FE 315 INSTRUMENTAL ANALYSIS
High Performance Liquid Chromatography (HPLC)

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High Performance Liquid Chromatography (HPLC) is one mode of chromatography, the most widely used analytical technique. Chromatographic processes can be defined as separation techniques involving mass-transfer between stationary and mobile phases. Present day liquid chromatography that generally utilizes very small packing particles and a relatively high pressure is referred to as high performance liquid chromatography (HPLC).

HPLC utilizes a liquid mobile phase to separate the components of a mixture. These components are first dissolved in a solvent, and then forced to flow through a chromatographic column under high pressure. In the column, the mixture is resolved into its components. The amount of resolution is important, and is dependent upon the extent of interaction between the solute componentsand the stationary phase. The stationary phase is defined as the immobile packing material in the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and has the ability to easily separate a wide variety of chemical mixtures.

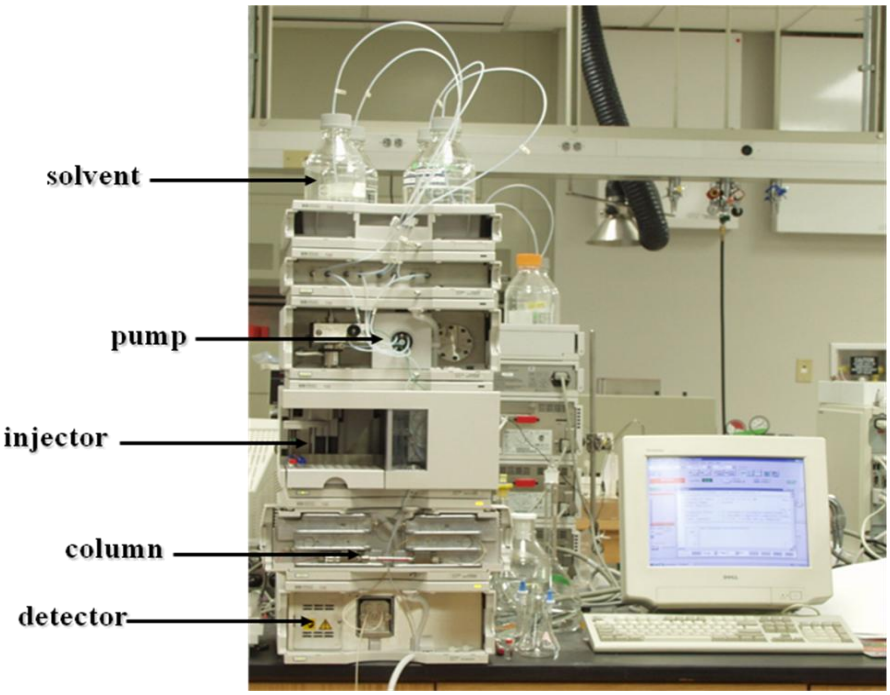
In the HPLC technique, the sample is forced through a column that is packed with irregularly or spherically shaped particles or a porous monolithic layer (stationary phase) by a liquid (mobile phase) at high pressure. HPLC is historically divided into two different sub-classes based on the polarity of the mobile and stationary phases. Technique in which the stationary phase is more polar than the mobile phase (e.g. toluene as the mobile phase, silica as the stationary phase) is called normal phase liquid chromatography (NPLC) and the opposite (e.g. water-methanol mixture as the mobile phase and C18 = octadecylsilyl as the stationary phase) is called reversed phase liquid chromatography (RPLC). Ironically the "normal phase" has fewer applications and RPLC is therefore used considerably more.

High performance liquid chromatography (HPLC) makes use of a high pressure pump to deliver a mobile phase solvent at a uniform rate at pressures that are typically from 500 to 5000 psi. The most obvious advantage of HPLC over gravity liquid chromatography is that samples can be separated much more quickly. In addition, samples that are not volatile or that would thermally decompose in gas chromatography can be rapidly and routinely separated in HPLC. Consequently, this powerful analytical method is a great complementary instrument to gas chromatography.

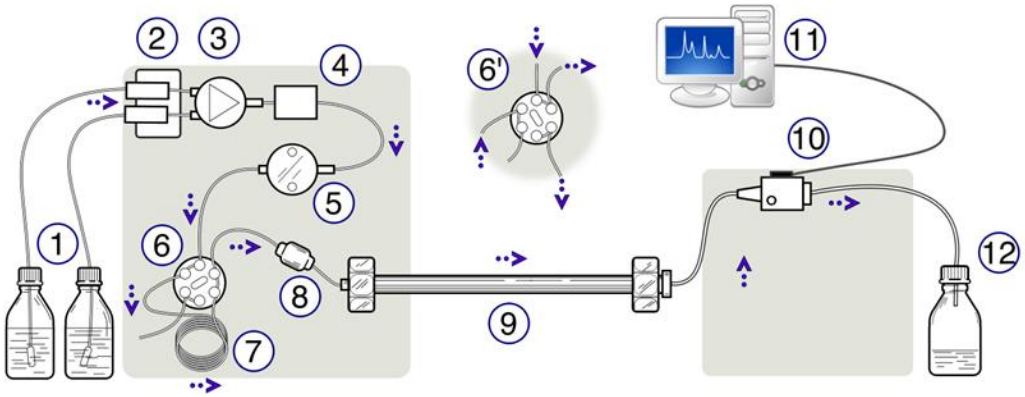
High-performance liquid chromatography (or **high-pressure liquid chromatography**, **HPLC**) is a chromatographic technique that can separate a mixture of compounds, and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

