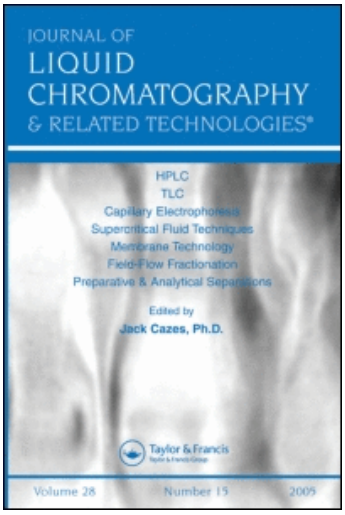

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REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC STUDIES FOR HOMOLOGOUS SERIES OF POLYUNSATURATED FATTY ACIDS ON A COMMERCIAL μBONDAPAK FREE FATTY ACID COLUMN

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ABSTRACT

Reversed phase high performance liquid chromatography (RPHPLC) has been proven to be one of the most efficient and reproducible separation methods for fatty acids since 1974. Particularly, in the case of homologous series studies, RPHPLC is an exclusively unavoidable method[1]. The thermodynamic and kinetic studies for free fatty acids, oleic acid (octadecenoic acid 18:1Δ9c), linoleic acid (octadecadienoic acid, 18:2Δ9c, 12c), linolelaidic acid (octadecadienoic acid, 18:2Δ9t, 12t), linolenic acid (octadecatrienoic acid, 18:3Δ9c, 12c, 15c), γ-linolenic acid (octadecatrienoic acid, 18:3Δ6c, 9c, 12c), eicosadienoic acid (eicosadienoic acid, 20:2Δ11c, 14c) and eicosatrienoic acid (eicosatrienoic acid, 20:3Δ11c, 14c, 17c) were performed on a 10μ commercial alkyl phenyl free fatty acid column. The mobile phase was acetonitrile, water and tetrahydrofuran in the ratio of 45:35:20 v/v%. The isocratic elution method was adopted in these studies using differential refractometer as detector. The retention data was obtained and thereby capacity factors, enthalpies of adsorption were calculated. Finally the retention mechanism was explained.

INTRODUCTION

Long chain polyunsaturated fatty acids can be divided into two categories, essential fatty acids (EFA) and non-essential fatty acids. The essential fatty acids are those belonging to linoleic (C18:2ω6) and linolenic (C18:3ω3) family groups. According to R.T.Holman[2], these essential fatty acids are those which are active in the growth and the maintenance of human skin. Linoleic acid and linolenic acids are the precursors for arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA), which are the synthesizers of eicosanoids and docosanoids, a hormone like substances such as prostaglandins (PG), thrombaxanes (TX) and luekotrienes (LT). These eicosanoids are the regulators for certain human health disorders like inflammation, heart diseases and cancer[3]. The importance of these unsaturated fatty acids was recognized by Dyerberg and his colleagues[4] in 1978 by conducting experiments on GreenLand Eskimos, whose diet contains high content of cold water marine fish. They reported that these people experienced low incidence of death from ischemic heart disease compared to western population.

For the past 20 years, the liquid chromatographic technique was well applied for the analysis and separation of fatty acids and their derivatives. Normal and reverse phases are general and the most popular methods in liquid chromatography. In normal phase chromatography, silica gel impregnated with silver nitrate is used as stationary phase. The separation was achieved based on the degree of unsaturation of the solutes in this method. Although it is a routine method for the separation of fatty acids and their derivatives, it is not suitable for the separation long chain fatty acids from complex natural oils and fats. Normal phase liquid chromatography is highly selective for resolving geometrical isomers of fatty acids whereas reverse phase liquid chromatography is more effective for the separation of long chain fatty acids[5]. The octadecylsilyl (C_{18}) and octylsilyl (C_8) are more commonly employed silica stationary phases for the separation of fatty acids and triglycerides. Previously Pei, et. al.[6] performed retention studies for saturated and unsaturated free fatty acids and their esters and also triglycerides using VYDAC octadecylsilyl column with methanol and water as the mobile phase

