Instrumental Techniques

Chromatography

Chromatography is a collective term for different techniques to separate mixtures.
A carrier/vehicle, which is called the mobile phase, take the sample mixture through a permanent structure with/without a chemically modified material called the stationary phase. The various constituents of the sample mixture travel at different speeds and interact indifferently with the stationary phase, causing them to separate. The separation process is based on differential partitioning between the mobile and stationary phases.

We have differences in the choice of:
- stationary phase carrier or base material
- composition of the mobile phase
- physical parameters (such as temperature, pressure, different sizes in length, diameter)

We can differentiate between the techniques based on the mobile and the stationary phase.

- Gas Chromatography (GC):	gas + column
- Liquid Chromatography (LC):	liquid + column
- Thin layer Chromatography (TLC):	liquid + plate

If you have a big number of samples with a complex matrix and wish to use minimum sample preparation, you can apply thin layer chromatography. If you are interested in volatile compounds, gas chromatography.is the preferred technique. If you wish to check contaminants in low concentration, ultra/high performance liquid chromatography (HPLC/UHPLC) is a viable alternative as it provide very high separation efficiency and will elute the molecules of interest in sharp peaks to allow sensitive detection.

Following the chromatographic step, there has to be a tool for the detection and quantitative determination of the compounds. Different sort of molecules and different concentration ranges require different detection techniques.

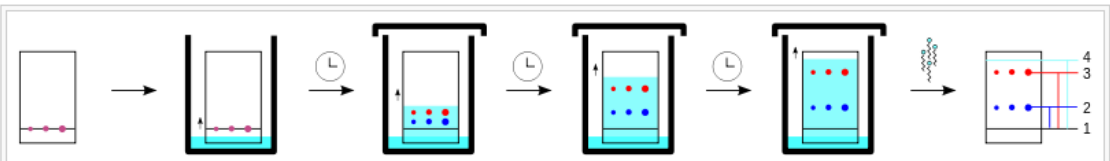
To select the most appropriate detection mode, four important parameters should be taken into consideration; chemical nature of the analytes, potential interferences, limit of detection (LOD) and limit of quantitation (LOQ) required, linearity range, availability and/or cost of detector.

Instrumental Techniques

Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a technique in which compounds are separated on a thin layer of adsorbent material, typically a coating of silica gel on a glass, aluminum foil, or plastic sheet.

TLC is a straightforward and extremely cost-efficient technique allowing sample preparation and chromatographic separation in one step due to its high sample matrix tolerance.



Development of a TLC plate, a purple spot separates into a red and blue spot

A small spot of solution containing the sample is applied to a plate. A small amount of an appropriate solvent (eluent) is poured into a separation chamber. The TLC plate is then placed in the chamber so that the spot(s) of the sample do not touch the surface of the eluent in the chamber, and the lid is closed. The solvent moves up the plate by capillary action, meets the sample mixture and carries it up the plate.

Compounds are separated by the differences in their attraction to the stationary phase and because of the differences in solubility in the solvent. In some cases, if you have colored components you can visually detect the different compounds on the plate.
More frequently used options are:

1. using a special solution, which gives a color reaction with the analyte (such as iodine)

2. UV light (366nm to see some fluorescent organic compounds or 254 nm using a plate with an adsorbent layer fluorescent at this wavelength, on which the spots of the analyte quench this fluorescence)

3. after elution of the spot, any other known analysis technique can be used to identify the compound (like MS)

TLC is often used as a manual technique, but there are different possibilities to automatize the application, the detection, or the evaluation and documentation.

Instrumental Techniques

Thin layer chromatography with mass detection (TLC-MS)

Over the last few years, several coupling techniques for mass spectrometry have emerged to broaden the scope of TLC-MS analysis enabling unequivocal substance identification.

The most widely used technique for coupling TLC to MS is the elution-based sampling.
It is a semi-automatic system to extract analytes from the TLC plate and transfer them online into the mass spectrometer. It is suitable for all thin layer materials and every eluent that is LC-MS compatible. The interface can be easily connected to any kind of LC-coupled mass spectrometer. Therefore the fields of application are quite broad.

Perhaps the strongest feature of elution-based coupling is its complete independence from the mass spectrometer – any MS system can be used, in principal. Plus, offline extraction into vials is possible, if additional analysis (with NMR, for example) is needed. In online TLC-MS mode, the interface sits between the HPLC pump and the MS system, and is pretty much "plug and play". It's fair to say that such systems offer the lowest entry barriers into TLC-MS, and can be used with almost any kind of plate.