HPLC utilizes different types of stationary phase (typically, hydrophobic saturated carbon chains), a pump that moves the mobile phase(s) and analyte through the column, and a detector that provides a characteristic retention time for the analyte. The detector may also

provide other characteristic information (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase.
With HPLC, a pump (rather than gravity) provides the higher pressure required to propel the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.



HPLC System



Schematic Diagram of a High Performance Liquid Chromatograph. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column or guard column, (9) Analytical column, (10) Detector (i.e. IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

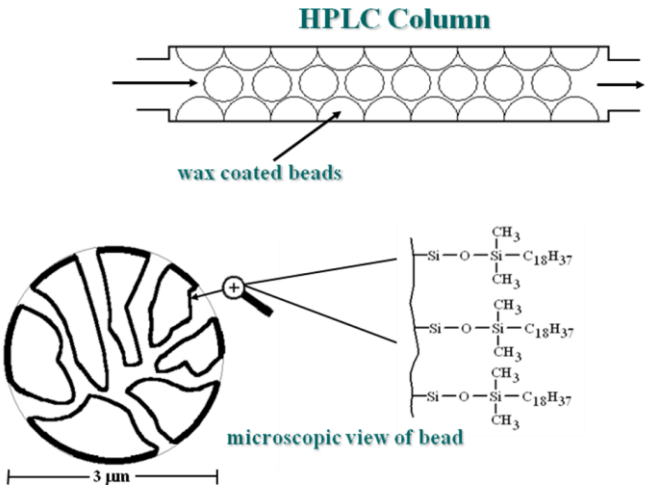
HPLC is accomplished by injection of a small amount of liquid sample into a moving stream of liquid (termed the mobile phase) that passes through a column packed with particles of a stationary phase. As in gas chromatography, separation of a mixture into its components

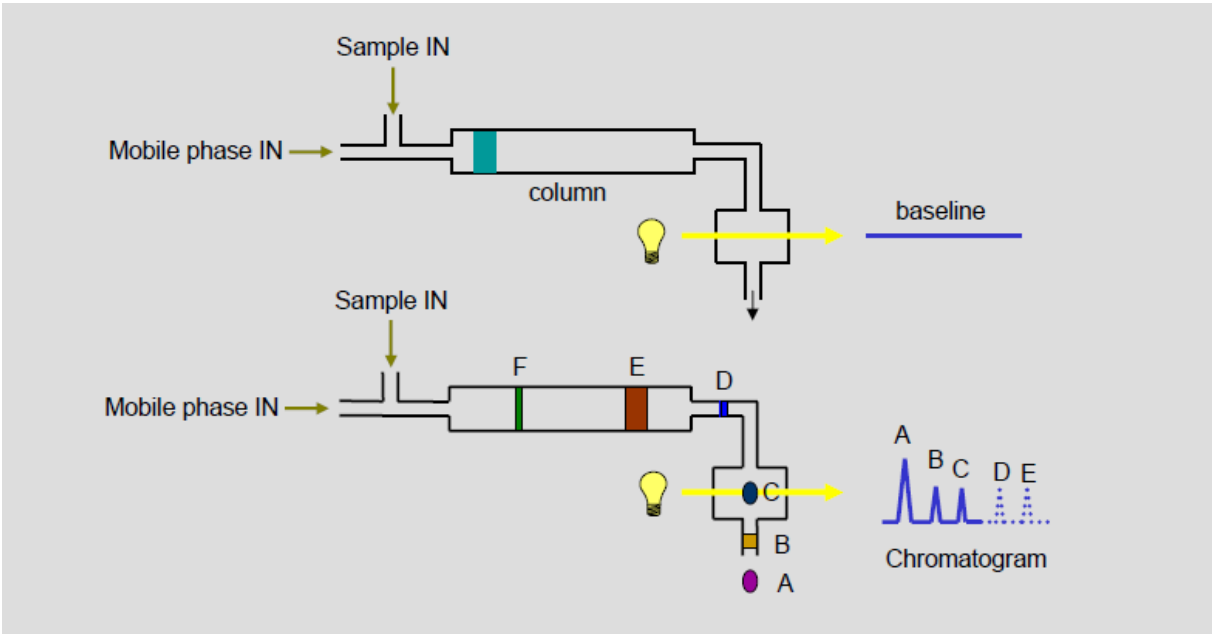
depends on different degrees of retention of each component in the column. The extent to which a component is retained in the column is determined by its partitioning between the liquid mobile phase and the stationary phases. A variety of HPLC separation techniques that utilize different stationary and mobile phases have been developed.

