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Chromatography

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http://tera.chem.ut.ee/~koit/arstpr/krom_en.pdf

Chromatography is a method (group of methods) for separating components of mixtures. A system consisting of a stationary and a mobile phase is necessary for chromatographic separation. The stationary phase is a substance that binds and shortly releases the molecules moving through the system. The particles can move through the system due to the mobile phase, which can be for example a liquid (eluent) or a gas (carrier gas) that carries the molecules through the stationary phase. The chromatographic separation process is based on the different mobility of different components in the chromatographic system (column, plate, etc.). The compounds that are more like the stationary phase (have higher affinity towards it), move slower that the compounds that are more like the mobile phase. The time spent on going through the chromatographic system is called the retention time (t_R). Due to the different mobilities different compounds also have different retention times.

1 Classification of chromatographic methods

Chromatography may be classified according to its aim, technical details, state of mobile phase or other parameters.

The aim of the chromatographic procedure can either be a preparative or an analytical chromatography. Preparative chromatography is used for separation of individual substance, so that the substance can be further used for some purpose (e.g. for use in pharmaceutical preparation). For example, purification of a reaction product from the reaction mixture in order to use it in pharmaceuticals. The aim of analytical chromatography is to detect presence (qualitative analysis) and amount (quantitative analysis) of certain component in the mixture.

The variation of the properties of stationary and mobile phases enables to use very wide range of technically different chromatographic systems. Therefore, the stationary phase may be a sheet of paper (paper chromatography) or a thin layer of porous material on a surface of metallic or glass plate (thin layer chromatography, TLC), a granular filling in a tube (column chromatography) or as a layer in a capillary tube (capillary chromatography). Mobile phase can be liquid (liquid chromatography, LC), gaseous (gas chromatography, GC) or supercritical fluid (supercritical fluid chromatography, SFC). The mobile phase in liquid chromatography is also called the eluent.

The detection of the compounds after the chromatographic separation is carried out visually or by using a special device for detection.

2 Column chromatography

The chromatographic column consists of glass or metal column filled with porous sorbent. Surface of the sorbent acts as a stationary phase. Gravity or a special eluent pump forces the eluent flow through the column. A detecting device must follow the column in order to detect the separated substances. Simple photometer can serve as a detector (Figure 1).

The same column at different times is depicted in Figure 1. At time t_0 the analytes are introduced at the top of the column. As the mobile phase flows, it carries the substances with it. Every substance has its characteristic velocity, which depends on the time that particles spend in mobile phase *vs.* the time they spend on stationary phase. The graph on the lower part of Figure 1 shows the absorbance, measured by the photometer, during the elution process. Such graphs are called chromatograms (Figure 2).

At time t_1 components A and B have moved some distance in the column. At the same time, the solvent used to introduce those substances has passed the column and reached the detector. The change in detector output signal when a substance passes it, is called chromatographic peak. A peak is characterized by the time at its maximum.

The moment at which the maximum of solvent peak exits is called column dead time (t_m). Dead time is time it takes for the unretained species (move at the same velocity as eluent) to reach the detector.

At t_2 the substances have moved along the column. The further they move the more they separate from each other. Substance A reaches the end of column at t_3. The detector registers a peak which's maximum time is called the retention time of substance A – t_A. Substance B reaches the detector at t_4 – retention time of B is t_B.

