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ORIGINAL

Peak Fraction Purity and Chromatographic Resolution: Gaussian Peaks Revisited

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Abstract

For incompletely resolved peak pairs, the purity of the chromatographic or fractographic fractions is oftentimes underestimated by the common user. This results in wasted time and effort while trying to achieve higher resolution than needed for the intended use. While a choice regarding acceptable fraction purity is ultimately up to the user and will be dictated by the purpose for which the separation is being conducted, knowledge of fraction purity as a function of chromatographic resolution R_s can help make an informed decision in this regard. To this effect, we revisit here the relationship between peak fraction purity and R_s for pairs of Gaussian peaks, equal pairs ranging in R_s from 0.42 to 1.68 and unequal pairs of various analyte ratios and R_s values. Employing sophisticated yet highly accessible commercial software, we calculate, to a greater precision than previously reported, the purity resultant from midpoint or valley cuts of peak pairs, and also show the improvement gained from performing these cuts at either the maxima of the cumulative peak or at the locations in this peak corresponding to the centers of gravity of the individual component peaks. The methodology employed and equations given are applicable to R_s values other than those investigated here and can be employed to calculate cut-point estimates for virtually any arbitrary desired purity.

Keywords Chromatographic resolution · Peak fraction purity · Gaussian peaks · Computer simulations

Introduction

Along with plate number and plate height, chromatographic resolution R_s is one of the key metrics traditionally employed to describe the performance of chromatographic columns in particular, and of separation systems in general. Oftentimes, the goal of an experiment is to fit in a chromatogram (or fractogram, henceforth implied when discussing separations) as many well-resolved peaks as possible, "well-resolved" being understood as each peak pair having an R_s of a particularly agreed upon value [1, 2]. For example, the term "critical resolution" is used to denote separations in which the least resolved pair, *i.e.,* the "critical pair," has $R_s \geq 2.0$, meaning that all other peaks in the chromatogram will be resolved at least equally well from one another as is this pair [3]. Other times, the desire might be for only one particular pair of peaks to be well resolved. Yet another goal might be to calculate the "true" area of incompletely resolved peaks. This last point has received much attention over the decades (see, *e.g.* [4–8],), as summarized by Meyer [9], who has also pointed out that these studies have generally not attracted the attention of the greater separations community. For preparative fractionations, inter

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alia, an additional intent can be identified, namely to separate peaks from each other with a resolution sufficient so that a "cut" from each peak is pure enough ("fit-for-purpose") for subsequent analysis or end-use.

The subject of peak fraction purity was initially broached by Glueckauf [10], who introduced expressions for estimating the equal-purity cut-off point (*i.e.,* the point at which peak fractions on either side have the same purity as each other) for both equal and unequal peak pairs. Over three decades later, an approximate expression relating the sensitivity of the impurity fraction in a given peak to the retention volume at which a cut is made was given by Karol [11]. A rapid (for its time) approach to estimating initial sample resolution was provided by Snyder in 1972 [12], via a graphical approach combined with numerical estimates.

Here, we revisit the relation between R_s and peak fraction purity for pairs of Gaussian peaks. Our approach is more reminiscent of Snyder's than of either Glueckauf's or Karol's and is aided by modern, generally available peak fitting and integration software, and thus easily implementable in most laboratories. Results include the purity of fractions collected by center- or valley-point cut-offs for both equal and unequal peak pairs and also by peak maxima and center-of-gravity cut-offs for both types of pairs. In all cases, the purity of the fractions, which is generally underestimated by the everyday chromatography user, has been determined to a greater precision than previously reported. We hope the results presented herein provide users with some intuitive feel as to the purity of peak fractions for a given R_s as well as with an approach to determining said purity in their own studies.

Experimental

All graphing and calculations were conducted using the Peak Analyzer and Integrate functions in OriginPro 2021b (OriginLab, Northampton, MA). This software will fit data points to a Gaussian; as such, "seed" data such as the *x,y*-coordinates of a chromatogram, are needed to create Gaussian curves in OriginPro. Here, Gaussian peaks were based on an at least 100 data point fit of the size-exclusion chromatogram, obtained using a differential refractometer, of a non-mutarotating methyl-α-D-mannopyranoside in aqueous solution [13]. The choice of size-exclusion data is arbitrary, as essentially any chromatogram (RP, NP, HILIC, etc.) would do. The fits followed the relation:

$$y = y_0 + \frac{S \exp\left[\frac{-4\ln(2)\left(x-x_c\right)^2}{w^2}\right]}{w\sqrt{\frac{\pi}{4\ln(2)}}}, \tag{1}$$

where y_0 is the *y*-offset (zero in all cases here), x_c and y_c are the *x*, *y* coordinates of the peak center of gravity, w is the peak width (full width at half maximum of peak height, *i.e.,* $w = (y_c - y_0)/2$), and S is the peak area.