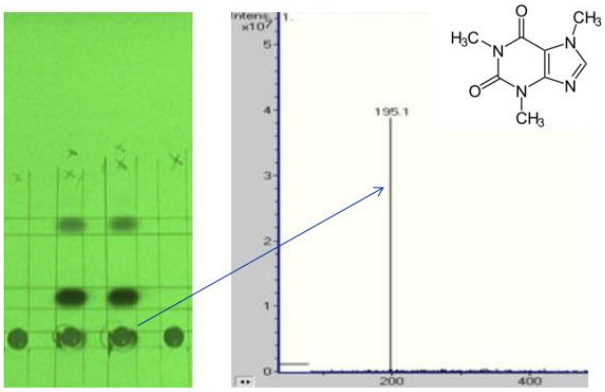


plate after extraction

MS identification of caffeine

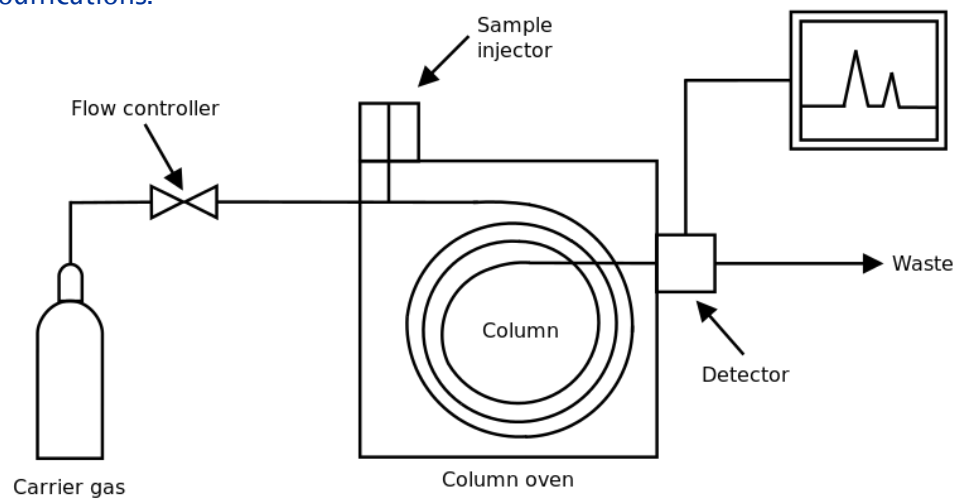
Instrumental Techniques

Gas Chromatography (GC)

Gas chromatography (GC) is a technique for the separation of volatile, thermally stable compounds. It is simple, easy to use, and can be highly automated.

In gas chromatography, a gas is used to carry a mixture across a bed of material, using pressure as force. Therefore you have as part of the system a source for gas (tank or gas generator) including regulators to control the gas flow. The most often used carrier gases are Helium, Hydrogen, Nitrogen.

You also need an oven, an injector for sample input, the column and a detector. The injection has to be done under pressure. The stationary phase is a capillary column, with different inside surface modifications.



The most commonly used detectors are the flame ionization detector (FID) and the thermal conductivity detector (TCD).

TCDs are essentially universal and can be used to detect any component other than the carrier gas (as long as their thermal conductivities are different from that of the carrier gas, at detector temperature), FIDs are sensitive primarily to hydrocarbons.

In recent years mass spectrometers (MS) are getting more common in connection with GC. They are highly effective and sensitive, even in a small quantity of sample.

Instrumental Techniques

Liquid Chromatography (LC)

High Performance Liquid chromatography (HPLC) is a separation technique where you select the most suitable mode of separation, depending on sample solubility and how the analytes of interest differ from other compounds or matrix in sample. In reversed phase (RP) mode the mobile phase is polar and the stationary phase is non-polar. The major distinction between analytes is their hydrophobicity where samples should be soluble in water or a polar organic solvent.

In normal phase (NP), the mobile phase is non-polar while the stationary phase is more polar, and where the major distinction between analytes is not their hydrophobicity, and where the samples should be soluble in a hydrophobic solvent like hexane and the mobile phase is a weak to moderate solvent for the sample.

In HILIC mode, the mobile phases are the same as for reversed phase, but with the opposite elution strength. The major distinction between analytes is their hydrophilicity and the sample should be soluble in a polar organic solvent or organic solvent - water mixtures. For polar and hydrophilic compounds the traditional approach utilized reversed phase ion-pairing and were used for analytes being ionic or potentially ionic. In this situation the mobile phase contains a buffer, an ion-pair reagent and a polar organic solvent. Typical ion-pair reagents are; alkyl sulfonates (heptane sulfonic acid, octane sulfonic acid) and used for bases; and where quaternary amines (tetrabutylammonium chloride) are used for acids.

