Chem 551
Separation Techniques

1. MODERN HPLC FOR PRACTICING SCIENTISTS
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   Chapters 1-5

2. MODERN PRACTICE OF GAS CHROMATOGRAPHY
   Fourth Edition
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   Chapters 1, 2, 3 & 6

Chromatography permit the scientist to separate closely related components of complex mixtures. In all chromatographic separations the sample is transported in a mobile phase, which may be a gas, a liquid, or a supercritical fluid. This mobile phase is then forced through an immiscible stationary phase, which is fixed in place in a column or on a solid surface. The two phases are chosen so that the components of the sample distribute themselves between the mobile and stationary phase to varying degrees.

Classification of Chromatographic Techniques

Chromatographic methods can be categorized in two ways. The first classification is based upon the physical means by which the stationary and mobile phases are brought into contact. In column chromatography, the stationary phase is held in a narrow tube through which the mobile phase is forced under pressure. In planar chromatography, the stationary phase is supported on a flat plate or in the interstices of a paper; here, the mobile phase moves through the stationary phase by capillary action or under the influence of gravity.

Classification of Chromatographic Methods

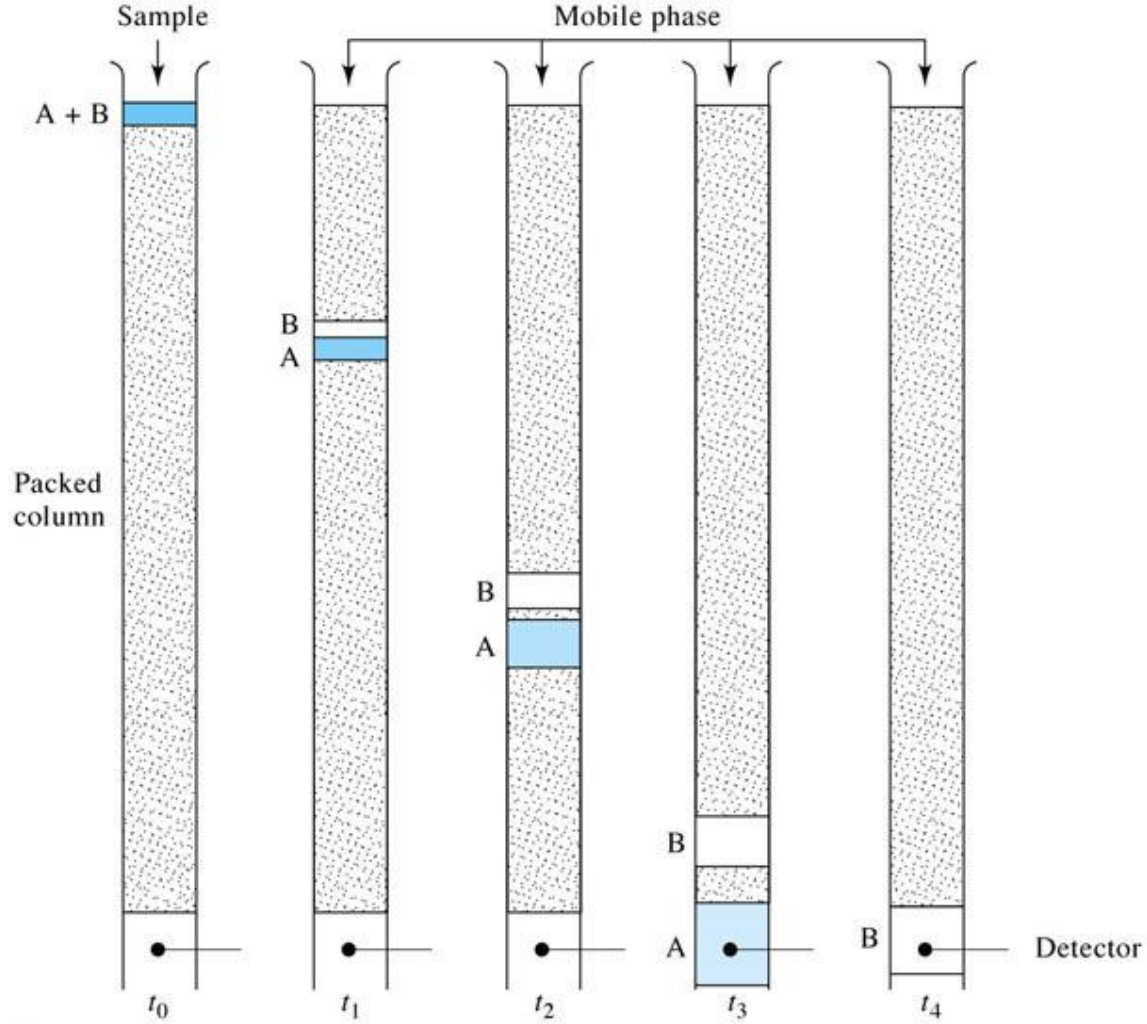
A more fundamental classification of chromatographic methods is one based upon the types of mobile and stationary phases and the kinds of equilibria involved in the transfer of solutes between phases. **Three** general categories of chromatography: **(1)** liquid chromatography, **(2)** gas chromatography, and **(3)** supercritical-fluid chromatography. The mobile phases in the three techniques are liquids, gases, and supercritical fluids respectively.

