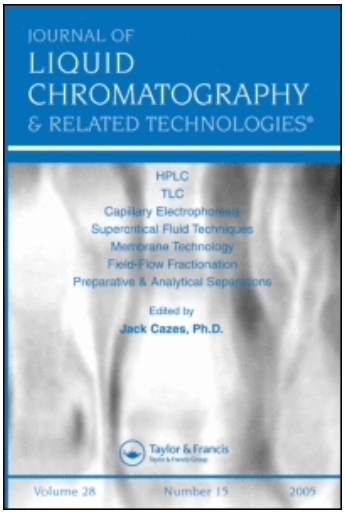

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HIGH RESOLUTION LIQUID CHROMATOGRAPHY

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Development of high resolution liquid chromatography can be traced to the use of columns with particle size of less than 30 μm (1). As anticipated from theoretical considerations, this has produced a significant improvement in resolution over previously used larger particle size columns; therefore, based on the subjective judgement of the chromatographer, the technique is designated either high performance or high resolution liquid chromatography. Discussion is provided below as to what is currently considered high resolution and how one might reasonably define it.

A. Common Misconceptions About High Resolution

Numerous examples of high resolution abound in liquid chromatography. These comprise separations of a large number of compounds from a homologous series as well as separations of compounds with different functional groups. Therefore, there is a tendency to equate high resolution with separation of a large number of components or high number of theoretical plates. Examples include separation of 150 polynuclear aromatic hydrocarbons (2) or a large number of components from coal fractions (3) or 300 peptides from a single tryptic digest (4). It must be noted, however, that there is no meaningful definition or fixed rules to determine when a given separation should be designated high resolution separation.

1. High N, α, or k'

The following equation defines resolution in terms of N, α, or k' for a separation of two given components:

$$\text{Resolution} = R_S = 1/4\sqrt{N}\left[\frac{\alpha-1}{\alpha}\right]\left[\frac{k'}{1+k'}\right]$$

Where:

N = number of theoretical plates
α = separation factor
k' = capacity factor

It is clear that, individually, none of these terms (N, α and k') is sufficient to describe resolution. Therefore, it is erroneous to describe separation power of a column, as is frequently done, on the basis of the number of theoretical plates (N) or peak capacity (k'). Perhaps these situations can be best described as high plate number or high peak capacity separations. Even though α, the separation factor, is an important component of resolution, it is limited in this equation to define only separation of one pair of peaks, generally the closest separation. Hence, no assurance exists on separation of other components.

B. Various Routes to High Resolution

For difficult chromatographic separations, it is common to look for high N or k'; however, what is really needed is high selectivity. An excellent example as to why it is not always necessary to produce a high number of plates is provided in the separation of toluene, nitrobenzene and m-dinitrobenzene (5). A 6 m x 1 mm i.d. column packed with Zorbax BP Sil 7-8 μm provided 270,000 theoretical plates with a baseline separation of these three components in 300 minutes with methylene chloride as mobile phase; however, the same components can be separated in 15 seconds on 10 cm x 1 mm i.d. column packed with the same material (less than 1500 theoretical plates) with hexane:methanol (99:1) mobile phase. Not only can one obtain a reasonable separation in 15 seconds, a run for 60 seconds can separate 5 additional components with various substituents. The important difference in the above separation is the selectivity provided by the mobile phase.

High selectivity is achieved by optimal selection of mobile phase which is unique to HPLC as compared to GLC; the end result is separation in a more reasonable time. This is accomplished through appropriate selections of particle size (for packed columns), column diameter/length, pressure drop, flow velocity, sorption properties of the reversed-phase column, solvent viscosity, peak capacity, and total number of theoretical plates.

1. Optimum Column Selection (Conventional vs Small Particle vs Microbore Columns)

High resolution in HPLC can be achieved by use of conventional columns (<30 μm), small particle columns (3 μm) or microbore columns. However, column selection is dependent upon whether one wants to optimize N, k' or analysis time. To maximize the effective peak number achieved in a given analysis time at constant N, the largest calculated value of k' is approximately equal to 6 (6). At k' ≅ 6, the highest peak

capacity of 74 was offered by the conventional column and lowest analysis time by the small particle column with a peak capacity of 54 and analysis time one-fifth that of a conventional column (7).

