Retention mechanism of a cholesterol-coated C18 stationary phase: van't Hoff and Linear Solvation Energy Relationships (LSER) approaches

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ABSTRACT

This study examines the effect of temperature on the dynamic cholesterol coating of a C18 stationary phase and the effect of this coating on the retention mechanism. In general, an increase in temperature results in a decrease in the mass of cholesterol coated on the stationary phase. Typically, an increase in temperature from 25 °C to 55 °C results in a nearly 60% reduction in the mass of cholesterol loaded. The inclusion of temperature, along with loading solvent composition and cholesterol concentration in the loading solvent, allows for loading a targeted amount of cholesterol on the stationary phase over an order-of-magnitude range. In addition to loading studies, the retention mechanism of small non-ionizable solutes was examined on cholesterol-coated stationary phases. A van't Hoff analysis was performed to assess retention thermodynamics, while a LSER approach was used to examine retention mechanism. With 50/50 water/organic mobile phases, the addition of cholesterol results in an increase in the entropic contribution to retention, with a decrease in the enthalpic contribution. The opposite trend is seen with 40/60 water/organic mobile phases. LSER system constants are also affected by a cholesterol coating on the stationary phase, with some changing to favor elution and others changing to favor retention.

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1. Introduction

1.1. Stationary phases containing cholesterol

A variety of schemes for incorporating cholesterol into a chromatographic stationary phase have been examined. Recently, Ogden and Coym described the use of a dynamic coating of cholesterol onto a C18 stationary phase [1]. In their work, frontal analysis was used to determine the amount of cholesterol coated on the phase, and a variety of selectivity tests were performed to examine the effect of a cholesterol coating on chromatographic behavior. It was found that the cholesterol coating had an effect on shape-type selectivities but not on hydrophobic (methylene) selectivity. In addition, the stability of a cholesterol coated phase was assessed. These results were similar to those reported by Cole [2]. In addition, it was found that the cholesterol coating was stable, even when cholesterol was removed from the mobile phase, for at least 250 column volumes when mobile phases of less than 70% methanol were used.

Initial work with bonded cholesterol stationary phases was communicated by Pesek and co-workers [3–7]. Their work focused on the preparation of these phases on a silica hydride based

material, and their characterization via spectroscopic and chromatographic methods. Their work described the use of these phases for a variety of separations, most significantly, for the resolution of isomers and enantiomers. Work by Delaurent and co-workers [8–10] examined various bonding chemistries for preparation of cholesterol bonded phases. Using a variety of chromatographic approaches, they determined the origin of the unique selectivity of these phases is due to the presence of the cholesteric moiety than to differences in bonding chemistry. In addition, Buszewski and co-workers have published a series of papers examining a variety of phase preparation approaches and ligand chemistries [11–16]. Their work focused on the differences in chromatographic behavior between cholesteric phases and alkyl phases, noting that both provided reversed-phase behavior.

This work focuses on evaluation of the effect of temperature on the behavior of a cholesterol-coated alkyl stationary phase and on chromatographic retention mechanisms when such phases are employed. By examining retention as a function of temperature, differences in retention thermodynamics between uncoated and cholesterol-coated phases can be compared. Retention mechanisms between coated and uncoated phases can be examined directly via an LSER approach.

1.2. The van't Hoff approach

The process of chromatographic retention is associated with an entropy and enthalpy change, and the magnitude of these changes

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can be assessed via van't Hoff analysis. Such an analysis allows for description of the retention process as enthalpically or entropically driven. In addition, van't Hoff analysis can be performed on two different chromatographic systems to evaluate the difference in retention thermodynamics when a chromatographic variable is changed, such as the addition of an additive to the mobile phase or changing the identity or structure of the stationary phase.

In brief, a van't Hoff analysis is performed by measuring the retention factor, k, of a probe solute at a variety of temperatures. The natural logarithm of the retention factor, $\ln k$, is regressed against inverse temperature, according to the equation:

$$\ln(k) = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln(\Phi) \qquad (1)$$

In this equation, k is the retention factor, ΔH° and ΔS° are the standard-state enthalpy and entropy change associated with the retention process, R is the gas constant, T is the absolute temperature, and Φ is the phase ratio, which is the volume of the stationary phase divided by the volume of the mobile phase.

The van't Hoff analysis has been used by a variety of authors for investigation of retention mechanism [17–23]. For example, Cole, Dorsey, and Dill [17,18], used van't Hoff analysis in their investigation of the hydrophobic model for retention. In a study by Ranatunga and Carr [20], van't Hoff analysis was used to examine individually the contributions of the stationary and mobile phase to retention thermodynamics of small non-polar molecules. They determined that enthalpic changes in the stationary phase, based on the formation of lipophilic interaction between solutes and the stationary phase ligands, were the primary driving force for retention under most reversed-phase conditions.

One problem with the use of the van't Hoff approach is appropriate estimation of the phase ratio. While the retention factor is easily calculated, the phase ratio is unavoidably complex to determine since the stationary phase volume cannot be simply defined [21,24–26]. It can be reasonably assumed, however, that the phase ratio does not change significantly between similar compounds and over a modest temperature range [21]. Therefore, the difference between the natural logarithms of the retention factor between adjacent members in a series of compounds, such as toluene and ethylbenzene will remove the phase ratio term from the van't Hoff equation. This difference or separation factor (α) is also known as the selectivity, and the thermodynamics based on the selectivity can now be determined from the line of a plot of $\ln(\alpha)$ vs. inverse temperature as shown in Eq. (2) [21]. In this equation, ΔH_α° and ΔS_α° represent the differences in molar enthalpy and entropy of retention between the two solutes examined.

$$\ln(\alpha) = \frac{-\Delta H_\alpha^\circ}{RT} + \frac{-\Delta S_\alpha^\circ}{R} \qquad (2)$$

This use of selectivity van't Hoff plots eliminates the phase ratio from the calculation. In a second approach, the phase ratio term ($\ln \Phi$) can be taken as an entropic contribution to retention (which it truly is as it represents the entropy of dilution) and compare van't Hoff plot intercepts as the total entropic contribution to retention [21,23]. This has the advantage of allowing for comparison of retention thermodynamics without an estimation of the phase ratio, but with the drawback of convoluting various entropic contributions (dilution and cavity formation).

