Retention behaviour of polyunsaturated fatty acid methyl esters on porous graphitic carbon

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Abstract

Retention with porous graphitic carbon was investigated with 25 structures of fatty acid methyl esters (FAMEs) with two different mobile phases: $CH_3CN:CHCl_3$ 60:40 (v/v) and $CH_3OH:CHCl_3$ 60:40 (v/v) with both 0.1% triethylamine (TEA) and an equimolar amount of HCOOH. Preliminary results showed that the use of TEA/HCOOH led to the response increase of saturated FAMEs with evaporative light scattering detection. No increase was observed for unsaturated one. These modifiers may slightly reduce the retention of FAMEs but did not significantly modify the separation factor with porous graphitic carbon. Thermodynamic parameters were calculated for each structure using Van't Hoff plot measured over the temperature range from 10 to 50 °C, with the both mobile phase conditions. All the studied compounds were found linked by the same retention mechanism on porous graphitic carbon. Quantitative *in silico* analysis of the retention using a molecular mechanics calculation demonstrated a good correlation between the retention factors and the molecular interaction energy values ($r > 0.93$). Especially the Van der Waals energy was predominant, and the contribution of electrostatic energy was negligible for the quantitative analysis of the retention. The results indicate that Van der Waals force, hydrophobic interaction, is predominant for the retention of FAMEs on this packing material. The relative retention for highly unsaturated homologues can be changed by the selection of the weak solvent CH_3CN or CH_3OH. Then isomers differing only in the position of the carbon double bond on the alkyl chain can be separated and their behaviour is summarised as the closer the carbon double bonds to the FAME polar head, the more the retention decreases. Finally, the more important the number of carbon double bonds in the alkyl chain is, the smaller the retention is.

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Keywords: Porous graphitic carbon; Polyunsaturated fatty acid methyl esters; Enthalpy–entropy compensation; ELSD; Computational chemical analysis

1. Introduction

Carbon adsorbent for HPLC separations is greatly underutilized due to the poor chromatographic properties of the earliest commercially available materials and the limited understanding of solute interactions with the solid surface.

Porous graphitic carbon (PGC) is an extremely strong adsorbent due to the large dispersion forces between the solute and the rigid planar graphite surface [1,2]. PGC exhibits important retention of polar solutes mainly caused by specific interactions with the polarizable surface of graphite [3]. However, the retention mechanism is still under discussion. The "polar retention effect of graphite" is generally observed with hydro-organic eluent [1]. The PGC has the remarkable property of separating isomers or closely related compounds [4–6].

PGC was underused for separation of very apolar compounds due to the requirement of non-aqueous mobile phase for their elution [7]. Ceramides [8,9], glyceroglycolipids [10], and fatty acid methyl esters (FAMEs) [11,12] were analysed with PGC using important fraction of chlorinated solvent. The same behaviour was encountered for all these molecules, i.e. increasing the alkyl chain length led to the increase in retention, which was similar to reversed phase mechanism with octadecyl grafted silica. PGC provides further advantages that octadecyl

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grafted silica lacks, that is the separation of *cis–trans* isomers [12] and different retention between molecular species with the same partition number [10].

Polyunsaturated FAMEs are an interesting group of compounds to study different PGC properties since these lipids possess functional groups able to be involved in polarizable interactions and/or in molecular geometry recognition. Although the retention was firstly due to dispersive interactions with PGC, these two PGC retention properties were suspected to be responsible for a particular behaviour of polyunsaturated FAMEs. Viron et al. [11] have described two different retention behaviours of polyunsaturated FAMEs. Retention decreases with the increase in carbon double bond number in the alkyl chain of FAMEs up to three unsaturations, and retention increases with the increase in the number of carbon double bonds beyond four unsaturations.

This work presents the retention of closely related compounds (i.e. polyunsaturated FAMEs) in non-aqueous liquid chromatography with PGC. Thermodynamic study using enthalpy and entropy measurements, and computational chemical analysis using a model phase were approaches envisaged to describe the retention mechanism. This study involved various chemical funtions such as alkyl chain lengths and number of carbon double bonds as well as position of these carbon double bonds. Ten different mobile phase conditions were tested.

2. Experimental

2.1. Chemicals

The saturated FAMEs (C16:0, C18:0, C20:0, C22:0), the monounsaturated FAMEs (C18:1 *cis*-6, C18:1 *cis*-9, C18:1 *cis*-12, C20:1 *cis*-8, C20:1 *cis*-11, C20:1 *cis*-13, C22:1 *cis*-13), the diunsaturated FAMEs (C18:2 *cis*-9,12; C20:2 *cis*-11,14; C22:2 *cis*-13,16), the polyunsaturated (C18:3 *cis*-9,12,15; C20:3 *cis*-5,8,11; C20:3 *cis*-8,11,14; C20:3 *cis*-11,14,18; C22:3 *cis*-13,16,19; C20:4 *cis*-5,8,11,14; C22:4 *cis*-7,10,13,16; C20:5 *cis*-5,8,11,14,17; C22:5 *cis*-7,10,13,16,19; C22:6 *cis*-4,7,10,13,16,19) and the ricinoleic acid methyl ester (C18:1 *cis*-9,12-OH) were purchased from Sigma (St. Quentin Fallavier, France).

All solvents were HPLC grade and were obtained from Fisher Scientific (Elancourt, France).

2.2. Apparatus

2.2.1. HPLC apparatus devoted to column comparison

Chromatographic measurements were carried out with an Ultra-plus II HPLC pump (Micro-Tech Scientific, CA, USA) connected to a 5 µL sample loop injection valve. Detection was performed with a DDL 31 evaporative light scattering detector (Eurosep, Cergy, France) where the drift tube temperature was set at 45 °C, the nebulizer temperature at 30 °C and nitrogen pressure at 1.5 bar. The chromatograms were recorded with Kroma 3000 integration software (Biotek Instrument, Milan, Italy). The flow-rate was set at 0.4 mL/min. The columns were Hypercarb (5 µm), 100 mm × 2.1 mm I.D. (Hypersil, Runcorn,

UK), and were thermostated at 30 °C with an oven Crocosil (Cluzeau, Sainte Foy-la-Grande, France).

