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Retention behaviour of ceramides in sub-critical fluid chromatography in comparison with non-aqueous reversed-phase liquid chromatography

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Abstract

This study was devoted to the development of an analytical method for ceramide analysis in packed subcritical fluid chromatography (pSubFC). Monofunctional grafted silica support was found to be more suitable for ceramide analysis. Five Kromasil columns were coupled and the parameters, temperature, pressure and percentage of organic modifier in CO_2 were optimised, considering selectivity and analysis time. The final conditions were 31°C, 6% of methanol (MeOH) and 13 MPa. In these conditions the selectivity for structural differences (methylene group, unsaturation or two different bases) were studied. As classically observed, the methylene selectivity decreased with the increase of the eluotropic strength. Moreover, unlike in non-aqueous reversed-phase liquid chromatography (NARP–LC), adding a further unsaturation and two further methylene groups on ceramide results to an increase of retention in pSubFC. Moreover, this last technique allowed to separate ceramides with the same total number of carbons containing unsaturated fatty acids, when the distribution of carbon number of the two chain is very different. These results had enabled to plot retention chart in order to predict ceramide structure in view to identify additional ceramide. This retention chart was finally compared with the one already obtained in NARP–LC.

Keywords: Subcritical fluid chromatography; Retention behaviour; Retention chart; Ceramides

1. Introduction

The analysis of cutaneous ceramides is of particular interest, because ceramides are the major class of lipids in the stratum corneum involved in the lipid matrix which surrounds the corneocytes and therefore provides the epidermal barrier [1–3]. The epidermis contains potentially a thousand of various ceramide structures. This magnitude of various structures comes from two origins of structural differences [4,5]: the variability of the sphingoid base (Fig. 1) and the functionalities in the fatty acid (saturated, unsaturated or α-hydroxyl) which occur together with the variability of the chain length of the fatty acid and the base moieties.

The few similar chemical structures commercially available led us to consider an original approach for identification of ceramides: establishment of a retention chart which represents the relationship between the retention and the structure of ceramides.

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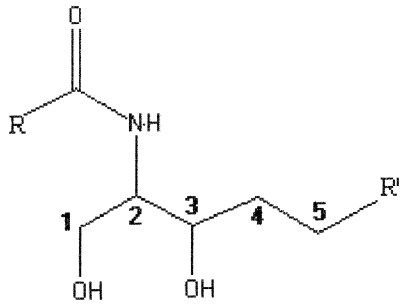

Fig. 1. General structures of ceramides. R and R′ are respectively fatty acid and base chain length. The represented structure corresponds to ceramide with a Dihydrosphingosine base (C_n:x$D_{n'}$). An OH on position 4: structure of ceramides with Phytosphingosine base (C_n:x$P_{n'}$). A double bond between carbons 4 and 5: structure of ceramides with the Sphingosine base (C_n:x$S_{n'}$). n=number of carbons of the fatty acid chain length. n'=number of carbons of the base chain length. x=number of unsaturation.

This strategy has the advantage to allow the identification of a ceramide structure in spite of the absence of a standard. And furthermore this retention chart can be upgraded as soon as new ceramide structures will be encountered.

Ceramides are hydrophobic compounds. Therefore, high-temperature gas chromatography (GC) [6,7] and non-aqueous reversed-phase liquid chromatography (NARP–LC) [8] can be used to develop analytical methods. An alternative is packed subcritical fluid chromatography (pSubFC) [9]. Compared with NARP–LC, pSubFC often improves separations. It has been successfully applied for carotenoid pigments [10–12], triglycerides [9,13–18] and waxes [19]. Relevant properties of SubFC are the low viscosity which allows high flow-rates and column coupling [20], and the high eluotropic strength which favours the high-molecular-weight compound solubilisation [21]. Consequently, a high efficiency is often reached in a short analysis time. Moreover, when organic modifier such as acetonitrile (ACN) or methanol (MeOH) are added, different selectivity changes may occur [18,22–24]. Finally as retention order may be different compared with NARP–LC or GC, additional compounds can be separated by SubFC [9,25].

The goal of this study was to evaluate the potential of pSubFC in discriminating ceramides, to select the chromatographic parameters by studying the retention behaviour of ceramides and, finally, to plot a chart of ceramide retention versus their structure and compare this retention behaviour with this observed in NARP–LC.