in different volume ratios. The separation was achieved within 10 minutes. Scholfield[7], obtained similar separations with aqueous acetonitrile in the place of methanol. Later Perkins, et. al.[8] isolated octadecatrienoic fatty acids from partially hydrogenated soyabean oil (PHSBO) on a 5μ LC18 octadecylsilyl column using acetonitrile and water as mobile phase. In 1976, Bidlingmeyer et. al.[9] reported the development of a μBondapak free fatty acid column. Bailey et. al.[10] used Waters μBondapak free fatty acid and Lichrosorb RP-8 columns for the analysis of the underivatized fatty acids in margarines and also for the separation of geometrical isomers of linoleic acid. The mobile phase used in their experiment was acetonitrile, tetrahydrofuran and water with 0.1% acetic acid. King et. al.[11] analyzed mixtures of saponified fatty acids of natural oils like soyabean, coconut and also alkyd resin samples by using μBondapak free fatty acid column. They used acetonitrile, water and tetrahydrofuran mixture as the mobile phase and they succeeded in separating the fatty acids within ten minutes.

In this experiment, the studies were done by using μBondapak free fatty acid (alkyl phenyl bonded silica) column with a mobile phase consisting of acetonitrile, tetrahydrofuran and water ternary mixture in the ratio of 45:20:35 v/v respectively. The experiments were performed at three different flow rates and temperatures. The standard fatty acids belonging to linolenic (ω3), linoleic (ω6), oleic (ω9) family groups were used in this experiment and their retention data were obtained. The capacity factors, heats of adsorption and HETP values were estimated from the experimental values. Finally the retention mechanism was explained.

INSTRUMENTATION AND MATERIALS

The system consists of Waters M-45 isocratic solvent delivery system (Water associates, Milford, Masaachusetts), R-401 Waters differential refractometer, Valco sample injector (Valco instruments, Houston, TX) and R02 Rikadenki electronic chart recorder (Rikadenki Kogyo, Japan). The column was Waters μBondapak free fatty acid column (Millipore, Milford, MA) and fatty acids were obtained from

Sigma Chemicals. The mobile phase chemicals were acquired from Fisher. The datalogging was done using Phillips P-3200 personal computer.

EXPERIMENT

Fatty acids (each 50 mg) dissolved in 5 ml mobile phase, were injected to the column by using 100μl sample loop. The signal from the detector was converted to digital form and this was processed by the personal computer. The response curves were integrated numerically to determine the first moments by using the equation given below.

$$\mu = \frac{\int_0^\infty c(t)tdt}{\int_0^\infty c(t)dt}$$

The retention times were calculated by subtracting dead time from the first moments. The capacity factors, k', was calculated by using the formula given below.

$$k' = \frac{t_R - t_o}{t_o}$$

The column voidage was determined by injection of a pulse of Blue Dextran, a very high molecular weight compound that do not penetrate through the small pores of the stationary phase.

RESULTS

The column voidage, ε was determined from the retention time measurements with blue dextran molecule by using the wave equation given below.

$$\omega = \frac{L}{\tau} = \frac{U}{1+\frac{(1-\varepsilon)}{\varepsilon}K}$$

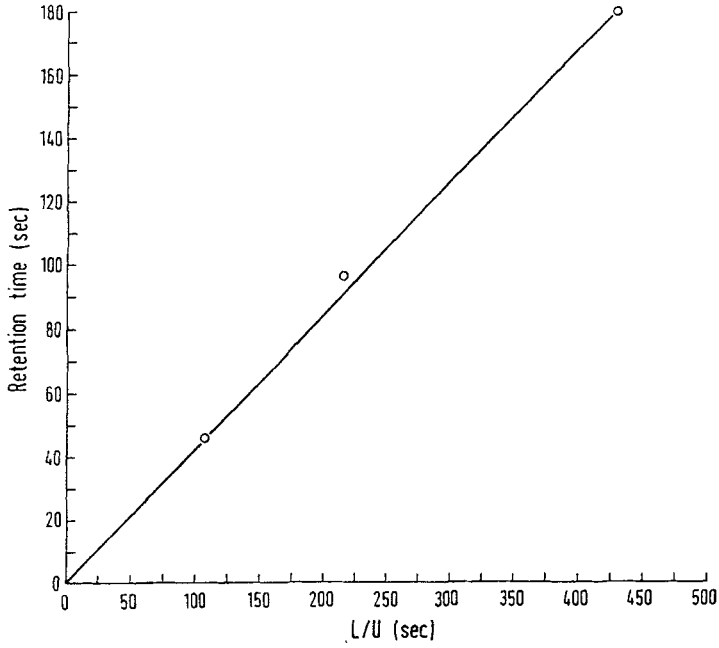


FIG. 1 Retention time vs L/U for blue dextran.