2.2.2. HPLC apparatus devoted to retention and thermodynamic study

Chromatographic measurements were carried out with a Thermo Separation Products P1000 XR gradient pump with a TSP SCM1000 vacuum membrane degasser (Thermo Separation Products, San Jose, CA, USA) connected with a Kontron autosampler 360 (Bio-Tek Kontron Instruments, Milan, Italy) equipped with a 5 µL sample loop injection valve. Detection was performed with a Cunow DDL 11 Evaporative Light Scattering Detector (Eurosep, Cergy Saint Christophe, France) where the drift tube temperature was set at 35 °C and nitrogen pressure at 1 bar. The chromatograms were recorded with a Personal Computer-integrator KromaSystem 2000 1.60 (Bio-Tek Kontron Instruments). The flow-rate was set at 0.4 mL/min. The column was Hypercarb (5 µm), 100 mm × 2.1 mm I.D. (Hypersil), and was kept thermostatic with a Jetstream 2 temperature controller (Thermotechnics Products, Vienna, Austria).

2.3. Molecular mechanics calculations

The molecular properties of analytes, model phases and molecular interactions were calculated by molecular mechanics (MM2) from version 5 of the CAChe program from Fujitsu, Tokyo, Japan. The standard parameters used were bond stretch, bond angle, dihedral angle, improper torsion, Van der Waals energy, hydrogen bond and electrostatic energy (MM2 bond dipoles). The Van der Waals cut-off distance was 9 Å.

3. Results and discussion

3.1. Selection of the chromatographic conditions

3.1.1. Solvent selection for the mobile phase

The set of FAMEs selected for this study differs in the position and the number of carbon double bonds (from 0 to 6) and by the number of carbon atoms of the alkyl chain (from 16 to 22) which constitutes a group of 25 molecules. The mobile phase condition was selected in order to analyse this important structural diversity in the same run. The mobile phase selection consists in selection of strong and weak organic solvents [7,11,13]. Strong solvents mainly have an effect on eluotropic strength whereas weak solvents play role in the selectivity between unsaturated FAMEs [12]. As the present study focuses on the retention behaviour of polyunsaturated compounds, two mobile phase compositions were envisaged to differ in the weak solvent nature. Consequently, chloroform (CHCl₃) was selected among strong solvents since it provides the greater eluotropic strength with PGC [7]. Thereby, the fraction of weak solvent in binary mobile phases is thus as large as possible to emphasize influence on selectivity between polyunsaturated FAMEs. Methanol (CH₃OH) and acetonitrile (CH₃CN) were selected as weak solvents. The elution of this important set of compounds in the same run required 40% of chloroform with 60% of weak solvent regardless of its nature.
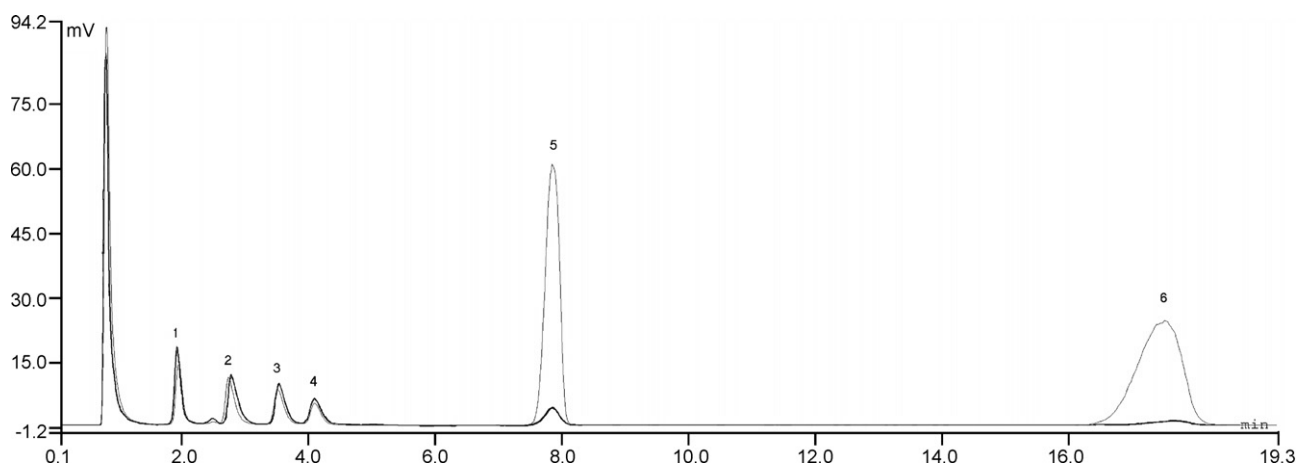
Fig. 1. Comparison of the ELSD response for different structures of FAMEs. Isocratic elution: 0.4 mL/min CH$_3$CN:CH$_3$OH:CHCl$_3$ 30:30:40 (v/v/v) at 25 °C. Detection ELSD: air gas 1 bar, drift tube 30 °C. Conditions with 0.1% (v/v) TEA/HCOOH 1:1 mol/mol and without these organic modifiers correspond to the solid thin line and the thick line chromatograms, respectively. C18:1 *cis*-9,12-OH (1), C22:6 *cis*-4,7,10,13,16,19 (2), C22:5 *cis*-7,10,13,16,19 (3), C20:2 *cis*-11,14 (4), C18:0 (5), C20:0 (6) at 0.3 mg/mL each in the sample mixture.

