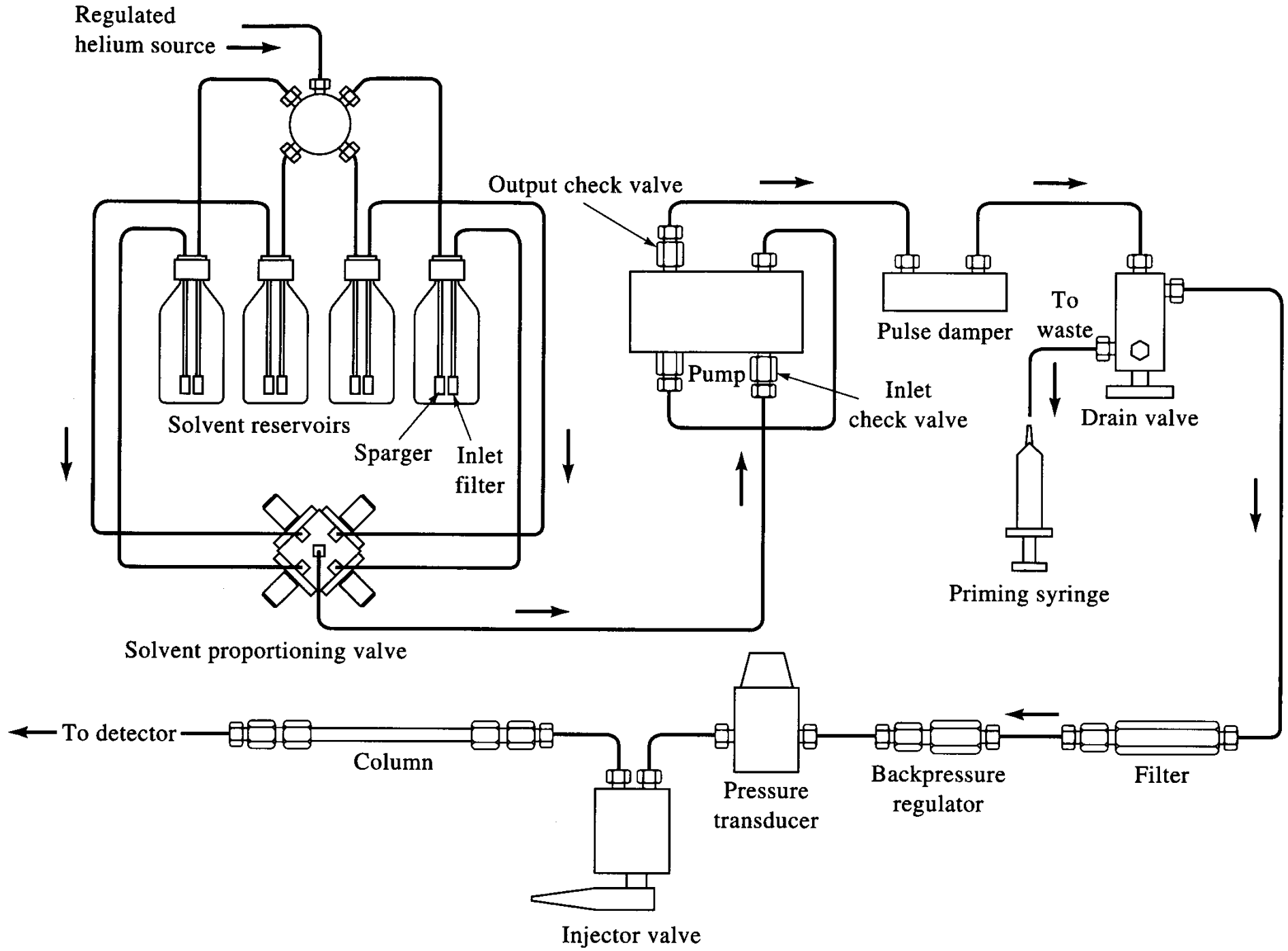
BAB 8
KROMATOGRAFI CAIR:
High Performance Liquid Chromatography (HPLC)

Tipe Kromatografi



Komponen Dasar pada HPLC:

1. **Pompa.** Mobil phase pressures up to 600 psi are necessary to achieve reasonable column elution times (~ minutes). Typical flow rates are 0.1 to 10 mL/minute.
2. **Sistem Injeksi.** Used to introduce small samples (0.1 to 500 µL) into the carrier stream under high pressure.
3. **Reservoirs (Solvents).** Multiple solvents are necessary for performing gradient elution's (i.e. changing the polarity of the mobil phase during a run).
4. **Kolom Kromatografi** Typically 10-30 cm in length containing a packing of 5-10 µm diameter. Many types of columns are available, depending on the type of liquid chromatography desired.
5. **Detektor.** Many types are available including UV, IR, refractive index, fluorescence, conductivity, mass spectrometry, and electrochemical. Diode array detectors are used when wavelength scans are desired.


Regulated
helium source
Output check valve
Pulse damper
To
waste
Pump
Inlet
check valve
Drain valve
Solvent reservoirs
Sparger
Inlet
filter
Priming syringe
Solvent proportioning valve
To detector
Column
Pressure
transducer
Backpressure
regulator
Filter
Injector valve

Pompa

Desirable Features:

- Must generate pressures up to 6,00 psi
  - To allow for separation in reasonable time frames
- Flow-rates range from 0.1 to 10 mL/minute
- Limited pulsing in the system
  - Many HPLC systems have a dual pump system to minimize pulsing
- Corrosion resistance

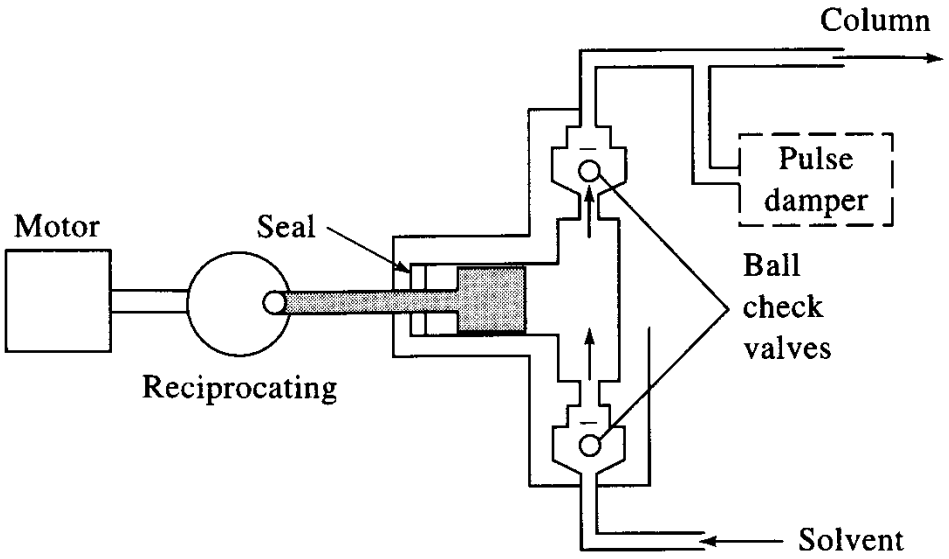


Figure 28-6 A reciprocating pump for HPLC.

