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Modelling of ceramide interactions with porous graphite carbon in non-aqueous liquid chromatography

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Abstract

Interactions of solutes on porous graphitic carbon (PGC) with non-aqueous mobile phases are studied by the linear solvation energy relationship (LSER). Studies have been carried out with eight binary mixtures composed of a weak solvent (acetonitrile or methanol) and a strong solvent (tetrahydrofuran, *n*-butanol, CH_2Cl_2, 1,1,2-trichloro-2,2,1-trifluoroethane). The systematic analysis of a set of test compounds was performed for each solvent mixture in isocratic mode (50:50). The results were compared to those obtained on PGC with hydro-organic liquids and supercritical fluids. They were then correlated with the observed retention behaviour of lipid compounds, more particularly ceramides.

Keywords: Non-aqueous reversed-phase liquid chromatography (NARP-LC); Linear solvation energy relationship (LSER); Ceramides; Porous graphitic carbon (PGC)

1. Introduction

Different separative methods can be applied to the analysis of hydrophobic compounds such as lipids. Depending on the molecular weight of the compounds, gas or liquid chromatography is selected [1,2].

HPLC is well suited for triglycerides, sphingolipids, phospholipids, carotenoid pigments and direct analysis of tocopherols. Several types of packed columns are used in high performance liquid chromatography: silica or diol to obtain separations on the basis of the polar part of the compounds, silver (Ag^+) coated silica to separate compounds mainly following the unsaturation number, octadecyl bonded silicas (ODS) to reach separation of compounds mainly differing by their hydrocarbonaceous volume or by their unsaturation number [1,2]. Recently, porous graphitic carbon (PGC) has been used for lipid separation [3–5] because of its greater methylene selectivity than ODS [6,7] and its ability to develop charge transfer interactions [8].

Due to the low solubility of most lipids in water, non-polar mobile phases are required, hexane/isopropanol for instance, in normal phase, or non-aqueous mobile phases in reversed-phase liquid chromatography (NARP-LC). Supercritical fluids such as CO_2 with modifiers are also especially well suited to ensure the solubility of such compounds, both with polar [9,10] or apolar [11–13] stationary phases, and promote the use of isocratic conditions when eluting gradients are often needed in HPLC [13].

For numerous lipid families, because the compounds mainly differ in their methylene or methyl group number, double bond and hydroxyl group number, apolar stationary phases are used for fine separations, when polar ones are preferred for class fractionation.

The relationships ruling separations are well known on polar phases: the retention increases following the unsaturation number or the polar group number. On apolar stationary phases, generally, the increase in methylene group number

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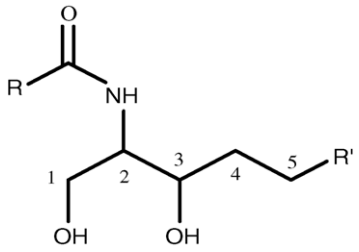

Fig. 1. General structure of ceramides. R is the fatty acid chain (length ranging from 16 to 26 methylene groups) and R′ is the base chain (length ranging from 11 to 25 methylene groups). The represented structure corresponds to ceramides with the dihydrosphingosine base. When a —OH is on position 4, the structure corresponds to ceramides with the phytosphingosine base. When a double bond is between carbons 4 and 5, the structure corresponds to ceramides with the sphingosine base.

favours retention while the increase in unsaturation number decreases the retention time of the compounds.

However, for ceramides, the retention behaviour depends on the nature of the apolar stationary phase. Ceramides (Fig. 1), which are made of a fatty acid chain (R) and of a sphingoid base carrying an alkyl chain (R′), are structurally widely varied and polarities spread over a large range. On ODS phases, ceramides containing phytosphingosine base having three hydroxyl groups always eluted before those with sphingosine having two hydroxyl groups and a double bond, whereas on PGC this elution order depends on the mobile phase composition [4].

A better understanding of retention mechanisms can be achieved by linear solvation energy relationships (LSERs) using Abraham's parameters. According to the LSER theory, the retention of a compound can be related to specific interactions through this relationship [14]:

$$\log k = c + eE + sS + aA + bB + vV \quad (1)$$

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, while lower case letters represent the system constants, related to the complementary effect of the phases on these interactions. E is the excess molar refraction (calculated from the refractive index of the molecule) and models polarizability contributions from n and π electrons; S is the solute dipolarity/polarizability; A and B are the solute overall hydrogen-bond acidity and basicity; V is the McGowan characteristic volume in units of $cm^3\,mol^{-1}/100$. Thus e reflects charge transfer interactions; s dipole–dipole interactions; a and b H-bond interactions; v represents both the cavity formation and dispersive interactions. c is a constant, depending on specific column parameters such as phase ratio. The system constants (c, e, s, a, b, v) are obtained through a multilinear regression of the retention data for a certain number of solutes with known descriptors. They reflect the magnitude of difference for that particular property between the mobile and stationary phases, such as:

$$x = x_{\text{stationary}} - x_{\text{mobile}}$$

where $x_{\text{stationary}}$ represents the interactions of type x between the solute and the stationary phase and x_{mobile} represents the interactions of type x between the solute and the mobile phase. System constants with a positive sign indicate that the characterized interactions are more favourable for the stationary phase than for the mobile phase, therefore lead to an increase in retention, and vice-versa. Consequently, system constants also reflect the system's relative selectivity towards a particular molecular interaction.