The largest number of plates/meter were obtained on the 3 μm column (119000 plates/m). This column also generated the largest number of plates/unit time (99 plates/second); however, it was poor in terms of plates generated per unit pressure drop. At "optimum practical" flow (2 x optimum flow), conventional columns provided the largest number of plates/unit time and highest column performance as measured by impedance. Several lengths of individually packed microbore columns can be connected to produce columns with a high plate count - a distinct advantage at a fixed pressure of 5,000 p.s.i. Under these conditions, the microbore column produces 318,000 theoretical plates with analysis time of 33 hours (k' = 64) and a peak capacity of 282. The conventional column provides a peak capacity of 125 and analysis time of <1 hour. For a fixed number of theoretical plates (10,000), the column length and analysis time are shortest for 3 μm particle columns (84 mm, 224 sec.).

2. Optimum Analysis Time

Comparison between the resolving power of a packed and open tubular column indicates that open tubular columns have better resolving power than packed columns in a given analysis time, if column diameter for the open tubular column is the same as particle diameter in the packed column (8). The speed of analysis is strongly dependent on the particle size of the packing materials for the packed column and column diameter for open tubular columns. It can be calculated that open tubular columns with i.d. $\geqq$ 31 μm would never meet the separation speed of a 5 μm particle column having h (reduced plate height) = 2 and v (reduced velocity) = 5. For k' between 0 - 10, a $\leqq$ 7.5 μm i.d. open tubular column with the same peak capacity as 5 μm particle size column should be used if the faster analysis time is desired.

It is possible to generate a large number of plates, e.g. 1 x 10^6 plates with the smaller i.d. columns. However, in practice, it is much easier to achieve the gain in column resolving power by using microparticles (3 - 5 μm) packed columns than by using microbore open tubular columns. The columns packed with 3 μm particle size provide shorter analysis time than those packed with 5 and 10 μm particles for solute pairs with α of about 1.03.

The following equation can assist in optimization of analysis time (9):

Where: $t_{R(n)} = \frac{1}{\Delta P} \frac{\phi}{d_p^2} (1 + k_n')(L_{req})^2 \eta$

$t_{R(n)}$ = retention time of the strongest retained component
ΔP = pressure at which the system is operated
ϕ = column resistance parameter
d_p = particle size of the column packing
k'_n = capacity factor of the last eluting component
L_{req} = minimum column length required for the separation
η = viscosity of the mobile phase

To obtain optimum analysis time, the operating pressure should be the maximum allowable pressure with the given equipment. The particle size (d_p) should be the smallest available except for certain separations. L_{req} is mainly dependent on the selectivity of the HPLC system; if L_{req} is the same for two, then the system with the smallest $(1 + k'n)\ \eta$ factor, should be favored.

C. High Resolution Evaluations

Comparability of separation between HPLC and capillary GC, both in terms of resolution and time, has been demonstrated (10); ~36 components can be resolved in 60 minutes within $k' \cong 6$, some with low α (<1.1). Since the thrust of chromatography is to resolve the largest number of components in the shortest possible time, it would be desirable to consider both analysis time and the number of components separated in any separation. The time scale is better than the commonly used theoretical plate scale because the same resolution obtained over a very long time span is obviously less desirable than that obtained in a short time span. Theoretical plates/second attempts to address this question but misses the mark since the large number of theoretical plates/second does not necessarily assure separation of a variety of components.

What is really needed then is a large selection of molecular probes that can evaluate a system for selectivity (11) since this term is not sufficiently evaluated in the resolution equation. Selectivity as evaluated by the separation factor (α) between the closest pair does not fully assure separation of multifunctional compounds. Therefore, it is apparent that even though the current resolution equation is able to reasonably define the separation ability of a homologus series of compounds, it does very poorly with multifunctional compounds which may also be acidic, basic or neutral.

From the above discussion, it is clear that high resolution generally entails separations of closely resolved components (generally >10) in a reasonable period of time ($\leqq$ 60 minutes). To determine whether optimum peak number has been achieved, $k' \cong 6$ can be used to measure resolution power or separation ability (SA) of a system. Since high resolution implies the largest number of compounds that can be separated with small α values, separation ability or resolution power can be derived at constant N from summation of $\left[\frac{\alpha - 1}{\alpha}\right]$ values of the number of pairs resolved.

$$S_A = \sum_{1 \to n} \left[\frac{\alpha - 1}{\alpha}\right]$$

S_A = Separation ability of a system
α = Separation factor of various pairs

To determine separation ability on the basis of time (S_{At}) would merely require multiplying S_A by C/T, where:

C = Number of multifunctional compounds resolved within k' = 6 and T = Time, generally ≤ 60 minutes

The calculated value of S_A or S_{At} would provide a resasonable measure of the resolution power of a given system.

Conclusion

Separation of a large number of components in HPLC mainly requires optimization of selectivity. This is especially true when it is necessary to separate compounds with multifunctional groups in a short period of time. Discussion has been provided to assist evaluations of high resolution separations and allow optimization of select parameters to obtain a short analysis time.

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