Detecting Coeluted Impurities by Spectral Comparison

Impurities can go undetected during chromatographic analyses if the resolution is too low for single-wavelength detectors to reveal shoulders and valleys. Complete spectral data, obtained by using a photodiode-array detector, provides a greater level of information about seemingly homogeneous peaks. Coeluted compounds in these peaks can be detected by comparing the apex spectrum mathematically with the spectra at each time point across a peak. These spectral comparisons take into account changes in spectral shape caused by noise and other nonideal effects inherent in the measurement process, thereby identifying only real spectral changes caused by the presence of more than one compound. This article presents specific examples showing the sensitivity and the limits of coelution detection.

Marc V. Gorenstein, Jeanne B. Li, John Van Antwerp, and Dudley Chapman
Waters Corporation, 34 Maple Street, Milford, Massachusetts 01757, USA

Address correspondence to Marc V. Gorenstein.

Coelution is a fact of life in liquid chromatography and so is the requirement to detect coeluted impurities. New techniques for collection and comparison of complete spectral data can make purity assessment much easier for chromatographers.

High performance liquid chromatography (HPLC) systems equipped with a UV–vis absorbance detector tuned to a single wavelength can detect a coelution if it causes a shoulder or a valley in a chromatogram. However, if the chromatographic resolution, the peak-height ratio, or both are too small, then the shape of a fused peak will be practically indistinguishable from that of a baseline-resolved peak.

Photodiode-array detectors can record a complete UV–vis absorbance spectrum at each time point during a chromatographic separation. A pure, baseline-resolved compound forms a peak with all spectra having the same shape. In contrast, two compounds that are coeluted (and have different spectral shapes) will form a peak containing spectra that gradually change shape across the elution profile. Thus, comparing the spectra within a peak can reveal coeluted compounds. Many researchers have proposed methods to detect coelutions by the mathematical comparison of spectra from a chromatographic peak (1).

We developed a mathematical approach to spectral comparison that provides a means for detecting coelution quantitatively (2–4). Called the *spectral-contrast technique*, our method uses all absorbance information between peak lift-off and touchdown to analyze the spectral heterogeneity within a peak. Furthermore, the technique estimates the magnitude of spectral heterogeneity resulting from noise and other nonideal effects. The presence of coelution is indicated only when the observed spectral differences are greater than those of the measurement process. These comparisons are sensitive enough to reveal coelution at a chromatographic resolution that is too low to produce a shoulder or valley feature in single-channel detection.

DETECTING COELUTION

Limits of single-channel methods:

The detection of coelution using a single-wavelength UV–vis absorbance detector depends on visual identification of a valley or shoulder feature. Three numerical-simulation examples show the effect of coelution on peak profiles. Figure 1 shows a valley that is the product of a 2:1 absorbance–height ratio between compounds with a chromatographic resolution of 0.7. At a height ratio of 5:1, the valley vanishes, but a shoulder remains. At height ratio of 30:1, the shoulder is gone, and a bell-shaped profile remains. Hence, chromatographic resolutions that yield evidence for coelution at one height ratio may not reveal coelution at a more extreme height ratio.

Figure 2 shows the relationship between chromatographic resolution, peak height ratio, and coelution profile for a two-compound coelution. Again, we derived these results from numerical simulations in which each peak was modeled as an exponentially modified Gaussian peak, with time constant equal to the root mean square width of the underlying Gaussian peak. This plot shows that the minimum peak-height ratio necessary for revealing a coelution increases dramatically as resolution decreases. More importantly, no shoulder or valley appears, regardless of peak-height ratio, when the chromatographic resolution is <0.4.

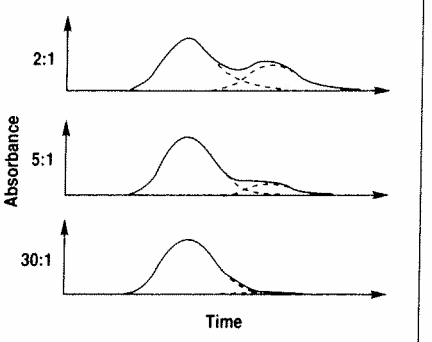

FIGURE 1: Single-channel detection at varied height ratios. The solid line is the trace that a single-channel detector would record. The dashed lines are the absorbance traces that each compound would produce in the absence of the other.

