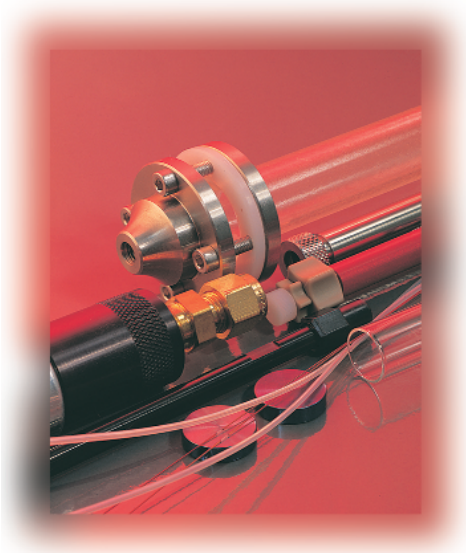
What Drives a Separation — the Stationary Phase or the Mobile Phase?



The driving force of a liquid chromatography separation is determined by solute partitioning between the mobile and stationary phases. Chromatographers must study a wide range of mobile-phase compositions and stationary-phase bonding densities to determine the thermodynamic driving force. The author investigated methanol and acetonitrile as organic modifiers throughout a wide range of bonding densities (1.74–4.4 μmol/m^2) with nitroalkanes and alkylbenzenes as homologous solute series. She found that acetonitrile solvated the alkyl chains more efficiently than methanol and that she needed approximately 20% organic modifier to sufficiently wet the stationary phase and solvate the alkyl chains. The chromatographic system showed nonlinear enthalpy and entropy of solute transfer throughout the entire composition range for both organic modifiers, but she obtained a linear free energy of solute partitioning at organic modifier concentrations greater than 20%. All solute partitioning was enthalpically driven on a monomeric C18 stationary phase.

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Previous work investigating the solvent's role in reversed-phase liquid chromatography (LC) separations was limited by the range of solvents chosen (1–10). Even with the limited number of solvent compositions studied, researchers detected significant changes in retention caused by a change in mobile-phase composition in both methanol and acetonitrile hydro-organic mobile phases. One unanswered question is whether the driving force of solute partitioning throughout the entire solvent range affects solute partitioning energetics. The intent of this article is to expand on earlier investigations by encompassing the entire hydro-organic mobile-phase composition range from 0:100 (v/v) to 100:0 (v/v) for both organic modifiers.

Within this composition range many degrees of solvent associations (both self-association and association with water molecules) exist, and these associations influence the separation. However, the extent of their influence is unknown. Little is known about how these solvent differences change partitioning thermodynamics and ultimately influence the driving force. Thermodynamic quantities determined from homologous series retention throughout the entire composition range may lead to a better understanding of the driving force and fundamental retention mechanism that results from the inherent differences in chromatographic solvation between acetonitrile and methanol. Tan and co-workers (11) showed that stationary-phase composition also has been important in determining a separation's driving force; therefore, I investigated bonding densities ranging from high to low surface coverage.

Many have investigated the various hydro-organic solvent interactions that occur in reversed-phase LC (2,8,9,12–19). Differences in intermolecular interactions of the solute between mobile- and stationary-phase components are the driving force of a separation. Tan and Carr (9) found that interactions between the stationary phase and solute are affected by the composition of the mobile phase. How do differences in interactions alter retention and possibly change the fundamental retention mechanism in a reversed-phase LC separa-

tion? This question has long plagued chromatographers. I'll start by looking at the inherent differences between the popular reversed-phase LC solvents.