3.1.2. ELSD response modifier

All mobile phases used for the retention study contained 0.1% of TEA and an equimolar amount of HCOOH. These modifiers have the particular properties of increasing ELSD responses for lipids [14], without significantly modifying the retention [15,16]. Fig. 1 illustrates this influence with six different FAMEs representing the main structural difference encountered in our set of molecules. First, in the absence of these modifiers, ELSD response appears similar for all the FAMEs which are injected at the same concentration. Secondly, the retention was not altered by the presence of ELSD modifier in the mobile phase as previously demonstrated. Finally, difference between ELSD response of saturated and unsaturated FAMEs was observed. The ELSD response of unsaturated FAMEs remained identical whereas that of saturated FAME was significantly increased by the presence of TEA and HCOOH. In addition, ELSD response of ricinoleic acid methyl ester (i.e. peak 1) was not modified although this molecule contains a hydroxyl group. Until now all species of many various lipid classes undergo this enhancement, i.e. phospholipids [16], ceramides [15], glycolipids [17]. Gaudin et al. had demonstrated that ELSD response enhancement always occurred for ceramide species independently of the presence of unsaturation with TEA and HCOOH as modifiers [15]. However, ceramide molecules possess two alkyl chains, among which one remains generally saturated. The intensity of this phenomenon is strongly related to the solvent condition and the solute volatility [14].

Although the response enhancement occurred only for saturated FAMEs, the presence of these modifiers was necessary since the saturated FAMEs were the more retained compounds with PGC. Thereby, response enhancement ensures their detection in the case of very long time analysis. In spite of an important rinsing procedure of PGC column to remove these modifiers, no modification of the retention was observed in the absence of the modifiers. However, the column was mainly used with TEA and HCOOH for 10 years. PGC is a strong adsorbent [4]. For example, ionic modifier like trifluoroacetic acid had exhibited modification of the selectivity with PGC in hydro-organic con-

dition [18,19]. Therefore, to assert that no modifier remained adsorbed on PGC surface and did not influence the retention of FAMEs, retention of these products was carried out with a new column.

3.1.3. Influence of ELSD response modifier on the retention

In order to ascertain that TEA and HCOOH addition was not responsible for particular retention behaviour with polyunsaturated FAMEs, three PGC columns were tested without TEA and HCOOH modifiers. These experimentations were carried out with another chromatographic apparatus which was never employed with these organic modifiers. The columns were labelled with their date of commissioning. Among them, the one commissioned in 2002 was new. These columns were tested using the complete homologous series of C22 FAMEs which contained structures having 0–6 unsaturation(s) in the alkyl chain.

The retention values were globally comparable except for the new column (2002) inducing higher retention factors for all of the homologues (Fig. 2A). However, the elution order and selectivity values were similar between these columns even if TEA/HCOOH mixture was used (Fig. 2A and B). The difference in retention between columns may be due to irreversible adsorption of TEA and HCOOH on the polar sites at the edges of graphite layer. However, since 10 years, the manufacturing process of PGC synthesis has changed. For example, the nature of silica used for the skeleton has changed. Therefore, these results may highlight this evolution instead of the influence of TEA and HCOOH on the retention. In conclusion, the trend of retention behaviour was similar in all the different conditions.

3.2. Retention study

Viron et al. [11] described a particular behaviour of polyunsaturated FAMEs which were strongly retained by PGC due to π–π interaction. However, this study was only performed with seven different FAME molecules. Therefore, the investigation
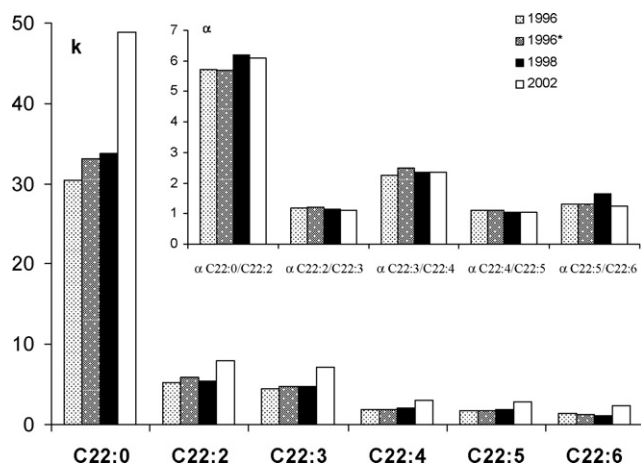
Fig. 2. Retention factor and selectivity comparison between three different PGC columns. (*) Chromatographic apparatus for these data corresponds to Section 2.2.1 because TEA and HCOOH were present in the mobile phase.

of such retention behaviour is herein envisaged with a larger set of structures.

3.2.1. Thermodynamic study

A more detailed understanding of retention behaviour and retention mechanisms can be gauged by enthalpy–entropy compensation (EEC) [20]. The Gibbs free energy of the solute molecule transfer from the mobile phase to the PGC surface, ΔG°, can be linked to its equilibrium constant K with the help of the following equation:

$$\ln K = \frac{-\Delta G^\circ}{RT} \tag{1}$$

where R is the gas constant and T is the absolute temperature.

Since $k = \varphi K$, where k is the solute retention factor and φ the phase ratio of the column, Eq. (1) can be developed as follows:

$$\ln k = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \varphi \tag{2}$$

where ΔH° and ΔS° are the enthalpy and the entropy of transfer of the solute from the mobile phase to the PGC surface.

If the plot of $1/T$ versus $\ln k$, which is commonly described as a Van't Hoff plot, is a straight line, its slope and its origin allow the calculation of the enthalpy and the entropy of the solute transfer from the mobile phase to the stationary phase, respectively. The entropy calculation requires the value of the column phase ratio φ, which is delicate to establish. Therefore, the apparent entropy (ΔS^*) was calculated [21]:

$$\Delta S^* = \frac{\Delta S^\circ}{R} + \ln \varphi \tag{3}$$

ΔS^* and ΔS° follow the same trend because the phase ratio can be considered as a constant in case of PGC stationary phase. This material is a rigid and mechanically strong adsorbent, thus its phase volume should remain unchanged under different temperature conditions. Therefore, the calculation of ΔS^* is sufficient to study the retention.