The distribution coefficient, K, for blue dextran in the above equation becomes zero, since there is no adsorption. Then the above equation reduces to,

$$\tau = \frac{L}{U}\varepsilon$$

The retention times measured for blue dextran over a range of eluent flow rates and plotted against L/U was shown in the Figure 1. The slope of the linear plot yields ε, that was 0.41. The retention times data for these fatty acids at various flow rates and temperatures are tabulated in Tables I and II respectively.

The capacity factors at various flow rates and temperatures are shown given below in Tables IIIa and IIIb.

TABLE I

RETENTION TIMES OF THE FATTY ACIDS AT DIFFERENT MOBILE PHASE FLOW RATES AND AT ROOM TEMPERATURE 24°C

Fatty acid	Retention times at various flow rates (minutes)		
	0.5 ml/min	1.0 ml/min	2.0 ml/min
Oleic acid (18:1)	19.19	10.05	5.31
Linoleic acid (18:2)	15.10	7.38	4.52
Linolelaidic acid (18:2)	16.32	8.17	4.56
Linolenic acid (18:3)	13.10	6.59	4.18
γ-Linolenic acid (18:3)	13.26	7.14	4.45
Eicosadienoic acid (20:2)	18.04	9.04	5.57
Eicosatrienoic acid (20:3)	16.02	8.15	4.59

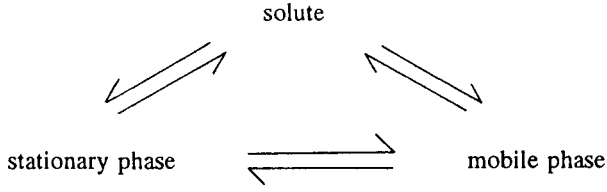
DISCUSSION

From the retention times results shown in Table I, it is observed that the elution of these free fatty acids was in the order of increasing carbon number and for the homologous series, the greater the unsaturation, the earlier is the elution. The trans acids are eluted after cis acids. This can be explained that cis unsaturated fatty acid isomers will have a bend at each double bond compared to trans isomers of the same fatty acid. This irregularity in structure leads to the unsaturation among the cis fatty acids and thereby the earlier was the elution. The estimated molecular size calculations for these fatty acids shows that the chain length of the fatty acid is shorter by 0.226Å for each additional double bond in the molecule in the homologous series. Hence the higher the unsaturation, the shorter the molecule and thereby the earlier is the elution.

The column packing material is alkyl phenyl bonded silica. An important feature about this aryl ligate is its resonance ring. It is this phenyl bonded phase that enables the separation of the long chain fatty acid on the basis of like-like bonding characteristic. In addition to that, the resonance ring formed by the π

orbital, is a delocalized electron rich zone. The affinity to the unsaturated solute drastically drops since the π bond in the double bond "repels" the π orbital in the phenyl silica. This resulted in the decrease in the retention time for a corresponding increase in the degree of unsaturation.

In general in RPHPLC, the mobile phase plays a dominant role in the separation. It has been reported that the induced dipole interactions between the double bonds in the fatty acids and triple bonds in the nitrile group of acetonitrile may play a key role in the separation[12]. The tetrahydrofuran should not be used more than 25 volume parts in order to prevent the precipitation of the fatty acids. The water content should be kept at high volume parts, since free fatty acids are more polar compared to methyl and ethyl esters of the corresponding fatty acids. The interaction involved between these three phases can be shown below as



In terms of hydrophobicity, the fatty acids becomes more hydrophobic as their size increases because solvation takes longer in RPHPLC. As a result the longer the alkyl group, the more hydrophobic it is and therefore a longer retention is expected. The fatty acids in this experiment follows the same criteria. The principle of solvophobicity is based upon the tendency of the mobile phase to minimize the site occupied by the solute molecules in the hydrorganic mobile phase. Solutes which have polar substituents can interact more strongly with polar hydrorganic mobile phase, leading to shorter retention time as compared to similar compound with no polar moiety. For fatty acid, the only slightly polar end is carboxyl group. The rest of the chain is practically nonpolar. Also the double bond position in the C18:3 group leads the molecule far from the symmetry compared to C18:2 family, hence C18:3 is more polar than C18:2. The same argument can

