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Effect of spectral resolution, detector linearity and chromatographic resolution on peak purity calculations

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Abstract

The detection of inhomogeneity in chromatographic peaks is one of the principal benefits of photodiode array detection for HPLC. The peak homogeneity can be estimated by comparing the angles between the vector representations of the instantaneous spectrum and the peak apex spectrum, and the instantaneous spectrum and the spectral noise. When the spectral or purity angle exceeds the noise angle, the peak is not homogeneous because the differences between the instantaneous spectra and the apex spectrum cannot be explained by statistical variation of the apex spectrum. In this paper, we consider the impact of detector linearity and slit width on the purity (homogeneity) measurements of a separation of a series of compounds related to vanillin. The peak purity angle, i.e., the spectral contrast of spectra within a chromatographic peak, varies with the maximum absorbance. At small maximum absorbances (less than 0.1 AU), the variation in purity angle is dominated by system noise. At large maximum absorbances (greater than 0.5 AU), the variation in purity angle is dominated by the photometric uncertainty. The smallest measurable purity angle for a homogeneous peak is inversely proportional to the slit width and spectral bandpass of the polychrometer of the photodiode array detector.

1. Introduction

The principal benefits of photodiode array detection (PDA) in high-performance liquid chromatography include confirmation of peak identity by comparison with reference spectra [1] and detection of co-elution of compounds which have different UV–Vis absorbance spectra [2,3]. The application of PDA to the validation of pharmaceutical analyses has recently been reviewed [4].

Several measures of spectral dissimilarity have been proposed to aid in the recognition of co-elution. These include comparison of absorbance ratios at one or more pairs of wavelengths [5], monitoring changes in the absorbance-weighted average wavelength [6], overlaying spectra taken on the upslope, apex and downslope of the peak [7], and computing the angle (or its sine and/or cosine) between the n-dimensional vector representations of the spectra to be compared [8,9]. The purity angle, i.e., the angle between the n-dimensional representations, has several advantages which include: (a) all of the wavelengths contribute to the spectral comparisons, (b) the spectral noise can also be represented as a noise vector which allows the use of hypothesis testing for the significance of differences in purity angle and (c) the angle is a linear function of the difference between spectra while the sine (dissimilarity index) and cosine (similarity index) are non-linear. Library matching of UV–Vis

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spectra, i.e., comparison of stored spectra of authentic materials with apex spectra is commonly performed by applying the same algorithms [4].

The spectral bandpass, linear dynamic range, stray light, and noise define the performance of spectrophotometers. When operated as an HPLC detector, the time constant, speed of spectral acquisition, flow cell path length, and flow cell dispersion are additional critical performance characteristics. The relationship between spectral bandpass, linear dynamic range and noise is complex; the most significant challenge to photodiode array detector design is to maximize the linear dynamic range and spectral fidelity by minimizing spectral bandpass while simultaneously maintaining a high energy throughput to minimize noise. The resolution of PDA can be described by reporting the number of nm/diode by dividing the spectral range of the polychrometer by the number of diodes or by reporting the spectral bandpass of the polychrometer. The spectral bandpass of the polychrometer may exceed the nominal diode resolution of the instrument. However, no additional information is provided by the excess diodes.

Although several authors have commented on the limited information content of UV–Vis absorbance spectra [10,11] there are no reports describing the relationship between spectral bandpass of PDA spectrophotometers and the spectral differences which can be measured. Ryan [12] and Ebel and Mueck [13] noted the necessity of collecting spectra which have good signal-to-noise ratio and are within the linear dynamic range of PDA. The fundamental assumption of all of the methods of spectral comparison is that the absorbances of all of the components are strictly additive and that Beer's law is obeyed at all of the wavelengths selected for comparison. Special care must be exercised in the low UV where molar absorptivities are generally large and the absorbance can exceed the linear dynamic range of PDA [6].

The collection of a blank injection as a source of data for estimating the system noise for inclusion in purity and homogeneity calculations was recommended by Ebel and Mueck [13]. The noise models which have been included in purity calculations have assumed that the noise is constant over the entire absorbance range. While this assumption is convenient, it is true only when the absorbance values are small, i.e., less than 0.4 AU. When the absorbance exceeds that value, the principal noise source changes from a fixed noise, typically the read noise of the diode array, to shot and/or source flicker noise. The relative uncertainty in absorbance follows the familiar photometric error curve which has a broad minimum at ca. 0.5 AU because the principal noise source changes as absorbance increases and less energy reaches the PDA system. Comparisons of spectra with absorbance values that are greater than 1 AU will be limited by the uncertainty in the absorbance values. Ebel and Mueck did recommend that absorbance values be limited to less than 1 AU, but did not discuss the rationale for such a limit [13].

