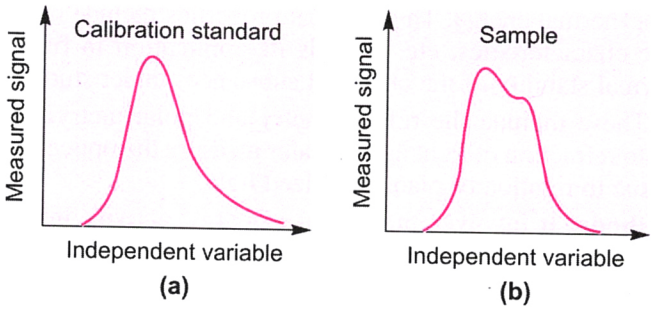

Fig. 1.2. Illustration of how multi-channel data allow for the detection of interferences. Comparison of the multi-channel signal of the (a) the calibration standard, and (b) the sample reveals that there is interference in the latter.

possibility is that the sample matrix alters the response of the analyte, giving rise to an altered peak shape.

More than just identifying the presence of an interfering substance, multi-channel data often allows the analyst to correct for its presence. For example, if it is suspected that the altered peak in Fig. 1.2(b) is due to an additional component, then a channel can be chosen for quantitation where the interfering substance does not contribute. The left side of the peak looks unaltered, so perhaps the data in one of these channels can be used to estimate analyte concentration. An important point, although, multi-channel methods are capable of collecting measurements on multiple channels (e.g. different wavelengths), it is possible to use them in "single-channel" mode. In other words, to decrease measurement time, the analyst has the option of measuring the response on only a single channel (e.g., the wavelength corresponding to the peak response). If the nature of the sample or standard is well known, this may be perfectly acceptable. However, the analyst must realize that a lot of information is being thrown away – the advantages of multi-channel data described above (multicomponent analysis and detection/correction of interferences) will be lost. As a general guideline, it is always a good idea to collect the multi-channel response of at least one of the calibration standards to see what the analyte response looks like, and then to collect the multi-channel response of at least one of the samples to ensure that no interferences are present.

One last item: There is another way of classifying analytical techniques according to the measurement data produced. Whether it is single- or multi-channel techniques, we may check the order of the analytical technique. The order is equal to the number of independent parameters that are controlled as the data is collected for each sample. Thus, single-channel techniques would be *zeroth order methods*, since only a single data point is collected. If absorbance is measured as a function of wavelength, as in molecular absorption spectroscopy, the technique is labelled *first order*. Examples of second order techniques include the following:

- Gas chromatography with mass spectrometric detection (the two independent parameters are retention time and ion mass/charge ratio);
- Liquid chromatography with UV-visible spectrophotometric detection (signal is determined as a function of retention time and wavelength); and
- Molecular fluorescence (signal measured as a function of both excitation wavelength and emission wavelength).

Relative vs. absolute techniques

Another way of classifying analytical techniques is according to the method by which the analyte concentration is calculated from the data:

Overview

Size-exclusion chromatography (SEC), also called gel-filtration or gel-permeation chromatography (GPC), uses porous particles to separate molecules of different sizes. It is generally used to separate biological molecules, and to determine molecular weights and molecular weight distributions of polymers. Molecules that are smaller than the pore size can enter the particles, and, therefore, have a longer path and longer transist time than larger molecules that cannot enter in the particles. Typically, when an aqueous solution is used to transport the sample through the column, this technique is known as **gel filtration chromatography,** versus the name **gel permeation chromatography**, which is used when an organic solvent is used as a mobile phase.

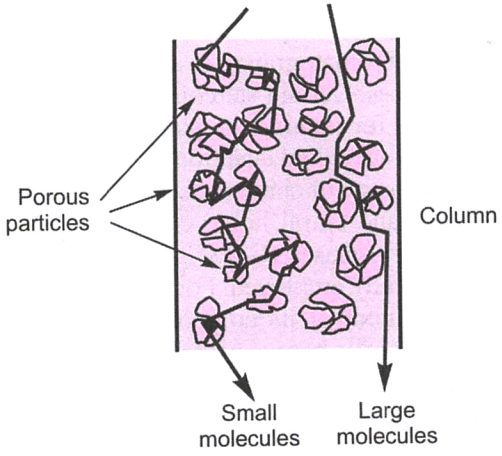

Fig. 2.3. Schematic diagram of a size-exclusion chromatography column.

