High Performance Liquid Chromatography (HPLC)

Presented by: Dr.Qomi

2010

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What Does a Chemist Do?

- ***Studies the atomic composition and structural architecture of substances***
- ***Investigates the varied interactions among substances***
- ***Utilizes natural substances and creates artificial ones***
- ***Comprehends the marvelous and complex chemistry of living organisms***
- ***Provides a molecular interpretation of health and disease***

How Does S(He) do it?

Main Divisions of Chemistry

Organic Chemistry

Inorganic Chemistry

Physical Chemistry

Analytical Chemistry

Industrial Chemistry (Chemical Engineering and Applied Chemistry)

Biochemistry

Materials Chemistry

Environmental Chemistry

Forensic Chemistry

What is Analytical Chemistry?

QUALITATIVE ANALYSIS

Deals with the detection of elements or compounds (analytes) in different materials.

QUANTITATIVE ANALYSIS

Refers to the measurement of the actual amounts of the analyte present in the material investigated.

Chemical and Biochemical Methods

- ***Gravimetry***
- ***Titrimetric Analysis***
- ***Enzymic Analysis***
- ***Inmunochemical Analysis***
- ***Instrumental Analysis***

Instrumental Analytical Chemistry

- ***Atomic and Molecular Spectroscopic Methods***

  - ***Nuclear Magnetic Resonance (NMR)***
  - ***Electron Spin Resonance (ESR)***
  - ***Mass Spectrometry (MS)***
  - ***Vibrational Spectroscopy (IR, RAMAN)***
  - ***X-Ray Fluorescence Analysis (XPS)***
  - ***Electronic Spectroscopy (UV, VIS, Luminiscence)***
  - ***Atomic Spectroscopy (AA, ICP)***
  - ***Rotational Spectroscopy (Microwave, FIR)***

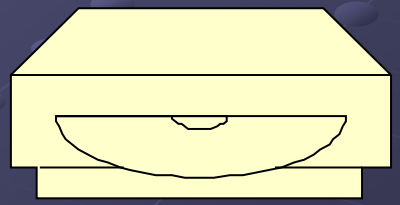
Instrumental Analytical Chemistry

- ***Thermal Methods***
  - *Thermogravimetry (TG)*
  - *Differential Thermal Analysis (DTA)*
  - *Differential Scanning Calorimetry (DSC)*
  - *Thermomechanic Analysis (TMA)*
- ***Electrochemical Methods***
  - *Electrogravimetry*
  - *Electrophoresis*
  - *Conductimetry, Potentiometry*
  - *Polarography*
  - *Voltammetry*

Instrumental Analytical Chemistry

Chromatographic Methods ***(Partition equilibrium)***

- *Gas Chromatography (GC)*
- *High Performance Liquid Chromatography (HPLC)*
- *Gel Permeation Chromatography (GPC)*
- *Thin Layer Chromatography (TLC)*
- *Ion Chromatography*



High

Performance

Liquid

Chromatography

High

Pressure

Liquid

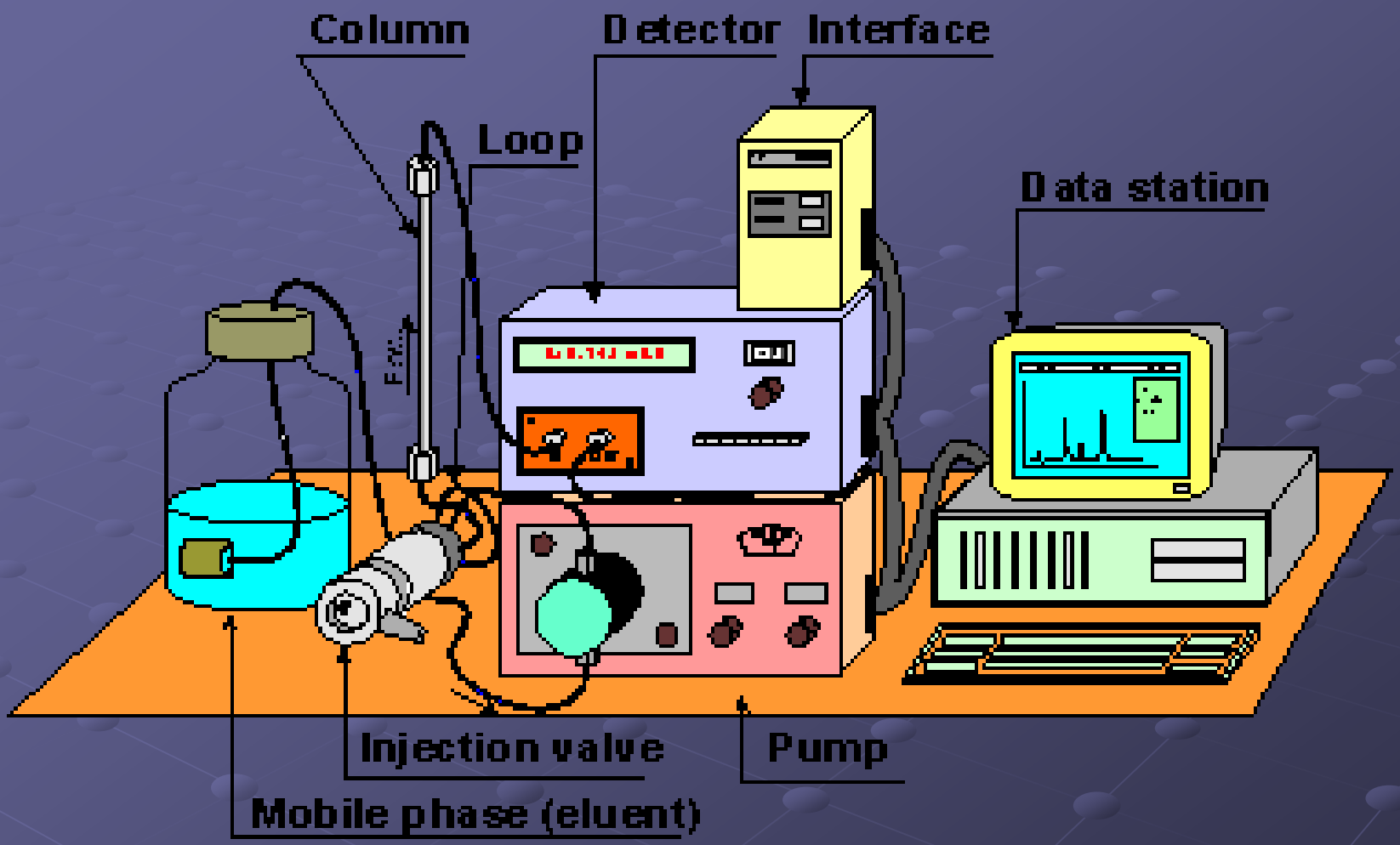
Chromatography

High

Priced

Liquid

Chromatography


Column
Detector
Interface
Loop
Data station
Injection valve
Pump
Mobile phase (eluent)

Aims and Objectives

- To give a brief History of Liquid Chromatography
- To compare and contrast High Performance Liquid Chromatography (HPLC) with Gas Chromatography (GC)
- To introduce the Liquid Chromatograph (LC) and explain the function of each component
- To introduce the Chromatogram and explain the information it gives
- To outline the main separation mechanisms used in HPLC
- To explain the fundamental principles of separation
- To highlight the different "modes" of chromatography and explain their uses and applications

Origins of Liquid Chromatography

The Russian botanist Mikhail Tswett first used the term 'Chromatography' (Greek for 'coloured drawing') in 1906, to describe the separation that occurred when solutions of plant pigments were passed through columns of calcium carbonate or alumina, using petroleum ether.

HPLC was first presented by Huber, J.F.K. and Hulsman, J.A.G., in 1967.

Why Choose Liquid Chromatography?

The two main chromatographic techniques used in modern analytical chemistry are Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC).

HPLC uses a liquid mobile phase to transport the analytes (sample) through the column, which is packed with a stationary phase material.
In contrast, Gas Chromatography uses a gaseous mobile phase to transport sample components through either packed columns or hollow capillary columns. In most cases, GC columns have smaller internal diameter and are longer than HPLC columns.

GC has developed into a sophisticated technique since the pioneering work of Martin and James in 1952, and is capable of separating some very complex mixtures.

However, due to limitations of volatility and thermal stability, it is only capable of separating around 23% of known substances.

So ? under what circumstances would we chose HPLC to separate our sample components?

Suitable Samples for HPLC

GC

- Samples analysed by GC must be volatile (significant vapour pressure below 250°C).
- Derivatisation to increase analyte volatility is possible but cumbersome and introduces possible quantitative errors.
- Most GC analytes are under 500 Da Molecular Weight for volatility purposes.

HPLC

- HPLC analysis has no volatility issues, however the analyte must be soluble in the mobile phase.
- HPLC can analyse samples over a wide polarity range and is able to analyse ionic samples. Mobile phase components are selected to ensure sample solubility.
- HPLC has no real upper molecular weight
- limit and large proteins of many thousands of Daltons may be analysed. Solubility in the mobile phase may preclude the analysis of very large molecules.

Comparison with Gas Chromatograpy

GC

- Temperatures in GC can exceed 350°C and samples that are thermally unstable (labile) may decompose.
- Many GC detectors such as the Flame Ionisation Detector (FID) are destructive and the analyte does not survive analysis in-tact and therefore cannot be recovered.
- GC samples are prepared in organic solvents and extraction of analytes from aqueous samples will be necessary.
- Sample size is usually between 1 and 5µl with typical detector sensitivity between nanograms (ng) and picograms (pg) on column.

HPLC

- HPLC is usually carried out at (or around) room temperature and most HPLC detectors apart from the Mass Spectrometer are non-destructive.
- HPLC samples are prepared in a solvent system that has the same or less organic solvent than the mobile phase and volumes of 1 to 50 µl are common (1-10µg of analyte per 1g packing material).

Typical HPLC Data

- HPLC - Even though the Flame ionisation detector may be more universal and sensitive, the UV detector is non-destructive, relatively sensitive (nanograms of analyte on column), and also has the capability of producing spectra associated with sample components. This can aid qualitative analysis and assist with identification of sample components.

- HPLC and GC can both use Mass Spectrometers (MS) as detection systems to assist with analyte identification - although MS detectors are destructive. HPLC-MS is a less mature technique and there are no spectral libraries available for compound identification as there are with GC instruments. However, HPLC-MS (LC-MS) is a burgeoning technique that can assist in the characterisation of sample components in a wide variety of application types.

Summary

If the sample cannot be analysed by Gas Chromatography without lengthy sample preparation (indicating issues with volatility), then HPLC should be the technique of choice. HPLC is the best choice for higher molecular weight analytes and analytes which may potentially degrade when heated.

