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QUANTITATIVE COMPUTER RESOLUTION OF SEVERELY OVERLAPPING LIQUID CHROMATOGRAPHIC PEAKS

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SUMMARY

Severely overlapping peaks in high-performance liquid chromatography have been resolved by using a mathematical technique to extract quantitative information. This technique is applicable even in the worst situation, that in which the retention volumes of two peaks are identical, as long as the peak shapes are different. Conversely, even in the rare situation in which the peak shapes are identical, the method is applicable if the retention volumes of the peaks differ somewhat. Changes in the mobile phase flow-rate affect the chromatographic peak positions, and corrections for these variations must be made. Correction is also made for minor changes in the retentive ability of the chromatographic column as a function of aging. An accurate mobile phase flow-rate monitor and the associated computer routines were developed to make the necessary corrections. Overlapping computer-generated peaks as well as experimentally measured chromatograms have been resolved to demonstrate the applicability of this procedure to liquid chromatography. Overlapping peaks exhibiting slight shoulders were resolved with $< 13\%$ error, and quantitative information with $< 18\%$ error was obtained from completely overlapped peaks (almost identical retention times).

INTRODUCTION

The quantitative analysis of severely overlapping chromatographic peaks has been a major problem for chromatographers since chromatography was first developed. This problem continues, especially as analyses and samples become more complex, in spite of the numerous improvements that have been made in instrumentation and theory. Only two options are available for achieving quantitative information from unresolved peaks. These are: (1) to improve the resolution of the peaks by choosing an alternate column packing or mobile phase and by optimizing experimental parameters, or (2) to perform a mathematical resolution of the components of interest. In many laboratories the first approach is most often done in an empirical manner. This approach therefore generally leads to an undesirable increase in the analysis time, and, in fact, with complex mixtures it often does not succeed[1,2]. The second approach, involving mathematical resolution by such techniques as the perpendicular drop, triangulation, curve-fitting assuming a pre-defined shape, principal-

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component analysis and linear simultaneous equations, is successful under certain experimental conditions of resolution, but all these techniques are prone to progressively worse results as the resolution of the overlapping peaks decreases, and all these methods fail when there is complete overlap.

Lundeen and Juvet[3] recently reviewed the problems associated with previously published mathematical approaches and proposed an alternative method, which gave promising results when using computer-simulated chromatographic peaks. The present paper applies this theoretical treatment to experimental verification for the case of liquid chromatography. Pairs of chromatographic peaks with varying degrees of overlap have been evaluated for quantitative information. The detector response for each component at various times (or more exactly, at various volumes of mobile phase) throughout the peak is fitted to a second-order polynomial of each component's concentration. A series of non-linear simultaneous equations is then solved for the concentration of each component in the mixture. The complete theory for this analysis has been discussed in detail by Lundeen and Juvet[3]. The only requirement for a successful analysis is differing peaks shapes if the compounds have approximately identical retention volumes, or somewhat differing retention volumes if the components have almost identical peaks shapes. Gas chromatographic (GC) peaks obtained isothermally generally meet these requirements[3–7]. In this work it will be shown that these requirements are also met when using high-performance liquid chromatography (HPLC).

The proposed resolution method requires that parameters affecting peak position (such as mobile phase flow-rate) either be very closely controlled throughout the experiment or else these parameters must be accurately measured and the peak position corrected to what it would have been had the parameters been held constant. We chose to use the latter approach. The mobile phase flow-rate was observed to change slightly, but significantly, over the time of analysis; pump pulsations also occurred. Unless corrections were made, these changes shifted the positions of the peaks, and large errors in quantitative measurements were introduced. An accurate, computer-monitored and continuous-flow monitor was developed to record flow-rate fluctuations for the computerized correction procedure. For additional improvement in accuracy, the data may also be corrected for the slight changes in column retention behavior that are occasionally observed during an analysis. These changes may arise, for example, from the slow hydrolysis of the chemically bonded stationary phase by the mobile phase. Although this change in retention behavior is usually small, for best results it should be considered, since both the peak shapes and their positions are involved.

