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Structure–retention diagrams of ceramides established for their identification

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Abstract

Molecular species analysis of ceramides was carried out using porous graphitic carbon with gradient elution: chloroform–methanol from 45:55 to 85:15 with a slope at 2.7%/min. These conditions gave a linear relationship between retention data and structure of ceramides. It was demonstrated that linearity occurred when a high slope value of linear gradient elution was used. Thereby the linear diagram was evolved by plotting the adjusted retention time against the total number of carbon atoms of ceramide molecules. Each line represents one ceramide class. Such a Structure–Retention Diagram describes ceramide retention and thus constitutes an identification method using only retention data. This Structure–Retention Diagram was assessed and compared to another obtained from octadesyl-grafted silica in terms of their reproducibility, precision and ability to provide ceramide identification. Better identification was obtained using the results from both Structure–Retention Diagrams. This approach with a two-dimensional separation system allowed to take advantage of the specificity of both identification models.

Keywords: Structure–retention relationships; Retention behaviour; Ceramides; Porous graphitic carbon

1. Introduction

Ceramides are lipids involved in the permeability barrier function of the skin [1–4]. Structural studies indicate that cutaneous ceramides are constituted by an amine (sphingosine, phytosphingosine or 6-hydroxy-sphingosine) linked with a fatty acid (non OH fatty acids, α-OH fatty acids or ω-OH fatty acids) [5,6]. This natural structural heterogeneity is widely increased by the variability of the length and degree of unsaturation of the alkyl chain of the fatty acid and the base moieties. However, the role of this heterogeneity in the stratum corneum is not established and not studied with regard to the skin properties. One reason could be the lack of a simple routine analytical method applicable for a large number of skin samples. For this purpose, several chromatographic techniques were investigated: gas [7], subcritical [8] and liquid chromatography [9]. The main differences of these methods were: (1) gas chromatography analysis needed the derivation of ceramide which is time consuming; (2) the reproducibility of data in supercritical chromatography requires greater control of chromatographic parame-

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ters, and (3) the liquid chromatographic method prevented the drawbacks of both previous techniques but the system using octadecyl bonded silica (ODS) and gradient mobile phase was found to be less discriminating. However, liquid chromatography was selected as this technique is classically encountered in laboratories and presents more advantages for its application in routine analysis. In liquid chromatography (LC), the large hydrocarbon volume of ceramides led to the investigation of the potential of non-aqueous reversed-phased (NARP) chromatography for their separation. However with ODS [10], the choice of solvents was limited and thus the modulation of the selectivity. The idea to improve the separation of ceramide species was then to select another stationary phase sufficiently different to allow the use of other solvents or at least different solvent compositions such that different selectivities can be reached. Thereby the porous graphitic carbon (PGC) packing material [11] was then selected for its property of greater discrimination of methylene groups than ODS [12,13]. A previous chemometric study of ceramide analysis with PGC by principal component analysis [14] had allowed, among the various studied parameters, to roughly classify solvents according to their eluotropic strength and described the consequences on the selectivity. The greater potential of PGC compared to ODS for ceramide separation was underlined. Moreover few similar chemical structures are commercially available which complicate method development. From this statement, a structural approach was required. Consequently, the appropriate technique appeared to be liquid chromatography associated with mass spectroscopy [15–19]. However such identification was correctly achieved only if the separation method was based on the skill of the retention behaviour. Therefore the strategy developed herein was the establishment of a separation method associated with a data treatment which consists to link together the structures of ceramides with their chromatographic retention. This approach led to a very simple tool for ceramide identification which still allowed hyphenation with mass spectrometry.

On the basis of previous separation studies, a simple system was achieved and devoted to the identification of molecular species of ceramide molecules. In order to evolve this identification tool, equations were developed which described how the adjusted retention time varied with the hydrocarbonated volume of the ceramide. Thereby the experimental conditions were selected leading to linear representation of this relationship. This model was examined and compared to another established from a liquid chromatographic method with ODS. The reliability of the ceramide identification was reached from the combination of two retention measurements obtained from two different chromatographic systems.

2. Experimental

2.1. Structural abbreviations

dn:0 cn′:x, tn:0 cn′:x and dn:1 cn′:x correspond, respectively, to ceramides with dihydrosphingosine phytosphingosine and sphingosine base. n and n′ are, respectively the number of carbon atoms of the base and the fatty acid alkyl chains. x is the number of carbon double bond(s) of the fatty acid moiety. Ceramides with α-hydroxylated fatty acid were symbolised by dn:1 hn′:x (e.g. in the case of ceramide with sphingosine base).

2.2. Chemicals

Ceramide type III (commercial mixture of non-hydroxy ceramide with mainly sphingosine base), Ceramide type IV (commercial mixture of α-hydroxy ceramide with mainly sphingosine base), *N*-palmitoyl-D-sphingosine (d18:1 c16:0), *N*-stearoyl-D-sphingosine (d18:1 c18:0), *N*-palmitoyl-DL-dihydrosphingosine (d18:0 c16:0), *N*-oleoyl-D-sphingosine (d18:1 c18:1), *N*-lignoceroyl-DL-dihydrosphingosine (d18:0 c24:0), *N*-nervonoyl-D-sphingosine (d18:1 c24:1) and fatty acid methyl esters were all purchased from Sigma (St. Quentin Fallavier, France). Ceramide III (t18:0 c18:0) and ceramide IIIB (t18:0 c18:1) were a generous gift from Cosmoferm (Delft, Netherlands). Type III and Type IV were prepared at 0.5 mg ml^{-1}. All of the solvents were HPLC-grade from Fisher (Elancourt, France) and contained 0.1% triethylamine and an equivalent amount of formic acid [20,21] (Prolabo, Nogent sur Marne, France).