TABLE 26-1 Classification of Column Chromatographic Methods

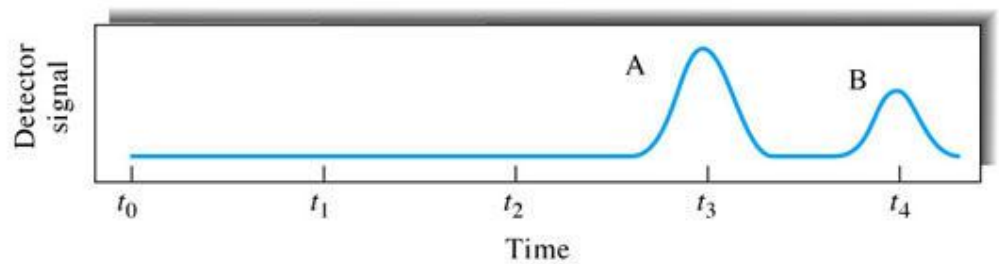
General Classification	Specific Method	Stationary Phase	Type of Equilibrium
1. Gas chromatography (GC)	a. Gas-liquid chromatography (GLC)	Liquid adsorbed or bonded to a solid surface	Partition between gas and liquid
	b. Gas-solid	Solid	Adsorption
2. Liquid chromatography (LC)	a. Liquid-liquid, or partition	Liquid adsorbed or bonded to a solid surface	Partition between immiscible liquids
	b. Liquid-solid, or adsorption	Solid	Adsorption
	c. Ion exchange	Ion-exchange resin	Ion exchange
	d. Size exclusion	Liquid in interstices of a polymeric solid	Partition/sieving
	e. Affinity	Group specific liquid bonded to a solid surface	Partition between surface liquid and mobile liquid
3. Supercritical fluid chromatography (SFC; mobile phase: supercritical fluid)		Organic species bonded to a solid surface	Partition between supercritical fluid and bonded surface

Elution Chromatography on Columns

Elution involves washing a species through a column by continuous addition of fresh solvent. The sample is introduced at the head of a column, whereupon the components of the sample distribute themselves between the two phases. Introduction of additional mobile phase (the eluent) forces the solvent containing a part of the sample down the column, where further partition between the mobile phase and fresh portions of the stationary phase occurs.