Scope of HPLC

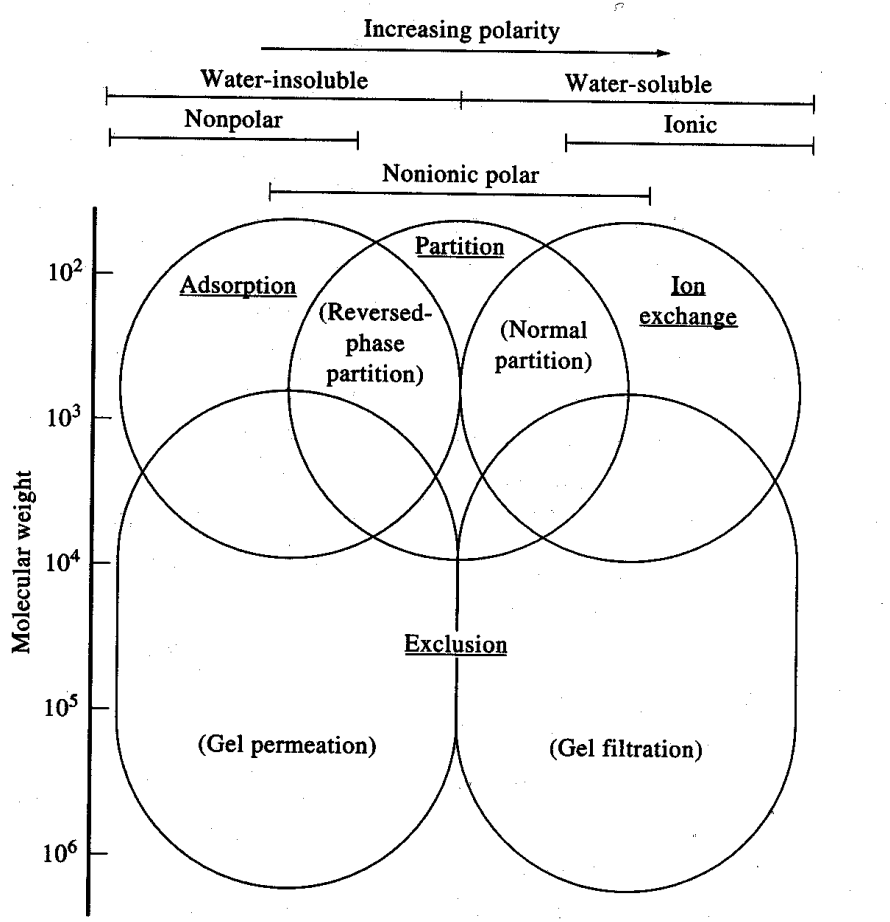


Figure 28-1 Applications of liquid chromatography. *(From D. L. Saunders, in* Chromatography, *3rd ed., E. Heftmann, Ed., p. 81. New York: Van Nostrand Reinhold, 1975. With permission.)*

- Sensitive
- Quantitative
- Nonvolatile Compounds
- Thermally Fragile Compounds
- Broad Applicability
  - Biochemical Species
  - Pharmaceuticals
  - Pesticides
  - Inorganic and Organometallics
  - Industrial Chemicals

Chromatography Separation Mechanisms

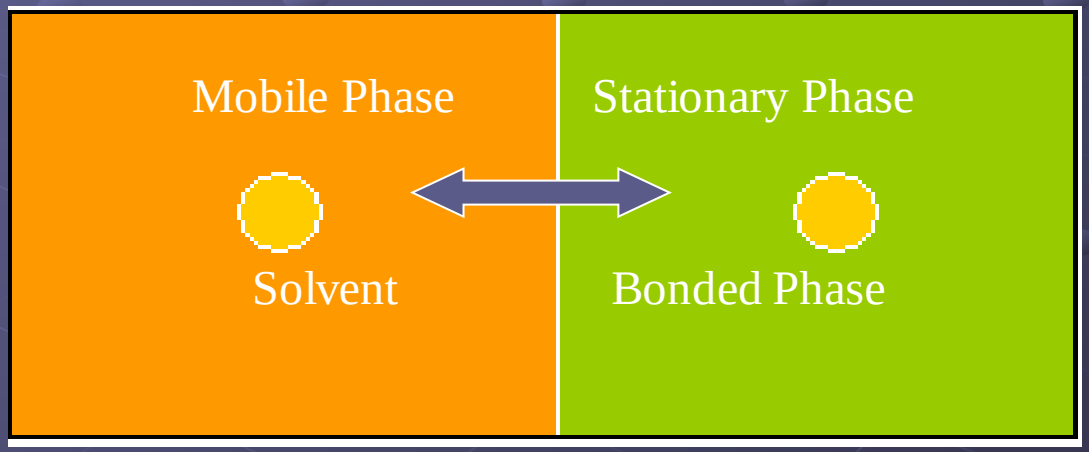
- HPLC separations involve both the mobile phase (a liquid) and the stationary phase (usually materials of varying hydrophobicity chemically bonded to a solid support). In contrast, GC separations do not involve the mobile phase, which is only used to carry the analyte through the column.

- As an illustration - the amount of water in an HPLC mobile phase will determine how strongly a hydrophobic analyte is repelled into the stationary phase - and how well retained it is. The chemical nature of the stationary phase will also govern how strongly the analyte is retained. For this reason, HPLC is a technique that is driven by the 'selectivity' achieved using two interacting phases.

- In contrast, analytes in a capillary GC column will only be retained due to their interaction with the stationary phase (usually an immobilized polymeric liquid of varying hydrophobicity) coated onto the inner walls of the GC column. There are less options for improving selectivity in GC, however, as it is very highly efficient - this is often enough to achieve the desired separation.

Chromatography Separation Mechanisms (Partitioning)

Separation is based on the analyte's relative solubility between two liquid phases



Mobile Phase: The compound of interest to be analysed by injection into and elution from an HPLC or GC column.

Stationary Phase: The stationary phase is one of the two phases forming a chromatographic system. It may be a solid a gel or an immobilised polymeric liquid. if a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid ("Bonded Phase"). The expressions "Chromatographic Bed" or "Sorbent" may be used as general terms to denote any of the different forms in which the stationary phase is used. A stationary phase which is covalently bonded to the support particles or into the inside wall of the column tubing is known as a "Bonded Phase".

Hydrophobic: Meaning "water-fearing", hydrophobic compounds do not dissolve easily in water and are ususally non-polar. Oils and other long hydrocarbons are hydrophobic.

.

- **Selectivity**: "Selectivity" is also callned the "Separation Factor" (alpha), which describes the separating "power" of the system.
  The relative retention value calculated for 2 adjacent peaks (VR2' > VR1'):

  alpha = t_{R2}'/t_{R1}'

  By definition, the value of the separation factor is always greater than 1.

- **Efficient**: "Efficiency" is also called the plate Number (N), which describes the broadening of the chromatographic band by using the chromatographic peak width. The Plate Number is indicative of column performance, calculated from the following equations, which depend on the selection of the peak width expression (see 'Peak Width').

  $N = 16(t_R/w_b)^2$ $H=L/N$

  TR = peak apex retention time, wb = width of the peak at the base, measured using tangents to the peak sides

The Liquid Chromatograph

In HPLC, several instrument and column chemistry parameters need to be optimised in order to generate a satisfactory separation.

Each of the following items needs to be optimised in order to generate a chromatogram that is suitable for qualitative or quantitative purposes.

- ✓ Mobile Phase Composition
- ✓ Bonded Phase Chemistry
- ✓ Injection Volume
- ✓ Sample Pre-treatment and concentration
- ✓ Mobile phase Flow Rate
- ✓ Column Temperature
- ✓ Detector Parameters

The Liquid Chromatographic Process

The mobile phase is continuously pumped at a fixed flow rate through the system and mixed (if required) by the pump. The injector is used to introduce a plug of sample into the mobile phase without having to stop the mobile phase flow, and without introducing air into the system.

The Chromatogram

TECHNIQUES

1. Solvents
2. Pumps
3. Sample Injection
4. Column
5. Detectors

Solvents

- Low viscosity
- Be free of particles and dissolved air

- Degassing
- ✓ Helium purge
- ✓ Vaccum
- ✓ Ultrasonic bath

Modes of Chromatography

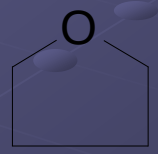
Normal Phase.

- Polar stationary phase and non-polar solvent.

Reverse Phase.

- Non-polar stationary phase and a polar solvent.

Common Reverse Phase Solvents

- Methanol CH_3OH
  - Acetonitrile CH_3CN
  - Tetrahydrofuran 

  - Water H_2O

Pumps

- Pressure drop:

$$P = L\eta\mu/\Theta d^2$$

L = column length, η=solvent viscosity, μ=flow rate, Θ= constant, d=particle diameter

- Reciprocating pumps
- Positive Displacement pumps (Syringe pumps)

Sample Injection

- Sample volume: 5-50 µL
- Never use a gas chromatographic syringe in HPLC injection block

Injection Systems

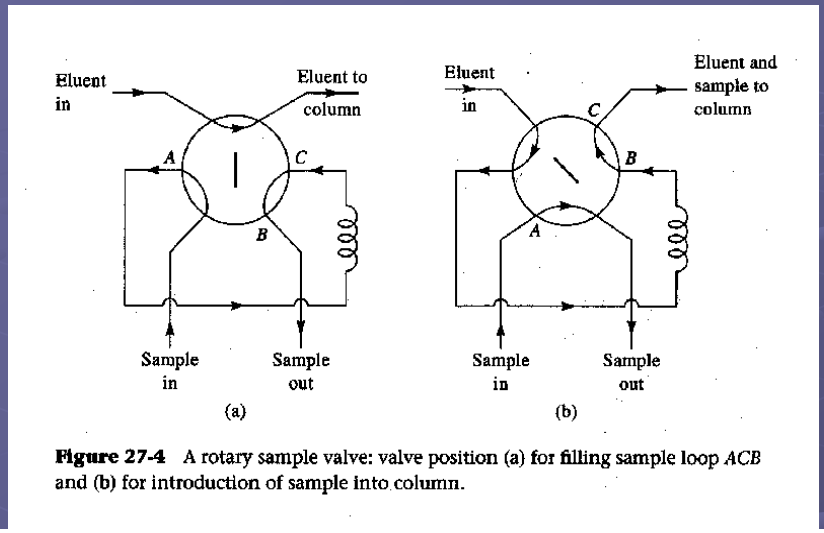


Figure 27-4 A rotary sample valve: valve position (a) for filling sample loop *ACB* and (b) for introduction of sample into column.