In this paper we consider the impact of spectral bandpass, detector linearity, and the photometric error curve on peak purity measurements for the separation of vanillin and several related compounds. We demonstrate that the photometric error curve correlates the observed spectral contrast angle with the noise-to-signal ratio.

2. Experimental

2.1. Chemicals and reagents

The mobile phase was a 88:12 (v/v) mixture of 1.0% (v/v) H_3PO_4 in water and HPLC-grade acetonitrile (both obtained from Fisher Scientific, Boston, MA, USA) pumped at 1.0 ml/min at ambient temperature. The water was purified with a Milli-Q™ system (Millipore, Bedford, MA, USA). Stock solutions (1 mg/ml) of vanillin, ethyl vanillin, *m*-anisic acid, and 4-hydroxy-3-methoxy benzoic acid (vanillic acid) in methanol were prepared and serially diluted with mobile phase. Vanillin and the related compounds were obtained from Sigma (St. Louis, MO, USA). The column used was a 150 × 3.9

mm C_{18} NovaPak™ column obtained from the Waters Chromatography Division of Millipore (Milford, MA, USA). The column was operated at ambient temperature. A typical separation is shown in Fig. 1.

2.2. Instrumentation

The chromatograph used in this study consisted of a Model W600 solvent-delivery system, Model 715 Ultra WISP™ and Model 996 photodiode array detector, all obtained from Waters. A Millennium 2010™ data system, also obtained from Waters, was used for control of the chromatograph and acquisition of the data.

2.3. Data processing and procedures

Samples of 25 μl were injected unless otherwise noted. The detector was operated with the following parameters: wavelength range: 195–400 nm; sampling rate: 2 points/s; resolution: 1.2 nm, i.e., no diode bunching; slit width: 50 μm, unless otherwise noted.

The PDA data were processed using Millennium 2010 software which supports the use of a derived channel which contains the largest instantaneous absorbance value for each data point over a user-defined wavelength range, the maxplot. The principal peak purity calculations were performed on the maxplot (200–400 nm) channel. Quantitation was performed on both the maxplot channel and the 290 nm extracted channel.

Results of peak purity, peak area and peak height measurements were exported to Microsoft Excel™ (Microsoft Corp., Redmond, WA, USA) for further processing and to SigmaPlot™ (Jandel Corp., San Rafael, CA, USA) or TableCurve™ (Jandel) for curve fitting and/or plotting.

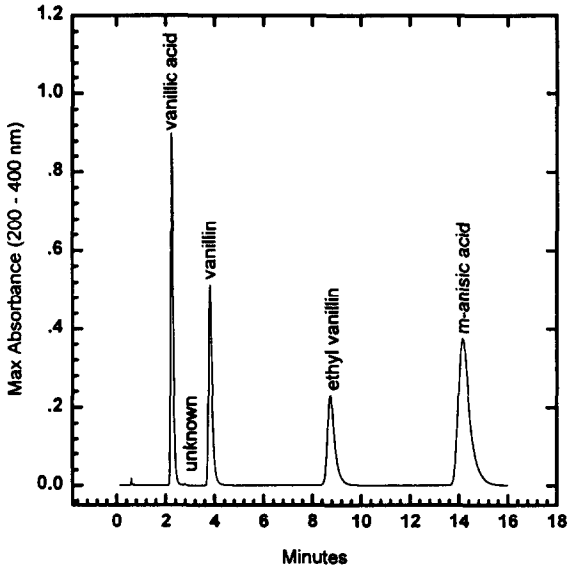

Fig. 1. Typical separation of vanillin, ethyl vanillin, vanillic acid and *m*-anisic acid, maxplot channel (200–400 nm), 25-μl injection containing 40 μg of each component.

3. Results and discussion

3.1. Detector linearity

The linear dynamic range of photometric detectors can be measured by determining the value at which the measured absorbance is 5% less than the value predicted by extrapolating a linear calibration curve which passes through the origin [14]. Dorschel et al. [15] proposed a less subjective test of linearity, i.e., that the instantaneous slope of the calibration curve be constant. We have described a more general approach to determining the goodness of fit of various chromatographic calibration models; the residuals from the regression equation should be randomly distributed over the range of calibrants [16]. The advantage of this approach is that it is applicable to all calibration models and that the residual distribution can be submitted to significance testing through the χ^2 test [17].