Results using the Gaussian fit from OriginPro described above were checked employing Gaussian peaks, of similar mean and standard deviation as those in OriginPro, created using the normal distribution function NORMDIST in Excel. The data for these Excel peaks were then imported into the OriginPro Peak Analyzer. To the level of precision reported here, there was no difference in results when using Gaussian peaks created in Excel using NORMDIST versus those created by fitting experimental data in OriginPro.

Chromatographic resolution R_s was calculated by

$$R_s = \frac{2\left(x_{c,B} - x_{c,A}\right)}{w_A + w_B}, \tag{2}$$

where the subscripts *A* and *B* represent the first (earlier-eluting) and second (later-eluting) peaks, respectively, in each pair (these will be referred to below as "Peak A" and "Peak B"). No allowance was made for differences in band broadening as a function of retention time, as this was not deemed germane to the present discussion. The center of gravity (first zero-point moment) of each peak was determined through:

$$m_1' = \frac{1}{m_0}\int_0^\infty F(x)x\,dx, \tag{3}$$

where m_0 is the zeroth moment (area) of the peak, defined as

$$m_0 = \int_0^\infty F(x)\,dx. \tag{4}$$

Results and Discussion

Figure 1 shows the cumulative fits of thirteen peak pairs, ranging in R_s from 0.42 to 1.68. In all cases, both peaks in each pair are present in a 1:1 ("1/1") ratio, *i.e.,* they constitute equal peak pairs, and detector response is assumed to be the same for both peaks. This latter assumption is maintained through all examples given here, for both equal and unequal pairs. The cut-off point in each case is denoted by an arrow; this is the reference point for peak purity: The percent purity of Peak A is defined as the percentage of analyte A relative to that of analyte B contained in the portion of the cumulative fit ending at the cut-off point (*i.e.,* contained in the section of the cumulative fit to the left of the cut-off point), the percent purity of Peak B as the percentage of analyte B relative to that of analyte A contained in the portion of the cumulative fit starting at the cut-off