THEORETICAL

Fig. 1A is a series of chromatograms of 2-octanone at different concentrations. If the response at a particular mobile-phase volume is plotted *vs.* concentration, non-linear calibration curves similar to those shown in Fig. 2 are produced. These curves are linear only in the region near the peak maximum, demonstrating that peak shapes change with concentration in HPLC as has been previously demonstrated for GC[3,4,6]. The calibration curves are concave upward before the peak maximum and concave downward after the maximum. The directions of curvature can vary with the chromatographic system and experimental conditions, since opposite curvatures are

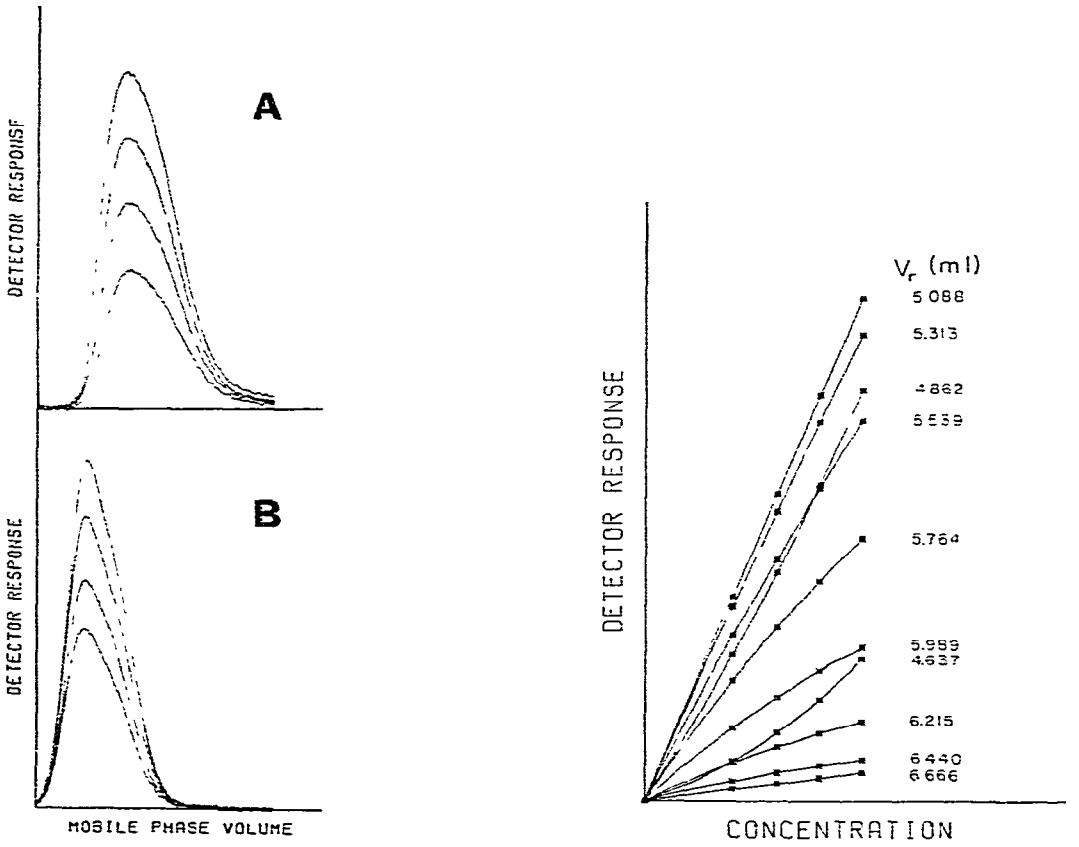


Fig. 1. A, 2-Octanone standards (5.159, 4.140, 3.132, 2.068%, w/w) in methanol–water (55:45) mobile phase. B. Toluene standards (2.133, 1.796, 1.410, 1.109%, w/w) in methanol–water (55:45) mobile phase.
Fig. 2. Calibration curves of 2-octanone standards. V_r = Retention volume at point *i*.

generally obtained for GC systems[3]. Typically, 200–300 second-order equations relating the concentration to the detector response are utilized for the resolution calculations. A least-squares procedure is then performed on these equations in order to calculate the concentrations of each component in the unknown by minimizing the variance between the sum of the detector response of the components and that of the unknown.