A series of samples containing from 1 to 100 μg/25 μl were injected, in triplicate, into the chromatograph. The maxplot (maximum instantaneous absorbance from 200 to 400 nm) and 290 nm chromatograms were extracted from the PDA data. Vanillic acid was the analyte which eluted first and had the largest peak height; its peak absorbance for the 100 μg injection was 2.2 AU. Fig. 2 shows a plot of the distribution of

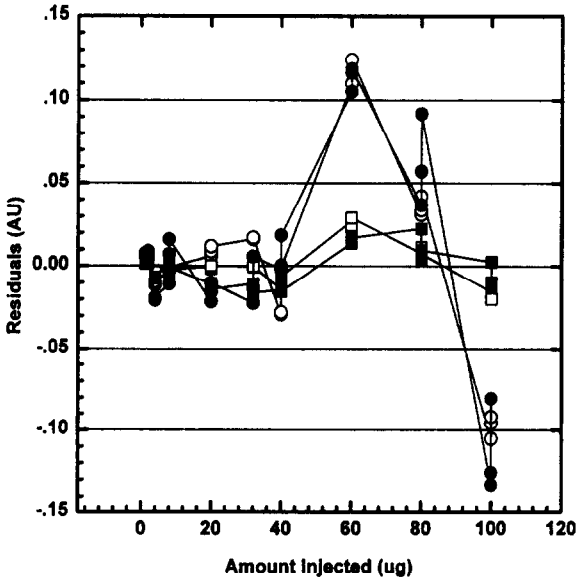

Fig. 2. Residual plots from vanillic acid calibration. ○ = Maxplot, 50-μm slit; □ = 290 nm, 50-μm slit; ● = maxplot, 150-μm slit; ■ = 290 nm, 150-μm slit.

residuals from a linear regression (zero intercept) for both the maxplot and 290 nm channels. The experiment was repeated with a 150-μm slit. Fig. 2 also shows the residual plots for that experiment. In both cases, the distribution of the residuals shows no obvious pattern, and the calibration curves are linear.

The maxplot calibration data for vanillic acid was fitted to an apparent stray light model, $A_{obs} = \log_{10}[(1+s)/(10^{-\epsilon bC}+s)]$, where s is the fractional stray light, ϵ is the molar absorptivity, b is the path length and C is the molar concentration. The apparent stray light was 0.2% and 0.1% for the detector operated with the 50-μm and 150-μm slits, respectively. These values of apparent stray light correspond to an upper limit of the linear dynamic range (as defined by the ASTM procedure E685-79 [14]) in excess of 2.2 AU.

3.2. Photometric error curve

Because the noise of photometric absorbance detectors is relatively constant over several orders of magnitude, most noise measurements are performed under conditions corresponding to the chromatographic baseline. For PDA, this means that the charge measured at the individual diodes is large and can be read with good precision and accuracy. We have examined the variation of noise, which was measured in accordance with the ASTM procedure E685-79 [14], as a function of wavelength. If the energy spectrum is normalized by the maximum energy of the deuterium lamp, i.e., 232 nm, an apparent absorbance scale can be calculated from $A = \log_{10}$ (1/transmittance). Fig. 3 shows a plot of the observed noise vs. the apparent absorbance; it also contains a plot of the noise/signal ratio for the same data set. The relative uncertainty in absorbance measurements is given by

$$s_A/A = 0.434 s_T/T \log T$$

where s_A is the standard deviation of the absorbance, A is the absorbance, s_T is the standard deviation of the transmittance and T is the transmittance. The standard deviation of the transmittance can be treated as the sum of terms corresponding to fixed noise and shot noise

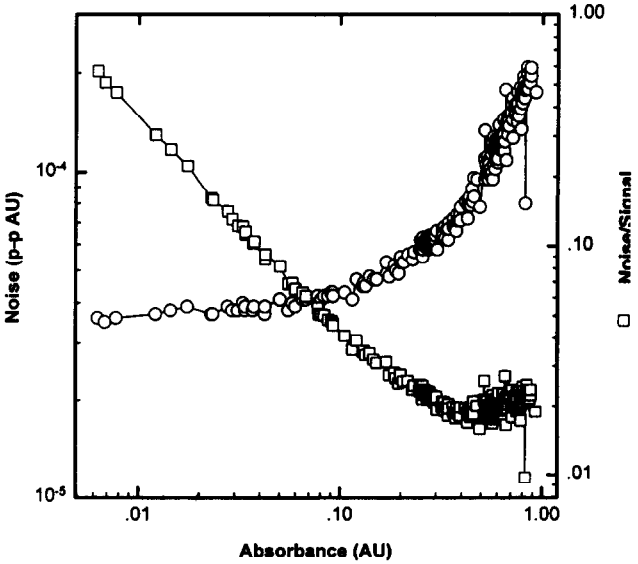

Fig. 3. Photometric error plots. ○ = Peak-to-peak noise, AU; □ = noise/signal ratio.