Molecules larger than the pore size can not enter the pores and elute together as the first peak in the chromatogram. This condition is called **total exclusion.** Molecules that can enter the pores will have an average residence time in the particles that depends on the molecules size and shape. Different molecules, therefore, have different total transit times through the column. This portion of a chromatogram is called the **selective permeation region.** Molecules that are smaller than the pore size can enter all pores, and have the longest residence time on the column and elute together as the last peak in the chromatogram. This last peak in the chromatogram determines the **total permeation limit.**

Affinity chromatography

This is the most selective type of chromatography. It utilizes the specific interaction between one kind of solute molecule and second molecule that is immobilized on a stationary phase. For example, the antibodies in a serum sample specific for a particular antigenic determinant can be isolated by the use of affinity chromatography.

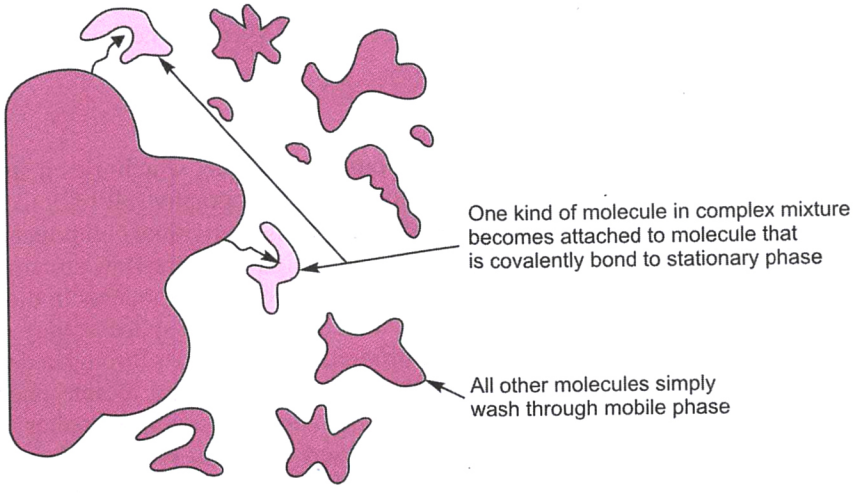

Fig. 2.4. Schematic representation of affinity chromatography.

The detecting techniques can also be categorized as:

- **Destructive technique**, e.g. specific spray reagents, where the samples are destroyed before detection. e.g. ninhydrin reagents.
- **Non-destructive technique** like UV chamber method, iodine chamber method, densitometry method, etc. where the sample is not destroyed even after detection.

For radioactive materials, detection is done by using autoradiography or Geiger Muller counter.

For antibiotic, the chromatogram is laid on nutrient agar inoculated with appropriate strain and the zone of inhibition is compared.

9. Interpretation of chromatogram

The R_f value for each spot is calculated. R_f stands for "ratio of fronts", or "retardation factor", and is characteristic for any given compound on the same stationary phase, using the same mobile phase for development of the chromatogram. Hence, known R_f values can be compared to those of unknown substances to aid in their identifications.

Note: R_f values often depend on the temperature, solvent, and type of paper used in the experiment; the most effective way to identify a compound is to spot known substances next to unknown substances on the same chromatogram.

In addition, the purity of a sample may be estimated from the chromatogram. An impure sample will often develop as two or more spots.

If a sample develops as only one spot (Fig. 2.7) it may or may not be pure. The sample may contain another compound, which did not separate under the conditions of the experiment. Purity of samples is often determined in conjunction with other techniques, such as measuring a sample's melting point or recording its nuclear magnetic resonance spectrum.

$$R_f = \frac{\text{Distance from start to centre of substance spot}}{\text{Distance from start to solvent front}} = \frac{x}{y}$$

The chromatogram is developed by allowing the mobile phase to travel over the surface of the paper in number of ways. These are mentioned in the following text.

