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V. Gradient Techniques

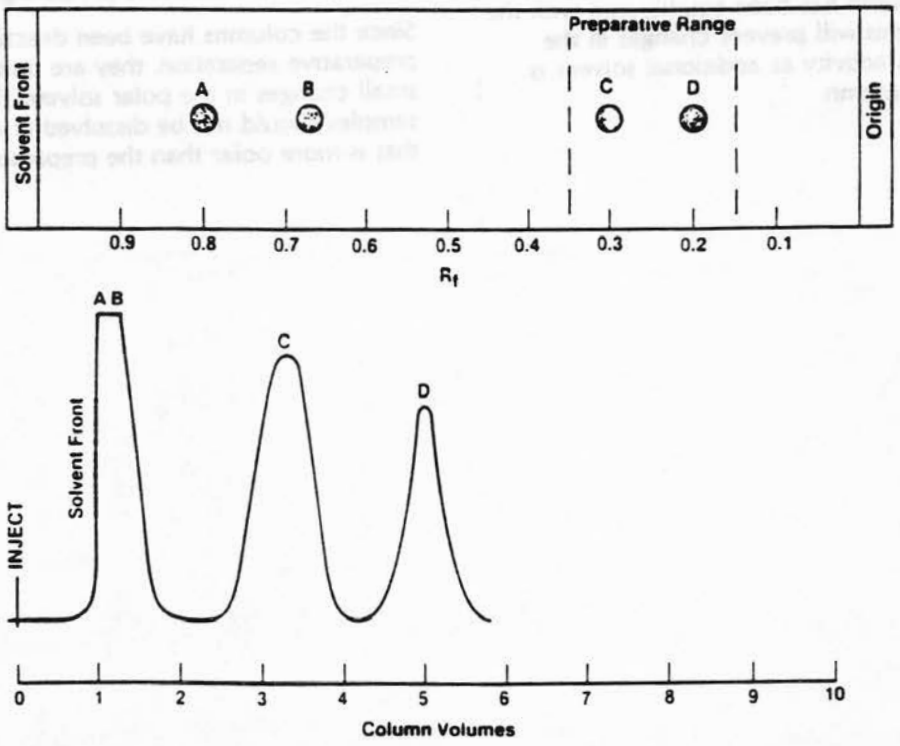
In column chromatography, the continuous flow of solvent through the column elutes the solutes from the stationary phase through the column. As this occurs, new equilibria are established between the adsorbent, solutes, and the solvent. When the solutes move through the column at different rates, because of their differing affinity for the adsorbent and the solvent, separation occurs. Some separations require only a single solvent, but others may require a mixture of two or more solvents to achieve the desired separation.

Often one must start the elution with a nonpolar solvent to remove relatively nonpolar compounds from the column and then increase the solvent polarity to elute compounds of greater polarity from the column. By changing the solvent polarity, the equilibrium in the column is altered. The more polar solvent will absorb on the column, displacing the polar compound from its site of adsorption, and minimize tailing. In preparative LC, both techniques are employed, but use of the step gradient is more prevalent, especially when it results in a significant decrease in the total solvent required over isocratic (constant) solvent mixtures.

A. Step Gradients

In Section I the relationship of TLC R_f, LC column volumes and the reasons to adjust R_f values between 0.15 and 0.35 were discussed. Many samples contain components of interest which cannot be maintained between R_f = .15 and 0.35 on the TLC plates because of widely differing polarities. An isocratic mobile phase cannot elute all the components within a reasonable volume (Fig. 13).