2.3. HPLC apparatus

Chromatographic measurements were carried out with a Thermo Separation Products P1000 XR gradient pump with a TSP SCM1000 (Thermo Separation Products, San Jose, CA, USA) vacuum membrane degasser connected to a Kontron autosampler 360 (Bio-Tek Kontron Instruments, Milan, Italy) with a 5-μl sample loop injection valve. Detection was performed with a Cunow DDL 11 evaporative light scattering detector (Eurosep, Cergy, France) where the drift tube temperature was set at 35 °C and nitrogen pressure at 1 bar. The chromatograms were recorded with a PC-integrator KromaSystem 2000 1.60 (Bio-Tek Kontron Instruments, Milan, Italy). The flow-rate was set at 0.4 ml min^{-1}. The column was Hypercarb (5 μm), 100×2.1 mm I.D. (Hypersil, Runcorn, UK) and was thermostated at 50 °C with a Jetstream 2 temperature controller (Thermotecnics Products, Austria).

The chromatographic conditions and the retention model in NARP-LC with ODS were described elsewhere [10].

2.4. Data processing

The computer program for Student's *t*-test and the calculation of the total number of carbons from the adjusted retention time value was written in Pascal using Borland Delphi.

3. Results and discussion

The model envisaged for ceramide identification was based on chromatographic conditions which allowed a linear relationship between the adjusted retention time values and the total number of carbon atoms of ceramides. The advantage of this approach was that the representation of the model required a reduced number of data. As the separation of ceramides with the same polar head and containing the same total number of carbon atoms cannot be achieved, the contributions of the two alkyl chains were considered together by the total number of carbon atoms per molecule. Therefore a linear relationship per ceramide class was obtained which entirely described ceramide structures according to the non-polar and polar parts, respectively due to the two-alkyl chains and the polar head. In this study, a Structure-Retention Diagram (SRD) designed such modelling.

3.1. Establishment of the SRD

The first step for SRD development was the selection of chromatographic conditions. At this stage, the criteria were the best discrimination between ceramides associated with the shorter analysis time. From chemometrics results [14], the best condition in term of separation per time unit was obtained with binary gradient elution of chloroform–methanol. A gradient elution was necessary because of the large range of ceramide polarity.

In a second step, the gradient elution conditions were adjusted to reach a linear relationship between structure and retention of ceramides. For a homologous series analyzed with PGC, the logarithm of the methylene selectivity is assumed to vary linearly with the number of carbon atoms of homologues in isocratic elution [12,13] as follows:

$$\mathrm{Log}(k_{N_C}) = \log \beta + \log \alpha_{CH2} N_C \quad (1)$$

where can be described as:

$$\mathrm{Log}\ \alpha_{CH2}(\varphi) = \alpha_0 - \alpha_1 \varphi \quad (2a)$$

$$\mathrm{Log}\ \beta(\varphi) = \beta_0 - \beta_1 \varphi \quad (2b)$$

where φ is the volume fraction of strong solvent in the mobile phase.

From the retention of homologous series (fatty acid methyl esters) with isocratic mobile phase: chloroform–methanol (φ ranging between 0.4 and 0.9), log β and log α_{CH2} were calculated [22]:

$$\mathrm{Log}\ \alpha_{CH2}(\varphi) = 0.210 - 0.142\varphi \text{ with } r = -0.9988$$

$$\mathrm{Log}\ \beta(\varphi) = -2.228$$

The mean value of log $\beta(\varphi)$ obtained from each φ value was calculated because β_1 was found to be not significantly different from zero at 5% risk.

The ceramide series was not used for this evaluation because of the reduced number of homologues available in each series in order to accurately establish these equations.

Using these equations, the adjusted retention time of the solute with N_C carbon number eluted with a linear gradient of slope b was given by (see Appendix A):

$$tg_{(N_C,b)} = \frac{1}{2.3(\beta_1 + \alpha_1 N_C)b}(\ln(2.3(\beta_1 + \alpha_1 N_C)(t_0 k_{\varphi_0,N_C} - t_D) + 1) + 2.3(\beta_1 + \alpha_1 N_C)bt_D) \quad (3)$$

led to the plots in Fig. 1 for three gradient slopes $b = 1$, 2 and 4%/min and species from $N_C = 12$ to 30.

As expected from Eq. (3), the adjusted retention times of homologues did not increase linearly with carbon number but rather with a sigmoidal shape. At least, the curvature of the *tg* versus N_C curve tends to be less important when increasing the gradient slope. Fig. 2 shows the difference in adjusted retention times between two solutes differing by one methylene unit. Rapid gradients were preferred since discrepancy in differential retention is less marked. Therefore as demonstrated by the graph in Fig. 2, the retention interval between two successive homologues: methylene increment (i.e. the difference between adjusted retention time values of two consecutive homologues) is not constant during linear gradient elution but can be minimised.

In order to apply this approach for ceramides, important preliminary experiments were required for each ceramide class. Consequently, the selection of the gradient conditions was carried out by following the important points underlined by the theoretical development: (1) choose the slope of the gradient elution with the highest value compatible with the separation; and (2) elute the first compound after the effective start of the gradient. During previous SRD development, the same elements were empirically established [10]. The compromise between the separation and a high value of the gradient elution slope was obtained with the following conditions: chloroform–methanol from 45:55 to 85:15 with a slope at 2.7%/min. In this case, the mean value of the methylene increment was 0.82 with a relative standard deviation equal to 9% (Table 1).