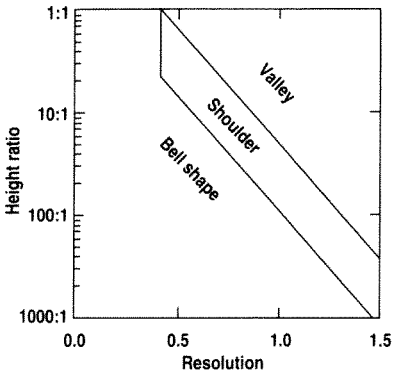

FIGURE 2: Plot of peak-height ratio versus resolution for a two-compound coelution.

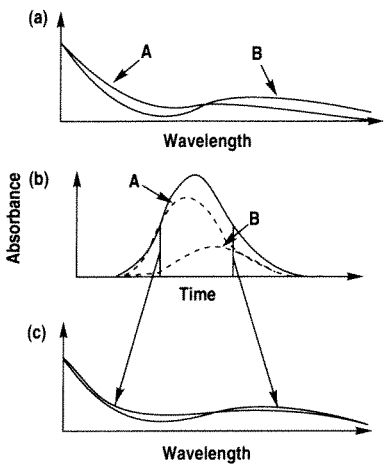

FIGURE 3: Numerical simulation of spectral shape variation resulting from coelution. Shown are plots of (a) absorbance spectra of compounds A and B; (b) a chromatographic profile from the elution of those compounds, in which the dashed lines are the individual profiles of A and B and the solid line is the fused-peak profile; and (c) two spectra from the peak, one from the up slope, and one from the down slope (indicated by arrows). The peak-height ratio and resolution for this separation are 2:1 and 0.2, respectively.

Detecting coelutions by spectral comparisons: If two compounds have different spectral shapes and are eluted with low, nonzero, chromatographic resolution, they will produce a peak that has uniquely shaped spectra at different time points. Comparing spectra within a peak can be a sensitive method for detecting coelution. As illustrated in Figure 3, two coeluted compounds (with only modest differences in spectral shape) are distinguishable by comparing the shape of the fused peak's up- and down-slope spectra.

Detector noise, photometric error, and solvent effects can also reshape spectra. Because of these nonideal effects, all absorbance spectra collected by a photodiode-array detector have different shapes at some level. To verify a detected coelution, the observed level of spectral heterogeneity must exceed that produced by nonideal effects.

Spectral-contrast and purity angles: We used a two-step spectral-contrast technique. First, we normalized and compared the peak spectra quantitatively, obtaining values termed the spectral-contrast angles and the purity angle. The purity angle is a measure of the spectral heterogeneity of a peak. A spectral-contrast angle measures the shape difference between two spectra. Next, we quantified the nonideal effects, which provided a value called the threshold angle, which can verify a detected coelution when compared with the purity angle. All of these mathematical calculations are based on vector algebra.

We used a series of four calculations to determine the spectral-contrast angles and the purity angle. First, we performed baseline correction for each spectrum within a peak. We interpolated lift-off and touchdown spectra to obtain a series of baseline spectra. Then, we subtracted the resulting baseline spectra from corresponding peak spectra to remove absorbance caused by the mobile phase.

We can view these baseline-corrected absorbances as elements of a matrix B, where the absorbance at wavelength i and sample time j is the matrix element B_{ij}. The index i (which ranges from 1 to N) refers to one of N wavelengths recorded by the photodiode-array detector, and the index j (which ranges from 1 to M) refers to one of the M spectra recorded in the peak. Equivalently, we can describe B as a collection of column vectors, where the vector $\vec{B}_j$ contains the elements of the jth column of B, corresponding to the spectrum collected at the jth time point.

Second, we compared the apex spectrum with all the other spectra in the peak. We chose the spectrum from B with the highest absorbance at a reference wavelength as the apex spectrum. We designated this spectrum as $\vec{A}$, where the absorbance elements of $\vec{A}$ are labeled A_i. To compare the shape of spectrum $\vec{A}$ with the shape of spectrum $\vec{B}_j$ we first computed a normalizing factor s_j. This factor, when applied to $\vec{A}$, normalized this spectrum so that it matched $\vec{B}_j$ in a least-squares sense.