In HPLC, this model has been developed first to describe relationships occurring on octadecyl bonded silica with hydro-organic mobile phases [15]. The model was later applied to PGC with hydro-organic mobile phases [16] and supercritical fluids [17].

However, no studies were carried out in NARP-LC with PGC. The aim of this paper is to apply this model to improve the characterization of the binary non-aqueous mobile phases used on PGC. Moreover, these studies will be used to explain the retention order of ceramides depending on the organic solvent nature. Thus, a better understanding of the interactions taking place will be helpful to choose a well suited chromatographic system as well controlled elution order can favour the separation of minor compounds and their quantification.

2. Experimental

2.1. Chemicals

Fifty-one test compounds (Table 1), benzene and naphthalene derivatives were used for modelling investigations, and two sphingoid bases (phytosphingosine and sphingosine, Fig. 1) for the elution order studies of ceramides. All compounds were supplied by Sigma Aldrich (L'Isle d'Abeau, France) and were dissolved into THF before analysis, except the phytosphingosine which was a generous gift of Cosmoferm (Delft, The Netherlands).

2.2. HPLC apparatus

Depending on the detection required, different chromatographic systems were used.

For benzene and naphthalene derivatives, measurements were made with a PU Intelligent HPLC pump Jasco 880 (Jasco France, Nantes). The injector valve was supplied with a 20 μL loop (model 7125 Rheodyne, Cotati, CA, USA). The chromatograms were recorded with a Shimadzu C-R6 A Chromatopac manual integrator (Shimadzu, Kyoto, Japan). Detection was performed with a UV Detector Jasco UV 975 (Jasco France, Nantes). Wavelength was set at 254 nm. Column temperature was fixed through two heating equipments, a Croco·Cil oven (Cluzeau, St Foy la Grande, France) and a Cryostat Julabo 25. A thermocouple allowed checking out the temperature in the oven.

Table 1
Chromatographic solutes and LSER descriptors

	Test compounds	*E*	*S*	*A*	*B*	*V*
1	Toluene*	0.601	0.52	0.00	0.14	0.8573
2	Ethylbenzene	0.613	0.51	0.00	0.15	0.9982
3	Propylbenzene*	0.604	0.50	0.00	0.15	1.1391
4	Butylbenzene	0.600	0.51	0.00	0.15	1.2800
5	Hexylbenzene	0.591	0.50	0.00	0.15	1.5620
6	Aniline	0.955	0.94	0.26	0.50	0.8162
7	Benzoic acid	0.730	0.90	0.59	0.40	0.9317
8	*N,N*-Dimethylaniline*	0.957	0.84	0.00	0.47	1.0980
9	Phenylethanol	0.784	0.83	0.30	0.66	1.0570
10	Benzyl alcohol*	0.803	0.87	0.39	0.56	0.9160
11	Benzaldehyde*	0.820	1.00	0.00	0.39	0.8730
12	Acetophenone*	0.818	1.01	0.00	0.48	1.0139
13	Butylbenzoate*	0.668	0.80	0.00	0.46	1.4953
14	Benzonitrile*	0.742	1.11	0.00	0.33	0.8711
15	Nitrobenzene*	0.871	1.11	0.00	0.28	0.8906
16	Chlorobenzene*	0.718	0.65	0.00	0.07	0.8288
17	Bromobenzene	0.882	0.73	0.00	0.09	0.8910
18	Phenol	0.805	0.89	0.60	0.30	0.7751
19	*o*-Chlorophenol*	0.853	0.88	0.32	0.31	0.8980
20	2,4-Dimethylphenol*	0.840	0.80	0.53	0.39	1.0570
21	2,5-Dimethylphenol*	0.840	0.79	0.54	0.37	1.0570
22	2,6-Dimethylphenol*	0.860	0.79	0.39	0.39	1.0570
23	3,4-Dimethylphenol*	0.830	0.86	0.56	0.39	1.0570
24	Resorcinol	0.980	1.00	1.10	0.58	0.8340
25	*o*-Nitrophenol*	1.045	1.05	0.05	0.37	0.9490
26	*m*-Nitrophenol*	1.050	1.57	0.79	0.23	0.9490
27	*p*-Nitrophenol	1.070	1.72	0.82	0.26	0.9490
28	*o*-Xylene*	0.663	0.56	0.00	0.16	0.9980
29	*m*-Xylene*	0.623	0.52	0.00	0.16	0.9980
30	*p*-Xylene*	0.613	0.52	0.00	0.16	0.9980
31	Phenylurea*	1.110	1.40	0.77	0.77	1.0730
32	Benzophenone	1.447	1.50	0.00	0.50	1.4810
33	Biphenyl	1.360	0.99	0.00	0.26	1.3242
34	Phenylnaphtalene	1.910	1.08	0.00	0.30	1.6932
35	Naphthalene*	1.400	0.92	0.00	0.20	1.0854
36	1-Methylnaphthalene*	1.344	0.90	0.00	0.20	1.2260
37	2-Methylnaphthalene*	1.304	0.92	0.00	0.20	1.2260
38	1-Ethylnaphthalene*	1.371	0.87	0.00	0.20	1.3670
39	2-Ethylnaphthalene*	1.331	0.87	0.00	0.20	1.3670
40	1-Aminonaphthalene*	1.670	1.26	0.20	0.57	1.1850
41	Naphthalenemethanol*	1.640	1.19	0.27	0.64	1.2850
42	Naphthaleneethanol*	1.670	1.21	0.23	0.72	1.4259
43	1-Naphtylaldehyde	1.470	1.19	0.00	0.47	1.2420
44	1-Naphtylacetate*	1.130	1.25	0.00	0.62	1.4416
45	1-Naphtylacetonitrile*	1.430	1.44	0.00	0.53	1.3810
46	1-Cyanonaphthalene	1.190	1.25	0.00	0.41	1.2401
47	1-Nitronaphthalene*	1.600	1.51	0.00	0.29	1.2596
48	1-Fluoronaphthalene*	1.320	0.82	0.00	0.18	1.1030
49	1-Chloronaphthalene*	1.540	0.92	0.00	0.15	1.2078
50	1-Bromonaphthalene*	1.670	0.97	0.00	0.17	1.2604
51	2-Naphtol*	1.520	1.08	0.61	0.40	1.1440