When using methanol–water (55:45) mobile phase, a mixture of toluene and 2-octanone will be eluted from a Bondapak C_{18} Corasil column as an overlapping pair of peaks with a resolution of only 0.34, thus producing a single peak with a shoulder. Fig. 1B is a series of chromatograms of toluene standards at different concentrations, which have the same mobile-phase-volume axis as Fig. 1A. When comparing the two chromatograms of Fig. 1, it can be seen that both the retention time and the peak shapes differ. Thus, this pair of compounds easily meet the requirements for resolution described previously. The only basic assumption necessary for the success of this method is that the composite signal is equal to the sum of the responses of each of the

components. This implies that there is negligible interaction between the solutes at the low concentrations normally used in chromatography.

Small changes in the mobile phase flow-rate can be continuously measured with the flow monitor described below. Observed changes are caused by various factors, including pump variations and pulsations, gradual clogging of the submicron frits in the system, and build-up of particulate matter on the column. Therefore, to ensure an identical mobile-phase-volume axis for all chromatograms, the data are corrected for these perturbations.

Three different methods of flow correction were studied. The least accurate, the peak-shift method (PSM), compares the average flow-rate of the chromatogram of interest with the average for the entire data set. The chromatogram is then transposed the necessary amount along the mobile-phase-volume axis without modifying the peak shape. Thus, the new response, R_i', is,

$$R_i' = (R_{j+1} - R_j)M' + R_j \tag{1}$$

where M' is the ratio of the two flow-rate averages and R_j and R_{j+1} are the detector responses of the chromatogram that is being corrected.

In the average-volume method (AVM), the mobile phase volume corresponding to each detector response measured in the chromatogram is evaluated by summing the mobile phase volumes eluted between all previous detector response measurements. The summation is performed for both the unknown and the standards. The mobile phase volumes of the unknown are the reference volumes used when correcting all standards. The computer searches for a contiguous pair of volumes, V_j and V_{j-1}, in the standard's array, that will bracket the reference volume, V_i'. The new standard response, R_i', corresponding to the reference volume is then given by

$$R_i' = \frac{(R_{j+1} - R_j)(V_i' - V_j)}{V_{j+1} - V_j} + R_j \tag{2}$$

where R_j and R_{j-1} are the detector responses that are paired with their respective volumes. By averaging up to and including each point, this method can account for all perturbations in the mobile phase flow-rate, such as pumping pulsations, surges and dips, and it will modify the peak shape accordingly.

In the regression-volume method (RVM), the logic used is similar to that of the average volume method, but calculates the mobile phase volume in a different manner. A linear regression analysis is performed on a plot of the flow-rate as a function of time for the unknown. Knowing the slope and y-intercept of the flow-rate trend, a reference volume, V_i', can be calculated for each unknown detector response. In a similar manner, volumes are calculated for each standard sample response from its flow-rate trend. As in the AVM, the computer searches for a pair of contiguous volumes, V_j and V_{j+1}, in the standard's array that bracket the reference volume. The new standard response is calculated by using eqn. 2. This method effectively smoothes out the effect of pump pulsations and stray noise and will also modify the peak shape. Since 200–300 points per peak are used, these methods assume a straight line between successive points on the chromatographic peak with little introduction of error. Use of a second-order, least-squares fit to approximate the peak shape requires approxi-

mately twice the execution time of a straight line approximation and does not improve the results sufficiently to justify the extra computing time.