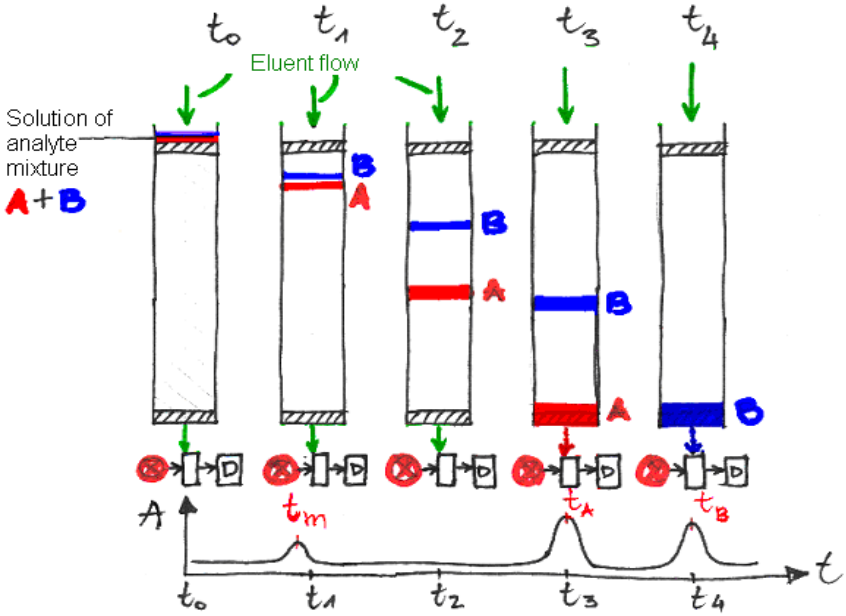

Figure 1. Principle of column chromatography and the chromatogram.

Identification of substances. Retention times are characteristic to substances, meaning that two different substances have different retention times. So knowing the retention time of a substance under specific experimental conditions enables to identify the peaks.
Quantitative determinations. The more of the substance is introduced into the column, the higher is the peak corresponding to that substance (and the larger is the area under the peak). So measuring the peak height (or area) enables to determine the concentration of analyte in mixture if appropriate calibration graph is available.

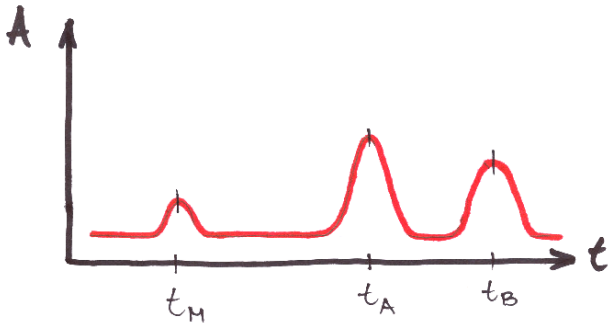

Figure 2. A chromatogram.

3 Thin layer chromatography (TLC)

TLC is a type of chromatography, where stationary phase is a thin layer of sorbent (paper, metallic plate coated with sorbent, etc.). Eluent moves through the stationary phase due to capillary forces.

Figures 3-5 depict TLC separation of mixture *x*:

1. The starting line is drawn close to the one end of the chromatographic plate. Spots of the substances to be determined (*a*, *b*, *c*) and the mixture *x* are transferred onto the starting line (Figure 3).
2. The plate is placed in the elution chamber with small amount of eluent (mobile phase) in it (Figure 3).

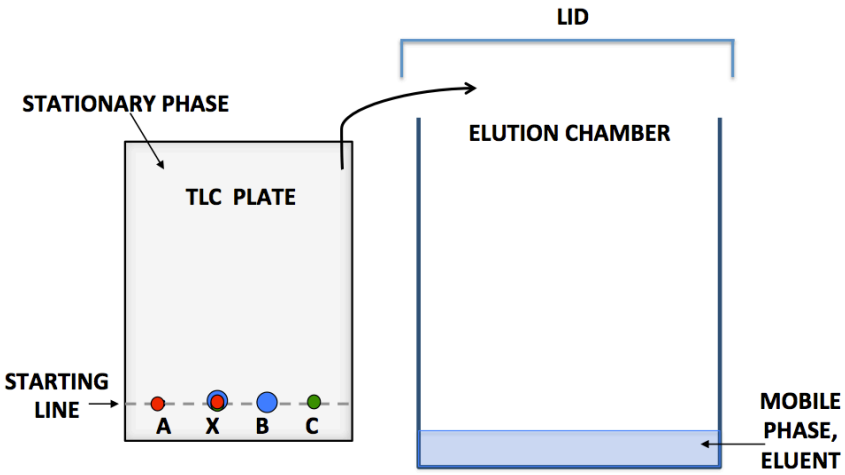

Figure 3. Spots of substances on chromatographic plate; elution chamber.

3. The lower edge of the plate is immersed in the eluent so that eluent does not reach the spots on the starting line. To avoid evaporation of eluent away from the plate the elution chamber is covered with a lid. Eluent starts moving upward through the solvent layer due to capillary forces (Figure 4).
4. As the eluent flows, it carries the analytes with it. The velocities at which analytes move are different and characteristic to the analyte. The velocity of substace depends on their affinity towards stationary phase. Figuratively speaking: substances which "like" to be in liquid phase move faster and the substances which prefer stationary phase move slower. This way the analytes move different distances from the starting line (Figure 5).

