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Quantitation of unresolved chromatographic peak using overlapping ratio of profiles of standard chromatographic peaks

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Abstract

This paper is intended as an investigation of a new method for extracting information about overlapping ratios from the profile of a standard substance and to sort the unresolved chromatographic peak into individual peaks. Several cases of overlap are tested by using the prepared profile of standard substances. This method has been applied to analyse an actual two-component chromatogram and three-component chromatogram, results showing that the proposed method is capable of accurate quantitation of the unresolved chromatographic peaks.

Keywords: Peak overlap; Hydroxyindoleacetic acid; Deoxyepinephrine; Dihydroxyhydrocinnamic acid

1. Introduction

For the quantitation of a multi-component peak, much work on improvement has been reported. Extracting accurate information from real chromatographic peaks has been challenging research.

Progress has been made on the ratio technique with the original concept of Hirschfeld [1]. Koenig et al. used the ratio of sequential spectra to determine scale factors for subtracting reference spectra from mixture spectra, and to estimate constituent spectra [2]. Synovec and co-workers reported a technique for data analysis in chromatography, based on taking the point-by-point ratio of a sequential chromatogram [3], and studied the sequential chromatogram ratio technique to characterize and correct for retention time variation and peak shape change [4].

Jeasonne and Foley proposed a method based on the exponentially modified Gaussian technique for the calculation of statistical moments via the measurement of peak width, asymmetry and peak height, which was applicable to the calculation of moments of overlapped peak pairs [5]. However, it is difficult to resolve more than two overlapped peak pairs. Later, progress was made in the resolution of a chromatogram containing three overlapped peaks [6]. A method of heuristically evolving latent projections was described using the detection and resolution of two-component mixtures of drug isomers [7], and for quantitation and error avoidance in the resolution of chromatograms of closely eluting peaks [8].

Wu and Gu employed a method based on the ratio of two apparent heights and an empirical correction factor for quantitation of peak areas of severely overlapped pairs [9]. Lin and Lu have improved the perpendicular drop method by calculating correction factors which are based on an exponentially modified Gaussian model [10]. Both methods require a valley between the peaks and are difficult to apply to closely eluting peaks.

With the development of multi-wavelength spectra, the techniques for resolution of several over-

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lapped peaks by the least-squares method was reported [11–14]. The results were independent of degree of overlap, peak shape or retention time, but were sensitive to a high degree of spectral similarity among overlap components.

The relative advantages of several types of resolution methods have been reported, such as the orthogonal polynomial analysis of chromatogram segments [15,16], the factor analysis method [17,18], an inverse diffusion model [19], curve fitting method [20], and a straightforward method for resolving chromatograms [21]. The reported methods have optimal applications.

In this study, the ideal method is different from previously reported methods. I extract overlapped information from the chromatographic peaks of standard substances. The method can produce simple and accurate quantitative results. This paper reports the theory, resolution process, discussion of overlapping conditions, and application to resolve chromatographic peaks containing two and three known components.

2. Theory

Fig. 1 depicts a model that contains two isolated chromatographic profiles and an unresolved chromatographic profile described using the following equations. Peaks A and B are actual chromatographic profiles obtained by electrochemical detection. The overlapping peak is an artificial profile manufactured by peaks A and B.

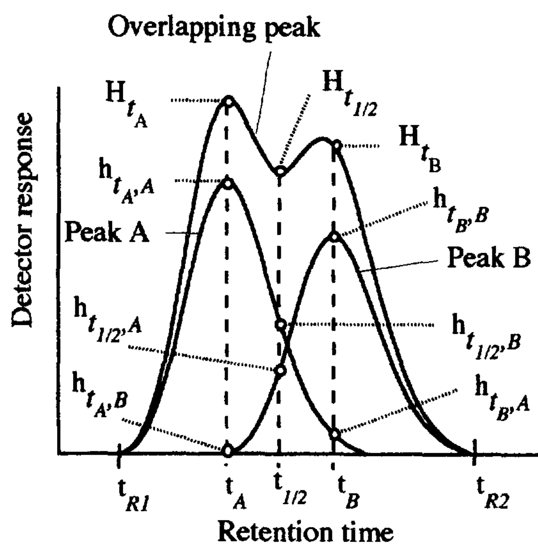


Fig. 1. Peak profile model.