Sistem Injeksi

Used to introduce small samples (0.001 to 0.5 mL) into the carrier stream under high pressure

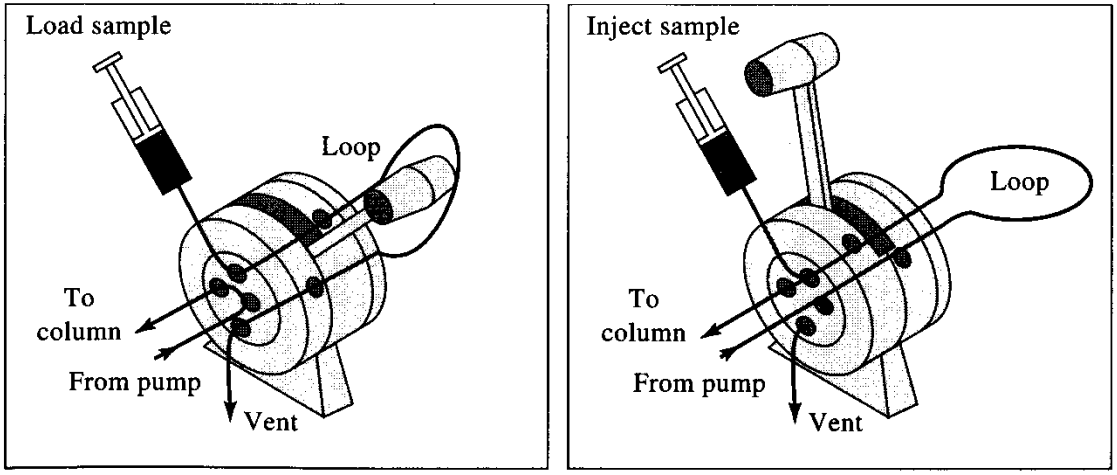


Figure 28-7 A sampling loop for liquid chromatography. *(Courtesy of Beckman Instruments, Fullerton, CA.)* With the valve handle as shown on the left, the loop is filled from the syringe, and the mobile phase flows from pump to column. When the valve is placed in the position on the right, the loop is inserted between the pump and the column so that the mobile phase sweeps the sample onto the column.

Detektor

- Tipe
  - General – respond to mobil phase bulk properties which vary in the presence of solutes (e.g. refractive index)
  - Specific – respond to some propert of the solute (not possessed by the mobil phase (e.g. UV absorption)
  - “Hyphenated” detector – LC-MS

DETEKTOR

- Detektor umum : deteksi pada properti-keadaan pelarut/F_M (contoh perubahan indeks bias dalam pelarut, sehingga kurang sensitiv (limit deteksi <<))

  - tidak sesuai untuk elusi bergradien

  - dapat mendeteksi solut 10^{-6} gram

  - dapat mendeteksi semua solut yang keluar dari kolom karena berdasarkan perubahan indeks bias pelarut

- Detektor selektif/khusus : deteksi pada properti-keadaan/sifat molekul cuplikan/zat terlarut

  - sesuai untuk elusi bergradien

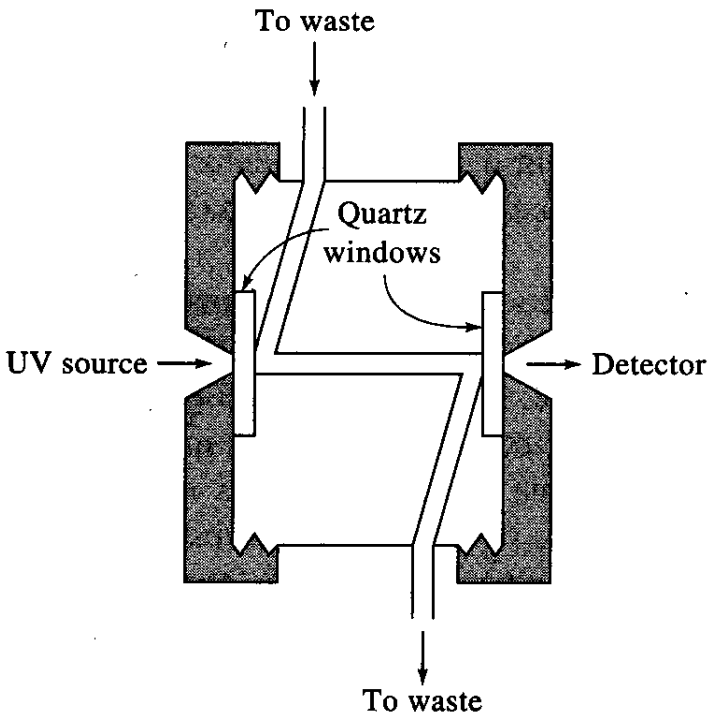
  - dapat mendeteksi solut 10^{-9} gram

  - untuk analisis rutin, dengan solut yang runut

  - hanya tepat untuk mendeteksi solut yang memiliki sifat optis/dapat menyerap rentang panjang gelombang UV

Absorbance Detectors

- The UV/Vis source usually comes from a monochromator so the wavelength can be selected, or scanned.
- Absorbance increases as eluate passes through the cell.
- If wavelength scanning is desired, the flow is stopped long enough for the scan to take place.
- It's possible to have the same setup using IR light, although not as common since many useful solvents are not IR transparent.



Diode Array Detector

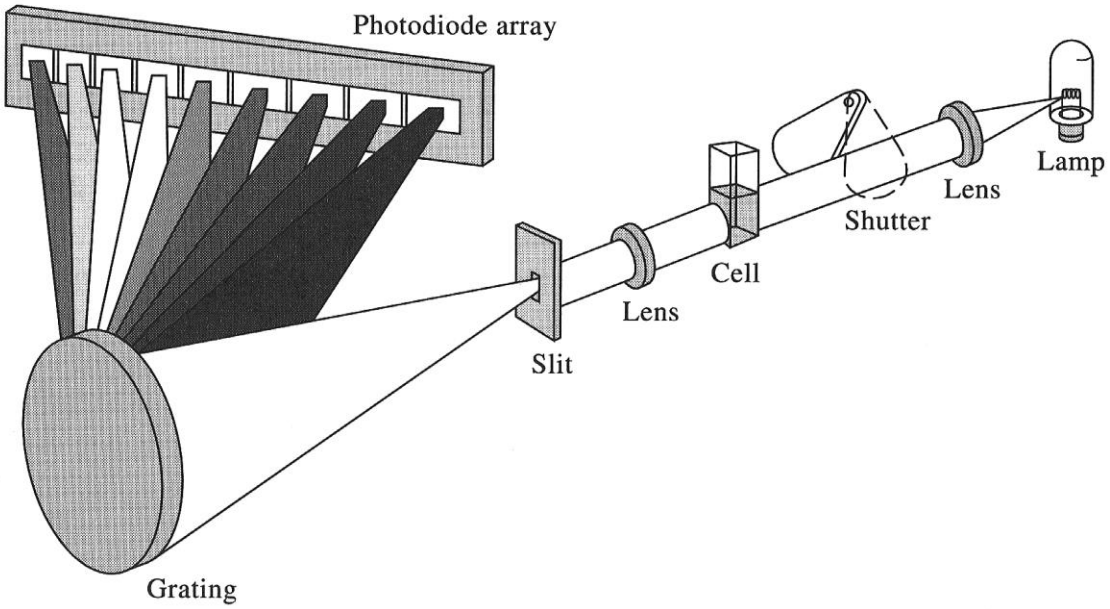


Figure 13-22 A multichannel diode array spectrometer; the HP 8452A. *(Courtesy of Hewlett-Packard Company, Palo Alto, CA.)*