Figure 13

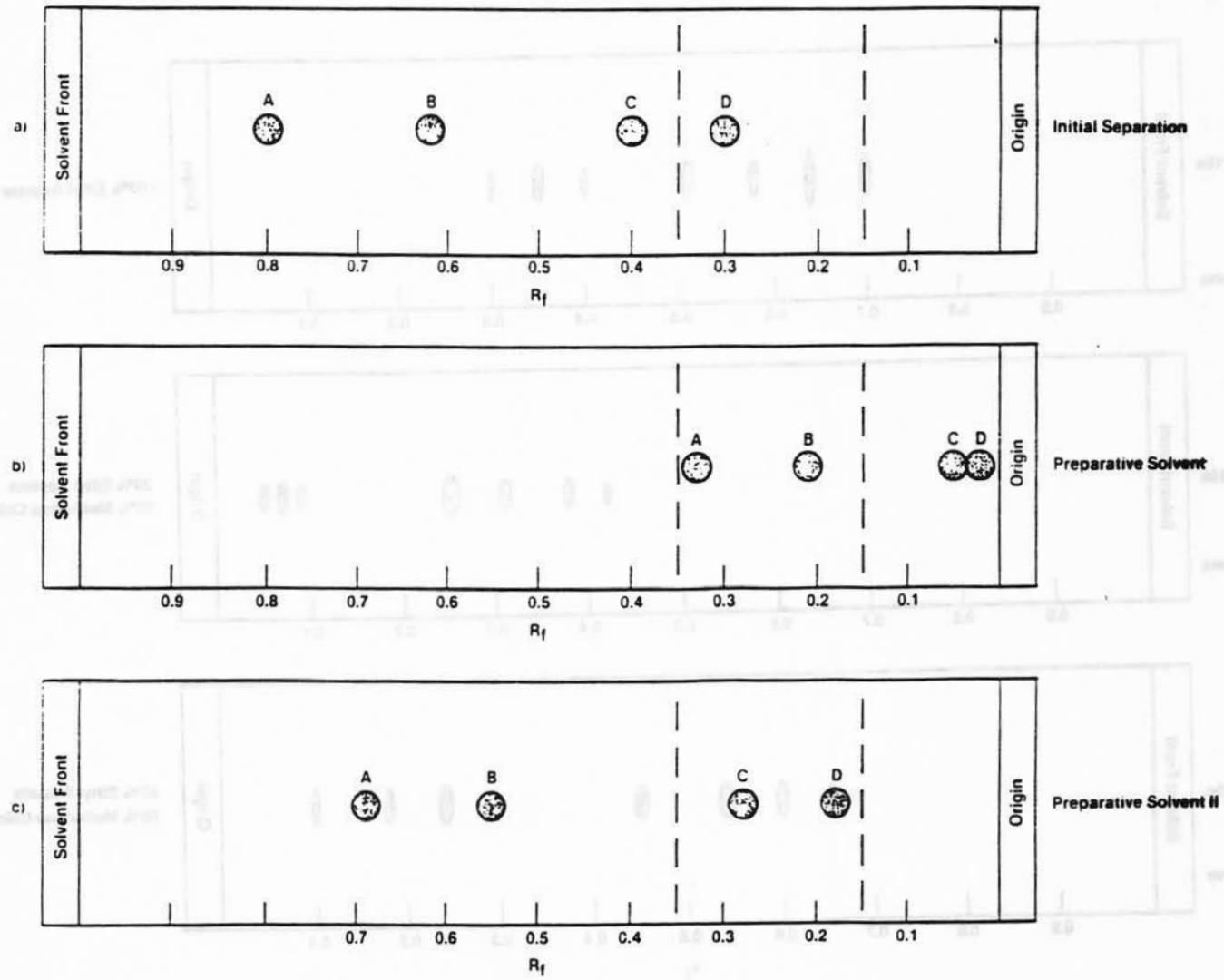


After eluting the less polar components, the more polar components are eluted with more polar solvents in a step-wise fashion. Two techniques have been developed to select the appropriate solvent steps. Both techniques are equally successful, and the choice between them depends on the complexity of the sample, sample mass, desired purity, and the difference in polarity between the individual components.

Either the refractive index detector or a UV detector can be employed to monitor the column effluent during a gradient run. The UV detector is less sensitive to solvent changes, but it is more sensitive to certain compounds and may become saturated when a highly concentrated sample elutes. The RI detector is quite sensitive to solvent changes (i.e. RI changes), and the reference cell must be flushed with the new solvent for each step gradient before a baseline can be established.

Technique 1: When the initial TLC separation indicates two or more distinct regions on the plate containing multiple components (Fig. 14a), this technique is employed. The solvent that elutes the least polar components between R_f = 0.15 and 0.35 is the initial preparative solvent. The second is the solvent which moves the more polar components compounds between R_f = 0.15 and 0.35 (Fig. 14 b, c). If the sample complexity warrants, additional solvent steps may be determined in a similar manner.