The thermodynamic parameters of the transfer of the solute from the mobile phase to the PGC surface were determined

with the following protocol. Five temperatures between 10 and 50 °C, with a range of 10 °C, were tested for all compounds in both the mobile phase conditions. The enthalpy and the apparent entropy changes are calculated for each FAME and the results were summarised in Table 1. Both ΔH° and ΔS^* are negative in all conditions for all solutes, which is classically observed in reversed phase HPLC [22]. The negative enthalpy values suggest an exothermic retention process. The ΔH° values decrease with the apolarity increase of FAME (e.g. $\Delta H^\circ_{C22:0} < \Delta H^\circ_{C20:0} < \Delta H^\circ_{C18:0} < \Delta H^\circ_{C16:0}$). For each compound the enthalpy of transfer is found to be smaller with CH_3OH mobile phase than with CH_3CN one. Thus transfer of the FAMEs from the mobile phase to the stationary phase was enthalpically less favourable with the $CH_3CN:CHCl_3$ mobile phase. All ΔS^* values are negative, which means that FAME molecules are more ordered at the surface of the stationary phase than in the mobile phase. The ΔS^* values increase with the increase in the number of carbon double bonds in the molecule. The ΔS^* values are higher for the CH_3CN mobile phase than for the CH_3OH phase, thus the retention process is more entropically driven in the case of the CH_3CN mobile phase. This mobile phase condition allows a more significant discrimination between FAMEs, which may relate to a better geometry recognition.

An extra-thermodynamic approach for the analysis of physicochemical data is the EEC [20,23]. This investigation tool has been previously used in chromatographic procedures to analyse and compare the retention mechanism [24]. The EEC is described by the following equation:

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G^\circ_\beta \tag{4}$$

where ΔG°_β is the Gibbs free energy of a physicochemical interaction at a compensation temperature, β, ΔH° and ΔS° are the corresponding standard enthalpy and entropy, respectively. According to Eq. (4), when EEC is observed for a group of compounds in a particular chemical transformation, all of the compounds have the same ΔG°_β at the compensation temperature, β, although their temperature dependencies may differ.

Combining Eqs. (2) and (4), the following equation is obtained:

$$\ln k = \frac{-\Delta H^\circ}{R} \left(\frac{1}{T} - \frac{1}{\beta} \right) - \frac{\Delta G^\circ_\beta}{R\beta} + \ln \varphi \tag{5}$$

Eq. (5) shows that if ΔH° versus $\ln k_T$ plot at constant temperature, T (harmonic temperature), is a linear function, the compensation temperature, β, can be calculated from the slope. The similarity of the values for the compensation temperature, β suggests that the solutes are essentially retained by an identical mechanism.

Fig. 3 plots ΔS^* versus ΔH°. The linearity testing points out linear relationships. In fact, the data from both mobile phases are aligned closely to one another. By measuring the slope of the data for each composition in Fig. 3, the compensation temperature (β) can be compared. For $CH_3CN:CHCl_3$ and $CH_3OH:CHCl_3$, β was 399 ± 8 K and 409 ± 17 K, respectively. Since the β values are the same within the errors of the experiment, this indicates that despite the differences observed in the degree of retention,

Table 1
Values of enthalpy (ΔH°) and apparent entropy (ΔS^*) of the transfer of FAMEs from the mobile phase to the stationary phase, and Interaction energies between the optimised FAME structure and a 195 aromatic ring structure for PGC

Fatty acid chain	CHCl$_3$:CH$_3$OH (40:60)		CHCl$_3$:CH$_3$CN (40:60)		Δ VW[a] (kcal/mol)	Δ ES[b] (kcal/mol)	MI[c] (kcal/mol)
	ΔH° (J/mol)	ΔS^* (J/mol/K)	ΔH° (J/mol)	ΔS^* (J/mol/K)			
C16:0	−28223.25	−81.28	−25320.95	−74.98	31.26	−0.01	31.06
C18:0	−34164.68	−93.78	−30109.71	−84.06	34.65	−0.01	34.44
C18:1 (9), OH (12)	−18207.62	−56.24	−15778.16	−50.47	28.23	0.01	26.82
C18:1 (6)	−23554.31	−65.97	−19437.29	−57.31	29.23	−0.01	29.05
C18:1 (9)	−23407.05	−66.30	−19229.52	−57.18	29.02	−0.01	29.15
C18:1 (12)	−23916.54	−67.13	−19721.12	−58.15	29.09	0.00	28.98
C18:2 (9,12)	−19759.87	−57.19	−15038.79	−47.11	26.81	0.00	25.21
C18:3 (9,12,15)	−20306.65	−59.42	−13393.02	−42.64	26.99	−0.02	24.62
C20:0	−39165.43	−103.33	−35860.23	−96.11	38.54	−0.01	38.58
C20:1 (8)	−25590.61	−66.38	−25078.11	−69.40	35.62	−0.01	35.01
C20:1 (11)	−25707.27	−67.43	−25359.02	−70.96	37.71	−0.01	36.02
C20:1 (13)	−25395.51	−65.62	−25177.92	−69.44	37.71	−0.01	35.98
C20:2 (11,14)	−24034.13	−64.82	−20301.26	−57.67	32.64	−0.01	31.55
C20:3 (5,8,11)	−20686.46	−57.92	−15374.56	−46.89	28.93	−0.03	29.12
C20:3 (8,11,14)	−21133.11	−58.10	−17527.86	−52.50	30.69	0.02	29.56
C20:3 (11,14,17)	−21694.01	−57.93	−18311.30	−52.94	32.24	−0.02	30.48
C20:4 (5,8,11,14)	−17238.08	−50.01	−10385.72	−34.75	26.47	−0.17	24.49
C20:5 (5,8,11,14,17)	−15822.66	−45.41	−10368.05	−35.31	25.43	−0.02	24.19
C22:0	−47035.35	−122.46	−45055.85	−119.49	41.54	−0.01	41.69
C22:1 (13)	−31471.26	−79.72	−28442.51	−74.26	39.20	−0.01	38.50
C22:2 (13,16)	−27233.24	−69.65	−24462.77	−65.94	33.83	−0.01	33.77
C22:3 (13,16,19)	−26249.41	−67.33	−22934.44	−62.26	34.39	−0.01	32.44
C22:4 (7,10,13,16)	−19968.34	−52.99	−14964.14	−43.78	29.19	−0.05	27.10
C22:5 (7,10,13,16,19)	−20530.14	−54.99	−14383.41	−42.66	29.98	0.00	28.13
C22:6 (4,7,10,13,16,19)	−18390.97	−50.01	−11208.51	−34.55	26.90	−1.14	26.77

