According to the U.S. Pharmacopeia (USP), specificity is the ability to unequivocally assess an analyte in the presence of other components in the sample matrix, such as impurities and degradation products (1). Depending upon a method’s intended use — as an assay, purity, or identification test — analysts can approach method validation different ways. Peak-purity tests using photodiode-array or mass spectrometry (MS) spectra are useful to show that an analyte response cannot be attributed to more than one component.

Method validation hinges upon demonstrating the specificity of an analytical high performance liquid chromatography (HPLC) method (2–5). Specificity in HPLC suggests that a given analyte peak is indeed the desired analyte structure and is 100% pure. Each response should be for a single compound of a single chemical structure with no detectable impurities coeluted; it should be the intended and assumed analyte structure. All these assumptions must be fully demonstrated unequivocally to the U.S. Food and Drug Administration’s satisfaction using the latest HPLC equipment, detectors, associated software, and spectral information.

In reality, HPLC alone cannot prove peak purity or identification, even if authentic external standards are available for the desired analyte. Chromatographers always must rely on ultraviolet (UV), photodiode-array, and MS data to prove a peak to be pure and of the correct, known structure. Specificity means that the HPLC conditions and detectors have isolated the desired analyte peak of interest and, ideally, separated from all other components of the sample matrix in a 100% pure form that is ready for identification and quantitation. It is inadvisable to quantitate a peak that is not 100% pure nor of the proven structure. In most cases, accurate and precise quantitation will rely upon an external standard calibration plot in which the external standard also is 100% pure and of the same structure as the analyte peak in the samples of interest. Therefore, the chromatography and detector spectral properties of the external standard must match those of the sample analyte peak.

In today’s world, peak purity, homogeneity, and structure determinations rely almost completely upon computerized methods, commercial software, and spectral library searching. Analysts today no longer need to understand the correlation between an unknown compound’s UV and MS spectra and its structure, rather they need to know how to use their spectra in automated search and interpretation routines to determine the most likely structural fit. If an unknown analyte’s peak provides spectra that cannot be found in any library database, then analysts must use spectral interpretation software as well as their understanding of how to use spectra to predict chemical structures (6–10).

The art and expertise of manually interpreting spectra for structure derivation is slowly passing away, because computerized methods have become very sophisticated, rapid, extensive, and almost foolproof. The combination of using spectral software and databases and software that predict spectra based upon structures or vice versa, obviates analysts’ need to understand how this determination was performed manually. This type of structure elucidation software now is commercially available from several sources, including Advanced Chemistry Development Laboratories (Toronto, Ontario, Canada) and Bio-Rad Laboratories (Hercules, California).

Figure 1 shows a typical application of the spectra–structure elucidation software, which can take an MS spectrum and predict a likely structure or take a likely structure and predict its mass spectrum. The software also can compare the original spectra with predicted spectra and determine if they are identical, in which case the structure is defined and confirmed. This software also can be used with proton and 13C nuclear magnetic resonance (NMR) and Fourier transform infrared (FTIR) spectra. We can argue whether this software represents an advance in human knowledge or...
yet another total dependency on computers and software.

**The Role of HPLC and Photodiode-Array and MS Spectra in Demonstrating Peak Purity**

Chromatography alone never proves the purity of any peak. Even a combination of multidimensional HPLC, gas chromatography (GC), thin-layer or supercritical fluid chromatography, or capillary electrophoresis methods cannot prove peak purity or identity. Specificity can be derived from software interpretation of both photodiode-array and MS data and the demonstration that these spectra remain unchanged across the peak’s width. There should be no change in the normalized, overlapping spectra derived at any point in the chromatographic peak. Both photodiode-array and MS spectra can demonstrate peak purity and peak identity or spectral matching.

Today’s software is better able to demonstrate peak purity, or lack thereof, than any analyst’s eye or experience. Even coelution of small impurities should be discernible with the latest photodiode-array and MS spectral software routines (11–14).

If an impurity perfectly overlaps the desired analyte peak, then the peak is homogeneous, but it is not pure nor a single entity. In that case, the normalized photodiode-array and MS spectra taken at all points throughout the peak should exhibit differences. The problem is greatly simplified when a small difference in retention time of the two coeluted peaks is present. Disparate levels of an impurity that overlaps the main peak also should be discernible, especially when looking at the edges and middle of the peak. The software might fail to recognize differences in the photodiode-array and MS spectra throughout the peak after normalization only when two components under a single peak are close in concentration or absorbance response.