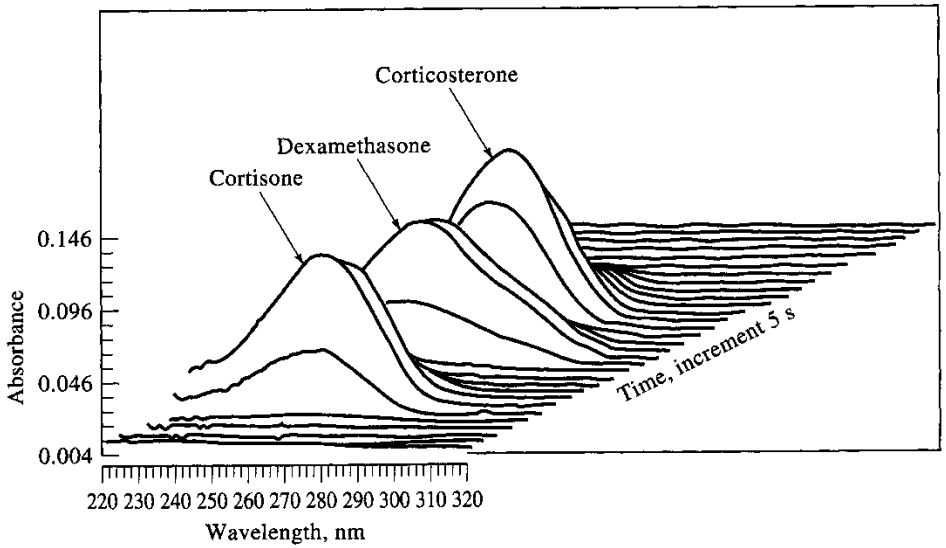
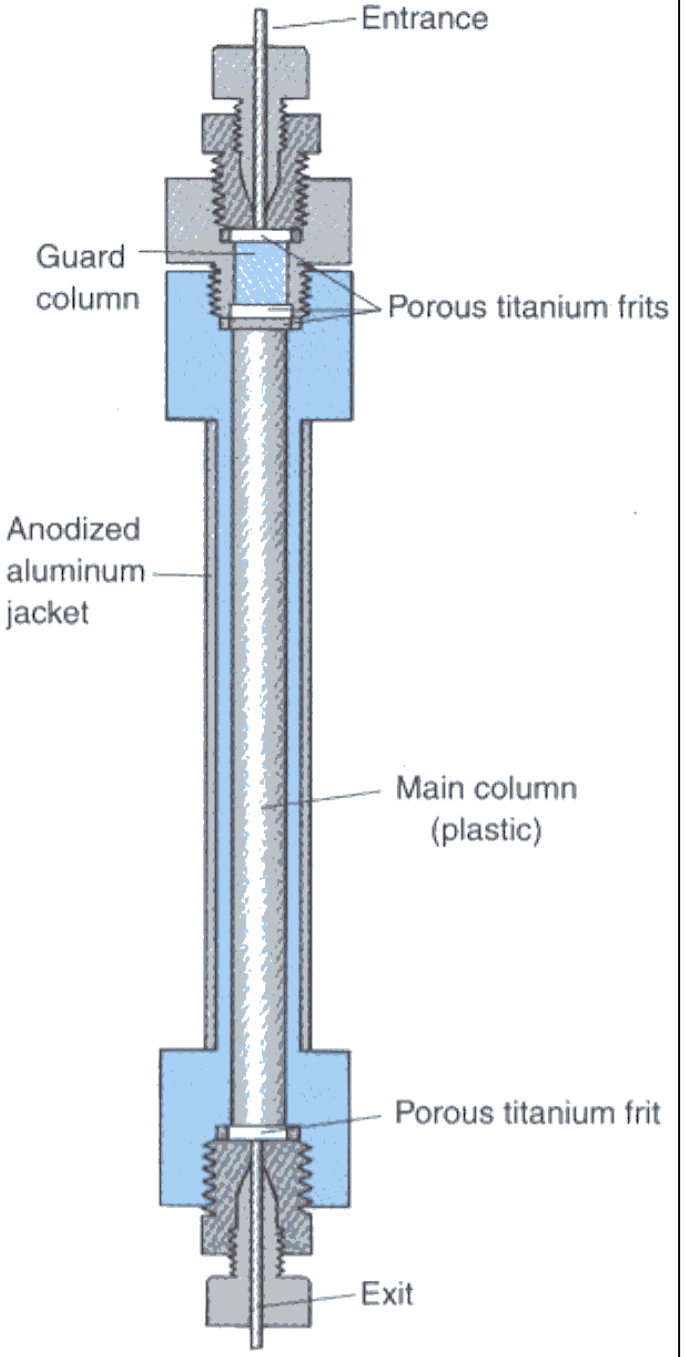


Figure 28-10 Absorption spectra of the eluent from a mixture of three steroids taken at 5-second intervals. *(Courtesy of Hewlett-Packard Company, Palo Alto, CA.)*



