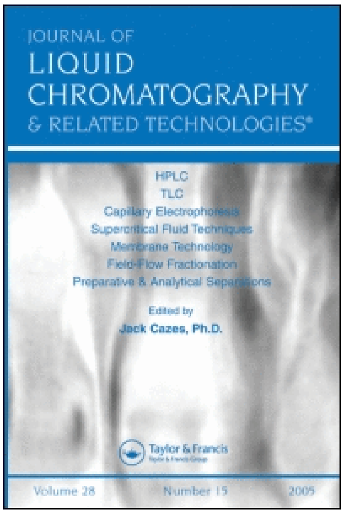

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An Evaluation of Expressions Used for Chromatographic Resolution in the Case of Skewed Peaks of Varying Peak-Heights

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AN EVALUATION OF EXPRESSIONS USED FOR CHROMATOGRAPHIC RESOLUTION IN THE CASE OF SKEWED PEAKS OF VARYING PEAK-HEIGHTS

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ABSTRACT

The classical way to express resolution can not be used as measure for the separation in the case of tailing peaks or peaks with strongly varying peak heights. A number of different expressions for resolution are evaluated with respect to peak overlap, i.e. the classical resolution, resolution based on the individual peak widths, resolution based on the first and second moments, the discrimination factor and an adjusted resolution which determines the peakwidth relative to the second peak. Attention is given to the application of these expressions for prediction of overlap in subsequent chromatograms, used in interpretive optimization techniques.

+ on leave from Veszprem University of Chemical Engineering, Hungary

INTRODUCTION

The problem how to describe the concept 'separation' has been one familiar to physists and chemists for many years (1-8). Especially in the field of chromatography, a separation science by definition where we usually refer to separation as resolution, this quality is of fundamental importance. In the past, many different ways have been proposed to define resolution, ranging from almost emperical solutions (1) to theoretical descriptions based on general separation science (2,3). However, many are still dissatisfied with these definitions, as expressed in more recent publications (7,8).

In the first place, the applied definition should be connected to the goal we are pursuing. On the one hand we have preparative forms of chromatography, where the true, physical separation of chemical species is the primary goal. Here classical definitions of separation, such as the extent of separation (2), can be applied. On the other hand we have forms of modern analytical chromatography, where we are interested in the accuracy of measured concentrations, something which is connected to, but not directly correlated with physical separation. By applying the correct integration techniques, one is capable of determining the concentrations of minor components quite accurately in the presence of large tailing matrix peaks, even if the separation is not complete. Furthermore, with the use of modern data analysis and detection techniques (GC-MS, LC-UV) the concept of 'sufficient separation' gets a completely new meaning.

One of the fields where the definition of resolution is of fundamental importance, is the optimization of chromatographic separations (9). By a systematic variation of the experimental circumstances, one tries to optimize the separation of a given sample. The quality of the separation is expressed in the form of a criterion, which is determined for chromatograms already measured. One form of optimization, the interpretive strategies (9), tries to describe the chromatographic behaviour of the solutes, in order to predict the chromatograms and the corresponding criterion to determine the optimal values of the experimental parameters. Since one of the aspects of a chromatogram is the quality of the separation, this quantity must be part of the criterion. In fact, the only common aspect in the multitude of optimization criteria proposed in the literature (10) is the application of some form of resolution, ranging from the separation of a single, critical peak-pair (minimal resolution) to a complex combination (e.g. product) of all resolutions between subsequent peak pairs (11). Until now, in the case of interpretive optimization strategies the classical definition of chromatographic resolution is often applied:

$$Rs = \frac{t_2 - t_1}{2\ (\sigma_1 + \sigma_2)} \qquad (1)$$

The resolution Rs between two components is expressed as the ratio of the difference between the respective retention-times t_1 and t_2 and the sum of the standard deviations of the peaks, σ_1 and σ_2. In the case of two Gaussian peaks of components in equal concentrations this corresponds to a 'complete' separation (peak-overlap of 0.3%) at Rs= 1.5.

Unfortunately in chromatographic practice we almost always observe more or less tailing peaks of unequal peak heights and hence the overlap between subsequent peaks will differ even when the resolution as defined by equation (1) is constant. As a consequence, the optimum predicted on the basis of criteria using this definition will differ from the chromatogram which we intuitively feel to give a better separation. This is illustrated in figure 1. The first chromatogram is optimal when we base our optimization on the normalized resolution product (11), calculated at a fixed plate-count. This criterion is aimed at an even distribution of all peaks over the available space in the chromatogram. However, the components are much better separated in the second chromatogram where more space is reserved for tailing and small peaks, resulting in an uneven distribution of the peaks. Chromatogram A will also be prefered when the minimal resolution calculated at a fixed plate-count is used. It will be clear that problems like these especially arise in more complicated, less ideal forms of chromatography, e.g. ion-pairing chromatography, where interaction between the ionized solutes and stationary phase often gives rise to tailing peaks.

Another area where we wish to have a better expression for resolution, is the evaluation of multivariate data analysis techniques as applied in hyphenated separation techniques such as multiwavelength detection in liquid chromatography by means of a photodiode-array detector (12). A number of advanced methods using this form of detection (13,14) require only minimal assumptions on the peak shape to derive elution profiles of individual, overlapping components. However, the performance of these techniques is strongly dependent on the amount of overlap, especially in the case of tailing peaks (12). In order to judge the derived results correctly a more accurate expression for resolution is required.