An unresolved chromatographic peak includes two or more components. At a retention time t, detector response H_t is expressed as the sum of responses over all individual component (n):

$$H_t = \sum_{n=1}^{k} h_{t,n} \qquad (n = 1,2,...,k) \tag{1}$$

where $h_{t,n}$ is a response to a component. The response ratio of a component in the total response, $R_{t,n}$, is defined by

$$R_{t,n} = \frac{h_{t,n}}{H_t} \times 100 = \frac{h_{t,n}}{\sum_{n=1}^{k} h_{t,n}} \times 100 \tag{2}$$

The sum of all ratios is:

$$1 = \sum_{n=1}^{k} R_{t,n} \tag{3}$$

A component response may be calculated from multi-component response by the ratio $R_{t,n}$ as follow:

$$h_{t,n} = H_t R_{t,n} \tag{4}$$

A component area, C_n, can be calculated from a multi-component area if every $R_{t,n}$ in the peak elution region is known. The mathematical calculation area is given by

$$C_n = \int_{t_{R1}}^{t_{R2}} [(H_t R_{t,n}) + E_{t,n}]\, dt \tag{5}$$

where $E_{t,n}$ is error due to noise, t_{R1} and t_{R2} are a peak elution region. In this study, in order to investigate the proposed method, a higher concentration sample was used, so the $E_{t,n}$ value was seen as zero. Eq. (5) was performed as the following approximation:

$$C_n = \int_{t_{R1}}^{t_{R2}} (H_t R_{t,n})\, dt \tag{6}$$

3. Method

I prepared a peak A profile and a peak B profile by respectively injecting the solution of standards A and B. Although the prepared peaks are higher (or lower) than the original overlapping peak, we can reduce (or magnify) those prepared peaks to be equal with overlapping peaks by using a magnification factor (F).

For a peak with greater than two-components ($n=1$ to k), at time t, the overlapping-ratio may be calculated by:

$$R_{t,1} = 1-\sum_{n=2}^{k} R_{t,n}$$

$$R_{t,2} = 1-\left(\sum_{n=3}^{k} R_{t,n} + R_{t,1}\right)$$

$$R_{t,3} = 1-\left(\sum_{n=4}^{k} R_{t,n} + \sum_{n=1}^{2} R_{t,n}\right)$$

$$\vdots$$

$$R_{t,k} = 1-\left(\sum_{n=1}^{k-1} R_{t,n}\right)$$

At the centre between the vertices of an overlapping-peak pair, time t is shown as $t_{1/2}$ (See Fig. 1), and the relationship of the ratio between peaks A and B is:

$$R_{t_{1/2},A} = 1 - R_{t_{1/2},B}$$

For resolving a two-component overlapping peak (see Fig. 1), I use a factor to magnify $h_{t_{1/2},A}$ and $h_{t_{1/2},B}$ of profiles of standard solutions, and then calculate $h_{t_A,A}$ $h_{t_A,B}$ $h_{t_B,A}$ and $h_{t_B,B}$ by using those magnified $h_{t_{1/2},A}$ and $h_{t_{1/2},B}$. The operations are performed repeatedly until three position (t_A, t_B and $t_{1/2}$) offer Eqs. 7a, b and c. New $h_{t_{1/2},A}$ *and* $h_{t_{1/2},B}$ are obtained, and are named $h'_{t_{1/2},A}$ and $h'_{t_{1/2},B}$. Computating $h_{t,A}$ by using $h'_{t_{1/2},A}$ gives $h'_{t,A}$, computating $h_{t,B}$ by using $h'_{t_{1/2},B}$ gives $h'_{t,B}$, over t_{R1} to t_{R2}, and two new profiles are manufactured. Using those new profiles to calculate the overlapping ratio by Eq. (2), the ratios thus obtained are used to resolve the overlapping peaks by Eq. (4), and then integration is performed by Eq. (6).

$$h_{t_{1/2},A} + h_{t_{1/2},B} = H_{t_{1/2}} \tag{7a}$$

$$h_{t_A,A} + h_{t_A,B} = H_{t_A} \tag{7b}$$

$$h_{t_B,A} + h_{t_B,B} = H_{t_B} \tag{7c}$$

A simplified algorithm is presented below:

1. Read eluted-profile (H_t) at every time point from multiple-component chromatogram, and list those values in the order of t_{R1} to t_{R2} using Microsoft Excel.
2. Prepare profile of standard chromatogram by injecting standard solution. Read eluted-profile (h_t) at every time point from the prepared chromatogram, and list those values in the order of t_{R1} to t_{R2} using Microsoft Excel.
3. Align multiple-component chromatographic peak with the prepared chromatographic peak of standard solution.
4. Magnify (or reduce) the prepared profile of standard solution to complete 7a, b and c.
5. Manufacture new elution profiles $h'_{t,A}$ and $h'_{t,B}$ ($t = t_{R1} \sim t_{R2}$).
6. Resolve multi-component chromatographic profile and calculate peak-area.