Stationary Phases

Silica Based Stationary Phases

The most popular column is a octadecyl carbon chain (C18) bonded silica. This is followed by C8 bonded silica, pure silica, cyano bonded silica and phenyl bonded silica. Note that C18, C8 and phenyl are dedicated reversed phase packings while cyano columns can be used in a reversed phase mode depending on analyte and mobile phase conditions. It should be noted at this point that not all C18 columns have identical retention properties. Surface functionalization of silica can be performed in a monomeric or a polymeric reaction with different short-chain organosilanes used in a second step to cover remaining silanol groups (end-capping). While the overall retention mechanism remains the same subtle differences in the surface chemistries of different stationary phases will lead to changes in selectivity.





Mobile Phase

Mixtures of water or aqueous buffers and organic solvents are used to elute analytes from a reversed phase column. The solvents have to be miscible with water and the most common organic solvents used are acetonitrile, methanol or tetrahydrofuran (THF). Other solvents can be used such as ethanol, 2-propanol (iso-propyl alcohol). Elution can be performed isocratic (the water-solvent composition does not change during the separation process) or by using a gradient (the water-solvent composition does change during the separation process). The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Charged analytes can be separated on a reversed phase column by the use of ion-pairing (also called ion-interaction). This technique is known as reversed phase ion-pairing chromatography.

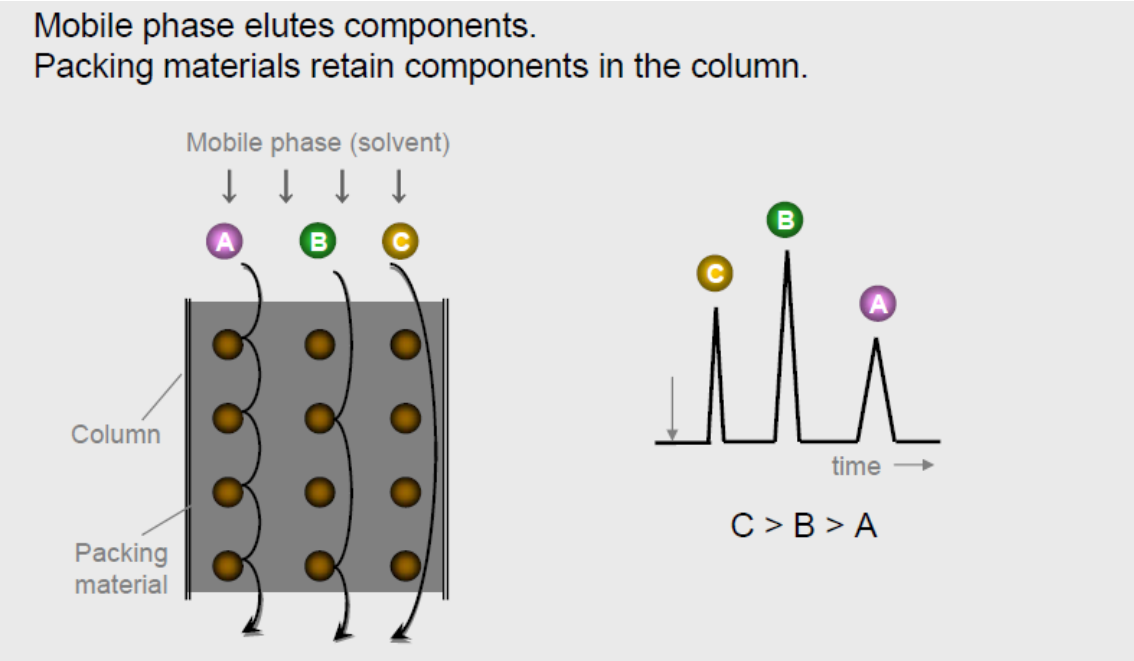


Table 1. Polarity Scale for Various Classes of Compounds in order of increasing polarity and retention.

Fluorocarbons
Saturated hydrocarbons
Olefins
Aromatics
Halogenated compounds
Ethers
Nitro compounds
Esters ≈ ketones ≈ aldehydes
Alcohols ≈ amines,
Amides
Carboxylic acids

Retention also can be controlled by the polarity of the mobile phase, which competes with sample components for adsorption sites. Thus, a more polar mobile phase will more

effectively displace adsorbed solute molecules and cause the retention time to decrease. Table 2 ranks solvents in the order of their strength of adsorption on the adsorbent alumina. Such a scale is called an eluotropic series. A solvent of higher polarity will displace one lower in the polarity scale.