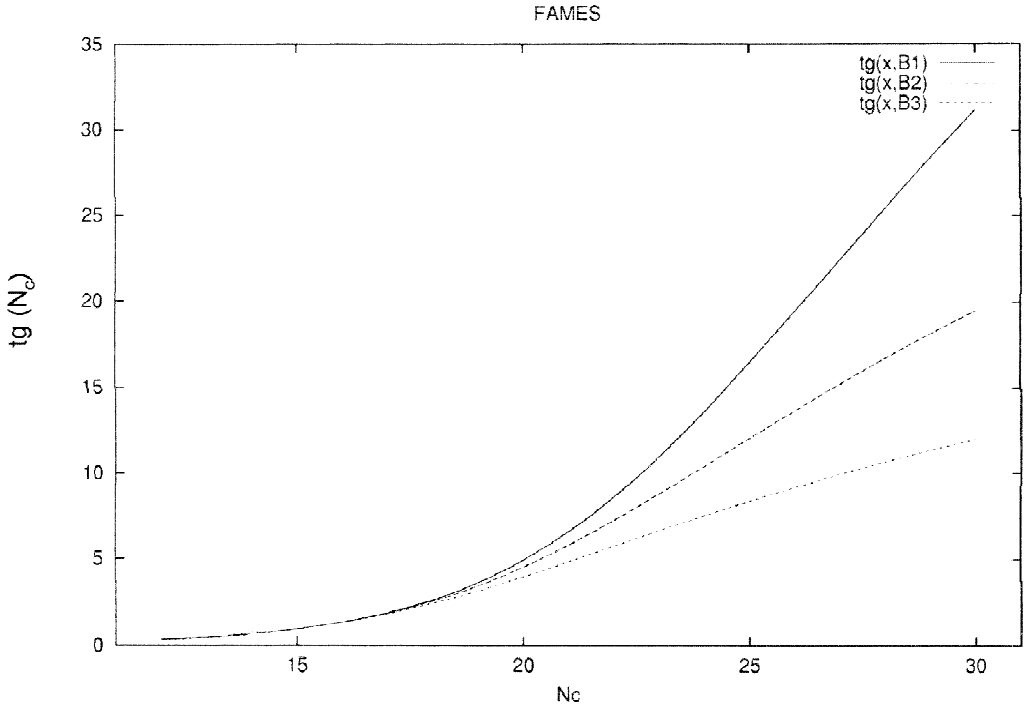

Fig. 1. Adjusted retention time calculated with Eq. (3) for species with methylene units ranging from $N_C = 12$ to 30 and three gradient slopes: $b = 1$%/min (solid line), 2%/min (dashed line), 4%/min (dotted line). Gradient starting at 40% chloroform in methanol.

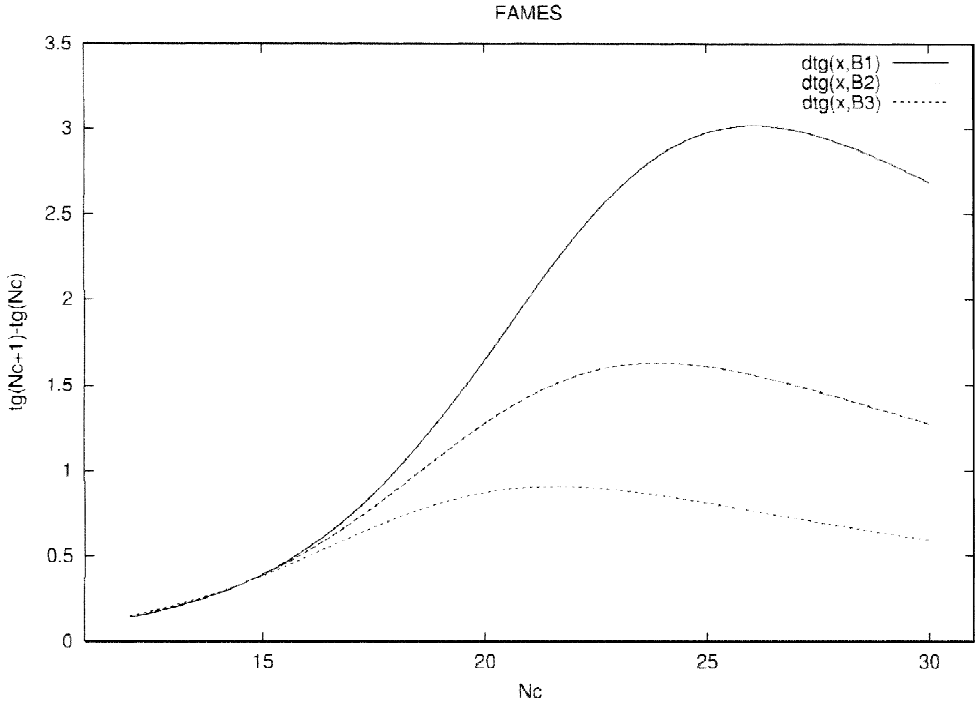

Fig. 2. Difference between calculated adjusted retention time of species with N_C+1, N_C methylene units for three gradient slopes $b=1\%/\text{min}$ (solid line), $2\%/\text{min}$ (dashed line), $4\%/\text{min}$ (dotted line). Gradient starting at 40% chloroform in methanol.

The SRD with this chromatographic system was achieved using ceramide standards: single compounds and complex mixtures (Type III and IV). The structures contained in each sample were rigorously identified in gas chromatography coupled with mass spectrometry (GC–MS) [7]. The identification of the chromatographic peaks in LC (Fig. 3) was achieved using these results and the calculation of the increment values (Δ) for each structural variation encountered in ceramide molecules (Table 1). Using all ceramide commercial samples, SRD was achieved containing five linear relationships (Fig. 4A and Table 2). The linear relationship plotted for dn:1 hn′:0 ceramides constitutes an interesting example of ceramide identification in the absence of standard since these structures were only available in one sample (ceramide Type IV).

3.2. Retention behaviour

Such application [23–25] of PGC with high molecular mass compounds and non-aqueous mobile phase remains scarce. Therefore the retention mechanism is not completely understood.

Table 1
Calculated values of increments from adjusted retention time of ceramide standards

Structural variations	Increment (min)	Standard deviation	Relative standard deviation (%)	Number of data
ΔCH_2	0.82	0.07	9	11
ΔC=C	−2.05	0.18	9	4
Δα-OH	−1.38	–	–	2
ΔS/P	−0.55	0.07	13.1	5
ΔD/S	0.67	–	–	1