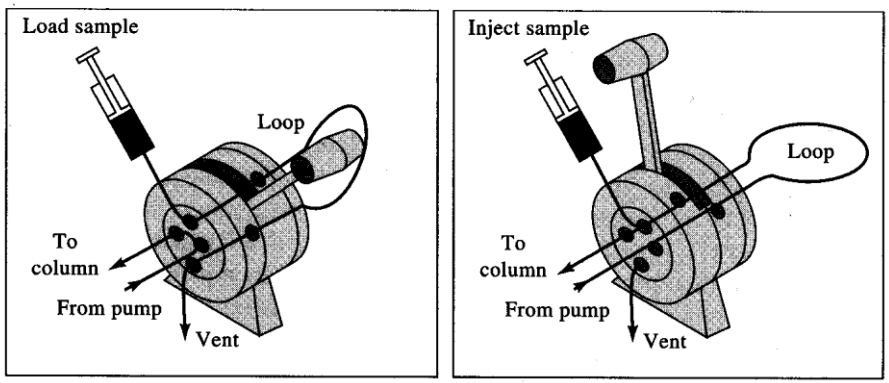


Figure 28-7 A sampling loop for liquid chromatography. *(Courtesy of Beckman Instruments, Fullerton, CA.)* With the valve handle as shown on the left, the loop is filled from the syringe, and the mobile phase flows from pump to column. When the valve is placed in the position on the right, the loop is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

- Syringe Injection
  - Possible
  - Rarely Used
- Standard Method - Loop Injector
  - Diagrams:
    - Figure 27-4 (better)
    - Figure 28-7
  - Interchangeable Loops
    - Standard 5 to 500 μL
    - Micro 0.5 to 5 μL
  - Operating Pressures to 7000 psi

LC Columns

- Construction
  - Mainly Smooth Bore Stainless Steel (Pressures to 10,000 psi)
  - Occasionally Heavy Wall Glass (Pressures to 600 psi)
- Costs:
  - $200 to $500
  - More Expensive Available
- Analytical Columns
  - 10 to 30 cm
  - Extend Length by Coupling Additional Columns
  - Inside Diameters: 4 to 10 mm
  - Packing Diameters: 5 or 10 mm
  - Common: 25 cm long; 4.6 mm inside diameter; 5 mm particles; N = 40,000 to 60,000

Column Efficiency in HPLC

- Mobile Phase Mass Transfer
  - $C_M \propto d_p^2$
    - C_M - MP mass transfer coeff.
    - d_p - particle diameter
  - Effect shown in Fig 28-2

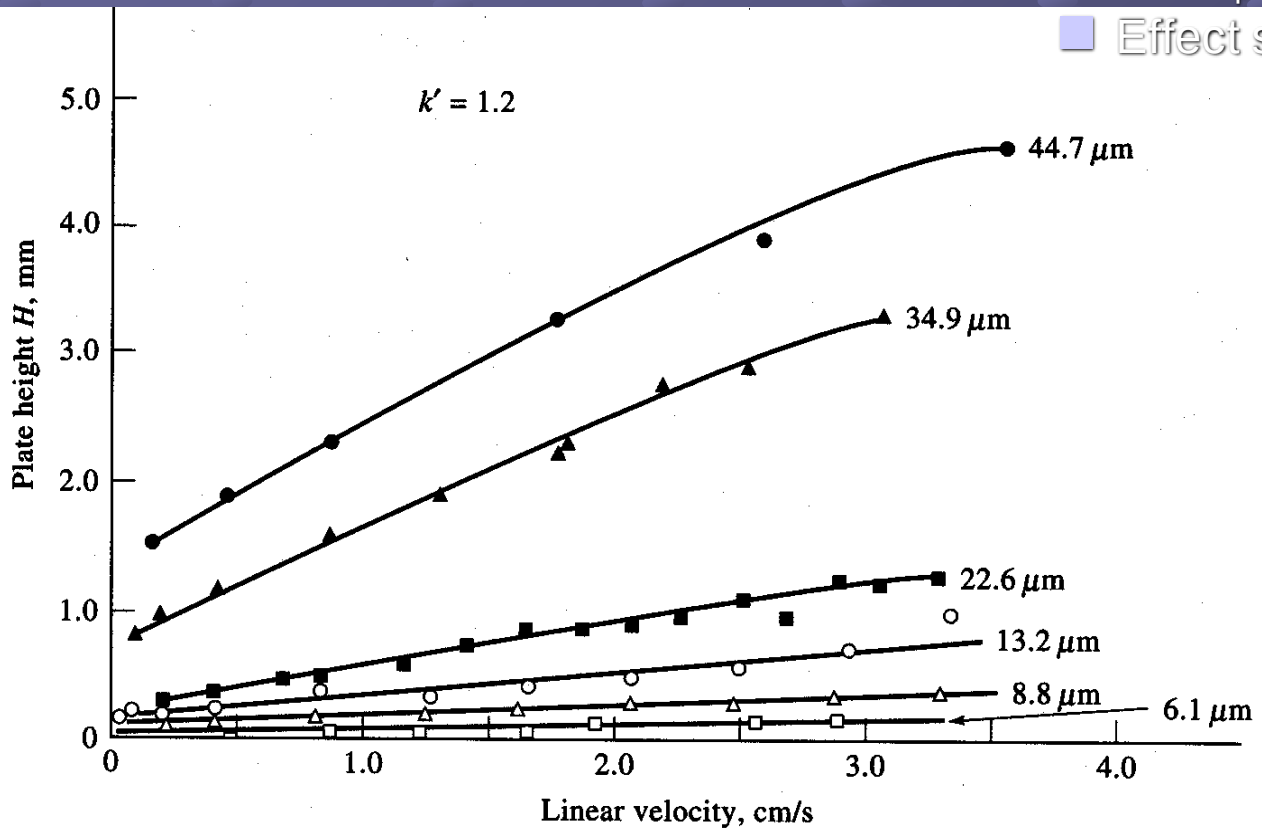


Figure 28-2 Effect of particle size of packing and flow rate upon plate height H in liquid chromatography. Column dimensions: 30 cm × 2.4 mm. Solute: N,N-diethyl-*n*-aminoazobenzene. Mobile phase: mixture of hexane, methylene chloride, isopropyl alcohol. *(From R. E. Majors,* J. Chromatogr. Sci., ***1973, 11, 92. With permission.)***

Picture of an HPLC column

LC Columns

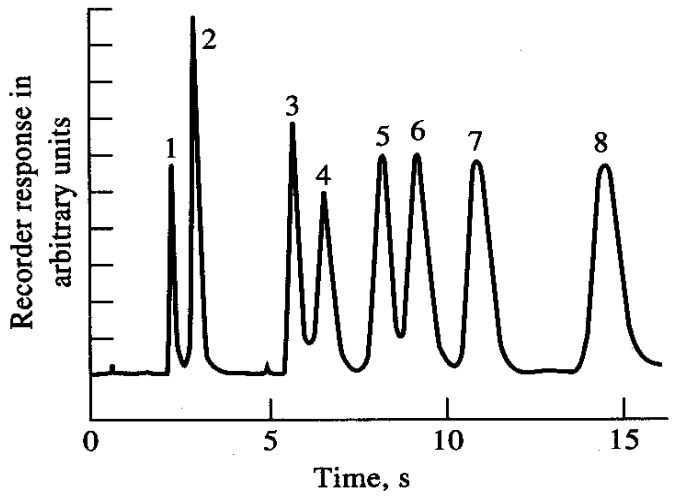


Figure 28-8 High-speed isocratic separation. Column dimensions: 4 cm length; 0.4 cm i.d. Packing: 3-μm sperisorb. Mobile phase: 4.1% ethyl acetate in *n*-hexane. Compounds: (1) *p*-xylene, (2) anisole, (3) benzyl acetate, (4) dioctyl phthalate, (5) dipentyl phthalate, (6) dibutyl phthalate, (7) dipropyl phthalate, (8) diethyl phthalate. *(From R. P. W. Scott,* Small Bore Liquid Chromatography Columns: Their Properties and Uses, *p. 156. New York: Wiley, 1984. Reprinted with permission of John Wiley & Sons, Inc.)*

- High Speed, High Performance
  - Smaller than Standard Columns
  - Lengths: 3 to 7.5 cm
  - Inside Diameters: 1 to 4.6 mm
  - Particle Diameters: 3 or 5 mm
  - Number of Plates: Up to 100,000
  - Advantages:
    - Speed - Much Faster Elutions
    - Minimum Solvent Usage
  - Figure 28-8
    - 8 Components in 15 s
    - 4 cm long
    - 4 mm inside diameter
    - 3 mm particle diameter

LC Columns

- Guard Columns
  - Extend Analytical Column Life
  - Removes:
    - Particulate Matter
    - Solvent Contaminents
    - Sample Components Which Bind Irreversibly to SP
  - Liquid-Liquid Chromatography
    - Saturates MP with SP
    - Minimizes Analytical SP Loss
  - Composition Matched to Analytical
  - Pressure Drop Minimized with Larger Particle Size
  - Repacked or Discarded When Contaminated
- Column Thermostats
  - Temperature Control
    - Unimportant for Most Applications
    - Constant Temperature Improves Chromatograms
  - Modern HPLC's Have Column Heaters
    - Range: Ambient to 100° to 150° C
    - Control: a Few 0.1° C
  - Column Water Jackets
    - Provides Heating or Cooling
    - Uses Standard Constant Temperature Baths

HPLC Column Packings

- Silica and bonded-phase silica
  - Classic, Type II, and hybrid silica
- Polymer reverse phase
- Zirconium and MS bonded-phase
- Ion exchange: polymer and silica
- Size separation: polymer and silica

Type of Particles

- Fully porous particles
- Pellicular particles
- Microporous particles

LC Columns

- Column Packings
  - Pellicular Beads
    - Spherical
    - Nonporous
    - Glass or Polymer
    - Diameters: 30 to 40 μm
    - Thin Porous Layers Deposited
      - Silica
      - Alumina
      - PS-DVB Resin
      - Ion-Exchange Resin
    - Sometimes Additional Liquid SP Applied to Porous Layer
    - Surface Chemically Modified
      - Yeilds Organic Surface
    - Mostly for Guard Columns
  - Porous Particles
    - Microparticles
    - Diameter: 3 to 10 μm
    - Diameter Ranges Minimized
    - Compostion:
      - Silica
      - Alumina
      - PS-DVB Resin
      - Ion-Exchange Resin
    - Silica Most Common
    - Particles Coated with Organic Films
      - By Physical Adsorption
      - By Chemical Bonding

Columns

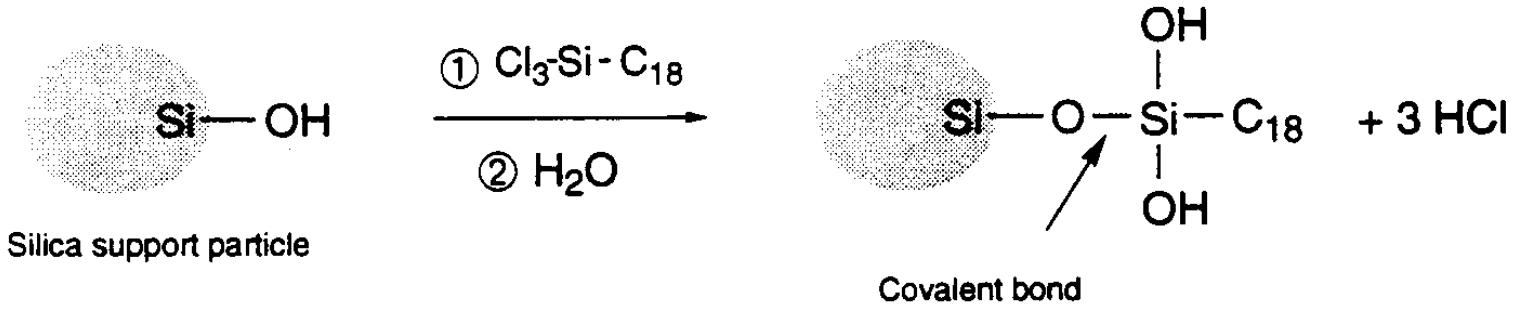
- Solid Support - Backbone for bonded phases.
  - Usually 10µ, 5µ or 3µ silica or polymeric particles.
- Bonded Phases - Functional groups firmly linked (chemically bound) to the solid support.
  - Extremely stable

