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Analysis of unresolved chromatograms by the absorbance ratio and sequential chromatogram ratio techniques coupled with peak suppression

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Abstract

The sequential chromatogram ratio (SCR) technique was successively applied or was coupled with the absorbance ratio (AR) technique to facilitate analysis of a peak of interest that was overlapped with two other peaks. First, the AR or SCR technique was used to suppress one undesired peak. This created or elongated a region of pure-component elution for the peak of interest. Then the appropriate ratio technique was applied to this region for qualitative and quantitative analysis. The AR technique allows suppression and qualitative analysis of unknown components via the absorptivity ratio. For the SCR technique, peak identity can be deduced prior to suppression and quantitative analysis. A statistical peak matching procedure that employs user-selected standards is described for the SCR technique, by which components in sequentially injected samples may be identified based on differences in retention time, t_R, or in diffusion coefficient, D_m, which controls peak width. For two similarly retained analytes in sequentially injected samples, having a factor-of-two difference in D_m, the problem of reduced resolution, R_s, with a third peak was investigated by manipulating the data to simulate a reduction in selectivity factor. Below a limit, $R_s = 0.38$, the original two analytes could no longer be qualitatively distinguished. At this same R_s limit, the two-times difference in D_m, for two analytes having essentially zero t_R-based resolution, provided equivalent qualitative discrimination of peaks as a t_R-based resolution of 0.12 for two analytes having equal D_m. The classical problems of inaccurate baseline correction and run-to-run retention variation were examined, and the latter was more limiting for the SCR technique. Still, small t_R shifts were adequately corrected by selecting and aligning a common peak in sequential chromatograms.

1. Introduction

Chromatography necessarily involves an overall compromise between analysis time, resolution of peaks, and instrumental limitations. For application of chromatography to industrial process monitoring [1], the instrumentation should be made simple and reliable, for example by avoiding complicated gradient systems and the increased maintenance and safety concerns associated with high-pressure, high-efficiency analytical HPLC columns and pumps. Further, rapid chromatographic analysis in the high- and superspeed regimes [2] facilitates effective process control. Unfortunately, these desirable characteristics are achieved at the sacrifice of resolution, which increases the likelihood of peak overlap due to bounded peak capacity [3] and

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inadequate thermodynamic selectivity factors. Even for laboratory applications, especially moderately well defined systems such as routine assays, it is often preferred to use a chemometric technique to extract analytical information from overlapping peaks rather than expend the resources of time and instrument complexity needed to achieve baseline resolution. Therefore, suitable data analysis techniques are continually sought that enable identification and quantification of an analyte or interferent in the case of partial or complete overlap with other peaks.

The most challenging data analysis situation involves the necessity to use single-channel detection, such as refractive index, because minimally, the analyst is merely alerted to the presence of unknown overlapped interferent(s). Quantification of overlapped peaks, including unknowns, requires additional information [4], for example by making assumptions about the chromatographic peak shapes, as is done for simple geometric methods [5] such as perpendicular drop, as well as for non-linear curve-fitting techniques [6]. More powerful data analysis techniques, that do not require peak shape assumptions about unknown components, involve expanding the dimensionality of the data [4]. This is usually achieved using UV–Vis or MS multichannel detection, but was recently done using two sequential conductometric detectors [7]. Two techniques of increasing dimensionality that are simple and work well for analysis of unresolved peaks, including unknown interferents, are the absorbance ratio (AR) technique [8–11] and the sequential chromatogram ratio (SCR) technique [12–14]. Both techniques employ the point-by-point ratio of two baseline-corrected chromatogram data vectors, as obtained from simultaneous dual-wavelength detection of a single sample (AR) and from two single-channel chromatograms obtained by sequential injections of related samples (SCR). Both techniques use the flat, pure-elution region of the ratiogram for peak-purity assessment and peak identification. The AR technique has greater power for unknown identification by means of the absorptivity ratio, but lacks the capability for quantification. For the SCR technique, identification is accomplished by a procedure, described herein, that matches an unknown peak with chromatograms of user-selected candidate standard solutions. Once peak identity is verified in sequential samples, the SCR technique immediately provides quantitative information, the ratio of injected concentrations.

Often, the peak of interest is completely overlapped by more than one other peak and does not possess an adequate pure-elution region within the chromatogram. In such situations, the AR and SCR techniques may be combined in stages, by suppressing the response of an unwanted peak that is overlapped with the peak of interest. The initial stage, suppression, uses a ratio technique with two chromatogram data vectors to calculate a scaling constant that allows the signal contribution of one component, common to both vectors, to be subtracted away. Using the familiar spectral suppression technique [15–17], a component may be suppressed without the need for identification, by virtue of its spectral properties. We will show that a component may similarly be suppressed using two single-channel chromatograms when it is known or can be ascertained from the data that the component is common to both chromatograms. The process of suppression uncovers or elongates a pure-elution region for the desired component. Following suppression, the analysis stage consists of applying the appropriate ratio technique to this pure-elution region to extract qualitative and quantitative information as outlined in the previous paragraph.

In this paper, coupling of the SCR and AR techniques through suppression is applied to a challenging test separation in which the peak to be analyzed is largely overlapped with two other peaks. We begin with successive application of the SCR technique, which requires either advance knowledge of analyte identity or a short list of likely analytes and suspected interferents. In this context, we first digress to evaluate the classical problems of inaccurate baseline correction, common to both techniques but previously unreported for the SCR technique, as well as run-to-run retention time variation, which is

more limiting for the SCR technique. By preselecting a common peak in the chromatograms as a timing standard, run-to-run retention variation can be corrected sufficiently to permit subsequent analysis. After dealing with the classical problems, we demonstrate the peak matching procedure for identification of peaks in sequential chromatograms, subsequent peak suppression, and quantification using the SCR technique. The ability to qualitatively discriminate similarly retained components in sequential chromatograms is enhanced when the components have different diffusion coefficients, D_m, because of the resulting difference in peak width. This result will be established using calculations which compare the sensitivity of differences in D_m and in t_R for distinguishing different components in sequential chromatograms when resolution is below 0.5. The significance is that selectivity due to peak shape differences, which occur among similarly retained analytes having different D_m, is not considered useful for analytical chromatography [18,19]. Recent calculations of higher statistical moments in linear non-ideal chromatography [20,21] suggest otherwise. Note, the data manipulations and plots within this section do not reflect the simple application and interpretation of ratio-based techniques [8–14]. Next, SCR-based suppression of a known analyte followed by AR analysis is demonstrated for obtaining a more confident absorptivity ratio by which to identify an unknown component. Finally, the reverse situation, AR-based suppression followed by SCR analysis, is demonstrated for improved quantification of an identified analyte after conveniently suppressing an unknown peak without requiring identification. Successive application of the AR technique, which has been demonstrated for complete deconvolution of unknown mixtures [22], will not be covered.

2. Theory

A brief review of the AR and SCR techniques is useful to illustrate their mathematical similarity and complimentary nature. Both ratio techniques begin with a detected chromatographic signal, $S^{\lambda 1}(t)$, of an initial sample, S, containing M solutes. Assuming linear, concentration-sensitive absorbance detection and temporarily ignoring noise, the data vector, $S^{\lambda 1}(t)$, is given by

$$S^{\lambda 1}(t) = \sum_{j=1}^{M} \epsilon_{j,1} b C_{j,S} G_j(t) \tag{1}$$

where $\epsilon_{j,1}$ is the absorptivity (molar or specific) at wavelength 1 for analyte j, b is the optical pathlength of the detector, $C_{j,S}$ is the injected concentration of analyte j within sample S, and $G_j(t)$ is the dispersed concentration profile, sensed at the detector, for a unit concentration of solute j injected in a small, constant volume. For all chromatograms, $S^{\lambda 1}(t)$ is the result of digitizing the detected signal at uniform sampling intervals from the point of sample injection, and then applying a suitable algorithm to center the baseline at zero [23]. Use of computer-automated sample injection and data acquisition is necessary for precise timing of events.