Computer measurements were accurate enough to detect a gradual loss of column efficiency and peak retention when repetitive samples were injected. This was verified by computer calculation of peak moments, which showed that the peak shape and retention volume change slightly with time, perhaps due to slow hydrolysis of the column packing material. Since peak shape and position are both important in this method, corrections for changes in column characteristics are desirable. These corrections were performed by measuring the difference in first moments (retention volume) of the first and the last chromatograms (identical samples in the scheme used) and proportioning the correction for the change in column characteristics linearly over the entire analysis run, which consists of the unknowns and calibration standards.

EXPERIMENTAL

The liquid chromatograph was built in our laboratory. All connecting tubing was 1/16-in. O.D. and either of stainless steel or PTFE (Alltech Assoc., Los Altos, CA, U.S.A.).

The mixed solvent was constantly refluxed in a 2-l, round-bottomed Pyrex flask to eliminate dissolved gases and to maintain constant composition.

Pump pulsations from a Model 396 Minipump (Laboratory Data Control, Rivera Beach, FL, U.S.A.) were easily detected with the monitor, and these fluctuations were reduced by means of a pulse dampener similar to the systems described by Nikelly and Ventura[8,9]. Acting as a bellows, a Swagelok short flexible metal hose connector (Crawford Fitting Co., Solon, OH, U.S.A.) was connected between the pump and a Li-Chroma II pulse dampener (Handy and Harman Tube Co., Norristown, PA, U.S.A.). A pressure gauge was installed before the pulse dampener to monitor the approximate pressure of the system and the pump pulsations. These modifications reduced pulsation noise by at least 50%.

An Alltech Model 9200 0.5-μm HPLC filter (Alltech Assoc.) was installed after the pulse dampener to remove any particulate matter that may have been present in the mobile phase. The flow monitor, described in detail below, was positioned after this filter.

A Rheodyne Model 7120 injector valve (Rheodyne, Berkeley, CA, U.S.A.), equipped with a 10-μl sample loop was used for reproducible sample injection. Syringe injection was not acceptable, owing to the non-uniformity of sample introduction and inaccuracies in timing encountered when injecting against a head pressure of 500 to 1000 p.s.i. The valve was so mounted that a micro switch was closed automatically at the instant of injection. This switch was monitored by software to start or stop computer sampling.

The analytical column was Bondapak C_{18} Corasil (Waters Assoc., Milford, MA, U.S.A.) contained in a 30 cm $\times$ 3.9 mm I.D. stainless-steel tube. To reduce dead volume, the 1/4- to 1/16-in. reducing unions used as end fittings were bottomed out with a 1/4-in. drill so ground that the tip was almost flat rather than cone-shaped. The fitting was then reamed through with a No. 52 drill to allow passage of 1/16-in. O.D. PTFE tubing. A 1/4-in.-diameter stainless-steel frit with 0.5-μm pores (Alltech Assoc.) was placed into the flat bottom of the fitting, and both the column tubing and the

connecting tubing (0.3 mm I.D.) were inserted into the column fittings until they butted against the frit.

A Waters Associates Model R401 refractive index detector (RID) was used to monitor the eluting peaks. An Analog Devices Model 610L instrumentation amplifier boosted the RID signal to the 0–5-V range required by the computer. Any amplification gain from 5 to 1000 could be achieved by use of a resistor bank and DIP switch arrangement. For visual monitoring of the amplified RID response, a Rikadenki Model B-181 recorder (Rikadenki Kogyo, Tokyo, Japan) and a Hewlett-Packard Model 3465A digital multimeter (Hewlett-Packard, Palo Alto, CA, U.S.A.) were used.

Earlier work in our laboratory by Werho and Juvet[10] involving the development of a streaming-potential detector for HPLC had demonstrated that the detector response changed with the flow-rate. Pursuing this observation, Werho pioneered the development of a flow monitor that produced an output voltage proportional to the flow-rate of the mobile phase with a high degree of accuracy. Modifications were made to the original design, and a monitor was built for use in this research.