In **absorption chromatography**, the stationary phase is a solid of a polar nature such as particles of hydrated silica or alumina. The mobile phase and the solute (components in the sample) are in competition for active adsorption sites on the stationary phase particles. Thus, more strongly adsorbed components are retained longer than weakly adsorbed components. Because more polar compounds adsorb on a polar surface to greater degree than do less polar compounds, retention in the column is related to sample polarity. A generalized polarity scale for various classes of compounds is shown in Table 1.

In **partition chromatography**, the solute is distributed between the liquid mobile phase and a second, immiscible liquid that is coated on or bonded to solid particles as the stationary phase. Compounds that partition more strongly into the stationary liquid phase are retained longer in the column. This type of chromatography is termed normal phase if the stationary phase is more polar than the mobile phase and reverse phase if the mobile phase is more polar than the stationary phase. The stationary phase can be a liquid coated on solid support particles. Bonded phase columns have the stationary phase chemically bonded to the solid support and are the most popular column for partition chromatography. For example, *n*-octadecane can be bonded directly to silica by attachment to surface hydroxyl groups to form what is termed a C18 column. The extent of partitioning of a solute into the stationary phase can be controlled by varying the solvent polarity.

Ion exchange HPLC is based on the partition of ions between a polar liquid phase and a stationary phase with ion exchange sites. The ion exchange sites are typically immobilized in small beads of resin that are formed by a cross-linked polymer. Bonded phase columns in which the ion exchanger is bonded to small particles of silica also are available. Cations are separated on cation exchange resins which contain negatively charged functional groups such as SO^{3-} and $–COO^{-}$. Anions are separated on anion exchange resins which contain positively charged functional groups such as $CH_2N^{+}(CH_3)_3$, a quaternary ammonium ion. Separation is based on ions partitioning into the ion exchange phase to varying degrees. The selectivity of a resin for an ion is determined primarily by the charge on the ion and its hydrated radius. Resin affinity increases with increasing charge density.

Table 2: Eluotropic Series for Alumina and Silica

Solvent	Relative Polarity	Solvent	Relative Polarity
n-Pentane	0.00	Tetrahydrofuran	0.45
Isooctane	0.01	Acetone	0.56
Cyclohexane	0.04	Ethyl acetate	0.58
Carbon tetrachloride	0.18	Aniline	0.62
Xylene	0.26	Acetonitrile	0.65
Toluene	0.29	Propanol	0.82
Benzene	0.32	Ethanol	0.88
Ethyl ether	0.38	Methanol	0.95
Chloroform	0.40	Acetic acid	large
Methylene chloride	0.42		

A schematic drawing out a typical high performance liquid chromatograph is shown in Fig. 1. The apparatus consists of a container of mobile phase, a pump capable of pressures up to 4000 psi or greater, a valve for injecting the sample (usually 10 to 500 μL volumes), the column (sometimes thermostatted), a detector, electronics associated with the detector, and a recorder.

Isocratic flow and gradient elution

A separation in which the mobile phase composition remains constant throughout the procedure is termed **isocratic** (meaning *constant composition*).

The mobile phase composition does not have to remain constant. A separation in which the mobile phase composition is changed during the separation process is described as a **gradient elution**. One example is a gradient starting at 10% methanol and ending at 90% methanol after 20 minutes. The two components of the mobile phase are typically termed "A" and "B"; **A** is the "weak" solvent which allows the solute to elute only slowly, while **B** is the "strong" solvent which rapidly elutes the solutes from the column. Solvent **A** is often water, while **B** is an organic solvent miscible with water, such as acetonitrile, methanol, THF, or isopropanol.
In isocratic elution, peak width increases with retention time linearly according to the equation for N, the number of theoretical plates. This leads to the disadvantage that late-eluting peaks get very flat and broad. Their shape and width may keep them from being recognized as peaks.

Gradient elution decreases the retention of the later-eluting components so that they elute faster, giving narrower (and taller) peaks for most components. This also improves the peak shape for tailed peaks, as the increasing concentration of the organic eluent pushes the tailing part of a peak forward. This also increases the peak height (the peak looks "sharper"), which is important in trace analysis. The gradient program may include sudden "step" increases in the percentage of the organic component, or different slopes at different times - all according to the desire for optimum separation in minimum time.

UV-visible absorbance is the most commonly used mode of detection. Such detectors enable the effluent from the column to flow through an 8 to 10 μL spectrophotometric cell for detection of compounds at a particular wavelength (often in the ultraviolet, < 400nm, where essentailly all organic molecules absorb). Electrochemical and fluorescence detectors often are used to achieve lower detection limits. The other commonly used detector is based on measurement of the differential refractive index.