1.3. Linear Solvation Energy Relationships (LSER)

The LSER model is a particular subset of thermodynamic relationships known as linear free energy relationships. This model relates the solute partitioning measure (SP) to solute dependent input parameters as a linear relationship with each parameter multiplied by a system coefficient. This relationship also includes a constant or intercept term c, which is independent of the solute [27–34].

$$SP = c + eE + sS + aA + bB + vV \qquad (3)$$

Eq. (3) for the LSER model is presented by Abraham and is currently the most accepted, symbolic representation [28]. The measure of the solute partitioning between the stationary and mobile phases that is commonly used in chromatography is $\log k$. The solute input parameter S represents polarizability and dipolarity, relative to the dipolar and polarizability interaction of cyclohexane. Parameter E represents the excess polarizability that is not included in the S parameter, due primarily to the presence of n and π electrons. Parameters A and B represent the hydrogen bond acidity and hydrogen bond basicity, respectively. Parameter V accounts for the unfavorable (endoergic) process of cavity formation and more specifically represents the ease of cavity formation by the stationary phase as compared to the mobile phase.

Each of the lower case letters represents the corresponding coefficients and reflects the differences between the stationary and mobile phases. Collectively, these are called system constants and are a measure of the type and extent of interactions occurring in the chromatography column. More specifically, their magnitudes reflect the degree of difference in the solvent interaction abilities with the stationary and mobile phases, and thus the extent to which each interaction dictates the overall solute property. The signs of the coefficients determine whether there are favorable or unfavorable interactions of that input parameter with retention for that solute. Positive values result in increased solute retention while negative values result in reduced solute retention. The c term represents solute-independent influences on retention (such as the phase ratio) as well as any interactions not included in the solute descriptors used for the regression. For the calculation of these coefficients, $\log k$ for a series of varying analytes are measured and a multi-parameter linear least-squares fit is performed, using Eq. (3) as the model.

2. Experimental

2.1. Reagents

Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Water was purified in-house using a Continental Water Systems Modulab Analytical Research Grade water purification system. Cholesterol (99% purity) was obtained from Aldrich Chemical Company (St. Louis, MO, USA). For van't Hoff analysis, acetophenone, 3,4-dichlorophenol, p-chlorobenzene, and a series of alkyl parabens (methyl to butyl) were obtained from Aldrich. The solutes used in the LSER analysis, along with their solute descriptors, are shown in Table 1. These were also obtained from Aldrich. Solutions of the analytes were made up in methanol. Uracil was used as an unretained marker to estimate the mobile phase volume.

2.2. Equipment

A Phenomenex (Torrance, CA, USA) Luna C18(2) column, 150 mm × 4.6 mm with 5 μm stationary phase particles, was used for all aspects of this study. According the manufacturer, this is a monomeric, endcapped stationary phase with a pore size of 100 Å, a surface are of 400 m^2/g, a carbon load of 17.8%, and bonding density of 3.25 μmol/m^2.

Two chromatographic systems were used in this work. The first system was used for the study of cholesterol loading on the stationary phase at different temperatures, unloaded and loaded LSER, and van't Hoff analyses for the 50/50 methanol/water mobile phase. This system consisted of a Shimadzu (Columbia, MD, USA) LC-20AD

Table 1
Solute descriptor for LSER study. Values are from Refs. [33,34].

Solute	V	B	A	S	E
Benzene	0.7176	0.144	0	0.511	0.608
Toluene	0.8573	0.139	0	0.499	0.606
Ethylbenzene	0.9982	0.139	0	0.499	0.613
Propylbenzene	1.1391	0.134	0	0.502	0.610
Acetophenone	1.0139	0.503	0	1.026	0.806
3,4-Dichlorophenol	1.0199	0.030	0.850	1.140	1.020
p-Dichlorobenzene	0.9612	0.020	0	0.750	0.825
Acetone	0.5407	0.490	0.040	0.700	0.179
Benzyl alcohol	0.9160	0.557	0.400	0.882	0.803
p-Chlorophenol	0.8975	0.205	0.886	0.794	1.016
Phenol	0.7751	0.319	0.716	0.759	0.769
m-Cresol	0.916	0.340	0.570	0.880	0.822
Theophylline	1.2223	1.340	0.540	1.600	1.500
n-Benzyl formamide	1.1137	0.630	0.400	1.800	0.990
3-Phenyl-1-propanol	1.1978	0.669	0.354	0.892	0.821
Phenyl ethyl alcohol	1.0569	0.648	0.351	0.819	0.823
Benzonitrile	0.8711	0.331	0	1.135	0.742
Methyl benzoate	1.0726	0.439	0	0.923	0.738
Anisole	0.9160	0.311	0	0.768	0.712
p-Nitrotoluene	1.0315	0.264	0	1.194	0.918
Benzophenone	1.4808	0.576	0	1.330	1.224
Bromobenzene	0.8914	0.089	0	0.723	0.882
p-Xylene	0.9982	0.160	0	0.494	0.615
Nitrobenzene	0.8906	0.269	0	1.138	0.846
Caffeine	1.3632	1.232	0.039	1.726	1.518

pump, LabAlliance (Sci-Con, Winter Park, FL, USA) Model 500 UV-Vis detector, and SRI PeakSimple (Alltech, State College, PA, USA) Model 302 A/D converter with PeakSimple v. 3.29 software. A column jacket (Alltech) and Fisher Isotemp 3016S circulating water bath or the Torrey Pines Scientific C050 HPLC column chiller/heater was used to maintain a constant column temperature. Switching between neat mobile phase and mobile phase containing cholesterol was done using a manual t-valve.

The second system was used to run the LSER and van't Hoff analyses for the 40/60 water/methanol and 40/60 and 50/50 water/acetonitrile mobile phases. This system was a Shimadzu Prominence system consisting of a model LC-20AT pump, model DGU-20A5 degasser, a Rheodyne 7725i manual injector, a model CTO-10AS column oven, model CBM-20A system controller, and LCSolution software.