The flow monitor was constructed as shown in Fig. 3. A metal box (4 × 6 × 8 in.) was used to house the flow-monitor components and to act as an electrical earth. A Swagelok 1/16-in. nut, cut such that the threaded portion was only 1 mm thick, was used to secure a 1/16-in. union to the box in bulkhead fashion, while also ensuring a good earth for the union. A Swagelok nut was ground slightly at the threaded end so that it could be used to seal PTFE tubing to the union. The PTFE support was machined into a cylindrical shape, 4 cm in diameter at the edges and center, with the remainder of the support being 3 cm in diameter. Holes were drilled and tapped to accept 10-32 screws, which held the support away from the box and were anchored to the box with nuts. In order to reduce dead volume and enable work at the high pressures necessary for acceptable flow-rates and retention times, PTFE tubing of I.D. 0.3 mm was used. The longer length (*ca.* 10 cm) of PTFE tubing was just long enough to reach from the inlet to the union secured in the PTFE support, avoiding contact with the earthed aluminium box or support screws. The shorter length of tubing was about 2.5 cm long and was connected to the outlet union with stainless-steel tubing. A multi-strand 18-gauge wire was silver-soldered to a 1/16-in. union

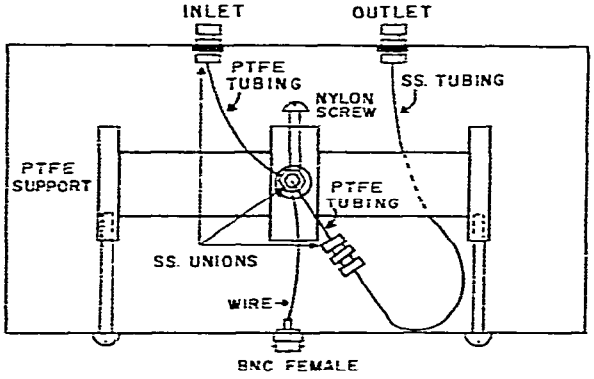


Fig. 3. The flow monitor. SS = Stainless steel.

secured inside the PTFE support. This wire conducted the voltage difference developed across the two unequal lengths of PTFE tubing inside the monitor to the BNC connector. A nylon screw was used to prevent the union inside the support from vibrating and generating electrical noise. The box, the terminating unions and the BNC female connector were earthed.

The unit was tested at 1500 p.s.i. The box was sealed by using a silicone rubber glass-and-ceramic adhesive and was repeatedly dipped into melted Parowax (Amoco Oil Co., Chicago, IL, U.S.A.) to seal any remaining leaks and to insulate the box from stray charges. This apparatus was then submerged in an aqueous constant-temperature bath controlled by a mercury thermoregulator and heated with a 50-W heater. Water was circulated constantly around the monitor by a pump. This procedure ensured constant humidity and a temperature that was held constant to within $\pm 0.01^{\circ}C$ at approximately 30°C (helpful in reducing voltage fluctuations and drift).

Since the flow monitor had low output impedance, a Keithley Model 601A electrometer (Keithley Instruments, Cleveland, OH, U.S.A.) was used to measure and amplify the output voltage.

A Digital Equipment Corporation PDP-8/E computer system (Digital Equipment Corp., Maynard, MA, U.S.A.) was used for data taking and resolution calculations. The computer was equipped with 16K of memory, a KL8/E asynchronous data control board for the Teletype (Teletype Corp., Skokie, IL, U.S.A.), and a DK8-EP real-time programmable clock. An analogue-to-digital converter (ADC) (Phoenix Data, Phoenix, AZ, U.S.A.) was incorporated into a data interface. This included a multiplexer that could sample up to eight different devices at times dictated by software and convert the input voltages into binary numbers for computer utilization. A DEC RXO2 dual-density floppy-disc system fulfilled the need for a mass-storage device and a medium to run the DEC OS/8 V3D operating system.

To accomplish the resolutions presented in this paper, the minimum requirements are a 16K computer with an operating system such as the DEC OS/8 and BASIC, a mass-storage device with more than 300 free blocks for data and calculation files, a programmable clock, and an ADC–multiplexer interface. Any system with similar capability could be used to execute the BASIC programs described below.