The accuracy, expressed as the relative error (%ER), was calculated according to the following equation:

$$\%ER = \left(\frac{C_n}{C_{STD}} - 1\right) \times 100$$

where C_n is the mathematical area measured by the proposed method and C_{STD} is the original area from the peak of the standard.

Computation studies on the magnification factor, the retention time, the ratio of peak-height and the overlapping-ratio have been done in order to avoid quantitative error.

4. Experiment

The tested materials, 5-hydroxyindole-3-acetic acid (5HIAA), deoxyepinephrine (DEP) and 3,4-dihydroxyhydrocinnamic acid (DHCA) were obtained from Wako (Osaka, Japan). Chemicals were obtained from Wako. The mobile phase was 0.03 *M* citric monohydrate–0.04 *M* phosphate–5.15 m*M* 1-

heptanesulfonic acid–0.11 mM Na_2EDTA–7.5% acetonitrile–4% methanol at pH 3.10. The standard materials were prepared to 0.992 mg/l (5HIAA), 1.564 mg/l (DEP), and 1.125 mg/l (DHCA) in 0.2 M perchloric acid.

The chromatographic system consisted of an intelligent pump 880 (JASCO., Tokyo, Japan), an L-ODS analytical column (150×4.6 mm) (Chemicals Inspection and Testing, Tokyo, Japan) and an electrochemical detector (ESA, Bedford, MA, USA). An LC-100 data process system (Yokogawa, Tokyo, Japan) and a PC-9801-RX personal computer (NEC, Tokyo, Japan) were employed to save the chromatographic data. Software edited in our laboratory was used to read chromatograms. A Macintosh LC630 personal computer (Apple, USA) with installed software of Microsoft Excel was used for mathematical calculation.

Chromatograms were monitored under −350 mV. The obtained chromatogram of standard solution (5HIAA, DEP, DHCA) was employed as a model (Fig. 1). Finally, the actual multiple-component chromatogram was prepared by injecting a mixture of 5HIAA, DEP and DHCA, and then was resolved by the proposed method.

5. Results and discussion

5.1. Elution profile of standards

The researcher's ultimate purpose was to resolve an overlapping-peak into individual peaks which resemble the original peak in both shape and area. That is, one resolved profile, which is resolved from a multi-component overlapping peak by an excellent method, should agree with the profile of its single chromatographic peak. Because the chromatographic peak of a standard solution supports accurate information of the profile, I use the profile of chromatographic peaks of standard solutions to resolve their overlapping chromatographic peaks. In this study, it has become much easier to use the profile of standard chromatographic peaks, and it yielded better results.

It is well known that peak shape is asymmetrical, i.e., tailing edge. If the peak height of the prepared standard chromatogram is very much smaller than the original overlapping peak, the trailing edge possibly trails into zero. The zero can not be calculated. The more the peak size of standards approximates that of overlapping peak, the less the error from calculation. In this study, the peak height of standards was about 0.9×overlapping peak.

5.2. Overlapping ratio at $t_{1/2}$

Because profile is asymmetrical, and there is a difference in size between the overlapping pair, $h_{t_{1/2},A}$ and $h_{t_{1/2},B}$ are not the intersections of peak A and peak B. The $h_{t_{1/2}}$ shows a higher overlapping ratio for both peaks of the overlapping pair at $t_{1/2}$ than at another time t, and can be easily used. The several degrees of overlap at $t_{1/2}$ were tested in three cases of height, shown in Fig. 2. As in Fig. 2, $h_{t_{1/2}}$ is the ability to express the degree of the overlap between the peak A and the peak B.

An overlapping pair (ratio of height is 2 to 1) was resolved favourably by using $h_{t_{1/2}}$ (Table 1). It has be seen that any one of either an extensively overlapping peak or a slightly overlapping peak has been resolved successfully.