[a] Δ VW (Van der Waals energy) = VW (FAME) + VW (PGC) − VW (complex); VW (PGC) = 340.003 kcal/mol.
[b] Δ ES (electrostatic energy) = ES (FAME) + ES (PGC) − ES (complex); ES (PGC) = 0 kcal/mol.
[c] MI = FS (FAME) + FS (PGC) − FS (complex).

both of these mobile phases undergo the same type of retention mechanism. Thus, FAMEs with a significant number of carbon double bonds do not induce a different retention mechanism with the PGC surface compared to the saturated ones.

3.2.2. Computational chemical analysis

In order to argue about the nature of the interactions between polyunsaturated FAMEs and PGC surface, computational chemical approach was envisaged.
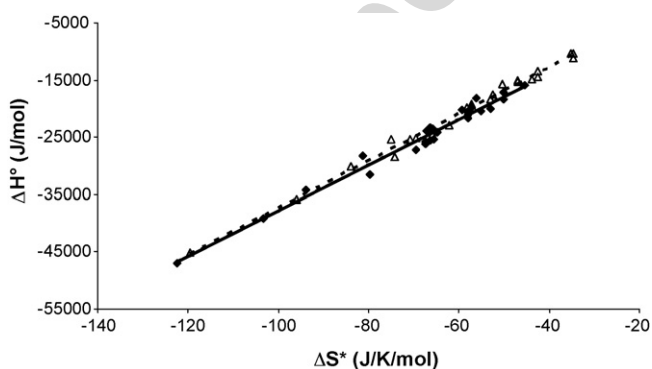

Fig. 3. EEC investigation plots according to Eq. (4) for the data in Table 1. ($\triangle$ and dashed line): CH$_3$CN:CHCl$_3$ 60:40 (v/v); ($\blacklozenge$ and thick line): CH$_3$OH:CHCl$_3$ 60:40 (v/v); Each mobile phase contained 0.1% TEA and an equimolar amount of HCOOH.

The graphitic carbon surface acts as a Lewis base towards polar solutes and is involved in Lewis acid–base interactions and dispersive interactions with aromatic solutes. 10^5 carbon arrangement is the expected number of atoms involved in graphitic carbon from electric conductivity study. However, Hosoya [24] from theoretical calculation indicated that one hundred carbon atoms were enough to demonstrate graphite property. After analysis by theoretical calculation of the model, the localized electrons could be observed at the edge of the graphite material. This may mean that the centre of the graphite carbon is electron poor and thus neutral except for induced effects [25]. Such flat model was considered for modelling the study of the retention in gas chromatography [26,27]. The graphite surface was considered homogeneous based on the liquid chromatographic data of heparin and chondroitin dissacharides [28]. To verify this model, in this study, a 195-ring polycyclic aromatic hydrocarbon was constructed as a model of graphitic carbon. The molecular interaction energy between a FAME and the 195-ring polycyclic aromatic hydrocarbon was calculated using molecular mechanics (MM2) to quantitatively analyse the retention of FAMEs.

The molecular interaction (MI) energy value was calculated as the sum of the energy values of the individual molecule FS (FAME) and PGC model phase FS (PGC) subtracted by the energy value of the complex FS (complex) (Table 1). Retention depends on the contact surface area of the molecules. The alkenes with multiple double bonds had less contact surface
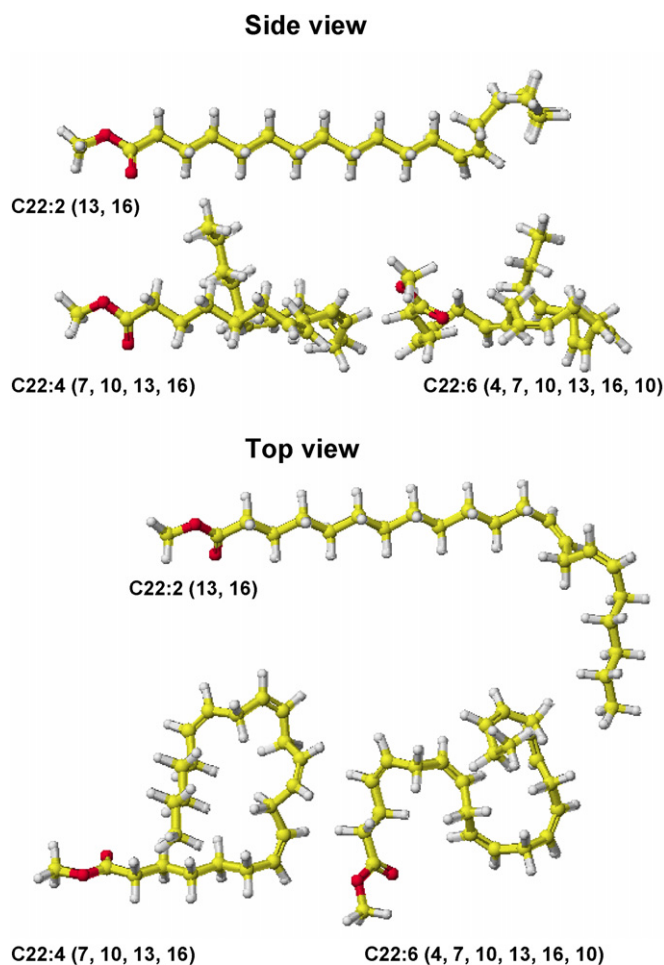
Fig. 4. Optimized molecular structure of FAMEs.