Figure 14



After equilibrating the cartridge with the initial solvent, the sample is injected, and the least polar compounds are eluted (2-6 C.V.). After collecting these compounds, the solvent is changed to the second one and the more polar compounds elute in the next 2-6 column volumes. A preparative separation utilizing this technique is illustrated in Fig. 15.

This step gradient technique requires the use of a detector to indicate when a compound elutes from the column. Generally, there is sufficient time to rebalance the refractive index detector, since the compounds are retained 2-6 column volumes after each step.

Figure 15 Establishing Appropriate Solvents for a Step Gradient

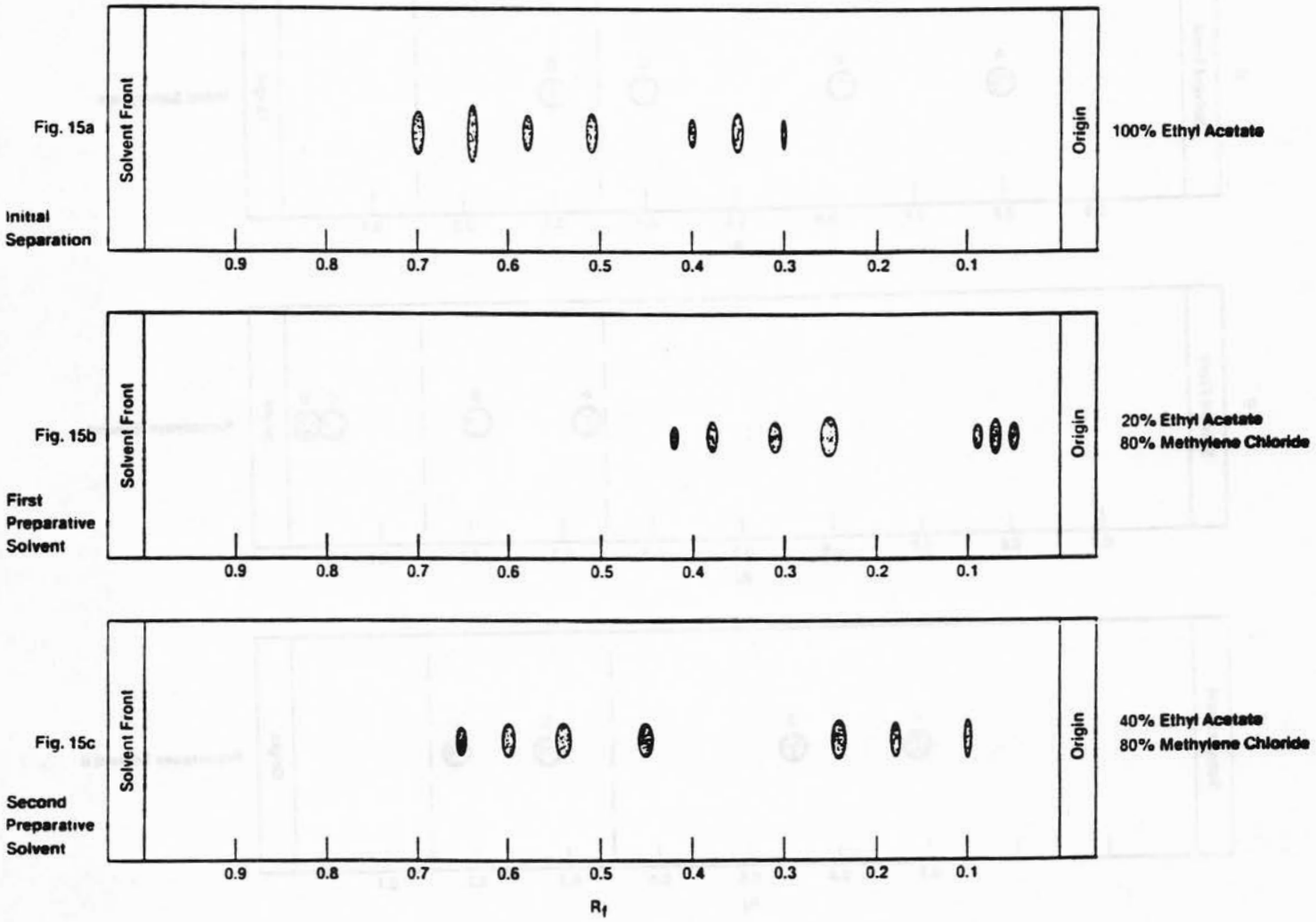
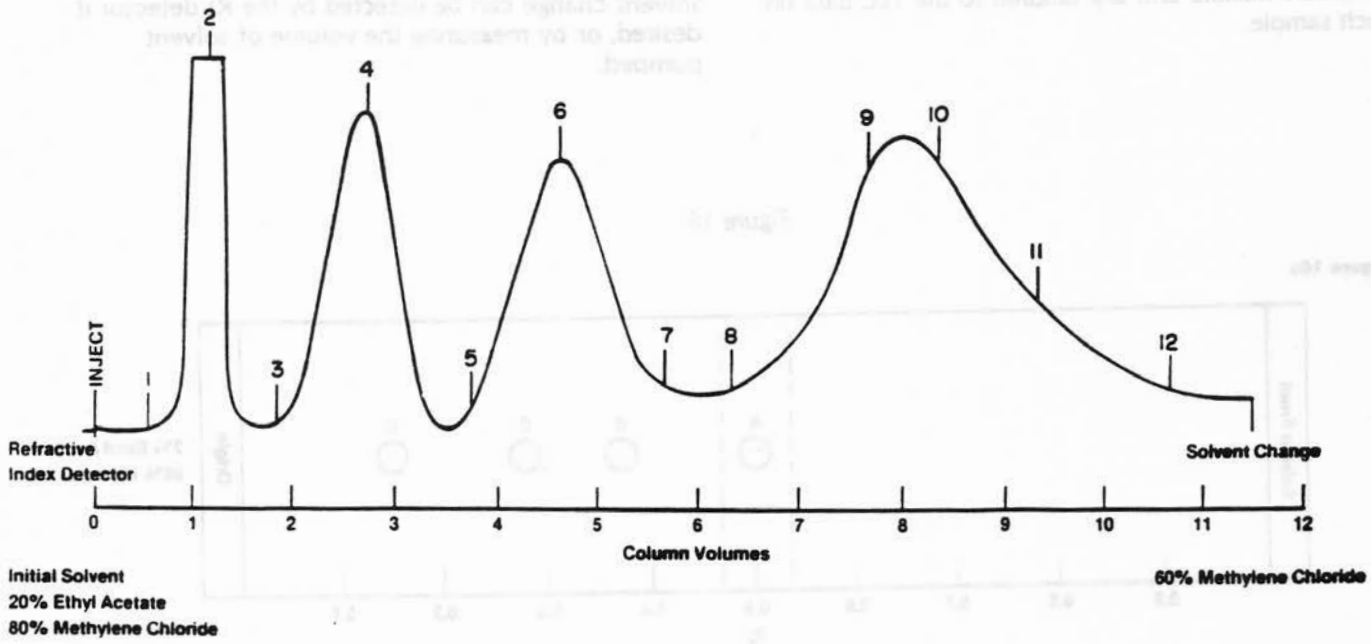
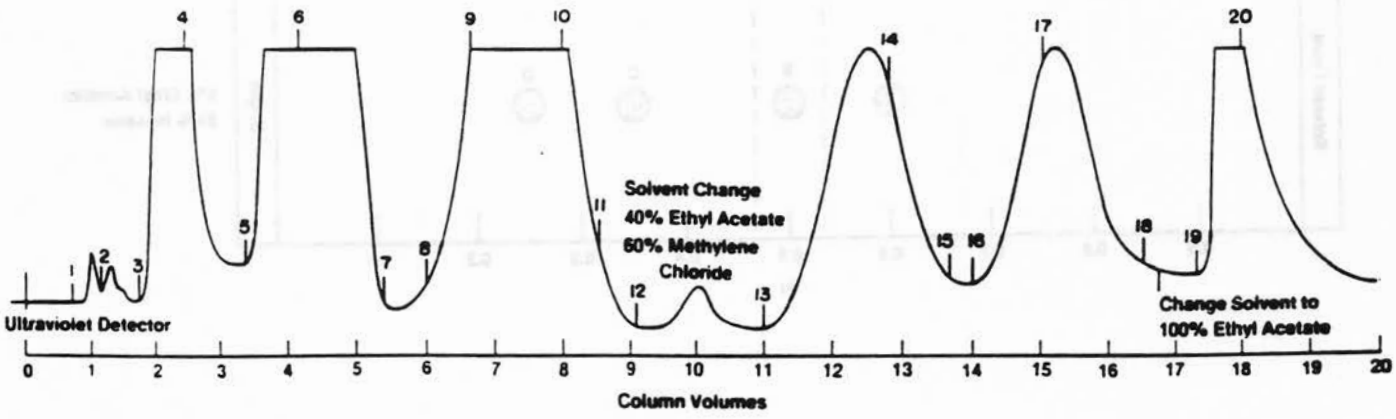