The two most popular organic modifiers are methanol and acetonitrile. These two modifiers differ in their hydrogen-bonding abilities — methanol is able to form hydrogen bonds by accepting or donating protons, whereas acetonitrile is unable to form hydrogen bonds to any appreciable extent. Most reversed-phase LC hydro-organic mixtures are thought to be a binary mixture on the macroscopic level but actually consist of a ternary mixture on the microscopic level because of the microheterogeneities formed (13). This ternary mixture comprises free or self-associated organic molecules, water- and organic solvent–associated molecules, and free (self-associated) water molecules. Hydrogen-bonding solvents such as water and methanol have higher polarity and more solvent microheterogeneities, so they are unable to solvate the nonpolar alkyl chains as well as acetonitrile. Scott and colleagues (20) showed that the stationary-phase alkyl chains move freely within the mobile phase at high organic-solvent concentrations. Acetonitrile solvates the alkyl chains the best and enables free movement of the entire alkyl chain at relatively low compositions. This ability is in contrast to the same alkyl chains in pure water; these chains are associated by dispersive interactions and form clumps of alkyl chains on the silica surface.

Lochmuller and Hunnicutt (21) hypothesized that acetonitrile becomes trapped within the narrow silica pores by a collapsed chain structure in highly aqueous mobile phases. Marshall and McKenna (22) found that increased associations between stationary-phase alkyl chains and water in highly organic solvent compositions are a result of water forming hydrogen bonds with residual silanols and also forming acetonitrile–water mixed species. When added to bulk solution, acetonitrile enters cavities within the water structure until all sites are occupied (18). Acetonitrile then becomes increasingly more self-associated as more is added. This causes differences in solute interactions with the mobile phase and changes the retention of solute molecules in this composition range (18).

The methanol concentration within the stationary phase increases slightly with increasing volume percentage of methanol (13,20). In contrast, acetonitrile saturates the stationary phase at relatively low acetonitrile volumes and remains constant in subsequent regions (23). Stalcup and coworkers (1) determined that as water content increases, nonpolar solutes preferentially interact with nonpolar alkyl chains and retention increases.

Researchers have performed little work looking at water as a mobile phase for reversed-phase LC because it is the weakest solvent and retention is extremely high, even for small molecules. One exception to this statement is the work performed by Gangoda and Gilpin (24), who found that bonded alkyl chains are in a rigid environment when solvated with pure water. The changes they observed with highly polar solutes as a function of bonding density most likely are due to the reorganization of the water-rich region associated with residual silanols (25). Lower temperatures can increase stationary-phase rigidity because of the increased water structure at these temperatures, and, therefore, they can cause an exclusion of the organic modifier (25).

Variations of stationary-phase solvation and chromatographic retention mechanisms can be explained by differences in solvent structural information. The stationary phase has been described as a quaternary mixture of bonded organic chains, residual silanol groups, absorbed water, and organic modifier molecules (9). Sorption studies indicate that formation of the stationary phase seen by the solute is a dynamic process under the control of the mobile-phase composition (9,26–30). Both organic modifiers intercalate into the stationary phase to some extent; therefore, the amount of organic modifier within the stationary phase is thought to change throughout the mobile-phase composition range of 0:100–100:0 (v/v), though remaining relatively constant after approximately 50% organic modifier (15,17, 31–33).

Thermodynamics are bulk properties that measure net interaction effects (34). Thermodynamics usually can be assigned to different interactions, but numerous effects usually are present, and individual interactions cannot be separated easily (35). In the partitioning mechanism, both the mobile and stationary phases play important roles in the separation; the solute of interest has certain specific interactions with the stationary phase, which allow the separation to occur. Solutes spend the same amount of time in the mobile phase, which enables the interactions between the stationary phase and solute to be the main contributor in retention of the solute of interest in a partitioning retention mechanism. Thermodynamically, a separation occurs because of differences in the free energies of the solutes partitioning through the mobile phase and interacting with the stationary-phase alkyl chains. One class of solutes will have more interactions with the stationary phase (longer-retained compounds), and another class will have more affinity for the mobile phase (shorter-retained compounds). The greater the differences in these interactions, the larger the difference in free energy (ΔG) of solute partitioning from the mobile phase to the stationary phase.