Table 2
Contribution of final structure energy for the molecular interaction

MI energy	Weak solvent	Column temperature (°C)	a^a	b^a	r^a
MI	CH$_3$OH	50	0.126	−2.760	0.935
		40	0.139	−2.881	0.931
		30	0.158	−3.139	0.939
		20	0.174	−3.314	0.944
		10	0.194	−3.564	0.941
	CH$_3$CN	50	0.142	−3.810	0.968
		40	0.160	−4.113	0.968
		30	0.177	−4.403	0.965
		20	0.200	−4.824	0.967
		10	0.226	−5.314	0.968
MI$_{vw}$	CH$_3$OH	50	0.132	−3.090	0.930
		40	0.146	−3.228	0.922
		30	0.165	−3.534	0.930
		20	0.182	−3.720	0.930
		10	0.201	−3.970	0.921
	CH$_3$CN	50	0.148	−4.144	0.954
		40	0.166	−4.471	0.951
		30	0.184	−4.785	0.946
		20	0.208	−5.246	0.946
		10	0.235	−5.791	0.948

^{a} $\ln k = a(\text{MI}) + b$.

area and the molecular interaction energy was smaller than that expected from the carbon atom numbers. The electrons of the double bond and the hydroxyl group did not affect the retention from *in silico* experiment of the relationship between MI energy values of alkyl alcohols, alkanes, and alkenes and the carbon atom numbers. Increasing the number of carbon double bonds reduced the MI energy values, especially for the multi *cis* compounds. The predominant interaction force was the Van der Waals force. The electrostatic energy did not change after formation of the complex. No π–π interaction influenced the retention of graphite surface [29]. The behaviour was the same for all FAME structures. The structures of all *cis* polyunsaturated FAMEs were neither flat nor straight (Fig. 4). Increasing the number of carbon double bonds decreased the contact surface area with the model phase. This phenomenon is supported by the difference in Van der Waals energy values. The Van der Waals energy values decreased by increasing the number of carbon double bonds, and the contribution of electrostatic energy values for the interaction was not observed. Then MI values were correlated with the retention factors (Table 2), indicating that such model molecular mechanics calculation using a model phase was feasible, and Van der Waals force energy (MI$_{vw}$) was predominant. The contribution of hydrogen bonding energy is zero and that of electrostatic energy is less than 0.1 kcal/mol. The above results support Hosoya's observation [25], and π–π inter-

action was not predominant for the retention of polyunsaturated FAMEs on the PGC.

A semi-empirical molecular statistical theory of adsorption based on an atom–atom approximation for the potential function to study intermolecular adsorbate–adsorbent interaction was applied to obtain Henry's constant based on gas chromatography data using graphitic thermal carbon, even if porous adsorbents are heterogeneous. The simple quantitative correlation of the thermodynamic characteristics of adsorption was applied in liquid chromatography [28]. The molecular interaction, hence a molecular mechanics calculation, was performed to obtain Henry's constant using a flat model [27]. The MI energy values can be obtained by a molecular mechanics calculation as demonstrated for the analysis of FAME's retention. The latter method is simple to analyse a variety of chromatographic data; however, it is not simple to synthesize the model phase, except a graphite carbon phase. Chromatographic phases are synthesized homogeneously but the steric structure is heterogeneous. The original computer software is used for the conformation analysis of proteins; therefore, such approach can be applied for the analysis of chromatographic retention if a prospective model is designed.

3.2.3. Retention behaviour description

The retention behaviour appeared qualitatively similar between both the mobile phase compositions differing by the nature of the weak solvent, i.e. increasing the number of carbon double bonds decreased the retention (Fig. 5). However, the relative retention between unsaturated homologues was more important with mobile phase containing CH$_3$CN even if the retention was weaker with this chromatographic condition. This observation was in agreement with the previous study involving FAMEs with up to three unsaturations [12].
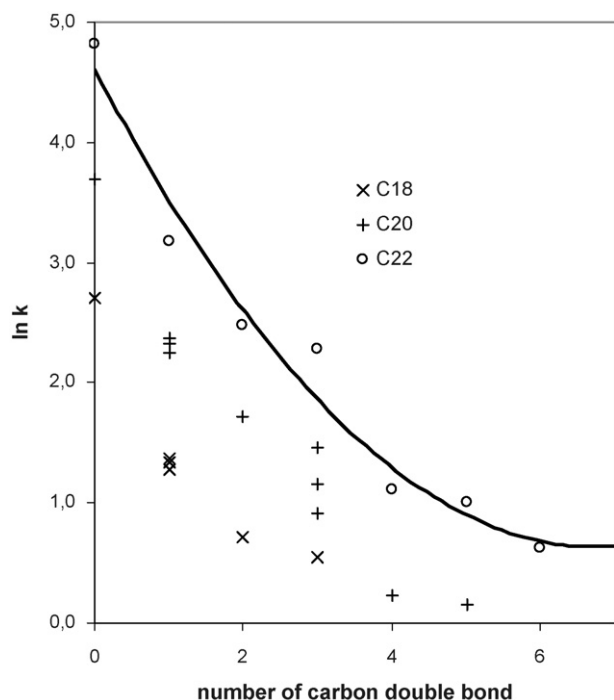
Fig. 5. Retention related to the number of carbon double bonds for $CH_3CN:CHCl_3$ 60:40 (v/v); 0.1% TEA and an equimolar amount of HCOOH, at 10 °C.

The addition of a first unsaturation decreases the retention (Figs. 6 and 7). Three positions of this unsaturation were studied with two different homologous series (Fig. 6). The more central position of the unsaturation in the alkyl chain (i.e. cis-9 and cis-11, respectively, for C18:1 and C20:1) induces weaker retention values; however, this different in retention is little.

The addition of a second carbon double bond on the alkyl chain also decreases the retention, however, less significantly than in the case of the first one. This observation was previously reported [12,30]. The three homologues with two carbon double bonds have the additional second carbon double bond on the opposite side of the polar head in the alkyl chain relative to the position of the first carbon double bond (i.e. C18:1 (9) and C18:2 (9,12); C20:1 (11) and C20:2 (11,14); C22:1 (13) and C22:2 (13,16)).