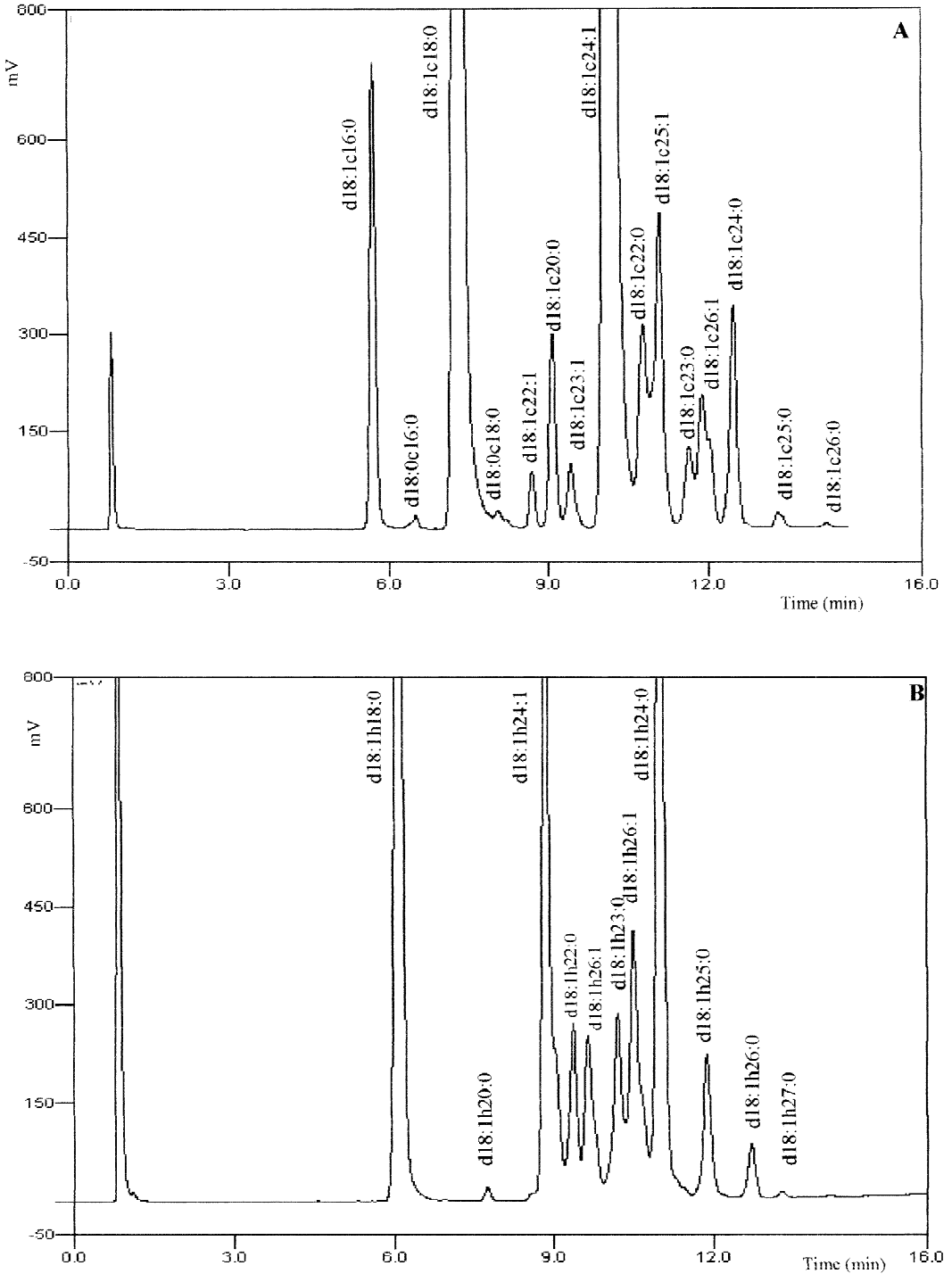

Fig. 3. Chromatograms of Type III (A) and Type IV (B), with PGC condition gradient elution.