A series of seven BASIC programs entitled RESOL was written to do the resolution calculations; these programs are modular, in order to keep the memory required by each to a minimum. This design allows large data arrays to be used, and these programs will chain to each other automatically as required. Programs for either a PDP-8/E or a PDP-11/70 are available from the authors.

Methanol and toluene were "Baker Analyzed" (J.T. Baker, Phillipsburg, NJ, U.S.A.). Other reagents were 99% pure 2-octanone and diethyl *n*-butylmalonate, and 97% *o*-xylene (Aldrich, Milwaukee, WI, U.S.A.). Methanol was used as solvent for all samples. The composition of the distilled water–methanol mobile phase was varied to change the degree of resolution between the overlapping peaks.

Since the slope of the flow monitor calibration curve changes with the mobile phase composition, it is necessary to calibrate the device each time the mobile phase composition is changed. An assembly-language program, DATATK, was written to calibrate and sample the flow monitor and RID voltages, and store the data on floppy disc. After the flow monitor was calibrated, the unknowns were injected, and the

detector-response and flow-rate pairs were stored on disc with the latter being converted from flow-monitor voltages by using the calibration curve. Eight standards were then injected in a routine fashion —four standards of varying concentrations of sample A and four of sample B. Finally, the first unknown was re-injected for later evaluation of possible changes in column retention behavior. The entire set of complete chromatograms was stored as a permanent file for calculations with the RESOL series of BASIC programs.

RESULTS AND DISCUSSION

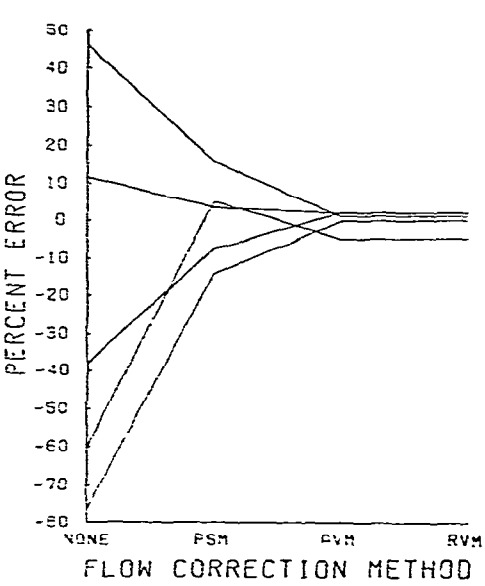
The ADC–computer system was calibrated and found to be linear to within $\pm 0.05\%$. The electrometer, coupled with the ADC–computer system, was evaluated and found to be accurate within $\pm 0.12\%$. The reproducibility of the HPLC–ADC–computer system was $\pm 0.2\%$ in retention time and $\pm 1\%$ in area when using repetitive injections of the same sample and after correcting for flow variations by either the average volume method or the regression volume method.

With constant temperature control, the no-flow output voltage of the flow monitor varied by ± 2 mV. The calibration curve of the monitor was linear in the range used in these studies (0.5–3 ml/min) to better than $\pm 0.3\%$. With changes in the methanol–water mobile phase composition, the y-intercept of the flow calibration curve remained reproducibly at zero, while the slope varied directly with the concentration of water in the mobile phase. The output voltage was typically 6 V at a flow-rate of 1-ml/min when methanol–water (1:1) was used as mobile phase. The error introduced by digitization of the flow monitor output was small (± 0.01 to 0.05%) compared to the short- and long-term pump variability of ± 0.3 and $\pm 0.9\%$, respectively. These perturbations made flow corrections necessary.

Synthetic studies were used to evaluate the necessity for making flow corrections. Five independent studies with a random $\pm 1\%$ variation in the flow-rate and $\pm 0.3\%$ variation in pump stroke were used to simulate experimental chromatographic conditions. These five studies are shown in Fig. 4 with a line tracing the errors obtained with the three flow-correction methods. This plot demonstrates the effect that flow variations have on the results and the effectiveness of the three types of flow correction. Errors as much as 70% with no flow correction were reduced to less than 3% when flow corrections were employed. Thus, for accurate results, flow corrections to adjust the peak shapes and positions are important.

