Liquid Chromatography Problem Solving and Troubleshooting

Question

Recently, I joined a new company and I have been trying to develop an assay for separating palmitic, elaidic, and oleic acids. The decision has been made not to use derivatization gas chromatography because of validation issues in the QC department. To date, I have been trying to develop an HPLC method but have not been able to obtain adequate resolution on the C18 and C8 columns that I have tried. What do you think about trying other columns or do you have any other suggestions that I might try?

Answer

The use of derivatization followed by high-resolution capillary gas chromatography is a useful technique for the types of analytes you are trying to separate. However, since your company prefers not to use a derivatization method because of validation issues, I will attempt to help you solve your problem using HPLC. Although you could try a number of other columns, an alternate and perhaps better solution to your separation problem involves a slightly different approach, the use of a mobile phase additive that will selectively alter the retention of the unsaturated fatty acid while having little to no influence on the retention of the saturated fatty acid. Such an additive is silver ion.

Early accounts of the use of silver ion as an eluent additive date back to the mid seventies. Since that time, Ag^+ has been employed in both normal-phase and reversed-phase chromatography and has been applied to a number of different types of analytes. It has been useful, especially for the separation of positional isomers and complex alkene mixtures. When employed in the normal-phase mode, the surface of silica is dynamically modified with Ag^+ and subsequent separations are carried out using standard types of nonaqueous eluents. The silver ions deposited on the surface act as highly selective adsorption centers that specifically interact with the π-orbitals in double-bond containing compounds such as alkenes. In the second instance, Ag^+ is added to the eluent in which it forms weak π-complexes with the solute. The result is to render the solute slightly charged thus reducing its interaction with the bonded alkyl chains of hydrophobic surfaces such as C8 and C18 materials. The stability constant for these complexes is low and the formation and strength of them depend on the number, position, geometry, and steric hindrance of the double bonds in an analyte. As such, retention differences are observed as a function of structural features in the solute such as *cis* and *trans* arrangements and placement of the double bond within a molecule.

There are a number of manuscripts that have appeared in the literature that discuss various aspects of the use of silver ion in both normal-phase and reversed-phase chromatography. Sometimes this technique is referred to as silver ion chromatography and in other instances as argentation chromatography. A relatively recent review has been published that deals with various aspects of the topic (1). However, if you are interested in carrying out a literature search electronically then a good search strategy is "liquid chromatography" and "silver" or "argentation". Using this, you should be able to retrieve a number of articles that directly relate to the types of compounds you are separating.

Since you have expressed an interest in carrying out your analysis in the reversed-phase mode, the remainder of this note will concentrate on this approach. As such, there are three important aspects to keep in mind. First, since your solutes are organic acids, you should use eluent conditions that assure they are protonated and thus maximize interactions of the solutes with the stationary phase. This topic has been discussed in an earlier troubleshooting note (2). Experimentally, you will need to use a hydroorganic eluent that is buffered to approximately 1.0 pH unit below the pK_a values of the organic acids. A pH 3.5 eluent is a useful working condition that will assure appropriate equilibria and will not be too severe on your bonded phase in terms of degrading it. Secondly, you will need to take advantage of

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Associate Editor

(i.e., enhance) the structural differences in the solutes you are separating. These are the two fewer carbons in the palmitic acid and the 9-position double bonds in oleic acid (9-*cis*-octadecenoic acid) and elaidic acid (9-*trans*-octadecenoic acid). As previously discussed, this can be accomplished by the addition of silver ion to the eluent. Third, same as controlling the pH, formation of the alkene complexes is an equilibrium process and appropriate conditions must be selected to obtain the desired separation (i.e., the appropriate concentration of Ag^+ in the eluent).

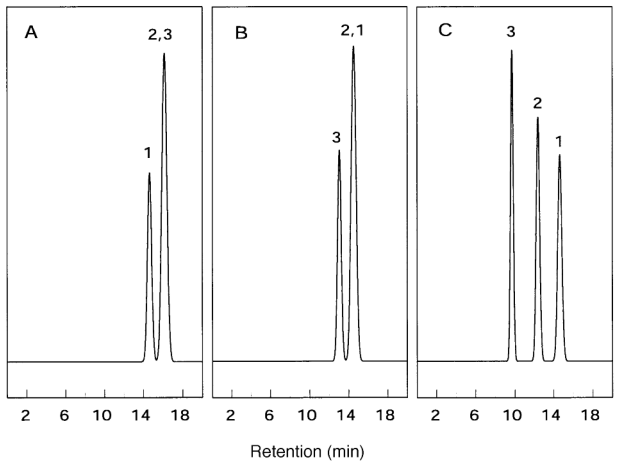

Figure 1. Simulated separations of palmitic (1), elaidic (2), and oleic (3) acids using reversed-phase silver ion chromatography.

Shown in Figure 1A is the separation of palmitic acid, elaidic acid (9-*trans*-octadecenoic acid), and oleic acid (9-*cis*-octadecenoic acid) carried out on a C18 column with no silver ion added to the eluent. This chromatogram is a computer simulation that was generated from the retention and selective data presented elsewhere (3) and assumes no peak tailing, a column that generates 5000 theoretical plates, a void time of approximately 2 min, and equal concentrations of the three analytes. The elution order for the three compounds is palmitic acid (1), elaidic acid (2), and oleic acid (3). Although this particular separation can resolve palmitic acid from oleic acid on columns with slightly different selectivities, this may or may not be possible on all C18 or C8 columns.

The important aspect of Figure 1 is to illustrate how changes in selectivity can be obtained among the three analytes via the use of silver ion in the eluent. The simulations shown in Figures 1B and 1C are the result of the addition of 30mM and 60mM Ag^+ to the eluent. The chromatograms were again generated using the same assumptions discussed previously. Figure 1B, which looks nearly equivalent to Figure 1A, is experimentally deceptive (i.e., the elution order of the peaks changes but the overall chromatographic profile appears to be similar) and clearly shows that adequate levels of silver ion are necessary to assure that the three analytes are completely resolved. In designing your separation, you will need to evaluate different levels of silver ion in the eluent.

If you decide to use silver ion as an eluent additive, from an operational standpoint one of the problems that has been reported for this reagent is the tendency for frit clogging because of the formation of AgCl precipitate from the presence of trace amounts of chloride ion. This can be minimized by prefiltering your eluent, preparing new eluent on a regular basis, and thoroughly rinsing your column before storing it.

References

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