Figure 15 Preparative LC Separation





Technique 2: This is designed to separate a complex sample rapidly into several fractions, each of which may contain one or two components (Fig. 16a). The number of steps and the solvent polarity range are extremely flexible and are tailored to the TLC data on each sample.

This step gradient does not require the use of a detector. By choosing the appropriate solvent changes, the components will elute at the solvent change minimizing the need for a detector. The solvent change can be detected by the RI detector if desired, or by measuring the volume of solvent pumped.

Figure 16

Figure 16a

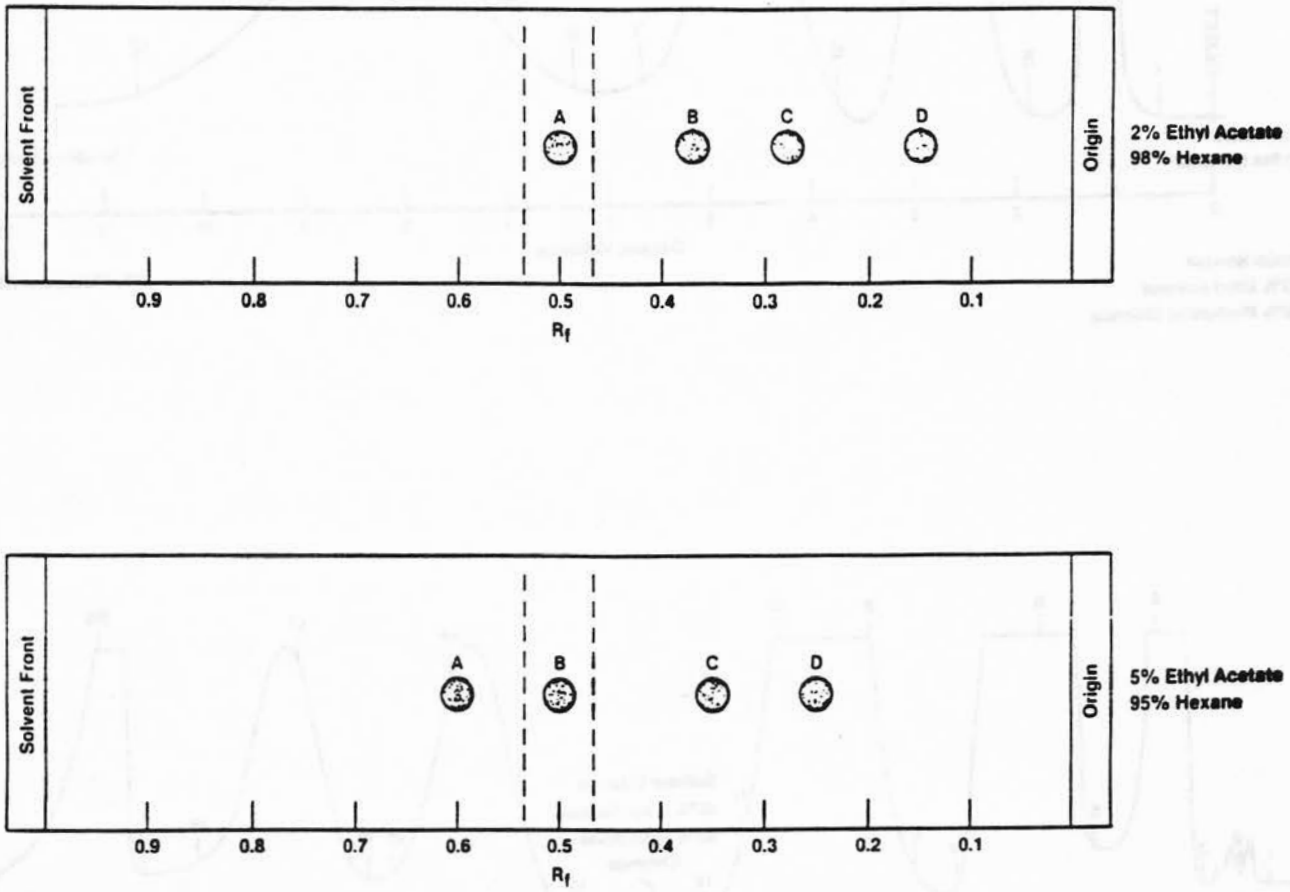
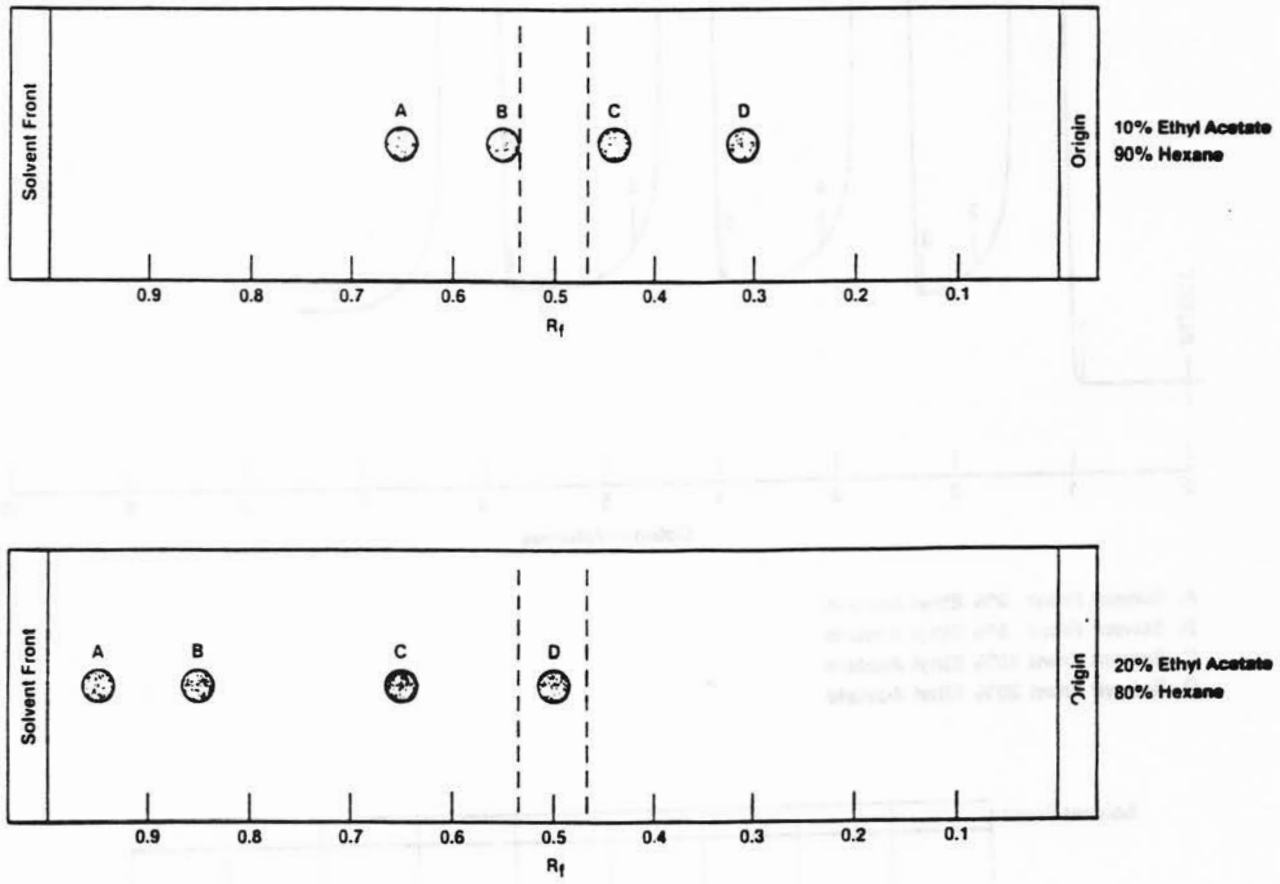
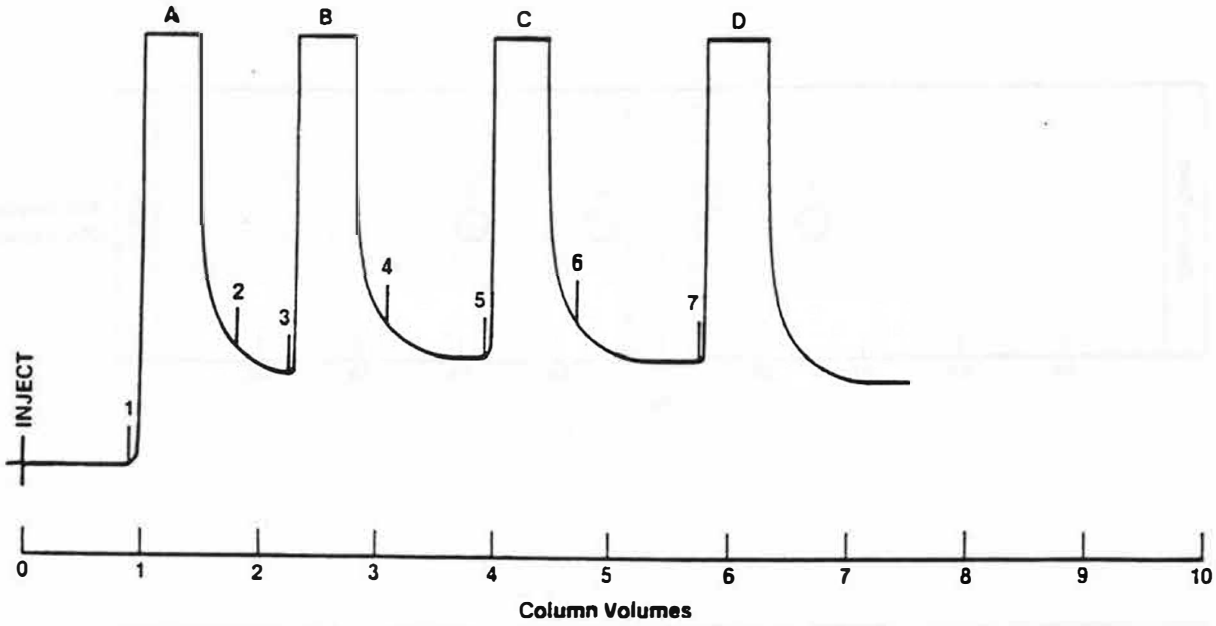