The peak-shift flow-correction method proved to be inferior to the average-volume and regression-volume methods, each of which gave comparable results. A pair of peaks showing only a slight shoulder was resolved with *no* flow correction (Fig. 5) and had an error in the quantitative results of -16% for peak A and $+19\%$ for peak B. The resultant poor fit of the peaks, as shown by the non-zero risidual plot, is due to an increase in flow-rate of 0.8% over the entire period of analysis (approximately 1.5 h). The residual was calculated by subtracting the sum of the responses of the fitted peaks from the response of the mixture at each point in the data window.

However, when flow corrections were made on the same chromatograms by using the average-volume method, the fit was markedly improved, as shown in Fig. 6A. The residual was reduced considerably, and was mostly reflective of the noise in



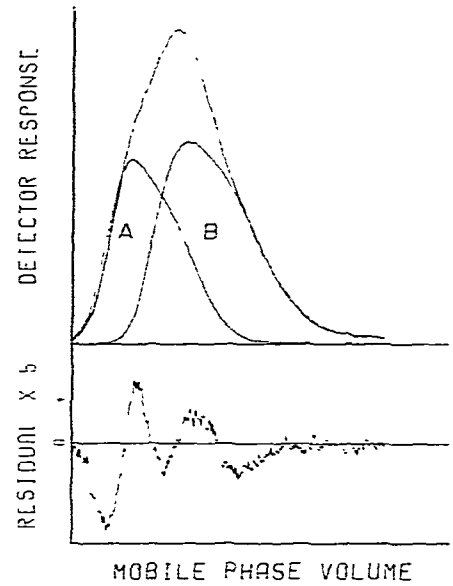


Fig. 4. Effect of flow-correction methods on synthetic data. PSM = peak-shift method; AVM = average-volume method; RVM = regression-volume method.

Fig. 5. Resolution of overlapping peaks with a shoulder with no flow correction, peak A, 1.409% of toluene and peak B, 3.106% of 2-octanone in methanol–water (55:45) mobile phase.

the unk. wn and the fitted peaks in addition to slight imperfections in the flow-correction rocedures. In this analysis, results exhibited an error of only −7% for peak A and one of +9% for peak B, acceptable errors considering the poor resolution of the pair. Average errors encountered when resolving peaks with a similar resolution of 0.34 were −12% for peak A and 13% for peak B. If the flow had decreased rather than increased over the sampling interval, all positive residuals would be negative and *vice versa*. Since the average-volume method most correctly accounts for pumping pulsations and other short- and long-term variations, results obtained with use of this method are presented below. If the first moment of the unknown in the first chromatogram, and the first moment of the same mixture repeated as the last chromatogram differed by more than 0.75%, corrections for changes in column characteristics were also made, as described under Theoretical.

Chromatographic peaks showing a valley and a resolution of 0.46 (Fig. 6B) were resolved with an average error of 7% and 9% for toluene (peak A) and 2-octanone (peak B), respectively. A small peak (2-octanone, peak B) overlapped by a large peak (toluene, peak A) was resolved with an average error of 1% and 8% for the smaller and larger components, respectively (Fig. 6C); the resolution for this pair of peaks was only 0.32. Completely overlapped peaks (resolution of 0.20), showing no shoulder, were resolved with an average error of 18% for both diethyl *n*-butylmalonate (peak A) and *o*-xylene (peak B) as shown in Fig. 6D. Average reproducibility for all analyses was ±3.7%. Undoubtedly, errors could be reduced by using a detector that is more sensitive and less noisy than the RID. A solvent-delivery system with less