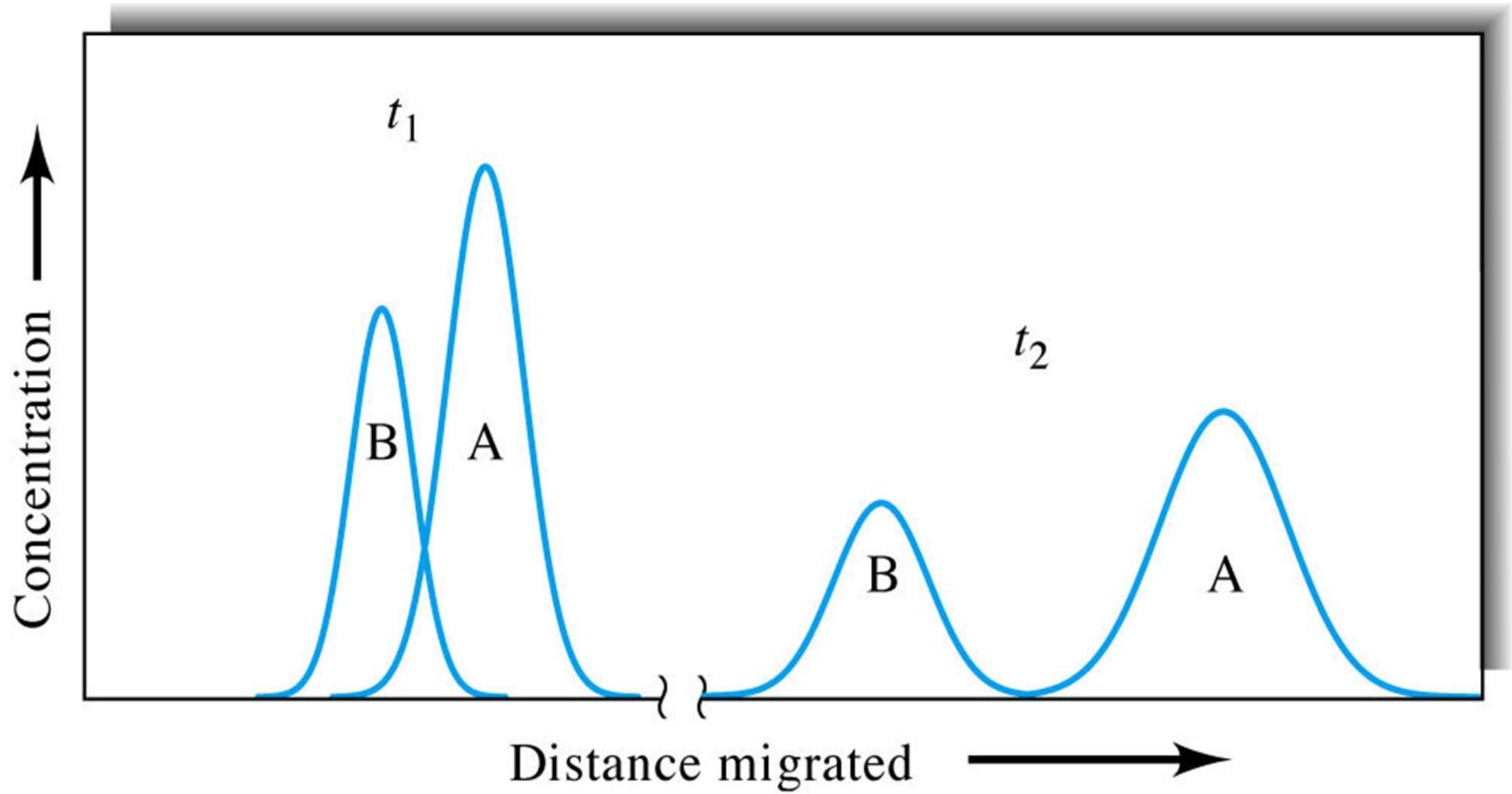
(a)



(b)

Chromatograms

If a detector that responds to solute concentration is placed at the end of the column and its signal is plotted as function of time (or of volume of the added mobile phase), a series of peaks is obtained. Such a plot, called a chromatogram, is useful for both qualitative and quantitative analysis. The positions of peaks on the time axis may serve to identify the components of the sample; the areas under the peaks provide a quantitative measure of the amount of each component.



What Is HPLC?

