BCH 309 - BIOCHEMICAL METHODS (2 UNITS)

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CHROMATOGRAPHY- Principle, techniques and applications

The basis of all forms of chromatography is the partition or distribution coefficient (Kd) which describes the way in which a compound distributes itself between two immiscible phases. For two such immiscible phases A and B, the value for the coefficient is a constant at a given temperature and is given by the expression:

Kd = concentration in phase A/concentration in phase B

The term effective distribution coefficient is defined as the total amount, as distinct from the concentration of substrate present in one phase divided by the total amount present in the other phase.

Basically, all chromatographic systems consist of the stationary phase, which may be solid, gel, liquid or a solid/liquid mixture that is immobilized, and the mobile phase may be liquid or gaseous and which flows over or through the stationary phase.

Modes of chromatography:

Chromatographic separation may be achieved using three contrasting modes namely:

(i) Column chromatography
(ii) Thin-layer chromatography
(iii) Paper chromatography

Types of column chromatography are:

(i) gel filtration or molecular sieve column chromatography
(ii) ion-exchange chromatography
(iii) affinity chromatography
(iv) adsorption chromatography
(v) high performance liquid chromatography
(vi) hydroxylapatite chromatography
(vii) reversed-phase liquid chromatography
(viii) gas-liquid chromatography
(ix) partition chromatography

Matrix materials

The matrix is the material used to support the stationary phase and its choice is vital to the successful chromatographic procedure. Generally, a matrix needs to have:

(i) high mechanical stability to encourage good flow rates and to minimize pressure drop along the column
(ii) good chemical stability
(iii) available in the range of particle sizes
(iv) porous structure of correct size and shape
(v) inert surface to minimize the non-selective adsorption of analytes.

In practice, six (6) most commonly used types of matrices are:

(i) agarose
(ii) cellulose
(iii) dextran
(iv) polyacrylamide
(v) silica
(vi) polystyrene

The chemical nature of the stationary phase depends upon the particular forms of chromatography to be carried out. Most stationary phases are available attached to the matrices in a range of sizes and shapes. The larger the particle, the faster the flow rate but, conversely, the smaller the particle the larger the surface area-to-volume ratio and potentially the greater their resolving power.

Column Packing

This is one of the most critical factors in achieving a successful separation by any form of column chromatography. It is commonly carried out by gently pouring slurry of the stationary phase in the mobile phase into a column that has its outlet closed, whilst the upper part of the slurry in the column is stirred and /or the column is gentle tapped to ensure that no bubbles are trapped and that the packing settles evenly. Once the required height has been obtained, the flow of mobile phase through the packed column is started by opening the outlet, and continued until the packing has completely settled. The top of the column should not be allowed to dry and this is achieved by connecting it to reservoir containing the eluting buffer. Precautions to follow while packing a column includes among others:

(i) erect the column in a straight form
(ii) stir the slurry gently and tap the column to ensure that no air bubbles are trapped
(iii) ensure that the top of the column is not dried.

Application of sample.

The simplest way for sample application involves the removal of most of the mobile phase from above the column by suction and allowed the remainder into the column bed. The sample will then be applied gently by pipette and allowed to run into the column. A small volume of mobile phase is then applied in a similar manner to wash final traces of the sample into the bed. Thereafter, mobile phase is then carefully added to the column to a height of 2-5cm. The column is then connected to a suitable reservoir that contains more mobile phase so that the height of the phase in the column can be maintained at 2-5 cm.

N.B: Care must be taken to avoid overloading of the column with sample, so that there would not be irregular separation. Not more than 10% of the total bed volume should be layer as sample.

EXCLUSION (PERMEATION CHROMATOGRAPHY)

This method is based on the saparation of molecules on the basis of their molecular size and shape. The principle involved a column of gel particles or porus glass granules which is in equilibrium with a suitable mobile phase for the molecules to be separated . Large molecules that are completely excluded from the pores will pass through the interstitial spaces and will appear in the effluent first. Smaller molecules will be distributed between the mobile phase inside and outside the molecular sieve and will then pass through the column at a slower rate, hence appearing last in the effluent. The three stages in such a column are represented thus:

Material often used in gel filtration include cross-linked dextran (e.g sephadex), agarose (sepharose, Biogel A), polyacrylamide (Bio gel P).

APPLICATIONS OF EXCLUTION CHROMATOGRAPHY

(1) Purification: It is commonly used in the purification of biological macromolecules by facilitating their separation from larger and smaller molecules. Diiferent enzymes, hormone, antibodies nucleic acids and polysaccharides have been separated and purified by using appropriate gels
(2) Relative molecular weight determination: It have been shown that over considerable range of relative molecular mass, elution volume is approximately linear function of logarithm of molecular mass. Thus, construction of a calibration curve , with proteins of known molecular mass and elution volume will enables the molecular weight value of other (unknown) proteins to be estimated.
(3) Desalting: By the use of a column of sephadex G-25 for instance, solution of high molecular weight compounds may be desalted. This is because a high molecular weight substance move with the void volume whereas the low molecular weight component will be distributed between the mobile and stationary phases and hence moves slowly.
(4) Solution concentration: Addition of sephadex G-25 (coarse) to solution of high molecular weight substance may results in concentration of such solution in that, water and low molecular weight substances will be absorbed by the swelling gel while high molecular weight substance remain in solution. Thereafter (about 10 min.), the gel will be removed by centrifugation, leaving the high molecular weight materials in solution whose concentration has increased but whose pH and ionic strength are unaltered.

Reference:

Keith Wilson and John Walker (1995) Practical Biochemistry: Principle and Techniques of Practical Biochemistry, 4th Edition pp 206-