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# Thermodynamic parameters of the chromatographic equilibrium distribution process of amphiphilic compound by HPLC. Part I: Fatty acid

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## Abstract

The retention behaviour of fatty acids on a reverse phase HPLC column is reported. Retention increases as the acetonitrile/methanol mobile phase becomes richer in acetonitrile. For a given solvent mixture, retention increases (linearly) with the acid chain length. Retention factor (k) is dimensionless and independent on any geometrical parameters of the column or HPLC system. It could be considered to be a thermodynamic characteristic of the adsorbent-compound-eluent system. HPLC experiments carried out at different temperatures allow to plot the variation of the ln k vs. 1/T, and to calculate the $\Delta G°$, $\Delta H°$ and $\Delta S°$ of transfer of one molecule from the mobile phase to the stationary phase. According to a Van't Hoff expression, the retention decreases as temperature increases. Both $\Delta H°$ and $\Delta S°$ are negative, a result that corroborates that the adsorption of the n-alkyl chains onto the stationary phase is favourable from the energetic point of view, and that it increases the molecular order.

**Key words:** Fatty acids; HPLC; Van't Hoff expression; thermodynamic parameters.

# Parámetros termodinámicos del proceso de distribución de equilibrio cromatográfico de compuestos anfifílicos por HPLC. Parte I: Ácidos grasos

## Resumen

En este trabajo se evaluó el comportamiento de retención de diferentes ácidos grasos en columnas de fase reversa por HPLC. La retención aumenta con la proporción de acetonitrilo en la fase móvil acetonitrilo/metanol. Para una mezcla de solventes dada, la retención se incrementa linealmente con la longitud de la cadena alquílica del ácido. El factor de retención (k) es adimensional e independiente de los parámetros geométricos de la columna o del sistema HPLC en general. Esta variable puede ser considerada como una característica termodinámica del sistema adsorbente-compuesto-eluente. Se realizaron experimentos por HPLC a diferentes temperaturas para obtener la curva del ln k contro 1/T, y calcular así el $\Delta G°$, $\Delta H°$ y $\Delta S°$ de transferencia de una molécula de ácido de la fase móvil a la fase estacionaria. De acuerdo a la

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expresión de Van't Hoff, la retención disminuye con el aumento de la temperatura. Tanto ΔH° como ΔS° son negativos, un resultado que corrobora que la adsorción de las cadenas n-alquílicas en la fase estacionaria es favorable desde el punto de vista energético, y que esto incrementa el orden molecular.

**Palabras clave:** Ácidos grasos; HPLC; expresión de Van't Hoff; parámetros termodinámicos.

## Introduction

Analysis of biological or nutritional samples for fatty acids (FAs) seeks to produce a diversity of data. These include: the absolute or relative concentration of a specific FA; the carbon number of the each analyte; the number, position and configuration of double bonds; branching of alkyl groups and in the case of the hydroxyl fatty acids (HFAs), the position of the hydroxyl group. Their analysis (with one straight chain and one acid group) is usually carried out by liquid-gas chromatography (GLC) but in special cases it may be necessary to process HPLC (high performance liquid chromatography) separations. Things become even more complex in presence of a mixture of fatty acids, which is the most common case in practice, as for instance in pharmaceuticals, cosmetics, and food stuffs (1, 2). Carboxylic acids of this or other types are also found in natural substances and metabolites produced by living organisms (3) or slowly ripened products such as petroleum. Thus, there is a widespread interest in their quantitative analysis, particularly in mixtures.

Gas chromatography (GC) is the currently most common chromatographic method (4-6) to analyse fatty acids as methyl ester derivatives in a capillary GC column. Methylation and GC conditions, such as programmed-temperature, split-injection, as well as type of capillary column, carrier gas, and detector, are all important determinants of high accuracy and precision, however with an inherent limit because of the volatility decrease with molecular weight. The HPLC analysis of underivated fatty acids, therefore, offers a useful alternative to GC for accurate quantitative routine analysis where GC is not available or inefficient or where confirmation of peak identity is required (7-11). Any HPLC analysis is of course limited by the detection method, and in the case of fatty acids, the sensitivity of absorbance in the ultraviolet range can approach $10^{-9}$ g (12). However, the wavelength has to be selected as a compromise between the optical properties of the sample and the background absorption by the solvent. The carboxyl chromophore of organic acids has a weak absorption band near 200 nm due to a n-$\pi^*$ transition by the valence electrons (13). Therefore carboxylic acids they can be detected at 214 nm.