Liquid chromatography (LC) is a physical separation technique conducted in the liquid phase. A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). For example, the flowing liquid can be an organic solvent such as hexane and the stationary phase can be porous silica particles packed in a column. HPLC is a modern form of LC that uses small-particle columns through which the mobile phase is pumped at high pressure.

Advantages and Limitations of HPLC

Advantages:

- Rapid and precise quantitative analysis
- Automated operation
- High-sensitivity detection
- Quantitative sample recovery
- Amenable to diverse samples

Limitations:

- No universal detector
- Less separation efficiency than capillary GC
- More difficult for novices

These limitations have been largely minimized through instrumental and column developments.

MODES OF HPLC

1. Normal-Phase Chromatography (NPC)
2. Reversed-Phase Chromatography (RPC)
3. Ion-Exchange Chromatography (IEC)
4. Size-Exclusion Chromatography (SEC)
5. Other Separation Modes

1. Normal-Phase Chromatography (NPC)

Also known as liquid-solid chromatography or adsorption chromatography, based on adsorption/desorption of the analyte onto a polar stationary phase (typically silica or alumina). Polar analytes migrate slowly through the column due to strong interactions with the silanol groups(Si-OH). NPC is useful for the separation of nonpolar compounds and isomers. One major disadvantage of this mode is the easy contamination of the polar surfaces by sample components. This problem is partly reduced by bonding polar functional groups such as amino- or cyano-moiety to the silanol groups.