Third, we computed the difference in direction between the normalized apex spectrum, $s_j\vec{A}$, and the spectrum, $\vec{B}_j$. This calculation yields a spectral-contrast angle θ_j for that particular time point on the peak.

We evaluated the angle according to the following relation:

$$\sin\theta_j = \frac{\sqrt{\sum_{i=1}^{N}\left(B_{ij} - s_j A_i\right)^2}}{\sqrt{\sum_{i=1}^{N} B_{ij}^2}} \qquad [1]$$

The angle θ_j measures the shape difference between the two absorbance spectra. The smaller the angle, the smaller the shape difference; the larger the angle, the larger the shape difference. The spectral-contrast angle is 0° when the shapes of the apex spectrum and the spectrum j are virtually identical. When the absorbances have no overlap — that is, when the spectral shapes are maximally different — the spectral-contrast angle is at its maximum value, 90°.

The least-squares solution adjusts s_j minimizing the length of the vector $\vec{R} \equiv \vec{B}_j - s_j\vec{A}$. After minimization, the length of $\vec{R}$ measures any shape variation between $\vec{B}_j$ and $\vec{A}$ within the wavelength range of the measurement. For small shape differences, the angle θ_j, as defined by equation 1, is proportional to the length of $\vec{R}$, and thus, θ_j is a linear measure of dissimilarity. Figure 4 illustrates the relationships between $\vec{A}$, $s_j\vec{A}$, $\vec{B}_j$, $\vec{R}$, and θ_j.

In practical terms, spectral-contrast angles correspond roughly to an average fractional difference in peak shape. For example, a spectral-contrast angle of 10° for spectrum $\vec{B}_j$ means that the absorbances of spectrum $\vec{B}_j$ differ in value from the normalized absorbances in the apex spectrum on average by ~10% at the corresponding wavelengths. The point where we could see a visual difference between overlaid spectra was at a 1° spectral-contrast angle.

We then computed an overall purity angle, ϕ, by combining the results from all the comparisons involving the apex spectrum, as follows:

$$\sin\phi = \frac{\sqrt{\sum_{j=1}^{M-1}\left|\vec{B}_j\right|^2\sin^2\theta_j}}{\sqrt{\sum_{j=1}^{M-1}\left|\vec{B}_j\right|^2}} \qquad [2]$$

where

$$\left|\vec{B}_j\right|^2 \equiv \sum_{i=1}^{N} B_{ij}^2 \qquad [3]$$

is the square of the euclidean length of $\vec{B}_j$. The summation is for all spectra within the peak, except for the apex spectrum. This expression shows that contrast angles from highly absorbing spectra are weighted more heavily in the computation of the purity angle. Again, a purity angle of 0° represents an absence of spectral heterogeneity within the peak, and a purity angle of 90° indicates the maximum possible degree of heterogeneity. Equation 1 can be used to compare an analyte spectrum to library spectra. For such comparisons, the sensitivity and reproducibility of the spectral-contrast angle can be 0.1° or less (5).

EXPERIMENTAL

Calculating the purity angle for a two-compound coelution: Whether implemented by single-channel or spectral methods, coelution detection is an exclusionary technique that can prove that a peak contains a coelution but not that it is pure. To be useful, an exclusionary technique must be able to detect low-level interferences. To illustrate how the purity angle responds to a low-level coeluted compound, we performed experiments that used a series of two-compound mixtures (6). In all samples, we fixed the concentration of one compound, ethyl-PABA (ethyl-*p*-aminobenzoate, Sigma Chemical, St. Louis, Missouri), so its absorbance was 0.5 AU at 195 nm, corresponding to its absorbance maximum. We varied the concentration of the other com-

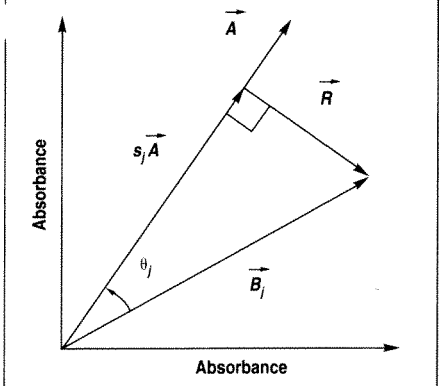

FIGURE 4: Spectral vectors and spectral-contrast angles.

pound, ethylparaben (ethyl-*p*-hydroxybenzoate, Aldrich Chemical Co., Milwaukee, Wisconsin), by factors of 2 over the height-ratio range of 1:1 to 10,000:1. We computed the height ratios from the compounds' absorbances, as measured at their respective wavelengths of maximum absorbance, in the absence of coelution.