As a first step towards a better understanding of resolution and an improved application to the afore mentioned techniques, we

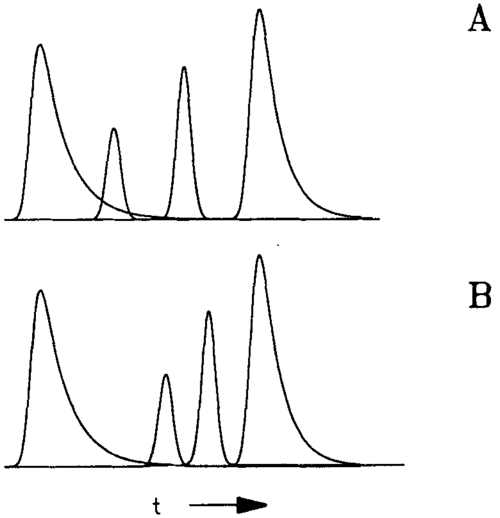

FIGURE 1
Two chromatograms containing four components. See text for discussion.

evaluated a number of expressions for the chromatographic resolution published in the literature. This evaluation is based on computer simulations, with special emphasis on asymmetric peaks and peak pairs with different peak heights.

THEORY

Before we give a more detailed description of the six expressions which were evaluated, there are two general, related aspects of the determination of the resolution which require some attention. An important characteristic of a given definition is the predictability of resolutions in subsequent chromatograms. If this is the case it will be possible to apply the definition for an interpretive optimization technique. In practice this means that the resolution must be based on a limited number of invariant or predictable quantities connected with either the chromatographic system or the components in the mixture. Another important aspect is whether the resolution can be determined from the combined one-dimensional chromatogram or whether the separate elution profiles are required. In the latter case the opportunity for separate injections c.q. a specific detection method is assumed.

1. Resolution Based on a Fixed Plate Count Rs_N

This method is probably the most wide-spread one to calculate or predict the amount of separation. The calculation is based on equation (1), while the standard deviation of the peaks is determined on the basis of the retention time of component i, t_i, and the plate count N:

$$\sigma_i = t_i / \sqrt{N} \qquad (2)$$

Equation (1) now reduces to:

$$Rs_N = \sqrt{N} * \frac{t_2 - t_1}{2\,(t_1 + t_2)} \qquad (3)$$

Based on general peak broadening mechanisms, this expression assumes a Gaussian peak shape and hence can not be expected to perform optimally in the case of more or less distorted peaks. The major advantage is the ease of calculation and predictability: assuming a constant plate count for a given column, the shape of every peak is determined by one parameter only, i.e. the retention time.

2. Resolution Based on the Individual Peak Widths Rs_w

An improved performance can be expected by an individual treatment of every peak. Although a Gaussian peak shape is still assumed for every component, the plate count may differ and hence an additional degree of freedom is added to the determination. In a practical situation the standard deviation of a peak can be determined from the full width at half-height, w_h:

$$\sigma_i = 0.43 * w_{h,i} \tag{4}$$

In this way equation (1) is replaced by:

$$Rs_w = 1.16 * \frac{t_2 - t_1}{w_{h,1} + w_{h,2}} \tag{5}$$

The additional parameter can be determined from the individual elution profiles of the components, which is equivalent with the determination of an individual plate count for every component. If this individual plate count is assumed to be constant for different retention times of the same component, this equation can be used to predict the resolution in advance.

When we have, as is often the case, only a combined one-dimensional chromatogram of a given sample, we might still want to give an estimate on the resolution. When we use peak widths observed in the combined profile, we can apply equation (5). In order to cover as large a number of applications as possible, we included this resolution, designated by Rs_o, in the evaluation. Obviously this estimate will be less accurate than Rs_w, especially for low resolutions where a valley is only just visible and peak widths must be estimated on the basis of left- or right-slope of the peaks (figure 2). It will be impossible to describe the resolution in cases where the individual peak maxima are no longer visible.

3. Resolution Based on Statistical Moments Rs_M

Since we are dealing with peaks with undefined shape, a more general form of equation (1) uses the statistical moments of the individual peaks:

$$Rs_M = \frac{M_{1,2} - M_{1,1}}{2\left(\sqrt{M_{2,1}} + \sqrt{M_{2,2}}\right)} \qquad (6)$$

Instead of using retention times, the first, or central, moment $M_{1,i}$ is used, and the peak width is described by means of the second moment $M_{2,i}$. These moments are defined according to:

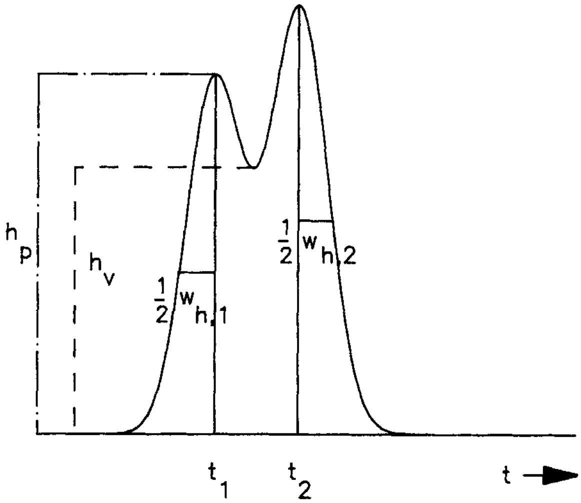

FIGURE 2
Different characteristics of a coeluting peak pair: the half width at half height, $\frac{1}{2}\,w_{h,1}$ and $\frac{1}{2}\,w_{h,2}$, and the height of the valley, h_v, and the smallest peak, h_p.

$$M_{1,i} = \int_{t=t_1}^{t_2} t * A(t)\,dt \;/\; \int_{t=t_1}^{t_2} A(t)\,dt \qquad (7)$$

$$M_{2,i} = \int_{t=t_1}^{t_2} (t - M_{1,i})^2 * A(t)\,dt \;/\; \int_{t=t_1}^{t_2} A(t)\,dt \qquad (8)$$

In other words, the chromatogram of a single component is treated as a frequency table A(t) over the time-interval t_1 to t_2. Because of the quadratic term in the calculation, tailing peaks will show an increased second moment, hence a more realistic presentation of the peak width is obtained. Needless to say the availability of individual elution profiles is a prerequisite for this method.