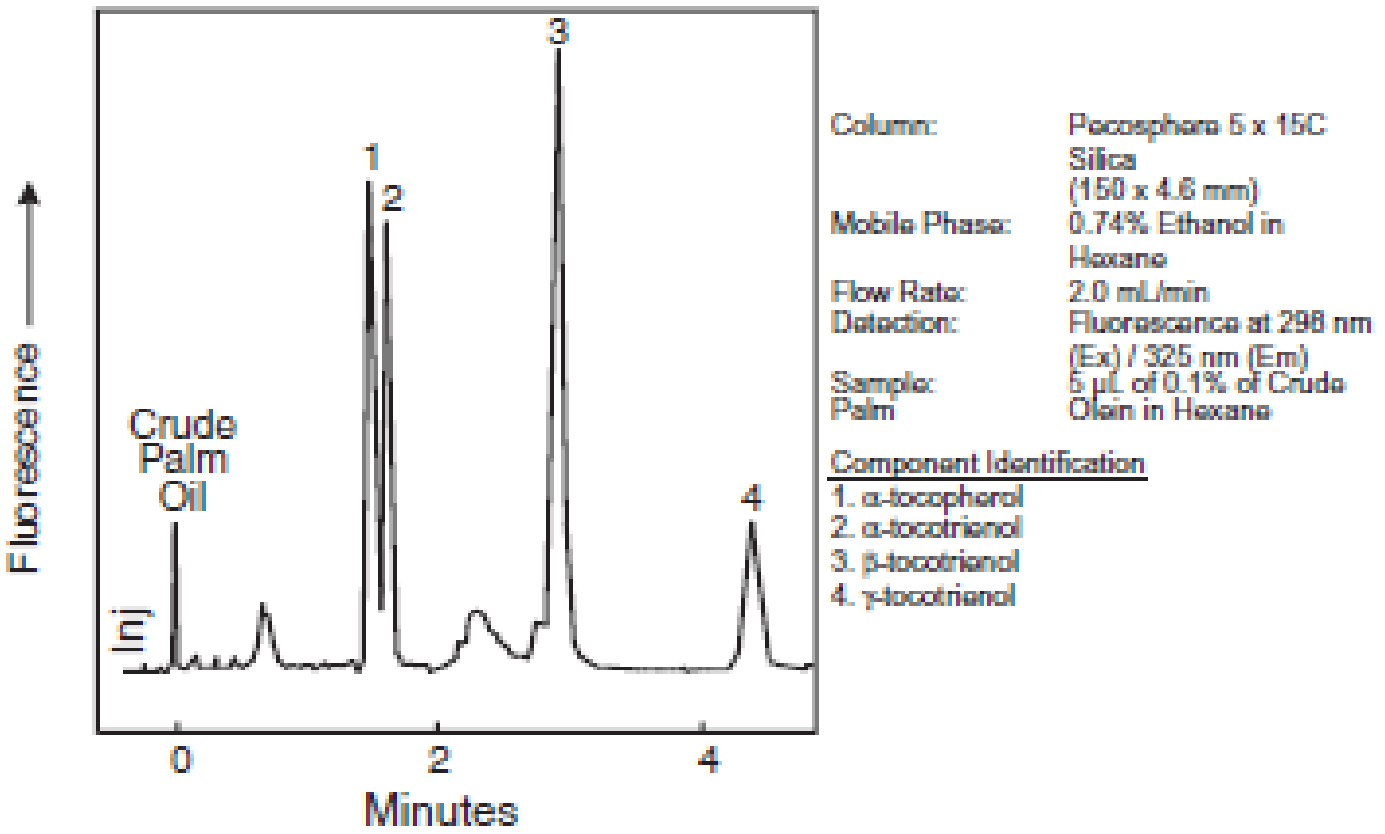


Figure 1.4. A normal-phase HPLC chromatogram of a palm olein sample showing the separation of various isomers of vitamin E. Chromatogram courtesy of PerkinElmer.

2. Reversed-Phase Chromatography (RPC)

Separation is based on analytes' partition coefficients between a polar mobile phase and a hydrophobic (nonpolar) stationary phase (solid particles coated with nonpolar liquids). Polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic C18 groups that form a "liquidlike" layer around the solid silica support. This elution order of "polar first and nonpolar last" is the reverse of that observed in NPC. RPC uses a polar mobile phase such as a mixture of methanol or acetonitrile with water. RPC is the most popular HPLC mode and it is suitable for the analysis of polar (water-soluble), medium-polarity, and some non-polar analytes.

3. Ion-Exchange Chromatography (IEC)

The separation mode is based on the exchange of ionic analytes with the counter-ions of the ionic groups attached to the solid support. Typical stationary phases are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. Mobile phases consist of buffers, often with increasing ionic strength, to force the migration of the analytes.

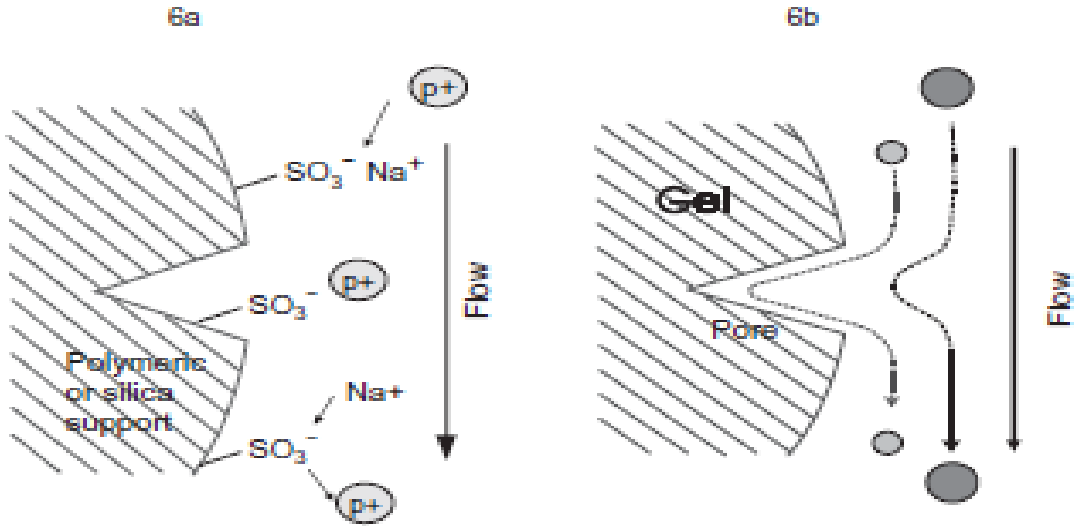


Figure 1.6. a. Schematic diagrams depicting separation modes of (a) ion-exchange c
matography (IEC), showing the exchange of analyte ion p^+ with the sodium counter ions o
bonded sulfonate groups; (b) size-exclusion chromatography (SEC), showing the faster m
tion of large molecules.