  -Reproducible

Bonded Phases

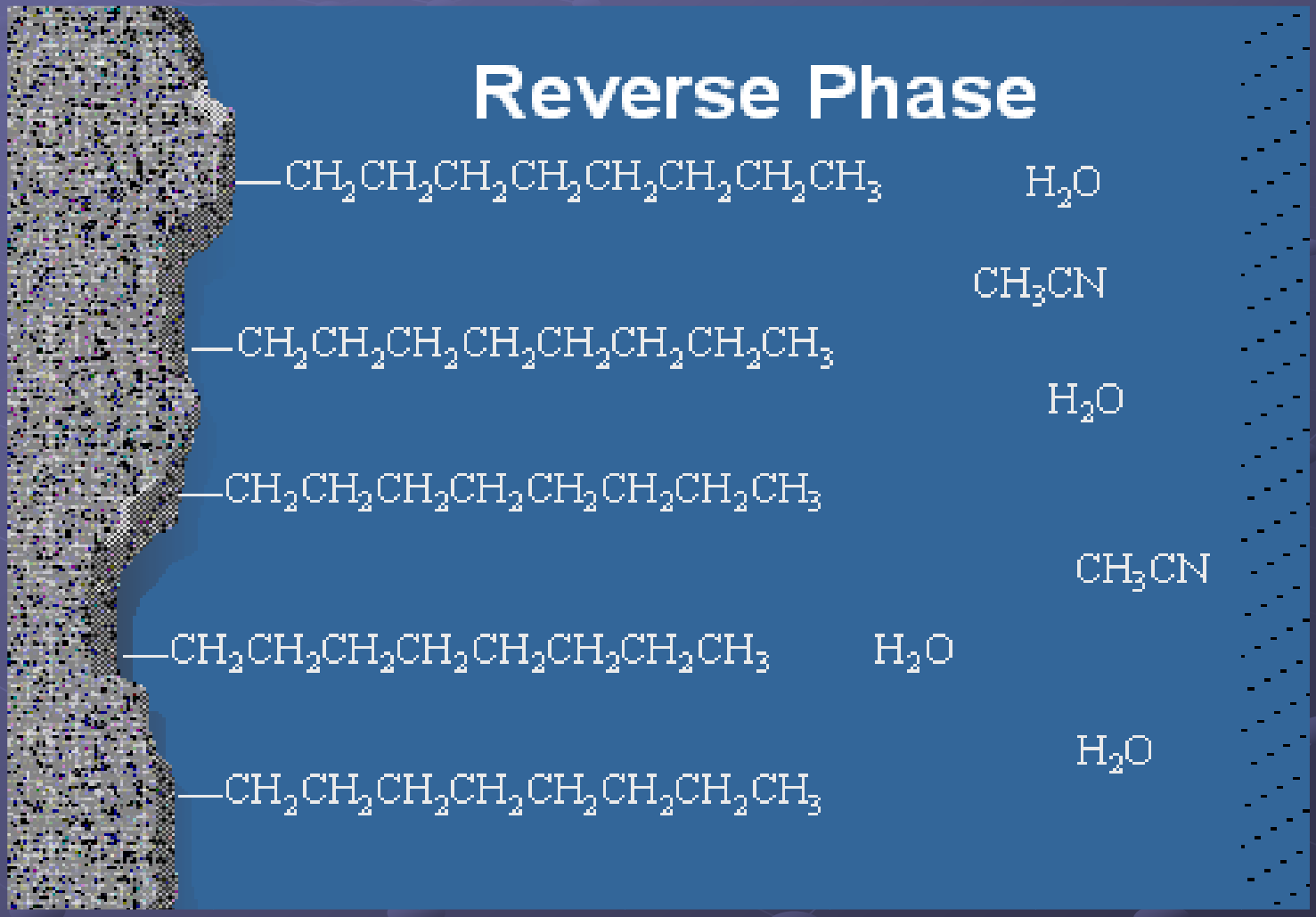
- C-2 Ethyl Silyl -Si-CH_2-CH_3
- C-8 Octyl Silyl -Si-$(CH_2)_7$-CH_3
- C-18 Octadecyl Silyl -Si-$(CH_2)_{17}$-CH_3
- CN Cyanopropyl Silyl -Si-$(CH_2)_3$-CN

Synthesis of RP Packings

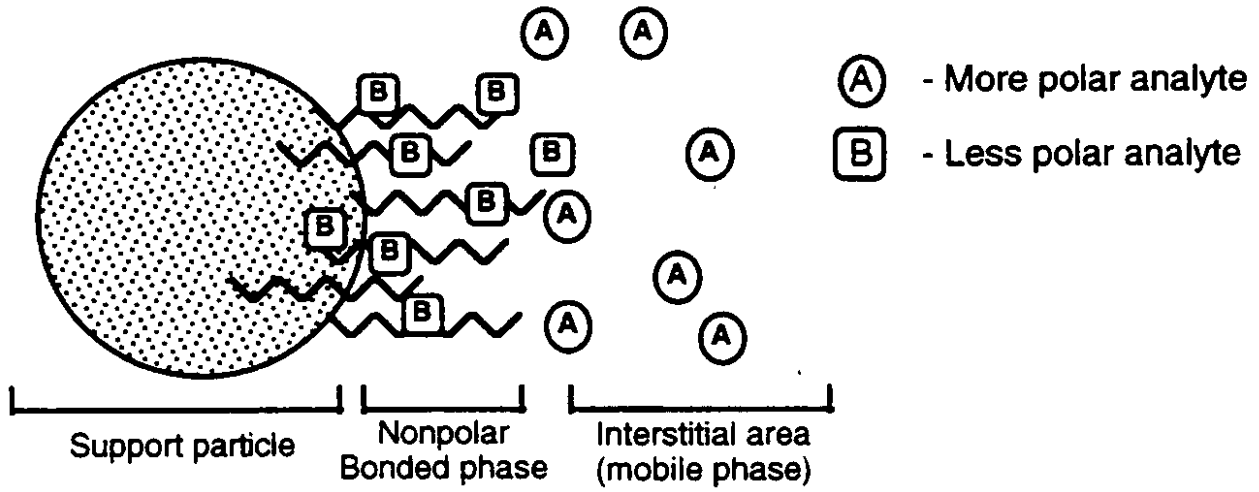


- **Covalent attachment of the stationary phase yields a thermally and hydrolytically stable *bonded phase*.**

Reverse Phase



RP Mechanism (Simple)



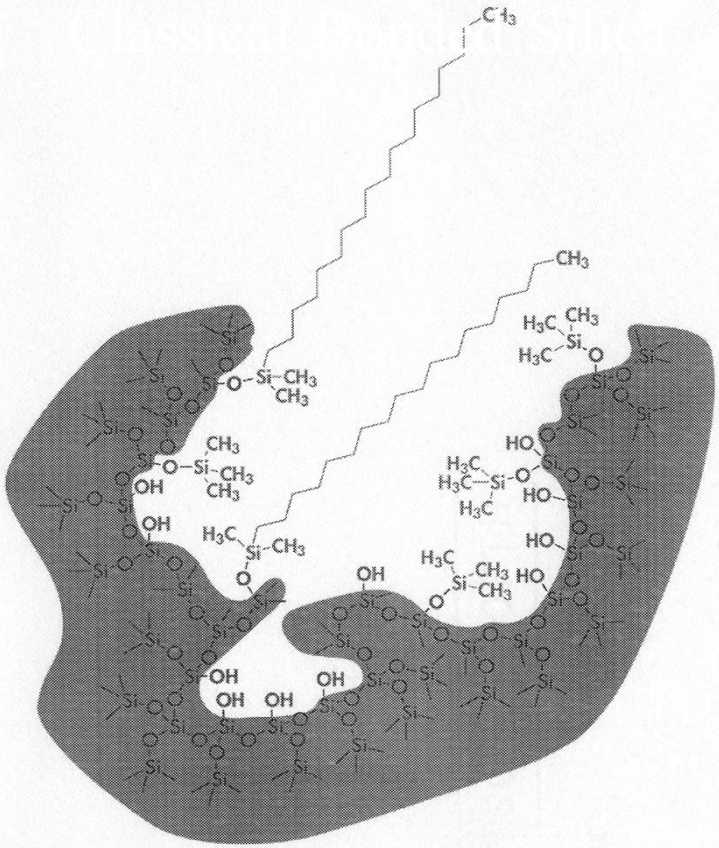
- **Less polar (more hydrophobic) analytes are more attracted to the hydrophobic bonded phase...**
- **...more hydrophobic spends more time associated with the bonded phase...**
- **...and are eluted last. Methanol is active solvent.**