E: excess molar refraction, *S*: dipolarity/polarizability, *A*: hydrogen-bond acidity, *B*: hydrogen-bond basicity, *V*: McGowan's characteristic volume.

Table 2
Solvent properties

	π^*	α	β
Weak solvents			
MeOH	0.60	0.93	0.62
ACN	0.75	0.19	0.31
Strong solvents			
THF	0.58	0.00	0.55
CH_2Cl_2	0.82	0.30	0.00
n-BuOH	0.47	0.79	0.88
TTE	na	0.00	0.00

π^*: bulk phase dipolarity/polarizability, α: bulk phase hydrogen-bond acidity, β: bulk phase hydrogen-bond basicity.

For the sphingoid bases analysis, a PU-980 Jasco pump was used. The injector valve 7125 was provided with a 10 μL loop. The chromatograms were recorded with a PC-integrator Kromasystem 2002 (BioTek Instruments, Milan, Italy). The column was thermostated with a Jetstream 2 temperature controller (Thermotecnic Produit Gmbh, Langenzersdorf, Austria). Detection was performed with a Cunow DDL 11 (Eurosep Instrument, Cergy Pontoise, France) evaporative light scattering detector. Nitrogen Pressure was set at 1.5 bar and the drift tube temperature at 40 °C. This detector is well suited for compounds without chromophores as ceramides.

The column was Hypercarb porous graphitic carbon (100 mm × 4.6 mm I.D.; 5 μm) supplied by Thermo-Hypersil Keystone (Runcorn, UK). Temperature was set at 30 °C and flow rate at 1 mL/min.

All solvents were HPLC-grade: methanol (MeOH, Prolabo), tetrahydrofuran (THF, Carlo Erba), 1-butanol (*n*-BuOH, LiChrosolv, Merck), Acetonitrile (ACN, Chromanorm), dichloromethane (CH_2Cl_2, Carlo Erba), 1,1,2-trichloro-2,2,1-trifluoroethane (TTE, Carlo Erba). These solvents were chosen because of their very distinct physicochemical properties, represented in Table 2 by the solvatochromic parameters [18,19]. They provide a large range of hydrogen bonding ability (α varying from 0 to 0.93 and β from 0 to 0.88) and polarity (π^* ranging from 0.47 to 0.82).

In order to obtain mobile phases with varied eluotropic strengths and different physical characteristics, binary mobile phases were considered. The mixtures were composed of a weak eluotropic strength solvent (MeOH or ACN) and a strong eluotropic strength one (THF, CH_2Cl_2, *n*-BuOH, or TTE).

The isocratic composition was set at 50:50 (v/v) to ensure that properties of each solvent would act on retention and would be reported in the model. Naturally, despite this identical composition, eluotropic strength of the studied mixtures was not equal.

When changing the mobile phase studied, the column was conditioned until repeatability of retention time was achieved, in order to reach the equilibrium state of the chromatographic system. The hold up time t_0 was marked with the dilution solvent for each injection.

2.3. Data analysis and modelling

From t_0 and t_r, respectively the dead time and retention time of a solute, the retention factor *k* was calculated in each mobile phase for each compound.

Multiple linear regression analysis and statistical tests were performed using the program SuperANOVA (Abacus Concept, Berkeley, CA, USA, 1989) based on Eq. (1).

The solute descriptors used in the solvation parameter model were taken from varied sources [15,20,21] and are presented in Table 1. The system constants for each mobile phase composition were obtained by multiple linear regression analysis of the measured retention factors against the descriptors (*E*, *S*, *A*, *B*, *V*). To obtain chemically meaningful coefficients, the solute parameters must be varied over a wide range. Consequently the probe solute set was carefully chosen to have a uniform distribution of each descriptor within a chosen space (Fig. 2a–e).