4. Size-Exclusion Chromatography (SEC)

It is a separation mode based solely on the analyte’s molecular size. A large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate the pores and migrates more slowly down the column. It is often called gel-permeation chromatography (GPC) when used for the determination of molecular weights of organic polymers and gel-filtration chromatography (GFC) when used in the separation of water-soluble biological materials. In GPC, the column is packed with cross-linked polystyrene beads of controlled pore sizes and eluted with common mobile phases such as toluene and tetrahydrofuran.

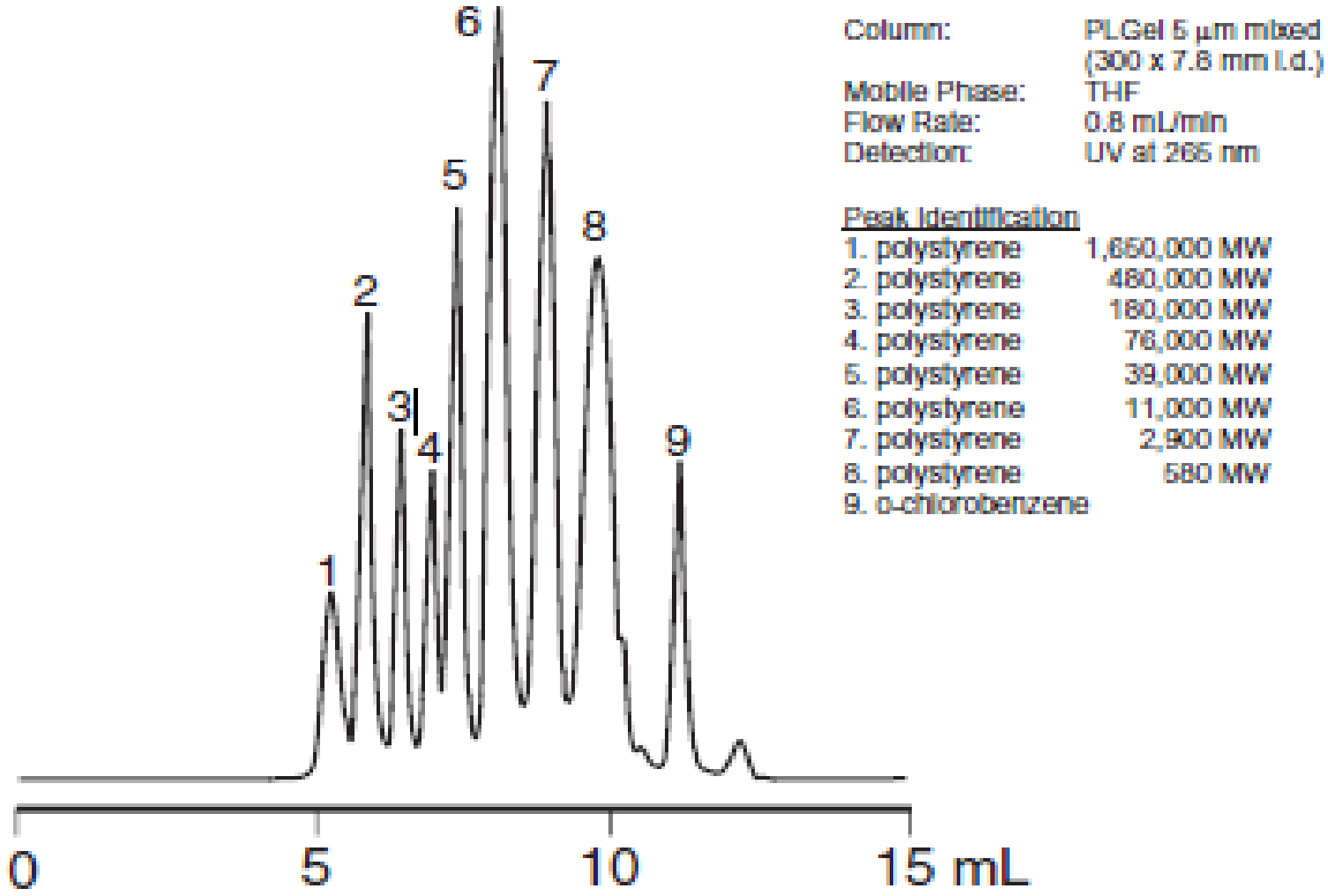


Figure 1.8. A GPC chromatogram of polystyrene standards on a mixed-bed polystyrene column. Chromatogram courtesy of Polymer Laboratories.

5. Other Separation Modes

Besides the four major HPLC separation modes, several others often encountered in HPLC or related techniques are noted below.

- *Affinity chromatography*: Based on a receptor/ligand interaction in which immobilized ligands (enzymes, antigens, or hormones) on solid supports are used to isolate selected components from a mixture. The retained components can later be released in a purified state.
- *Chiral chromatography*: For the separation of enantiomers using a chiral-specific stationary phase. Both NPC and RPC chiral columns are available.
- *Hydrophilic interaction chromatography (HILIC)* : It is somewhat similar to normal phase chromatography using a polar stationary phase such as silica or ion-exchange materials but eluted with polar mobile phases of organic solvents and aqueous buffers.

- *Hydrophobic interaction chromatography*: Analogous to RPC except that mobile phases of low organic solvent content and high salt concentrations are used for the separation of proteins that are easily denatured by mobile phases with high concentrations of organic solvents used in RPC.
- *Electrochromatography*: Uses capillary electrophoresis (CE) equipment with a packed capillary HPLC column. The mobile phase is driven by the electromotive force from a high-voltage source as opposed to a mechanical pump. It is capable of very high efficiency.
- *Supercritical fluid chromatography (SFC):* Uses HPLC packed columns and a mobile phase of pressurized supercritical fluids (i.e., carbon dioxide modified with a polar organic solvent). Useful for nonpolar analytes and preparative applications where purified materials can be recovered easily by evaporating the carbon dioxide. HPLC pumps and GC-type detectors are often used.