Our HPLC system consisted of a model 996 photodiode-array detector, Millennium 2010 Chromatography Manager software, a model 717 autosampler, and a model 600 solvent-delivery system (all from Waters, Milford, Massachusetts). We used a 7.5 cm × 3.9 mm C18 Nova-Pak column (Waters). The photodiode-array detector collected 1 spectrum/s from 190 to 350 nm at 1.2-nm resolution. We used a 300:700:0.2 acetonitrile–water–phosphoric acid mobile phase with a 1 mL/min flow rate and an injection volume of 15 µL. We obtained the HPLC-grade acetonitrile and the phosphoric acid from J.T. Baker (Phillipsburg, New Jersey) and used water purified by a Milli-Q water-purification system (Millipore Corporation, Bedford, Massachusetts).

We chose these chromatographic conditions to ensure that the compounds were eluted with a fixed 0.3 chromatographic resolution. Figure 5 shows an overlay of the ethyl-PABA and ethylparaben peak profiles and spectra. The spectral-contrast angle between pure ethyl-PABA and pure ethylparaben is 53°.

Figure 6 shows purity plots from the analysis of pure ethyl-PABA standard (Figure 6a) and a mixture with a 10:1 height ratio (Figure 6b). In each plot, the spectral-contrast angles between the apex spectrum and the peak spectra are plotted as a solid line. The purity plot of the standard shows little spectral heterogeneity within the heart of the peak, whereas the purity plot of the mixture shows significant heterogeneity on the down slope. The dashed line represents a prediction of the contrast angle caused by detector noise alone, as discussed below. The purity angle is 0.11° for the pure ethyl-PABA and 4.1° for the mixture. For both peaks, we compared the respective purity angles with the threshold angle of 0.14°, as discussed below.

We performed a series of dilutions comprising three replicate injections for each of 12 dilutions. Figure 7 shows these 36 purity angles plotted against the respective height ratios of the coeluted compound. In addition to the dilution series, we made six replicate injections of the pure PABA standard and plotted their purity angles to the left of the break. Below a height ratio of 500:1, the purity angles from the dilution series are essentially constant in value and equal to the purity angles of the pure PABA standard. Above the height ratios of ~500:1, the purity angles increase as the concentration of the coeluted compound increases. The data demonstrate that the coeluted ethylparaben can be detected at a height ratio of 300:1 because at this ratio (and above) the purity angle exceeds the threshold angle, as described below.

Next, we examined the two major features of this curve: the nearly linear increase in purity angles above the height ratio of 250:1 and the nearly constant value of purity angles below the height ratio of 500:1.

Theory of purity-angle calculation for a two-compound coelution: For a two-compound coelution, a peak's purity angle depends not only on the compounds' height ratio but also on the chromatographic resolution and spectral-contrast angle. An approximate relationship between purity angle ϕ, chromatographic resolution R, height ratio H, and spectral-contrast angle θ_0 is

$$\phi = 2HR\theta_0 \qquad [4]$$

This approximation is valid for small values in each quantity — for example, $0 < H < 0.4$, $0 < R < 0.4$, and $0 < \theta_0 < 60°$. For the dilution series in which we fixed R at 0.3 and θ_0 at 53°, Equation 4 gives the relationship $\phi \approx 30H$. This linear relationship between purity angle and height ratio is confirmed by the data presented in Figure 7 over the range of height ratios from 250:1 to 10:1. For example, for a 10:1 height ratio, H is 0.1, and ϕ is 3°.

Purity angle and nonideal measurement effects: The purity angles obtained from the replicate injections of the pure ethyl-PABA standard vary from 0.107° to 0.120°. How could baseline-resolved peaks of a standard have nonzero purity-angle values? Because even after baseline correction, absorbance spectra will always be somewhat distorted due to the combined effects of detector noise and photometric error. Because of these nonideal measurement effects, all chromatographic peaks are spectrally heterogeneous at some level (7).