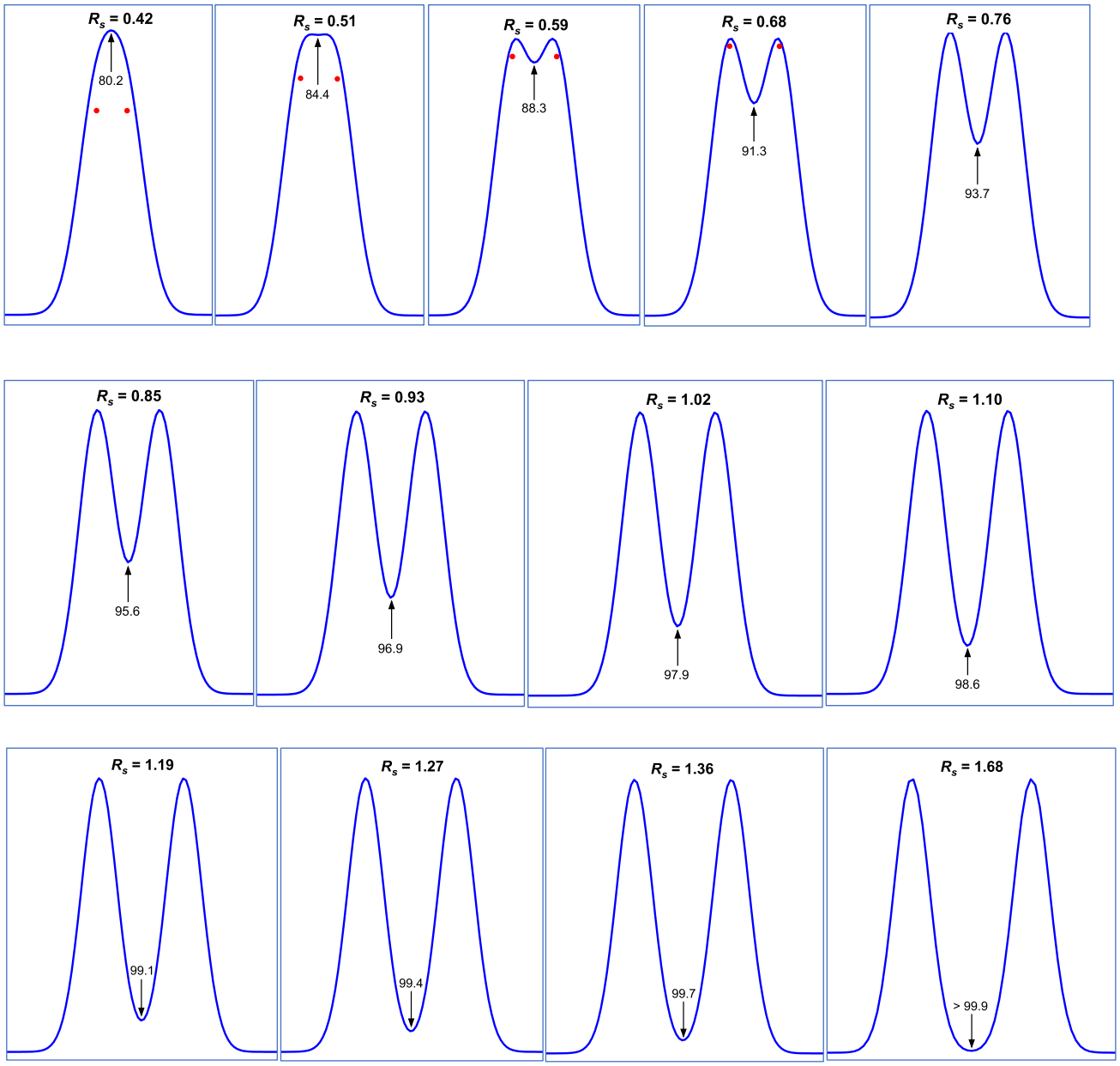

Fig. 1 Percent purity of peak fractions, at thirteen different values of R_s, for pairs of Gaussian peaks with a 1:1 ratio, assuming equal detector response for both analytes. Red dots denote center of gravity of individual peak components, shown where visually distinguishable from peak maximum or maxima. Arrows denote location of cut producing fractions of equal percent purity, *i.e.,* relative percentage of analyte A with respect to that of analyte B in left-hand side of cut is equal to relative percentage of analyte B with respect that of analyte A in right-hand side of cut. These relative percentages are given either above or below the arrows

point (contained in the section of the cumulative fit to the right of the cut-off point). Red dots denote the centers of gravity of each component; these are only shown when they are visually distinguishable from the peak maximum or maxima (*i.e.,* when the difference between the maximum and center of gravity of a peak is greater than the size of the red dot) in the examples given. In the figure, the existence of two individual peaks does not become obvious until $R_s = 0.59$.

The numbers either below or above the arrows in Fig. 1 denote the percent purity of each peak fraction. These were obtained using Eq. (5):

$$\%\text{Purity} = \left(\frac{I_{A,CumMin}}{I_0}\right) \times 100\% = 100\% - \left(\frac{I_{B,CumMin}}{I_0}\right) \times 100\%, \tag{5}$$

where $I_{A,CumMin}$ and $I_{B,CumMin}$ are the values of the integrates (areas under the peaks at given x-axis values) of Peaks A

and B, respectively, at x-axis values corresponding to that of the valley minimum of the cumulative peak fit (when two peaks can be distinguished in the cumulative fit) or to that of the cumulative peak center (when two peaks cannot be distinguished); and I_0 is the maximum of the peak integrates. Because we are dealing with equal peak pairs (*i.e.*, the peaks in each pair are present in a 1:1 ratio), $I_0 = I_{A,0} = I_{B,0} = (I_{A,CumMin} + I_{B,CumMin})$. As shall be seen below, these equalities will not hold when dealing with an unequal peak pair. The various terms in Eq. (5) are shown graphically in Fig. 2a, where it is also seen that a perpendicular from the baseline passing through the cumulative minimum also passes through the point of intersection of the individual peaks (this latter point is shown enclosed within a black box in the figure).