5.3. Magnification (or reduction) factor (F)

Magnification means that a profile is repeatedly multiplied by a micro factor. If a factor is 1 or more

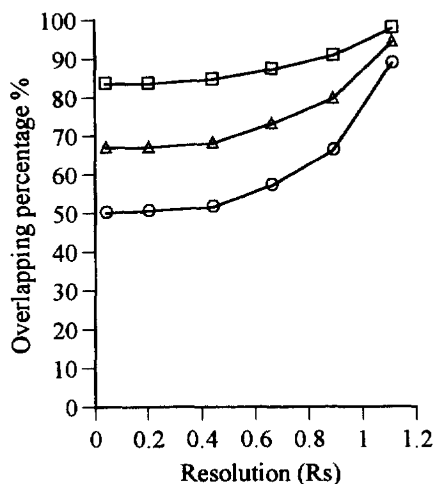


Fig. 2. The degree of the overlap in several resolutions (R_s). The ratios of height between peak A and peak B are 1:1 (○), 2:1 (△) and 5:1 (□), where y-distance is a percentage of peak A ($R_{1/2,A}$). The percentage of peak B ($R_{1/2,B}$) is $(1-R_{1/2,A})$.

Table 1
The effect of resolution (R_s) between the overlapping pair on accuracy

Resolution (R_s)	Peak A area error (%)	Peak B area error (%)
0.01	0.52	−1.04
0.04	0.53	−1.06
0.20	−0.15	0.31
0.44	−0.40	0.76
0.66	0.26	−0.70
0.89	0.44	−0.17
1.11	0.11	−1.77

($F \geq 1$), the multiplied profile could not precisely overlap with the original overlapping profile, producing error.

The effect of the factor F on accuracy was tested. Table 2 shows some factors and the corresponding calculation error, which used a two-component overlapping-peak, ratio of height was 1 to 1, and R_s was 0.67. For peak A, the area error was 0.08 when factor F was in a range of 0.001 to 0.002, the area error was 0.43 when the factor F was in a range of 0.005 to 0.04. A factor of 0.005 was employed in this work.

5.4. *Affect of peak retention time (Dt)*

Although the Δt value is small compared with the retention time, it is sensitive in the proposed method as the calculation depends on the corresponding time. Retention time shifts need to be considered when the proposed technique is applied.

The detector response was monitored periodically at intervals of 0.2 s. There are five data points in 1 s. The time variation in elution position of the relative peak was aligned according to the profile top. When a profile top contains two or more identical data points, one point in the centre is chosen. After peak A has been aligned to the overlapping peak, the calculation error, produced by time shift of peak B, was tested (Table 3). When the shifts were in range of 3 s (fifteen data), the calculation error in area was within 5%.

The elution time error from a standard peak is directly proportional to the time error of the resolved peak (Fig. 3). While the elution time error is improved to zero or is corrected, the accuracy of time of the resolved peak is increased.

Table 3
The effect of retention time variability (Δt) in the authentic chromatogram on accuracy

$t_2 - t_1$ (Δt)	Peak A area error (%)	Peak B area error (%)
−10	−15.6	14.0
−8	−12.4	11.4
−5	−15.3	13.8
−3	−4.6	4.5
−1	−1.5	1.5
−0.6	−0.8	0.8
−0.2	−0.3	0.3
0	0.0	0.0
0.2	0.4	−0.4
0.6	1.1	−1.1
1	1.8	−1.8
3	5.0	−5.0
5	8.4	−8.4
8	13.6	−13.6
10	16.0	−16.3

5.5. *Affects of ratio of peak-height*

The results shown in Table 4 suggest that error is not sensitive to the ratio of peak height of the overlapping pair, even several times. The results give us indications that the analysis of a minimum peak which is enclosed in a large peak is possible.