Choose the right HPLC Column

Chromatographic resolution is mainly affected by the selectivity (α). Changing the mobile phase composition or the stationary phase, is the most powerful way of optimizing selectivity whereas the particle size, pore size, length of the column, temperature, mobile phase strength have much less effect. Therefore, if satisfactorily results are not met, or no retention is achieved, it is better to change to another selectivity using a different column type and/or a different mobile phase.

Resolution (Rs or R) can be expressed in terms of three parameters (k, α, and N) which are directly related to experimental conditions. k is the average retention factor for the two bands, N is the column plate number and α is the separation factor (or selectivity factor). The parameters k and α is determined by the experimental conditions (composition of the mobile phase; stationary phase chemistry and temperature), and where N is affected by column length, particle size and pore size.

Instrumental Techniques

Liquid Chromatography (LC)

Choosing the Right Column Format

Find the best column configuration for minimum analysis time, with high efficiency and resolution. Match up method goals to make sure that the chosen format has the ability to produce resolution that fits the purpose of the application, e.g. choose right column length, column inner diameter, particle size and pore size. If you need high mass loadability, a larger column (both in length and diameter) is recommended as it can accommodate more mass. If you work with traditional detectors like UV, RI, FL etc 4.6 or 3.0 mm i.d. columns are suitable but for MS generally a 2 mm or smaller i.d. column is recommended.

If you have sufficient resolution, it is possible to speed up the separation by increasing the flow rate or shorten the column length. Silica-based materials are physically strong and will not shrink or swell, being compatible with a broad range of polar and non-polar solvents, and therefore often the initial choice. Most silica based columns are stable from pH 2-7.5, and historically, polymeric packing materials provided better column stability under pH extremes. A polymer-based packing material is compressible and may shrink or swell with certain solvents. Therefore care must be taken if a polymeric column is used, and upper backpressure limit is lower than corresponding silica based stationary phases. Newer high-purity silica based phases, like Purospher STAR, are stable at pH 1.5-10.5, with the surface functional groups bound to the base silica particle at multiple attachment points via polymeric modification.

Particle Size

Smaller particle sizes provide higher separation efficiency and higher chromatographic resolution than larger particle sizes. However, larger particle sizes offer faster flow rates at lower column back-pressure, and are less prone to clogging, and of these reasons more tolerant to matrix effects. Typical particle sizes range from 3-20 µm, but new 2 µm particle sizes are available to maximize resolution on short Purospher STAR columns. A 5 µm particle size represents the best compromise between efficiency and back-pressure for most non-high through put applications.

Carbon Load

For silica-based reversed-phase packing materials, carbon load indicates the amount of functional bonded phase attached to the base material. Phases with lower carbon loads are more weakly hydrophobic, which may significantly reduce retention times over phases with higher carbon loads. However, a higher carbon load will give higher capacity and often greater resolution, especially for compounds of similar hydrophobicity.
Carbon load is not a relevant parameter for columns used in normal phase or HILIC mode.

Instrumental Techniques

Liquid Chromatography (LC)

Pore Size

Choose a pore large enough to completely enclose your target molecule. If your molecule is larger than the pore, size exclusion effects will be seen and it will be difficult or impossible to retain. In general, packing materials with a smaller pore size have higher surface areas and higher capacities than packing materials with larger pore sizes. A larger surface area typically indicates a greater number of pores, and therefore a higher overall capacity. Smaller surface areas equilibrate faster, which is important for gradient elution analyses. Larger pores are better for interaction with large compounds, such as proteins.

Endcapping

Silica-based reversed-phase packing materials have free silanol groups that will interact with polar compounds. Endcapping the bonded phase minimizes these secondary interactions. Choose endcapped phases if you do not want interactions with polar compounds. Choose non-endcapped phases if you want enhanced polar selectivity, for stronger retention of polar organic compounds.

Mobile phase selection – solvents and buffers

The mobile phase solvent strength is a measure of its ability to elute analytes of the column. It is generally controlled by the concentration of the solvent with the highest strength; for example, in reverse phase HPLC with aqueous mobile phases, the strong solvent would be the organic modifier; in normal phase and HILIC, it would be the most polar one. Worth pointing out is that cyano-bonded phases are easier to work with than plain silica for normal phase separations.
The aim is to find the correct concentration of the strong solvent. With many samples, there will be a range of solvent strengths that can be used within the aforesaid capacity limits.
Other factors (such as pH) may also affect the overall retention of analytes.

Detection

Fluorescence, electrochemical or mass detectors can be used for trace analysis.
UV detectors are most commonly used as it is a robust, inexpensive and versatile detection technique. Most compounds absorb light, especially at low UV wavelengths. It is possible to use a Diode Array Detector (DAD) and allow monitoring at multiple wavelengths simultaneously.
The downside is that UV detectors are not analyte specific and requires that the analyte absorb more light than sample matrix at the set wavelength

Further information can be found in the Chrombook.