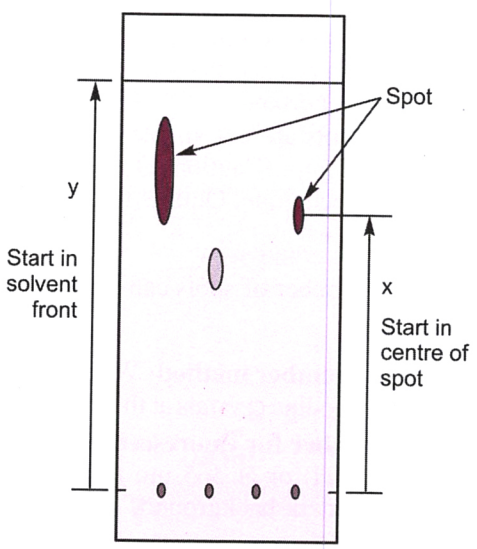

Fig. 2.7. A separated sample.

Types of paper chromatography

Various types of paper chromatographic techniques are as follows:

1. Ascending paper chromatography

Like conventional type, the solvent flows against gravity. The spots are kept at the bottom portion of paper and kept in a chamber with mobile phase solvent at the bottom (same as Fig. 2.8).

In a chromatographic chamber, the solvent rises up the paper by capillary action and allows a separation of the components as it ascends. Very simple equipments are required. However, the solvent will rise only 20 to 25 cm and separations of the analytes are limited. Ascending technique is relatively a slow process.

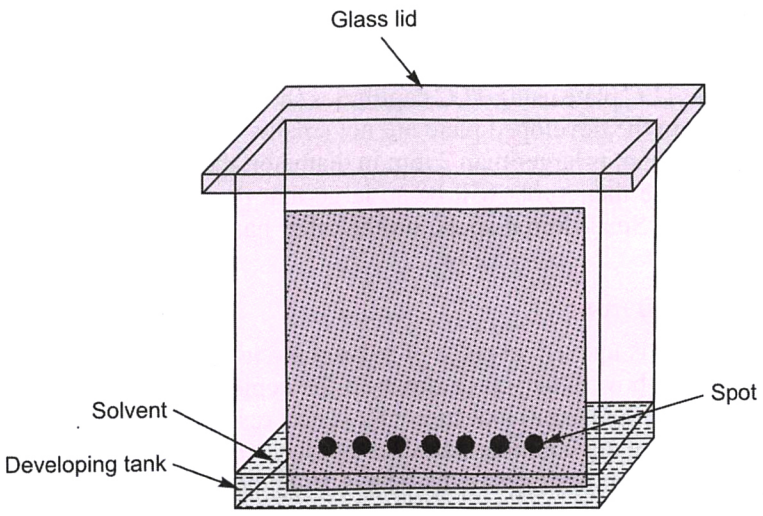

Fig. 2.18. Schematic representation of thin layer chromatography.

The bottle is filled with a small amount of the mobile phase and capped with a cork glass plate. In addition, a piece of filter paper is put around the inner side of the bottle to help in developing an atmosphere saturated with solvent vapor. Also make sure the origin spots are not below the solvent level in the chamber. If the spots are submerged in the solvent, they are washed off the plate and lost. Once the solvent has run within a centimeter of the top of the plate, remove it with tweezers. Using a pencil, immediately draw a line across the plate where the solvent front can be seen. The proper location of this solvent front line will be important for later calculations.

7. Visualization

Some organic compounds are colored. If you are fortunate enough to be separating organic molecules that are colored such as dyes, inks or indicators, then visualizing the separated spots is easy. However, since most organic compounds are colorless, this first method does not always work.

In most cases observing the separated spots by UV light works well. TLC plates normally contain a fluorescent indicator, which makes the TLC plate glow green under UV light of wavelength 254 nm. Compounds that absorb UV light will quench the green fluorescence yielding dark purple or bluish spots on the plate. Simply put the plate under a UV lamp, and the compounds become visible to the naked eye. Lightly circle the spots, so that you will have a permanent record of their location for later calculations.

Another useful visualizing technique is an iodine (I_2) chamber. Iodine sublimes and will absorb to organic molecules in the vapor phase. The organic spots on the plate will turn brown and can be easily identified. It is a universal visualizing agent for organic compounds. Also circle these observed spots, since the color stain will eventually fade from the plate. Sometimes, a combination of both a UV lamp and iodine is needed to observe all the spots. Some compounds are not "UV active", that is, they do not absorb light at the wavelength of 254 nm. Using both methods will ensure correct identification of all the spots on the TLC plate.

8. Solvent system

It is better to try the mixture of solvent randomly according to the compound. Mixture of two or more solvent of different polarity give better separation than the single solvent.