Table 2
The effect of the magnification factor (F) on accuracy

Magnification factor (F)	Peak A area error (%)	Peak B area error (%)
0.001	0.08	−0.1
0.002	0.08	−0.1
0.005	0.43	−0.57
0.01	0.43	−0.57
0.02	0.43	−0.57
0.04	0.43	−0.57
0.05	2.17	−2.91
0.06	3.92	−5.25

Table 4
The effect on accuracy of peak-height ratio between a co-eluting pair

Peak-height ratio of two-component	Peak A area error (%)	Peak B area error (%)
1:1	−0.48	0.49
2:1	0.18	−0.36
5:1	−0.56	0.28
10:1	0.40	−0.13

5.6. Application

A two-component chromatogram and a three-component chromatogram were prepared, shown in Fig. 4 A,B. The resolution (R_s) was 0.7 for the two component profile. The resolution was 0.9 between peaks of 5HIAA and DEP, and was 0.7 between peaks of DEP and DHCA, in a three component profile. The two multi-component chromatographic profiles were clearly resolved into individual chromatographic peaks by using the developed method (Fig. 5A,B). The area errors were 1.0% and 1.6% from peak pair of DEP and DHCA in figure 5A. The area errors were −0.9%, 0.8% and −3.2% form peaks of 5HIAA, DEP and DHCA in Fig. 5B.

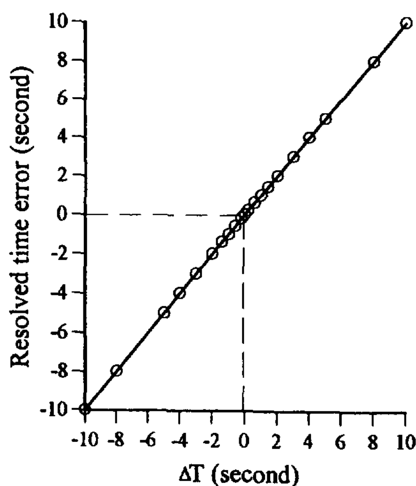


Fig. 3. The effect of elution time variation (Δt), comparing the authentic peak with the overlapping peak, on time error of the resolved peak (y-distance). The time error of the resolved peak achieves zero after variation has been corrected.

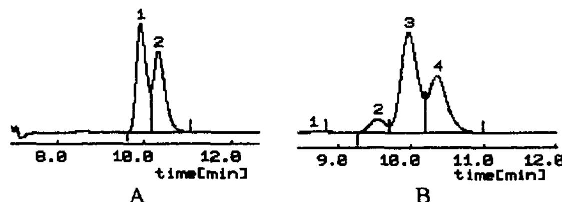
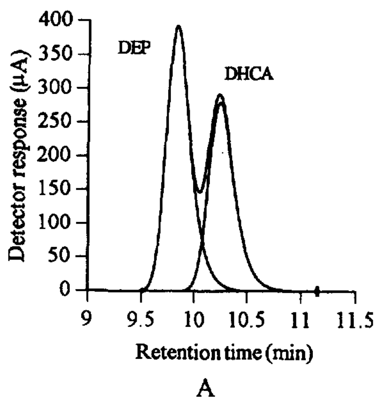


Fig. 4. The actual chromatograms. The chromatographic conditions are described in the text. A: two-component chromatogram (consisting of 557 data; 1 = DEP, 2 = DHCA). B: three-component chromatogram (consisting of 750 data; 2 = 5HIAA, 3 = DEP, 4 = DHCA).



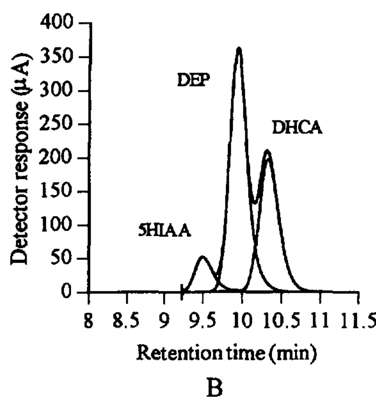


Fig. 5. Resolution of the multi-component chromatograms. The multi-component chromatographic profiles are copied from the chromatogram described in Fig. 4. These chromatographic profiles are resolved into the individual peaks.

6. Conclusion

A method for resolving the unresolved chromatogram has been described. Its performance has been examined by studying several cases of the eluted profile, and its applicability is discussed. Although the result is directly affected by the retention time shifts, the proposed method is capable of resolving actual unresolved chromatograms. The chief limitation is that it is necessary to know every co-eluting peak. Many studies are still required to be done, such as what are the errors when a known peak co-elutes with a unknown peak, when the peaks are highly asymmetric, when affected by noise, or when a small peak is hidden between two peaks.

This is a first report on the method which is both useful and valuable for study.

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