4. The Discrimination Factor d_0

In recent publications Schoenmakers (9) and El Fallah and Martin (7) define an alternative measure of separation, the socalled peak-to-valley ratio or discrimination factor. This factor was developed specifically with samples in mind which produce chromatograms with varying peak heights. This factor is defined as follows (figure 2):

$$d_0 = (h_p - h_v) / h_p \qquad (9)$$

h_p is the height of the smallest of two peaks and h_v is the height of the valley. This definition is closely related to earlier measures of separation such as the Kaiser and Christophe factors (4) and varies between 0, when a valley is not visible, and 1 for completely separated peaks. An important difference with the other expressions explained so far is the necessity of the combined experimental chromatogram to determine the value of the factor. In principle, this enables this factor to handle varying peak heights more efficiently, but has as major drawback with respect to interpretive optimization methods that the factor will be more difficult to predict in subsequent chromatograms.

5. The Adjusted Resolution Rs_a

Finally, Schoenmakers, Naish and Hunt (8) developed an adjusted resolution to define the separation of small solute peaks from large solvent peaks or other matrix peaks. This adjustment is based on strongly varying heights and widths of these peaks in comparison with the solute peak. As a consequence, the width of the large peak is determined on the basis of the width of the small peak, as illustrated in figure 3. The width w of an ideal gaussian peak can be expressed as four times the standard deviation and is observed at 13.5 % of the height of the peak. In the case of a large tailing peak it is better to use the half width observed on the left or right side of the peak (w_b or w_e), depending on the location of the smaller peak, and measured at the 13.5 % level of the smaller peak (full width w_o). All this assumes that the smaller of the two solute peaks is the one of interest. When these values are substituted in equation (1) the following equation results:

$$Rs_a = \frac{t_2 - t_1}{w_e + w_o/2} \qquad (10)$$

Generally speaking, especially in cases of severe overlap and equal peak heights, the value of w_e must be derived from the individual profile. By expressing the additional width of the matrix peak by means of a correction factor, the authors developed an expression to predict the adjusted resolution in subsequent chromatograms (8).

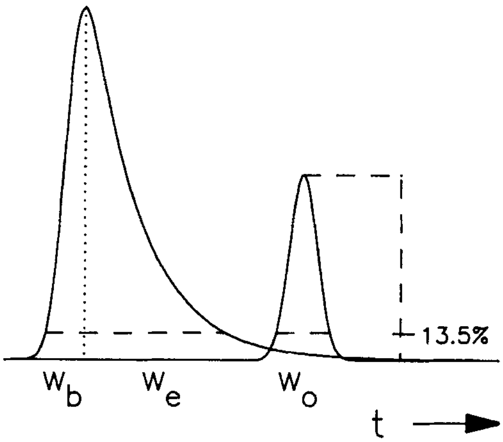

FIGURE 3
The characteristic quantities used for the adjusted resolution: w_o is the full width of the small peak at 13.5% of the height of this peak, while w_b and w_e are the widths on the left and right side of the perpendicular from the peak maximum of the large peak at the same level.

EXPERIMENTAL

In order to evaluate the different expressions, we have to define some objective measure of separation. Although, as expressed in the introduction, a true objective criterium will depend on the actual goal of the separation, we propose to use the relative peak overlap of the components as a basis of comparison. This will satisfy most separation problems and it is relatively easy to determine from the individual chromatographic profiles. The relative peak overlap of component i, RO_i, is defined by:

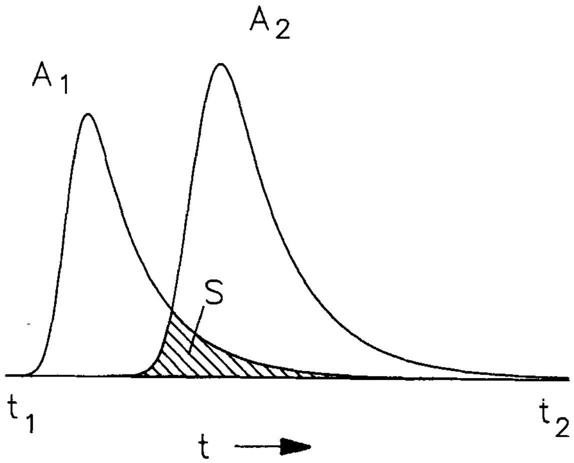

FIGURE 4
The common area S of two chromatographic signals, A_1 and A_2, used to define the relative overlap (equation 11).

$$RO_i = \int_{t=t_1}^{t_2} \min\,(A_1(t), A_2(t))\, dt \;/\; \int_{t=t_1}^{t_2} A_i(t)\, dt \qquad (11)$$

min $(A_1(t), A_2(t))$ denotes the minimum of the two individual chromatographic signals A_1 and A_2 observed at time t. This is illustrated in figure 4: the common area S is compared with the total area of one of either components. As a consequence this value will vary from 0, when the overlap is negligible, to either 1 (for the smaller of the two peaks), or to the ratio of the two peak areas (for the larger peak).