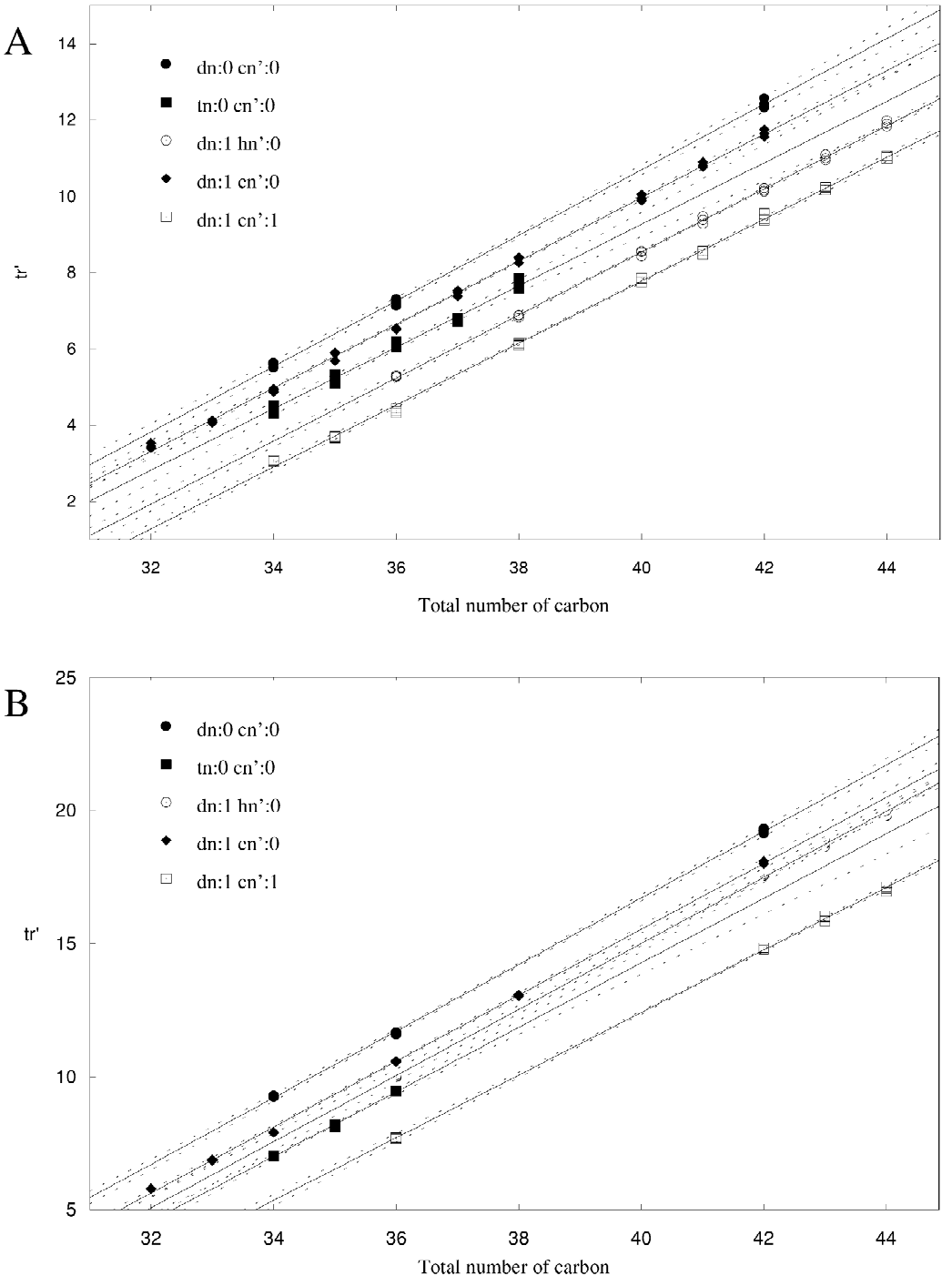

Fig. 4. A and B are, respectively, SRDs in conditions with PGC and ODS (for ODS–SRD data from Ref. [9]). ———, Linear relationship; - - -, Confidence interval.

Table 2
Equation of linear relationships of the SRD obtained from PGC system

Series	Slope	Intercept	R^2	Number of points
dn:0 cn′:0	0.8599	−23.7021	0.99914	9
dn:1 cn′:0	0.8322	−23.3231	0.99890	30
tn:0 cn′:0	0.8060	−22.9767	0.99138	15
dn:1 hn′:0	0.8266	−24.5231	0.99906	21
dn:1 cn′:1	0.8116	−24.6925	0.99892	27

With the PGC chromatographic conditions developed for the SRD, the greatest influence on the retention was due to the addition of one carbon double bond on the fatty acid alkyl chain that importantly decreases the retention (ΔC=C). Interaction between the carbon double bond and the surface of PGC can be expected due to its polarisable nature. However as an important retention decrease was observed, this phenomenon seemed to be widely compensated by the increase in solubility in the mobile phase provided by this functionality and/or the conformational modification. The addition of a *cis*-unsaturation modifies the flexibility of the molecule and decreases the contact surface of the whole molecule with the plane surface of the stationary phase. Such behaviour was already observed in various mobile phases with fatty acid methyl esters [23].

The addition of one α-hydroxyl group on the fatty acid chain also led to a decrease in the retention (Δα-OH) but less important than an unsaturation. The polarity of the ceramide polar head is increased which mainly increases the removal of this part of the molecule from the planar surface of PGC may be due to its increase in solubility in the mobile phase.

A ceramide possessing a phytosphingosine base was less retained than one with a sphingosine base with the same hydrocarbonaceous volume (ΔS/P). The structural difference consists, starting from the dihydrosphingosine structure, of a further hydroxyl in the case of phytosphingosine and a further unsaturation in the case of sphingosine. In this example, the contribution to the decrease in retention was found to be greater with the addition of the hydroxyl group. The further unsaturation was next to a hydroxyl group which modified its polarisability and its accessibility according to the graphite surface. However reverse order of retention was also observed using other solvents [14]. Therefore the relative solubility of each ceramide seems to be an important factor in modifying the retention.

With PGC, two types of solute–adsorbent interactions were reported: ''Polar Retention Effect of Graphite'' (PREG) and dispersive interactions [11]. In the few studies with non-aqueous mobile phase with PGC [14,23–25], the increase in the hydrocarbonated volume of the molecule always induced an increase in retention, whereas the contribution of the polar molecular part was versatile. In this study, the order of retention regarding the nature of the ceramide polar head was found similar to that obtained with the ODS chromatographic system [9]. Therefore no PREG phenomenon was underlined and the chromatographic retention behaviour was comparable with that obtained with ODS and non-aqueous reversed-phase chromatography.

The differences in terms of retention between both the PGC and ODS systems were illustrated by the differences in relative distance between the linear relationships of the SRDs due to the difference in selectivity.

3.3. Assessment of SRD

In order to assess this SRD for ceramide identification, the reliability of the chromatographic data and the precision of the linear relationships were studied and compared with another obtained from the ODS system [9].

The reproducibility of the chromatographic method was then first assessed. The chromatographic data were obtained from the measurement of three injections done on three different days with a thermostated column system. From this experimental approach, the relative standard deviations of the retention values were, respectively inferior at 3 and 1.5% for PGC and ODS. The ODS method led to less dispersed data. However more data were avail-

able for the SRD achievement of PGC system than ODS (respectively 20 and 34), due to the more discriminating chromatographic method in the case of PGC. Therefore this latter point compensated the smaller data precision of this method. The precision of the straight lines was assessed by the confidence intervals (Fig. 4). Near the experimental values, confidence intervals were narrow due to the precision of the chromatographic data in both the SRDs. In the area of extrapolation of the linear relationships, the confidence intervals became larger because of the absence of data.

The exactitude of the straight lines was discussed in regard to the sources of ambiguity which can occur when the identification was carried out using the SRDs. There were three types of ambiguity:

1. If the straight lines possess slopes significantly different, there exists a part of the SRD where the straight lines cross;
2. If the confidence intervals possess zone(s) of overlap;
3. If the adjusted retention time of a peak leads to different values of abscissa equal to integer values of carbon atom number.

The significance of the slope for each straight line was tested for both SRDs (Table 3). In the case of the PGC method, there was one straight line of dn:0 cn′:0 ceramides which presented a slope statistically different to all other straight lines of the SRD (Table 3). However in the domain of carbon number representative of reported cutaneous ceramides of total carbon number from 30 to 52 [4], the intersection between two lines should not occur. In the case of the SRD obtained with the ODS method, the linear relationship representing the dn:1 cn′:1 ceramides presented a slope statistically different to almost all other linear relationships. However, this line was far enough from other lines so as to expect no intersection in the retention domain of cutaneous ceramides.

