Peak Purity Determination with a Diode Array Detector

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INTRODUCTION

Peak purity analysis is an evaluation technique for detecting the presence of coeluting impurities in high-performance liquid chromatography (HPLC) data. Running a peak purity check prior to analytical quantitation helps to ensure accuracy. In the development of analytical methods, peak purity analysis can reveal the presence of contamination during standardization and, by doing so, can prevent the subsequent generation of false analytical data.

Peak purity analysis is also a useful addition to routine quality control procedures, especially in the analysis of pharmaceuticals and food products, for which contamination and quality of results are critical.

PEAK PURITY ANALYSIS WITH DIODE ARRAY DETECTOR

Peak purity analysis is designed to detect the presence of an impurity that is coeluting with the analyte peak. For impurity detection with a single-wavelength UV/Vis detector, one must see a shoulder, valley, or excessive tailing to suspect the presence of an impurity. The absence of these features on the chromatographic peak is not a foolproof assurance of peak purity. The impurity may not be seen simply because the chromatographic resolution is low (Fig. 1). A photodiode array detector can provide additional information by using the acquisition of UV/Vis spectra to determine peak purity.

A proper peak purity determination requires access to a significant portion of the eluting compound’s spectrum without interrupting the separation. For this reason, peak purity analysis is performed using a multisignal UV/Vis diode array spectrophotometer as the HPLC detector. Unlike the diode array detector (DAD), the traditional variable-wavelength detector examines only a single wavelength of the sample spectrum, providing insufficient information for peak purity determination. The diode array spectrometer illuminates the sample with the entire spectrum of wavelengths emitted by the light source. Light transmitted by the sample is then broken into its component wavelengths by a diffraction grating and directed to a bank of photodiodes, each of which is dedicated to measure a narrow band of the spectrum. As no mechanical scanning is required, spectral acquisition can be accomplished in as little as 12 msec, well within the precision limits for HPLC peak elution. The rapid spectral acquisition makes it possible to perform peak purity determinations using selected multiple spectra as inputs. Therefore, the absence of any mechanical action in the acquisition of spectra enhances the reproducibility and the accuracy of the peak purity analysis. For coeluting peaks, a DAD makes it possible to differentiate both compounds, even when their spectral absorption overlaps the entire range of captured wavelength data.[1,2]

DIFFICULTIES OF PEAK PURITY CONFIRMATION

Before the quantitative information contained in a chromatographic peak can be used, the purity of the peak should be confirmed. Only after we are sure that no coeluting impurity was present, which could have contributed to the peak response, can we convert the peak’s area or height into quantitative information based on the equivalent response of a pure standard. This peak purity analysis can be based on the comparison of the various spectra recorded during the elution of the peak. If the peak is pure, then, apart from concentration differences, the spectra taken at several points during a peak’s elution should all be identical and the match scores obtained should be very close to the perfect match scores. If significant deviations are encountered, this can be seen as an indication of impurity.

Unfortunately, the inverse is not necessarily true. If the spectra are identical, based on the algorithm used for comparison, the peak can still be impure for one or several of three possible reasons:

1) The impurity is present at a much lower concentration than the main compound and is not detectable.
2) The impurity has the same or a very similar spectrum, compared to the main compound.
3) The impurity exhibits the same peak profile as does the main compound; that is, it completely coelutes with the main peak, across the entire peak.

One of the most important aspects of peak purity analysis that is often overlooked is the fact that any peak purity algorithm can only confirm the presence of impurities, but can never unambiguously prove that a peak is pure.

HPLC SIGNAL OVERLAY FOR PURITY ANALYSIS

One way of uncovering contributions because of impurities in an HPLC peak is to overlay peak profiles acquired at several wavelengths. As two different compounds are unlikely to exhibit identical absorption over multiple wavelengths, the presence of an impurity is revealed by the deviation of the profiles. To compensate for the differences in spectral intensity at different wavelengths, the signals to be compared are first normalized to the maximum absorbance value or to equal areas. Peaks free of impurities exhibit good overlap, but the presence of an impurity is indicated by a shift in the retention time maximum at different wavelengths (Fig. 2).