A typical liquid chromatogram is shown in Figure below. Each component in a mixture can be qualitatively identified by its retention time t_R which is the time between injection and detection.

As with gas chromatography the retention time of a particular compound is constant for a fixed set of chromatographic conditions (flow rate, temperature, column condition). Qualitative identification is made by comparing t_R of the unknown with retention times of standards that have been injected into the chromatograph. This strategy works so long as components have unique retention times.

The area under each peak is proportional to the concentration of that component in the original mixture. If the peaks are reasonably sharp and the flow rate is carefully controlled, the peak heights are approximately proportional to concentration. Thus a calibration curve can

be prepared by plotting either peak height or peak area as a function of concentration for a series of standards.

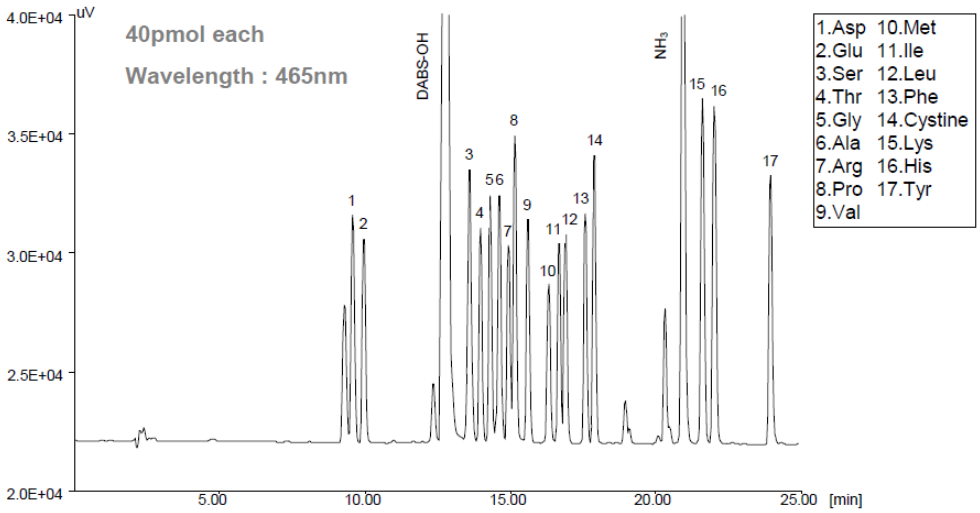


Figure 2. Reverse Phase (C18) Separation of Amino Acids.

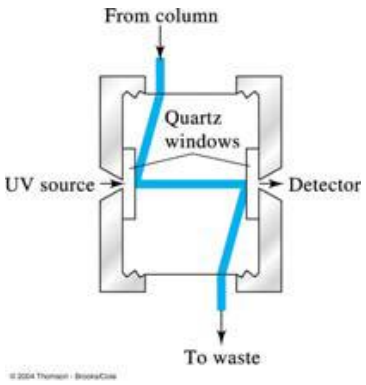
Detector

HPLC Detector	Available	(typical)	(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

Absorption detectors:

– UV-Vis: Most widely used

- Z-shape, flow-through cell (V, 1 ~ 10 μLand b, 2 ~ 10 mm)
- Photometer: Hg 254 nm and 280 nm linefor organic, D2 or W filament + interferencefilter
- Spectrophotometer: more versatile

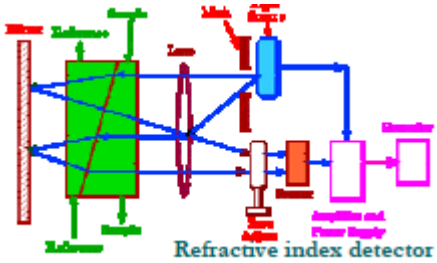


– IR: filter instrument or FTIR
  - Similar cell (V, 1.5 ~ 10 μL and b, 0.2 ~ 1.0mm)
  - Limit: no suitable solvent, special optics

– Fluorescence: Hg or Xe lamp
  - Fluorometer and spectrofluorometer
  - Fluorescing species or fluorescent derivatives

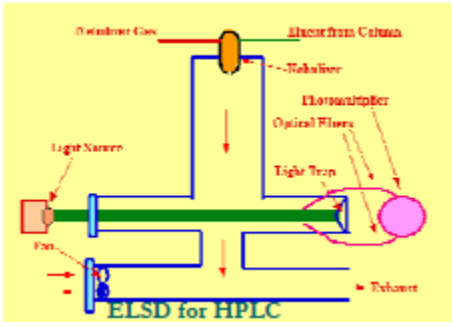
Refractive index detectors (RI):

– General, unaffected by flow rate
– DA: limited sensitivity (1 ng/μL),highly T dependent (0.001°C), nogradient elution
– Half solvent/eluant, η difference bending of the incident beam