The addition of a third unsaturation also decreases the retention. However, according to the location of this further unsaturation in the alkyl chain, this decrease is equal to or less important compared to the addition of the second one (Fig. 6). For the three tested homologous series, when the further third unsaturation is added to the opposite part of the alkyl chain relative to the polar head, the influence on the retention is the weakest (i.e. C18:2 (9, 12) and C18:3 (9, 12, 15); C20:2 (11, 14) and C20:3 (11, 14, 17); C22:2 (13, 16) and C22:3 (13, 16, 19)).

The presence of a fourth unsaturation shows (Fig. 6) an important decrease in retention in both the tested cases. However, the unsaturations present on these polyunsaturated FAMEs are much closer to the polar head than their homologues with less unsaturation.

The fifth unsaturation decreases the retention which is more important CH_3CN mobile phase condition. This further unsat-

uration is at the apolar extremity of the alkyl chain compared to the homologues with four unsaturations. This unsaturation position weakly influences the retention.

Only one case of the presence of a sixth unsaturation is tested with the homologue with 22 carbon atoms (Fig. 6E and F) which induces a relatively important decrease in the retention. However, this unsaturation is located close to the polar head.

This retention description gave several very interesting information about the behaviour of polyunsaturated FAMEs with PGC. First, with 25 different structures from three different homologous series, we always observe a decrease in the retention with an increasing number of carbon double bonds for homologues with the same carbon number. This observation is in opposition with previous results suggested by Viron et al. [11]. Their mobile phase conditions were also with a high amount of weak solvent (CH_3CN) associated with dichloromethane in the absence of TEA and HCOOH. Furthermore, they had studied seven structures which were C14:1, C16:1, C18:1, C18:2, C18:3, C20:5 and C22:6, and found the following retention order: C14:1, C16:1, C18:3 co-eluted with C20:5, C18:2 (close to C18:3 and C20:5) and then C18:1 co-eluted with C22:6. We have found with our $CH_3CN:CHCl_3$ mobile phase condition the following order of retention: C20:5, C18:3 co-eluted with C22:6, C18:2 (close to the previous structures) and then C18:1; and with the $CH_3OH:CHCl_3$ mobile phase condition the following order of retention was found: C20:5, C18:3 (close to the previous structure), C18:2, C22:6 and C18:1. In the second case, the observation is similar to that of Viron et al. Both these studies agree about the retention result. However, we have demonstrated that the increase in unsaturation number does not induce an increase in the retention for FAMEs with number of carbon double bonds higher than 4.

Moreover, in reversed phase systems, the molecular species (here FAMEs) elute roughly in the order of increasing partition number (PN). The PN value is obtained from the relationship $PN = CN - 2n$, where CN is the carbon number in the aliphatic residues and n the number of carbon double bonds [31,32]. With the mobile phase containing CH_3CN, C16:0 does not co-elute with C18:1, whereas they elute closer with the one containing CH_3OH. Thus by changing the weak solvent nature, PGC allows to discriminate such compounds efficiently. This difference in behaviour of alkyl grafted silica and PGC was previously shown [10].