Because these are equal peak pairs (and remembering the assumption of identical detector response for both components), both purity percentages are equal. For example, at the lowest resolution plotted, $R_s = 0.42$, where Peaks A and B coalesce into a single, broad peak, if fractions of this latter peak were collected individually to the left and right of the cumulative peak center, each fraction would be 80.2% pure in its respective component. At a slightly higher R_s of 0.51, where a "dimple" is observable at the coalesced peak center, the left and right fractions are each now 84.4% pure. By an R_s of 0.68, each fraction is > 90% pure and by an R_s of 0.76 the centers of gravity of the component peaks are visually indistinguishable from the maxima in the cumulative fit. By an R_s of 1.19, each fraction is 99% pure. By an R_s of 1.68, fraction purity exceeds 99.9%.

Shown in Fig. 3 are the cumulative fits for unequal peak pairs, with peak ratios of 2/1, 4/1, and 8/1, each at R_s values of 0.76, 1.02, and 1.19. Peak purities in these cases were calculated using Eqs. (6) and (7) for the left-hand side of the cut-point ("Peak A") and the right-hand side ("Peak B"), respectively:

$$(\%\text{Purity})_{PeakA} = \left(\frac{I_{A,CumMin}}{I_{A,CumMin} + I_{B,CumMin}} \right) \times 100\% \tag{6}$$

$$(\%\ \text{Purity})_{PeakB} = \left[\frac{I_{B,0} - I_{B,CumMin}}{(I_{B,0} - I_{B,CumMin}) + (I_{A,0} - I_{A,CumMin})} \right] \times 100\%. \tag{7}$$

The various terms in Eqs. (6) and (7) are shown graphically in Fig. 2b. Because we are now dealing with unequal peak pairs, $I_{A,0} \neq I_{B,0}$, unlike the equal peak pair cases. Equations (6) and (7) both reduce to Eq. (5) in the case of an equal peak pair.

Equations (5) through (7), all of which are for cut-off points located at the cumulative peak minimum ("*CumMin*" in Fig. 2a, b), are special cases of the more general Eqs. (8) and (9), which serve to calculate peak purities at any arbitrarily located cut-off point x:

$$(\%\text{Purity})_{PeakA_x} = \left(\frac{I_{A_x}}{I_{A_x} + I_{B_x}} \right) \times 100\%, \tag{8}$$

