IL-4

Vacuum Liquid Chromatography (VLC) and Gel Permeation Chromatography in Natural Product Research

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The screening of natural products is an essential part of developing drugs, agrochemicals, nutraceuticals etc. However it is reported that approximately 50 % of approved drugs being derived from natural products or derivatives of natural products. These natural products come from complex biological systems such as plants, microorganisms, animals etc. Separation of these components in biological systems are essential in order to facilitate the screening assays. Among natural products separation methods, Vacuum Liquid Chromatography (VLC) and Gel Permeation Chromatography are two common techniques used in natural product research.

Vacuum Liquid Chromatography (VLC)

VLC is used as a separation step in natural product research. This is very similar to the technique used in Preparative Thin Layer Chromatography (PTLC) as TLC plates are dried after run and then separate the compounds. In VLC the column is run with solvent by applying vacuum and the column is allowed to dry after each fraction collected. However this is different to other chromatographic techniques as the column will be dried after collection of each fraction. In natural product research, VLC has been increasingly used to separate secondary metabolites because operation is less complicated when compared to other separation techniques. Chromatographic supports used in VLC are silica gel (both normal and reversed phase), Al_2O_3, diol, polyamide and CN. The mobile phase used are hexane, dichloromethane, ethyl acetate and methanol. Separation of secondary metabolites in plant extracts can be performed using VLC and this method is mainly employed as fractionation of crude extracts. Nevertheless there are occasions that VLC has been used to isolate pure compounds. It is reported that chloroform extract of *Cucurbita maxima* flowers was subjected to dry packed silica gel 60G column (60 mm) and the column was eluted with gradient of ethyl acetate in hexane followed by 50 % ethanol in ethyl acetate. This method has helped to isolate pure spinasterol crystals (94 % purity).

In VLC, Sintered - glass Buchner funnels or wide-mouth short columns can be used to pack silica gel (Figure 1). However if longer columns are used, resolution of separation

can be increased. Unlike packing of normal silica gel column, in VLC, the chromatography column is dry-packed with TLC grade absorbent under vacuum in order to obtain the maximum packing density and vacuum is released. Low polarity solvent is used to wet the silica gel (ex. Hexane) and the vacuum is re-applied. The solvent poured onto the surface of the silica gel is sucked dry and now dried crude extract of natural product (in powder form) can be introduced on to the column. First the column is eluted with a non-polar solvent (hexane) and the polarity of the solvent can be gradually increased by adding ethyl acetate followed by 100 % MeOH.

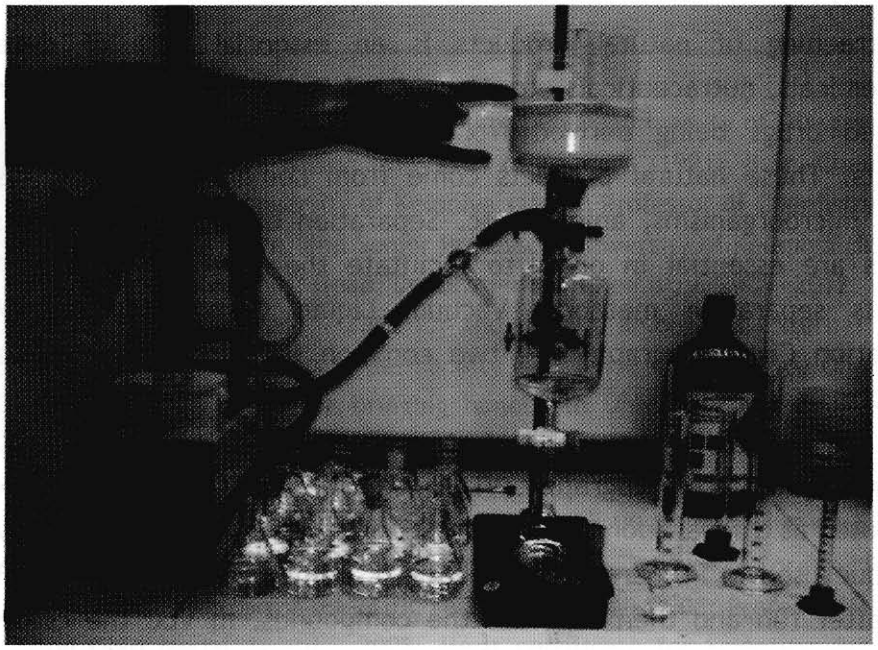

Figure 1 : Apparatus of Vacuum Liquid Chromatography

Gel Permeation Chromatography (GPC)

This is also known as size exclusion chromatography (SEC)or gel filtration chromatography (GFC). In this technique, liquid mobile phase and solid stationary phase are used. A polymer molecules (ex Sephadex) are used as the solid stationary phase and separation of natural products in a mixture is relied on size of the polymer molecule in solution rather than the chemical interaction between polymer molecules and chemical constituents in natural products. In this method, molecules are separated according to their size. The applications of this method in natural product research are analysis of synthetic polymers, oligomers, lipids, proteins, cellulose derivatives, crude oil alkanes and separation of small organic compounds in natural products.