This quantity and the expressions explained in the theoretical section were calculated for a large number of chromatograms of two components. These chromatograms were simulated and analysed on an IBM-PC (IBM Corp., Boca Raton, Florida), using programs developed in Turbo-Pascal (Borland, Scotts Valley, California). The simulated chromatograms were derived using exponentially modified gaussian (EMG) peakshapes, according to an approximation described by Foley and Dorsey (15).

The plate count of the system was kept at a constant value of 1000, and the first peak fixed at a retention of 15 minutes. The separation between the first and second peak was changed by varying the retention of the second peak. The subsequent retention times of the second peak were 15.8, 16.0, 16.2, 16.4, 16.6, 16.8, 17.0, 17.4, 18.0 and 19.0 minutes. The chromatograms were calculated between 13 and 33 minutes by determining the signal every 0.05 min (20 points/min). The ratio between the peak areas was defined as 1, 2, 4 and 8 for the first peak compared to the second one. The asymmetry of the first peak, described by the time constant τ was defined as 0 (a pure Gaussian), 1, 2, 3 and 4 minutes. The asymmetry of the second peak was varied from 0 to the asymmetry of the first peak in steps of one minute.

RESULTS AND DISCUSSION

In this section we will discuss the relative overlap observed in the simulations as compared to the resolution values derived by the expressions described in the theoretical section. The following figures are illustrative of the general trend in the results, but do not cover the simulations completely. As typical results, we concentrate on varying asymmetry of the first peak with a symmetric second peak (τ_2=0 minutes) and two area ratios, i.e. 1:1 and 4:1, with the first peak being the largest one. The relative overlap in all figures is related to the second peak. These results were selected because we feel the presence of small symmetric peaks in the presence of larger asymmetric ones constitutes the most serious problem.

1. Resolution Based on a Fixed Plate Count Rs_N

Figure 5a gives the values for Rs_N as a function of the relative overlap RO for different asymmetries of the first peak for peaks of equal area. Especially the range of moderate overlap, $RO < 0.2$, is noteworthy and illustrates the central problem of this discussion. Because there is no correlation between asymmetry and resolution a large variation of the overlap is observed for a given value of Rs_N: for an observed resolution of 1.0, the relative overlap may vary from 0.05 to 0.35. In other words, knowledge of the resolution does not guarantee a given separation. This effect is even more pronounced in the case of a much smaller second peak (figure 5b). For two symmetric peaks the deviation from the case with equal area is moderate, but especially for large asymmetry the overlap is much larger than expected on the basis of the resolution. One could go as far as to say there is really no relation between the calculated resolution and the separation of the peaks. The observations located at line RO=1 are caused by a complete overlap of the small peak by the large one.

One has to keep in mind that a comparison between figures 5a and 5b must be performed with some caution: since the resolution Rs_N is a single value characterizing the separation of two peaks in one number, a variation is to be expected in the value of the

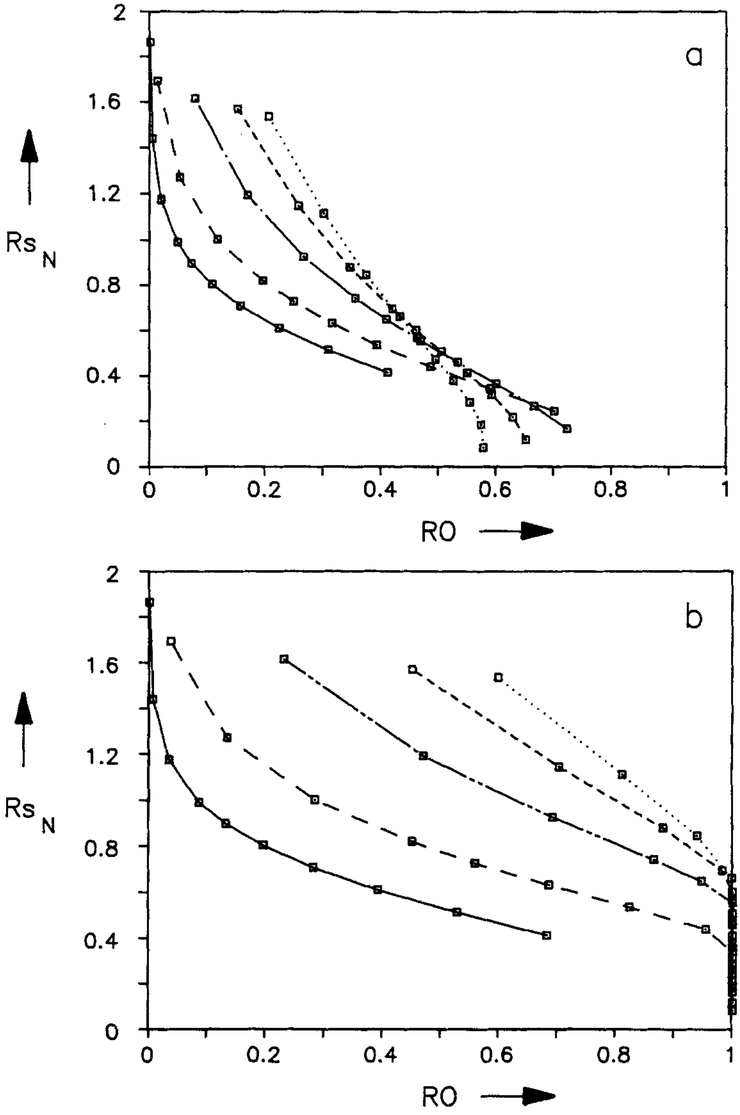

FIGURE 5
The relation between the resolution Rs$_N$ and the relative overlap RO of the second (symmetric) peak (τ_2=0). The time constant of the first peak varies according to: τ_1=0 (———), 1 (— — —), 2 (—·—·—), 3 (- - - - -) and 4 (··········).
a) peaks with equal area
b) peaks with 4:1 area ratio

relative overlap (which is specifically related to one component) when a given resolution is compared at different area ratios.