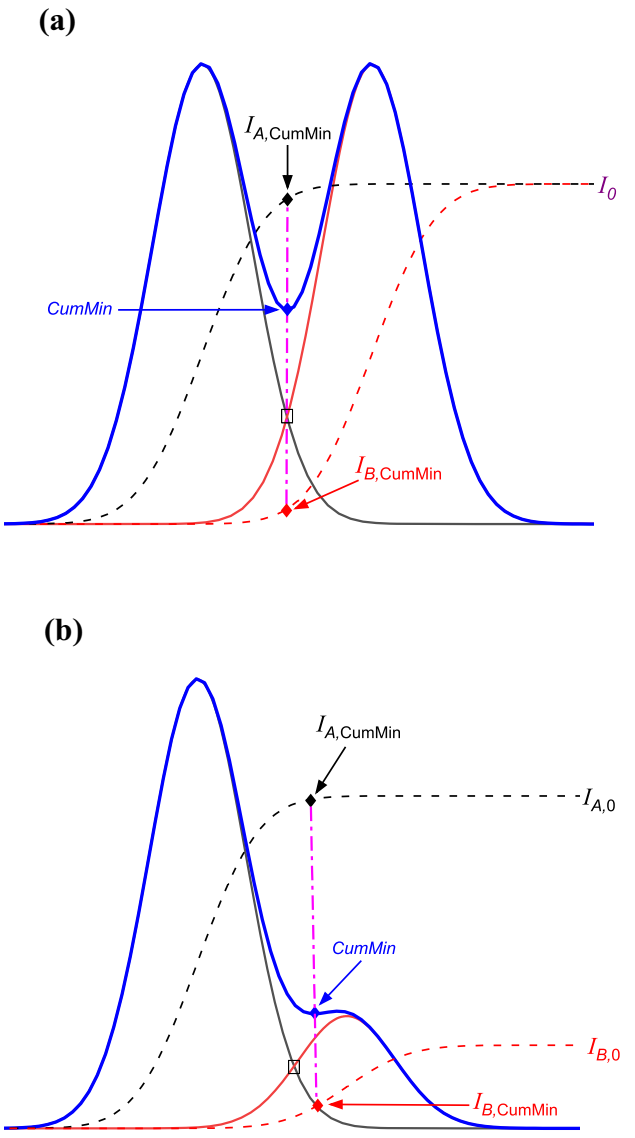

Fig. 2 Parameters related to calculation of percent purity in text. Blue solid curve is cumulative fit, black and red solid curves are the individual component peaks (Peak A and Peak B, respectively), dashed lines are integrates of the respectively-colored peaks. **a** Equal peak pair (1:1 ratio), with $R_s = 0.85$; here, $I_0 = I_{A,0} = I_{B,0} = I_{A,CumMin} + I_{B,CumMin}$. **b** Unequal peak pair (4:1 ratio), with $R_s = 0.76$. In both (**a**) and **b**, a black box has been placed around the point of intersection of Peaks A and B as a visual aid