FAs have been studied by various authors using HPLC analysis (14-16). The authors reported that the sensitivity of the analytical procedures varied considerably with each organic acid. In reversed-phase (RP) methods, the elution order of the fatty acids will be essentially based on the alkyl chain length. Numerous studies on retention behaviour and separation mechanism in reversed-phase have related the dependence of a retention parameter on the experimental conditions (17-19). It is relatively easy to accurately measure retention parameters such as the retention factor (k) and the adsorption equilibrium constant (K). The logarithm of this retention factor, is known to change in a linear fashion with the number of structural units in the molecule (20-21). There are two basic approaches for thermodynamic description of the HPLC retention phenomena, one is based on the partitioning theory and another is based on adsorption.

The chromatographic equilibrium distribution process of solutes between a sta-

tionary phase and a mobile phase, is related to changes of the standards free energy (ΔG°), enthalpy (ΔH°) and entropy (ΔS°) associated with the transfer of one mole of analyte from the mobile to the stationary phase (21):

$$\ln k = -\frac{\Delta G^\circ}{RT} + \ln\phi = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln\phi \qquad [1]$$

where, k= ($t_r$ - $t_0$) / $t_0$ ($t_r$ and $t_0$ are the retention time of the solute and the total dead time of the column, respectively), R is the gas constant (8.314 J $mol^{-1}$ $K^{-1}$), T is the absolute temperature (298.15 K) and $\phi$ is the phase ratio of the column (i.e. the volume of stationary to that of mobile phase). The thermodynamic characteristics can be calculated from the temperature-dependent retention data plotted as indicated by Equation [1]. If the ln k vs. 1/T, so-called Van't Hoff plot, is linear then the enthalpy and entropy are temperature-independent and the mechanism of the retention process is invariant over the range of temperature under investigation. ΔH° can be evaluated from the slope and ΔS° from the intercept of the regression line, provided that the column phase ratio $\phi$ is known. This procedure will be used to evaluate the retention behaviour of fatty acids by reverse-phase HPLC, and to determine the thermodynamic characteristics associated with the transfer one molecule of carboxylic acid from the polar mobile phase to the apolar stationary phase.

## Experimental Procedure

HPLC separations were performed with a liquid chromatography equipment consisting of a M600A pump and a U6K injector from Waters. A Waters 996 Photodiode Array Detector set to 214 nm was coupled to an PC loaded with Millennium software. The RP-18 bonded silica column (250 x 4.6 mm I.D.) was purchased from Merck. Fatty acid samples were first diluted with methanol down to the 0.05 mol per liter range, then aliquots (10 $\mu$L) of these methanol solutions were injected into the chromatographic equipment. Several acetonitrile volume fractions (X) in the methanol/acetonitrile mobile phase were tested, i.e., X = 0.05, 0.2, 0.4, 0.6 and 0.8.

The temperature of the column was varied from 288 to 323 K using an Eppendorf CH-30 column heater or immersing the column in a VWR heating/cooling circulation bath from Merck. Methanol and acetonitrile used as mobile phase were HPLC grade from Baker Chemicals. All mobile phase solvents were ultrasonically degassed. The flow-rate was set 1 mL $min^{-1}$. The studied samples were as follows: octanoic acid (C8), decanoic acid (C10), lauric acid (C12), myristic acid (C14), palmitic acid (C16) and stearic acid (C18), all provided by Merck. They are referred to as CN, where N indicates the number of carbon atom in the molecule.

All the measurements were carried out under isocratic conditions. The retention times ($t_r$) of fatty acids were obtained from at least five individual determinations. The relative standard deviation was lower than 1%. The retention factor (k) was calculated according to k= ($t_r$ - $t_0$) / $t_0$, were the dead time ($t_0$) was determined as the elution time of non-retained compounds (methanol and acetonitrile).