Both ratio techniques employ a second chromatogram data vector, defined presently, obtained using the same separation conditions and data acquisition procedures as for $S^{\lambda 1}(t)$. Ideally, each analyte occupies the same time axis region in both data vectors, which have the same number of points. Both ratio techniques involve calculating the point-by-point ratio of the two data vectors, yielding a ratiogram that is plotted *versus* the corresponding time, t, since injection. To increase interpretability and to prevent division by zero, the ratiogram is set to zero in regions where either data vector falls below a specified noise threshold [9,12,13].

The well-known AR technique employs simultaneous dual-wavelength detection of the single injected sample, S. The absorbance ratiogram, $AR(t)$, is defined as the point-by-point ratio of the two chromatogram data vectors corresponding to detection wavelengths 2 and 1 for sample S

$$AR(t) = \frac{S^{\lambda 2}(t)}{S^{\lambda 1}(t)} = \frac{\sum_{j=1}^{M} \epsilon_{j,2} b C_{j,S} G_j(t)}{\sum_{j=1}^{M} \epsilon_{j,1} b C_{j,S} G_j(t)} \tag{2}$$

where the notation emphasizes that the ratios are calculated at corresponding time points, t. Within a pure-elution region of the chromatogram for a particular solute, j, the quantities b, $C_{j,S}$ and $G_j(t)$ are identically equal for the same injected sample, S, detected at both wavelengths. Therefore, $AR(t)$ simplifies, in the absence of significant noise, to a flat, time-invariant form within the pure-elution region

$$AR(t) = AR_j = \frac{\epsilon_{j,2}}{\epsilon_{j,1}} \tag{3}$$

in which AR_j is equal to the ratio of absorptivities for analyte j between the two wavelengths used.

For the SCR technique, the second data vector is obtained from a subsequent chromatogram run, also detected at wavelength 1, for a similar injected sample, U

$$U^{\lambda 1}(t) = \sum_{j=1}^{M} \epsilon_{j,1} b C_{j,U} G_j(t) \tag{4}$$

where the injected concentrations, $C_{j,U}$, may differ from $C_{j,S}$ in Eq. 1. The sequential ratiogram, $R(t)$, is defined as

$$R(t) = \frac{U^{\lambda 1}(t)}{S^{\lambda 1}(t)} = \frac{\sum_{j=1}^{M} \epsilon_{j,1} b C_{j,U} G_j(t)}{\sum_{j=1}^{M} \epsilon_{j,1} b C_{j,S} G_j(t)} \tag{5}$$

Within the pure-elution region for a particular solute, j, the quantities ϵ_j and b are automatically equal, and $G_j(t)$ is ideally equal for sequential chromatograms $S^{\lambda 1}(t)$ and $U^{\lambda 1}(t)$. Therefore, $R(t)$ also simplifies, in the absence of significant noise, to a flat, time-invariant form within the pure-elution region

$$R(t) = R_j = \frac{C_{j,U}}{C_{j,S}} \tag{6}$$

in which R_j is equal to the ratio of injected concentrations for analyte j in the sequential samples, S and U.

For both ratio techniques, objective data analysis beyond visual interpretation requires calculation of the pure elution region ratio values, represented by Eqs. 3 and 6. The two boundaries of the pure-elution region may be located by the threshold process mentioned above or from the first derivative of the ratiogram (Experimental section). The variation in noise magnitude throughout a ratiogram [13,24] may be compensated by using the reciprocal local variance as a weighting factor. Within the pure-j region of a ratiogram, a variance-weighted, least-squares horizontal line is fitted. This procedure yields a weighted-average estimate for AR_j or R_j, as well as a weighted standard deviation, s_w [12,13].

The statistic, s_w, is comprised of two independent variance components that measure the noise-related imprecision of the ratio value and the horizontal flatness of the ratiogram, respectively. This latter component, which quantifies lack-of-fit of the ratio model (Eqs. 3 and 6), gives the s_w statistic several practical uses. Differences in analyte retention time or peak shape between $S^{\lambda 1}(t)$ and $U^{\lambda 1}(t)$ will curve the ratiogram (Eqs. 2 and 5), thus inflating s_w above the minimum value associated with random noise. Therefore, minimization of s_w is a precise method to align a given peak in two sequential chromatograms for elimination of small retention shifts [13]. The s_w statistic may also be used to objectively locate the boundaries between flat pure-elution regions of a ratiogram and curved coelution regions (Experimental section). Also, use of s_w for measuring the flatness of $R(t)$ assists in peak identification, both in confirming the presence of a component in both $S^{\lambda 1}(t)$ and $U^{\lambda 1}(t)$, and in matching an unknown peak with pure-component chromatograms of user selected candidate standard solutions. For this matching procedure, the value of s_w from the sequential ratio of an unknown peak, $U^{\lambda 1}(t)$, with a standard, $S^{\lambda 1}(t)$, should be divided by $\sqrt{1 + R_j^2}$, where this divisor is obtained from propagation of errors [13] and R_j is the weighted-average ratio value. The division adjusts for concentration differences between the unknown and standard solutions. This quotient is then compared to the corresponding quotient calculated from sequential ratiograms of replicate chromatograms for the same standard solution, S. If the quotients match within experimental uncertainty, esti-

mated by performing at least 3 to 5 replicates each, then the evidence for a match is strong. The strength or confidence of the peak match is increased by the existence of peak shape differences among coeluting analytes. This will be demonstrated later.

It is instructive to contrast the information provided by the AR and SCR techniques. For both techniques, observation of a horizontal flat region indicates peak purity and locates the pure-elution region for an analyte. The AR technique possesses greater capability for identification of a completely unknown peak because the absorptivity ratio provides spectral discrimination among solutes. Peaks may also be identified using sequential ratiograms by matching an unknown peak with selected candidate standards, as described above. The SCR technique has the advantage of immediately providing quantitative information once peak identity is established, because for an analyte, j, the quantity $(R_j - 1)$ is the relative concentration change between sequential samples S and U (Eq. 6). In contrast, all concentration information cancels from the pure-elution region of $AR(t)$ (Eq. 3).

A common analysis situation is that the peak of interest is overlapped with one or more peaks and does not possess an adequate pure-elution region. With three independent chromatogram data vectors (defined later by Eqs. 10 and 11), one vector (*i.e.* dimension) may be used to suppress one undesired peak common to all three vectors. The appropriate ratio technique is then applied to analyze the peak of interest within the pure-elution region that was uncovered by suppression. Previous suppression approaches sought to isolate the peak of interest for subsequent height or area analysis [15–17]. By using suppression merely to uncover a pure-elution region, fewer detection wavelengths or samples may be needed.

Suppression involves multiplicative scaling of one data vector by a suppression constant, k_q, so that the signal contribution of the component to be suppressed becomes equal with respect to a second data vector. Subsequent point-by-point subtraction of the two vectors yields a new vector, possessing no contribution from the suppressed component. Assuming linearity of the adsorption isotherm and the detector, the detected signal for a solute to be suppressed, q, is proportionally related, either between dual-wavelength chromatograms for the same injected sample, or between single-wavelength chromatograms for two sequential samples. Therefore, solute q may be suppressed using either dual-wavelength or sequential-chromatogram data as follows. If solute q is completely unknown and unsuspected, it may be suppressed spectrally without requiring identification. Employing dual-wavelength detection, the suppressed chromatogram, $S(t)_{\text{supr}}$, is given as

$$S(t)_{\text{supr}} = S^{\lambda 2}(t) - k_{q,2/1} S^{\lambda 1}(t) \tag{7}$$

Referring to Eq. 3, $k_{q,2/1}$ is equal to the ratio of absorptivities of solute q at wavelengths 2 and 1, and can be calculated from the pure-q region in $AR(t)$. Alternatively, the presence of solute q may be known or suspected in the two sequential samples, S and U. Once the identity of solute q is confirmed, as described earlier, it may be suppressed easily as follows. Using two sequential chromatograms, the suppressed chromatogram, $U(t)_{\text{supr}}$, is given as

$$U(t)_{\text{supr}} = U^{\lambda 1}(t) - k_{q,U/S} S^{\lambda 1}(t) \tag{8}$$

Referring to Eq. 6, $k_{q,U/S}$ is equal to the ratio of concentrations of solute q in samples U and S, and can be calculated from the pure-q region in $R(t)$.

