

the proper conditions can be found (18). Refer to Sec. 12 below further discussion on software method development.

### 10.1. Key Variables—Resolution Equation Parameters

In reversed-phase/ion-pair chromatography, there are essentially 8–10 key variables that affect the separation, as depicted in the resolution equation,  $R$ :

$$R = \frac{1}{4} \cdot N^{1/2} \cdot (\alpha - 1) \cdot \frac{k'}{1 + k'}$$

where  $N$ ,  $\alpha$  and  $k'$  are referred to as the efficiency, selectivity, and retention (capacity) factors, respectively affecting the resolution of the analyte from other components in the separation. The efficiency is affected by the nature of the column, and both selectivity and retention are affected by the solvent. Column variables include length, particle size, and flow. Solvent variables are the nature of the sample, the mobile phase, and the column surface, i.e., bonded-phase (adsorbent type) such as C18, phenyl or cyano, etc.

These key variables include mobile phase strength, solvent type, column type/size, pH, temperature, ion-pair reagent (type and concentration), buffer, and mobile phase flow rate.

### 10.2. Isocratic or Gradient Mode

Either isocratic or gradient mode may be used to determine the initial conditions of the separation, following the suggested experimental conditions given in Table 3. Depending on the number of active components to be resolved or separated, the more complex the separation, the more gradient elution would be advantageous over isocratic mode, which is akin to a brute force application when trying to separate a complex mixture. When faced with developing a method to separate a complex mixture, the use of computer software is useful. This is further discussed in Sec. 12.

In deciding whether a gradient would be required or whether isocratic mode would be adequate, an initial gradient run is performed, and the ratio between the total gradient time and the difference in gradient time between the first and last component are calculated. When the calculated ratio is  $< 0.25$ , isocratic is adequate; when the ratio is  $> 0.25$ , gradient would be beneficial (19) as shown in Figure 2.

For complex mixtures (separations), when there are many degradation products, a long gradient run may be needed. In this case, a compromise may have to be made, using an isocratic method for product release and a gradient method for stability assessment. The isocratic method has generally a shorter run time, say under 15 minutes, and no degradation product would be monitored, assuming that none are formed initially. With time the degradation products are formed and must be monitored, which requires a gradient method to resolve completely the mixture (15 minutes and longer depending on the complexity of the degradation mix). The gradient method, then, would be the stability or regulatory method.

### 10.3. Role of pH

pH is another factor in the resolution equation that will affect the selectivity of the separation. In reversed-phase HPLC, sample retention increases when the analyte