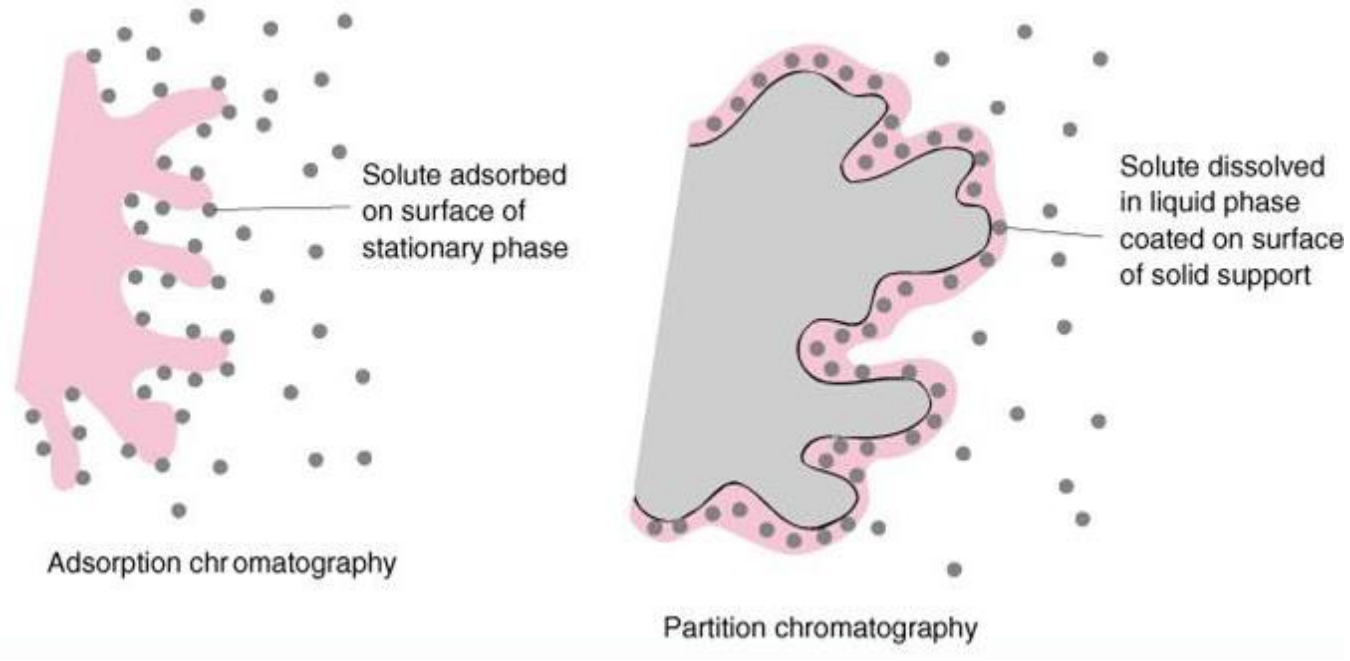
Kolom HPLC

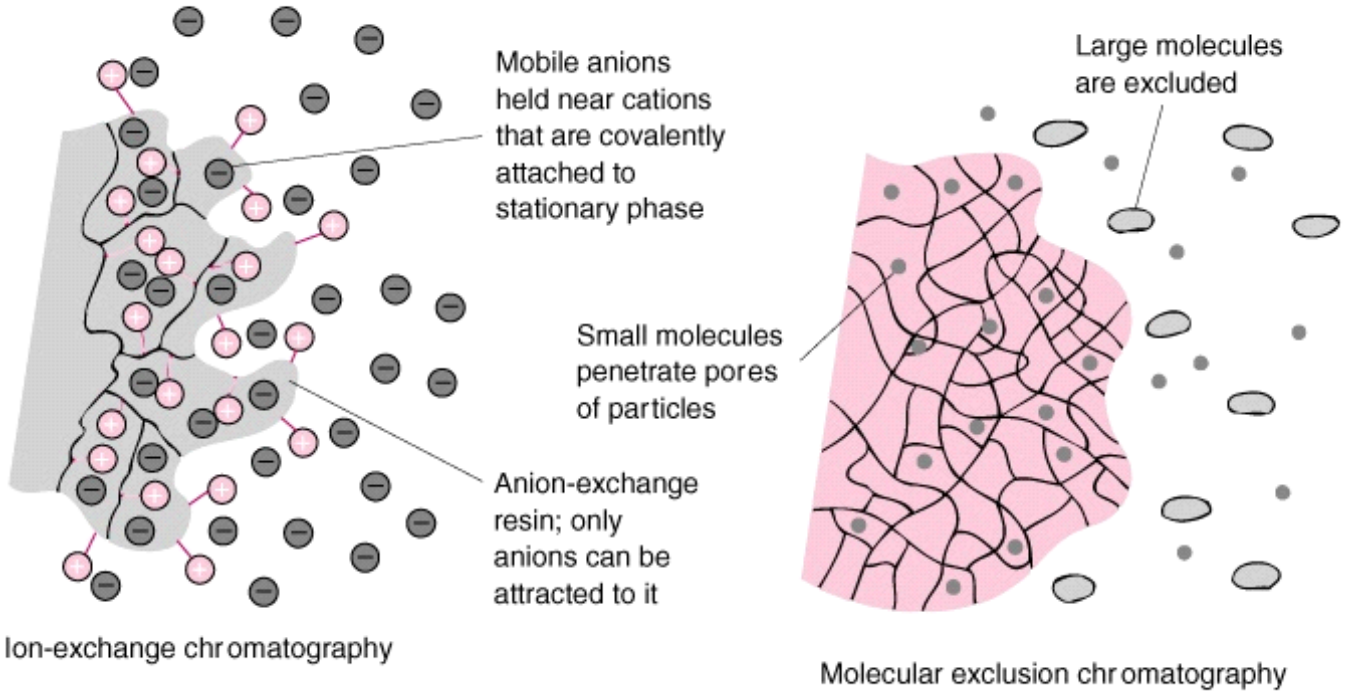
- Must operate in high pressure
  - Usually constructed of metals
- Typical dimensions
  - 10-30 cm long
  - 1-3 cm ID
- Contains packing material which holds the stationary phase
  - Many types exist
  - Typical packing materials are 5-10 µm in diameter
- Guard column used to extend life of main column

Jenis-jenis Kromatografi Cair

1. Liquid-Liquid or Partition Chromatography
   - The stationary phase is a liquid adsorbed on a solid
2. Liquid-Bonded Phase Chromatography
   - The stationary phase is an organic species bonded to a solid surface
3. Liquid-Solid or Adsorption Chromatography
   - The stationary phase is a solid
4. Ion-Exchange Chromatography
   - The stationary phase is an ion-exchange resin
5. Size Exclusion or Gel Permeation Chromatography
   - The stationary phase is a liquid in the intersticies of a polymeric solid

Types of LC


Solute adsorbed on surface of stationary phase
Adsorption chromatography
Solute dissolved in liquid phase coated on surface of solid support
Partition chromatography


Mobile anions held near cations that are covalently attached to stationary phase
Anion-exchange resin; only anions can be attracted to it
Ion-exchange chromatography
Large molecules are excluded
Small molecules penetrate pores of particles
Molecular exclusion chromatography

Separation Principles in HPLC

General Rule of Thumb:

Polarity of analytes ≈ polarity of stationary phase ≠ polarity of mobil phase

To achieve good separation, the analytes should interact with the stationary phase, but not too strongly or the retention time will become very long

Normal and Reversed Phased Liquid Chromatography

1. Characteristics of Normal Phase Chromatography

- Highly polar stationary phase
  - Silica or alumina oxides
- Relatively non-polar solvent
  - e.g. hexane or i-propylether
- Least polar solutes elute first
- Increasing mobil phase polarity decreases elution times (i.e. polar compounds remain in the mobil phase longer)

2. Characteristics of Reversed Phase Chromatography

- Non-polar stationary phase
  - e.g. a hydrocarbon
- Relatively polar mobil phase
  - e.g. water, methanol or acetonitrile
- More polar solutes elute first
- Penurunan kepolaran fasa gerak akan meningkatkan waktu elusi