A general limitation of the suppression technique is that it diminishes the S/N ratio for all other peaks in the chromatogram. Because suppression involves subtraction of two chromatograms that each contain noise, the noise level following suppression can increase by a factor of $\sqrt{2}$, assuming a random, independent noise distribution. More importantly, a fraction of the peak for each analyte is removed in the process of suppressing solute q. The percentage signal for a desired solute, j, remaining after suppression of solute q, is given by

$$\text{percentage } j \text{ remaining} = 100(1 - k_q/k_j) \tag{9}$$

For a successful analysis, the S/N ratio for the desired solute, j, must be sufficiently high after suppression. According to Eq. 9, this consideration requires that the suppression constants for solutes q and j be sufficiently different. For a single injected sample detected at two wavelengths, the restriction is

$$k_{j,2/1} = \frac{\epsilon_{j,2}}{\epsilon_{j,1}} \neq \frac{\epsilon_{q,2}}{\epsilon_{q,1}} = k_{q,2/1} \quad (10)$$

and for chromatograms of two sequential samples detected at the same wavelength

$$k_{j,U/S} = \frac{C_{j,U}}{C_{j,S}} \neq \frac{C_{q,U}}{C_{q,S}} = k_{q,U/S} \quad (11)$$

If either Eqs. 10 or 11 is violated, then the desired solute, j, is suppressed along with solute q. This necessitates care in wavelength selection for suppression using the AR technique (Eq. 10), which has been addressed [11,16,25,26]. For suppression using the SCR technique, the analogous cautions and criteria apply to analyte concentrations in sequential samples (Eq. 11). Often, when the peak to be suppressed is known or can be identified, it is potentially more feasible to select or adjust analyte concentrations than to select detection wavelengths. In particular, by employing a pure-component chromatogram of solute q as $S^{\lambda 1}(t)$ (Eq. 8), $k_{j,U/S}$ is theoretically infinite (Eq. 11) and no suppression of solute j occurs (Eq. 9). This advantage was recently demonstrated with high-speed HPLC analysis of high-fructose corn syrup for process monitoring applications [14].

3. Experimental

The chromatographic apparatus employed a Zorbax 150 × 4.6 mm, 5 μm C_{18} column (MacMod, Chadds Ford, PA, USA) and an LC-2600 syringe pump (ISCO, Lincoln, NE, USA). The detector was an ISCO V^4 single-channel UV–Vis absorbance detector, set to 0.02 or 0.05 AU sensitivity, with a flow cell having 5 mm pathlength and 3.5 μl illuminated volume. Dual-wavelength detection was approximated by recording all samples at 250 nm and then at 260 nm. These wavelengths were chosen by the method of absorbance index [25]. Injections were made using a valve (Valco, Houston, TX, USA) equipped with a 5-μl loop and an electric actuator. The eluent was approximately 8% (v/v) water in methanol, flowing at 1 ml/min. Sample mixtures were prepared by volumetric dilution of stock solutions of high-purity chemicals in HPLC-grade methanol. Typical injected concentrations are listed in Table 1.

Automated sample injection and 12-bit res-

Table 1
Solution data and results for ratio-based analysis

	Chlorobenzene	Toluene	Dibutyl phthalate
C_U (mM)	2.09	2.27	0.208
C_S (mM)	2.61	2.27	0.156
W_B (s)[a]	7.0	7.3	9.3
R_{true}[b]	0.80	1.00	1.33
R_{meas}[b,c]	0.797 ± 0.003	1.01 ± 0.01[f]	1.33 ± 0.05
AR_{ref}[d,e]	2.354 ± 0.003	2.39 ± 0.03[f]	0.358 ± 0.001
AR_{meas}[d,c]	2.34 ± 0.04	2.40 ± 0.03[f]	0.37 ± 0.04

All measured ratio values are listed ± one standard deviation (3 trials).
[a] Peak width at base.
[b] $R = (C_U)/(C_S)$ by Eq. 6.
[c] Measured value from pure-elution region in mixture chromatograms.
[d] $AR = (\epsilon_{250\ nm})/(\epsilon_{260\ nm})$ by Eq. 3.
[e] Reference value from pure-component chromatograms.
[f] After suppression of dibutyl phthalate.

olution data acquisition were described previously [13]. The S/N ratio was about 500. The data sampling rate was 54 Hz or 60 Hz, permitting small adjustments for alignment of a selected peak in sequential chromatograms [13]. Data post processing routines were written as MATLAB functions (The Math Works, South Natick, MA, USA). Baseline correction was performed as previously described [23]. The ratiograms were set to zero in regions where the detected signal fell below a noise threshold of about 3–4% of the tallest peak height. This located the pure-elution region boundary in the peak tail for both interferent suppression and analyte analysis (described later). The other "inner" pure-region boundary was located according to the increase in the s_w statistic when the boundary was extended into a coelution region. For the last study, this boundary was located instead where the first derivative of the ratiogram exceeded a threshold value indicative of horizontal (zero) slope [12]. The algorithm details for calculation of the ratio value and s_w were given previously [13]. Data post-processing requires several minutes per trial, although no attempt was made to assemble an all-inclusive, non-interactive package. All reported resolution values were obtained by measuring retention time and width at half height of isolated peaks.

4. Results and discussion

4.1. Suppression and analysis using the SCR technique

This section consists of studies that illustrate analysis of a peak of interest that is overlapped by two other peaks. We begin with the case that only single-channel detection is available, but where available knowledge of sample analytes and interferents permits identification, suppression, and subsequent analysis using the SCR technique. Two overlaid sequential chromatograms are shown in Fig. 1a, as obtained from two different samples, S and U, having analytes in common. The resolutions, R_s, were 1.10 for peaks C (chlorobenzene) and T (toluene), and 0.90 for peaks T and P (dibutyl phthalate). Although the overlap for peak T was not severe, this data will be used to examine the classical problems of ill-defined detector baseline and run-to-run changes in retention time.

The problem of baseline slopes and/or offsets for the AR technique [9,16] is equally relevant to the SCR technique. A sloping baseline correction [23] is most effective when the true baseline can be located before and after the peaks of interest. The SCR technique is also susceptible to difficulties and errors due to small run-to-run retention shifts. An adequate, albeit local retention correction may be achieved by selecting a peak, common to both chromatograms, to serve as a timing standard (TS). Provided that the TS peak is well-resolved ($R_s > 0.8$), it is easy to align the TS peak in both chromatograms, $S(t)$ and $U(t)$, by shifting them so as to minimize the weighted standard deviation, s_w, as calculated from the sequential ratiogram, $R(t)$ (Eq. 5) [13]. For this purpose, both samples in Fig. 1a contained benzene as TS (caution, known carcinogen!), but other similarly retained compounds would suffice. Deferred injection of the TS has also been practiced [27]. The sequential ratiograms in Fig. 1b show that typically occurring, uncorrected run-to-run retention shifts are usually more limiting for the SCR technique than are typical errors in baseline correction. The middle curve, offset downward by 0.35 for clarity, is $R(t)$ as obtained before chromatogram alignment using the TS peak. The retention shift shown, a representative value of only 0.1 s, severely distorted the pure-elution regions of this ratiogram. The top curve in Fig. 1b, for which the TS peak was aligned, shows good retention correction for peaks near the TS peak, as evidenced by the flat pure-elution regions for peaks C and P. In contrast, the bottom curve in Fig. 1b, offset by 0.7, is $R(t)$ as obtained from the aligned chromatograms, but where a large run-to-run error in baseline correction was simulated by first adding the dashed sloping baseline, shown in Fig. 1a, to the later chromatogram, $U(t)$. As expected [9,16], the pure-elution regions of this ratiogram were bowed upward. These baseline correction and chromatogram alignment procedures provided objective and satisfactory corrections for the reported analyses.