It follows that in order to evaluate separation correctly one has to discriminate between the two peaks. A separation which is quite sufficient for a large peak, could be insufficient for neighbouring small peaks. This suggests that the characterization of a complete chromatogram would need separate resolution values for each relevant peak.

2. Resolution Based on the Individual Peak Width Rs_w

Figure 6a is the equivalent of figure 5a for Rs_w, the resolution based on the peak widths of both peaks according to equation (3). Because of this additional parameter in the calculation, the derived resolution gives a more accurate picture of the true separation: compared with figure 5a the spread in the range of moderate overlap is clearly reduced, although still quite substantial. The same applies to figure 6b, the value of Rs_w for the 4:1 area ratio. However, the spread in the overlap is still too large for the Rs_w to characterize the separation.

It is clear that the difficulties encountered for the resolution derived from the widths of the individual peaks increase when these widths must be derived from the combined chromatogram (figure 7). An additional disadvantage is the limited range where the value of Rs_o can be calculated. Beyond an overlap of 0.3-0.4 there is no valley visible between the two peaks and one is unable to estimate the widths without additional information. Furthermore these estimates will be less accurate for closely overlapping peaks and will give rise to additional uncertainties in the calculated resolutions.

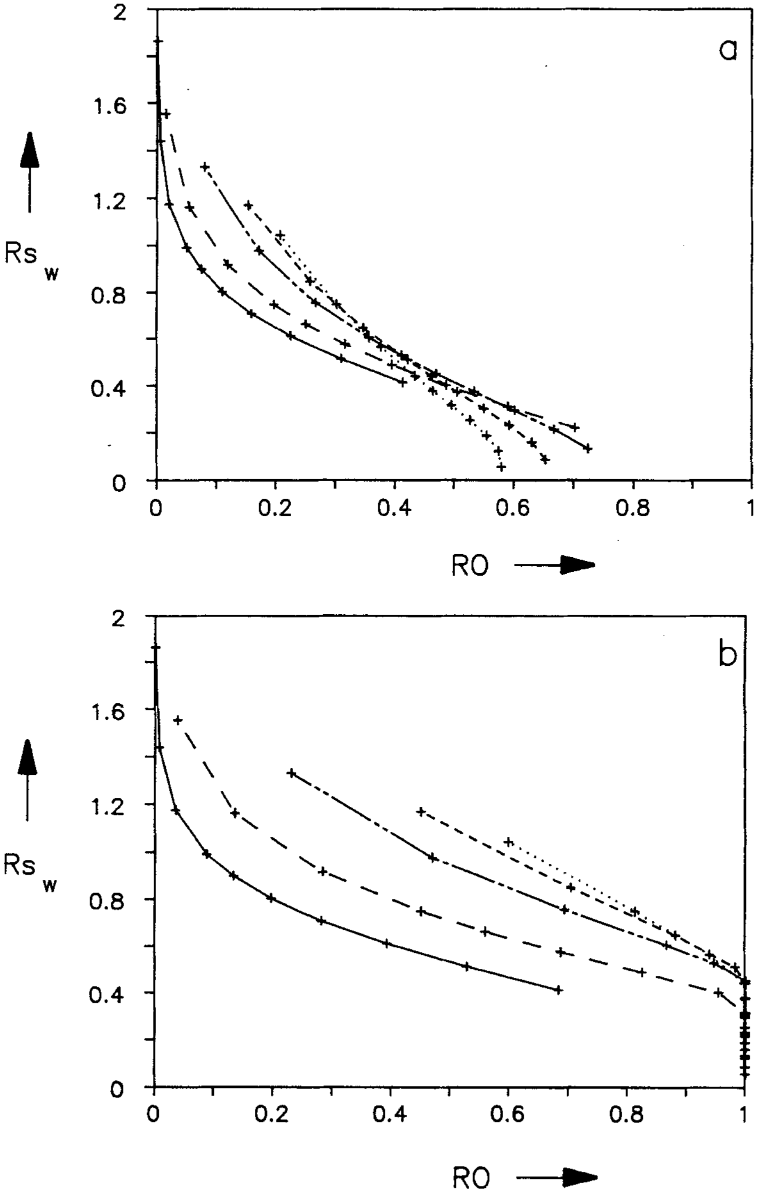

FIGURE 6
The relation between the resolution Rs_w and the relative overlap RO of the second peak. Time constants according to figure 5.
a) peaks with equal area
b) peaks with 4:1 area ratio