Evaporative light scatteringdetector:

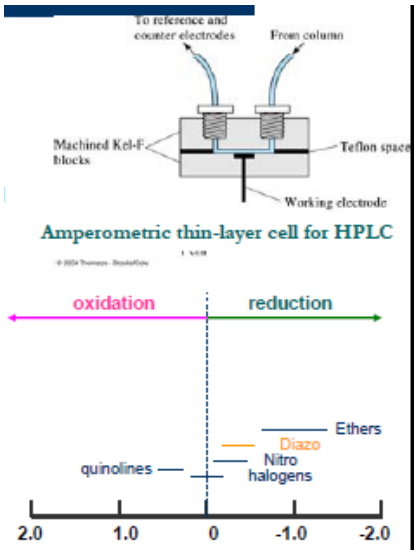
– ELSD: new, laser beam
– Nebulizer fine mist in N2 solvent evaporation in drift tube fine particles scattered radiation
– A: same for all nonvolatile solutes,more sensitive than RI, 0.2 ng/μL
– DS: mobile phase must be volatile



Electrochemical detectors:

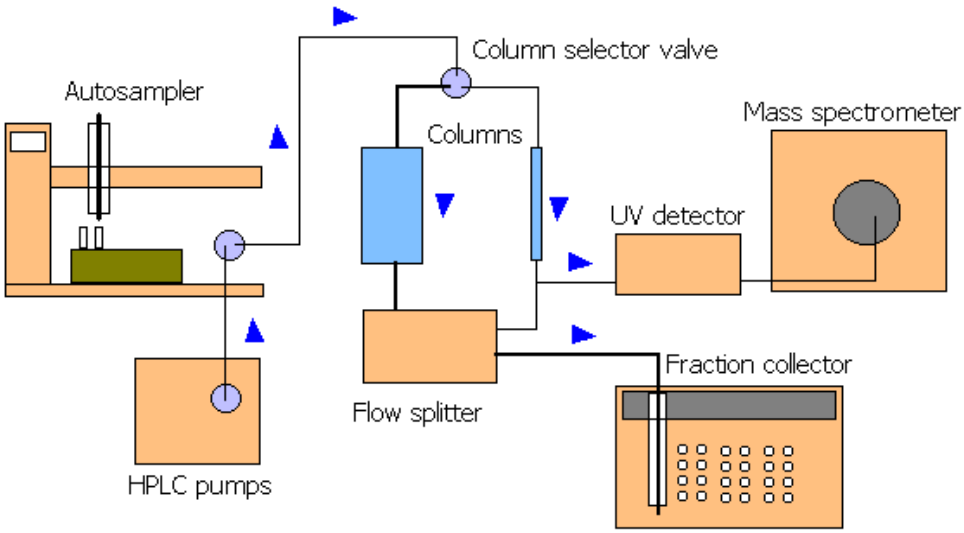
– Amperometry, voltammetry,coulometry and conductormetry

– A: simplicity, high sensitivity,convenience and wide-spreadingapplication
– Thin-layer flow cell of Teflon : 50μm thick, 1 ~ 5 μL volume
– Indictor E: Pt, Au, C
– RE and CE: down stream
– Multi-electrode: simultaneousdetection or sample purityindication



Mass Spectroscopy Detector

Liquid chromatography-mass spectrometry (**LC-MS**, or alternatively **HPLC-MS**) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity. Generally its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).



Mass spectrometry (MS) is an analytical technique that measures the mass-to-charge ratio of charged particles.It is used for determining masses of particles, for determining the elemental composition of a sample or molecule, and for elucidating the chemical structures of molecules, such as peptides and other chemical compounds. The MS principle consists of

ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios. In a typical MS procedure:

1. A sample is loaded onto the MS instrument, and undergoes vaporization
2. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions)
3. The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields
4. The ions are detected, usually by a quantitative method
5. The ion signal is processed into mass spectra

Analysis of Caffeine in Beverages

Reverse phase HPLC is used to determine the concentration of caffeine in coffee, tea, and soft drinks. The traditional method for the determination of caffeine is via extraction with spectrophotometric quantitation. Use of the liquid chromatography system permits a fast and easy separation of caffeine from other substances such as tannic acid. caffeic acid. and sucrose found in these beverages. Five standard solutions of caffeine are prepared and injected into the HPLC. In addition, the beverages coffee, tea, caffeine containing soft drinks are prepared as indicated in the following section and injected into the HPLC. From the resulting chromatograms, measurements of retention time tR and peak areas are made. If the flow rate and pump pressure are held constant throughout the entire experiment, tR may be used as a qualitative measure and the peak area as a quantitative measure. A calibration curve for peak area against concentration of the caffeine standards can then be employed to determine the concentration of caffeine in the four beverages. The solvent (mobile phase) in this experiment is 47% methanol / 53% water.