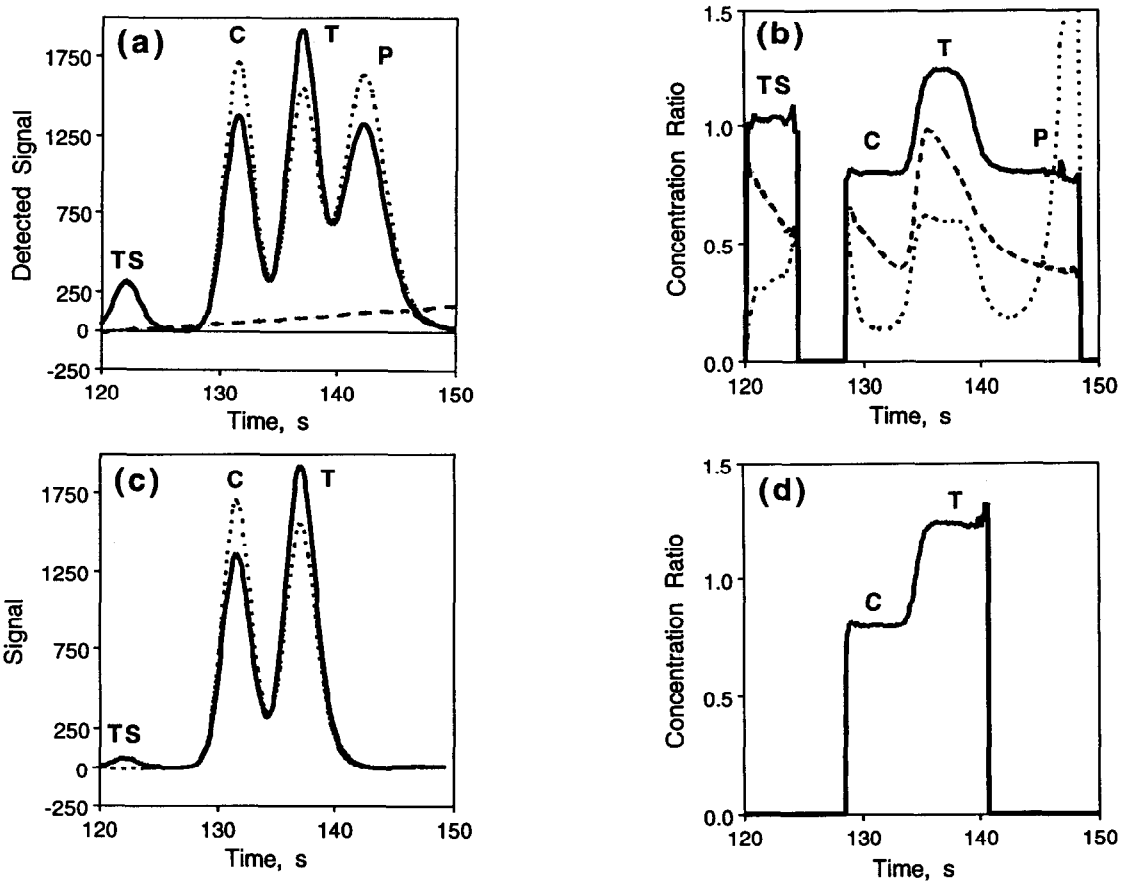

Fig. 1. (a) Overlaid sequential chromatograms of initial (S, dotted) and later (U, solid) samples. Analytes are chlorobenzene (C), toluene (T) and dibutyl phthalate (P), detected at 270 nm. The timing standard peak (TS, benzene) was used to align the chromatograms. The dashed diagonal line is a simulated baseline correction error. (b) Sequential ratiograms, $U(t)/S(t)$, from Fig. 1a after applying a noise threshold. Top curve (solid): proper baseline correction and alignment using the TS was performed prior to ratio; middle curve (dashed, offset downward by 0.35): effect of the typical 0.1-s retention shift that was then corrected (top curve) using the TS peak; bottom curve (dotted, offset by 0.7): effect of adding the sloping baseline error from Fig. 1a to chromatogram $U(t)$. (c) Suppressed chromatograms from Fig. 1a after using a pure-P chromatogram to suppress peak P via SCR. (d) Suppressed sequential ratiogram from Fig. 1c showing extended pure-elution region for analyte T.

Next, the desired analyte, T, was analyzed by first suppressing the overlapping peak, P, to extend the pure-elution region for peak T. A pure-P chromatogram (not shown) was used for suppression, which assumes that peak P was either known in advance or was identified, for example, by peak matching (demonstrated below). Following Eqs. 6 and 8, the suppression constants, $k_{P,U/S}$, were calculated from the pure-P region of sequential ratiograms for both chromatograms, $S(t)$ and $U(t)$, taken with the pure-P chromatogram. For example, the mean and standard deviation suppression constant (four replicates) for the initial solution, S, was 1.022 ± 0.003, which agreed adequately with the expected volumetric value of 1.00. The corresponding sequential chromatograms from Fig. 1a, after suppression of peak P, are shown in Fig. 1c, and $R(t)$ for these suppressed chromatograms is shown in Fig. 1d. After suppression, the pure-T region was extended from 2 to 5 s, about 70% of the baseline peak width. The relative concentration change for peak T was quantified by the weighted-average ratio value, R_j, 1.235 ± 0.002,

which agreed adequately with the expected volumetric value of 1.25. This quantitative result is resistant to errors in the interferent suppression constant. For example, if $R_s = 0.5$ for equal Gaussian peaks, the contribution of the interferent to the analyte peak height is only 13.5%. Residual interferent contribution due to suppression constant error is attenuated accordingly, and the increased residual interferent contribution in the analyte tail receives correspondingly less weight in the algorithm. In summary, suppression of peak P, using a pure-component chromatogram, improved the ability of the SCR technique to verify and then quantify the concentration increase for analyte T.

A second study examines use of single-channel data to qualitatively discriminate and identify two analytes in sequential chromatograms in the case that resolution based on differences in retention time, t_R, is essentially zero, but where significant selectivity exists because of differences in diffusion coefficient, D_m. The same analytes and conditions as in the first study were employed. For brevity, we begin with the sequential ratiogram, $U(t)/S(t)$ (Eq. 5), for two new samples, S and U. In this ratiogram, Fig. 2a, the distorted shape within the pure-T region (135 to 139 s) suggests a new interferent in sample U, rather than an increase in analyte T. The visually flat regions for components TS, C and P in Fig. 2a rule out uncontrolled baseline or timing problems, as studied in Fig. 1b. Similarly to the first study, suppression of overlapping peak(s) facilitates our goal of characterizing the composition change for the middle peak. For this simplified study, analytes C and P did not change concentration between samples S and U, consistent with the two respective flat pure-elution regions at 1.0 in $R(t)$ (Fig. 2a). Therefore, point-by-point subtraction [28] of chromatograms $U(t)$ and $S(t)$ suppresses peaks C and P (Eq. 8, $k_{q,U/S} = 1.0$). More generally, pure-component chromatogram(s) could be used to suppress peaks P and/or C from $U(t)$ and $S(t)$, as demonstrated in Fig. 1c, prior to subtraction. The difference chromatogram, labeled as DIFF in Fig. 2b (solid curve), demonstrates effective suppression of peaks C and P. Also shown in Fig. 2b are two pure-component chromatograms of analyte T (middle curve) and a suspected interferent, X (top curve, benzylbutyl phthalate). For visual clarity, these chromatograms were scaled by factors of 0.3 and 0.6 for components T and X, respectively. The t_R-based resolution of peaks T and X, 0.03, was negligible.

