Liquid Chromatography Problem Solving and Troubleshooting

Question

Recently, our laboratory purchased a fluorescence detector for carrying out a published HPLC method for a compound that we are interested in monitoring in one of our products. When we tried the original method, we found that it worked well in terms of the reported precision and limits of detection. However, the separation was excessively long for a routine quality assurance method. When we tried to optimize the chromatography in terms of the assay time by changing the eluent conditions, we were unable to obtain the same sensitivity. We would like to know why this is happening and if there is anything we can do to correct the problem?

Answer

In order to answer your question, it is important to briefly review a few of the basic principles that govern molecular fluorescence and how various conditions can affect them. First, the process involves an initial absorption of electromagnetic radiation, which is an extensive property that is related to the electronic energy level spacing in the molecule and the number of molecules present. In the case of molecular spectroscopy, there are a number of vibrational and rotational states associated with each of the allowed electronic transitions in the molecule. Hence, unlike atomic spectroscopy, molecules absorbing in the UV–vis range that are dissolved in solution have broad absorption bands. Mathematically, the simple process of absorption is described by the familiar Beer's Law expression. This is illustrated in the Jablonski energy level diagram shown in Figure 1.

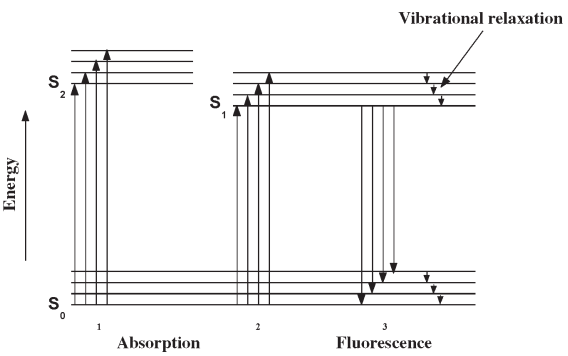

Figure 1. Energy level diagram illustrating the transitions involved when electromagnetic radiation in the UV–vis region is absorbed and emitted between the ground and excited singlet states.

However, unlike molecules that undergo simple absorption, in electronically-rich systems (i.e., more rigid molecules that have extended π-electron conjugation) many of the nonradiative rotational and vibrational deactivation mechanisms that depopulate the excited states are unavailable. These types of molecules, when excited via the absorption of electromagnetic radiation, relax back to the ground state by photoemission (i.e., fluorescence). During the time that the molecules are in the excited state, typically around 10^{-9} s, they can undergo vibrational relaxation (i.e., a process that occurs in the 10^{-12}-s timeframe) prior to returning to the electronic ground state. Under these conditions, the re-emitted light is at longer wavelengths than the original absorption transitions. These processes also are illustrated in Figure 1.

In answering your question, it is also important to note that the solvent environment in which fluorescence occurs plays a role secondary only to the actual molecular structure that is itself influencing the spectral positions (i.e., wavelengths) and intensities of the transitions (1). Stated in slightly different terms, changes in the solvent conditions can have a very pronounced influence on the spectral profile, typically resulting in either a

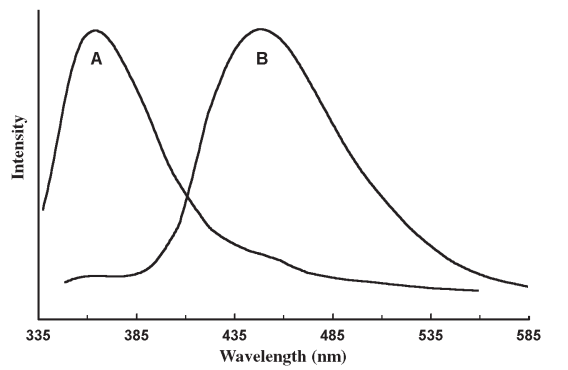

Figure 2. Influence of solvent conditions on the fluorescence spectrum of the 6-hydroxy-naphthalene-2 sulphonamide derivative of α-L-phenylalanine (Phe–NSOH). Spectrum A was obtained in 100% methanol and spectrum B in a binary solution that was approximately 70:30 methanol–water.