In both the SRDs (Fig. 4), there were some cases of confidence interval overlap. However, the main reason was an important widening of the confidence intervals due to a lack of data covering the entire domain of structures instead of an important proximity of the lines. Therefore the accuracy will be improved each time new structures of ceramides are encountered and included in the SRD.

In both the SRDs, the third source of ambiguity seemed to occur. In the SRD from the PGC system,

Table 3
Comparison of the straight-line slopes of the both SRDs

Stationary phase	Straight line of ceramides	dn:1 cn′:1[a]	dn:1 hn′:0	tn:0 cn′:0	dn:0 cn′:0
ODS	dn:1 cn′:0	5.67 (2.05) 26[b] S[c]	0.20 (2.05) 26 NS[d]	1.17 (2.07) 23 NS	1.03 (2.07) 23 NS
	dn:1 cn′:1		4.91 (2.08) 20 S	1.67 (2.11) 17 NS	6.53 (2.11) 17 S
	dn:1 hn′:0			1.23 (2.11) 17 NS	0.67 (2.11) 17 NS
	tn:0 cn′:0				1.7 (2.14) 14 NS
PGC	dn:1 cn′:0	2.75 (2.00) 53 S	0.71 (2.01) 47 NS	1.22 (2.02) 41 NS	2.55 (2.03) 35 S
	dn:1 cn′:1		1.89 (2.01) 44 NS	0.26 (2.02) 38 NS	4.43 (2.04) 32 S
	dn:1 hn′:0			0.95 (2.04) 32 NS	2.99 (2.05) 26 S
	tn:0 cn′:0				2.35 (2.09) 20 S

[a] Abbreviations are described in the Experimental section.

[b] Detailed explanation of the three values: experimental value of the Student's *t*-test; (theoretical value of the Student's *t*-test); number of degree of freedom n_1+n_2-4.

[c] S, slopes significantly different.

[d] NS, slopes not significantly different.

the three straight lines tn:0 cn′:0, dn:1 hn′:0 and dn:1 cn′:1 can lead to three different structural propositions for a similar adjusted retention time. In the SRD from the ODS system that concerns the dn:0 hn′:0 and tn:0 cn′:0 lines, this latter type of ambiguity was underlined by testing the SRD as identification method.

3.4. Ceramide identification with the SRDs

The result of ceramide identification provided by these SRDs was assessed with two commercial mixtures: ceramide Type III and Type IV. These samples were selected because of their complexity and known compositions [7]. Ceramide Type III contained only one structure in trace amount (d18:1 h24:1) not belonging to any linear relationships of the SRDs, whereas in ceramide Type IV, a third of its structures was dn:1 hn′:1 absent in the SRDs. Therefore, this latter sample was a useful example for testing the external validity of the SRDs (i.e. the ability to assign or not a ceramide structure to the adjusted retention time of a compound unknown by the SRDs).

With the retention time obtained for each sample in both chromatographic systems, the corresponding values of the total number of carbon atoms were calculated with linear regression according to the confidence intervals. As the ceramide identification led to total number of carbon atoms of the molecule, the results of ceramide identification were provided with sphingoid base containing 18 carbon atoms which is a length often encountered in natural ceramides [4].

The identification of ceramide type III sample with the PGC–SRD (Table 4) led to seven identified peaks out of 15 observed peaks. For the other peaks, several structures were assigned per peak. Even if the chromatographic discrimination was not complete, the result of identification was coherent since for each peak at least one of the proposed structures corresponded effectively to a compound present in the sample according to the GC–MS analysis [7]. The two types of ambiguity predicted in the previous section appeared: (1) between dn:1 cn′:0 and tn:0 cn′:0 (source of ambiguity type 2); (2) between dn:1 cn′:1 and dn:1 hn′:0 (source of ambiguity type 3). The identification with the ODS–SRD (Table 4) led to five identified peaks out of 12 peaks observed on the chromatogram. The ratio of identified peaks out of the total peak number was slightly smaller than those obtained with the PGC–SRD mainly due to the less discriminating chromatographic method obtained using ODS.

The identification of ceramide type IV sample with the PGC–SRD (Table 5) led to three unidentified peaks and eight peaks associated with several structures. The ambiguities were between: (1) dn:1 hn′:0 and tn:0 cn′:0; and (2) dn:1 hn′:0 and dn:1 cn′:1. These ambiguities were related to the ambiguity of type 3. The peaks (3, 5 and 7) were unidentified and corresponded effectively to dn:1 hn′:1 ceramide structures, which was in accordance with the absence of a linear relationship representing this class. The identification with the ODS–SRD (Table 5) led to three unidentified peaks, two ambiguities and three identified peaks. Although this chromatographic system involved more co-elution, it had allowed the identification of three peaks against none with the PGC–SRD. This underlines that better discrimination between ceramide species was not a guarantee of greater identification efficiency.

These results illustrated the limit of the SRD for complete ceramide identification because of the chromatographic methods themselves which were not discriminating enough and/or the presence of sources of ambiguity of type 3 and 2. This latter limitation can be gradually avoided in this concept of evolutive model when new structures can be introduced into the SRD. However, the interesting element of this comparison was in both cases identifications were performed and were different. Therefore the assimilation of the results from these two SRDs can take advantage of this complementarity.

3.5. Identification by crossing the results from both SRDs

The peaks identified directly with the SRDs were accounted for by structures identified by crossing. Table 6 summarizes these results.