How can we use the purity angle as a coelution indicator if its value is always greater than zero? The key is to realize that the resulting purity angles will cluster around a single value for a fixed method and replicate injections of a standard at a single concentration. A histogram of these purity angles from a single standard will show reproducible mean values and reproducible distributions about the mean.

If our goal is to assess the peak purity of a compound that is always injected at the same concentration, C, and for which we have a

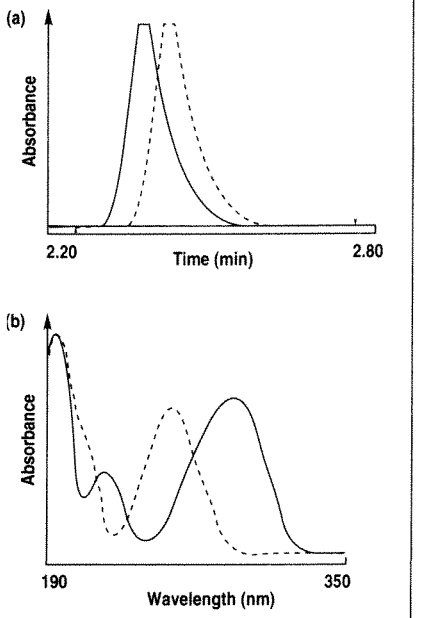

FIGURE 5: Overlay of (a) chromatograms and (b) absorbance spectra of pure ethyl-PABA (solid line) and pure ethylparaben (dotted line).

standard, then we can conceptualize a protocol for sensitive peak-purity determination. We could perform a large number of injections of the standard at the concentration C and adopt the largest purity-angle value from this population as a threshold angle. We could then use this threshold to assess the spectral homogeneity by comparing a sample's purity angle to the threshold. A purity angle larger than the threshold is positive evidence that the sample contains one or more coeluted compounds. A purity angle less than this threshold provides no evidence that the sample contains a coeluted compound because the purity angle is consistent with that of the standards.

DETERMINATION OF THE THRESHOLD ANGLE

We developed a practical method for obtaining threshold angles that analysts can apply to a range of sample concentrations, assuming a standard is available. Because a sample's peak height can vary, it is necessary to compute its threshold angle from the sum of two angles, one of which varies in inverse proportion to the sample's peak height and one of which is held constant.

The portion of the threshold angle that varies with peak height derives from the effect of detector noise on spectral heterogeneity. As the concentration of a chemically pure compound decreases, the purity angle of its peak increases because the detector noise–spectral absorbance (noise-to-signal) ratio increases as the spectral absorbance of the compound decreases. The correct computation of the variable angle requires the identification within the chromatogram of a baseline region that is free of peaks and contains only baseline noise. The

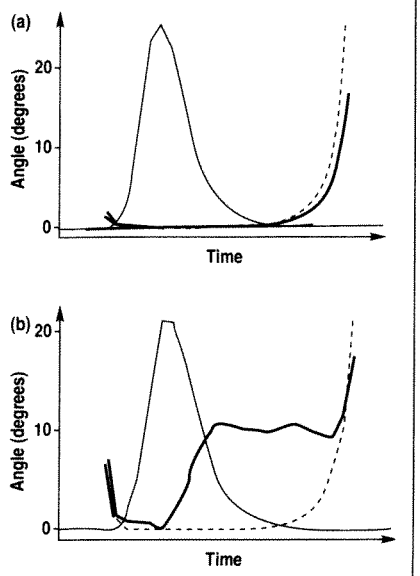

FIGURE 6: Purity plots for (a) one- and (b) two-compound peaks. Purity-angle (solid line) and noise (dotted line) data are plotted with chromatograms.

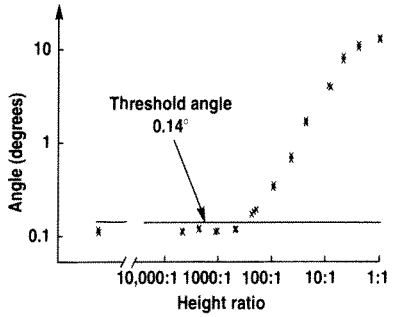

FIGURE 7: Plot of purity angles versus height ratio for a dilution series of ethyl-PABA and ethylparaben.