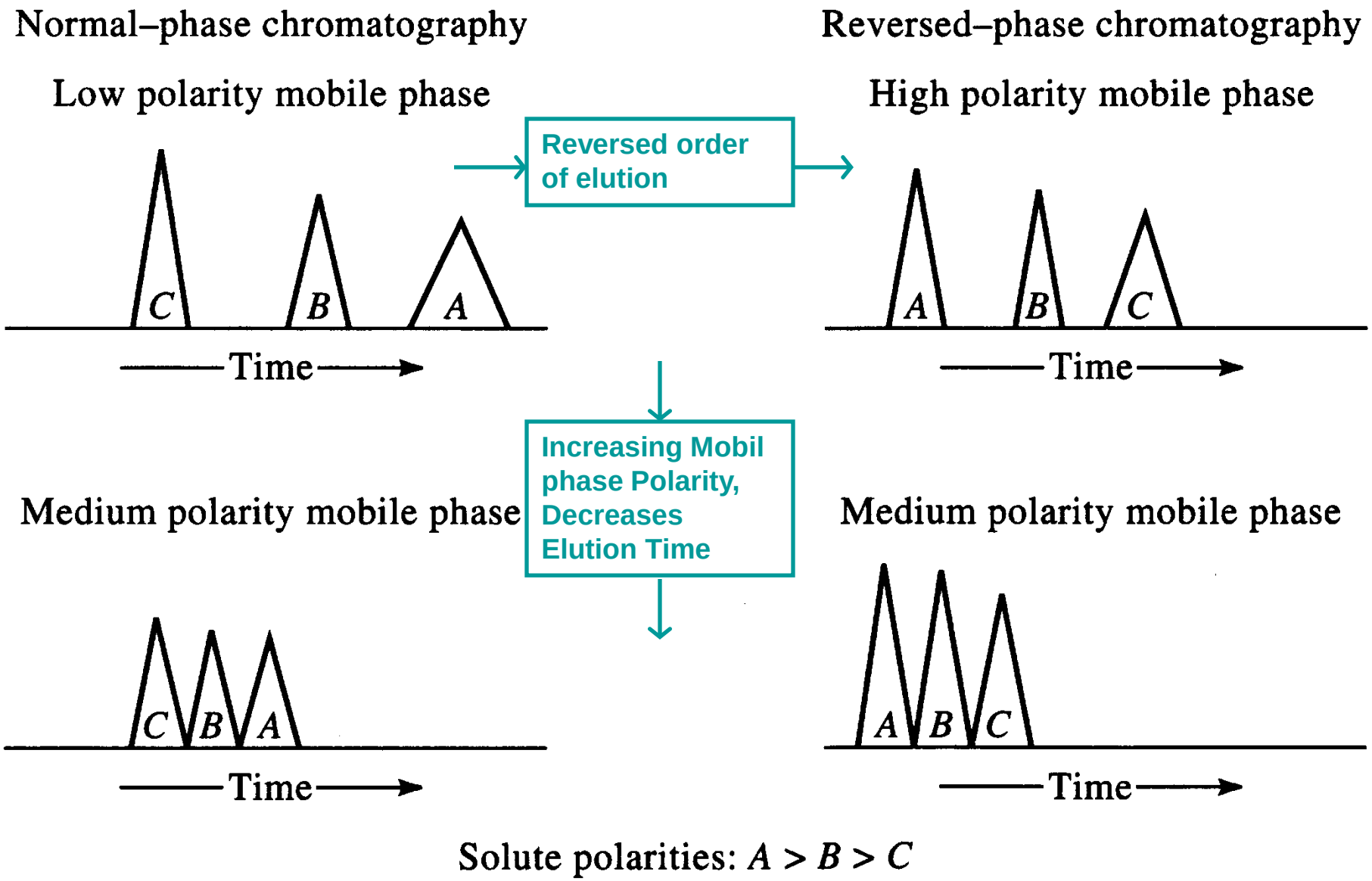


Figure 28-14 The relationship between polarity and elution times for normal-phase and reversed-phase chromatography.

1. Liquid-Liquid or Partition Chromatography

Characteristics

- A liquid stationary phase is retained on the surface of the packing.
- Separation is based on differences in the polarity of the solutes.
- One phase will be polar and the other non-polar.

Kromatografi cair-cair mengikuti prinsip ekstraksi cair-cair

- Diperlukan fasa diam yang tidak larut dalam fasa gerak (kenyataannya tdk ada yang benar2 tidak larut, karena dilewati berliter-liter fasa gerak dengan tekanan tinggi
- Fasa diam disalutkan secara fisis, sehingga mudah tererosi. Contoh Fs: Corbowax, Oksidipropionitril
- Biasanya diatasi dengan menjenuhkan fasa diam terhadap fasa gerak
- Fs Tidak tahan terhadap perubahan suhu karena akan mengubah kelarutannya
- K = Cs/Cm

Typical Applications of Partition Chromatography

Field of Application	Separation
Pharmaceuticals	Antibiotics, Sedatives, Steroids, Analgesics
Biochemical	Amino acids, Proteins, Carbohydrates, Lipids
Food Products	Artificial Sweeteners, Antioxidants, Preservatives
Industrical Chemicals	Condensed Aromatics, Surfactants, Propellants, Dyes
Forensic Chemistry	Drugs, Poisons, Blood Alcohol, narcotics
Clinical Medicine	Bile Acids, Drug Metabolites, Urine Extracts, Estrogens
Pollutants	Pesticides, Herbicides, Phenols, PCBs

Partition Chromatography – Example Chromatograms

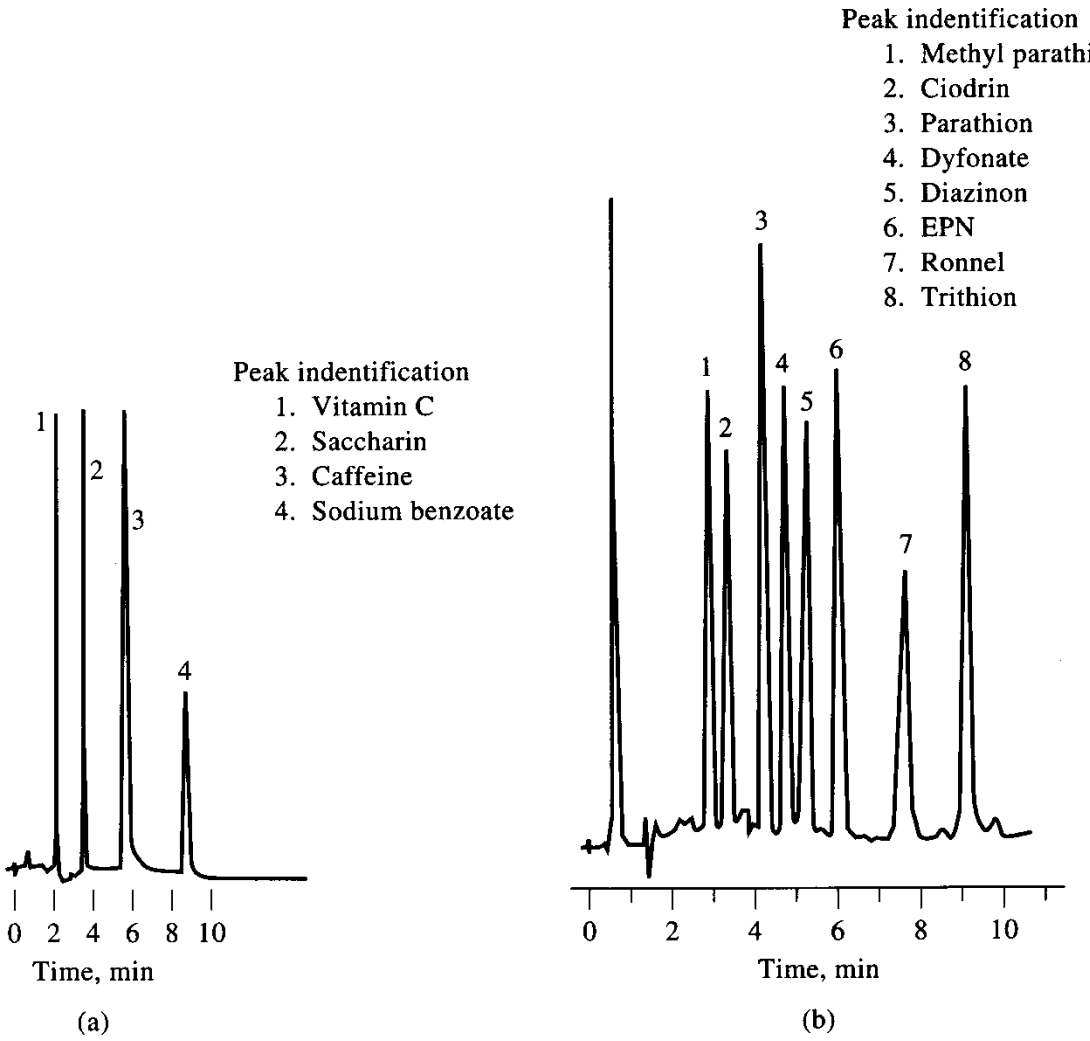


Figure 28-17 Typical applications of bonded-phase chromatography. (a) Soft-drink additives. Column: 4.6 × 250 mm paced with polar (nitrile) bonded-phase packings. Isocratic solvent: 6% HOAC/94% H_2O. Flow rate: 1.0 cm^3/min. *(Courtesy of DuPont Instrument Systems, Wilmington, DE.)* (b) Organophosphate insecticides. Column: 4.5 × 250 mm paced with 5-μm, C_8, bonded-phase particles. Gradient: 67% CH_3OH/33% H_2O to 80% CH_3/20% H_2O. Flow rate 2 mL/min. *(Courtesy of IBM Instruments Inc., Danbury, CT.)* Both used 254-nm UV detectors.