For ceramide type III, 15 structures were found, two of which were not present in this sample according to the GC–MS analysis: d18:1 h22:0 and t18:0 c26:0. t18:0 c26:0 can be eliminated because of the large confidence intervals of the tn:0 cn′:0

Table 4
Peak identification of ceramide Type III with the PGC and ODS SRDs

SRD	Peak	t_R' (min)	dn:1 cn':0	dn:1 cn':1	dn:1 hn':0	tn:0 cn':0	dn:0 cn':0
PGC	1	4.87±0.085	**d18:1 c16:0** [a] 33.87±0.004 [b]				
	2	5.65±0.090					**d18:0 c16:0** 34.14±0.009
	3	6.59±0.096	**d18:1 c18:0** 35.94±0.004				
	4	7.20±0.091					**d18:0 c18:0** 35.94±0.008
	5	7.84±0.105		**d18:1 c22:1** 40.08±0.005			
	6	8.24±0.100	**d18:1 c20:0** 37.92±0.004				
	7	8.57±0.105		**d18:1 c23:1** 40.98±0.005	d18:1 h22:0 40.04±0.005	←source of ambiguity (type 2)	
	8	9.43±0.108		**d18:1 c24:1** 42.04±0.005	d18:1 h23:0 41.08±0.005		
	9	9.96±0.086	**d18:1 c22:0** 39.99±0.004				
	10	10.25±0.097		**d18:1 c25:1** 43.06±0.005	d18:1 h24:0 42.07±0.005		
	11	10.82±0.074	**d18:1 c23:0** 41.02±0.004			t18:0 c24:0 41.95±0.025	←source of ambiguity (type 3)
	12	11.06±0.096		**d18:1 c26:1** 44.05±0.005	d18:1 h25:0 43.05±0.005		
	13	11.65±0.095	**d18:1 c24:0** 42.03±0.004			t18:0 c25:0 42.98±0.032	
	14	12.49±0.094	**d18:1 c25:0** 43.03±0.004			t18:0 c26:0 44.03±0.035	**d18:0 c24:0** 42.09±0.009
	15	13.36±0.106	**d18:1 c26:0** 44.08±0.005			t18:0 c27:0 45.04±0.039	d18:0 c24:0 43.10±0.013
ODS	1	8.06±0.032	**d18:1 c16:0** 33.96±0.005				d18:0 c15:0 33.07±0.005
	2	9.27±0.036		d18:1 c16:1 33.94±0.005			**d18:0 c16:0** 34.05±0.005
	3	10.66±0.031	**d18:1 c18:0** 36.06±0.005			t18:0 c19:0 37.02±0.020	
	4	11.89±0.064	**d18:1 c19:0** 37.05±0.005			t18:0 c20:0 38.03±0.04	
	5	12.24±0.060		**d18:1 c22:1** 39.87±0.004			
	6	13.29±0.061	**d18:1 c20:0** 38.17±0.005				
	7	13.49±0.061		**d18:1 c23:1** 40.92±0.004			
	8	14.96±0.064		**d18:1 c24:1** 42.15±0.006	d18:1 h22:0 39.94±0.007		
	9	15.97±0.104		**d18:1 c25:1** 43.04±0.006			
	10	17.07±0.110		**d18:1 c26:1** 43.96±0.006			
	11	18.07±0.156	**d18:1 c24:0** 42.05±0.007				d18:0 c23:0 41.09±0.011
	12	19.10±0.165				t18:0 c26:0 43.99±0.228	**d18:0 c24:0** 41.91±0.012

[a] Structures in bold were effectively present in the sample according to the GC–MS analysis.
[b] Total number of carbon atoms calculated from the linear regression and the confidence intervals.

Table 5
Peak identification of ceramide Type IV with the PGC and ODS SRDs

SRD	peak	t_R' (min)	dn:1 cn':0	dn:1 cn':1	dn:1 hn':0	tn:0 cn':0	dn:0 cn':0
PGC	1	5.29±0.032		d18:1 c19:1	**d18:1 h18:0** [a]	t18:0 c17:0	
				36.94±0.004	36.06±0.004 [b]	35.06±0.010	
	2	6.85±0.038			**d18:1 h20:0**	t18:0 c19:0	←source of
					37.95±0.003	37.01±0.011	ambiguity
	3	7.98±0.035					(type 3)
	4	8.48±0.056			**d18:1 h22:0**	t18:0 c21:0	
					39.93±0.004	39.04±0.015	
	5	8.74±0.070					
	6	9.33±0.055			**d18:1 h23:0**	t18:0 c22:0	
					40.95±0.004	40.09±0.018	
	7	9.61±0.045					
	8	10.16±0.038		d18:1 c25:1	**d18:1 h24:0**	←source of	
				42.95±0.004	41.979±0.005	ambiguity	
	9	11.00±0.056		d18:1 c26:1	**d18:1 h25:0**	(type 3)	
				43.10±0.005	42.98±0.005		
	10	11.82±0.070		d18:1 c27:1	**d18:1 h26:0**		
				45.01±0.005	43.99±0.005		
	11	12.49±0.150	d18:1 c25:0		**d18:1 h27:0**	t18:0 c26:0	
			43.05±0.006		44.90±0.006	44.04±0.050	
ODS	1	10.02±0.040		d18:1 c20:1	**d18:1 h18:0**		
				37.96±0.004	35.98±0.006		
	2	14.49±0.056					
	3	15.51±0.060	d18:1 c22:0			t18:0 c23:0	
			39.98±0.005			41.03±0.068	
	4	15.72±0.140					
	5	16.92±0.119					
	6	17.67±0.161			**d18:1 h24:0**		
					42.15±0.008		
	7	18.78±0.142			**d18:1 h25:0**		
					43.04±0.008		
	8	19.86±0.122			**d18:1 h26:0**		
					43.92±0.007		

[a]Structures in bold were effectively present in the sample according to the GC–MS analysis.
[b]Total number of carbon atoms calculated from the linear regression and the confidence intervals.

straight line around 44 total carbon atoms. The identification of d18:1 h22:0 remained which did not belong to this sample. That constituted an uncertainty of the result of about 5% (Table 7). Six compounds were unidentified because of lack of discrimination of the chromatographic method and their presence in weak amount: d18:1 c17:0, d18:1 c19:0, d18:1 c25:0, d18:1 c26:0, d18:0 c22:0, d18:1 c24:1.