The peak, DIFF, which is the isolated detected response corresponding to the composition change of interest for the middle peak, can be identified by matching the DIFF peak with peaks from sequential chromatograms of standards. After applying the noise threshold [12] to DIFF, $R(t)$ was calculated for the DIFF peak taken in turn with the unscaled chromatograms of components T and X. As shown in Fig. 2c, the bowed, dotted ratiogram is DIFF/T. The flat, solid ratiogram is DIFF/X, thus giving a visual match of the DIFF peak with the interferent, X. If analyte T had changed concentration as well, neither ratiogram in Fig. 2c would have appeared flat, but concentration changes in T only or X only could be excluded. Statistical assessment of ratiogram flatness is demonstrated in the following paragraphs.

Although analytes C and P are suppressed from Fig. 2c, this data may be used to examine use of the SCR technique for identification and quantification of the DIFF peak for the case that peak C is not suppressed and that its resolution with peak T decreases due to a reduction in selectivity factor. Resolution of adjacent peaks is important for both the AR and SCR techniques because it dictates the duration and S/N ratio of the available pure-elution region, which is used for identification, suppression, and quantification. The known pure-component retention times and peak widths were used to relate R_s for peaks C and T with a left-hand boundary of the available pure-T region in both ratiograms (Fig. 2c). For example, when $R_s = 1.10$ for peaks C and T, the right tail of peak C extended to 135.2 s, thus defining the initial left pure-region boundary, labeled α in Fig. 2c. Moving this boundary, α, to the right progressively shortened the pure-T region, as though peak C were artificially moved right toward peak T. For each value of R_s for peaks C and T, the flatness statistic, s_w/

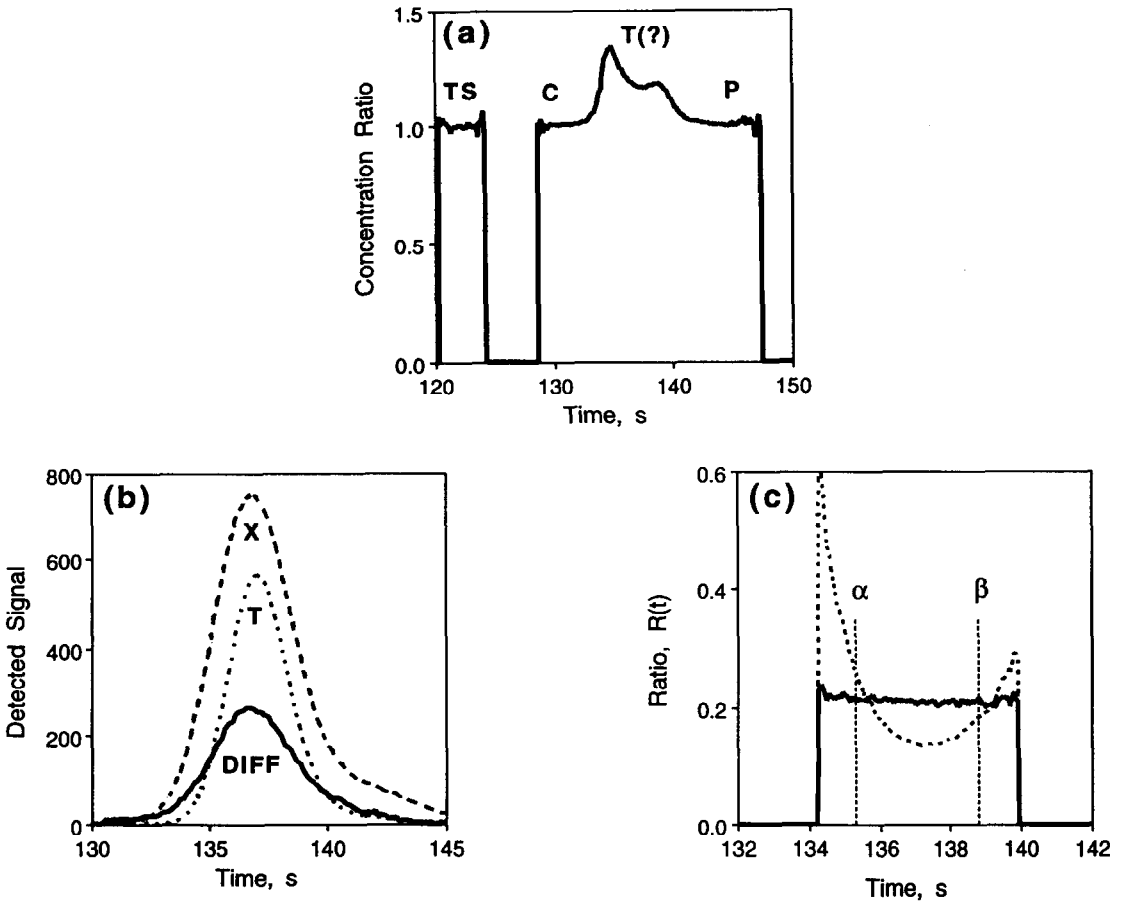

Fig. 2. (a) Sequential ratiogram, $U(t)/S(t)$, corresponding to Fig. 1b (top curve), but for different samples, S and U. The distorted appearance within the pure-T region (135–139 s) suggests a new interferent in $U(t)$. (b) Characterization of composition changes between samples S and U. Bottom curve (solid): point-by-point difference (DIFF) of the original chromatograms (not shown), which for this study suppresses peaks C and P. Also shown are scaled (see text) pure-component chromatograms of analyte T (middle curve, dotted) and a suspected interferent, X (top curve, dashed, benzylbutyl phthalate). Their t_R-based resolution was only 0.03, but the diffusion coefficient, D_m, for analyte T was twice as large. (c) Identification and subsequent quantification of DIFF, from Fig. 2b, via SCR. Solid curve: DIFF/X; dotted curve: DIFF/T. The statistical test of ratiogram flatness matched DIFF with X. Labels α and β are left boundaries of the usable part of the ratiograms for an investigation of reduced R_s of peaks C and T, for which $R_s - 1.10$ (α); 0.38 (β).

$\sqrt{1 + R_j^2}$, and the weighted-average ratio value, R_j, were calculated [13] using the portion of the ratiograms to the right of the boundary (Fig. 2c). These results are shown in Fig. 3a and b as a function of R_s for peaks C and T. In both Fig. 3a and b, the mean values (solid) are enclosed by 95% confidence intervals (dotted) for six replications. The peak identification results in Fig. 3a were that concentration changes for analytes T and X, which lacked significant t_R-based resolution relative to each other, were confidently distinguished by the significant increase in the flatness statistic that occurred for an incorrect peak match. For $R_s = 1.10$ for peaks C and T (α), the flatness statistic for DIFF/X, 0.006 ± 0.004, matched the value for replicate chromatograms of standards, 0.006 ± 0.003, consistent with the visual match of peaks DIFF and X in Fig. 2c. The corresponding value for DIFF/T was significantly larger (Fig. 3a), therefore ex-