2. Adsorption or Liquid-Solid Chromatography

- The classic form of liquid chromatography (introduced in the 1900's) → Michael Tswett dengan pemisahan klorofil
- Two common stationary phases
  - Silica (most common)
  - Alumina
- Highly polar stationary phase, less polar mobile phase
- Separation occurs based on the solvent strength for retaining the solute
  - Mobil phase discussion given later
- Use overlaps with normal phase chromatography
- Suitable for: non-polar compounds of low molecular weight

Mekanisme Kromatografi Cair Padat

- Molekul solut akan terikat pada gugus aktif fasa diam, dengan persamaan adsorpsi:

  $X_m + nS_{ads} == X_{ads} + nS_m$

  $K =$
- Fasa Diam dalam KCP: Silika $(SiO_2)_x$ dan alumina $((Al_2)_3)_x$
- Contoh elusi dalam KCP sesuai dengan kepolaran solut/analit:

  Sebuah sampel dengan komposisi: benzena+asetofenon+benzil alkohol. Maka yang akan terelusi lebih dulu dalam Fs silika:

  Benzena-asetofenon-benzil alkohol. MENGAPA ??
- Silika dan alumina mempunyai gugus hidroksil, sehingga sifat adsorpsinya mengikuti reaksi asam lewis
- Contoh memisahakan isomer hidrokuinon

Kemasan Kolom KCP

- Ada 2 kemasan dalam KCP:
  1. Bahan pelikel
  2. Bahan berpori

- Kapasitas kolom berbanding lurus dengan jumlah fase diam yang dipakai = luas penyerapan

- F_M dalam KCP harus menyesuaikan kepolaran dengan Fs, utamanya jika solut adalah polar -- supaya waktu retensi tidak terlalu lama. Untuk mengatasi sering ditambahkan modifier seperti air, alkohol dan asetonitril pada F_M terutama untuk F_M yang non polar
- Model elusi solut dapat juga dengan elusi bergradien

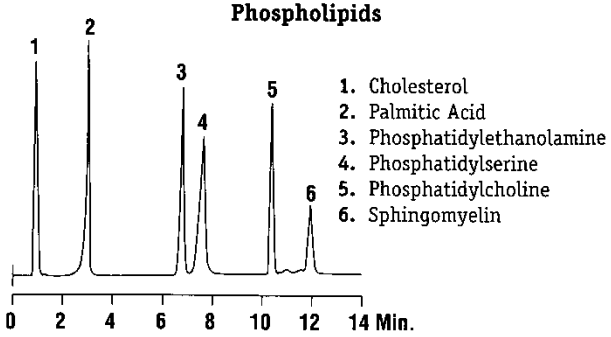
Adsorption Chromatography - Compounds That Can be Separated

- Olefins
- Aromatic hydrocarbons
- Halides, sulfides
- Ethers
- Nitro- compounds
- Esters, aldehydes, ketones
- Alcohols, amines
- Sulfones
- Sulfoxides
- Amides
- Carboxylic acids

listed in order of retention time; i.e. from least to greatest interaction with the stationary phase

Adsorption Chromatography – Example Chromatograms

Phospholipids



1. Cholesterol
2. Palmitic Acid
3. Phosphatidylethanolamine
4. Phosphatidylserine
5. Phosphatidylcholine
6. Sphingomyelin

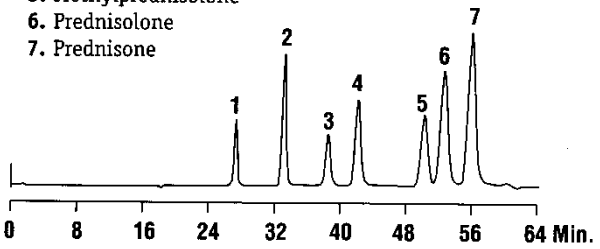
Column: Allsphere™ Silica, 3µm, 100 x 4.6mm
Mobile Phase: **A:** IPA **B:** Hexane **C:** Water
Gradient:

Time	0	7	15
%A	58	52	52
%B	40	40	40
%C	2	8	8

Flowrate: 1.25mL/min
Detector: 500 ELSD

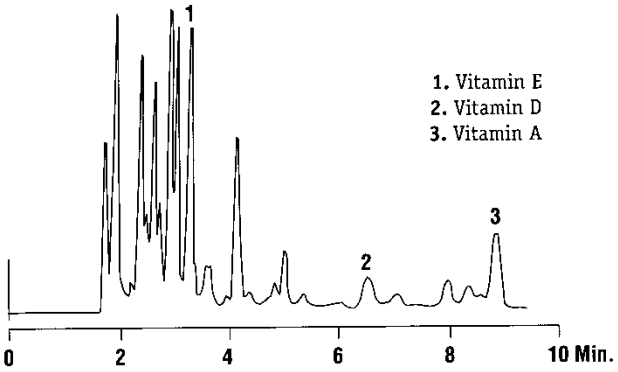
Steroids

1. Cortisone Acetate
2. Fluorometholone
3. Hydrocortisone
4. Hydrocortisone Hemisuccinate
5. Methylprednisolone
6. Prednisolone
7. Prednisone



Column: Adsorbosphere® Silica, 10µm, (2) 250 x 1mm
Mobile Phase: Butyl Chloride:THF:Methanol:Glacial Acetic Acid (80:10.5:5.3:4.2)
Flowrate: 25µL/min
Detector: UV at 254nm

Fat Soluble Vitamins in Powdered Milk

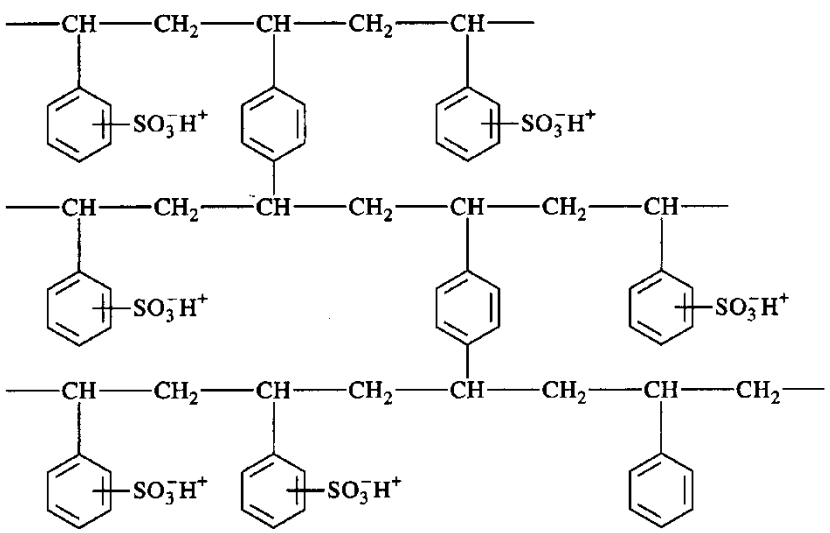


1. Vitamin E
2. Vitamin D
3. Vitamin A

Column: Ultrasphere® Si, 5µm, 250 x 4.6mm
Mobile Phase: Isopropanol:Hexane (1:99)
Flowrate: 1.5mL/min
Detector: UV at 265nm