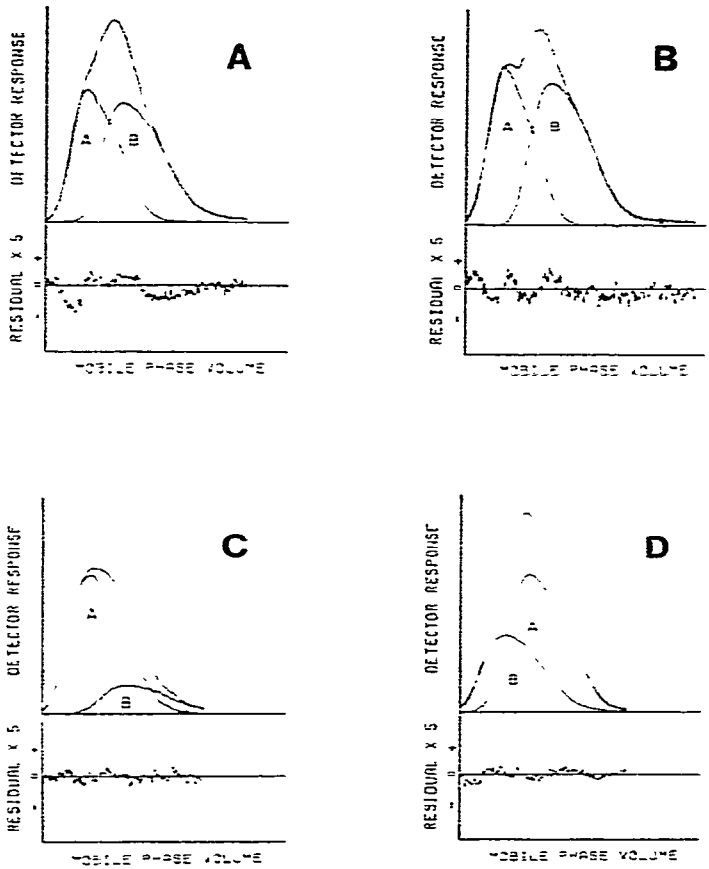


Fig. 6. Resolution of peaks with use of AVM for flow correction. A, Overlapping peaks with a shoulder: 1.409% (w/w) of toluene (peak A) and 3.106% (w/w) of 2-octanone (peak B) in methanol–water (55:45) mobile phase. B, Overlapping peaks with a valley: 1.409% (w/w) of toluene (peak A) and 3.106% (w/w) of 2-octanone (peak B) in methanol–water (50:50) mobile phase. C, Small component overlapped by a larger component: 1.780% (w/w) of toluene (peak A) and 0.6300% (w/w) of 2-octanone (peak B) in methanol–water (55:45) mobile phase. D, Completely overlapped peaks: 1.447% (w/w) of diethyl *n*-butylmalonate (peak A) and 3.629% (w/w) of *o*-xylene (peak B) in methanol–water (60:40) mobile phase.

fluctuations in the mobile phase would also be expected to improve reproducibility, although some corrections would probably still be necessary.

A good fit (small residual) does not necessarily ensure accurate results, although accurate results without a good fit are unlikely. Since this method minimizes the sum of the variance between both components and the unknown, the quantitative results will be balanced for the best fit possible. In instances in which the peaks are partially resolved, such as in Fig. 6B, there are large portions of the overlapped peak where the detector response is almost exclusively due to only one component in the mixture, which increases the probability of accurate results. However, the residual may be relatively large, as there is no other component over most of the peak to offset the detector response, which may be either too high or too low.

With completely overlapping peaks, such as in Fig. 6D, the opposite is true.

The quantitative values are the result of the best fit of the components, both of which contribute almost equally over most of the overlapped region. Therefore, errors of the size reported above should be expected, as detector response is due to one component only over a small portion of the peak. However, the residual may be small, as any positive error in the detector response of one component will be off-set by the negative error from the other. Therefore, residuals cannot strictly be compared between analyses with differing degrees of overlap. For a particular data set, of course, the best method of correction will always have the smallest residual and the most accurate results.

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