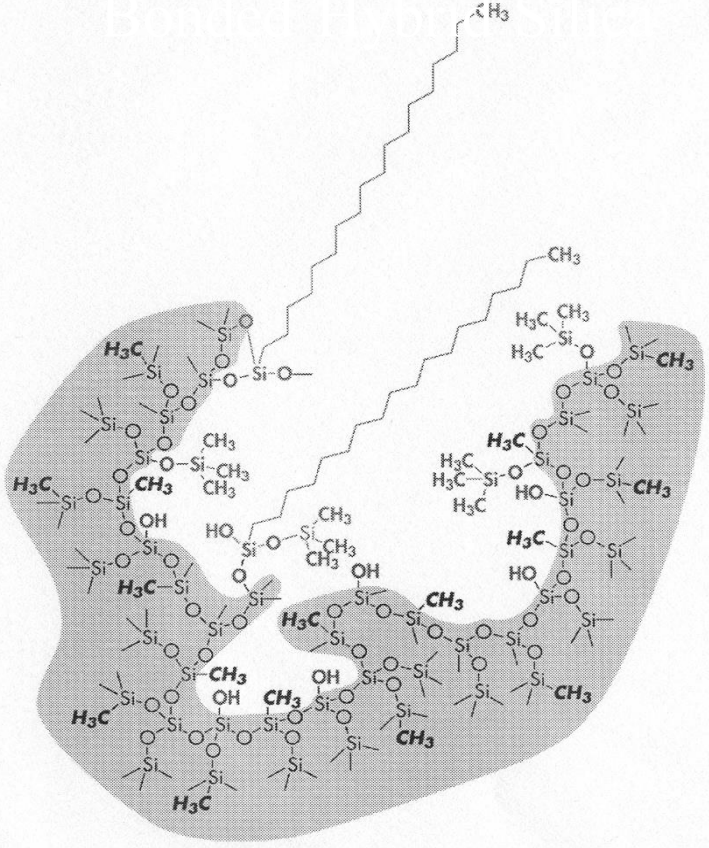
Common NP Packings

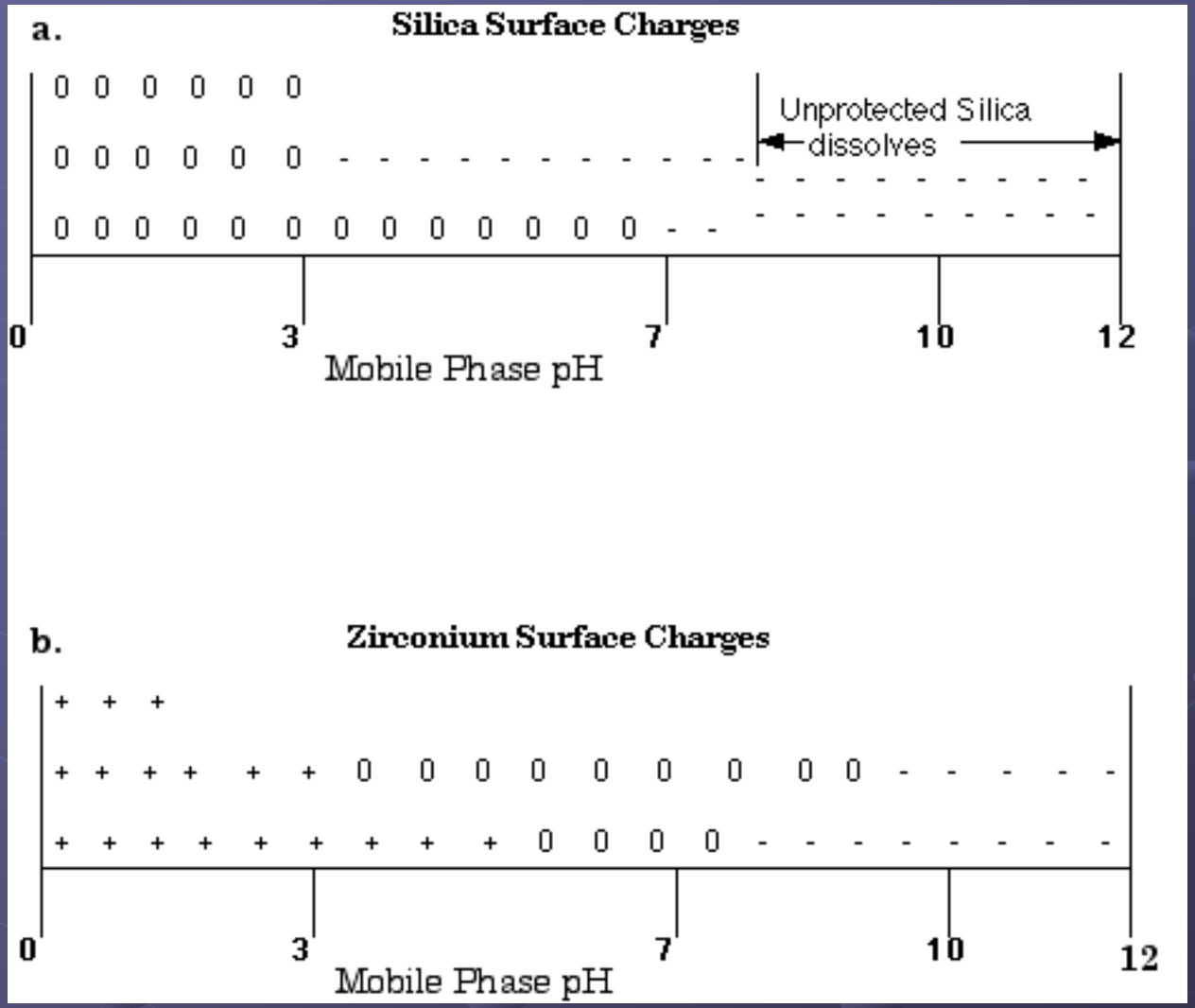
NORMAL PHASE:

Cyanopropyl - 

Aminopropyl - 






a.
Silica Surface Charges
Unprotected Silica
dissolves
0
3
7
10
12
Mobile Phase pH
b.
Zirconium Surface Charges
0
3
7
10
12
Mobile Phase pH

HPLC Pre-run Preparation

- Volatile Buffer: mobile phase pH control
- Sample and mobile phase filtration
- Sample solubilizing & deproteination
- SFE cartridge sample cleanup
- SFE cartridge windowing
- Dry System startup

Detectors

- Ultraviolet absorption (UV)
  - Single wavelength (filter)
  - Variable wavelength (monochromator)
- Fluorescence
- Refractive Index (RI)
- Electrochemical (EC)
- Mass Spectrometric

HPLC Detectors

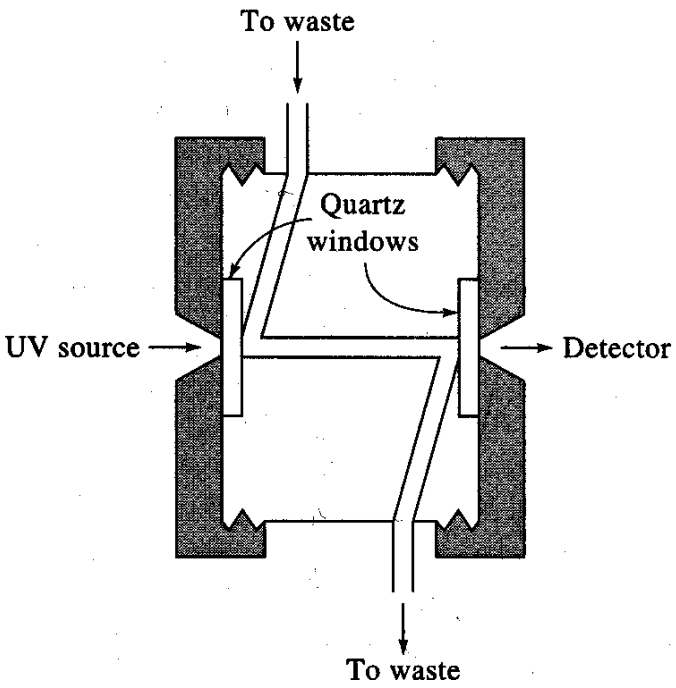


Figure 28-9 Ultraviolet detector cell for HPLC.

- Absorbance Detectors
  - Eluent Measurement Cell
    - Minimum Volume
      - Reduces Extra Column Broadening
      - Volume : 1 to 10 mL
      - Path Length (b): 2 to 10 mm
    - Pressure Limited
      - Maximum Typically 600 psi
      - Usually Requires Pressure Reducer
  - Instrument Configurations
    - Double Beam
      - Self Correction for Random Intensity Variations
    - Single Beam
      - Simpler, Less Expensive

Refractive Index (RI)

- The speed of light in a medium of refractive index n is C/n, where C is the speed of light in vaccum.

For vaccum n=1

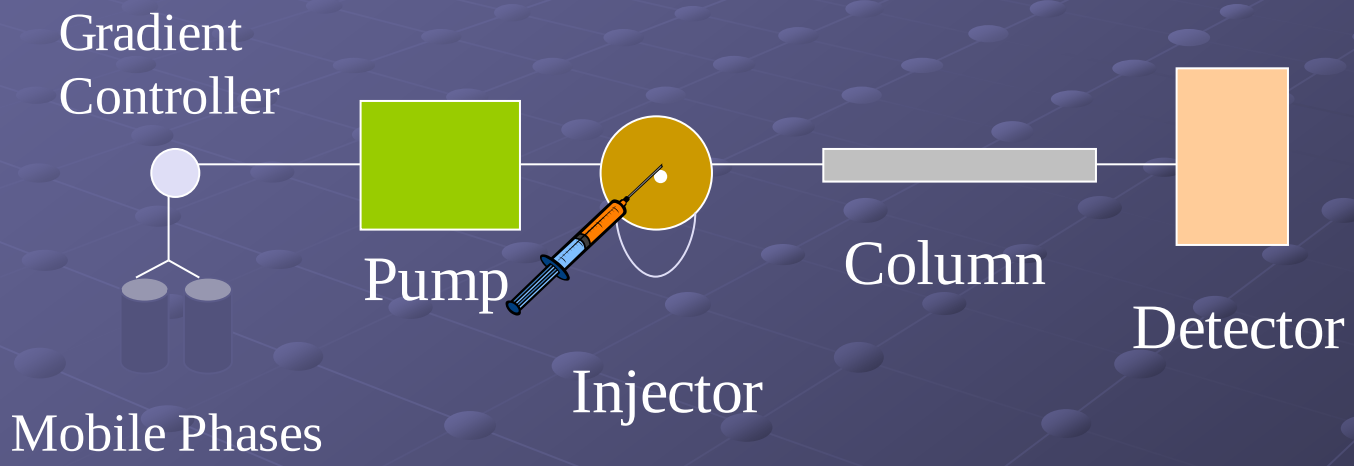
The refractive index of a material is a function of the wavelength of light and temperature

Making Sensitive Derivatives

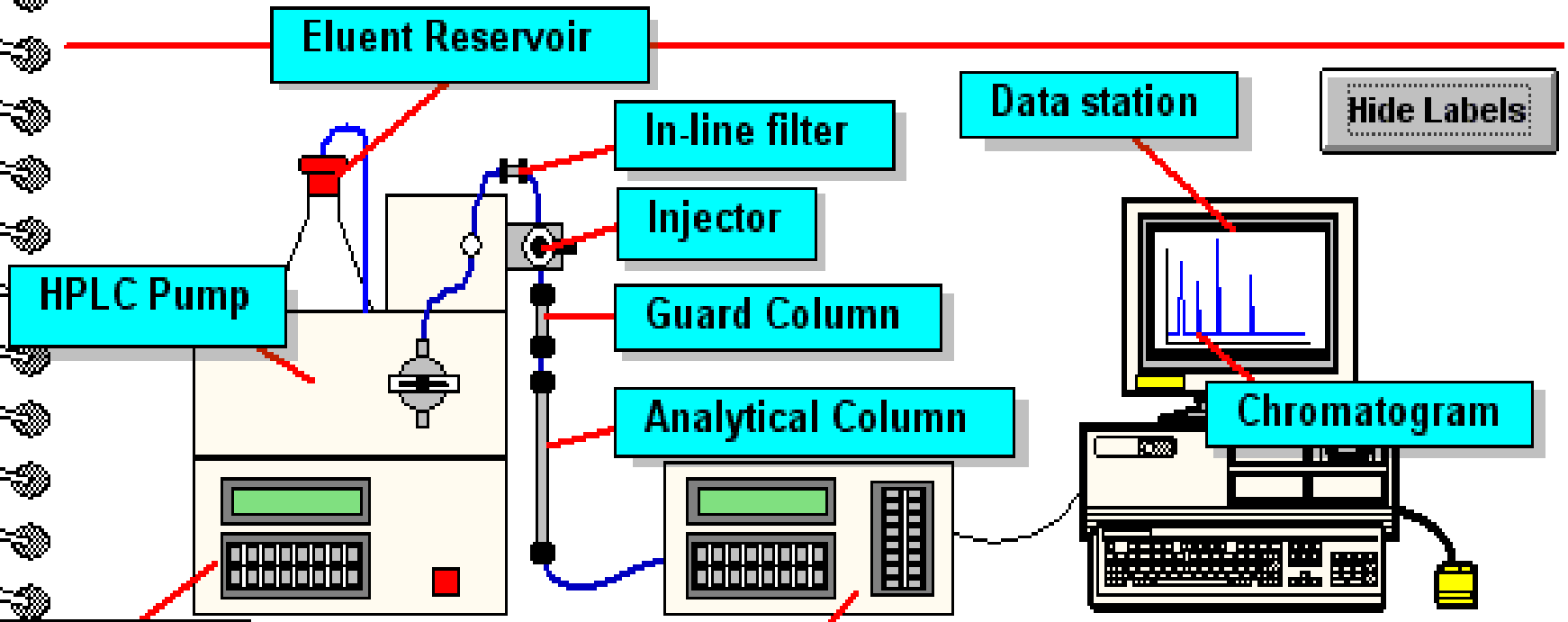
- Pre column reaction
- Post column reaction