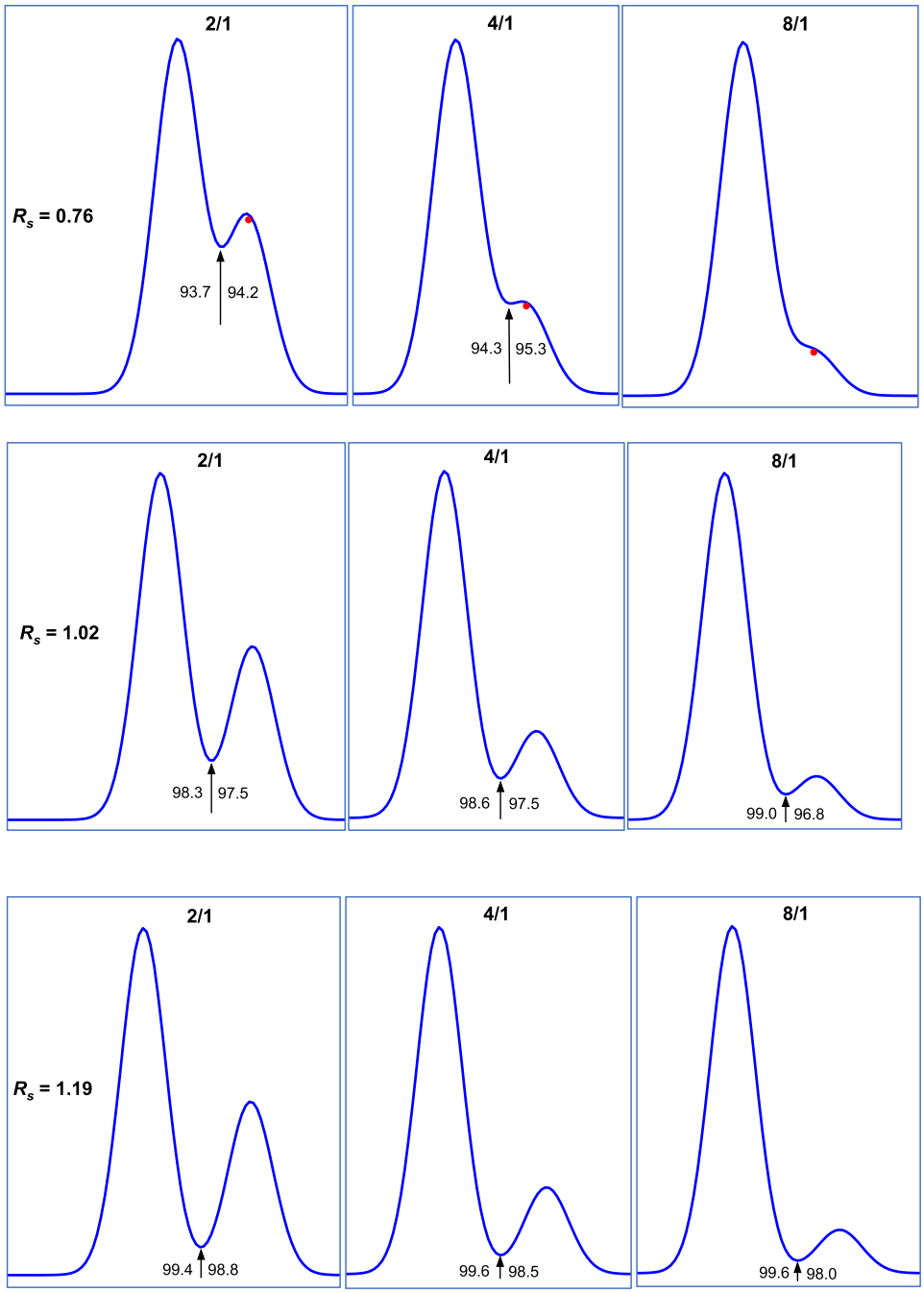

Fig. 3 Percent purity of peak fractions for unequal peak pairs with 2/1, 4/1, and 8/1 ratios of A/B. Top row: $R_s = 0.76$; middle row: $R_s = 1.02$; bottom row: $R_s = 1.19$. Red dots denote center of gravity of Peak B, where visually distinguishable from respective peak maximum in cumulative fit; there is no observable distinction between center of gravity of Peak A and respective peak maximum in cumulative fit in the cases shown. Arrows denote location of cut point, placed at valley minimum of cumulative fit

$$(\%\text{Purity})_{PeakB_x} = \left[\frac{I_{B,0} - I_{B_x}}{(I_{B,0} - I_{B_x}) + (I_{A,0} - I_{A_x})}\right] \times 100\%. \tag{9}$$

In the cases in Fig. 3, the fraction to the left of the cut-off point was enriched in analyte A, *i.e.,* the percent purity of Peak A was greater in the unequal than in the corresponding equal peak pairs of the same R_s shown in Fig. 1. (For $R_s = 0.76$ at a 2/1 ratio, the percent purity of Peak A in the equal and unequal cases is the same to the precision given). This, of course, results from the smaller relative amount of analyte B present in the unequal peak pairs as compared to their equal counterparts. This percent purity either increased or, within the precision of the calculations, remained identical as a function of increased ratio of analyte A to analyte B, reflecting the predominance of the former over the latter in the mix.

At the right-hand side of the cut-off point, the percent purity of Peak B follows less obvious trends. At $R_s = 0.76$, the right-hand side of the cumulative fit in the 2/1 case in Fig. 3 is enriched in analyte B as compared to the 1/1 case shown in Fig. 1. This remains so for the 4/1 case at the same

R_s, where an even further enrichment is observed (no results are shown for the 8/1 case, as the location of a cut-off point would be highly arbitrary here due to the lack of a valley in the cumulative fit combined with the unequal nature of the peak pair). The reason for these enrichments is that the distribution of analyte B has now been pushed farther to the right in the unequal peak pairs versus the equal peak pair, as evidenced by the fact that the center of gravity of Peak B is now visually distinguishable from its respective peak maximum in the cumulative fit (as in Fig. 1, centers of gravity are denoted as red dots in Fig. 3 and shown only when visually distinguishable from the respective peak maximum). By an R_s of 1.02, there is no discernible difference between the center of mass of Peak B and the maximum of its respective component peak in the cumulative fit. As a result, the right-hand side of the cut-off in the cumulative fit is no longer enriched in analyte A at $R_s = 1.02$ or 1.19 when comparing the unequal peak pairs to the equal ones at the same resolution. Also, because the centers of mass of both component peaks remain identical to their respective component peak maxima in the cumulative fit, the percent purity of Peak B tends to decrease as a result of increased A-to-B ratio in the mix.

Thus far, we have only examined the percent purity obtained as a result of collecting fractions to each side of a single, valley-located (or midpoint-located, where no observable valley is present) cut-off point. For peak pairs that possess a valley, two other obvious cut-off points exist, namely the peak maxima. Rather than indulging in the same exercise as above for all the R_s cases previously examined, we report here representative results for one equal and one unequal peak pair. These are shown graphically in Fig. 4a–d, respectively, at an R_s of 0.59 in the former and an R_s of 0.76 and an A-to-B ratio of 4/1 in the latter. In each case, we effect two cuts in the cumulative fit, one at each peak component maximum (the locations of these cuts are shown by the blue arrows in the figures). We are now left with three fractions, one to the left of the cut in the left-component peak (peak corresponding to analyte A, shown as a dashed green peak in the figures), one to the right of the cut in the right-component peak (peak corresponding to analyte B, shown as dashed red peak), and one in between these two cuts (shown as shaded region). For the equal peak pair case depicted, the percent purity of the left- and right-most fractions is 98.7% in its respective component (Fig. 4b), a substantial increase from the 88.3% obtained using a valley-point cut (Fig. 4a and Fig. 1). For the unequal peak pair shown in the figure, the same approach yields a percent purity of 99.9% for Peak A and of 98.4% for Peak B (Fig. 4d), up from 94.3% and 95.3%, respectively, employing a valley-point cut (Fig. 4c and Fig. 3). Naturally, less material is being collected in each relevant fraction when comparing the peak-maximum-cut to the valley-cut approach. It remains to the user to decide whether the trade off in collected material is compensated for by the increased purity of the collected fractions.