The quality of the fits was estimated using the overall correlation coefficient (R), adjusted correlation coefficient (R^2_{adj}), standard error in the estimate (SD) and Fischer F statistic. Statistical tests (Student's tests) were performed to assess which parameters were pertinent. Descriptors that were not statistically significant, with a confidence interval

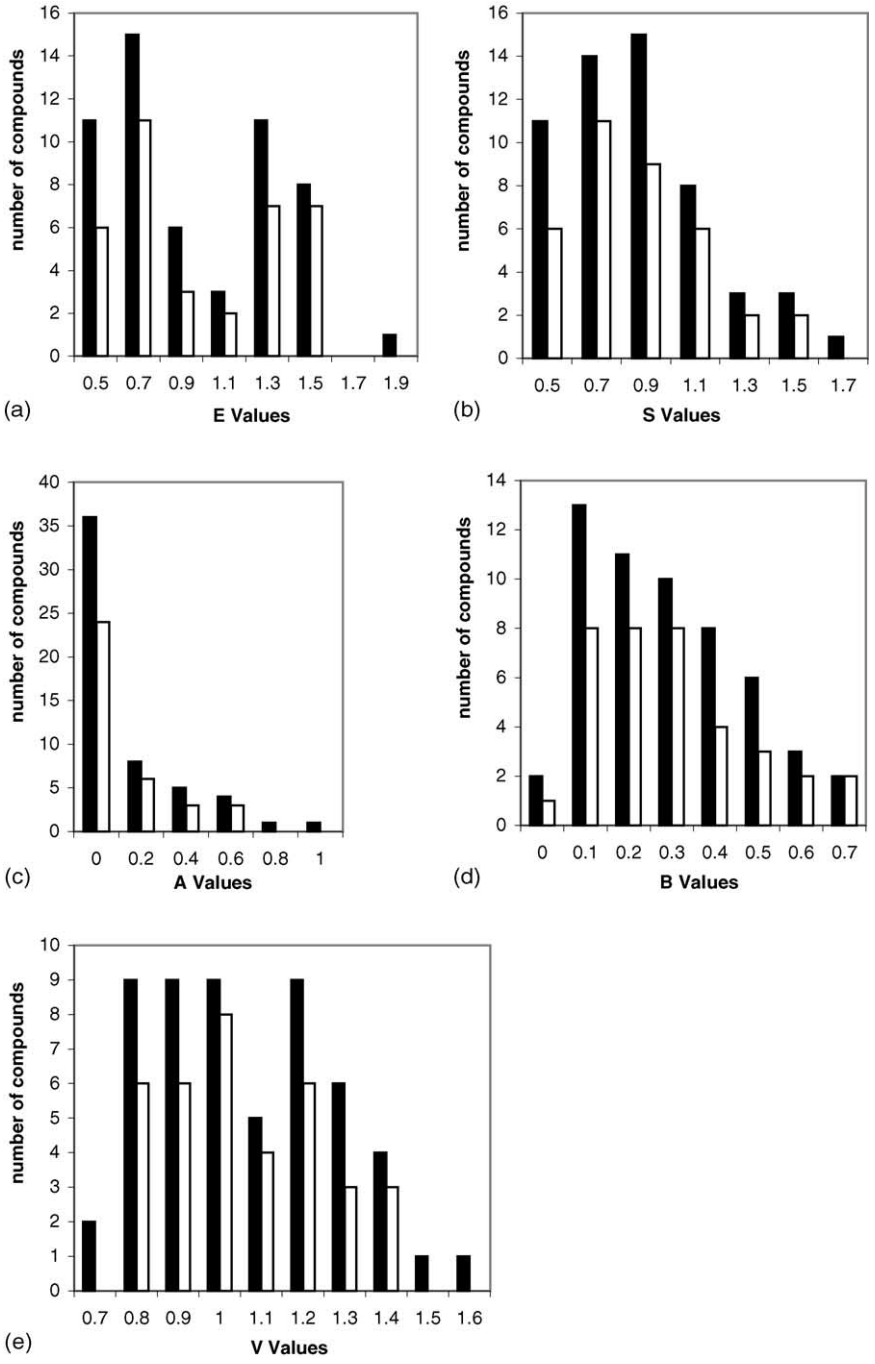

Fig. 2. Distribution of descriptor values. Black bars represent the initial set of $n_i = 51$ compounds; white bars represent the $n_f = 36$ compounds remaining in the ACN–*n*-BuOH set. (a) Descriptor *E*, (b) descriptor *S*, (c) descriptor *A*, (d) descriptor *B*, (e) descriptor *V*.