TABLE II

RETENTION TIMES OF THE FATTY ACIDS AT VARIOUS TEMPERATURES AND MOBILE PHASE FLOW RATE 1.0 ML/MIN

Fatty acid	Retention times at different temperatures (minutes)		
	30°C	40°C	50°C
Oleic acid (18:1)	9.10	9.00	8.52
Linoleic acid (18:2)	7.16	7.08	7.01
Linolelaidic acid (18:2)	7.32	7.15	7.04
Linolenic acid (18:3)	6.27	6.19	6.15
γ-Linolenic acid (18:3)	6.43	6.23	6.16
Eicosadienoic acid (20:2)	9.04	8.52	8.34
Eicosatrienoic acid (20:3)	8.08	7.59	7.24

also be applied to C20:2 and C20:3. This shows that the retention times for C18:3 and C20:3 are shorter than C18:2 and C20:2 respectively. The results conducted at three different flow rates, 0.5, 1.0 and 2.0 ml/min confirm this trend. In addition by operating the column at higher flow rates, a higher HETP is achieved which indicates a drop in column efficiency.

Results in Table II shows that the increase in the temperature lowers the retention times of the fatty acids. The temperature dependence of the distribution coefficient(K) can be shown by Van't Hoff equation as follow

$$\frac{d\ln K}{dT} = \frac{\Delta H_{M \to S}}{RT^2}$$

where $\Delta H_{M \to S}$ is the enthalpy of transfer of the solute molecules from the mobile phase to stationary phase. If the ratio of V_s/V_m is independent of temperature, the

TABLE IIIa

CAPACITY FACTORS AT VARIOUS FLOW RATES AND AT ROOM TEMPERATURE 24°C

Fatty acid	Capacity factors (k') at different flow rates		
	0.5 ml/min	1.0 ml/min	2.0 ml/min
Oleic acid (18:1)	5.43	5.28	6.19
Linoleic acid (18:2)	4.05	3.77	5.35
Linolelaidic acid (18:2)	4.52	4.18	5.44
Linolenic acid (18:3)	3.39	3.37	4.60
γ-Linolenic acid (18:3)	3.48	3.52	5.20
Eicosadienoic acid (20:2)	5.02	4.67	6.32
Eicosatrienoic acid (20:3)	4.34	4.16	5.50

TABLE IIIb

CAPACITY FACTORS AT DIFFERENT TEMPERATURES AND AT MOBILE PHASE FLOW RATE 1.0 ML/MIN

Fatty acid	Capacity factors (k') at different temperatures		
	30°C	40°C	50°C
Oleic acid (18:1)	4.73	4.63	4.55
Linoleic acid (18:2)	3.54	3.46	3.39
Linolelaidic acid (18:2)	3.70	3.54	3.38
Linolenic acid (18:3)	3.03	2.95	2.90
γ-Linolenic acid (18:3)	3.20	2.99	2.92
Eicosadienoic acid (20:2)	4.67	4.54	4.36
Eicosatrienoic acid (20:3)	4.08	3.99	3.63

TABLE IVa

ENTHALPIES AT DIFFERENT FLOW RATES AND AT ROOM TEMPERATURE 24°C

Fatty acid	Enthalpies at different flow rates (KJ/mole)		
	0.5 ml/min	1.0 ml/min	2.0 ml/min
Oleic acid (18:1)	4.18	4.10	4.50
Linoleic acid (18:2)	3.46	3.28	4.14
Linolelaidic acid (18:2)	3.72	3.53	4.18
Linolenic acid (18:3)	3.02	2.99	3.78
γ-Linolenic acid (18:3)	3.08	3.10	4.56
Eicosadienoic acid (20:2)	3.99	3.80	4.56
Eicosatrienoic acid (20:3)	3.63	3.60	4.20

TABLE IVb

ENTHALPIES AT DIFFERENT TEMPERATURES AND AT FLOW RATE 1.0 ML/MIN

Fatty acid	Enthalpies at different temperatures (KJ/mole).		
	30°C	40°C	50°C
Oleic acid (18:1)	3.92	3.99	4.07
Linoleic acid (18:2)	3.19	3.23	3.28
Linolelaidic acid (18:2)	3.30	3.29	3.27
Linolenic acid (18:3)	2.79	2.82	2.87
γ-Linolenic acid (18:3)	2.93	2.85	2.88
Eicosadienoic acid (20:2)	3.88	3.94	3.95
Eicosatrienoic acid (20:3)	3.63	3.60	4.20