Preparation of Caffeine Standards

Accurately weigh out 10.0 mg of caffeine. Transfer to a clean 100 mL volumetric flask. Dilute to the mark with HPLC grade water. Carry out a series of dilutions of the stock 10 mg / 100 mL solution to obtain standards of 1.0 mg /100 mL, 2.5 mg / 100 mL, 5.0 mg / 100 mL, and 7.5 mg / 100 mL. Make 10 mLs of each of the dilutions. Use HPLC grade water to make the dilutions. Shake the five caffeine solutions to insure adequate mixing.

Determination of UV Absorbance Spectrum of Caffeine

You must determine at which wavelength you will monitor the elution of your compound from the column. Caffeine is a colorless solution so the absorbance, if any, will be in the UV range. You should remember from Chem 105 how to acquire a UV spectrum. Obtain quartz cuvettes from the stockroom. Use the HPLC grade water as your blank and then measure the spectrum of your 1mg/100mL solution from 190 nm to 400 nm. What wavelength will you use to monitor the elution of caffeine from the HPLC column? Recall that many solvents absorb below 220 nm.

Operation of the Instrument

Injection techniques and solvent purity are important in this experiment.

1. Sample Preparation

Filter each solution through the syringe filter into a clean vial. Filter the least concentrated sample first. Wash the filter between samples by filtering 1 mL into waste before you collect the rest.

You will do this experiment in isocratic mode with 53% water and 47% methanol. The flow rate should be 0.8 mL/min. The procedure for setting the mobile phase composition is identical to that described for the paraben analysis.

2. Injection Procedure

1. Fill the syringe with at least 20 μL of the most concentrated caffeine standard.
2. Insert the syringe into the injection port, turn the valve to the **LOAD** position (counter clockwise), and then inject some of the standard solution until you see a few drops come out of the overflow port.
3. Next, start detector data collection as you did with the parabens. Remember, this is an isocratic run, so do not press "Start" on the pump.
4. Allow the peak due to the caffeine to be recorded (approximately 4 minutes). You may press "Reset" to terminate data collection without waiting the full 20 minutes. Do the same procedure (steps 1 through 4) again for the most concentrated sample. Continue until you have recorded three chromatograms for the most concentrated sample.
5. Repeat the above steps (1 through 4) for each of the remaining standard samples, going from the most concentrated to the least concentrated. Note that it is good practice to rinse the syringe out a few times with the methanol/water mixture when changing to a caffeine sample of different concentration. A blank can be run to be sure that the syringe is free of caffeine. Do three injections of each standard. Make sure that the peak heights are within 5% of each other for each standard. If they are not, repeat the injection until it is reproducible.

C. Treatment of Data

Plot the area of the caffeine peak against the concentration in milligrams per 100 milliliters, and the height of the caffeine peak against the concentration, for the caffeine standards. Comment on the relative merits of these plots.

D. Determination Of Caffeine In Various Beverages

Instrumentation: HPLC System with Reversed-phase (i.e. C18) column and UV detector

Chemicals:
Caffeine
1 L 50:50 Methanol:Water solution (volume %)
Coffee
Decaffeinated Coffee Samples
Tea
Soft Drink Beverages

Analyze three different caffeine-containing beverages that have been approved by the instructor. Follow the general instructions given below for each type of beverage.

Coffee

Pipette 5 mL of coffee into a CLEAN and DRY 50 mL volumetric flask and then dilute to the mark with HPLC grade water. Be sure to filter the sample into a clean vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each coffee sample.

Decaffeinated Coffee Samples

Pipette 25 mL of the decaffeinated coffee into a CLEAN and DRY 50 mL volumetric flask and then dilute to the mark with HPLC grade water. Be sure to filter the sample into a clean

vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each decaffeinated coffee sample.

Tea Samples

Pipette 10 mL of your tea sample into a clean and dry 50 mL volumetric flask. Dilute to the mark with HPLC grade water. Be sure that you filter your sample into a clean vial. Rinse the filter before collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each tea sample.

Soft Drink Beverages

If your beverage is carbonated, you must decarbonate it by pouring it back and forth between two beakers until the bubbling ceases. Now pipette 25 mL of your beverage into a clean and dry 50 mL volumetric flask and dilute to the mark with HPLC grade water. Be sure to degas this sample for 5 minutes. Also, be sure to filter the sample into a clean vial. Rinse the filter prior to collecting the sample by filtering the first 1-2 mLs to waste. Record three chromatograms for each beverage.