Instrumentation

Instrumentation



Instrumentation Overview

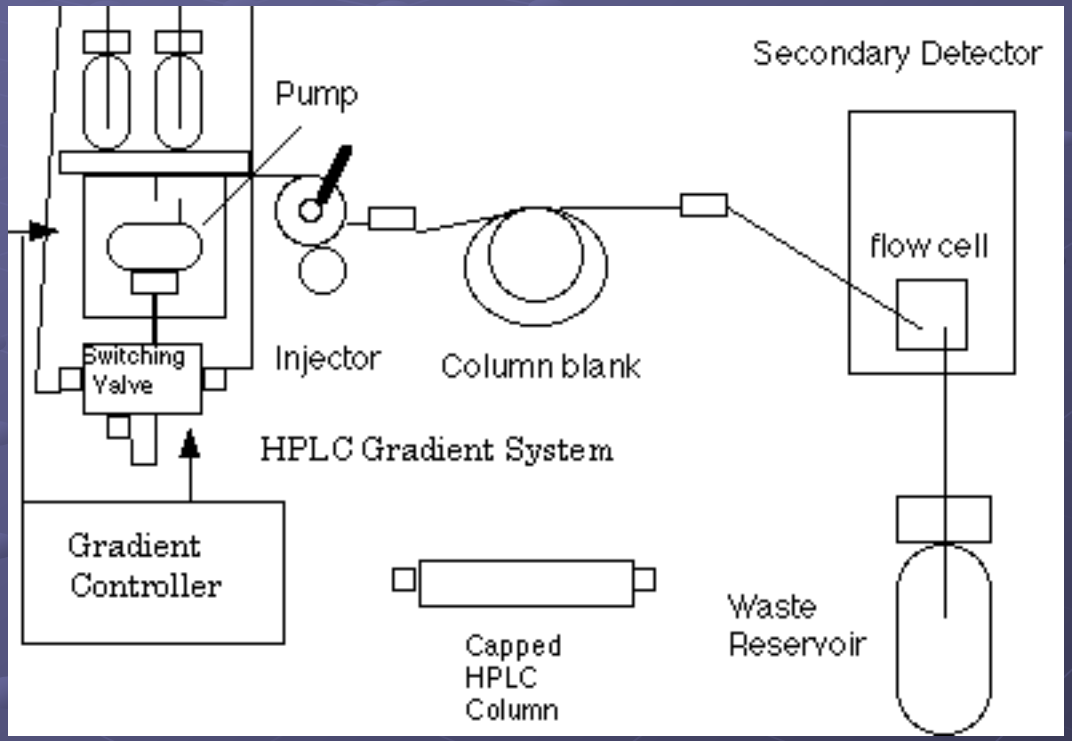


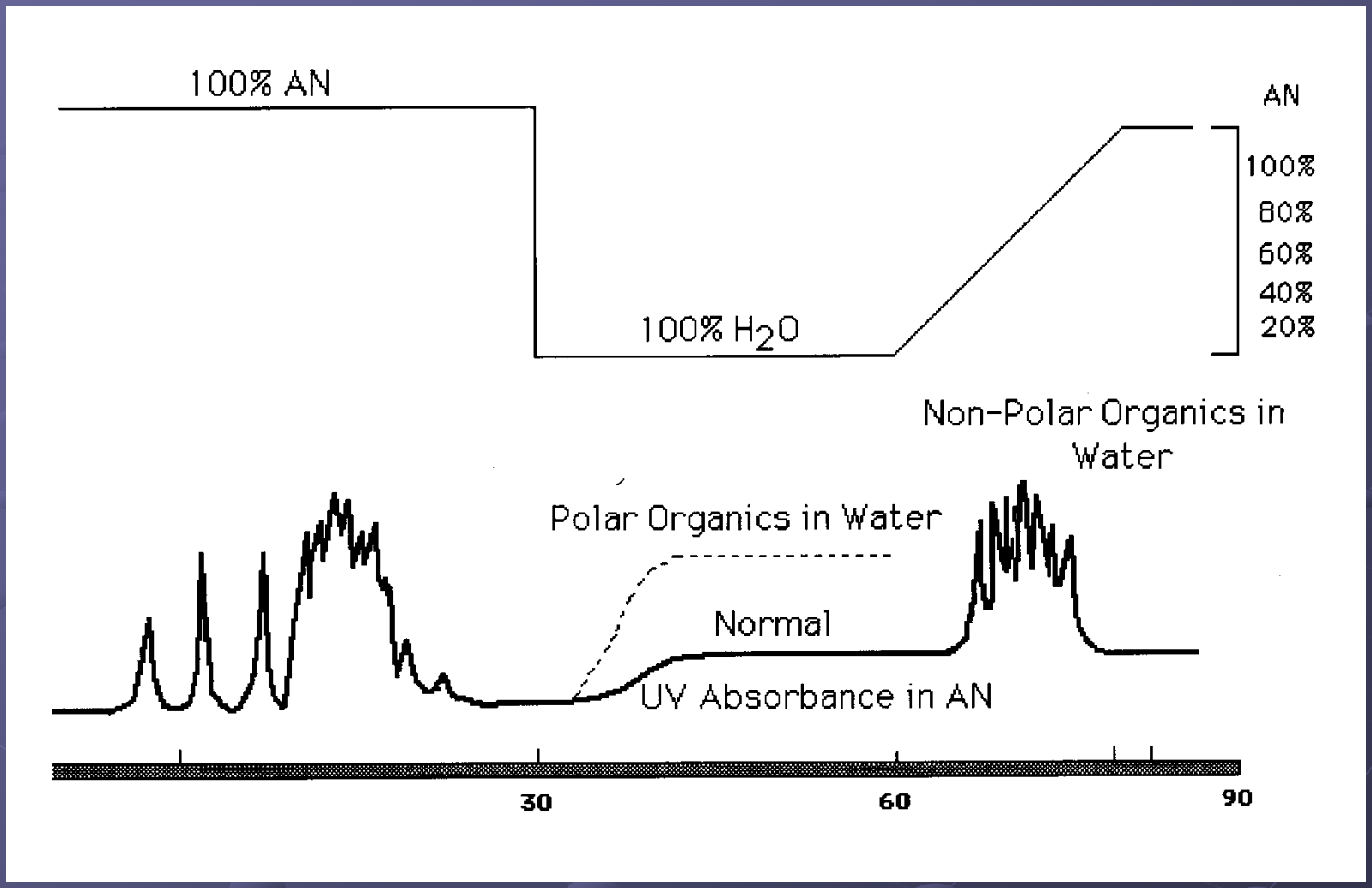
HPLC instrumentation comprises several components, as illustrated above. Each of the major components is described in more detail on the following pages. The choice of each component, (e.g. isocratic or gradient pumping system, type of column and detector), depends very much on the individual applications. Most instrument manufacturers supply an integrated system of components that are designed to work together under the control of one data system.

HPLC System Maintenance

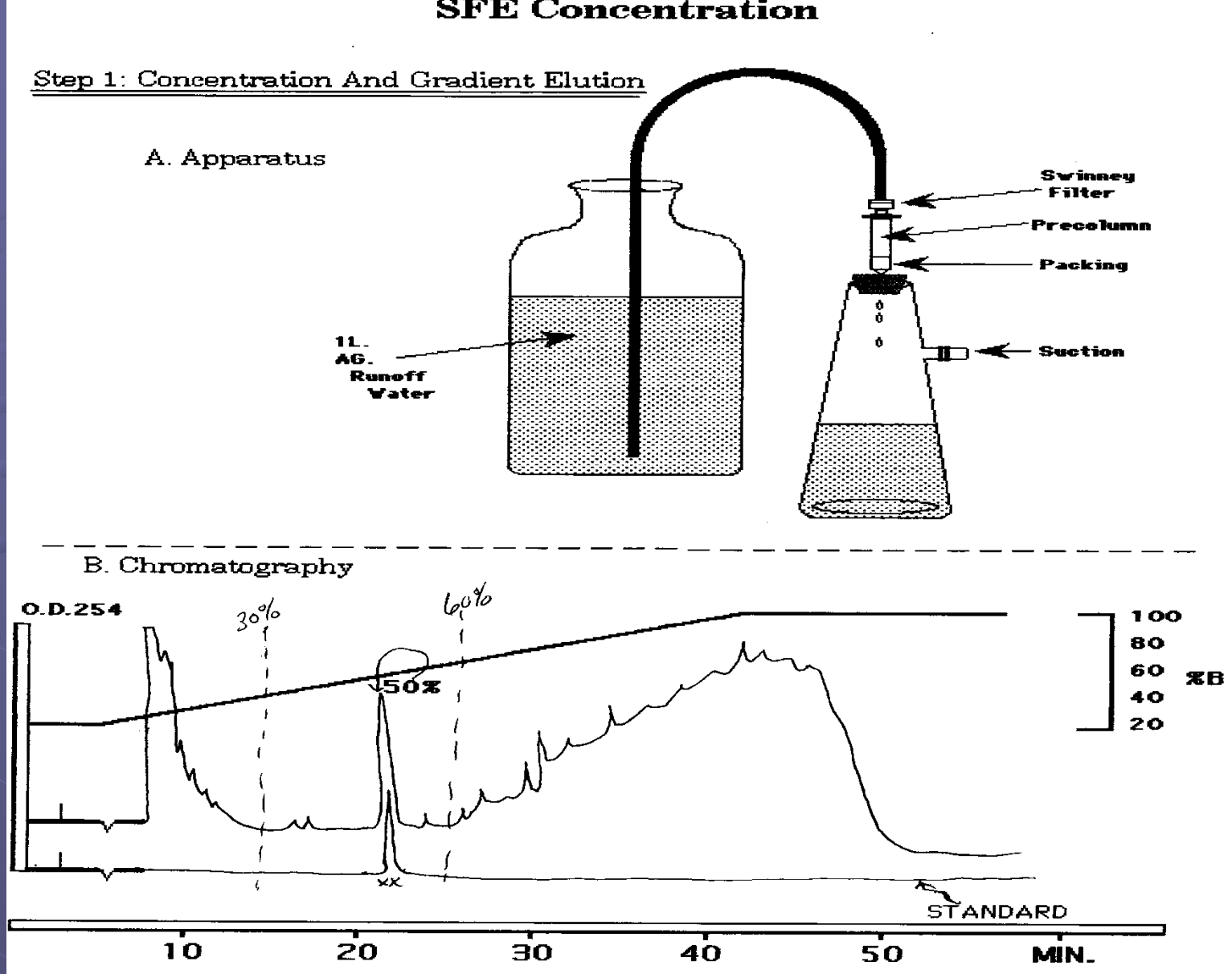
- Reverse Order Diagnostics
- Injector and tubing clearing
- Gradient checking with acetone spike
- Water Diagnostics
- Column cleaning and column killers