The purpose of *Chromatography Problem Solving and Troubleshooting* is to have selected experts answer chromatographic questions in any of the various separation fields (GC, GC–MS, HPLC, TLC, SFC, HPTLC, open column, etc.). If you have questions or problems that you would like answered, please forward these to the *Journal* editorial office with all pertinent details: instrument operating conditions, temperatures, pressures, columns, support materials, liquid phases, carrier gas, mobile phases, detectors, example chromatograms, etc. In addition, if you would like to share your expertise or experience in the form of a particular question accompanied by the answer, please forward to: JCS Associate Editor, *Chromatography Problem Solving and Troubleshooting,* P.O. Box 48312, Niles, IL 60714. All questions/answers are reviewed to ensure completeness. The *Journal* reserves the right not to publish submitted questions/answers.

Roger K. Gilpin
Associate Editor

red or blue shift depending on how the solvent's polarity changes. Likewise, depending on the acid-base character of the fluorescing molecule, changes in pH and hydrogen bonding also can produce large effects. Figure 2 shows an example of a large spectral shift that has been reported in the literature for a derivitized amino acid (2). Clearly, not all fluorescing molecules are influenced as dramatically as Phe–NSOH, and there are many hundreds of examples in which the observed shifts are much smaller. Even in these latter cases, if a fixed observation wavelength is initially selected near the edge of the emission band, a modest polarity shift in the solvent (i.e., modification of your chromatographic eluent conditions) could cause the original observation wavelength to be in a region where the emission intensity is significantly less intense.

In addition to spectral shift affects, the relative fluorescence intensity of the emission process (i.e., the quantum yield), which is defined as the number photons emitted relative to the number of photons absorbed, can vary widely depending on the conditions. For a perfect fluorescing system, the quantum yield would be one and many highly rigid molecules with extended π-electron conjugation have high quantum yields. However, quantum yields are also influenced by the solvent environment. References 1 and 3 are a good starting point to learn more about this and other related topics.

With all this in mind, let me now return to your specific question and make a few experimental suggestions. First, because your original problem involved excessively long retention times between peaks and not the detection sensitivity reported in the literature assay, you might try a shorter column if the method allows this. For example, if it uses a 25-cm column, try replacing it with a 15-cm column of the same bonded-phase type and from the same manufacturer. Alternately, if the assay employs a highly retentive surface such as an octadecyl bonded phase, try an octyl column. In either approach, when carrying out of these suggestions, you should not alter the originally reported eluent conditions. Instead, your approach will be to lower the retentivity of the column. A third suggestion, if the operating conditions described in the literature method will permit it, is to use higher flow rates. If the previously mentioned approaches or a combination of them work, then you will not need to worry about either spectral shift or decreasing quantum yield problems.

An alternate spectrometric approach is to optimize the detection wavelength in terms of the new assay conditions you have developed. If a scanning fluorimeter is available in your laboratory, you could do this directly by obtaining an absorption and emission profile using the eluent as the solvent. However, the more likely case is that you do not have this type of instrument, and you will need to obtain the spectral information indirectly. This is possible by repeatedly injecting your sample and obtaining a series of chromatograms at different emission wavelengths. Assuming you inject the same amount each time, a simple plot of peak area or height versus wavelength will provide a rough representation of the emission spectrum, assuming you carry out the experiment at enough wavelengths. A good starting point is the original wavelength, and then runs at emission wavelength settings that are 20 nm higher and 20 nm lower. Then try 40 nm on either side, etc.

In using the latter spectrometric optimization approach, you should keep in mind that the data you obtain will help you address only a spectral shift problem. However, if changing the solvent conditions (i.e., eluent composition) has resulted in lowering the quantum yield from a high value (e.g., one near 1) to a much lower value (e.g., 0.2), you will be stuck with the approximately 5-fold difference in sensitivity.

References

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