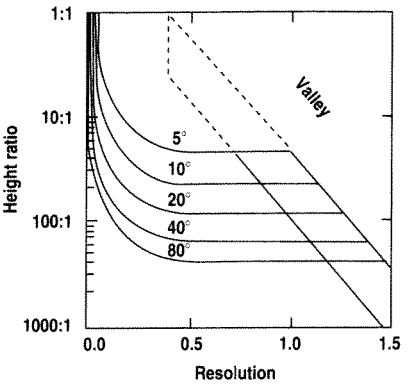

FIGURE 8: Two-compound coelutions that yield a purity angle of 0.25°. Each solid contour line corresponds to a spectral-contrast angle.

spectral-contrast algorithm implemented in the Millennium software uses these spectra and the spectra from the compound peak to compute this variable angle, which we called the peak's noise angle. This noise angle is a prediction of the largest possible purity angle caused by the effects of detector noise. Its value varies in inverse proportion to the sample's peak height.

The constant portion of the threshold angle corresponds to the purity angle derived from a combination of solvent effects and photometric errors. The second calibration step requires a chemically pure standard to assess peak purity. We recommend that chromatographers inject six replicates of the standard at the highest compound concentration for which they want a purity measurement. As a general rule, this maximum concentration should yield a maximum spectral absorbance of <1 AU (ideally ~0.5 AU) at all wavelengths within the spectral range of the measurements. We adopted the largest purity angle obtained from this series of calculations as the constant part of the threshold angle, which we called the solvent angle. The solvent angle represents the maximum heterogeneity that both photometric error and solvent effects can produce at all concentration levels lower than the concentration at which the standard was injected.

DETECTION OF COELUTION IN A TWO-COMPOUND MIXTURE

In the model system described above, the purity angles for the six replicate injections of the ethyl-PABA standard ranged from 0.107° to 0.120°. We used the highest value, 0.12°, as the solvent angle. For each sample injection, the software computed the threshold angle as a sum of the solvent and noise angles. At the height ratio of 250:1, we computed the noise angle to be 0.02°, resulting in a threshold angle of 0.14°.

In Figure 7, the computed value of the threshold angle is plotted for each injection and appears as a nearly horizontal line. This horizontal line intersects the curve traced by the purity-angle measurements at a height ratio of about 300:1. Thus, if coeluted ethylparaben exceeds this height ratio, the resulting purity angle will exceed the threshold angle, and the ethylparaben will be detected.

In our model system, we injected the samples at a fixed concentration that corresponded to the 0.5-AU absorbance maximum. However, the 0.12° solvent angle that we obtained from this system also is valid for injections of ethyl-PABA samples at concentrations with <0.5-AU maximum absorbance.

How sensitive is the purity angle for two-compound coelutions of compounds other than ethyl-PABA and ethylparaben and at chromatographic resolutions other than 0.3? A two-compound coelution produces a spectrally heterogeneous peak only if the compounds' spectra have different shapes and their peaks have nonzero chromatographic resolution. How different do two spectra have to be and how much chromatographic resolution do their peaks need to have to produce a peak containing a detectable level of spectral heterogeneity? To answer these questions, we performed a numerical simulation of a two-compound coelution to see what combinations of height ratio, chromatographic resolution, and spectral-contrast angle yielded a given purity-angle value.

Figure 8 shows the parameter combinations that yield a 0.25° purity angle. These contours can be interpreted as detection limits for a 0.25° threshold angle. For example, at a chromatographic resolution of 0.2, a coelution will produce a >0.25° purity angle if the spectral-contrast angle is ≥40° and the height ratio is ≥100:1. We overlaid the plot from Figure 2 to show the comparative limits of detection for single-channel methods. The results from the dilution series and from this simulation demonstrate how spectroscopic techniques exceed the capabilities of single-channel detectors for detecting coeluted impurities.

CONCLUSION

All measurement techniques contribute to our ability to understand a situation or to solve a problem. Collecting complete spectral data and comparing the spectra within a peak mathematically is an effective way to assess peak purity. By quantifying the spectral heterogeneity of a peak with a purity angle and establishing a threshold-angle value that accounts for non-ideal effects, we have developed a sensitive method for detecting low levels of coeluted impurities.

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