Lastly, we examine what happens if the two cut-off points are now placed, not at the component peak maxima, but at the centers of gravity of the two component peaks. The two points are denoted by the green and red arrows in Fig. 4b and d. For the equal peak pair case shown in the figure, the percent purity of the relevant fractions is now 99.1%, a modest (though perhaps important, depending on need) 0.4% gain over the maxima-cut approach. For the unequal peak pair case in the figure, cuts at the centers of gravity increase the percent purity of Peak B to 99.0%, an again relatively modest gain of 0.6% over the maxima-cut approach. There is no difference in the percent purity of Peak A when using the maxima- versus center-of-gravity-cut approaches for the case shown, nor for any of the other cases depicted in Fig. 3, because the center-of-gravity of analyte A's peak is indistinguishable from the maximum of this component's peak in the cumulative fit.

Conclusions

While it is always desirable that all peaks in a chromatogram or fractogram be as well separated from each other as possible, it is not always necessary for this to be so; lower R_s values than those traditionally regarded as optimal, such as those which define "critical resolution," may oftentimes be acceptable. This will depend on the purpose for which the separation is being performed, *e.g.,* to collect fractions of high enough purity for identification and/or subsequent end-use of the individual components. Previous studies in this regard, however, have not attracted the attention of the general separations community. As a result, most users highly underestimate the purity of incompletely resolved fractions, resulting in a waste of time and effort while trying to achieve a higher resolution than required for the intended purpose of a particular separation. Here, we have revisited, expanded, and more precisely shown how the purity of individual fractions in an equal peak pair depends on chromatographic resolution, over an R_s range spanning from 0.42 to 1.68 (always assuming identical detector response for all components), and have also demonstrated this for select unequal peak pairs of varying resolutions and component ratios. The unequal peak pair results also apply to the chromatograms of two analytes present in equal amounts, where the ratios in the figures would then correspond to the relative detector response to the analytes.

When fractions of an equal peak pair are collected to the left and right of a center cut, be it at the peak center for poorly resolved peaks or at the valley for better-resolved ones, fraction purity can be as high as 80% for a low R_s of 0.42 where both component peaks have coalesced into