## Results and Discussion

### Effect of the mobile phase acetonitrile content on the fatty acid retention

All chromatographic studies were performed under isocratic conditions. Since all fatty acids contain the same hydrophilic group, they are separated by reverse-phase HPLC after their hydrophobic group, i.e. their alkyl chain. The longer the alkyl chain is, the more hydrophobic the acid is, hence, the less soluble it is in the polar mobile phase, which has to be matched with the acid type. With pure methanol as mobile phase, fatty acids shorter than C10 merge into a single sharp peak. By adding acetoni-

trile, the solvent polarity may be adjusted to produce the appropriate separation, as indicated in Figure 1A. Figure 1B shows the variation of ln k versus the carbon atoms number (N) in the fatty acid.

Figure 1B data obey the following expression:

$$\ln k = a\,N + b \qquad [2]$$

In Figure 1 case $a$ = 0.18 and $b$ = -2.5. This means that the retention (as ln k) linearly increases with the length of the acid "tail" which interacts with the stationary phase. On the other hand intercept value $b$ depends on the acetonitrile/methanol proportion in the mobile phase and this indicated the retention characteristic of carboxylic group. Figure 2 indicates the variation of ln k as a function of the acetonitrile content

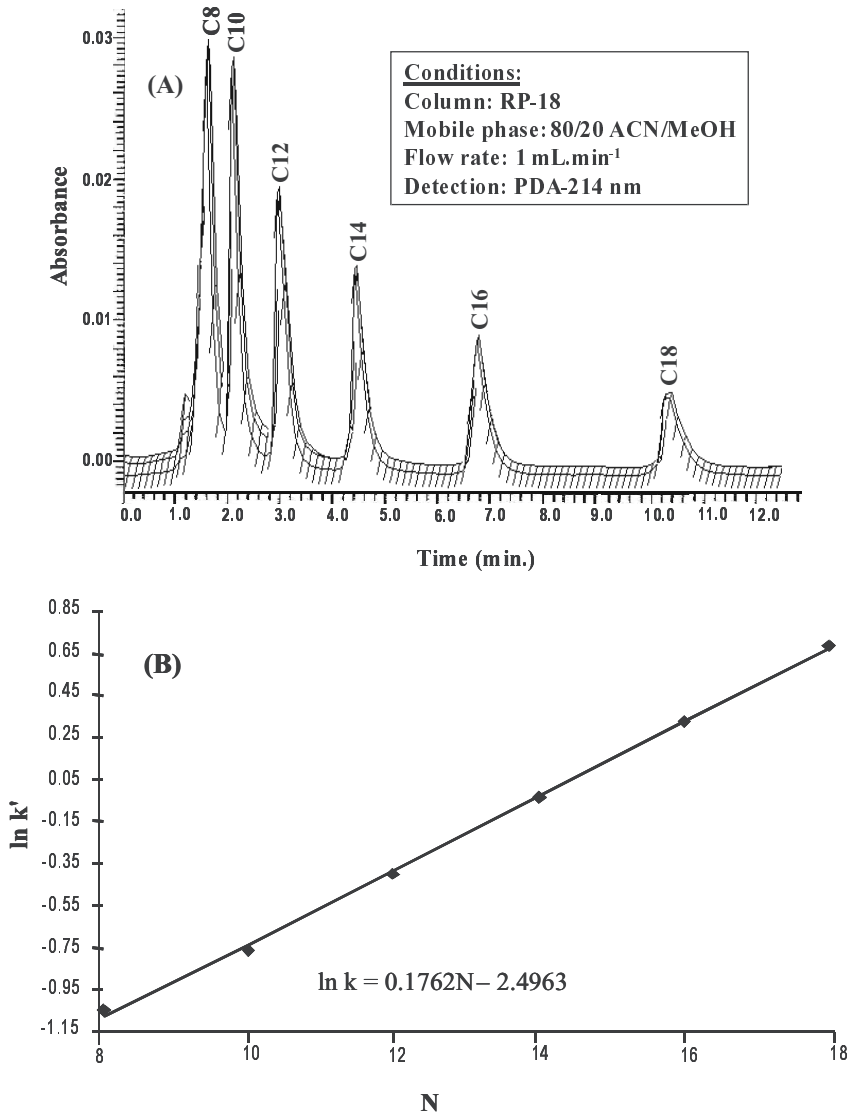


Figure 1. (A) HPLC chromatogram of FAs. (B) Variation of ln k versus the number of carbon atoms in the fatty acid (N), with pure methanol as the mobile phase, at 298 K. Conditions: RP18 column (5 µm, 250 x 4.6 mm I.D.) Flow-rate 1 mL $min^{-1}$, detection at 214 nm.