Because UV spectra often are quite similar for compounds derived from the same basic chromophores, these spectra are less useful for demonstrating the overall specificity of a method. Chemical structures cannot be proven by UV spectra alone; it can only suggest similarities of chromophores within a family of compounds based upon their UV spectral similarities. True analyte specificity of any HPLC method must be derived from the MS spectra for the compound of interest. Although it is possible that two compounds could have the same MS spectra, it occurs less often than with UV spectra. Many examples illustrate two different compounds that have virtually identical UV spectra (3,6–10). With MS, diastereomers, enantiomers, and positional isomers commonly may have the same MS spectra. Chromatographers can demonstrate overall analyte

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![Figure 1](image-url): Illustration of an on-screen assignment of fragments in the mass spectrum of the indicated compound. Each fragment can be assigned from its mass-to-charge ratio, and the sum of all fragments should produce the final, total structure. (Courtesy of Advanced Chemistry Development.)
specificity better by combining both UV and MS spectral data. To be specific and not just selective, combining these two types of spectra with NMR spectra would certify specificity definitely. This practice is not farfetched with modern HPLC–photodiode array–MS–NMR instrumentation.

Figure 2 illustrates a typical HPLC peak with slices taken throughout the peak. Photodiode-array and MS spectra were collected from each of these slices, and the peak apex spectrum was compared with every other spectrum collected across the peak. Shape differences in the photodiode-array spectra were calculated using software routines described elsewhere, and their weighted averages were then reported, for example, as purity angles (11–14). These terms, which are unique to one manufacturer’s software (Waters Corp.), vary somewhat from one manufacturer to another. In addition, one vendor’s software routines sometimes are not applicable to another vendor’s instrumentation. It is essential to learn the software for a brand of instrumentation and how to optimize its use.

In practice, chromatographers use multiple injections of the same sample or blanks to establish what can be called a threshold angle, which is a pseudo-detection limit for purity angles. This artificial threshold angle then sets the limit above which an impurity is suggested to be present in the analyte peak of interest. If the purity angle is less than the respective threshold angle, then the peak is considered to be uniform, homogeneous, and a single entity. When the software has detected a possible impurity in the HPLC peak, it can plot the purity angle versus elution time. A rise of this purity angle plot above the noise or threshold angle plot suggests the presence of an impurity (see Figure 3).

Photodiode-array peak-purity plots can be used in this way to examine the purity angle across an entire peak. A rise in the impurity angle indicates real spectral differences, and this information can be used to identify coelutions during method development or actual sample analyses. Peak-purity plots are incredibly useful to demonstrate peak purity, but they must be performed before any spectral matching routines or library searches are initiated. It is pointless to undertake additional structural interpretations or quantitation for any peak that is not homogeneous nor a single entity. Running the sample analyte under different HPLC conditions using peak-purity plots to again demonstrate purity of the analyte peak is further insurance against working with a possibly impure analyte peak. A lot of time, effort, and money can be lost in performing spectra interpretation, structure identification, and actual quantitation without first proving beyond a shadow of a doubt that the suspected peak of interest is indeed 100% pure.
The Role of HPLC and Photodiode-Array and MS Spectra in Demonstrating Peak Identity and Structure

After proving that an analyte peak in a real sample is 100% pure, chromatographers then can demonstrate the proof of structure. Several methods can determine structures — some are easier than others, and some are more automated than others. In the traditional approach, analysts collect chromatographic peaks from a GC or HPLC separation under semipreparative or actual preparative conditions (15). They reinject each fraction under analytical GC conditions to demonstrate supposed peak purity and then subject the fractions to separate IR, UV, NMR, and MS analyses. After obtaining those data, analysts can hit the books and begin a manual library search for identical or similar spectra. Spectral correlation charts were in vogue in the 1950s and 1960s, and these charts allowed analysts to use absorbance and transmittance wavelengths or frequencies to assume the presence of certain functional groups or collections of atoms. Functional groups also could be determined from IR spectra, protons present by proton NMR, or carbon atoms present by $^{13}$C NMR. Eventually, chromatographers used MS spectral interpretation with fragmentation patterns to determine parent ions, base peaks, metastable ions, and isotopic distributions of ions.

After many hours and missteps, all these approaches eventually led to a most-likely structure. To ensure that the mass spectrum was exhibiting the true molecular weight, chromatographers sometimes would perform a Rast molecular weight determination (6–8). A Rast molecular weight determination was performed using the melting point depression of camphor as an absolute method to derive molecular weights.

These approaches rarely are used today, and the mass spectrometer almost always is used to demonstrate a molecular weight of an unknown, assuming that the molecular (parent) ion is present. Of course, knowing the starting materials in a synthetic organic reaction often makes it simpler to deduce the possible reaction products from the manually obtained spectral data. An HPLC peak’s structure rarely is derived in this traditional — perhaps crude by today’s standards — manner. The traditional approach truly required analysts to understand the correlation between structures and spectra and to possess the ability to use spectral correlation charts in IR, UV, NMR, and MS.