Table 3
LSER coefficients and model fit statistics for each mobile phase composition

Mobile phase	c	e	s	a	b	v	n_f	R	R^2_{adj}	F	SD
MeOH–THF	−2.235	1.171	ns	ns	−0.832	0.939	46	0.952	0.901	137	0.170
	0.149	*0.084*			*0.146*	*0.147*					
ACN–THF	−2.141	1.203	ns	ns	−0.509	0.718	44	0.969	0.934	203	0.130
	0.115	*0.065*			*0.112*	*0.113*					
MeOH–CH_2Cl_2	−1.990	1.410	ns	−0.520	−0.813	0.566	38	0.982	0.959	219	0.120
	0.125	*0.067*		*0.087*	*0.134*	*0.130*					
ACN–CH_2Cl_2	−2.420	1.010	0.440	ns	−0.981	0.948	41	0.978	0.952	201	0.115
	0.122	*0.076*	*0.088*		*0.119*	*0.113*					
MeOH–TTE	−2.166	1.166	ns	−0.447	−0.923	1.183	39	0.972	0.937	143	0.155
	0.156	*0.083*		*0.101*	*0.177*	*0.159*					
ACN–TTE	−2.206	1.233	ns	ns	−0.745	0.899	37	0.959	0.913	126	0.165
	0.166	*0.086*			*0.167*	*0.163*					
MeOH–*n*-BuOH	−2.024	0.824	0.468	ns	−0.989	0.984	39	0.975	0.944	162	0.121
	0.132	*0.089*	*0.106*		*0.132*	*0.133*					
ACN–*n*-BuOH	−2.306	0.986	ns	ns	−0.682	1.180	36	0.977	0.951	228	0.112
	0.118	*0.067*			*0.110*	*0.134*					

n_f is the number of solutes considered in the regression, R is the multiple correlation coefficient, R^2_{adj} is the adjusted correlation coefficient, SD is the standard error in the estimate, F is Fischer's statistic and the numbers in italics represent 99% confidence limits. ns stands for "not significant".

of 0.01%, were eliminated from the model. Then in order to improve the fits, compounds with abnormally high residuals were excluded from the initial set. Graphs of the residuals (difference between the experimental and predicted log k values) plotted against the values of each individual descriptor showed no correlation. It was verified that no correlation between residuals and predicted log k values existed and that the points were randomly distributed. Moreover, absence of cross-correlation between the descriptors was checked.

The final models were obtained with n_f compounds (ranging from 36 to 46) retained from the initial set of n_i (51) compounds and contained only relevant coefficients. As a precaution, it was verified that the homogeneous distribution for each descriptor had been preserved in the final sets and that no biases were introduced by the elimination of outliers. This can be observed with the example in Fig. 2a–e, where the white bars represent the n_f (36) compounds (mentioned by an asterisk in Table 1) retained in the ACN–*n*-BuOH model and the black bars the initial set. For each descriptor, the outliers removed are equally distributed.

3. Results and discussion

3.1. Interaction model

The system constants and statistics obtained from the linear regression of log k are summarized in Table 3.

The LSER equations for the eight binary solvent systems showed reasonable statistics. R^2_{adj} was used to compare equations built up with a different number of data and a different number of variables. It ranged from 0.901 to 0.959 while SD varied from 0.112 to 0.170. These values are comparable to other studies carried out on PGC in HPLC [16].

Values of the system constants were both large and significantly larger than their uncertainty, therefore amenable to interpretation. Amongst these eight equations (Table 3) corresponding to the eight binary mixtures, the dominant contributions to retention within the chromatographic system studied are the e and v coefficients (positive contribution) and b coefficient (negative contribution). This indicates that PGC is particularly selective towards solutes able to develop those kinds of interactions, thus having high E, B and V values.

Positive e and v coefficients indicate that the charge transfer and dispersion interactions established between the solute and the stationary phase are more important than these same interactions between the solute and the mobile phase.

High values of the e coefficient (positive values superior to 0.9) indicate that solutes are developing strong charge transfer interactions with the stationary phase. These results corroborate the fact that PGC is also an electron-pair acceptor with non-aqueous mobile phases, as was observed with subcritical fluids and hydro-organic liquids.

The lowest e values are obtained for mixtures including *n*-BuOH. Lower values of e indicate that either solute/stationary phase interactions are lower with *n*-BuOH mixtures, or that solute/mobile phase interactions are greater. Judging by the low π^* value of *n*-BuOH, the lower solute/stationary phase interactions could explain the observed variations of e. This decrease of solute/stationary phase interactions can be induced by a strong adsorption of *n*-BuOH onto the stationary phase, reducing the charge transfer interactions between the solutes and the surface.

The values of the v coefficients with non-aqueous phases are dramatically smaller than those reported with

hydro-organic mobile phases [16]. It must be noted that the v coefficient results from both the negative cavity energy in the mobile phase and from positive dispersive interactions between the solute and the stationary phase. In aqueous mobile phases, the high cohesivity of water explains that the energy required to create a cavity is high and favours retention. Assuming that the solute-stationary phase dispersion interactions do not depend on the mobile phase nature, the lower v values obtained in non-aqueous mobile phases are explained by the lower cavity energy, because these fluids are less cohesive than hydro-organic ones. Consequently, in non-aqueous mobile phases, retention of non-polar compounds is driven by positive dispersion interactions rather than by the repulsive hydrophobic effect observed with hydro-organic mobile phases.