Big molecules cannot penetrate the small pores in the stationary phase, therefore they are eluted by a volume of solvent equal to the **void volume** (Vo) between beads

of the stationary phase and small molecules penetrate the small pores. They are eluted by a volume of solvent equal to the **total volume** (Vt) of mobile phase. Retention of molecules in SEC is characterized by partition coefficient (Kav),

$$K_{av} = \frac{V_r - V_0}{V_t - V_0}$$

V_r: retention volume for a solute

Unlike the other modes of liquid chromatography, the separation comes from the stationary phase only. The mobile phase should have no effect as long as the sample is well dissolvable. Separation is carried out on the pores which typically equals 40 % of the total column volume. As a result long columns, or several columns are required. Separation range of the column should be selected carefully. Stationary phase with small particle size (5µm) provides more theoretical plates small particle size can result on shear degradation of large polymers. Combination of packings with different separation range can be achieved by: columns of different porosity or mixed bed columns. When handle GPC column, a column should be always run in the same mobile phase, no air bubbles in columns during injection and assembly and always use a pre-column when in doubt about the quality of the sample.

Most commonly used is stationary phase in GPC is Sephadex and it is a bead-formed gel prepared by cross linking dextran with epichlorohydrin (Figure 2). Sephadex could filter molecules according to their sizes and Sephadex with different pore sizes are produced in GPC. Small molecules can enter into the pores of gels and travel down slowly indicating longer retention time whereas large molecules will be prevented to enter the pores and travel faster alone the column giving shorter retention time (Figure 3). The underlying principle of GPC is that particles of different sizes will elute (filter) through a stationary phase at different rates and particles of the same size should elute together. This method can be applied to separate water soluble macromolecules as well as small bioactive secondary metabolites soluble in organic solvents. Sephadex swells in aqueous solutions and in organic solvents. It should be noted that the degree of swelling in organic solvents or their mixtures will not be the same as in water alone. Sephadex LH-20 and Sepharose CL are recommended for GPC in organic solvents. When GPC is used to separate organic compounds soluble in an organic solvent, Sephadex LH-20 should be soaked in hexane overnight and eluted with hexane followed by hexane -CH_2Cl_2 (2:1), hexane- CH_2Cl_2 (1:1), hexane - CH_2Cl_2 (1:2), CH_2Cl_2 :acetone (3:2), CH_2Cl_2 :acetone (1:4), CH_2Cl_2 MeOH (1:1) and finally MeOH.

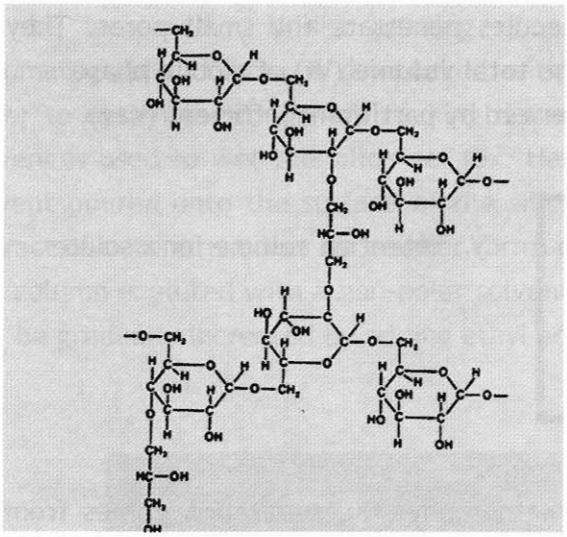

Figure 2. Partial structure of Sephadex

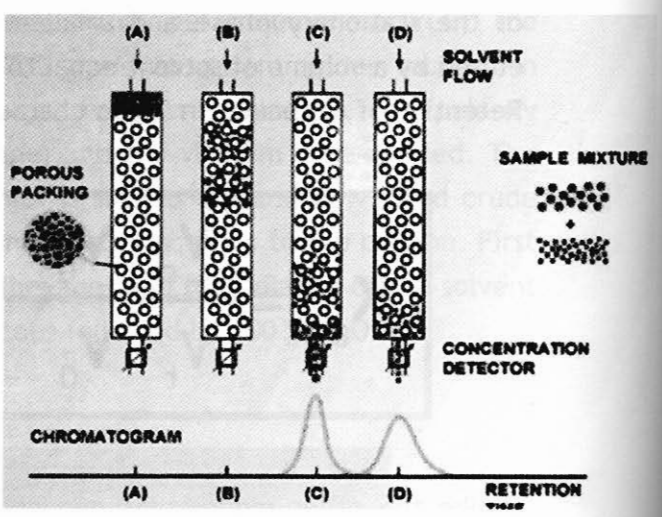

Figure 3. Schematic of a size-exclusion chromatography

A- Sample mixture before entering the column packing
B- Size separation begins
C- Collecting of the large molecule fraction
D- Collecting of the small molecule fraction

Advantages of this method are unlike ion exchange or affinity chromatography, molecules do not bind or interact with the medium, and hence composition of buffer or solvent does not directly affect resolution. This method is well suited for biomolecules that may be sensitive to changes in pH, conc. of metal ions or co-factors and harsh environmental conditions. Operating conditions can be varied to suit the type of sample or the requirements for further purification, analysis or storage without altering the separation. This method can be used after any chromatographic technique because components of any elution buffer / solvent will not affect the final separation.