3. Ion Exchange Chromatography

- Separation is based upon ion-exchange equilibria between the ions in solution and the ions of like charge on the surface of an insoluble packing.
- Discussed separately later



4. Bonded Phase Chromatography

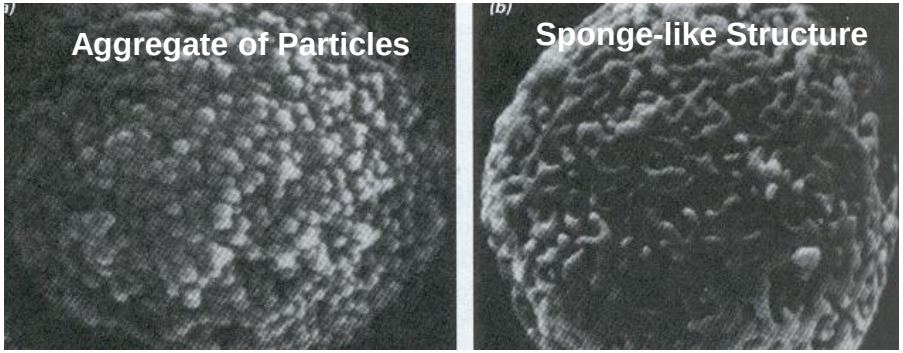
- Kromatografi Fase Terikat (FKT) memperbaiki kinerja kromatografi cair-cair karena Fs yang mudah tererosi
- Fs diikatkan secara kimiawi pada padatan pendukung
- K = Cs/Cm
- Jenis Fasa Diam:

  - Bahan pelikel

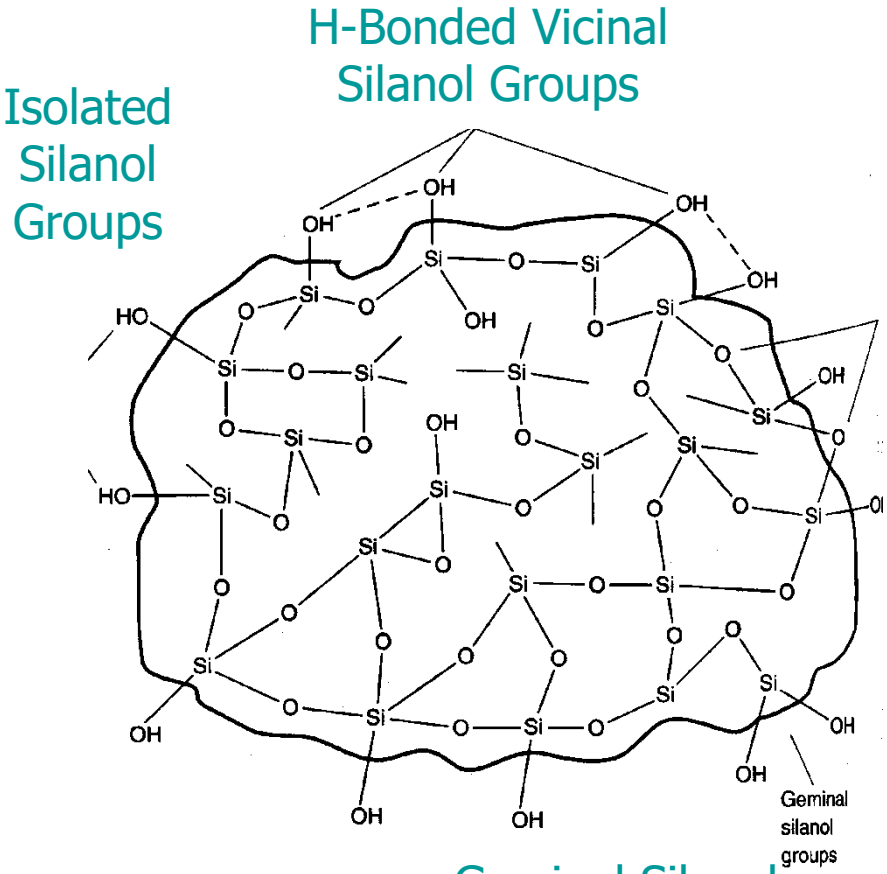
  - Bahan berpori/mikropori

Stationary Phase

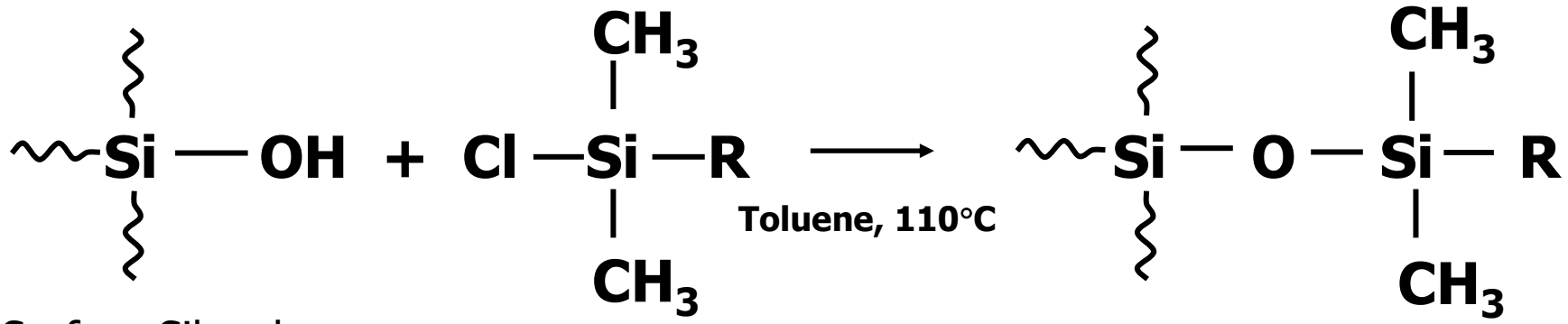
Microporous Silica Particles



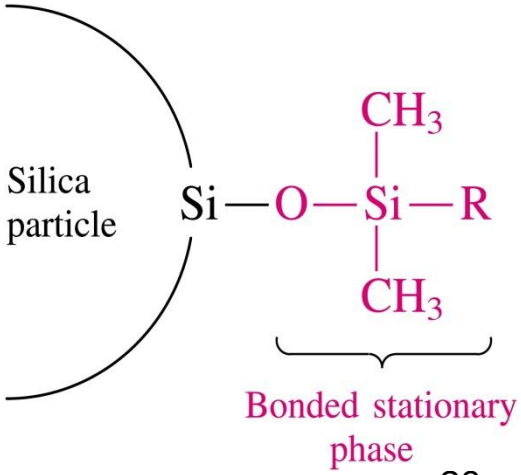
- Particles are permeable to solvent
- ~100 m^2/g of particles
- Only use for pH < 8.0
- Use polystyrene particles for pH 8-12