2.3. Procedures

2.3.1. Temperature dependent loading studies

The amount of cholesterol loaded on the stationary phase under a given set of conditions (mobile phase composition and cholesterol concentration) was assessed by the use of frontal chromatography [1,35]. The procedure for cholesterol addition and analysis was described in the previous work [1].

For each run, the mobile phase containing cholesterol was pumped through the column. Once the cholesterol fully saturated the stationary phase, the cholesterol would begin to elute from the column and be detected. Once the cholesterol curve reached maximum absorbance, the stationary phase was considered to be loaded. The amount of loaded cholesterol was then determined by integration of the area of the loading chromatogram. The column was maintained at a constant temperature throughout cholesterol loading. The resulting breakthrough curves were analyzed by numeric integration in Microsoft Excel with time increments of 0.2 s.

Loading studies were performed using a series of mobile phase compositions between 10/90 water/methanol and 100% methanol. Cholesterol concentration was varied from 0.5 mg/mL up to 1.0, 1.4, 1.8, 1.8, and 2.0 for mobile phases containing 90, 93, 95, 97, and 100% methanol, respectively. The temperatures examined for cholesterol loading were 25 °C, 35 °C, 45 °C, and 55 °C.

Breakthrough curves were collected in duplicate at each mobile phase composition/cholesterol concentration/temperature condition.

2.3.2. van't Hoff and LSER analyses

All analyses were performed with a flow rate of 1.00 mL/min, a detection wavelength of 254 nm, and an injection volume of 5 μL. The mobile phases tested were 40/60 and 50/50 water/methanol and 40/60 and 50/50 water/acetonitrile. Temperatures were varied from 25 °C to 55 °C in increments of 10 °C. Duplicate injections were made for each solute at each condition.

Once the uncoated van't Hoff analyses were completed, the column was loaded with 1.4 mg/mL concentration of cholesterol in a 5/95 water/methanol mobile phase using the previously described technique [1]. This resulted in a coating of 21.8 mg of cholesterol. After reaching the breakthrough point, the interstitial mobile phase was flushed out for approximately 3 min with the 5/95 water/methanol solvent with no cholesterol The van't Hoff analyses were then repeated with the loaded column under the same conditions. For the LSER study, a series of analytes with known solute descriptors were found. Each analyte was chromatographed at a temperature of 35 °C, in duplicate. The stationary phase was then coated with cholesterol using the same procedure as for the van't Hoff analysis, and the LSER study was repeated. For all retention factors, the extracolumn volume was measured and eliminated from the calculation.

2.3.3. Comments on stability of the cholesterol coating

In our previous work, cholesterol-coated stationary phases (using this same column) were shown to be stable when up to 250 column volumes of mobile phase were flushed through the column [1]. It was also shown that the column could be quickly cleaned of cholesterol by flushing it with neat methanol. In order to further assess the stability of the cholesterol coating, a chromatogram was obtained during the cleaning step to observe cholesterol removal. The area under the "cleanout curve" can be used to determine the amount of cholesterol removed from the column. This is done by calibrating detector response vs. cholesterol concentration in neat methanol, then using the calibration data to convert the area under the curve to a mass of cholesterol removed (similar to a loading

Fig. 2. Plot of cholesterol mass loading against temperature, for a variety of cholesterol concentration in the mobile phase. In all cases, the composition of the mobile phase was 5/95 water/methanol.
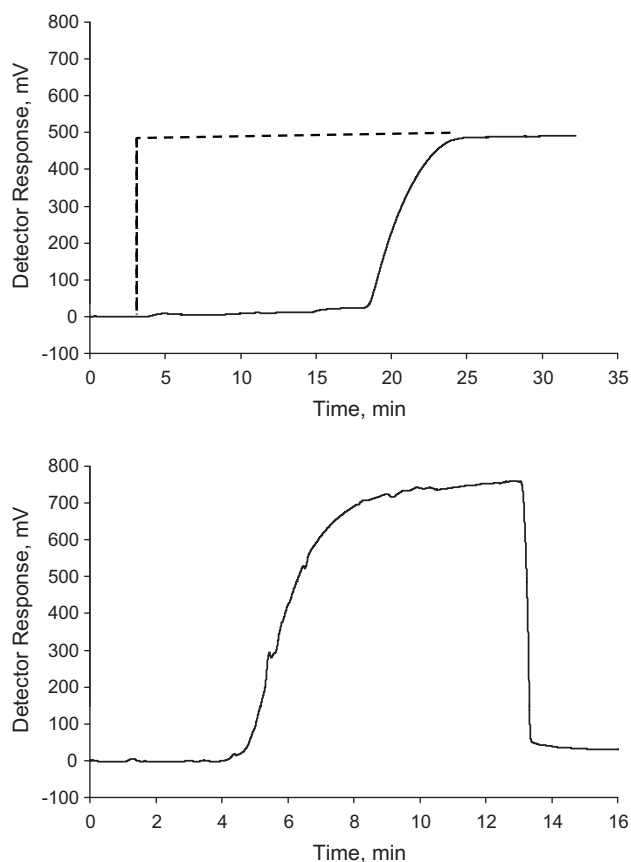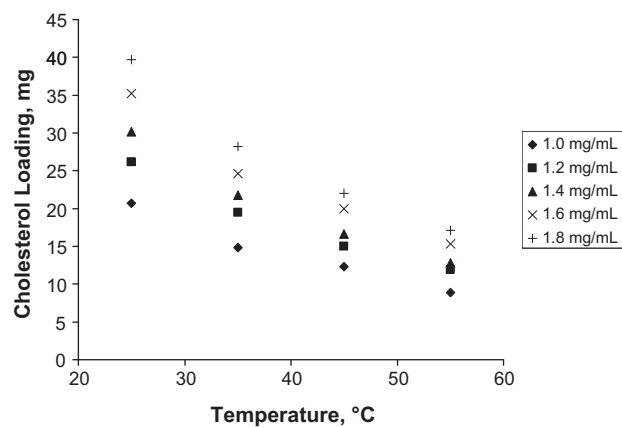
Fig. 1. Loading (top) and cleanout (bottom) curves for cholesterol. The integrated area of the loading curve indicates 21.8 mg of cholesterol was loaded onto the phase; the area under the cleanout curve indicates 20.0 mg was removed after the van't Hoff analysis was completed.

more easily, data can be examined while holding at least one of these variables constant.