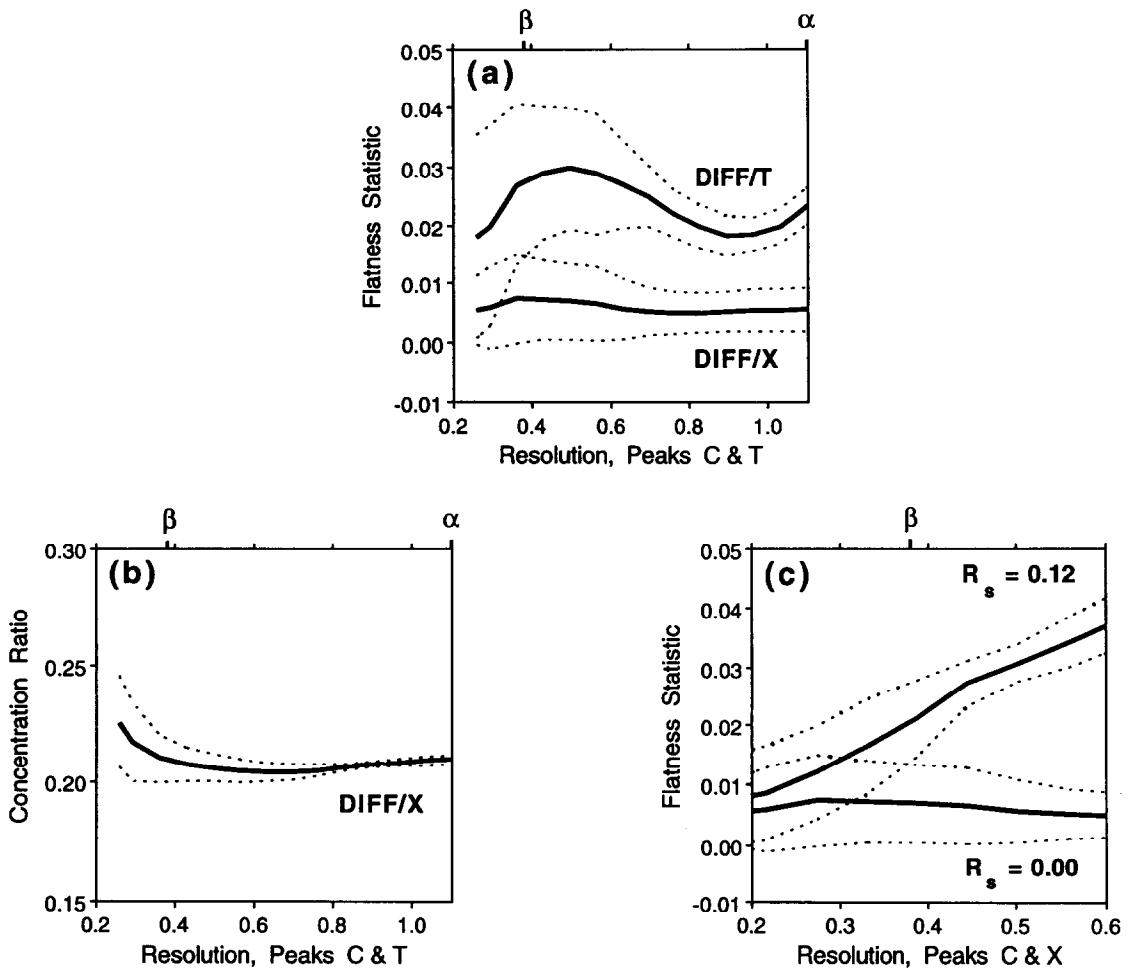

Fig. 3. Identification (a) and quantification (b) of DIFF, using the ratiograms from Fig. 2c, manipulating the data to investigate reduced R_s of peaks C and T. (a) When $R_s < 0.38$ for peaks C and T, the flatness statistic (solid) could not distinguish peaks X and T, because the 95% confidence intervals (dotted, 6 replicates) overlapped. (b) Quantification of DIFF by the weighted-average ratio value as calculated from DIFF/X (Fig. 2c). Good accuracy and precision (expected value 0.20) were maintained down to the R_s limit for identification, 0.38 (labeled β in Figs. 2c and 3). (c) Similar to Fig. 3a, except that the upper line corresponds to peak DIFF shifted to a R_s of 0.12 relative to peak X (Fig. 2b). The lower line is taken from Fig. 3a, where $R_s = 0.0$ for peaks DIFF and X. At the same R_s limit, β, for peaks C and X, the discrimination of the flatness statistic for the two-times difference in D_m for peaks X and T (Fig. 3a, $R_s = 0.03$) was equivalent to that for a t_R-based resolution of 0.12 (Fig. 3c, equal D_m).

cluding analyte T as the sole contribution to the DIFF peak. The ability to qualitatively discriminate peaks T and X became uncertain when R_s for peaks C and T fell below 0.38 (labeled β in Figs. 2c and 3), because the confidence intervals in Fig. 3a overlapped. At this point, β, the usable part of the ratiogram (Fig. 2c) was only one second wide, about 11% of the baseline peak width for X. The quantification results for the addition of interferent, X, are given in Fig. 3b by the ratio of injected concentrations, R_j, calculated over the same diminishing regions of DIFF/X as for Fig 3a. The accuracy and precision of R_j, relative to the expected volumetric value of 0.20, were satisfactory down to the observed R_s limit for identification, 0.38 (β, Fig. 3b). Drouen *et al.* [9] found a similar R_s limit for obtaining accurate absorptivity ratios and confi-

dent peak purity assessments using the AR technique. The limitations at low R_s arose from residual retention imprecision after aligning the TS peak and low S/N ratio in the peak tails, where uncertainty in locating the baseline as well as electronic or hydrodynamic "$1/f$" noise [29] can be relatively significant.

The ability to qualitatively discriminate peaks T and X, for which $R_s = 0.03$, is largely due to the peak width difference, which is a factor of 1.35, based on width at half-height. The upward bow for DIFF/T (Fig. 2c) suggests that peak X is wider than peak T, rather than merely having a small difference in t_R [13]. The larger peak width for X reflects increased band broadening due to slow mass transfer effects [30,31], because component X has a diffusion coefficient, D_m, half as large as for component T [30]. For the two solutes, T and X, having essentially equal capacity factors, k', the mass transfer contribution to peak width varies inversely with $\sqrt{D_m}$ [30,31], thus predicting a factor of 1.4 times larger peak width for component X.

This data may also be used to compare the sensitivity of discriminating peaks in sequential chromatograms for the two cases of differences in t_R and in D_m. By shifting the DIFF peak to the right to artificially create t_R-based resolution with respect to peak X, an exponential shape is produced in $R(t)$ for DIFF/X [13], which significantly increases the flatness statistic. With peaks DIFF and X shifted to a resolution of 0.12, reduced resolution of peaks C and X was investigated, using the same procedure as for Fig. 2c, which involved moving the left pure-region boundary of the ratiograms to the right from point α. The flatness statistic results for DIFF/X ($R_s = 0.12$) are plotted in Fig. 3c *versus* R_s for peaks C and X. For comparison, the results for the null case, DIFF/X ($R_s = 0.0$) from Fig. 3a, are repeated in Fig. 3c. Recall that the limit of resolution in Fig. 3a for peaks C and T, 0.38 (labeled β), referred to discrimination of peaks T and X essentially by their D_m difference. At this same resolution limit, β, for peaks C and X, we see in Fig. 3c that the t_R-based resolution introduced for peaks DIFF and X, 0.12, was just enough to yield a significant increase in the flatness statistic such that the confidence intervals did not overlap. Thus, the discrimination between peaks in sequential chromatograms due to a factor-of-two difference in D_m (Fig. 3a, $R_s = 0.03$) was equivalent to that of a t_R-based resolution of 0.12 (Fig. 3c, equal D_m). Clearly, both properties, t_R and D_m, contribute to the confidence of identification by peak matching. Considering the well-known likelihood of closely-spaced retention times for a complex mixture [3], the "extra" selectivity due to differences in D_m, especially for similarly retained non-homologous analytes or macromolecules, can increase the fraction of peaks in the mixture that are successfully analyzed [3].