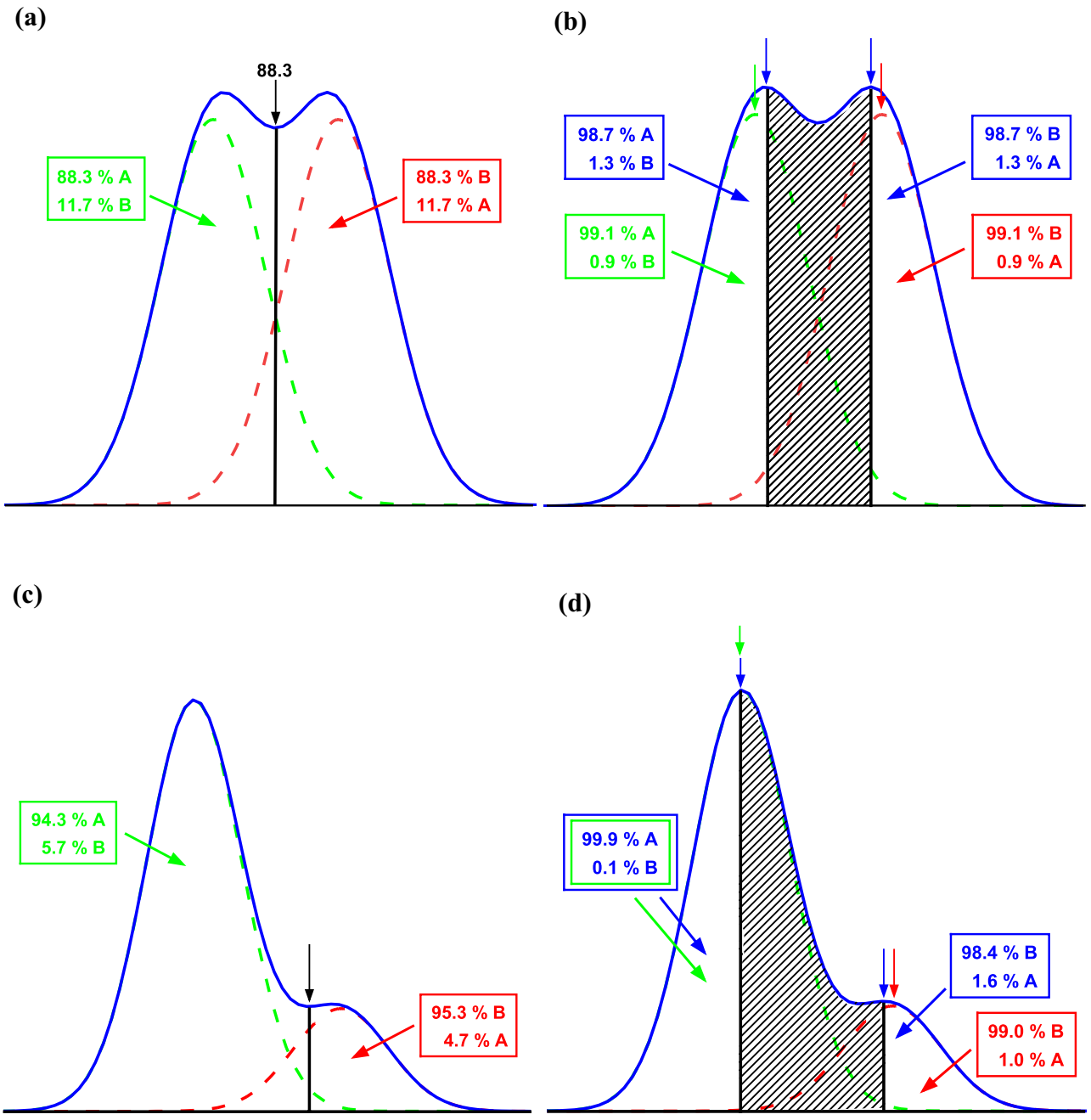

Fig. 4 Percent peak purity when using a valley-cut (black arrows) approach versus a peak-maxima cut (blue arrows) approach. Also shown, with green and red arrows for Peaks A and B, respectively, are the locations for center-of-gravity cuts. **a** and **b** $R_s = 0.69$, A-to-B ratio of 1/1; **c** and **d** $R_s = 0.76$, A-to-B ratio of 4/1

a single, broad peak. By an R_s of 1.10, a fractional purity of 99% is achieved. If a peak fraction purity of greater than 99.9% is necessary, this is found at $R_s = 1.68$. For unequal peak pairs, even at an R_s of 0.76 and a component ratio of 4/1, a valley cut produces fractions of 94% and 95% purity for the respective component fractions.

For both equal and unequal peak pairs, the purity of the collected fractions can be substantially increased by effecting two cuts, rather than a single valley- or center-cut, one at each peak maximum and then collecting the fraction to the left of the left-side cut and to the right of the right-side cut. This approach has been employed in both preparative and recycle chromatography as described in, *e.g.,* sections 15.2 and 15.3 of [14]. If an additional increase in purity is needed, a small gain over the peak-maxima-cut approach can be obtained by making the cuts at the centers of gravity

of the component peaks. For unequal peak pairs, this last strategy works better for the lesser than for the greater component in the pair.

The techniques described herein have been employed in one of our labs (AMS's) to identify the fractional purity of quantum dot peaks when analyzed by size-exclusion chromatography (SEC) with on-line quasi-elastic light scattering (QELS) detection and of gold nanoparticles characterized by both SEC and hydrodynamic chromatography with on-line QELS and inductively coupled plasma mass spectrometry detection [15, 16].

The approach presented can be employed to calculate cut-point estimates for virtually any arbitrary desired purity. Given the relative ease with which these methods can be applied through the use of sophisticated yet highly accessible commercial software packages, we hope the present work assists in disseminating the relation between fractional peak purity and chromatographic resolution throughout the separations community.

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Declarations

Conflict of interest There is no conflict of interest to report.

Ethical Approval This article does not contain any studies with animals or human participants.

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