Fig. 2 illustrates the effect of temperature on cholesterol loading when mobile phase composition is held constant. In this case, the mobile phase was 5/95 water/methanol. As expected, for a variety of cholesterol concentrations between 1.0 and 1.8 mg/mL, as temperature increases the amount of loaded cholesterol decreases. For larger cholesterol concentrations, the rate of this change is larger, but in relative terms, the decrease in amount loaded is the same.

This trend is shown in Table 2. Using the 25 °C loading values as a baseline, a 10 °C increase in temperature reduces the amount of cholesterol loaded by about 28%; a 20 °C increase reduces the amount loaded by about 43%, and a 30 °C increase in temperatures reduces the amount loaded by around 56%. This similarity in reduction of the amount of cholesterol loaded suggests that the thermodynamics of the loading process are independent of the cholesterol concentration in the mobile phase. To further investigate this idea, van't Hoff plots were constructed for this data by regressing ln(mg of cholesterol loaded) vs. $1/T$ for each of the cholesterol concentrations, as shown in Fig. 3. The slopes and intercepts of these plots are more-or-less constant, suggesting that the loading process is mechanistically independent of cholesterol concentration.

From a practical standpoint, however, the range of accessible loading values is larger at lower temperature. For example, at 25 °C, the loading values vary from 20.7 mg to 39.7 mg, or about 19.0 mg.

calculation). Example loading and cleanout curves are shown in Fig. 1, which was obtained after the van't Hoff analysis using the 40/60 water/methanol mobile phase. Cholesterol has been shown to have a higher solubility in methanol than acetonitrile, so this mobile phase should be the harshest one examined in terms of coating stability. In this case, the cleanout curve showed that nearly all the cholesterol remained on the column over the course of the experiment.

3. Results and discussion

3.1. Temperature dependence of loading

As shown in the previous study [1] on cholesterol loading at 35 °C, the strong solvent content of the mobile phase (% methanol) has a significant influence on the amount of cholesterol loaded onto the column. The amount of cholesterol loaded under isothermal conditions could be varied by up to an order of magnitude by varying the mobile phase composition between 85 and 100% methanol. As expected, weaker mobile phase compositions result in a higher amount of cholesterol loading [1].

For cholesterol loading at different temperatures, the amount of cholesterol coated on the column is expected to decrease with increasing temperature due to the increased solubility of cholesterol in water/methanol mobile phases. The inclusion of temperature as a variable in the cholesterol loading scheme allows for selection of three parameters to load a target amount of cholesterol: temperature, mobile phase composition, and cholesterol concentration. In order to examine the effect of temperature on loading
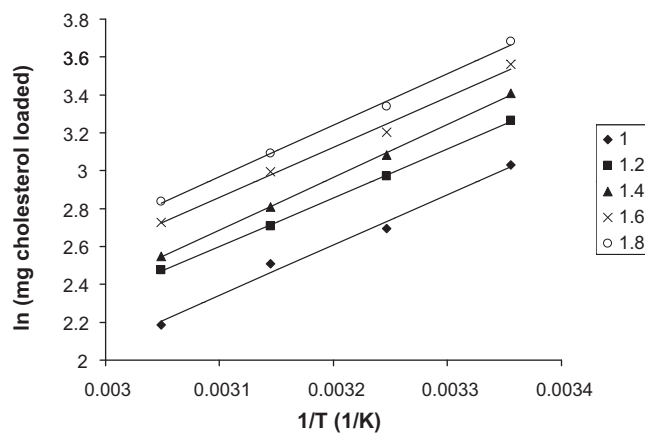

Fig. 3. van't Hoff plot of cholesterol loading, using a 5/95 water/methanol mobile phase and various cholesterol concentrations between 1.0 and 1.8 mg/mL. Slopes of the van't Hoff plots are given in Table 2.

Table 2
Relative changes in the amount of cholesterol loaded, using the amount loaded at 25 °C as a baseline. "Slope" and "Intercept" refer to a plot of the natural logarithm of the amount of cholesterol loaded vs. inverse temperature (a van't Hoff plot).

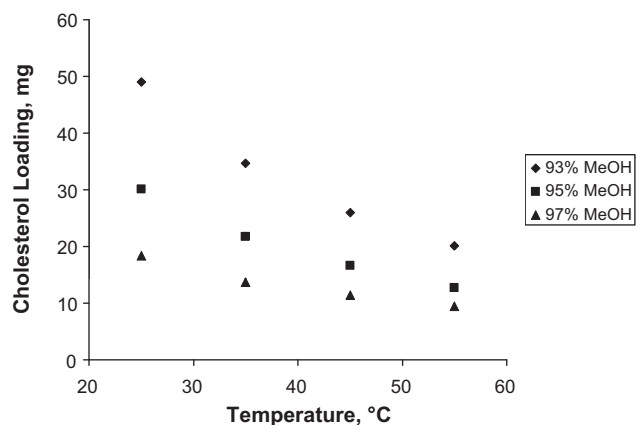|	1.0 mg/mL	1.2 mg/mL	1.4 mg/mL	1.6 mg/mL	1.8 mg/mL
Temperature					
25 °C	20.7	26.2	30.2	35.2	39.7
35 °C	−28.5%	−25.6%	−27.8%	−30.1%	−29.0%
45 °C	−40.6%	−42.7%	−45.0%	−43.2%	−44.6%
55 °C	−57.0%	−54.6%	−57.6%	−56.5%	−56.9%
Slope	2655 ± 202	2573 ± 47	2786 ± 53	2650 ± 169	2716 ± 107
Intercept	−5.9 ± 0.7	−5.4 ± 0.2	−6.0 ± 0.2	−5.4 ± 0.5	5.5 ± 0.3