Figure 16b

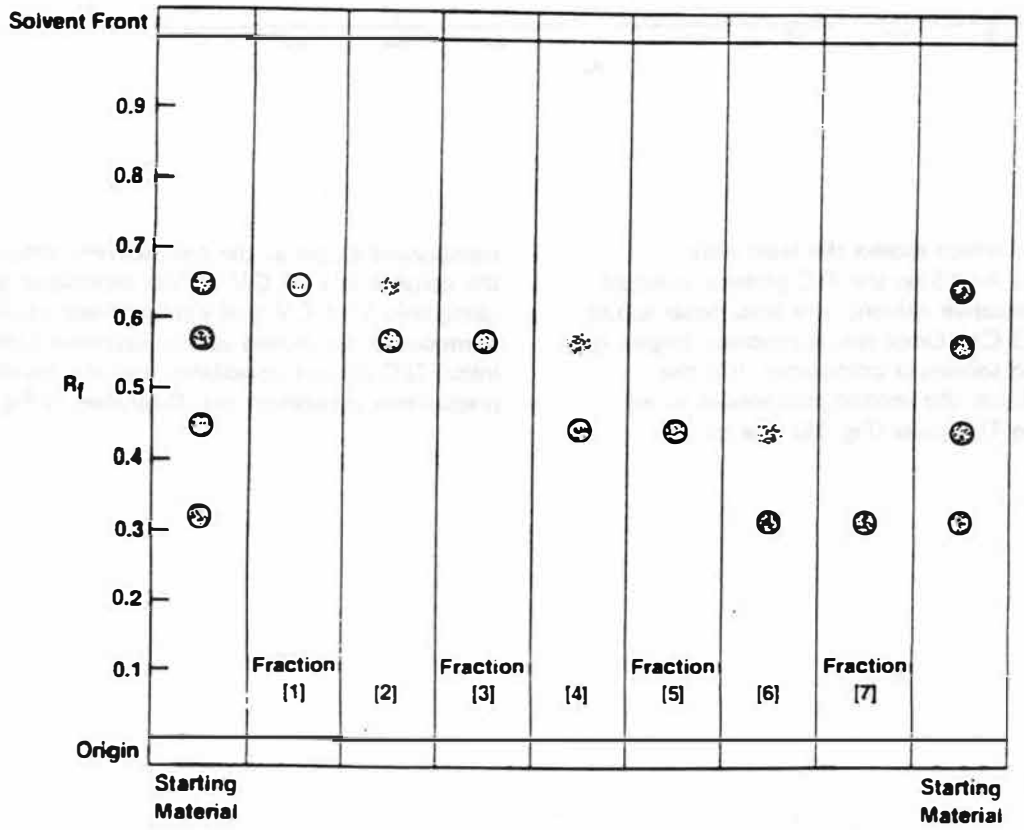


A solvent system which moves the least polar component to $R_f = 0.5$ on the TLC plate is selected for the first preparative solvent. The least polar solute will elute in 1-1.5 C.V. Once this component begins to elute, the second solvent is introduced. It is the solvent which eluted the second component to an $R_f = 0.5$ on the TLC plate (Fig. 16). The second component elutes as the new solvent emerges from the column in 1-1.5 C.V.'s. This technique is continued using only 1-1.5 C.V.'s of each solvent until all the compounds are eluted in this step-wise fashion. The initial TLC solvent conditions and the results from a preparative separation are illustrated in Fig. 17.

Figure 17



A. Solvent Front 2% Ethyl Acetate
B. Solvent Front 5% Ethyl Acetate
C. Solvent Front 10% Ethyl Acetate
D. Solvent Front 20% Ethyl Acetate



B. Continuous Gradients

Separating a complex mixture into its individual components is a tremendous benefit of preparative chromatography. In the last two sections, TLC information was combined with step-gradient techniques to form a powerful separations tool. The solvent polarity changes can also be generated continuously.

To evaluate if a sample should be separated preparatively by a step gradient or a continuous gradient depends upon:

1. **Complexity of the sample.** If 5 or 6 steps are required, it may be easier to do a continuous gradient.
2. **Solvent polarity.** If a polar modifier was required to move the components on a TLC plate, or if there was a large polarity difference in the solvents, the continuous gradient may better duplicate the separation.
3. **Analytical LC.** If the analytical separation required a gradient to separate a complex mixture, the preparative separation can be run similarly.

The LC can be easily adapted based on the criteria listed above. A continuous gradient technique using methanol is described below. This technique is also applicable with other polar or nonpolar solvents. To monitor the column effluent effectively during a continuous gradient, a UV detector must be employed. The solvent composition changes continuously, and will not allow a RI detector to become balanced.

Technique 3: As previously mentioned, if methanol or another polar solvent is required to provide a TLC separation, the solvent on the TLC plate is not of constant composition. The solvent at the solvent front has been depleted of polar solvent, while near the origin the composition is the initial TLC solvent (Fig. 12).

To achieve a similar separation, the solvent conditions can be duplicated by arranging the preparative instrument as shown in Fig. 18. Two identical solvent reservoirs are used, with the least polar solvent in Reservoir A. The more polar solvent (Reservoir B) generally is the solvent in the analytical TLC chamber. A syphon is formed between the two reservoirs, using a 1/4" teflon (not tygon) tube. During the preparative separation the solvent is drawn from the continuously stirred Reservoir A, while the more polar solvent is syphoned from Reservoir B to A. By operating the PrepLC System 500A at 250 ml/min, a linear gradient will be generated as represented in Fig. 19.

Figure 18 Gradient Elution with Waters PrepLC™ System 500A Preparative Liquid Chromatograph