The van't Hoff relationship ($\ln k$ versus $1/T$, where k is the retention factor and T is temperature) can provide valuable information about the retention mechanism for reversed-phase LC. This relationship is in part due to the temperature dependence of retention, which can be denoted as

$$\ln k = -\Delta H°/RT + \Delta S°/R + \ln \varphi \quad [1]$$

where k is the retention factor ($k = [t_R - t_0]/t_0$), $\Delta H°$ is the enthalpy of the transfer of the solute from the mobile phase into the stationary phase, $\Delta S°$ is the entropy of transfer of the solute from the mobile phase into the stationary phase, R is the gas constant, T is the temperature (in kelvins), and φ is the phase ratio (the volume of the stationary phase divided by the volume of the mobile phase). The slope of a linear van't Hoff plot is equal to $-\Delta H°/R$, and the intercept is $\Delta S°/R + \ln \varphi$. Assuming that the phase ratio is known and the van't Hoff plot is linear (that is, enthalpy is invariant with temperature), this relationship leads to a convenient and easy method of calculating the thermodynamic driving force of the separation, which in turn can provide information about reversed-phase LC's retention mechanism.

Most commercial C18 columns have an unspecified bonding density, although partitioning is affected by the surface coverage of the alkyl chains (11,36–40). Researchers have obtained linear van't Hoff plots for several commercially available monomeric and polymeric C18 phases (41–45). In all of these studies, $\Delta S°$ values could not be calculated because of the lack of knowledge about the phase ratio. Therefore, chromatographers lack information about the entropic driving force of the separation for most commercial phases. The driving force of a separation is determined by the Gibbs free energy equation:

$$\Delta G = \Delta H° - T\Delta S° \quad [2]$$

where the largest contribution to free energy comes from either $\Delta H°$ or $T\Delta S°$. Whichever

parameter has the largest contribution to the free energy is called the driving force of the separation. With this lack of knowledge about the entropy on commercial columns, it is difficult to determine the retention mechanism or to disprove if the retention mechanism is truly an adsorption mechanism that is entropically driven.

The other problem with thermodynamics derived from van't Hoff plots is that if the plots are nonlinear, users can derive little thermodynamic information from them. Researchers have obtained nonlinear plots in several different studies (36–38,46–57). These nonlinear plots may be indicative of a change in the solute retention mechanism within the studied temperature range. Cole and colleagues (36,38) have tentatively offered an explanation that phase transitions of the alkyl chains throughout the studied temperature range cause the deviation from linearity.

Although most van't Hoff studies have been performed within a narrow temperature range and at a single mobile-phase composition, several studies have been performed within a narrow composition range (8,12,42,58–65). I would like to expand on this previous work and investigate the entire hydro-organic mobile-phase composition range from 0:100 (v/v) to 100:0 (v/v) with both acetonitrile and methanol as the organic modifier. I hope that by determining the thermodynamics of solute transfer throughout the entire mobile-phase composition range, I will be able to learn more about the retention mechanism of reversed-phase LC and the fundamental driving force of separations.

Experimental

I synthesized the higher nitroalkane (C_7–C_{10}) homologues in my laboratory under previously described conditions (66,67) and took care that the silver nitrite was pure and unexposed to air to prevent oxidation before use or the reaction did not occur.

The detection wavelengths were 254 nm and 230 nm for alkylbenzene and nitroalkane homologues, respectively. Samples were injected using a manual injection valve equipped with a 20-μL sample loop. The flow rate was 1.0-mL/min. The temperature ranges studied for van't Hoff plots were 0 to 80 °C for acetonitrile and −5 to 80 °C for methanol. The columns were jacketed with a circulating water–ethylene glycol mixture thermostated to ±0.1 °C.

Monomeric stationary phases (1.74–3.52 μmol/m^2) were synthesized using Waters Microporasil silica (Milford, Massachusetts), which had irregularly shaped 6–10 μm particles, an average pore size of 125 Å, a specific surface area of 311 m^2/g, and varying surface coverages. High bonding densities (≥3.0 μmol/m^2) were ultrasonically driven, and all other syntheses were refluxed (68). The syntheses were refluxed for 24 h at 55 °C and the ultrasound syntheses were rotated for at least five days at 3 °C. I determined the bonding density from elemental analysis performed before column packing.