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Injection Date  :
Sample Name     :
Inj Volume      : 10 µl
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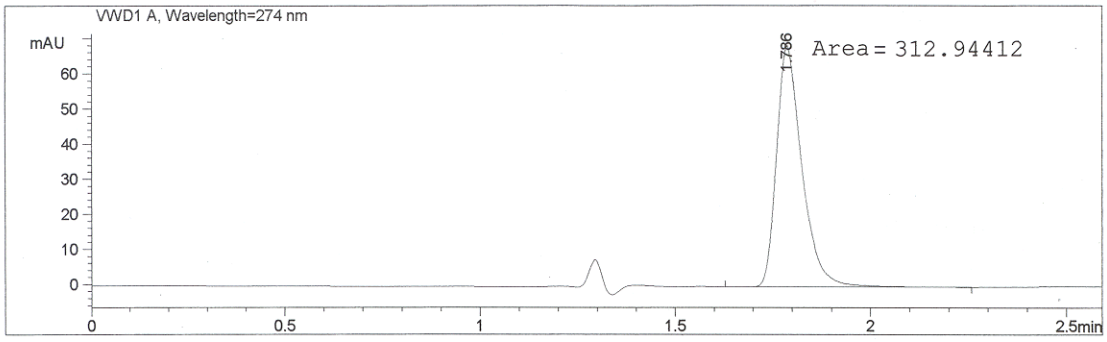

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
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Injection Date  :
Sample Name     :
Inj Volume      : 10 µl
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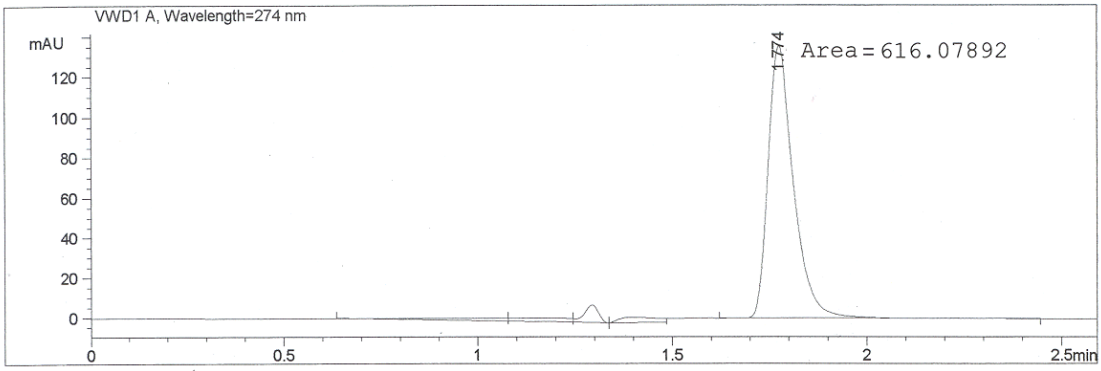

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
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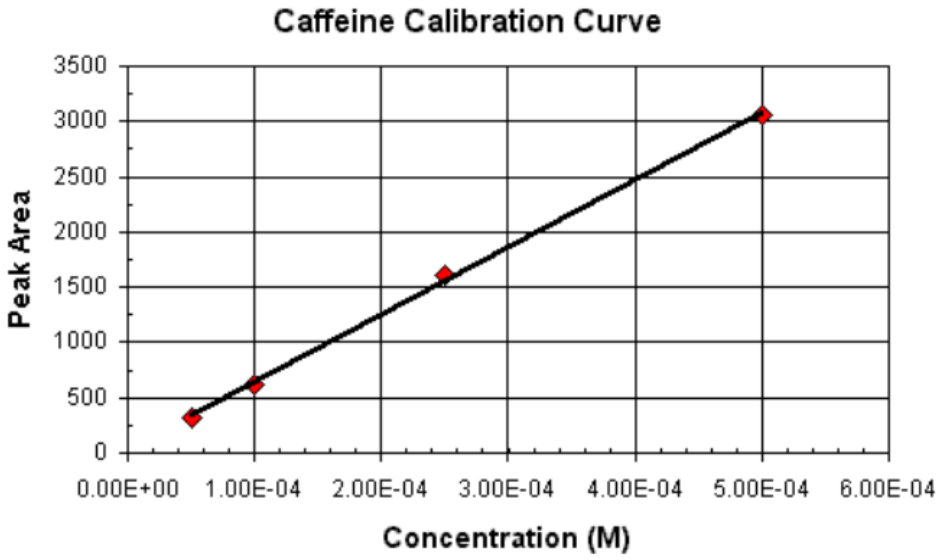

Treatment of Data

Determine and report the caffeine concentration in mg/ 100 mL (for each sample) using the calibration curve. This may be done by either taking the area of the peak or the height of the peak. (Or both. What are the advantages of each method?) Explain why you chose either the peak height or the peak area for your determination. A proper estimation of errors is expected in the report. Be sure you take into account the dilution of your samples. In your report, discuss how retention time depends on the methanol content and pH of the mobile phase. What factors determine the choice of mobile phase composition and pH in the present analysis?