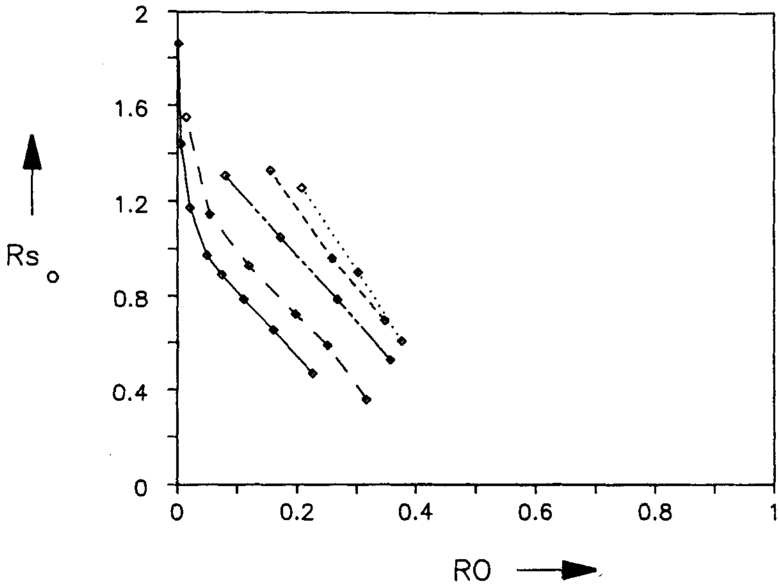

FIGURE 7
The relation between the resolution Rs_o and the relative overlap RO of the second peak. Time constants according to figure 5. Peaks of equal area.

3. Resolution Based on the Moments Rs_M

A substantial improvement with respect to the afore mentioned problems can be observed in figure 8a, describing the relation between Rs_M (the resolution based on the first and second moments of the individual peaks) and the relative overlap. Especially the range of moderate overlap (RO < 0.2) is defined much better, resulting in a one to one relation between resolution and overlap, even in the case of asymmetric peaks (τ = 1-2). This result can be explained by the fact that the complete

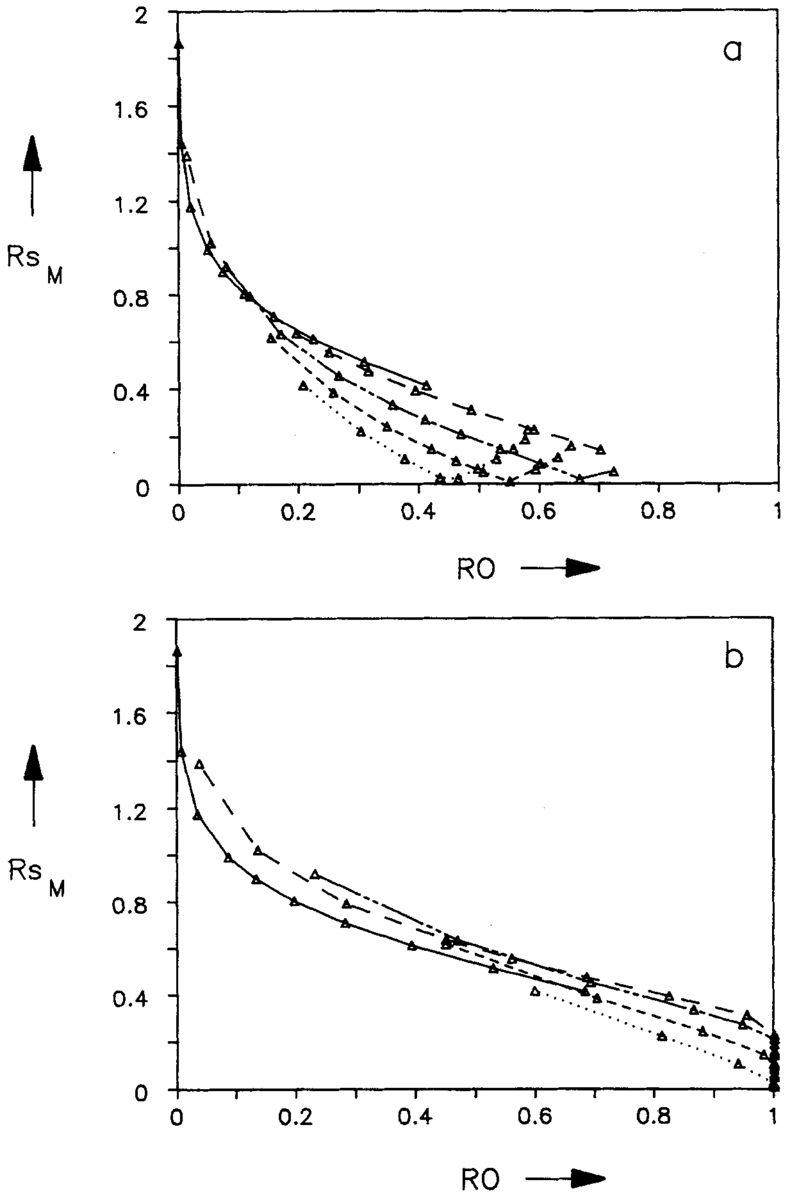

FIGURE 8
The relation between the resolution Rs_M and the relative overlap RO of the second peak. Time constants according to figure 5.
a) peaks with equal area
b) peaks with 4:1 area ratio

peak-shape is now utilized in the calculation, including the tail, hence a more representative number for the separation is derived.

In the case of a more severe overlap, the spread in the possible resolution values again increases. The minimum in the relation is caused by the fact that the absolute values of the resolution are plotted: when the asymmetry of the first peak increases, the first moment of this peak shifts to the right. If the difference in symmetry becomes very large and the peaks are close together, the first moment of the first peak becomes larger than the first moment of the second peak and the denominator of equation (6) becomes negative.