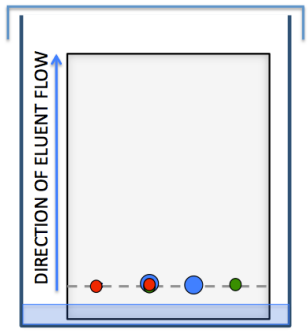

Figure 4. Eluent movement

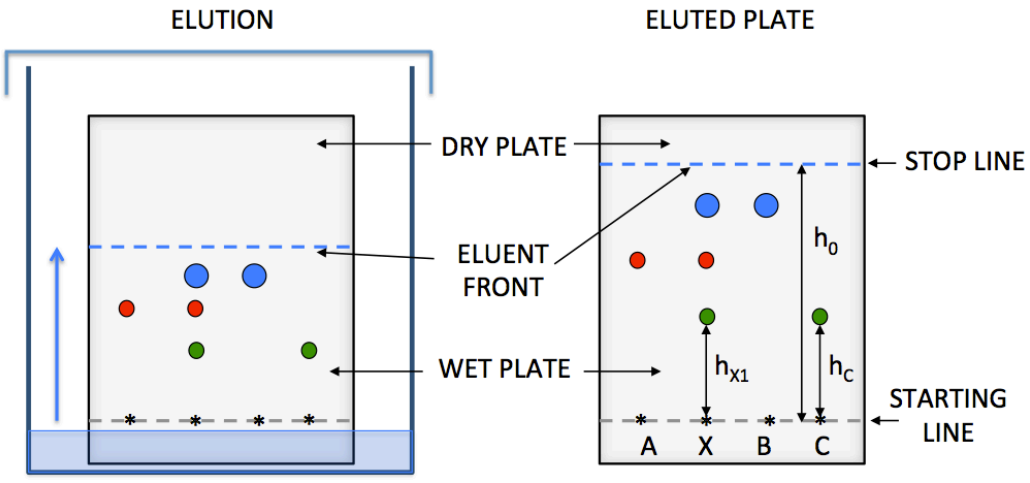

Figure 5. Separation of substances on chromatographic plate

5. The substances usually move slower than the eluent front. Before the eluent front reaches the upper edge of the chromatographic plate, the plate is removed from the elution chamber and is placed in horizontal position to let the eluent evaporate. As a result of movement at different speeds, at the end of the experiment, the substances are placed at different distances between the starting and the stop lines. Comparison of distances of spots of analyte and known substances from the starting line reveals the substances present in the mixture.

If the standard substance is not available, it may be possible to identify unknown spots according to its mobility value R_F (Equation 1).

$$R_F = \frac{V_X}{V_0} = \frac{h_X}{h_0} \quad (1)$$

Identification is based on assumption that under certain working method and conditions the ratio of spot's speed, V_x, to the speed of mobile phase front, V_0, is constant. This constant is characteristic to a substance. To calculate mobility of a substance, the distance between the starting line and the center of the spot (h_x) is measured and divided by the distance between starting line and the eluent front, h_0 (Figure 3 and Equation 1).

3.1 Developing, visualizing (detection)

Analytes are very often colorless substances and thus invisible on the chromatographic plate. In such cases the plate has to be developed after elution process. For developing different chemicals can be used which form colored substances upon reaction with analytes. Some substances which have no color appear as colored spots when observed under ultraviolet lamp.
In this course two practical works have set up which use TLC. "Determination of amino acids by TLC" employs paper as stationary phase and visualization is achieved by chemical reaction. "Determination of water soluble vitamins by TLC" uses aluminum foil coated with silica as stationary phase and the spots are analyzed under ultraviolet lamp.

4 Problems and exercises

1. Name some differences and similarities between thin layer chromatography and column chromatography?
2. The distance between start and finish line on a TLC plate is 6 cm. The spots of substances A and B had moved 2 and 4 cm, respectively. Calculate the mobilities of substances A and B! Which substance spent relatively more time in stationary phase?
3. Does the size of spot on chromatographic plate indicate anything about the analyte? Does it have influence on mobility? If yes, then how?
4. Why are the velocities of substances different in chromatographic column?