4.2. Coupling the AR and SCR techniques through suppression

These studies will demonstrate improved qualitative analysis of an unknown peak via the absorptivity ratio after using sequential chromatograms to suppress a known component. Also, more reliable quantification of concentration changes for an identified component will be demonstrated after performing AR-based suppression of an interferent without requiring its identification. The data consists of chromatograms of two samples, S and U, which have different concentrations. The chromatograms were recorded at two detection wavelengths: 250 nm (Fig. 4a) and 260 nm (Fig. 4b). The resolution between peaks C and T was 1.10; between peaks T and P, 0.63. The left peak, C, served as the internal timing standard for alignment of sequential chromatograms. The absorbance ratiogram, $AR(t)$, for the later solution, U (250 nm/260 nm), and the sequential ratiogram, $R(t)$, for solutions U and S at 250 nm are shown in Fig. 5a and b, respectively. In both Fig. 5a and b, the longer and lighter ratiogram was obtained from the original chromatograms in Fig. 4a and b, whereas the shorter and heavier ratiogram, offset for visual clarity, was obtained after suppression of peak P (described later). For both $AR(t)$ and $R(t)$ (unsuppressed), peaks C and P each had adequate pure-elution regions from which to calculate the absorptivity ratios

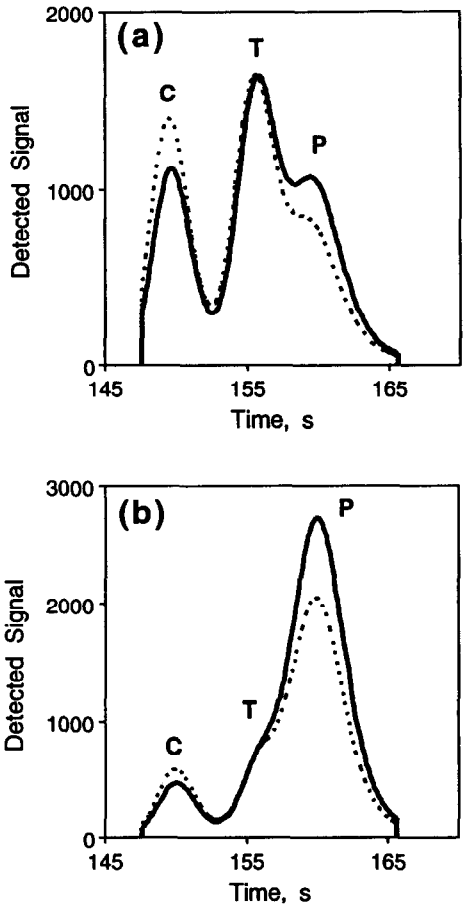

Fig. 4. Overlaid sequential chromatograms of initial (*S*, dotted) and later (*U*, solid) samples. Sample compositions are given in Table 1. Detection wavelengths, shown for both samples, were (a) 250 nm and (b) 260 nm. Application of the noise threshold is shown by the peak truncation. Peak C was used as an internal timing standard.

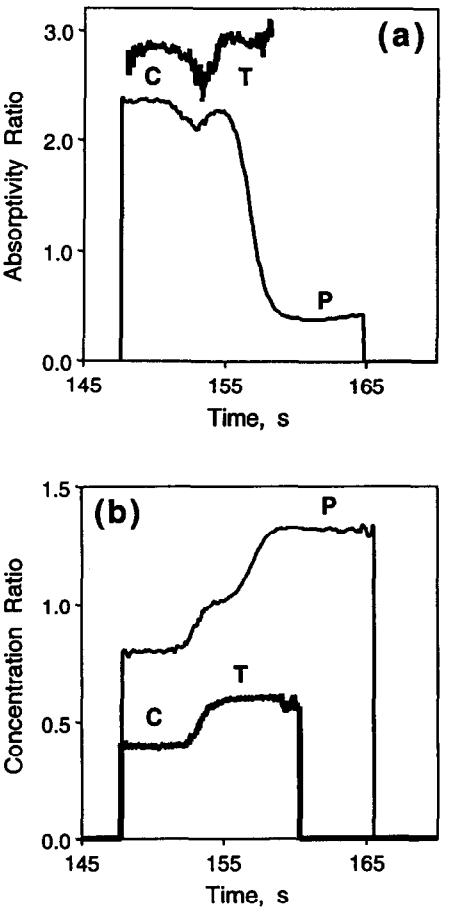

Fig. 5. Two overlaid ratiograms, before (long, light curve) and after (short, heavy curve) suppression of peak P, using only the data from Fig. 4a and b. The extended pure-elution region for peak T after suppression enables analysis of peak T: (a) absorbance ratiograms (250 nm/260 nm) for qualitative analysis, using Eq. 12 for sequential suppression (curve offset upward by 0.5); (b) sequential ratiograms ($U(t)/S(t)$ at 250 nm) for qualitative and quantitative analysis, using Eq. 13 for dual-wavelength suppression (curve offset downward by 0.4).

(Eq. 3) and the ratios of injected concentrations (Eq. 6). These results, collected in Table 1, agreed with the expected values.

Used without suppression, neither ratio technique provided qualitative or quantitative information for the middle peak, T, which lacked an adequate pure-elution region (Fig. 5a and b). By using the four chromatogram data vectors in Fig. 4a and b, any three of which are independent, peak P may be suppressed in order to facilitate analysis of peak T by extending its pure-elution region. This requires fewer runs than the earlier studies, which employed additional chromatograms of pure P for suppression. Analogous to a system of linear equations, suppression of additional peaks, if necessary, would require additional independent detection wavelengths (Eq. 10) or samples (Eq. 11). Sufficient resolution between adjacent peaks, studied in Fig. 3, would also be required for peak identification and subsequent calculation of the suppression constants.

Qualitative analysis of peak T in the dual-

wavelength domain (AR) is preferred when peak T is a true unknown and the absorptivity ratio is desired for identification. However, suppression of peak P· using sequential chromatograms (SCR) implies that peak P is known or can be matched in both chromatograms and that its concentration varies independently from that of peak T (Eq. 11). Combining Eqs. 2 and 8, the absorbance ratiogram after suppression of peak P, $AR_{supr}(t)$, is given as

$$AR_{supr}(t) = \frac{U^{250}(t) - k_{P,U/S}S^{250}(t)}{U^{260}(t) - k_{P,U/S}S^{260}(t)} \quad (12)$$

where the suppression constant, $k_{P,U/S}$, (1.33) was calculated from the pure-P region of the unsuppressed sequential ratiogram, Fig. 5b. For $AR_{supr}(t)$ (Eq. 12), shown in Fig. 5a (heavy line, offset upward by 0.5), the pure-T region has been extended from 1 to 3.5 s, about 48% of the baseline peak width. The dip in the signal at 154 s may be attributed to low signal in the valley between peaks C and T, especially for 260 nm (Fig. 4b). The absorptivity ratio for peak T obtained after suppression, 2.40 ± 0.03 (three replicates), agreed with the reference value obtained from chromatograms of pure T, 2.39 ± 0.03.

On the other hand, quantitative analysis of peak T in the sequential-chromatogram domain (SCR) implies that peak T is identified in both samples or that its presence can be confirmed, as demonstrated earlier by peak matching. In this case, suppression of peak P using dual-wavelength data (AR) does not require its identification, only that the absorptivity ratios of peaks P and T differ (Eq. 10). Combining Eqs. 5 and 7, the sequential ratiogram after suppression of peak P, $R_{supr}(t)$, is given as

$$R_{supr}(t) = \frac{U^{250}(t) - k_{P,250/260}U^{260}(t)}{S^{250}(t) - k_{P,250/260}S^{260}(t)} \quad (13)$$

where the suppression constant, $k_{P,250/260}$, (0.37) was calculated from the pure-P region of the unsuppressed absorbance ratiogram (Fig. 5a). For $R_{supr}(t)$ (Eq. 13), shown in Fig. 5b (heavy line, offset downward by 0.4), the pure-T region has been extended to 5.8 s, about 80% of the baseline peak width. The concentration ratio for peak T obtained after suppression, 1.01 ± 0.01, agreed with the expected volumetric value of 1.00.