limited cases, i.e., $s_T = k_1 + k_2(T^2 + T)^{1/2}$. The noise/signal vs. apparent absorbance curve was fitted to this equation and the residuals showed a random distribution. The minimum in this observed photometric error curve occurs at 0.5 AU and is about 2%. Consequently, uncertainty in the absorbance measurement will become significant for absorbance values greater than 0.5 AU. When the absorbance is less than 0.5 AU, the baseline noise will be the principal source of uncertainty. The most reliable comparisons of spectra will be performed at an absorbance corresponding to the minimum of the photometric error curve.

It should be noted that the photometric error curve predicts an increase in the relative uncertainty in absorbance which limits the confidence in spectral comparisons at an absorbance value which is one fourth of the upper limit of the linear dynamic range of the detector. Fortunately, the minimum in the photometric error curve is broad, and the relative uncertainty in absorbance does not change dramatically from 0.1 to 0.8 AU.

3.3. Peak purity (spectral homogeneity) measurements

We examined the variation in the purity angle for each of the peaks observed in the chromatograms used to measure the linear dynamic range of PDA. The results of those measurements for the vanillic acid and vanillin peaks are shown in Fig. 4. The curves in Fig. 4 are based on a combined fixed and shot noise model of the photometric error curve and have randomly distributed residuals.

Each plot of purity angle vs. maximum absorbance displays a minimum; for the 50 μm slit, that minimum occurs at 0.5 AU and a purity angle of 0.4°. When the 150-μm slit was used, the minimum occurred at 0.3 AU and 0.6°. The effect of increasing the slit width was to decrease the dynamic range of spectral homogeneity measurements.

The minima occurred at absorbance values much smaller than the upper limit of the linear dynamic range of the detector (greater than 2.2 AU). The maximum error in absorbance associated with the apparent stray light values of 0.2% and 0.1% corresponds to 0.3% and 0.2% at the minima of the peak purity curves which demonstrates that the comparison of spectra is limited by photometric uncertainty, not detector linearity.

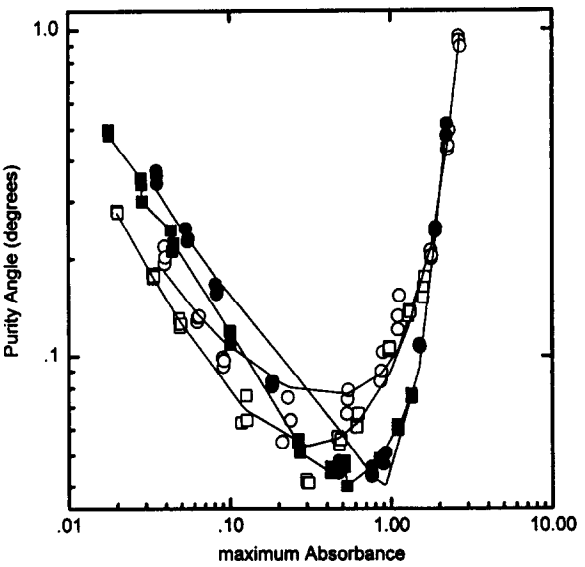

Fig. 4. Peak purity plots for vanillic acid and vanillin. ● = Vanillic acid, 50-μm slit; ■ = vanillin, 50-μm slit; ○ = vanillic acid, 150-μm slit; □ = vanillin, 150-μm slit.

4. Conclusions

Sensitive detection of spectral inhomogeneity within chromatographic peaks requires the use of the smallest spectral bandpass that the detector can support and limiting the maximum absorbance over the entire wavelength range used for comparing spectra to a value which corresponds to the minimum of the plot of purity angle vs. maximum absorbance. The use of a larger slit width, i.e., degrading spectral resolution, shifts the minimum of the purity angle vs. maximum absorbance curve to smaller values of absorbance and higher purity angles and decreases the PDA sensitivity to spectral inhomogeneity within a peak.

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