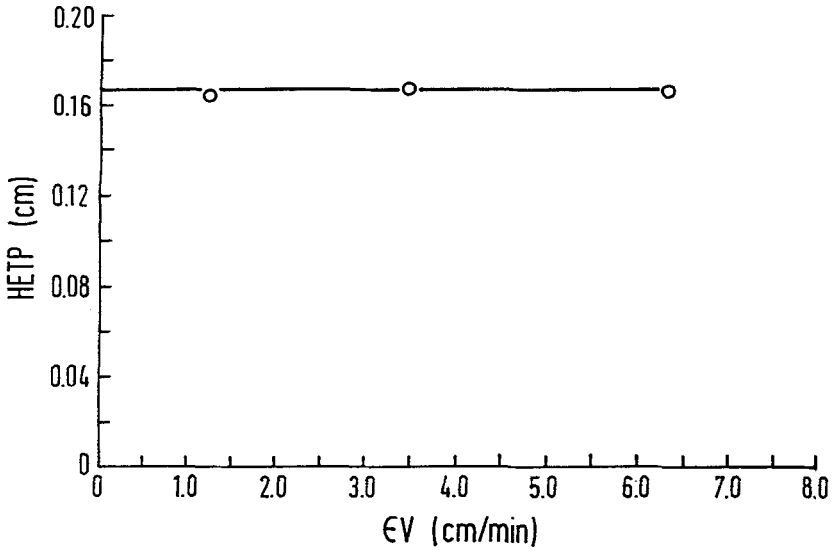


FIG. 2 HETP vs Superficial Velocity.

capacity factor, k', may be substituted for distribution coefficient, K, and the above equation becomes,

$$\frac{d\ln k'}{dT} = \frac{\Delta H_{M \to S}}{RT^2}$$

In the liquid chromatography, solute is transferred from the liquid mobile phase to a film of liquid layer adhering to the stationary phase. In general, the enthalpy involved in transferring solute from bulk liquid to liquid adhering to the stationary phase is not significant, so $\Delta H_{M \to S}$ can be assumed to be constant. By simple mathematical integration of the above equation, we obatin $\Delta H_{M \to S} = RT\ln k'$. The enthalpies at various temperatures including at room temperature were calculated by using above equation and tabulated in Table IVa and IVb. This

results in Table IVa and IVb shows that the enthalphies of adsorption was not varying widely within the homologous series of these fatty acids.

The plot of HETP versus εV in Figure 2 shows that HETP was independent of superficial velocity that indicates that only axial dispersion parameter was contributing in these experiments and there was no resistance to mass transfer effect on solute migration.

CONCLUSION

The fatty acids were eluting in increasing order of carbon number with increasing equivalent chain length values (ECL), which is defined as ECL = N - $2n_{c=c}$ where "N" is the number of carbons in the fatty acid and "$n_{c=c}$", is the number of ethylenic bonds. The ECL values of oleic acid, linoleic acid and linolenic acid are 16, 14 and 12 respectively. The ECL values for eicosadienoic acid and eicosatrienoic acid are 16, 14 respectively. Fatty acids having same ECL values are referred as "critical pairs". In this case oleic acid and eicosadienoic acid and also linoleic acid and eicosatrienoic acid have same ECL values, 16 and 14 respectively. It was observed that within the same ECL values, the most unsaturated acid will elute first. The chain length of the fatty acid is also an important factor in elution order. The elution order of these fatty acids was found to be linolenic acid < γ-linolenic acid < eicosatrienoic acid < linolelaidic acid < eicosadienoic acid < oleic acid.

The experimental results shows that the temperature was not an important parameter in this separation. The retention times for these fatty acids on this column are shorter compared to the separation on octadecylsilyl and octylsilyl bonded silica columns. This indicates a possible industrial scale separation of fatty acids with dimethyl phenyl silica bonded columns. In addition to that the alkyl phenyl bonded silica is less nonpolar than other reverse phase bonded packings,

and hence it enables separation to be performed with a lesser degree of denaturation of the products. The separation of highly unsaturated fatty acids can also be done more efficiently by using free fatty acid columns than octyldecylsilyl bonded silica columns[13].

NOMENCLATURE

$\Delta H_{M \to S}$	Enthalpy of transfer of solute from mobile phase to stationary phase
k'	Capacity factor
K	Distribution coefficient
L	Length of the column (cm)
R	Universal gas constant (8.31434 J/K-mol)
T	Absolute temperature (K)
t_o	Void time (min)
t_R	Retention time (min)
U	Superficial velocity (cm/sec)
V	Interstitial fluid velocity (cm/sec)
V_s/V_m	Phase ratio

Greek letter

ε	Bed voidage
μ	First moment (min)
τ	Retention time (min)
ω	Wave function

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