Note that the precisions of the R and AR values in Table 1 are for a limited data set. For instance, the average percent relative standard deviation (R.S.D.) for R_{meas} is 1.7% with a range of R.S.D. of 0.4 to 3.8%. A previous detailed examination of the precision of the SCR technique [13] revealed that the average R.S.D. was 3% for ten replicate pairs of an analyte peak. Since the SCR technique has the benefit of data averaging when a flat line is fit to a pure-elution region, the SCR technique precision should be limited in much the same way that peak area precision is limited. Comparison of the SCR technique precision level to conventional peak area and height measurements is useful in this regard. A cooperative study comparing the precision of peak height and area measurements in liquid chromatography indicated, as reported in Tables II and VII in ref. 32 as edited data for four test analytes, that the R.S.D. was $2.7\% \pm 1.0\%$ for peak area and $4.2\% \pm 1.1\%$ for electronic peak height measurements [32]. It is reasonable to conclude that the R.S.D. of the R_{meas} values in Table 1, while a bit low at 1.7% on average, are consistent with the precision results of the peak area cooperative study, $2.7\% \pm 1.0\%$ [32], and also with our previously reported precision for the SCR technique, 3% R.S.D. [13]. For the case of resolved chromatographic peaks, peak height and peak area measurements are easier to apply than the SCR technique. The role of the SCR technique is to facilitate analysis of ill-resolved small groups of peaks, possibly simplified by peak suppression, in which the remaining interferents are not known so that only the pure-elution region of the peak of interest is available for analysis, and peak height and peak area measurements are not feasible [12–14].

In comparing the two suppressed ratiograms (Fig. 5a and b), the implication of Eq. 9 is seen from the increased noise and the earlier peak T cut-off in $AR_{supr}(t)$ (Fig. 5a). The percentage

signal for peak T remaining after suppression of peak P, using Eq. 9 with suppression constants from Table 1, was 33% for $AR_{supr}(t)$ and 85% for $R_{supr}(t)$. This distinction reflects the large relative difference in absorptivity ratios for peaks P and T, whereas the concentration ratios were more similar (Table 1). Although the percentage signal for peak T, remaining after dual-wavelength suppression of peak P, was fixed by wavelength selection, the corresponding percentage, remaining after sequential-chromatogram suppression, would be increased if a pure-P chromatogram had been used for suppression.

These studies raise several issues for further work. Diffusion effects are important for qualitative analysis, as studied in Figs. 2c and 3a, and also for quantitative analysis, because diffusion-controlled peak width affects the duration and S/N ratio of the pure-elution region. Because k' and D_m describe somewhat independent thermodynamic and kinetic processes, respectively, the low resolution limits for any chromatographic data analysis technique depend on both analyte properties. Although the simple timing standard concept often works well as demonstrated, run-to-run variations in eluent temperature or flow rate can change peak-to-peak spacing in sequential chromatograms, becoming more severe with increasing distance from the TS peak. The relative importance of these detrimental effects increases with increasing plate count, holding k' constant. High-speed HPLC can reduce both the magnitude and the effect of flow-rate and temperature fluctuations, which are dominated by large low frequency components [29]. We are currently examining the trade-off of analysis time, resolution, and retention precision, as well as the role of solute diffusion rate, for extracting the needed qualitative and quantitative chemical information in the shortest analysis time.

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6. References

[1] J.B. Callis, D.L. Illman and B.R. Kowalski, *Anal. Chem.*, 59 (1987) 624A–637A.
[2] C.N. Renn and R.E. Synovec, *Anal. Chem.*, 60 (1988) 200–204.
[3] J.M. Davis and J.C. Giddings, *Anal. Chem.*, 55 (1983) 418–424.
[4] B.R. Kowalski and M.B. Seasholtz, *J. Chemom.*, 5 (1991) 129–145.
[5] A.N. Papas, *Crit. Rev. Anal. Chem.*, 20 (1989) 359–404.
[6] A.H. Anderson, T.C. Gibb and A.B. Littlewood, *J. Chromatogr. Sci.*, 8 (1970) 640–646.
[7] I. Berglund and P.K. Dasgupta, *Anal. Chem.*, 64 (1992) 3007–3012.
[8] A.F. Fell, H.P. Scott, R. Gill and A.C. Moffat, *J. Chromatogr.*, 282 (1983) 123–140.
[9] A.C.J.H. Drouen, H.A.H. Billiet and L. de Galan, *Anal. Chem.*, 56 (1984) 971–978.
[10] P.C. White and T. Catterick, *J. Chromatogr.*, 402 (1987) 135–147.
[11] E.L. Inman, M.D. Lantz and M.M. Strohl, *J. Chromatogr. Sci.*, 28 (1990) 578–583.
[12] R.E. Synovec, E.L. Johnson, T.J. Bahowick and A.W. Sulya, *Anal. Chem.*, 62 (1990) 1597–1603.
[13] T.J. Bahowick and R.E. Synovec, *Anal. Chem.*, 64 (1992) 489–496.
[14] D.R. Dunphy and R.E. Synovec, *Talanta*, 40 (1993) 775–780.
[15] G.T. Carter, R.E. Schiesswohl, H. Burke and R. Yang, *J. Pharm. Sci.*, 71 (1982) 317–321.
[16] P.A. Webb, D. Ball and T. Thornton, *J. Chromatogr. Sci.*, 21 (1983) 447–453.
[17] J.G.D. Marr, P. Horvath, B.J. Clark and A.F. Fell, *Anal. Proc.*, 23 (1986) 254–256.
[18] J.C. Giddings, in H.J. Cortes (Editor), *Multidimensional Chromatography —Techniques and Applications*, Marcel Dekker, New York, 1990, Ch. 1, p. 5.
[19] E.V. Dose and G. Guiochon, *Anal. Chem.*, 62 (1990) 174–181.
[20] M.S. Jeansonne and J.P. Foley, *J. Chromatogr.*, 461 (1989) 149–163.
[21] J.A. Jonsson, *Chromatographia*, 18 (1984) 427–433.
[22] N. Ostojic, *Anal. Chem.*, 46 (1974) 1653–1659.
[23] R.E. Synovec and E.S. Yeung, *Anal. Chem.*, 57 (1985) 2162–2167.
[24] M. Sharaf, G. Arroy and R. Perkins, *J. Chemom.*, 5 (1991) 291–298.
[25] A.F. Poile and R.D. Conlon, *J. Chromatogr.*, 204 (1981) 149–152.

[26] F.V. Warren, Jr., B.A. Bidlingmeyer and M.F. Delaney, *Anal. Chem.*, 59 (1987) 1897–1907.
[27] C.L. Guillemin, *J. Chromatogr.*, 441 (1988) 1–12.
[28] B. Wiese, *J. Pharm. Biomed. Anal.*, 7 (1989) 79–93.
[29] C.N. Renn and R.E. Synovec, *Anal. Chem.*, 60 (1988) 1829–1832.
[30] E.D. Katz and R.P.W. Scott, *J. Chromatogr.*, 270 (1983) 28–50.
[31] F. Erni, *J. Chromatogr.*, 282 (1983) 371–383.
[32] R.E. Pauls, R.W. McCoy, E.R. Ziegel, T. Wolf, G.T. Fritz and D.M. Marmion, *J. Chromatogr. Sci.*, 24 (1986) 273–277.