Liquid Chromatography Problem Solving and Troubleshooting

Question

I have an analyte that exists in two conformational states in solution. Unfortunately, I have been trying to separate them but have not been successful. I am pretty sure that I should be able to separate them because I have seen a few examples where other investigators have separated conformers. Do you have any suggestions that I might try?

Answer

The answer to your question is much more involved than: (*i*) a simple yes or no, (*ii*) a discussion of what you need to do to accomplish the separation, or (*iii*) explaining why it is not possible to resolve the conformers. The possibility of separating conformational isomers depends on both the thermodynamics and kinetics that control the system you are studying. In answering your question, it is important to discuss two basic parameters: (*i*) the barrier or activation energy of the process and (*ii*) the kinetics (i.e., forward and backward rates), which control the equilibrium ratio of the two isomeric forms.

When a compound exists in two conformational states, each of these represents potential energy minima in the coordinate system that describes the parameter controlling the system. This is illustrated in Figure 1 for a simple system where there are two rotational forms of the compound (i.e., forms A and B) that each have the same relative potential energy. In changing from one form to the other, the molecule must overcome unfavorable rotational states. Stated in a slightly different fashion, in a rotation of groups about a bond, the unfavorable barrier between the adjacent minima of the molecular entity as a function of torsion angle is the rotation barrier energy (1). This is illustrated in Figure 1 by the maximum in the relative potential energy versus rotational angle plot.

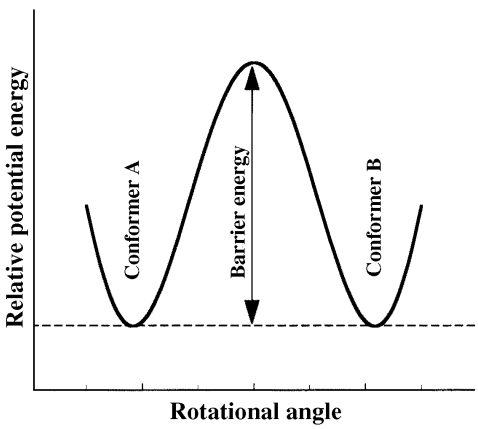

Figure 1. Illustration of an energy diagram for an analyte with two structural conformers. Each of the two preferred conformations is energetically equivalent. In the case shown, the process involves a simple bond rotation (i.e., rotomers). In some cases, A may be at a lower relative potential energy than B, and in other cases, it may be higher than B.

In addition to the barrier energy, the final equilibrium distribution is also influenced by the kinetics of the system, rate at which conformer A is changing to conformer B, and rate at which conformer B is changing to conformer A. If we refer to these two processes, respectively, as the forward and reverse rates, they can be described in terms of two rate constants: $k_{forward}$ and $k_{reverse}$. If one starts with either the pure form of A or B, the two will interconvert until a balance is obtained (i.e., the equilibrium state concentrations) as described by the following expression:

$$[A]k_{forward} = [B]k_{reverse} \qquad \text{Eq. 1}$$

It is important to keep in mind that the two rate constants in the expression are temperature-dependent. Likewise, in terms of actually carrying out an experiment, an additional and extremely important factor that must be considered is the time frame of the system being measured with respect to the time frame of the technique being used to make the measurement.

Thus the ability to separate conformers depends on whether the conversion kinetics (i.e., $k_{forward}$ and $k_{reverse}$) are slow or fast compared with the migration rate of the analyte through the column. It is easiest to describe what will be observed in the cases of the very slow and very fast systems. In the case of very slow interconversion kinetics, when the analyte is injected onto the column, it will start to separate based on their differential interactions with the stationary phase. Once one conformer begins to separate from the other, it will begin to reconvert to the other form based on the shown mathematical relationship. However, because the rate of conversion is very slow compared with the migration, little of it will be formed. Under these conditions, the end result will be a chromatographic profile that resembles the separation of any other two-component mixture.

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

Alternatively, if the interconversion kinetics are very rapid compared with the migration rate that the two forms, they will constantly be converting to the other, (i.e., form A to B and B to A). Therefore, under these conditions, the system will always be in equilibrium, and neither pure A nor B will move ahead of the other. The resulting chromatogram will contain only one band with a migration (retention time) that is a weighed average (i.e., based on their equilibrium distribution).

The more difficult case to accurately describe is when the interconversion kinetics and rate of migration are on similar time scales. Under this situation, the emerging band can be broadened, skewed, or distorted.

With all of this in mind, let us consider what can be done to help you solve your problem. The solution rests in your ability to take the last two case scenarios (i.e., the fast and intermediate conversion rate cases) and to change $k_{forward}$ and $k_{reverse}$ enough to produce the first case scenario (i.e., interconversion process is slow on the time scale of the chromatographic experiment). Sometimes this is possible and sometimes it is not, depending on the barrier energy. For larger barrier energies (i.e., a greater dependence of $k_{forward}$ and $k_{reverse}$ on temperature), it is possible by simply cooling the column/eluent. This concept is illustrated by the separation that is shown in Figure 2, in which the two rotational conformers of lactose (i.e., β- and α-isomers, peaks 1 and 2) are separated using a column and eluent temperature of approximately 4°C (1). Also shown are two additional sugars, maltose and sucrose (peaks 3 and 4). Although not shown, if this same mixture is separated at room temperature, the β- and α-lactose elute as a single peak, followed by peaks for maltose and sucrose.

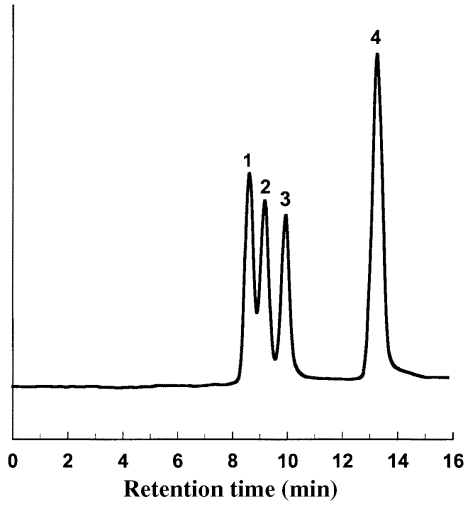

Figure 2. The low temperature separation of β- and α- lactose (peaks 1 and 2, respectively) along with maltose (3) and sucrose (4). At room temperature, peaks 1 and 2 will coalesce into a single band. Conditions for the separation are given in reference 2.

Thus, my suggestion in trying to solve your separation problem is to see if a much lower separation temperature will work. If you do start to resolve your compounds, you should keep in mind that it is a good idea to measure the rate constants as a function of temperature or verify in some other experiment that your measurements are not distorted by small amounts of interconversion (2).

References

1. A.D. McNaught and A. Wilkinson, Eds. *Compendium of Chemical Terminology: IUPAC Recommendations*, 2nd ed. Blackwell Science, Oxford, U.K., 1997, p. 2217.
2. J.M. Beebe and R.K. Gilpin. Determination of β- and α-lactose in dairy products by totally aqueous liquid chromatography. *Anal. Chim. Acta* **146:** 255–59 (1983).