The signal overlay method is not considered to be very sensitive, and is highly dependent on the resolution of analyte and impurity peaks. If care is taken to correct for solvent background and if the signals are normalized to the highest absorbance value in the time range plotted, the resulting ratiogram can provide conclusive information. It is usually recommended as an additional qualifier in conjunction with other peak purity analysis methods.

In addition to signal overlap, the ratios of signals acquired at different wavelengths can be calculated and plotted. The resulting ratiograms are good indicators of peak purity. Any significant distortion of the ratiogram's ideally rectangular form is an indication of differential absorption and the presence of an impurity (Fig. 3). Peak purity analysis based on signals is generally limited to instances for which the spectra of both analytes and impurities are well known, a requirement for selecting the wavelengths best suited for comparison. Typical applications for this information would be routine evaluations such as quality control checks.

PEAK PURITY USING SPECTRAL DATA

Comparison of spectra is the most popular method of peak purity determination. The primary advantage of this

![Fig. 1 Coelution of three compounds A, B, and C in the chromatographic peak. No shoulders, valleys, or excessive tailing are seen.](image)

![Fig. 2 Normalized signals for (a) pure and (b) impure peaks.](image)
approach is that prior knowledge of component spectra is
not required. However, information derived using these
techniques is not sufficient for determining the kind,
number, and level of impurities present.

A number of selection criteria and data manipulation
techniques can be applied prior to analysis to improve the
quality of the analytical result. These include setting
different spectrum acquisition modes, background correc-
tions, normalization, and threshold settings.

Selection of Spectra for Comparison

Peak purity analysis software allows users to sample
spectra at equidistant points across an HPLC peak. In
general, the poorer the resolution between potentially
coeleuting peaks is, the more desirable it is to use greater
numbers of data points to detect the impurity. Tradition-
ally, spectra have been sampled upslope, at the apex,
and downslope of the eluted peak. This selection pattern
may overlook the presence of impurities near the peak
extremities. However, acquisition of many spectra may
increase calculation and display time without adding
significant information.

Background Correction

A peak should not be labeled as impure because of
spectral background, or because of tailing interference
from a neighboring peak. Both effects can be corrected
for by suitable background correction, with the under-
standing that spectral subtraction increases the noise of a
spectrum and, thereby, lowers the ideal match factor.

Whether background correction needs to be applied
depends on the separation system employed. If the
instrument is balanced properly, then, for isocratic
separations, the solvent background will be eliminated
by the built-in automatic subtraction of the solvent
spectrum, as present at the beginning of the analysis, from
all recorded spectra. For gradient separations, background
corrections will have to be applied after the analysis.

Different methods for correction are possible; the
choice of method depends on the availability of spectra
resulting from specific modes of spectral storage for
different instruments. Ideally, all spectra are retained for
an analysis, but because of processing and storage
constraints, many instruments offer a mode of acquisition
where spectra are stored only during the elution of a peak.
Peak spectra can be limited to just the apex spectrum, or
to a number of additional spectra across the peak, most
commonly acquired at the baseline and the inflection
points. If only apex spectra are available, no background
correction is possible. For all other cases, the quality of
background correction will depend on the availability of
suitable baseline spectra.

In the simplest case, a single baseline spectrum is
subtracted from all analyte spectra. This will work only
for the elimination of constant spectral impurities. If
several baseline spectra are available, linear combinations
of those spectra can be used to generate a synthetic
background spectrum at any point in the chromatogram.
Ideally, the two spectra should be close to the beginning
and the end of the peak being analyzed. If this is not

Fig. 3 Ratiograms taken from (a) an impure and (b) a pure peak.
possible because of incomplete separation of the peak from its neighbors, baseline spectra could be taken from the beginning and the end of a peak group.

If only slope and apex spectra are available, background correction is still possible, in a limited fashion, by subtracting the apex spectrum from the two slope spectra. For isocratic separations or gradient with slow changes in solvent background, this approach will produce two baseline-corrected slope spectra with a loss in signal that is equal to the difference in amplitude between slope and apex spectrum.