Fig. 4. Plot of cholesterol mass loading against temperature, for a variety of mobile phase compositions. In all cases, the cholesterol concentration in the loading solvent was 1.4 mg/mL.
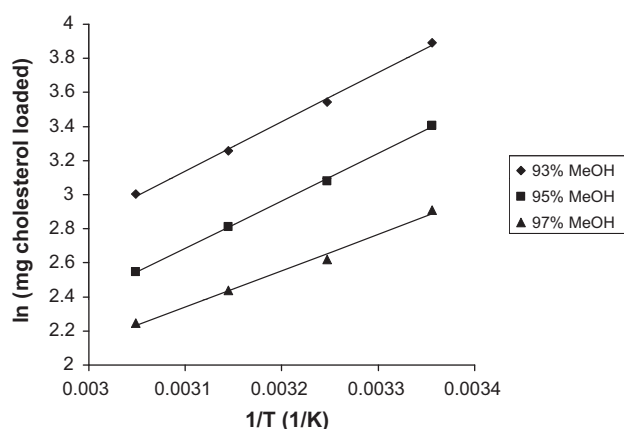

Fig. 5. van't Hoff plots of cholesterol loading using 93%, 95%, and 97% methanol mobile phases, and 1.4 mg/mL cholesterol. Slopes are given in Table 3.

At 55 °C, these values vary from 8.9 mg to 17.1 mg, or a range of 8.2 mg. This suggests that the cholesterol coating should be performed at lower temperatures.

Not surprisingly, the situation is different when the cholesterol concentration is held constant and the mobile phase composition is changed. Fig. 4 is a plot of amount of cholesterol loaded vs. temperature, for a variety of mobile phases, when cholesterol concentration is held constant at 1.4 mg/mL. As expected, as temperature increases the amount of loaded cholesterol goes down. However, the rate of that change is not conserved between the different mobile phase compositions. This is illustrated in Table 3. As the mobile phase becomes stronger, the change in loading mass with temperature decreases. Said another way, temperature has a larger effect on cholesterol loading when the mobile phase is weak, as compared to when it is strong. This effect is more pronounced when comparing the 95% methanol and 97% methanol mobile phases, and reflects the change in the partition coefficient between the stationary phase and loading solvent as the solvent composition varies. In comparing the van't Hoff slopes, the value decreases as the mobile phase composition becomes stronger, as

shown in Fig. 5. This is reflected in the lower loading values when stronger mobile phases are used.

3.2. van't Hoff analyses

Retention thermodynamics can be assessed by use of the van't Hoff analysis. In this work, the effect of a moderate cholesterol coating on retention thermodynamics was examined. This loading level was shown to produce clear changes in some chromatographic selectivities [1], particularly those related to molecular shape. For the retention van't Hoff analysis, acetophenone, 3,4-dichlorophenol, and p-dichlorobenzene were used as test solutes. These solutes were selected because of their significantly different LSER solute descriptors with regard to hydrogen bonding.

Four mobile phases were examined: 50/50 water/methanol and water/acetonitrile, and 40/60 water/methanol and water/acetonitrile. In performing the analysis, enthalpy values were calculated from the slopes of the van't Hoff plots. Entropies are compared as the intercept of these plots, so the value contains contributions from both cavity formation and any change in apparent phase ratio upon addition of cholesterol to the stationary phase. van't Hoff plots obtained with the cholesterol-coated stationary phases were linear, not exhibiting discontinuities or changes in slope. This is in contrast to bonded cholesterol stationary phases, where discontinuities in van't Hoff plot slopes have been reported [8].

Table 4 lists the retention enthalpies for the three test solutes with the four mobile phases on uncoated and coated stationary phases. Similar behavior is observed for both methanol containing and acetonitrile containing mobile phases. With the 50/50 mobile phases, retention enthalpy becomes less favorable (more positive) when the stationary phase is coated with cholesterol. This is observed for both methanol and acetonitrile, with all three test solutes. Surprisingly, a different trend was observed with the 40/60 mobile phases. For all three solutes with the water/methanol

Table 3
Relative changes in the amount of cholesterol loaded, using the amount loaded at 25 °C as a baseline. Cholesterol concentration was kept constant at 1.4 mg/mL; mobile phase composition (% MeOH) was varied as indicated. "Slope" and "Intercept" refer to a plot of the natural logarithm of the amount of cholesterol loaded vs. inverse temperature (a van't Hoff plot).

	93% MeOH	95% MeOH	97% MeOH
Temperature			
25 °C	49.0 mg	30.2 mg	18.3 mg
35 °C	−29.3%	−27.8%	−25.1%
45 °C	−46.9%	−44.8%	−37.6%
55 °C	−58.9%	−57.7%	−48.5%
Slope	2892 ± 83	2786 ± 53	2129 ± 133
Intercept	−5.8 ± 0.3	−6.0 ± 0.2	−4.3 ± 0.4

Table 4
Enthalpy comparison, kJ/mol. van't Hoff data were taken between 25 °C and 55 °C.

Solute	50/50, uncoated	50/50, coated	40/60, uncoated	40/60, coated
Methanol as organic modifier				
Acetophenone	-10.47 ± 0.26	-8.59 ± 0.99	-4.98 ± 0.30	-5.64 ± 0.06
Dichlorophenol	-22.33 ± 0.30	-18.54 ± 1.41	-11.28 ± 0.42	-12.47 ± 0.93
Dichlorobenzene	-18.24 ± 0.44	-14.90 ± 1.63	-9.44 ± 0.24	-9.91 ± 0.26
Acetonitrile as organic modifier				
Acetophenone	-6.92 ± 0.74	-4.13 ± 1.77	-3.98 ± 0.17	-4.32 ± 0.79
Dichlorophenol	-9.13 ± 0.31	-5.78 ± 1.40	-5.92 ± 0.62	-5.82 ± 0.38
Dichlorobenzene	-6.83 ± 0.62	-5.94 ± 1.31	-5.59 ± 0.57	-5.51 ± 0.23

mobile phase, and with acetophenone with the water/acetonitrile phase, changes in retention thermodynamics were much less pronounced, and possibility statistically insignificant. As a general rule, the differences between coated and uncoated phases, at least with regard to retention enthalpy, are more pronounced with the 50/50 mobile phases. Stated another way, coating the stationary phase with cholesterol results in a larger change in retention enthalpy with weaker mobile phases. This is not surprising, as when a weaker mobile phase is employed a solute spends more time interacting with the stationary phase, as compared to when a stronger mobile phase is used. For this reason, it is reasonable to expect that modifying the stationary phases has a more pronounced effect on retention mechanism when the mobile phase is weak.