This problem is not apparent in figure 8b, relating Rs_M to RO for the 4:1 area-ratio, because the overlap is 1 for all these cases and the corresponding points are consequently situated on the rightmost axis. The resulting figure shows a good relationship between resolution and overlap, although a certain spread of approximately 0.2 in overlap can still be observed, mainly because the slope of the relation is rather shallow. Although the calculation of the Rs_M is more complex, the resulting value gives a better presentation of the separation than expressions based on the peak width. Again it must be added that in order to interpret the resolution correctly with respect to overlap, the area ratio must be taken into account. This becomes apparent when figure 8a is compared with figure 8b.

4. The Discrimination Factor d_0

The spread which is observed in the plot related to the discrimination factor (figure 9a) is somewhat larger in the area

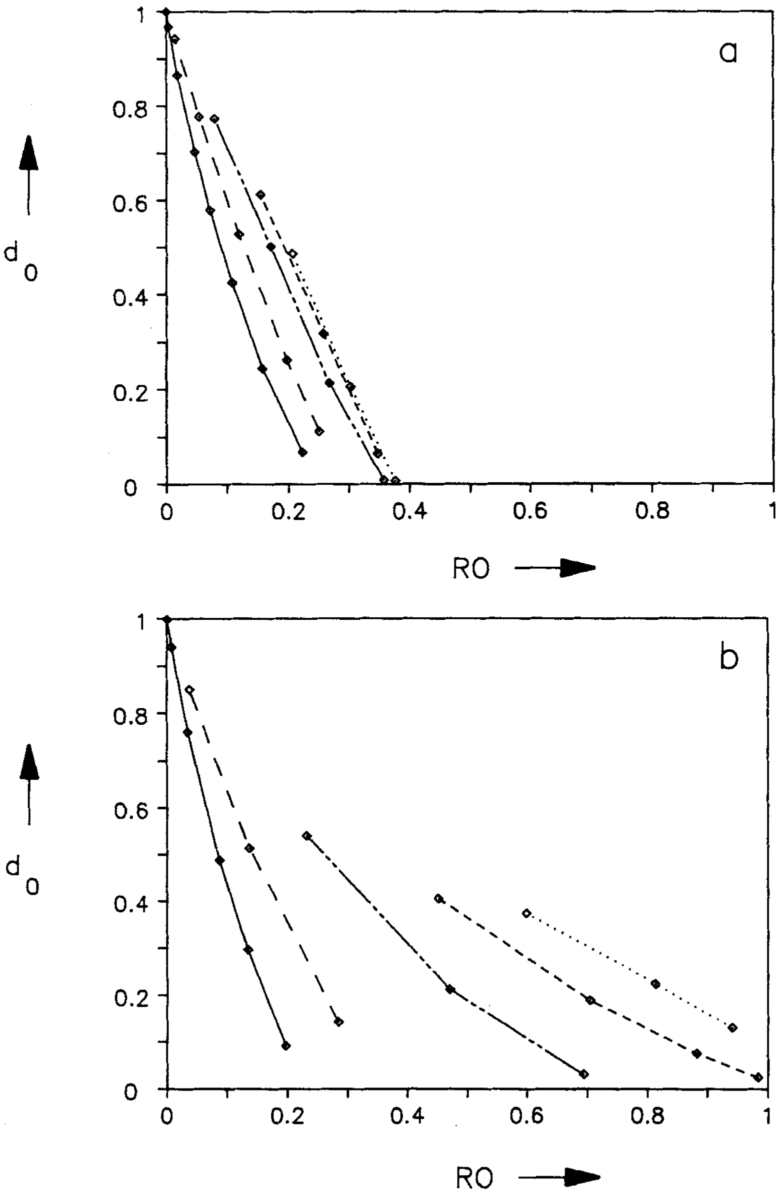

FIGURE 9
The *relation* between the discrimination factor d_0 and the relative overlap RO of the second peak. Time constants according to figure 5.

a) peaks with equal area

b) peaks with 4:1 area ratio

of moderate overlap than in the plot related to Rs_M (figure 8a). What is more apparent, however, is the limited applicability of the factor, because of the disappearance of the valley at higher RO.

This is less obvious in the figure related to the 4:1 area-ratio (figure 9b): in the case of tailing peaks, the overlap is more or less complete before the valey disappears. Consequently, for small peaks following large tailing peaks, there is little relation between de discrimination factor and the overlap, which is also observed in the spread in figure 13.

Noteworthy is the similarity between the two lines in figures 9a and 9b related to the symmetrical peaks, which are almost identical. As explained in the literature (8) the discrimination factor is less dependent on the peak ratio. This similarity is observed here because the value of the factor is always determined for the smaller of the two peaks, and as such comparable with the relative overlap in these plots. Consequently, if we compare the plots of the relative overlap with respect to the <u>larger</u> peak, we would see a marked difference. However, in the case of asymmetric peaks, this similarity is no longer apparent because of the above mentioned lack of correlation between overlap and valley-to-peak ratio.