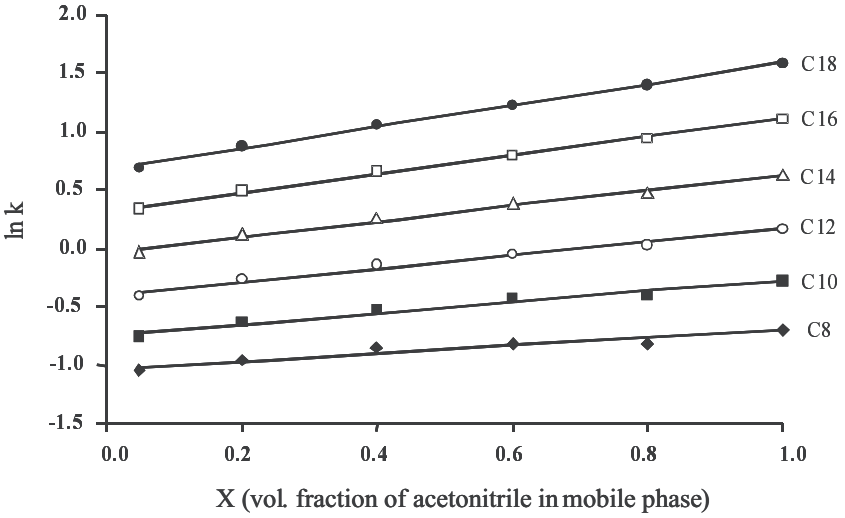


Figure 2. Dependence of the retention factor (as ln k) of the fatty acids on the concentration X (vol. fraction) of acetonitrile in mobile phase at 298 K. Other conditions as Figure 1.

in the mobile phase for the different acids. It is seen that the retention of all species increases as the mobile phase contains more acetonitrile, i.e. as it becomes more polar.

The data may be represented by a linear function such as:

$$\ln k = c\,X + d \qquad [3]$$

Where X is the volume fraction of acetonitrile in the mobile phase; c and d are parameters which are determined by linear regression and they depend on the other analysis conditions. They are found to vary quite linearly versus N. Hence, previous Equations [2] and [3] can be gathered as a bilinear expression of ln k as a function of X and N:

$$\ln k = \alpha + \beta X + \gamma N + \delta XN \qquad [4]$$

with $\alpha$= -2.45, $\beta$= -0.115, $\gamma$= 0.174 and $\delta$= 0.0555, at 298 K

**Thermodynamic characteristics of the fatty acid retention**

Thermodynamic equilibrium constant is an energetically parameter. Its logarithm is equal to the difference of the free Gibbs energy of the analyte and solvent in the adsorption system, $\Delta H$ is the difference of the enthalpy of adsorption of analyte and solvent and $\Delta S$ is the corresponding difference of their entropy. If the solvent interacts with the adsorbent surface stronger than analyte, it will be preferentially adsorbed. The free Gibbs energy value will be negative and the equilibrium constant will be less then 1.0.

From the Equation [1] we can conclude that the capacity factor of that analyte will be negative. This actually means that analyte will move through the column as faster than eluent. The analyte which have a negative adsorption will not penetrate in the adjacent to the adsorbent surface part of the volume (adsorbed eluent molecules will not allow it, as they have stronger surface interactions). As a result, this analyte will occupy less volume while it is moving through the column and will move faster than the eluent.

Capacity factor is dimensionless and independent on any geometrical parameters of the column or HPLC system. It could be considered to be a thermodynamic characteristic of the adsorbent-compound-eluent system. According the Equation [1], increasing the temperature will decrease the value of k',

thus the actual retention time will decrease. For most of the systems these decrease will not exceed 50% of the component reduced retention time at ambient temperature.

We using the following definition of the phase ratio (22):

$$\phi = \left(V_G - V_M\right) / V_M \qquad [5]$$

were $V_G$ and $V_M$ are the geometric (empty column) and dead volume of the column (it was determined using flow rate and the elution time of non-retained compound, i.e. methanol or acetonitrile). The phase ratio as defined in equation [5] is a dimensionless quantity and can be rapidly determined, because $V_G$ can be simply calculated from the length and inner diameter of the column (22). The $\phi$ value is found to be 0.54 for our RP18 column used. Figure 3 shows for the all acids the variation of ln k as a function of the reciprocal absolute temperature (1/T) when the solvent is pure acetonitrile. The data show that the variation closely matches equation [1] for all acids. The positive slope indicates a decrease of the retention as temperature increases.