The sum of the retention entropy terms for the three test solutes with the same test conditions is reported in Table 5. These values are the intercepts of the van't Hoff plots, and include entropic contributions from cavity formation in the stationary and mobile phases, as well as the entropy of dilution from the phase ratio. Because of the difficulty in both defining and calculating the phase ratio—especially with the cholesterol-coated stationary phase—the two entropic contributions are presented as one value. When the 50/50 mobile phases are examined, the entropic contribution to retention becomes more favorable (more positive) when cholesterol is added to the stationary phase. This is observed for all three test solutes, with both methanol and acetonitrile mobile phases. As was seen with retention enthalpies, a different trend is seen with the 40/60 mobile phases. With the 40/60 water/methanol mobile phase, retention entropy becomes less favorable after cholesterol is added. This is also seen for acetophenone with the 40/60 water/acetonitrile mobile phase. With the other two solutes, retention entropy does not change appreciably upon addition of cholesterol to the stationary phase.

In addition to examining van't Hoff plots based on retention, van't Hoff plots can be constructed based on selectivity [21]. These have the advantage of being independent of phase ratio. Table 6 lists the methylene selectivity on the various chromatographic systems examined, as well as the enthalpy and entropy change associated with the retention of a methylene group. As was previously reported [1], the overall methylene selectivity does not change significantly when cholesterol is added to the stationary phase. However, the $\Delta H°$ and $\Delta S°$ do change when cholesterol is added. With the 50/50 mobile phases, methylene selectivity becomes less enthalpically driven and more entropically driven. This is the same trend seen with retention van't Hoff values. The opposite is seen with the 40/60 mobile phases. In this case, when cholesterol is added to the stationary phase, the methylene selectivity enthalpy becomes more favorable, while the methylene selectivity entropy becomes less favorable.

In summary, the effect of a cholesterol coating on the stationary phase is dependent on the composition of the mobile phase. For 50/50 water/organic mobile phases, addition of cholesterol to the stationary phase makes retention less favorable enthalpically and more favorable entropically. This is not, however, seen with 40/60 water/organic mobile phases. In these cases, the opposite

thermodynamic change occurs: retention becomes more driven by enthalpy and less by entropy, or is not affected at all. This clearly illustrates the complex interplay between solute, stationary phase and mobile phase in determining retention thermodynamics.

3.3. LSER analyses

For the LSER study, the corrected retention factor was found for each analyte in the same manner as for the van't Hoff analyses. $\log k$ values were regressed against the solute descriptors to generate a set of system constants. The resulting parameters are displayed in Table 7 for uncoated and cholesterol-coated stationary phases, for each mobile phase examined. Comparison of system constants between two systems with a common mobile phase allows for an examination of changes in the chromatographic behavior of the stationary phase. Overall changes in the set of system constants can be used to evaluate overall changes in the selectivity of the chromatographic system, while changes in individual system constants can be used to describe how a specific interaction changes upon varying a chromatographic parameter. In this case, differences in the system constants are due to the presence of cholesterol in the stationary phase.

When mobile phases containing methanol are used, the most significant changes upon addition of cholesterol are observed with the v and e system constants. For both mobile phases examined, the v constant becomes more negative (favoring elution) while the e constant become more positive (favoring retention). In addition, with the 40/60 water/MeOH mobile phase, the s constant becomes more negative upon addition of cholesterol. This is observed for the 50/50 mobile phase as well, but to a much smaller extent. The other system constants (b, a, and c) do not change significantly upon addition of cholesterol to the stationary phase.

The v system constant represents the relative ease of cavity formation for insertion of a solute molecule in the two phases. Coating of cholesterol onto the stationary phase should not affect cavity formation in the mobile phase, so we can assume the differences in the v system constant arise in the stationary phase. The reduction in the system constant suggests that formation of a suitable cavity in the stationary phase is more difficult when the phase contains cholesterol. It is plausible that the presence of cholesterol reduces the mobility of the stationary phase chains, much in the same way that cholesterol interacts with the alkyl portion of the lipids of a membrane [36]. This restriction could make cavity formation more difficult, resulting in a reduction in the v system constant.

The e system constant represents excess polarizability, which is not included in the s constant, due to the presence of n and π electrons. There are several sites of unsaturation in the cholesterol molecule, which would enhance this type of interaction when cholesterol is added to the stationary phase. Although this system constant increases, favoring retention, changes in the other system constants are generally negative, and as a result, retention generally decreases upon addition of cholesterol to the stationary phase.

When acetonitrile is used as the organic modifier, significant changes in the v, b, and e system constants are observed when

Table 5
Entropy comparison, as $\Delta S°/R + \ln \Phi$ (van't Hoff plot intercept). van't Hoff data were taken between 25 °C and 55 °C.

Solute	50/50, uncoated	50/50, coated	40/60, uncoated	40/60, coated
Methanol as organic modifier				
Acetophenone	−3.15 ± 0.10	−2.47 ± 0.38	−1.62 ± 0.11	−1.95 ± 0.02
Dichlorophenol	−6.27 ± 0.11	−4.76 ± 0.54	−2.76 ± 0.16	−3.25 ± 0.36
Dichlorobenzene	−3.79 ± 0.17	−2.48 ± 0.63	−1.20 ± 0.09	−1.39 ± 0.10
Acetonitrile as organic modifier				
Acetophenone	−2.03 ± 0.29	−0.97 ± 0.69	−1.38 ± 0.07	−1.55 ± 0.31
Dichlorophenol	−2.26 ± 0.12	−0.97 ± 0.55	−1.68 ± 0.24	−1.65 ± 0.15
Dichlorobenzene	−0.92 ± 0.24	0.11 ± 0.51	−0.45 ± 0.22	−0.44 ± 0.09

Table 6
Methylene selectivity comparison, based on alkyl paraben series. van't Hoff data were taken between 25 °C and 55 °C.