Column Blank for System Pacification




100% AN
AN
100%
80%
60%
40%
20%
100% H_2O
Non-Polar Organics in Water
Polar Organics in Water
Normal
UV Absorbance in AN
30
60
90

SFE Concentration



FOUR TYPES OF LIQUID CHROMATOGRAPHY

- Partition chromatography
- Adsorption, or liquid-solid
- Chromatography
- Ion exchange chromatography
- Size exclusion, or gel, chromatography

Quantification in Chromatography

- Four methods
  - Normalizing peak areas
  - Internal standard
  - External standards
  - Standard addition

Normalizing Peak Areas

- Inject mixture with equal amounts of all components
- The area of each peak is measured precisely
  - Replicate (5+) injections
  - Need good precision
  - One component is chosen as the reference
- The areas of the others are normalized with respect to that one
  - Detector response factors (*DRF*)

Internal Standard

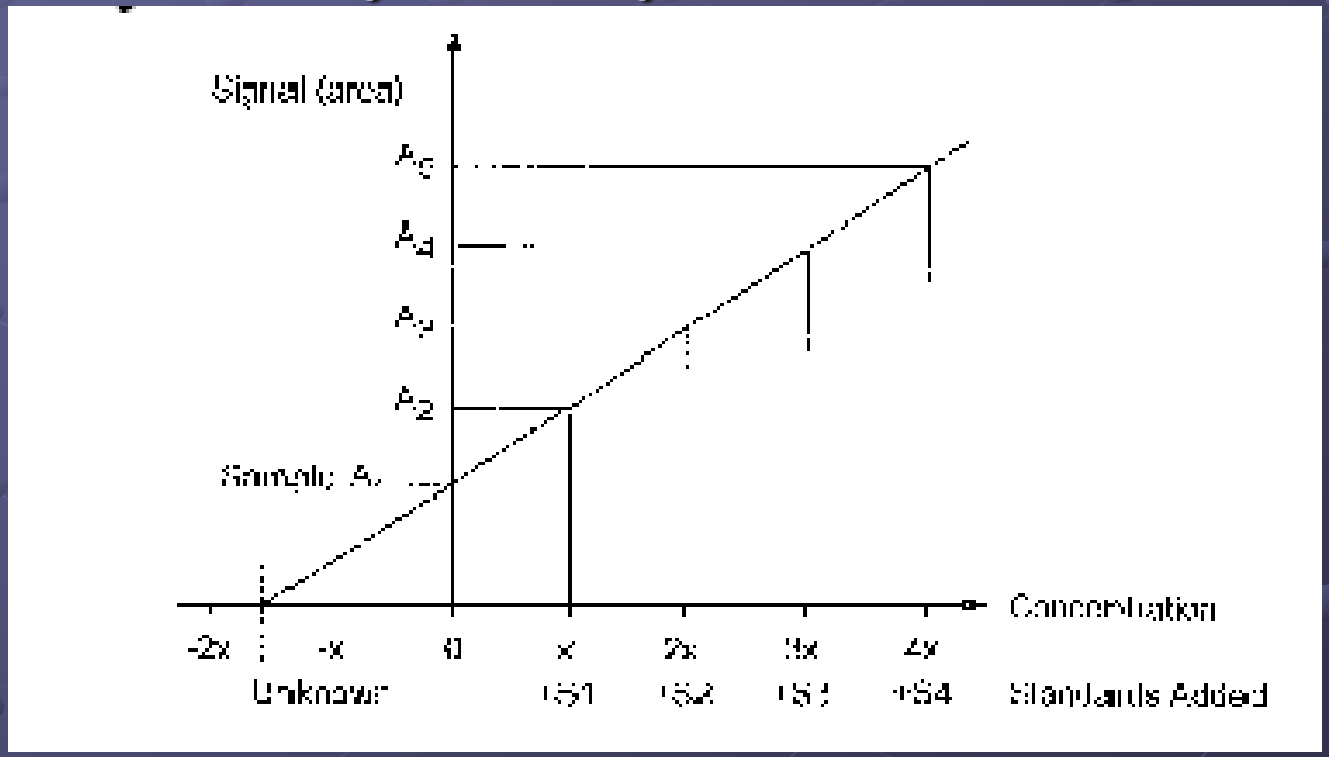
- Eliminate the need for accurate injections
- A reference standard is included in each sample *at accurately known concentration*
- Eluted in a gap in the chromatogram

External Standard

- Inject standard before and/or after analyzing the sample
- Standard will be on a different chromatogram
- Depends on good injection reproducibility
- HPLC multiport valves < 1% difference

Standard Addition

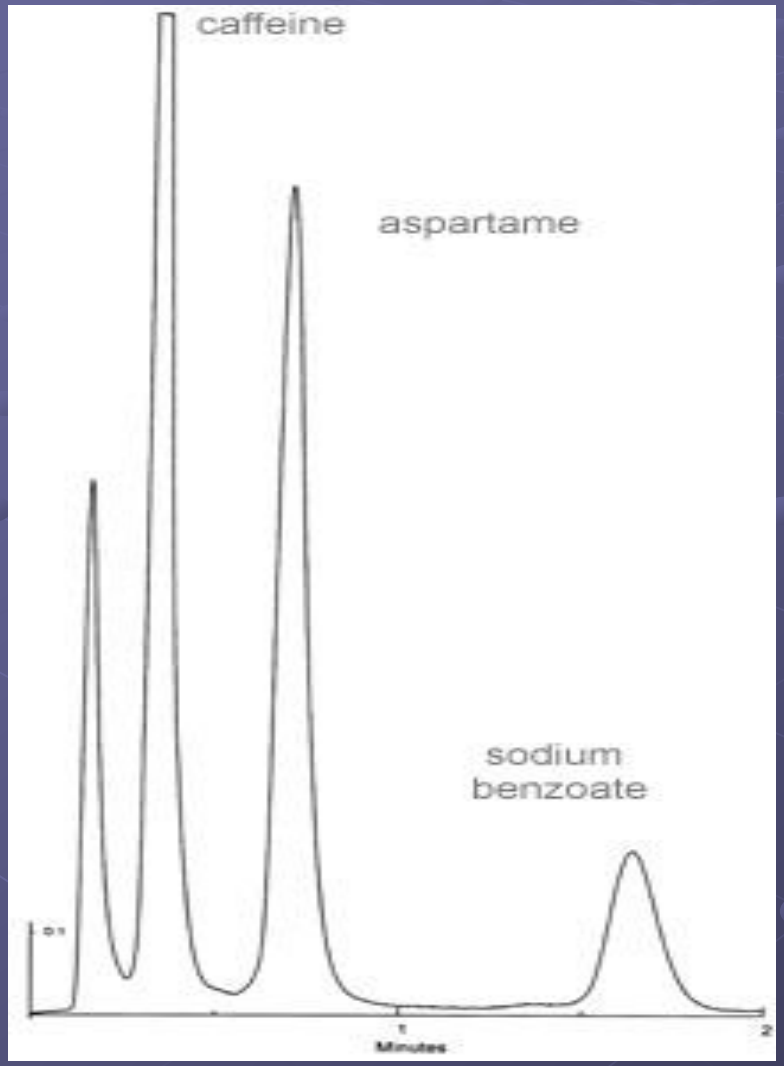
- Sample is analyzed
- Extra analyte is added, re-analysis
- Can use to verify linearity



HPLC Applications

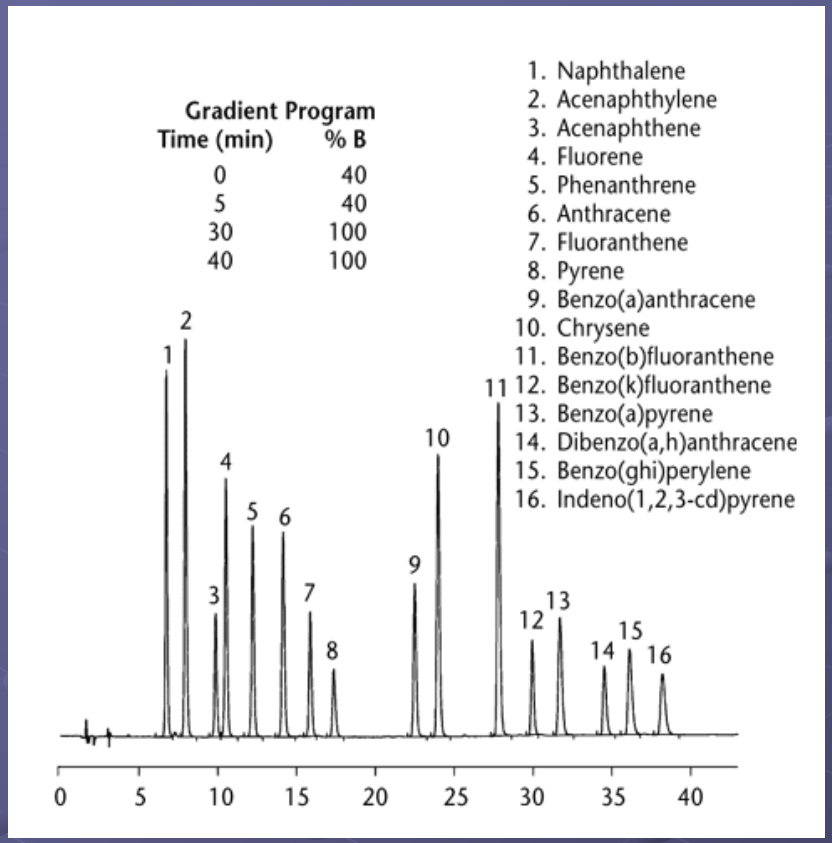
- Drug and compound discovery
- Proteomics and other biologicals
- Metabolites, Impurities, degradations
- Toxicology: Drugs of Abuse
- Clinical: Therapeutic Drug monitoring
- Arson residue determination
- Water and pesticides analysis

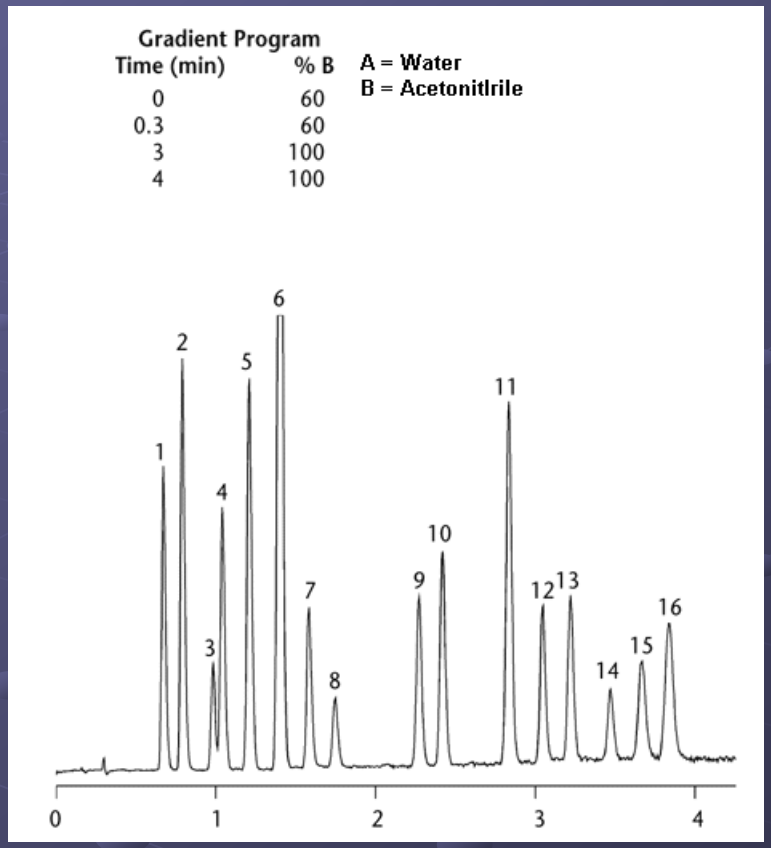
Analysis of Diet Cola Additives



- Conditions:
  - Cartridge column 20x2.1mm **HAIS*IL* HL** C_{18}
  - 200µL/min
  - 15% acetonitrile/ 10mM phosphate buffer (pH 2.2)
    Detector: 210nm

Chromatograms





A Supelcosil LC-PAH Columns. B

Conditions: A: 150mm x 4.6mm, 5µ.