The silica was packed under pressure into 10 cm × 4.6 mm stainless steel columns (68). I determined the stationary-phase volume, used in determining the phase ratio, by a calculation method (69). Each new mobile-phase composition underwent long equilibration times to ensure proper equilibration, especially at low organic solvent compositions. I also used a previously synthesized 15 cm × 4.6 mm column packed with 4.4-μmol/m^2 Whatman Partisil silica (Clifton, New Jersey) that had 10-μm spherical particles, an expected 350-m^2/g specific surface area, and an 85-Å average pore size. A decrease in carbon loading, which causes a decrease in bonding density, is prevalent as columns age; therefore, this stationary phase was sent out for elemental analysis upon completion of these studies. The analysis indicated that the bonding density had decreased to 3.60 μmol/m^2, assuming a nominal surface area of 350 m^2/g.

Results and Discussion

The most comprehensive solvent composition study to date was conducted by Alvarez-Zepeda and co-workers (8). Their results indicated that acetonitrile and methanol show different thermodynamic driving forces. They found that acetonitrile–water mobile phases showed a relatively constant enthalpy of solute transfer until they reached approximately 55% acetonitrile. At fixed compositions, $\Delta H°$ appears to be a linear function of the number of methylene groups (CH_2) in both hydro-organic systems, in which the enthalpy change is fairly constant at a given fixed composition (8). Stalcup and colleagues (1) showed that the enthalpic contribution to retention was independent of the methanol concentration for the stationary phases used in their study and that the entropy of solute transfer decreased with increasing water concentration.

Based on my van't Hoff plots, I saw that at concentrations of organic modifier greater than 20% the stationary phase was saturated with solvent and retention became linear. This result was more pronounced for higher bonding densities and the larger nonpolar alkylbenzene homologues. This increase in retention is caused by entropic expulsion of the solute from the stationary phase because of poorly solvated alkyl chains in their rigid environment that are unable to allow solute partitioning. The increase in retention is more noticeable for the alkylbenzene homologues because they are more hydrophobic than the nitroalkane series. Overall, I observed more retention-time variations for methanol, which does not solvate the alkyl chains as well as acetonitrile because of methanol's hydrogen-bonding abilities.

Figure 1 shows entropy of solute partitioning versus percentage of organic modifier for benzene and nitropropane for a mid-bonding density (2.34 μmol/m^2). Benzene is representative of the alkylbenzene homologous series, and nitropropane is representative of the nitroalkane series. All bonding densities showed the same general trends. For clarity, I will discuss only the 2.34-μmol/m^2 bonding density. A decrease in entropy is indicative of a loss of order of the solute in the mobile phase relative to the stationary phase. Frank and Evans (70) hypothesized that large negative entropies were a result of structural ordering around hydrocarbon molecules. In my study, I observed a decrease in entropy as the organic modifier concentration increased to 40–50%, which corresponds to this structural ordering around solute molecules. At highly aqueous compositions, the water hydrogen bond lattice is quite structured and remains so until approximately 40% organic modifier has been added to completely wet and solvate the stationary-phase alkyl chains. At concentrations of organic modifier greater than 40%, the alkyl chains are fully solvated and enable entropic expulsion of the solutes because the solutes are thermodynamically more stable in the mobile phase.

Nitroalkane homologues increase entropic contributions above approximately 60% organic modifier, most likely because of their hydrogen-bonding ability and because they form a stronger attraction to the mobile-phase hydrogen-bonding lattice than the stationary-phase alkyl chains (that is, entropic expulsion of the polar molecules from the nonpolar stationary-phase alkyl chains). Methanol mobile phases follow the same trends at concentrations less than

40%, as does acetonitrile for both solute classes with more broadcast data because of its poor solvating ability compared with methanol. At concentrations greater than 40%, the entropy increases for both homologous series with additional methanol, which indicates that approximately 40–50% methanol has the best solvating ability of all compositions for the solutes with this chromatographic system.