Parameter	50/50, uncoated	50/50, coated	40/60, uncoated	40/60, coated
Methanol as organic modifier				
α, 35 °C	2.104	2.086	1.881	1.883
$\Delta H°$, kJ/mol	−2.41 ± 0.03	−1.65 ± 0.07	−1.24 ± 0.17	−1.32 ± 0.02
$\Delta S°$, J/mol K	−1.64 ± 0.09	0.76 ± 0.23	1.27 ± 0.55	0.97 ± 0.08
Acetonitrile as organic modifier				
α, 35 °C	1.618	1.603	1.509	1.530
$\Delta H°$, kJ/mol	−0.148 ± 0.226	−0.097 ± 0.104	−0.181 ± 0.238	−0.307 ± 0.064
$\Delta S°$, J/mol K	3.49 ± 0.73	3.60 ± 0.34	2.89 ± 0.76	2.55 ± 0.21

cholesterol is added to the stationary phase. As with methanolic mobile phases, the ν term decreases, and the e term increases. The rationale for these changes is the same as with methanolic mobile phases: the ν term decreases due to restrictions on cavity formation in the stationary phase due to the presence of cholesterol; and the e term increases due to the π-electrons present on cholesterol. In contrast with the methanolic mobile phases, the b term becomes more positive with acetonitrile-containing mobile phases. This term represents the relative hydrogen-bond acidity of the stationary and mobile phases, an increase in which would increase retention of hydrogen bond bases. It is plausible that the OH-group on cholesterol could be serving as a hydrogen-bond acid site, so addition of cholesterol to the stationary phase would increase its hydrogen bond acidity resulting in an increase in the b system constant. This effect is muted when methanolic mobile phases are used because adsorbed methanol already provides –OH groups. In contrast to methanol, acetonitrile has no significant hydrogen bond acidity, so the presence of the –OH group from cholesterol should be more pronounced when mobile phases with acetonitrile rather than methanol are used.

As was the case with methanolic mobile phases, the s term did not change significantly when the 50/50 mobile phase was used, but did become significantly more negative (favoring elution) when the 40/60 mobile phase was used. This suggests that the incorporation of cholesterol has a more significant impact on the relative polarity

of the stationary phase when the mobile phase contains a larger amount of organic modifier.

An overall comparison of the LSER system constants can be used to describe how overall selectivity changes when a cholesterol coating is added to the stationary phase. One way in which system constants can be compared is to treat the v, b, a, s, and e constants as units of a 5-dimensional vector describing the chromatographic system. The "angle" between two such vectors, represents how "different" the two are. This method was introduced by Ishihama and Asakawa [37] and has been used by a variety of researchers to compare LSER system constants [23,32,37–39]. The angle between two vectors is calculated via Eq. (4), where **a** and **b** represent vectors and Θ is the angle between them:

$$\cos \Theta = \frac{\mathbf{a} \cdot \mathbf{b}}{\|\mathbf{a}\| \|\mathbf{b}\|} \tag{4}$$

An angle of 0° indicates collinear vectors (and identical selectivity); an angle of 90° indicates orthogonal selectivity. Using this approach, an overall difference in selectivity due to cholesterol coating can be assessed. For the methanolic mobile phase, the angles between the LSER vectors were 4.2° and 9.1° for the mobile phases with 50 and 60% methanol, respectively. For mobile phases containing acetonitrile, the angles are 6.7° and 9.9° for the 50% acetonitrile and 60% acetonitrile mobile phases. As an overall trend, the cholesterol coating has a more significant affect on selectivity

Table 7
LSER comparison.

System constant	50/50, uncoated	50/50, coated	40/60, uncoated	40/60, coated
Methanol as organic modifier				
v	2.257 ± 0.170	2.159 ± 0.220	1.879 ± 0.160	1.777 ± 0.152
b	−1.693 ± 0.088	−1.734 ± 0.102	−1.488 ± 0.083	−1.462 ± 0.079
a	−0.304 ± 0.079	−0.331 ± 0.092	−0.304 ± 0.074	−0.374 ± 0.070
s	−0.765 ± 0.096	−0.802 ± 0.114	−0.714 ± 0.091	−0.974 ± 0.087
e	0.414 ± 0.159	0.596 ± 0.187	0.412 ± 0.149	0.716 ± 0.142
c	−0.501 ± 0.117	−0.513 ± 0.139	−0.526 ± 0.110	−0.472 ± 0.105
Acetonitrile as organic modifier				
v	1.339 ± 0.178	1.185 ± 0.124	1.224 ± 0.132	1.144 ± 0.207
b	−1.439 ± 0.092	−1.260 ± 0.062	−1.253 ± 0.069	−1.146 ± 0.122
a	−0.524 ± 0.082	−0.588 ± 0.059	−0.522 ± 0.616	−0.555 ± 0.107
s	−0.554 ± 0.101	−0.547 ± 0.069	−0.522 ± 0.759	−0.674 ± 0.122
e	0.422 ± 0.166	0.577 ± 0.118	0.166 ± 0.124	0.423 ± 0.192
c	−0.126 ± 0.122	−0.152 ± 0.086	−0.113 ± 0.092	−0.107 ± 0.146

with mobile phases containing larger volume fractions of organic modifier.

3.4. Comparison of thermodynamic and LSER results

Comparisons of the results of the thermodynamic and LSER evaluation of cholesterol-coated stationary phases can be made. In general, changes in retention thermodynamics were more significant with weaker mobile phases, while differences in LSER system constants were more pronounced with stronger mobile phases. This suggests that the cholesterol coating had a more significant influence on retention with the 50/50 mobile phases, but a more significant influence on selectivity with the 40/60 mobile phases.