Today’s approach to deriving the correct structure of an HPLC peak relies upon spectral contrast software, which basically takes the UV or MS spectra for a peak and attempts to match (or contrast) them with reported, authentic, literature spectra already correlated with known structures (16). To best use this approach, chromatographers need to have available spectral libraries for photodiode-array and MS spectra. Several commercial MS spectral libraries are available; however, chromatographers also must derive their own UV libraries.

Because UV spectra are so dependent upon conditions such as the instruments, solvents, and pH used, finding a UV spectrum in a library that matches the eluted photodiode-array spectrum on-line may be problematic. To determine a true library match, users should extract the baseline corrected apex spectra from the chromatogram (see Figure 4) (16). Figure 4 makes two comparisons of where the compared UV spectra are very different with a large spectral contrast angle (24°) and of where the UV spectra have very small differences with a very small spectral contrast angle (2°). Clearly, the smaller the observed spectral contrast angle, the more likely that the two spectra and structures are identical.

The spectra next are mathematically normalized and represented as vectors in a multidimensional vector space (see Figure 5). Vector differences are calculated using linear algebra, and spectra that have the same patterns have vectors that point in the same direction, which results in a zero-angle match angle. Spectra that have different shapes point in different directions, which result in match angles that may be 0–30°. The smaller the match angle, the more likely that the two spectra — analyte peak and library spectra — may be identical. Figure 5 illustrates the use of match angles for spectra comparison purposes.

Chromatographers easily can observe differences in spectral contrast or match angles for small organic molecules. These large angles result from different functional groups, ring structures, or conjugation present in the molecules. In some compounds, the spectral differences are difficult to see. Smaller angles result from structural differences such as single atoms, positional isomers, or even diastereomers. For this reason, mathematically enhanced spectral analysis and contrast techniques have proven useful. Using library matching routines often leads to the correct structure for an analyte or an impurity peak, assuming that the compound’s spectra is in some homemade or commercial photodiode-array or MS library. As mentioned previously, however, photodiode-array UV spectra cannot be used by themselves to unequivocally prove either purity or identity. True purity is best proved by a combi-
nation of both UV and MS spectra, and true identity similarly is derived from a combination of these two spectral techniques as well. MS is still the best indicator of structure, because it is rare that two compounds will have the same MS fragmentation patterns, whereas they may have the same or similar UV spectra. High-resolution mass spectra only can better define or correlate an analyte’s identity with a library match.

What if no library contains the correct structure for an unknown impurity peak? Analysts can use the traditional approaches outlined above with a modern twist. Commercially available software can elucidate structures from routine spectral data. The software determines a structure from a variety of spectra — $^{13}$C and proton NMR, MS, FTIR, and photodiode array — collected on-line from an analytical system. The software also can take a known structure and predict the spectra most likely to be obtained. In this way, the software can confirm a suspected structure by comparing already-obtained spectra with the spectra it predicts. If users have proposed no structures, the software can predict the most-likely structures and rank them in order of greatest likelihood. The more spectra provided to the software, the more likely that it will determine the correct structure or structures. The software is useful for determining structures, even for trace impurities, and it does not require spectral libraries or library searches. It works independent of existing spectral databases, and this ability is important when working with totally unknown, previously unreported, or brand new compounds such as newly synthesized or natural product extracts. With a brand new compound, library database searching would prove pointless; however, structure elucidation software at least will provide the most likely structures, if not the correct one.

How to Prove an Unknown Structure’s Identity
Perhaps the specificity of a method to be validated is not yet fully proven. Someone has proposed a likely structure or structures for the peak of interest, but one ultimate structure has not been proven correct. To do so, chromatographers must obtain an authentic sample of the compound by synthesizing it in-house or by contract, by purchasing it, or by obtaining a certified reference material of it. Analysts must compare information about the authentic standard analyte with the analyte from a sample by any and all analytical techniques first obtained from the sample of interest.

Figure 4: Comparisons of (a) two UV spectra with very different characteristics and therefore a large spectral contrast angle (24°) and (b) two UV spectra having very small differences and thus a smaller spectral contrast angle (2°).

Figure 5: A plot comparing UV spectra as linear vectors. The UV spectra for B and B’ are identical and have the very same vector and a zero matching angle. A and B have very different UV spectra, their vectors do not overlap, and they have a spectral matching angle greater than zero.
Conclusion

One goal in all analytical method validation is to prove, by any or all of the above procedures, that an analyte of interest is pure and of a known structure. If an analyte is impure or if it has the wrong structure, then any additional work is wasteful and unproductive. In the end, a validated analytical method must show that the analyte response cannot be attributed to more than one component.

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Ira S. Krull
“Validation Viewpoint” co-editor Ira S. Krull is an associate professor of chemistry at Northeastern University in Boston, Massachusetts, and a member of LCGC’s editorial advisory board.

Michael E. Swartz
“Validation Viewpoint” co-editor Michael E. Swartz is a senior scientist at Waters Corp., Milford, Massachusetts, and a member of LCGC’s editorial advisory board.

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