Flow Rate: 1.5 mL/min

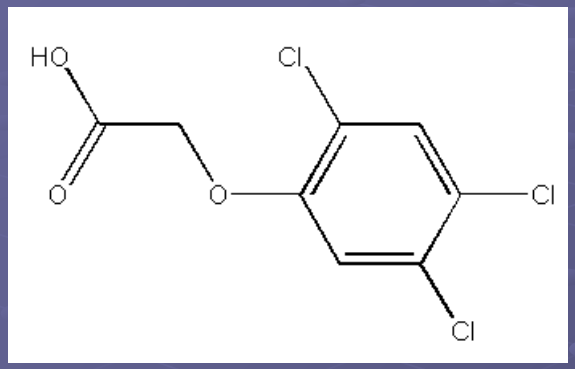
Conditions: B: 50mm x 4.6mm, 3µ.

Flow Rate: 3.0 mL/min

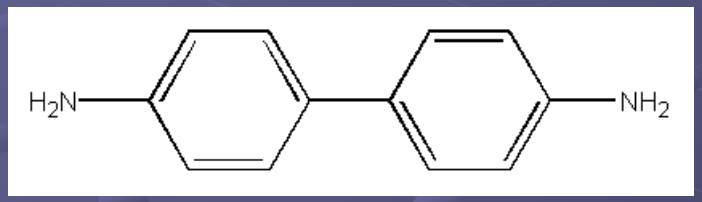
HPLC Methods

Parameter Group	Method	Compounds
SDW05.23000's	EPA 555	Cl-PhenoxyAcids
WPP05.06000's	EPA 605	Benzidines
WPP05.13000's	EPA 610	PAHs
SHW06.26000's	SW-846 8316	Acrylics
SHW06.28000's	SW-846 8330's	Explosives
SHW07.06000's	SW-846 8325	Benzidines and N- Pesticides

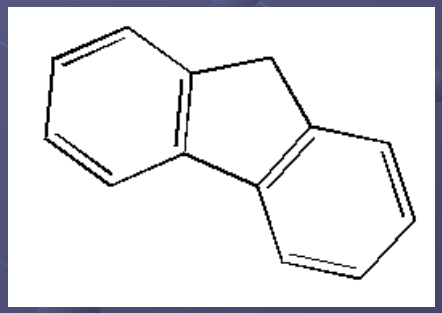
Compounds



2,4,5-T

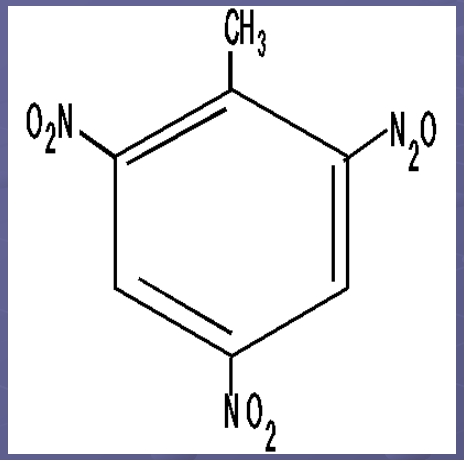


Benzidine



Fluorene

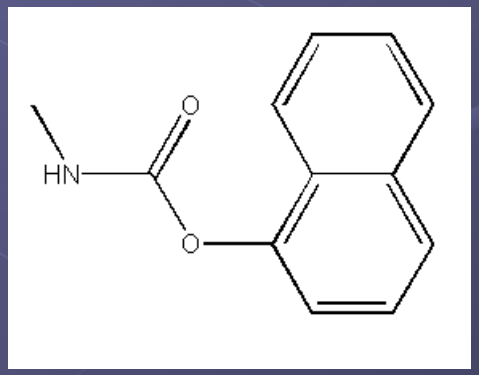
Compounds



TNT (2,4,6-Trinitrotoluene)

$H_2C=CH-CN$

Acrylonitrile



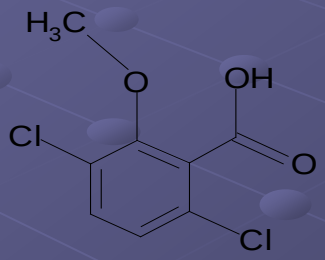
Carbaryl

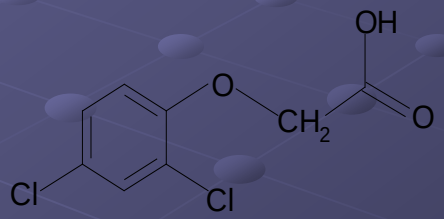
Determination of chlorophenoxy acid herbicides in water by liquid phase microextraction and high performance liquid chromatography detection

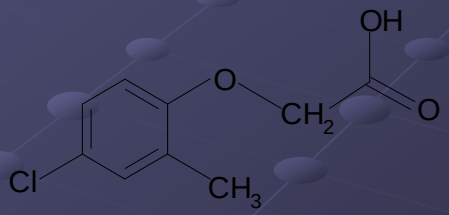
Peak No.	Chlorophenoxy acid hrebicide	Enrichment factor	LOD (ng/ml)	RSD%[a]	R^2	Linearity range (ng/ml)
1	Dicamba	192	0.4	3.9-6.8	0.9992	5.0-500.0
2	2,4-D	293	0.3	2.2-5.8	0.9994	5.0-500.0
3	MCPA	390	0.4	1.1-5.1	1.0000	5.0-500.0
4	2,4-DP	352	0.3	2.4-6.2	0.9990	5.0-500.0
5	Mecoprop	344	0.4	3.1-6.5	1.0000	5.0-500.0

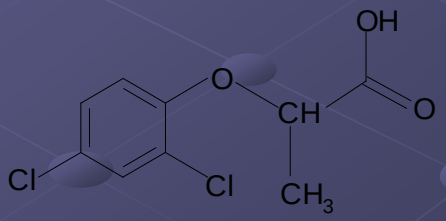
General structure of chlorophenoxy acid herbicides considered in this work

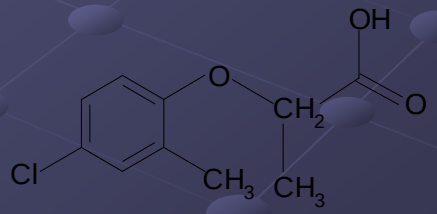
Chlorophenoxy acid	IUPAC name
Dicamba	(3,6-dichloro-2-methoxy) benzoic acid
2,4-D	(2,4-dichlorophenoxy) acetic acid
MCPA	(2-methyl-4-phenoxy) acetic acid
2,4-DP	2-(2,4-dichlorophenoxy) propanoic acid
Mecoprop	2-(2-methyl-4-chlorophenoxy) propionic acid



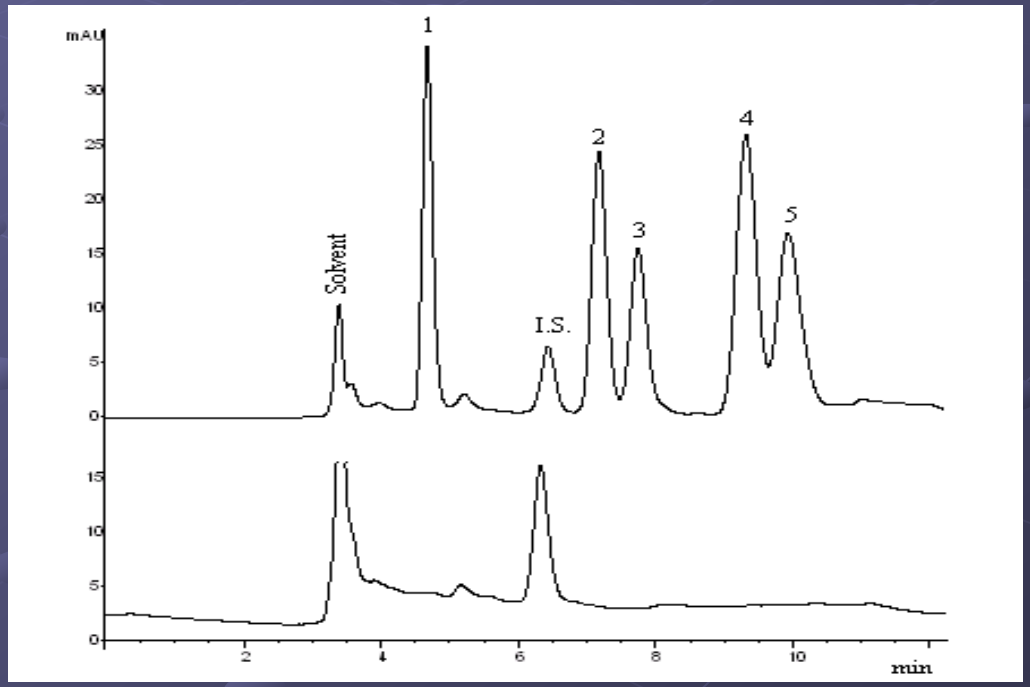








Injection procedures were carried out using a 25 µl Hamilton syringe, model 702 NR. Chromatographic analysis was carried out on a HP SERIES 1100 (USA) HPLC system. The chromatographic system consisted of injector equipped with a 20 ml sample loop, a HP SERIES 1100 pump, and a HP SERIES 1100 UV–vis spectrophotometric detector. A column (250mm×4.6mm I.D.) from Waters (CA, USA) packed with C18 (µBondPak™) was used. Data was collected and processed by ChemStation (Agillent Technologies, USA) data analysis software. A flow-rate 1.0 ml/min was applied in laboratory temperature of 20 (±2) °C. The mobile phase was methanol–25 mM phosphate buffer (60:40, v/v; pH = 2.75) and the detection wavelength was 240 nm.



Procedures for analysis

- *Step 1 – Extraction*
- *Step 2 – Cleanup*
- *Step 3 – Analysis*

Extraction methods

- Liquid-liquid
- Soxhlet
- Automated soxhlet
- Microwave
- Supercritical fluid
- Accelerated solvent extraction
- Ultrasonic
- Solid phase extraction (SPE)
- Blender

Thanks for your attention