5. The Adjusted Resolution Rs_a

Figure 10a displays the results with respect to the adjusted resolution Rs_a. This expression shows less divergence than both Rs_N and Rs_W. The divergence is somewhat worse in the area of moderate overlap when compared with the Rs_M but gives a better

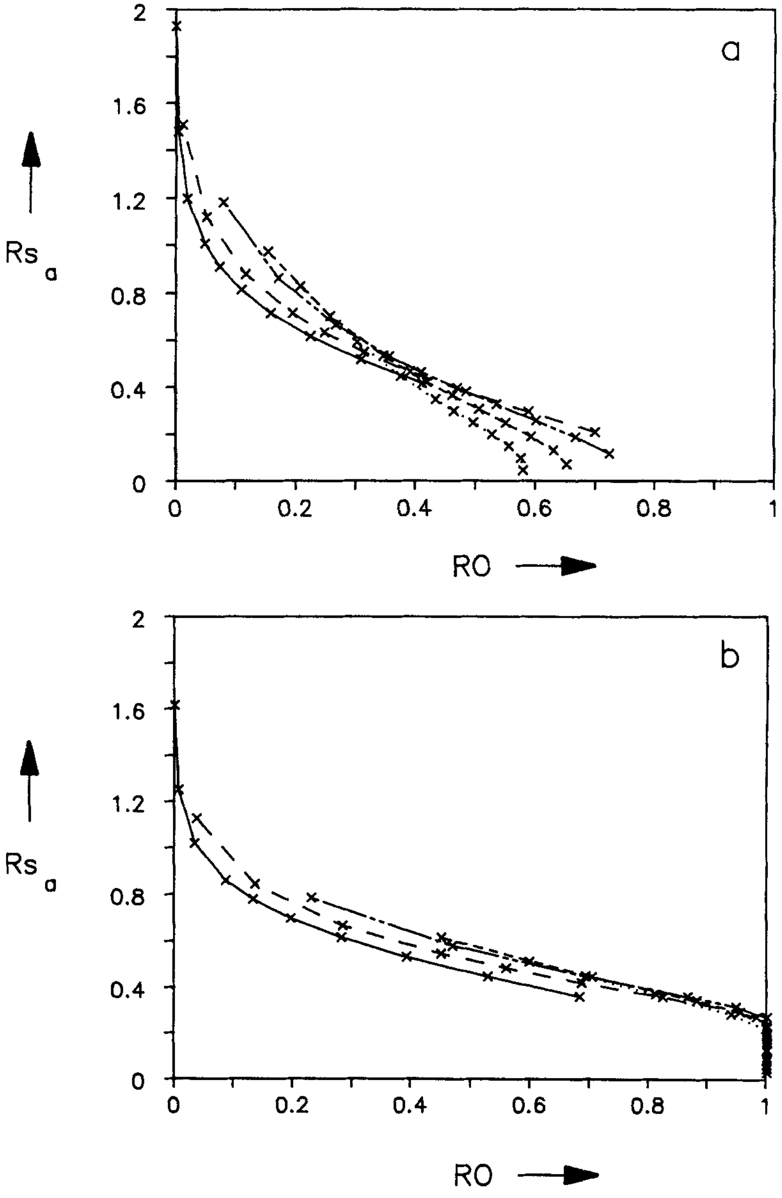

FIGURE 10
The relation between the resolution Rs_a and the relative overlap RO of the second peak. Time constants according to figure 5.
a) peaks of equal area
b) peaks of 4:1 area ratio

indication on the amount of separation if the overlap is larger (0.4-0.7).

In the case of the 4:1 area ratio very good results are obtained (figure 10b). Since the width of the large peak is determined with respect to the small peak, there is a close correlation between the calculated resolution and the relative overlap. This is especially clear when the area of strong overlap (0.8-1.0) is compared in the figures 8b and 10b.

As the expression of Rs_a is specifically derived for the small peak, the results in the figures 10a and 10b can be compared. In the area of moderate overlap, the adjusted resolution displays similar behaviour, independent of the area ratio. For higher overlap, however, the Rs_a values derived for peaks of equal area are located below the values derived for the 4:1 area ratio. The results in figure 10b show less divergence than the corresponding ones in figure 10a: when the width of the large peak is determined closer to the baseline, as is the case with a small peak following a large one, the tail of this large peak will be better defined and hence the resolution will show a better correlation with the overlap.

CONCLUSIONS

A comparison of different definitions of chromatographic resolution has shown that more classical ways to express separation by means of resolution do not give a true measure of the overlap in cases of asymmetry and/or varying peak heights.

When the peaks have different heights, the resolution based on a fixed plate count, Rs_N, does not reflect to the true overlap. As a result of the independent treatment of the two peaks in the calculation of the moments, the Rs_M suffers the same drawback. Definitions which consider the peaks with respect to each other, such as the discrimination factor d_0 and the adjusted resolution Rs_a, perform much better.

Resolution based on a fixed plate count also fails when the first peak is more or less asymmetric in comparison with the second one. When the individual peakwidths are taken into account (Rs_w) some improvement is observed because the deviating width of the asymmetric peak is now introduced into the equation. However, this advantage is limited even further when the individual peak widths must be derived from a combined elution profile (Rs_o). A well defined resolution is only possible when the tail of an asymmetric peak is defined more accurately, either by a complete statistical evaluation of the profiles (as is the case with Rs_M), or by determining the width of the peak at a relatively low level of the peak height (such as the 13.5% level used to determine Rs_a). Equations using a peak-to-valley ratio such as d_0 are less capable of observing the asymmetry, especially in the case of varying peak-heights.

Expressions like d_0 and Rs_M need the combined c.q. separate chromatographic profiles, consequently their values are difficult or impossible to predict and as such less suitable for interpretive optimization strategies. In principle the adjusted resolution also requires individual profiles to determine the required peak widths, but some simplifications (e.g. invariant asymmetry) enable a predictive approach (8).

The above conclusions indicate that the practical approach of the adjusted resolution offers the best compromise between a reasonable description of the separation, also for peak pairs with varying asymmetries and peak heights, and a relative ease of calculation.

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