The enthalpy of solute partitioning versus percentage acetonitrile in the mobile phase mimicked acetonitrile viscosity behavior with an enthalpy minimum at the viscosity maximum (14) (see Figure 2). Enthalpic contributions to solute partitioning come from the binding strength of the molecular interactions; therefore, it is plausible that the molecular interactions between the solute and mobile phase are at a minimum when the mobile-phase interactions themselves are at a maximum. Acetonitrile had more-or-less constant enthalpic contributions to retention regardless of mobile-phase composition (1). Stalcup and co-workers (1) also found that the solute has less contact with acetonitrile molecules in highly aqueous compositions; therefore, enthalpy and entropy of solute transfer decrease with increased aqueous composition in the mobile phase.

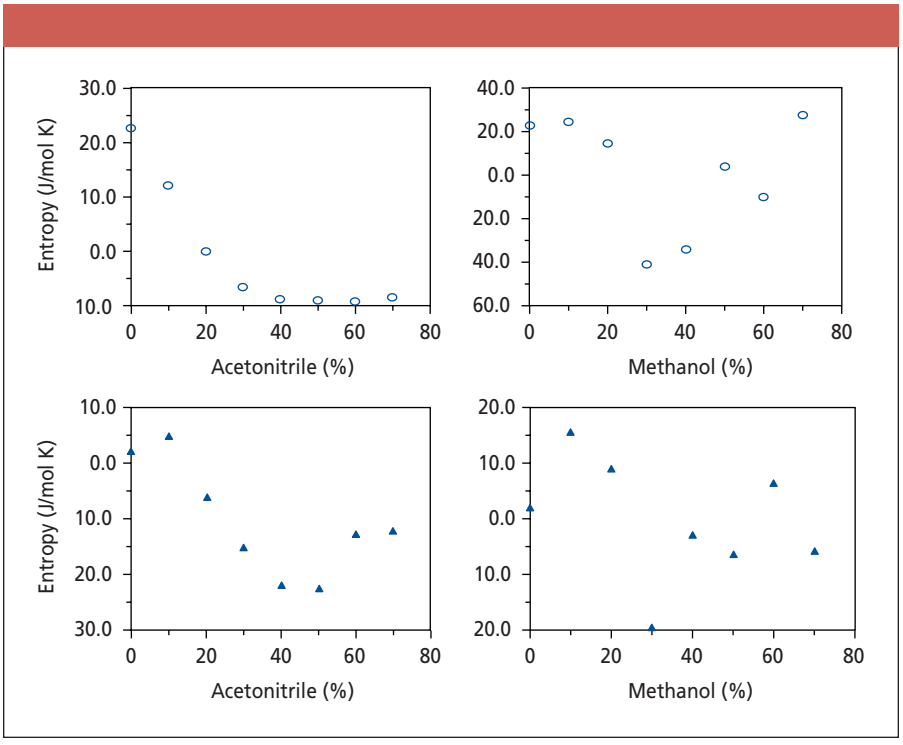

Figure 1: Entropy of solute partitioning versus percentage organic modifier in mobile phase for benzene (○) and nitropropane (▲) on 2.34-μmol/m^2 stationary phase.