The v values are also lower than those obtained in CO_2/methanol subcritical fluids. However, the pressure drop along the column when non-aqueous liquids are used is very similar to that observed with subcritical fluids, indicating that the viscosity of the fluids is close. This suggests that the mobile phase cohesivity is similar, leading to close cavity energies. Consequently, the difference of the v values between non-aqueous liquid and subcritical fluid indicates that dispersion interactions between solutes and mobile phases are greater with organic liquid than with carbon dioxide.

A negative b coefficient indicates that the mobile phase's acidity (H-bond donning ability) is always higher than that of the stationary phase. Thus solutes with highly basic character (B) should be less retained than non-basic solutes. However, basic compounds, having n electron pairs, also have a high molar refractivity (high E value). The e coefficient being high and positive, the effects of charge transfer (e) and acido-basic interactions (b) are opposite and the retention behaviour of these compounds can be complex.

A negative b coefficient was also observed with hydro-organic mobile phases, due to the high acidity of water. This term disappears when using subcritical CO_2/organic solvent mixtures, as this mobile phase is not very acidic.

Plot of experimental $\log k$ values of compounds in ACN–THF against experimental $\log k$ values of compounds in MeOH–THF (Fig. 3a) shows that the compounds having high H-bond acceptor properties (higher B values) are out of the general tendency. In comparison to other compounds, the basic solutes show a higher relative affinity for the stationary phase with ACN–THF as mobile phase than when MeOH–THF is the eluting phase. This is consistent with the values of the system constants obtained for these two systems: the ACN–THF system shows a higher b value than the MeOH–THF system (respectively -0.509 and -0.832), indicating that the former is less eluting towards basic solutes than the latter.

The a coefficient appears only in two systems (MeOH–CH_2Cl_2 and MeOH–TTE) and it is negative in both cases. Then, in MeOH–CH_2Cl_2 and MeOH–TTE, an increased acidity of the compound should contribute to decrease retention. A plot of experimental $\log k$ values in the ACN–TTE system against experimental $\log k$ values in the MeOH–TTE system displayed in Fig. 3b shows that solutes with particularly high acidic properties positively swerve from the general tendency. This plot demonstrates that compounds with H-bond donating abilities are more retained in ACN–TTE than in MeOH–TTE. Modelling results support this claim with respectively $a = 0$ and -0.447, supporting the fact that the eluting strength of the MeOH–TTE mobile phase towards acidic solutes is higher than that of ACN–TTE.

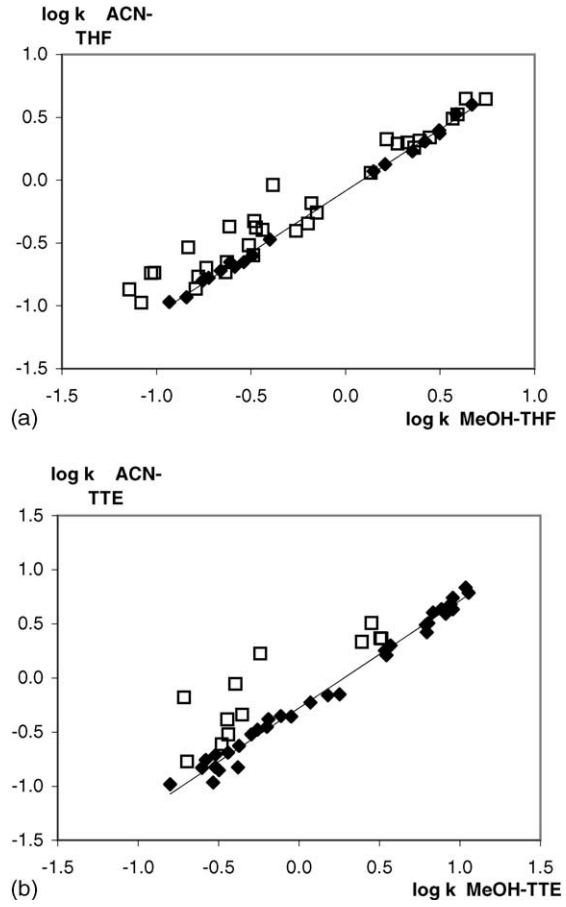

Fig. 3. (a) Plot of $\log k$ in ACN–THF mobile phase vs. $\log k$ in MeOH–THF mobile phase. Black diamonds represent low H-bond acceptor solutes; white squares represent high H-bond acceptor solutes ($B > 0.20$). (b) Plot of $\log k$ in ACN–TTE mobile phase vs. $\log k$ in MeOH–TTE mobile phase. Black diamonds represent non H-bond donor solutes ($A = 0.00$); white squares represent H-bond donor solutes ($A > 0.00$).

Negative values of the a coefficient for the two binary mixtures (MeOH–CH_2Cl_2 and MeOH–TTE) where it is significant, mean that those two mobile phases are more basic than the stationary phase. However, because the basic character (β) of the two strong solvents CH_2Cl_2 and TTE is equal to 0, their addition to MeOH should reduce the mobile phase basicity rather than increase it. Thus the negative a coefficient cannot be explained by the change in mobile phase properties but by a change in stationary phase properties, namely a decrease in its basic character. This decrease is suggested to be due to adsorption of the strong solvents on the surface.