Bonded Stationary Phase

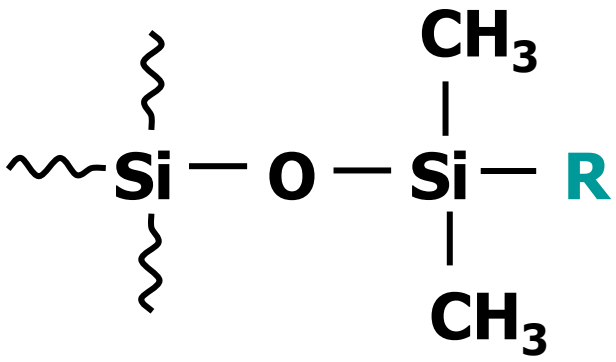


- Covalent attachment of the stationary phase yields a thermally and hydrolytically stable bonded phase



Common Functional Groups for Bonded Stationary Phase

Polar Phases (R =)		**Non-Polar Phases (R =)**	
$(CH_2)_3NH_2$	Amino	$(CH_2)_{17}CH_3$	Octadecyl
$(CH_2)_3CN$	Cyano	$(CH_2)_7CH_3$	Octyl
$(CH_3)_3OCH_2CH(OH)CH_2OH$	Diol	$(CH_2)_3C_6H_5$	Phenyl



Kemasan Kolom Kromatografi Fasa Terikat ada 2: Normal dan Terbalik

- Fasa Terikat Normal

Fasa diam yang terikat pada penyangga lebih polar daripada fasa gerak seperti pada Kromatografi cair padat.

Contoh Fs: gugus {(Si-$(CH_2)_n$CN)} alkil nitril dan {(Si-$(CH_2)_n$$NH_2$)} alkil amin

Contoh F_M: heksana, tetra hidro furan, metilena klorida

- Fasa Terikat Terbalik

Fasa diam lebih non polar daripada fasa gerak (fasa gerak lebih polar daripada fasa diam)

Contoh Fs: oktadekil, C18

Contoh F_M: metanol, air, asetonitril

KAIDAH UMUM SIFAT ELUSI: urutan elusi solut dalam fase terbalik berlawanan dengan elusi dalam fasa normal

Fasa Gerak pada LC

- Many Types are available
  - In contrast to GC
- The mobil phase interacts (electrostatically) with sample components
  - In contrast to GC
- Residence Time = f(solvent type)
- Polarity is important for solute, mobil phase and stationary phase

Gradient Elution

1. Phosphoserine
2. Aspartic acid
3. Glutamic acid
4. α-Amino adipic acid
5. Asparagine
6. Serine
7. Glutamine
8. Histidine
9. Glycine
10. Threonine
11. Citrulline
12. 1-Methylhistidine
13. 3-Methylhistidine
14. Arginine
15. β-Alanine
16. Alanine
17. Taurine
18. Anserine
19. β-Aminobutyric acid
20. β-Aminoisobutyric acid
21. Tyrosine
22. α-Aminobutyric acid
23. Methionine
24. Valine
25. Tryptophan
26. Phenylalanine
27. Isoleucine
28. Leucine
29. δ-Hydroxylysine
30. Lysine

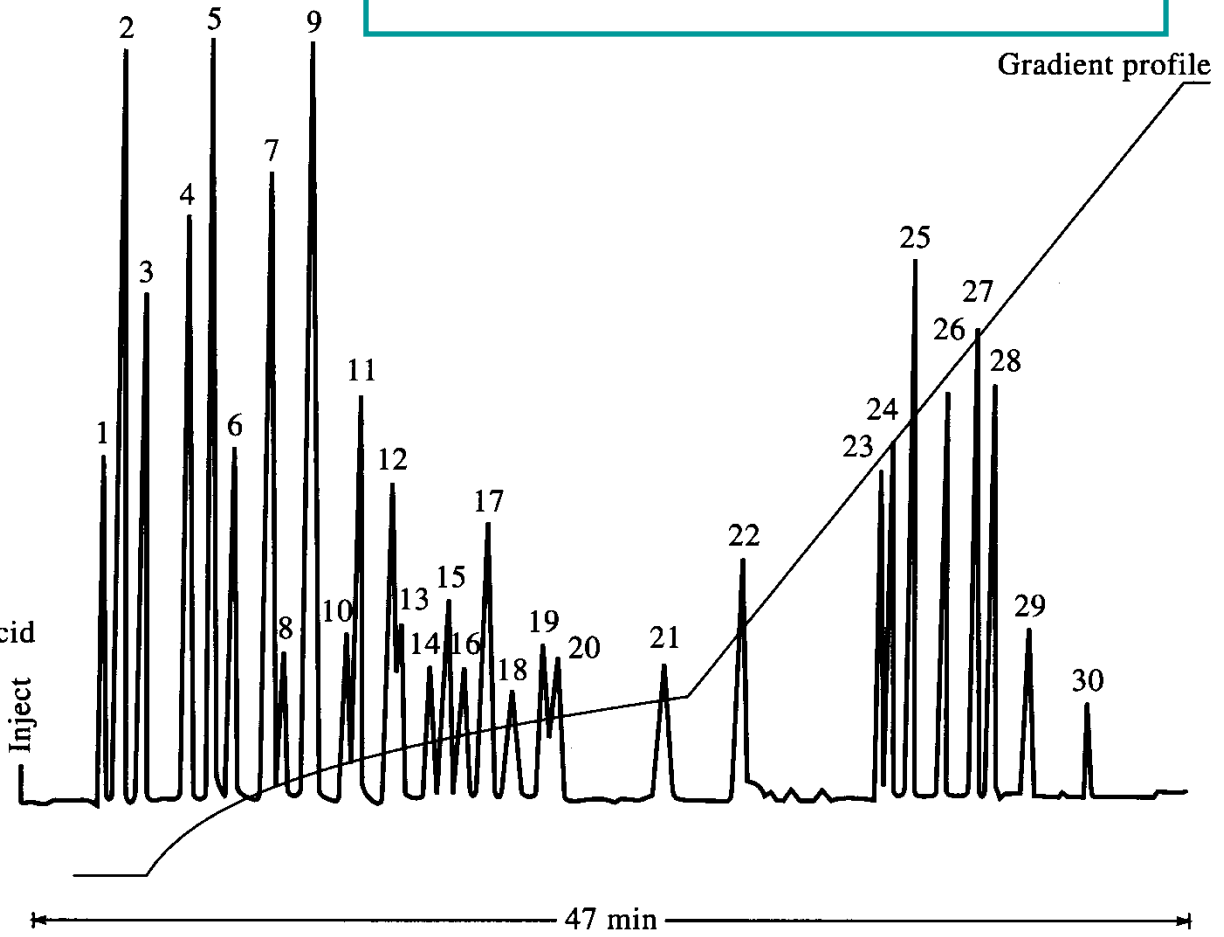


Figure 28-18 Chromatogram of orthophthalaldehyde derivatives of 30 amino acids of physiological importance. Column: 5 μm C_{18}, reversed-phase. Solvent A: 0.05 M Na_2HPO_4, pH 7.4, 96:2:2 $CH_3OH/THF/H_2O$. Fluorescence detector: excitation 334 nm; emission 425 nm. *(Reprinted with permission from R. Pfiefer et al.,* Amer. Lab., ***1983,*** *15(3), 86. Copyright 1983 by International Scientific Communications, Inc.)*