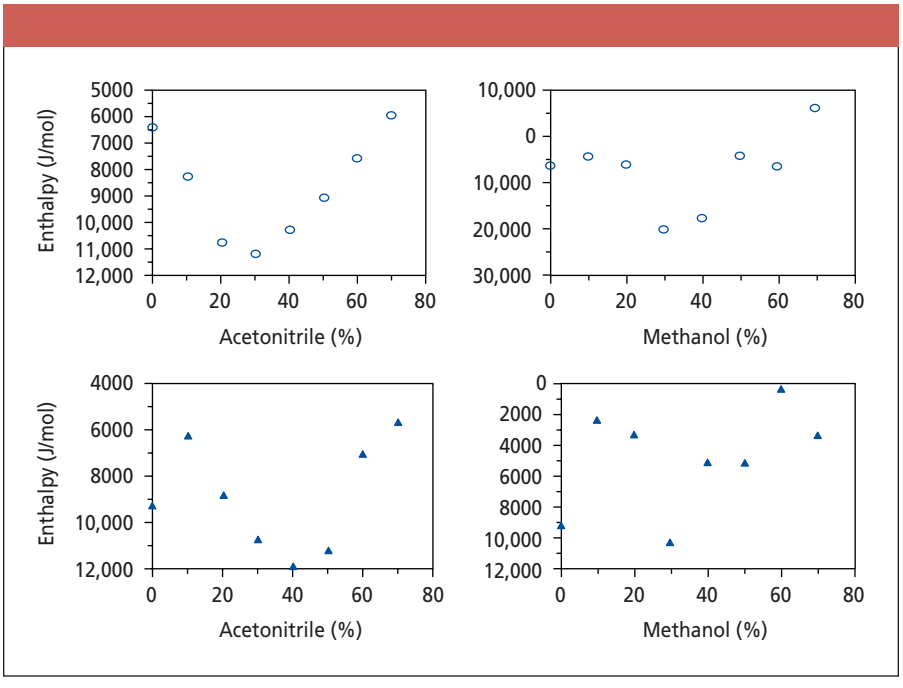

Figure 2: Enthalpy of solute partitioning versus percentage organic modifier in mobile phase for benzene (○) and nitropropane (▲) on 2.34-μmol/m^2 stationary phase.

Again, methanol follows the same trends but with more broadcast data, especially at concentrations greater than 40% for nitropropane. The scatter may be due to the polarity of the nitroalkane series and the lack of solvation of the alkyl chains by methanol or the fact that methanol was the second solvent studied and the columns were aging prematurely from excessive use at high aqueous compositions (that is, hysteresis from previous work with acetonitrile).

With my chromatographic system, I observed a change in both the entropy and the enthalpy of solute partitioning at each mobile-phase composition, which is in direct opposition to the theories of Alvarez-Zepeda (8) and Stalcup (1). The most likely explanation for the differences between my work and the previous work is either that the starting silica is very important to solute thermodynamics or that the stationary phase is monomeric. I used Microporasil silica as my base silica, and it has greater amounts of metal impurities and a larger pore diameter than other silicas currently in use commercially. Commercial columns also have proprietary synthesis techniques, and the manufacturers provide only percentage carbon information. Therefore, the previous work may have been performed using a polymeric stationary phase instead of my monomeric phase.

Figure 3 shows that the nonlinearity of entropy and enthalpy correspond to a rather linear free energy of solute partitioning based on the Gibbs free energy relationship. The alkylbenzene homologues showed a more linear free energy of solute partitioning across the entire composition range because of their large hydrophobic nature. The nitroalkanes, represented by nitropropane, had more deviations from linearity due to their small polar structures, which can efficiently interact with the mobile phase, and residual silanols. Therefore,

these compounds were solvated differently depending upon their organic composition, as Figure 3 shows. I found this chromatographic system to be enthalpically driven throughout the entire composition range — with all solutes at all monomeric C18 stationary-phase bonding densities and with both organic modifiers.

Conclusions

This study is the most comprehensive mobile-phase composition study to date, and it indicates a generally linear relationship observed for both retention and Gibbs free energy of solute partitioning for both homologous series studied with acetonitrile as organic modifier on a monomeric stationary phase. Both entropy and enthalpy of solute transfer were nonlinear where there was a minimum in enthalpy of solute transfer near the viscosity maximum for the hydro-organic mixture. The entropy of solute transfer was nonlinear, decreasing as the acetonitrile concentration approached approximately 40% and leveling off to a relatively constant value (approximately −10 J/mol K), which indicates that entropic expulsion of solutes occurred at acetonitrile concentrations greater than 40%.