Table 4
Retention factors and order of elution of the two sphingoid bases in each mobile phase

Mobile phase	log *k* (S)	log *k* (P)	Order of elution
MeOH–THF	−0.13	−0.23	P S
ACN–THF	0.44	0.90	S P
MeOH–CH_2Cl_2	−0.37	−0.45	P S
ACN–CH_2Cl_2	na	na	na
MeOH–TTE	−0.06	0.01	S P
ACN–TTE	0.67	0.36	P S
MeOH–*n*-BuOH	−0.09	0.12	S P
ACN–*n*-BuOH	0.45	0.68	S P

S stands for sphingosine and P for phytosphingosine. na stands for "not available".

These adsorption seem not occur for ACN/strong solvent mixtures.

The *s* coefficient appears only in two systems (ACN–CH_2Cl_2 and MeOH–*n*-BuOH) and is positive in both cases, indicating that the stationary phase, in these cases, develops higher dipole–dipole interactions with polar and polarizable solutes than the mobile phase.

Zero value of this coefficient for the other mobile phases at 50:50 (v/v) composition points out that the magnitude of this type of interactions must be of the same order between the solutes and PGC and solutes and mobile phases. Once again, adsorption of the mobile phase on the PGC surface must play a leading role in dipole–dipole interactions with PGC. Thus, on the sole basis of mobile phase properties, it is sometimes difficult to understand the behaviour of these mixtures.

3.2. Retention behaviour of ceramides

In previous studies [4], elution order variations were seen to be related to the phytosphingosine (P) and sphingosine (S) bases. Therefore, these two compounds only were studied in the eight chromatographic systems, and not the complete ceramide molecules. The retention factors and elution order of the two sphingoid bases in the various mobile phases are shown in Table 4.

The mobile phases are compared two by two, keeping one solvent constant.

The effect of the weak eluotropic strength solvent on the retention and separation is in good agreement with previous results: higher retention and selectivities were obtained with binary mixtures comprising ACN [4].

Inversions of the elution order between S and P are noticed when replacing MeOH by ACN. With THF as a strong solvent, the effect of the weak solvent on the elution order is clear. The MeOH–THF mobile phase elutes both compounds more rapidly than ACN–THF, but phytosphingosine is better solvated than sphingosine, thus resulting in an inversion of the elution order.

Another inversion appears in binary mobile phases composed with TTE, while changing the weak solvent nature, but the retention order is the opposite to the previous one.

No inversions were observed for binary mixtures with *n*-BuOH as strong solvent. The retention order seems surprising as *phytosphingosine* is more retained than sphingosine, while the *n*-BuOH (strong solvent) should favour the solubility of the compound having the greater hydroxyl group number. The greater retention of phytosphingosine could be due to higher interactions between this compound and solvent molecules adsorbed onto the stationary phase.

Table 5
Estimated solute descriptors for sphingosine and phytosphingosine

Compound	*E*	*S*	*A*	*B*	*V*
Sphingosine	0.680	0.959	0.748	1.217	2.8190
Phytosphingosine	0.708	1.021	0.971	1.502	2.9210
ΔX	0.028	0.062	0.223	0.285	0.1020

ΔX: differences between the two.

Moreover, other inversions occur between mixtures having identical weak solvents, when changing the strong eluent, for instance: replacing THF by TTE, whatever the weak solvent, leads to different orders of elution.

In order to understand the inversions of retention of S and P with mobile phase nature, the solvation parameter model was used. To use the models, knowing the descriptors of the sphingoid bases was required. Thus *V* was calculated by the summation of volumes of the atoms and bonds [15] and *E*, *S*, *A* and *B* were estimated with the fragment method [22]. The latter is a calculation model consisting in the summation of values of chemical properties (*E*, *S*, *A*, *B*) of functional group fragments forming the solutes. Possible intramolecular hydrogen bonds were taken into account in the descriptors calculation.

Table 5 shows sphingosine and phytosphingosine estimated descriptors, and the values of the differences for each descriptor between the sphingoid bases. In all cases, the descriptor values of phytosphingosine are higher than those of sphingosine. It can be seen that the most meaningful differences of properties between S and P are ΔB, ΔA, ΔV, and to a lesser extent ΔS and ΔE.

The assessment of the sphingoid base descriptors enabled the use of the linear solvation energy relationship. Retention order (Table 4) and regression coefficients of the solvation parameter model (Table 3) have been compared.

The first case we present in detail is that of MeOH–THF and ACN–THF systems, as illustrated in Fig. 4. Maintaining the strong solvent constant, the effect of the weak solvent on the elution order can be assessed. As reported previously, it can be noticed that both sphingosine and phytosphingosine are more retained in ACN–THF than in MeOH–THF. The

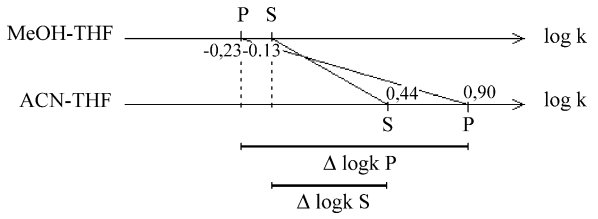

Fig. 4. Comparison of the retention factors and elution order of sphingosine (S) and phytosphingosine (P) in two different mobile phases.