- *Other forms of low-pressure liquid chromatography*:

— Thin-layer chromatography (TLC): uses glass plates coated with adsorbents and capillary action as the driving force. Useful for sample screening and semi-quantitative analysis.

— Paper chromatography (PC): a form of partition chromatography using paper as the stationary phase and capillary action as the driving force.

— Flash chromatography: a technique for sample purification using disposable glass NPC columns and mobile phase driven by gas-pressure or low-pressure pumps.

SOME COMMON-SENSE COROLLARIES

The goal of most HPLC analysis is to separate analyte(s) from other components

in the sample for accurate quantitation. Several corollaries are often

overlooked by practitioners:

1. *Sample must be soluble*: "If it's not in solution, it cannot be analyzed by HPLC." Solubility issues often complicate assays of low-solubility analytes or component difficult to extract from sample matrices.
2. *For separation to occur, analytes must be retained and have differential migration in the column*: Separation cannot occur without retention and sufficient interaction with the stationary phase. For quantitative analysis, analytes must have different retention on the column versus other components.

3. *The mobile phase controls the separation*: Whereas the stationary phase provides a media for analyte interaction, the mobile phase controls the overall separation.
4. *All C18-bonded phase columns are not created equal and are not interchangeable*: There are hundreds of C18 columns on the market. They vary tremendously in their retention and silanol characteristics.
5. *The final analyte solution should be prepared in the mobile phase*: The final analyte solution, should be dissolved in the mobile phase or a solvent of “weaker” strength than the starting mobile phase. Many anomalies such as splitting peaks or fronting peaks are caused by injecting samples dissolved in solvents stronger than the mobile phase.
6. *Every analytical method has its own caveats, limitations, or pitfalls*: An experienced method development scientist should identify these potential pitfalls and focus on finding conditions to minimize these problems areas for more reliable analysis.