4. Conclusions

This work examined the effects of cholesterol concentration, percent organic in the mobile phase and column temperature on the amount of cholesterol loaded onto an alkyl stationary phase. While increasing the cholesterol concentration in the loading solvent increases the amount of cholesterol loaded, the mechanism of the loading process is constant. However, the thermodynamics of the loading process do change if the composition of the mobile phase is changed. In addition, the effect of a cholesterol coating of the stationary phase on retention thermodynamics and mechanism were explored. A cholesterol coating does change the relative enthalpic and entropic contributions to retention, but the effect is dependent on mobile phase composition. Changes in a variety of LSER system constants were also noted upon addition of cholesterol to the stationary phase, indicating that the coating does change the relative magnitude of intermolecular interactions occurring between solutes, the stationary phase, and the mobile phase. Not all system constants change in the same direction, as some change to favor elution, while others change to favor retention. In some cases, particularly for the *s* system constant, which represents relative polar interactions in the mobile and stationary phases, the effect of cholesterol coating on retention mechanism is shown to be mobile phase dependent.

This work shows that retention and selectivity can be adjusted by the addition of a dynamic coating of cholesterol. This could be of use when alternate stationary phase selectivity is desired, but when column selection is limited. Future work will investigate the use of cholesterol-coated phases as biomembrane mimics, with comparison to C18 and IAM stationary phases, as well as comparison to bonded cholesterol phases.

References

[1] P.B. Ogden, J.W. Coym, J. Chromatogr. A 1216 (2009) 4713.
[2] S.R. Cole, Mobile phase additives for separation improvement in reversed-phase liquid chromatography and capillary electrophoresis, Ph.D. Dissertation, The University of Cincinnati, 1992.
[3] J.J. Pesek, M.T. Matyska, E.J. Williamsen, R. Tam, Chromatographia 41 (1995) 301.
[4] J.J. Pesek, M.T. Matyska, G.B. Dawson, A. Wilsdorf, P. Marc, M. Padki, J. Chromatogr. A 986 (2003) 253.
[5] J.J. Pesek, M.T. Matysak, M.T.W. Hearn, R. Boysen, J. Sep. Sci. 30 (2007) 1150.
[6] S. Bocian, M. Matyska, J. Pesek, B. Buszewski, J. Chromatogr. A 1217 (2010) 6891.
[7] B. Buszewski, S. Bocian, M. Matyska, J. Pesek, J. Chromatogr. A 1218 (2011) 441.
[8] C. Delaurent, V. Tamao, A.M. Siouffi, Chromatographia 45 (1997) 355.
[9] C. Courtois, G. Pagès, S. Caldarelli, C. Delaurent, Anal. Bioanal. Chem. 392 (2008) 451.
[10] C. Courtois, C. Allais, T. Constantieux, J. Rodriguez, S. Caldarelli, C. Delaurent, Anal. Bioanal. Chem. 392 (2008) 1345.
[11] B. Buszewski, M. Jezierksa, M. Welniak, R. Kaliszan, J. Chromatogr. A 845 (1999) 433.
[12] B. Buszewski, M. Jezierska, B. Ostrowska-Gumkowska, Mater. Chem. Phys. 72 (2001) 30.
[13] B. Buszewski, M. Jezierska-Switala, S. Kowalska, J. Chromatogr. B 792 (2003) 279.
[14] K. Krupczynska, P. Jandera, B. Buszewski, Anal. Chim. Acta 540 (2005) 127.
[15] M.A. Al-Haj, P. Haber, R. Kaliszan, B. Buszewski, M. Jezierska, Z. Chilmonzyk, J. Pharm. Biomed. Anal. 18 (1998) 721.
[16] B. Buszewski, S. Kowalska, P. Stepnowski, J. Sep. Sci. 29 (2006) 1116.
[17] L.A. Cole, J.G. Dorsey, Anal. Chem. 64 (1992) 1317.
[18] L.A. Cole, J.G. Dorsey, K.A. Dill, Anal. Chem. 64 (1992) 1324.
[19] C.S. Lee, W.J. Cheong, J. Chromatogr. A 848 (1999) 9.
[20] R.P.J. Ranatunga, P.W. Carr, Anal. Chem. 72 (2000) 5679.
[21] T.L. Chester, J.W. Coym, J. Chromatogr. A 1003 (2003) 101.
[22] S.D. Allmon, J.G. Dorsey, J. Chromatogr. A 1216 (2009) 5106.
[23] J.W. Coym, J. Chromatogr. A 1217 (2010) 5957.
[24] A. Alhedai, D.E. Martire, R.P.W. Scott, Analyst 114 (1989) 869.
[25] M. Wang, J. Mallette, J.F. Parcher, Anal. Chem. 80 (2008) 6708.
[26] P.R. Perry, J.W. Coym, J. Sep. Sci. 33 (2010) 2310.
[27] M. Vitha, P.W. Carr, J. Chromatogr. A 1126 (2006) 143.
[28] M.H. Abraham, A. Ibrahim, A.M. Zissimos, J. Chromatogr. A 1037 (2004) 29.
[29] J. Zhao, P.W. Carr, Anal. Chem. 71 (1999) 2623.
[30] A. Berthod, C.R. Mitchell, D.W. Armstrong, J. Chromatogr. A 1166 (2007) 61.
[31] A. Wang, L.C. Tan, P.W. Carr, J. Chromatogr. A 848 (1999) 21.
[32] S.K. Poole, C.F. Poole, J. Sep. Sci. 31 (2008) 1118.
[33] C.F. Poole, S.N. Atapattu, S.K. Poole, A.K. Bell, Anal. Chim. Acta 652 (2009) 32.
[34] T. Karunasekara, C.F. Poole, J. Chromatogr. A 1218 (2011) 809.
[35] F. Gritti, G. Guiochon, J. Chromatogr. A 1099 (2005) 1.
[36] D. Voet, J.G. Voet, C.W. Pratt, Fundamentals of Biochemistry: Life at the Molecular Level, 3rd ed., John Wiley & Sons, Hoboken, NJ, 2008.
[37] Y. Ishihama, N. Asakawa, J. Pharm. Sci. 88 (1999) 1305.
[38] C. West, E. Lesellier, J. Chromatogr. A 1191 (2008) 21.
[39] C. West, E. Lesellier, J. Chromatogr. A 1203 (2008) 105.