Secondly, an important retention variation between isomers was observed due to the position of unsaturation in the alkyl chain. This observation was observed especially in one case: the three isomers of C20:3. When there was only one unsaturation in the alkyl chain, a little retention variation occurred for homologues differing in the position of one unsaturation (i.e. C18:1 and C20:1). For the C20:3 isomers, the closer the unsaturations are to the polar head, the more the retention decreases. This description is reinforced by other retention behaviours. When a further unsaturation occurs in the apolar extremity of the alkyl chain, the decrease in the retention is weaker (e.g. C18:2 and C18:3; C20:4 and C20:5; C22:4 and C22:5) whereas in the alkyl chain part close to the polar head, higher decrease in retention
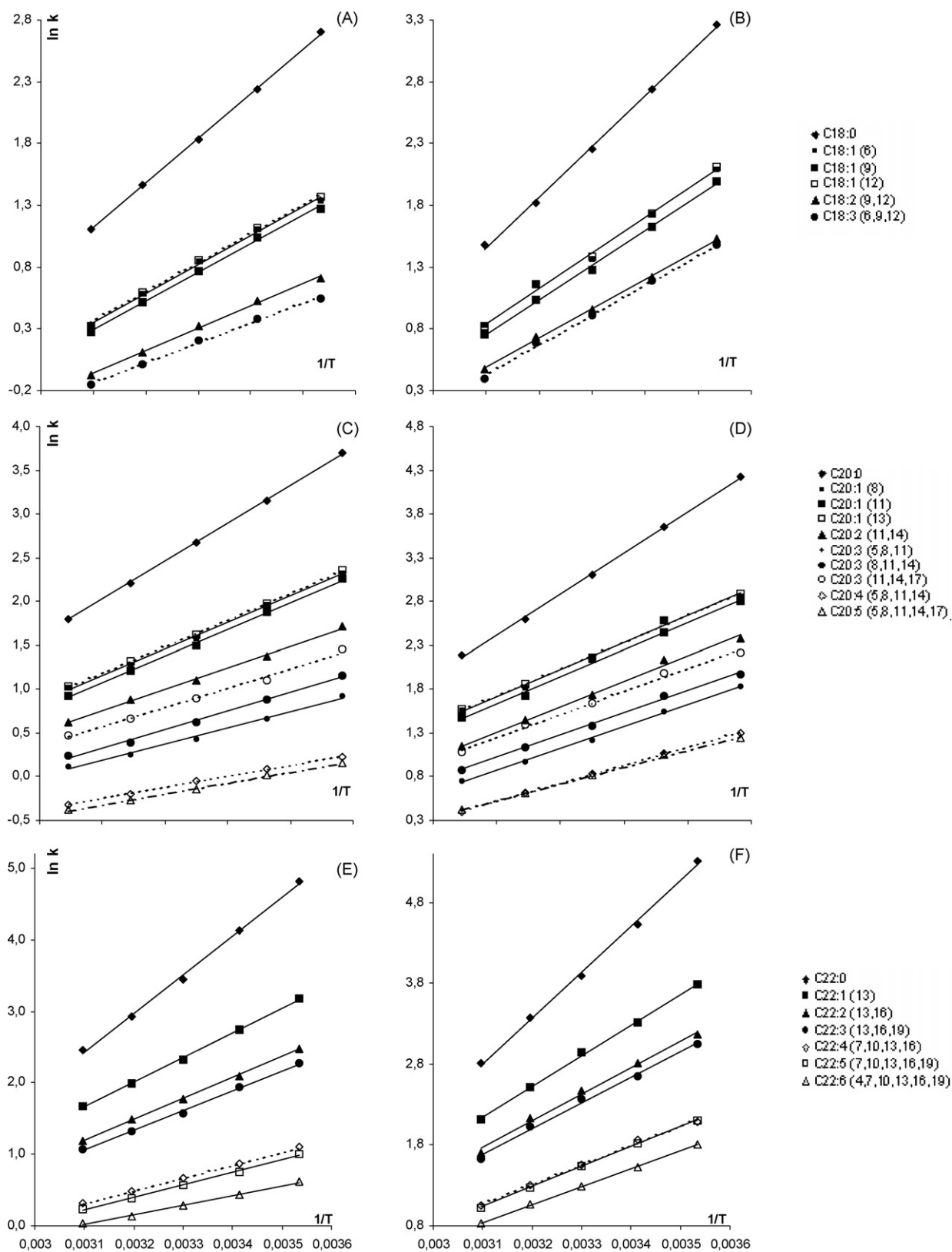
Fig. 6. Retention of homologous molecules. (temperature in K) (A and B) retention of 18 carbon alkyl chain FAMEs. (C and D) retention of 20 carbon alkyl chain FAMEs. (E and F) retention of 22 carbon alkyl chain FAMEs. (A, C and E) $CH_3CN:CHCl_3$ 60:40 (v/v); 0.1% TEA and an equimolar amount of HCOOH. (B, D and F) $CH_3OH:CHCl_3$ 60:40 (v/v); 0.1% TEA and an equimolar amount of HCOOH.
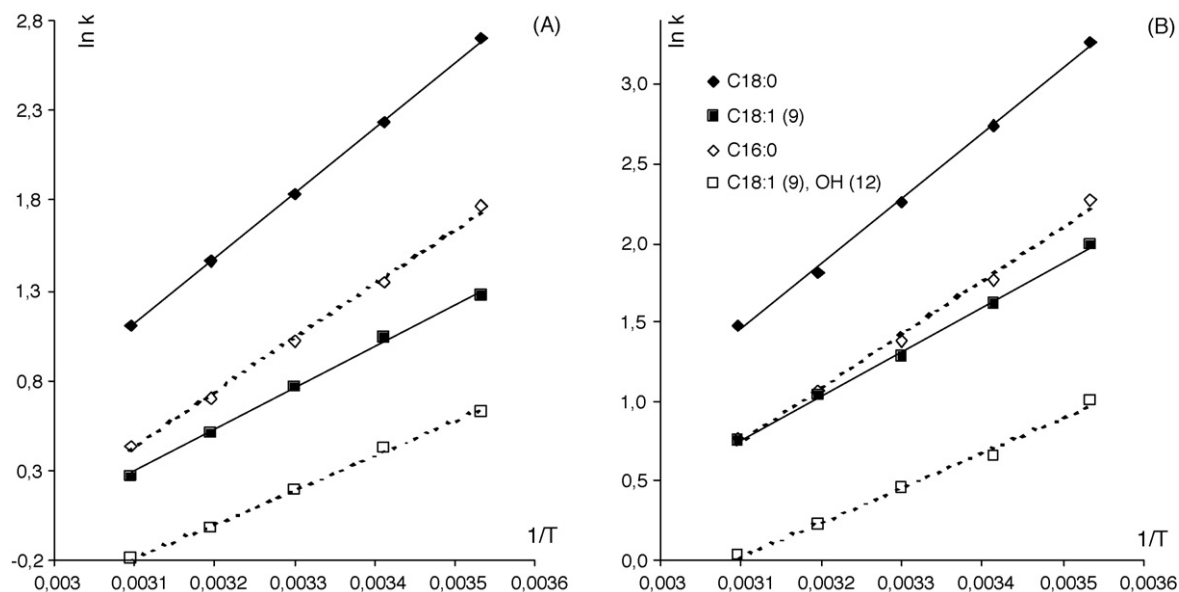
Fig. 7. Retention of homologous molecules of 18 and 16 carbon alkyl chain FAMEs. (Temperature in K) (A) $CH_3CN:CHCl_3$ 60:40 (v/v); 0.1% TEA and an equimolar amount of HCOOH. (B) $CH_3OH:CHCl_3$ 60:40 (v/v); 0.1% TEA and an equimolar amount of HCOOH.

occurs (e.g. C20:3 (8, 11, 14) and C20:4; C20:3 (11, 14, 17) and C20:4; C22:3 and C22:4; C22:5 and C22:6).

Another functionality was envisaged in this study: the presence of a hydroxyl group (Fig. 7), which importantly decreases the retention.

4. Conclusion

An original result was highlighted in this study about ELSD enhancement by organic modifier which may differently influence the molecule with regard to its structure, saturated or unsaturated. This feature may be useful for identification purpose.

Most significantly and most poorly appreciated is the ability of carbon sorbents to separate structurally similar species. The durability of this material coupled to its selectivity ought to make it extremely attractive for the chemical community and in particular to chromatographers. This study of the retention behaviour of FAMEs with PGC led to several remarks. First, the relative retention for highly unsaturated homologues can be changed by the selection of weak solvent, CH_3CN or CH_3OH. Then isomers of the position of the carbon double bond on the alkyl chain can be separated. The closer the unsaturation is to the polar head, the more the retention decreases. Finally, the more significant the number of carbon double bonds in the alkyl chain, the weaker the retention is.

This study suggested that the retention behaviour of this lipid class was mainly governed by the apolarity of the compounds and their molecular geometry. The high potentiality of PGC for the FAME discrimination represents a great advantage in the field of lipid control.

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