**Spectrum Normalization**

Spectra used for comparison during peak purity determination should be normalized to compensate for differences in concentration. Normalization can be based on setting equal absorbance maxima or maximum wavelength ranges, on setting equal area of spectra or spectral region of interest, or on using a matching algorithm that minimizes area differences by shifting and scaling spectra.

**Absorbance Threshold**

Setting an absorbance threshold improves the accuracy of spectral comparison by screening out spectra near the signal baseline. These spectra tend to have a relatively high degree of noise, which adversely affects the accuracy of both normalization and subsequent spectrum matching.

**Spectrum Overlay**

After spectra are acquired and processed, they are overlaid for visual evaluation (Fig. 4). Although significant deviations between the profiles can indicate the presence of an impurity, the converse is not necessarily true, and spectral profiles that match quite well may still mask the presence of an impurity. Factors that may contribute to nondetection of impurity include large concentration differences between analyte and impurity and either highly similar spectral profiles or identical chromatographic peak profiles and retention times for both analyte and impurity. As a rule of thumb, impurity concentrations in the 0.1–1% range may be detected when the spectra are dissimilar. However, if the spectra of the different components are highly similar and the HPLC peaks are not well resolved, the impurity detection limit is on the order of 5%.

**ADVANCED TECHNIQUES**

Simple peak purity analysis is relatively accurate when the impurity is present at significant concentration levels but, as the level of impurity diminishes, its impact on the target analyte spectrum becomes subtler and may require more sophisticated techniques. For this, statistical software routines are available for automated spectral comparisons. In these cases, peak purity determination and analysis of spectral differences are achieved using vector analysis algorithms. The more similar the spectra are, the closer the value is to 0.0; the more spectrally different they are, the larger the value. All the spectral data points across the peak are analyzed; the data are converted into vectors, compared, and graphically plotted so that the results can be visualized. These software routines provide both numerical results and graphical representations such as similarity and threshold curves.

Similarity curves are plots of retention times versus similarity factors computed by comparing spectra across an eluted peak with one or more selected spectra.

![Graphical display of similarity factors for different pairs of normalized spectra.](image)
Similarity curves improve the sensitivity of detecting impurities because they extract and highlight subtle impurity-generated anomalies in an analyte spectrum that might otherwise go unnoticed.

The threshold curve is a plot of retention time versus a similarity factor threshold, below which the presence of an impurity cannot be distinguished from spectral noise. The threshold trace may be computed automatically from the standard deviation of a number of user-selected pure noise spectra. Alternatively, the threshold may be set at a fixed value. Similarity and threshold curves tend to rise at the extremities of the eluted peak, even when no impurity is present. As signal strength decreases, a larger proportion of the spectral response is caused by noise. If an impurity is present at a detectable concentration, the similarity curve will intersect the threshold (Fig. 5).

Peak purity software incorporating routines for data acquisition and reduction, extraction and comparison of spectra, and display of analytical results can be utilized in either an interactive or automated fashion. A desired sequence of operations can be recorded in a peak purity analysis method file and can run unattended after input parameters are selected. As the quality of the determination is highly dependent on the preanalysis data treatment, results should be inspected visually to verify that peaks are properly baseline-separated and correctly integrated.

The evaluation of a peak purity analysis is assisted by a well-organized visual display of the output of various peak purity analysis techniques. [3–5]

**CONCLUSION**

Peak purity analysis is designed to detect the presence of an impurity that is coeluting with the analyte peak. A DAD, using the almost instantaneous acquisition of a considerable portion of the UV/Vis spectrum of eluted peak components, can give accurate information to determine peak purity. The spectral uniqueness of each compound is used to indicate when there are two or more components present in the peak, to identify peaks, and to assess purity.

Peak purity analysis is very useful in chromatographic method development, to confirm that all components have been chromatographically separated, and in quality control, to warn the analyst that an unexpected coeluting impurity has appeared.

**REFERENCES**