2. Experimental

2.1. Apparatus

Chromatographic separations were carried out using equipment manufactured by Jasco (Tokyo, Japan). Two Model 880-PU pumps were used, one for the carbon dioxide and the second for the modifier. The pump head used for pumping the carbon dioxide was cooled to −2°C by a cryostat (Julabo F10c, Seelbach, Germany, supplied by Touzart et Matignon, Les Ulis, France). After mixing the two solvents (modifier and CO_2), the fluid was introduced in a dynamic mixing chamber PU 4046 (Pye Unicam, Cambridge, UK), connected to a pulsation damper SEDERE (supplied by Touzart et Matignon).The injection valve was fitted with a 20 μl loop (Model 7125 Rheodyne, Cotati, CA, USA). The column was thermostated by using an oven (Crocosil, Cluzeau, Sainte Foy-la-Grande, France), regulated at 25°C by a cryostat (Haake D8 GH, Karsruhe, Germany). The outlet column pressure was controlled by a regulator Jasco 880-81 (Tokyo, Japan). The outlet regulator tube (internal diameter 0.25 mm) was heated to 80°C to avoid the ice formation during the CO_2 depressurisation.

Detection was carried out with a light scattering evaporative detector DDL 21 (Eurosep, Cergy-Pontoise, France). Since this detector was set up after the pressure regulator, no apparatus modification was required comparing to the liquid chromatography. The nebulisation gas was air, the nebulisation pressure 0.2 MPa and the nebulisation temperature 35°C.

Chromatograms were recorded using a Model CR 6A electronic integrator (Shimadzu, Kyoto, Japan).

2.2. Reagents

The solvents were HPLC-grade: MeOH (Carlo Erba, Milan, Italy) and ACN (SDS, Vitry sur seine, France). Carbon dioxide (N 45 grade, containing less

than 7 ppm of water) was purchased from Alphagaz (Bois d'Arcy, France).

2.3. Columns

The chromatographic columns were Octadecyl bonded silica (ODS) (250×4.6 mm i.d., 5 μm) except the Supelcosil which was an amido-propyl hexadecyl bonded silica: Kromasil UB 225 (Eka Nobel, Bohus, Sweden), Hypersil ODS (Hypersil, Sewickley PA, USA), Nucleosil 5 C18 AB (Macherey-Nagel, Düren, Germany), Vydac 201 TP 54 (The separation group, Hesperia, USA), Supelcosil Discovery RP amide 16 (Supelco, Bellafonte, USA).

2.4. Chemicals

Ceramide Type III, ceramide Type IV, *N*-Palmitoyl-D-sphingosine (C_{16}:0S), *N*-Stearoyl-D-sphingosine (C_{18}:0S), *N*-Palmitoyl-DL-dihydrosphingosine (C_{16}:0D), *N*-Oleoyl-D-sphingosine (C_{18}:1S), *N*-lignoceroyl-DL-dihydrosphingosine (C_{24}:0D), *N*-Nervonoyl-D-sphingosine (C_{24}:1S) were all purchased from Sigma (St. Quentin Fallavier, France). Ceramide III (C_{18}:0P_{18}) and ceramide IIIB (C_{18}:1P_{18}) were a generous gift of Cosmoferm (Delft, Netherlands). Ceramide Type III was prepared at 0.5 mg ml^{-1}. All ceramides structures in the commercial samples were identified by GC–MS [26].

3. Results and discussion

3.1. Selection of the stationary phase and the modifier for ceramide analysis in pSubFC

The high molecular weight of ceramides lead to envisage the initial choice of the temperature and pressure conditions close to those selected for carotenoids or triglycerides [9,11]. Therefore, the addition of modifier was essential to improve the solubility of these compounds and obtain symmetrical peaks from of the possible increase of the eluotropic strength of the mobile phase [21]. Among the two polar modifiers (MeOH and ACN) commonly used in pSubFC [9,11], MeOH was chosen with regard to the results obtained in NARP for ceramide solubility [27]. When 5% of MeOH was added to the CO_2, the retention times of ceramides were greatly reduced whereas with pure CO_2 the elution of ceramides did not occurred.

The temperature was set up at 25°C in order to keep subcritical conditions and to prevent thermal degradation of solutes. The outlet pressure was set up at 10 MPa.

3.1.1. Nature of the column

The apolar nature of ceramides led us to select bonded silica for their analysis. Five columns which present very different kind of bonded stationary phases, were tested for ceramide separation with a commercial mixture of ceramides (Type III, Sigma). All these columns are of the same dimension and particle sizes. Kromasil and Hypersil are monofunctional ODS with different bonded density respectively high (3.4 μmol m^{-2}) and medium (2.5 μmol m^{-2}). Nucleosil and Vydac columns are polyfunctional ODS, respectively with and without additional encapping treatment. Supelcosil Discovery column is a shielded amido-propyl hexadecyl bonded silica.