The deviations from linearity in both retention and free energy can be explained by the solute's small size, which causes it to be eluted close to the void volume of the system (and, therefore, to have more error in retention measurements), or the highly organic or aqueous mobile phases (extremely short or long retention, respectively). At both extremes of the composition range, deviations from linearity occur due to differences in solvation of the stationary-phase alkyl chains. In highly aqueous mobile phases, the alkyl chains are poorly solvated, and solvation of the alkyl chains occurs in highly organic mobile phases, but I observed more error in retention measurements caused by extremely short retention times, even for larger molecules. The alkylbenzenes behaved more regularly than did the nitroalkanes because of their nonpolar nature and their ability to intercalate into the stationary phase without interacting with residual silanols on the silica surface.

Methanol exhibited less linearity for the thermodynamics of solute partitioning as well as solute retention throughout the entire mobile-phase composition range. This result is an indication that methanol does not solvate the alkyl chains as well as acetonitrile does, thereby decreasing the thermodynamics of solute partitioning. The thermodynamics of solute partitioning depend greatly upon the amount of methanol in the mobile phase, and they can change dramatically with a small change in mobile-phase composition. Solute hydrogen bonding with the mobile phase could account for many of the deviations from linearity that I noticed for the nitroalkanes in the methanol–water system. Entropic expulsion was more noticeable for both homologous series with methanol as the organic modifier.

Both methanol and acetonitrile behaved nearly equivalently in this chromatographic system across the entire composition range. Thermodynamics of solute partitioning show that both modifiers create an environment that favors solute partitioning at low compositions and solute expulsion at higher compositions, which are related to solvation of both the alkyl chains and the solute molecules by the mobile-phase components.

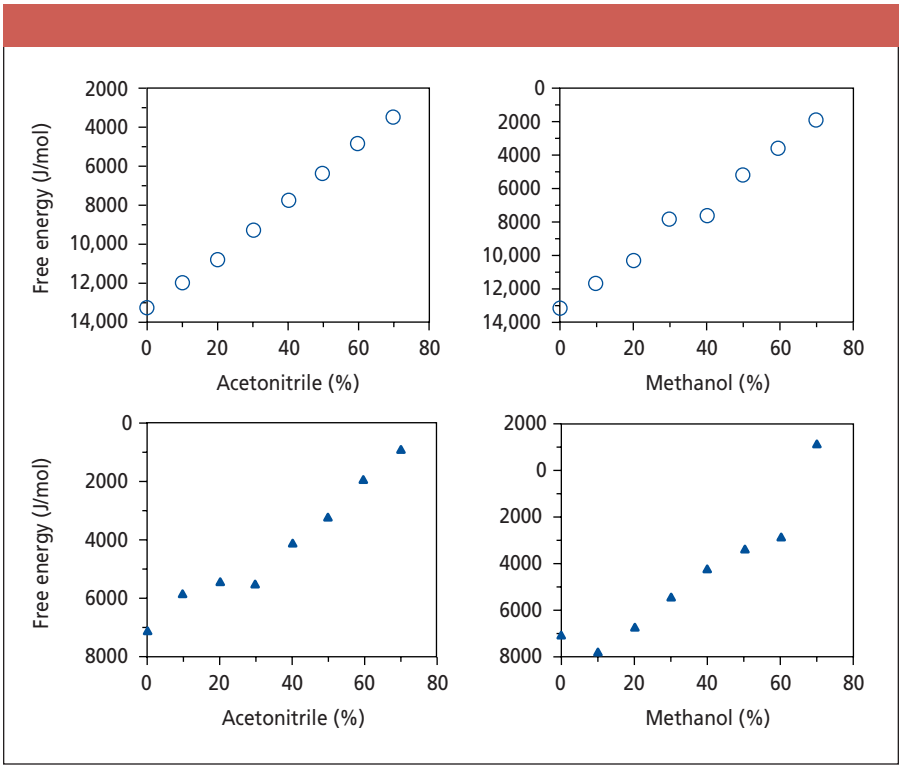

Figure 3: Free energy of solute partitioning versus percentage organic modifier in mobile phase for benzene (○) and nitropropane (▲) on 2.34-μmol/m^2 stationary phase.

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