The enthalpy and entropy of transfer from the pure acetonitrile mobile phase to the RP18 stationary phase, are calculated for the decanoic, lauric, myristic, palmitic and stearic acid. As seen in Table 1, both $\Delta H°$ and $\Delta S°$ are negative, a result that corroborates that the adsorption of the n-alkyl chains onto the stationary phase is favourable from the energetic point of view, and that it increases the molecular order.

For a given set of stationary and mobile phases, $\Delta H°$ becomes more negative as the fatty acid becomes more hydrophobic. On the other hand $\Delta S°$ is essentially independent of the acid. The average decrease in $\Delta H°$ and $\Delta G°$ per additional methylene group are 0.60 and 0.74 kJ $mol^{-1}$, respectively. These values were obtained from the data of the Table 1. The behaviour is consistent with the capability of reverse-phase HPLC to separate acid mixtures according to their hydrophobic "tail" length, since it has a major contribution in the relative affinity of the acid for the stationary phase.

## Conclusions

The retention time of fatty acid species on a RP18 column linearly increases with the length of the acid "tail" which interacts with the stationary phase and increases with acetonitrile content in the mobile

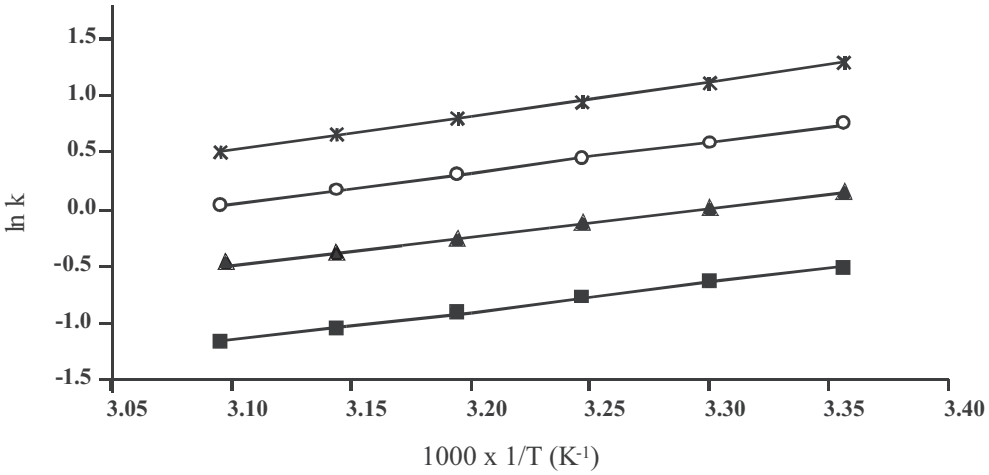


Figure 3. The change of retention factor of FAs with reciprocal temperature on the RP18 column with pure acetonitrile eluent: (■) lauric acid (▲) myristic acid (○) palmitic acid and (×) stearic acid. Other conditions as Figure 1.

Table 1
Standard enthalpy (ΔH°), entropy (ΔS°) and Gibbs energy (ΔG°) of transfer of acid from the pure acetonitrile eluent to the bonded phase of the RP18 column

| Fatty acid | ΔH° (kJ $mol^{-1}$) | ΔS° (J $mol^{-1}$ $K^{-1}$) | ΔG° (KJ $mol^{-1}$) |
|---|---|---|---|
| C10 | -21.428 | -0.071 | 0.597 |
| C12 | -21.157 | -0.070 | 0.584 |
| C14 | -21.553 | -0.066 | -1.105 |
| C16 | -22.859 | -0.065 | -2.561 |
| C18 | -24.729 | -0.067 | -3.864 |

phase. On the other hand, ΔS° is essentially independent of the acid and the ΔH° of transfer of an acid molecule from the mobile to stationary phase is negative and decreases when the acid alkyl chain gets longer, thus indicating an enhanced affinity for the RP18 substrate.

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