Table 6
Mobile phases compared and possible interactions leading to the inversion of the elution order of the sphingoid bases

Mobile phases	Interaction inducing the inversion
MeOH–THF ACN–THF	*bB*
MeOH–THF MeOH–*n*-BuOH	*sS*
MeOH–CH_2Cl_2 MeOH–TTE	*vV*
MeOH–TTE ACN–TTE	$aA + vV$?

most significant differences between the system constants of these two systems (see Table 3) appear in the *b* and *v* coefficients. However, the difference of *v* between the two systems cannot explain the increase in retention, as *v* is lower in the ACN–THF mobile phase. It can only be explained by the large value of the negative *b* coefficient in MeOH–THF ($b = -0.832$) compared to that of ACN–THF ($b = -0.509$), indicating, as expected, that the former has a higher eluting strength towards H-bond accepting solutes than the latter. Secondly, we note that the retention increase for phytosphingosine ($\Delta \log k$ P = 1.13) is higher than the retention increase for sphingosine ($\Delta \log k$ S = 0.57), causing the inversion of the elution order. This can be explained by the higher H-bond accepting ability of phytosphingosine ($B = 1.502$), compared to that of sphingosine ($B = 1.217$), meaning that phytosphingosine, having three hydroxyl groups, is more affected by a change in the H-bond donating ability (*b*) of the mobile phase than sphingosine, having only two hydroxyl groups. Thus, the difference in the acidic character of the mobile phase could explain the elution order of the sphingoid bases in these systems.

Another case is the inversion of elution order when comparing MeOH–THF and MeOH–*n*-BuOH systems. This would allow the comparison of the effect of the strong solvent when maintaining the weak solvent constant. When replacing THF by *n*-BuOH, coefficients *e* and *b* decrease and coefficients *s* and *v* increase. These opposite effects explain the fact that the variation in retention factors is not as important as in the first case presented. However, we notice that the retention factors increase. This is probably due to the major increase in the *s* value ($\Delta s = +0.468$). Similarly to the first case, the polarity–polarizability parameter of phytosphingosine ($S = 1.021$) being higher than that of sphingosine ($S = 0.959$), the former is more affected by changes in the dipole–dipole interacting ability of the mobile phase than the latter. Therefore, the increase in retention is higher, causing the inversion of the elution order (Table 6).

In the same manner, comparing MeOH–CH_2Cl_2 and MeOH–TTE systems allows to assess the effect of changing the strong solvent. The most significant difference in the system constants is the large increase of the *v* coefficient when replacing CH_2Cl_2 ($v = 0.566$) by TTE ($v = 1.183$). This indicates either a large increase in the dispersion interactions between the solutes and the stationary phase, or a large decrease in the dispersion interactions between the solute and the mobile phase. In any case, the result is a significant increase in retention of both sphingoid bases. Again, the larger volume of phytosphingosine ($V = 2.9210$) compared to that of sphingosine ($V = 2.8190$) explains the higher increase in retention of the former, causing the inversion of elution order.

However, some cases are more difficult to explain. In order to highlight the shortcomings of the predictive potential of the LSER method, the MeOH–TTE and ACN–TTE systems are compared. As in the first case, replacing MeOH by ACN causes a great increase in retention. This is probably due to the high increase in *a* values ($\Delta a = +0.447$), and the small increase in *b* values ($\Delta b = +0.170$). Nevertheless, the increase in retention of sphingosine is higher than that of phytosphingosine, which is in contradiction with our expectations. Thus the variation of acido-basic properties cannot explain the inversion of retention. Certainly, the opposite effects induced by the diminution of dispersion interactions ($\Delta v = -0.284$) should be considered for a better understanding of the retention behaviour in this case. Furthermore, the A descriptor values relative to the sphingoid bases may be inaccurate, due to possible intramolecular hydrogen bonds which are difficult to evaluate.

4. Conclusions

The linear solvation energy relationship was successfully applied to the study of non-aqueous binary liquids used as mobile phases on porous graphitic carbon.

Whatever the solvents, the charge transfer and the dispersion interactions are the major interactions involved in the retention mechanisms. The acidic character of the mobile phase has a great influence on elution. The polarity and basicity of the mobile phase act on retention only for two mixtures each one. As expected from results obtained with ODS stationary phases, the properties of non-aqueous liquid are closer to those of supercritical fluids than to those of hydro-organic liquid on PGC.

The elution order of two sphingoid bases can often be clearly explained on the basis of the difference in one solvation parameter between two mobile phases. Obviously, the interactions involved in these elution inversions depend on the mobile phase nature.

Initially, the inversion phenomenon was more imputed to the change of the weak solvent nature but this study exhibits also the role of the stronger solvent in this inversion phenomenon.

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