For ceramide type IV, the four proposed structures (Table 6) were effectively present in this sample according to the GC–MS analysis. Sixteen structures were found in GC–MS analysis in this sample. In these 12 remaining structures, four belonged to dn:1 hn′:0 and one to dn:0 hn′:0 which were not represented by a linear relationship. Therefore, seven structures were unidentified principally because of

Table 6
Identification results by crossing the results from both SRDs

Sample	Ceramide class	Structures
TIII	dn:1 cn′:0	d18:1 c16:0, d18:1 c18:0, d18:1 c20:0, d18:1 c22:0, d18:1 c24:0
	dn:1 cn′:1	d18:1 c22:1, d18:1 c23:1, d18:1 c24:1, d18:1 c25:1, d18:1 c26:1
	dn:0 cn′:0	d18:0 c16:0, d18:0 c18:0, d18:0 c24:0
	dn:1 hn′:0	d18:1 h22:0
	tn:0 cn′:0	t18:0 c26:0
TIV	dn:1 hn′:0	d18:1 h18:0, d18:1 h24:0, d18:1 h25:0, d18:1 h26:0

co-elution and their presence in weak amounts. From this sample no inexact identification was provided even the presence of peaks which cannot be identified because of the absence of the corresponding linear relationship.

Crossing the data had allowed to propose a correct identity for 18 peaks out of 26 and only one error. This approach has allowed overcoming the source of ambiguity type 3. Another solution would be to introduce a further criterion during the method optimisation to hinder this source of ambiguity. However, this latter required that the chromatographic method should be re-optimised if this source of ambiguity occurred again. Crossing the result presented the advantage to keep the iterative feature of the model by adding a new straight line if new ceramide structures were encountered.

4. Conclusion

In the present study, the two proposed methods were developed in liquid chromatography with two distinct types of hydrophobic stationary phases: octadecyl grafted silica and porous graphitic carbon. Ceramide separations based on their hydrocarbonaceous volume were thus achieved using two distinct retention mechanisms. Rapid and simple analyses of ceramide samples were achieved which constituted a method applicable in routine analysis and compatible with hyphenated techniques such as mass spectrometry.

The analysis of naturally occurring ceramides is an analytical challenge that cannot be resolved by a unique technique. Our previous studies focused on comparing different separation techniques such as liquid, subcritical and gas chromatography. Despite intrinsic advantages, none had sufficient discriminating power to ensure a total and unambiguous identification of molecular species. The data treatment as double structure–retention diagrams (SRD) constituted an identification tool for ceramides in the absence of standard molecule. In addition to its straightforward nature, the iteratively improved SRD

Table 7
Summary of the identification results

		Ceramide type III	Ceramide type IV
A	Total number of structures	20	16
B	Structures not represented in the SRDs	1	5
C=A−B	Structures represented by SRDs	19	11
D	Identification from each SRD	7/5[a]	0/3[a]
E	Number of identifications after crossing	14	4
F	Structures in trace amount	6	7
G	Unidentified traces	4	6
H	Error of identification	1	0
(E/C)×100	% identified	74	36
(H/C)×100	% error	5	0

[a] PGC–ODS.

required no further method optimisation in the case of new ceramide structures. This latter point allows our model to be capable of analysing any natural source of ceramides.

Appendix A

For a homologous series, the retention factor of species with N_C methylene units is given by:

$$\log(k_{N_C}) = \log \beta + \log \alpha_{CH2} N_C \quad \text{(A.1a)}$$

where β and α_{CH2} (methylene selectivity) describe, respectively the specific and the non-specific contribution to retention.

With linear gradient elution, solutes of homologous series would elute with more or less constant intervals depending on their number of methylene units. The theoretical reasons are developed hereafter.

In linear gradient elution, the adjusted retention time of a solute can be expressed as [26]:

$$\int_0^{tg} \frac{dt}{k(\varphi)} = t_0 \quad \text{(A.1b)}$$

For a linear gradient, the dependence of the strong solvent with time is given by:

$$\varphi(t) = \varphi_0 + b(t - t_D) \quad \text{(A.1c)}$$

where φ_0 is the volume fraction of strong solvent in the gradient starting mobile phase, b the gradient slope and t_D the gradient delay time.

As proposed by Jandera [27], the decrease in log α_{CH2} value with increasing strong solvent in the mobile phase can be accounted by a quadratic relationship, or linear when a restricted range of φ values was used. A similar model can be proposed to account for variations of log β.

Thus, if we restrict to a linear variation:

$$\text{Log } \alpha_{CH2}(\varphi) = \alpha_0 - \alpha_1 \varphi \quad \text{(A.2a)}$$

$$\text{Log } \beta(\varphi) = \beta_0 - \beta_1 \varphi \quad \text{(A.2b)}$$

Splitting Eq. (A.1b) into two terms accounting for an isocratic elution during the gradient delay time (t_D) before the effective start of the solvent program yields:

$$\int_0^{t_D} \frac{dt}{k(\varphi)} + \int_{t_D}^{tg} \frac{dt}{k(\varphi)} = t_0 \quad \text{(A.2c)}$$

By combining Eqs. (A.1a), (A.1b), (A.2a) and (A.2b), $k(\varphi)$ can be expressed as:

$$k(\varphi, N_C) = 10^{\beta_0 - \beta_1\varphi_0 - \beta_1 b(t-t_D) + (\alpha_0 - \alpha_1\varphi_0 - \alpha_1 b(t-t_D))N_C}$$
$$= k_{\varphi_0, N_C} 10^{-b(\beta_1 + \alpha_1 N_C)(t-t_D)} \quad \text{(A.2d)}$$

$k\varphi_{0,N_C}$ corresponds to the retention factor of the solute with N_C carbon number eluted with the gradient starting mobile phase. Substituting Eq. (A.2d) in Eq. (A.2c) gives:

$$\frac{t_D}{k_{\varphi_0, N_C}} + \frac{1}{k_{\varphi_0, N_C}} \int_{t_D}^{tg} 10^{b(\beta_1 + \alpha_1 N_C)(t-t_D)} = t_0 \quad \text{(A.2e)}$$

After integration of Eq. (A.2e), the adjusted retention time of the solute with N_C carbon number eluted with a linear gradient of slope b is given by:

$$tg_{(N_C, b)} = \frac{1}{2.3(\beta_1 + \alpha_1 N_C)b}(\ln(2.3(\beta_1 + \alpha_1 N_C)(t_0 k_{\varphi_0, N_C} - t_D) + 1) + 2.3(\beta_1 + \alpha_1 N_C) b t_D) \quad \text{(A.3)}$$

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