Table 1 reports the number of peaks obtained for ceramide Type III with one column of each type. In the previous analytical conditions, the two polyfunctional and the shielded bonded silicas failed to separate ceramides. However, the two monofunctional supports had enabled the separation of several ceramides. Moreover Kromasil led to higher retention factors and methylene selectivity than Hypersil due to its higher bonded density, that may explain the higher number of peaks observed with this stationary phase. Therefore, Kromasil was the selected stationary phase for ceramide separation.

3.1.2. Number of column

In these standard conditions with one column, the obtained separation in SubFC was already equivalent

Table 1
Number of peak obtained for ceramide Type III according to the nature of the stationary phase

Nature of the column	Number of peak
Hypersil	9
Kromasil	12
Nucleosil	2
Vydac	1
Supelcosil	1

Table 2
Number of peak of ceramide Type III according to the number of column

Number of column	Number of peak	Inlet pressure (MPa)	Analysis time (min)
1	12	145	11
2	13	172	17
4	18	232	30
5	19	260	40
7	19	330	49

at whose obtained after the optimisation of the chromatographic conditions in NARP–LC [8] (12 peaks), with a time analysis twice shorter. That emphasises the great power of SubFC technique (high flow-rate and efficiency) to separate hydrophobic molecules.

Moreover the low viscosity of sub-critical fluid allows coupling several columns. Standard conditions (10 MPa, 25°C, 5% MeOH) were used to test the influence of the number of column in regard to the number of peak obtained for sample ceramide Type III.

As seen from Table 2, increasing the number of columns from one to five, clearly improved the separation. Beyond five columns, for the use of seven, an important increase of analysis time and inlet pressure were obtained without providing an higher number of peaks. The retention factors and the selectivity decreased when the number of column increased (Fig. 2). Increasing the number of column increased the internal pressure in the further column which induces an increase of fluid density and thus increases its eluotropic strength. This phenomenon is counter balanced by an increase of the chromatographic efficiency due to the increase of the column number. Five columns were kept for the study as the maximum of peaks were obtained in this condition (Fig. 3A).

3.1.3. Nature of the organic modifier

The quality of the separation can be modified by the nature of interactions induced by the modifiers. Polar modifiers MeOH and ACN provide respectively two different kinds of interactions: hydrogen bonding and dipole–dipole, with the functionalities of the polar head of the ceramides. As expected [27], ACN was a very weak solvent which cannot lead to ceramide elution. Therefore, the influence of ACN was assessed by using various ratio of ACN/MeOH (see Table 3). In this assessment, the total amount of modifier added into CO_2 was increased to 10% in order to emphasise the effect of ACN. Furthermore, the temperature was increased to 30°C in order to

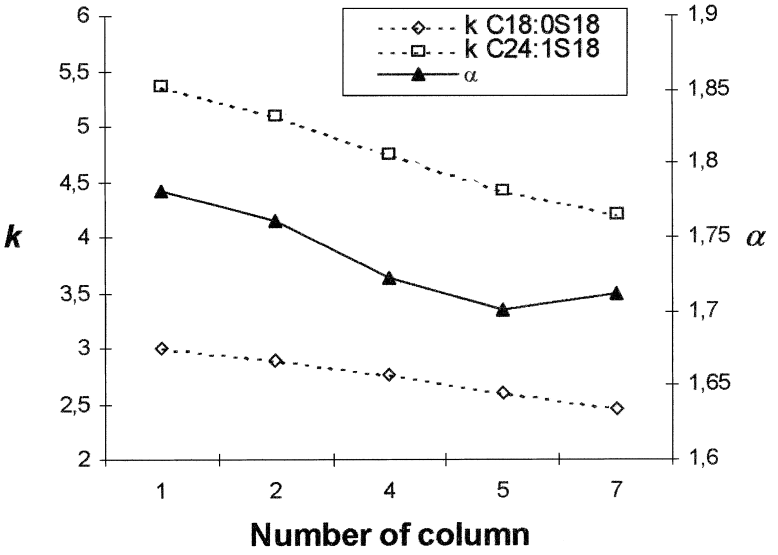

Fig. 2. Influence of the number of Kromasil column on retention factors (k) and selectivity. Retention factors of the two major compounds (k C_{18}:$0S_{18}$ and k C_{24}:$1S_{18}$) of ceramide Type III and selectivity (α) between them. 10 MPa, 25°C, 5% MeOH in CO_2.

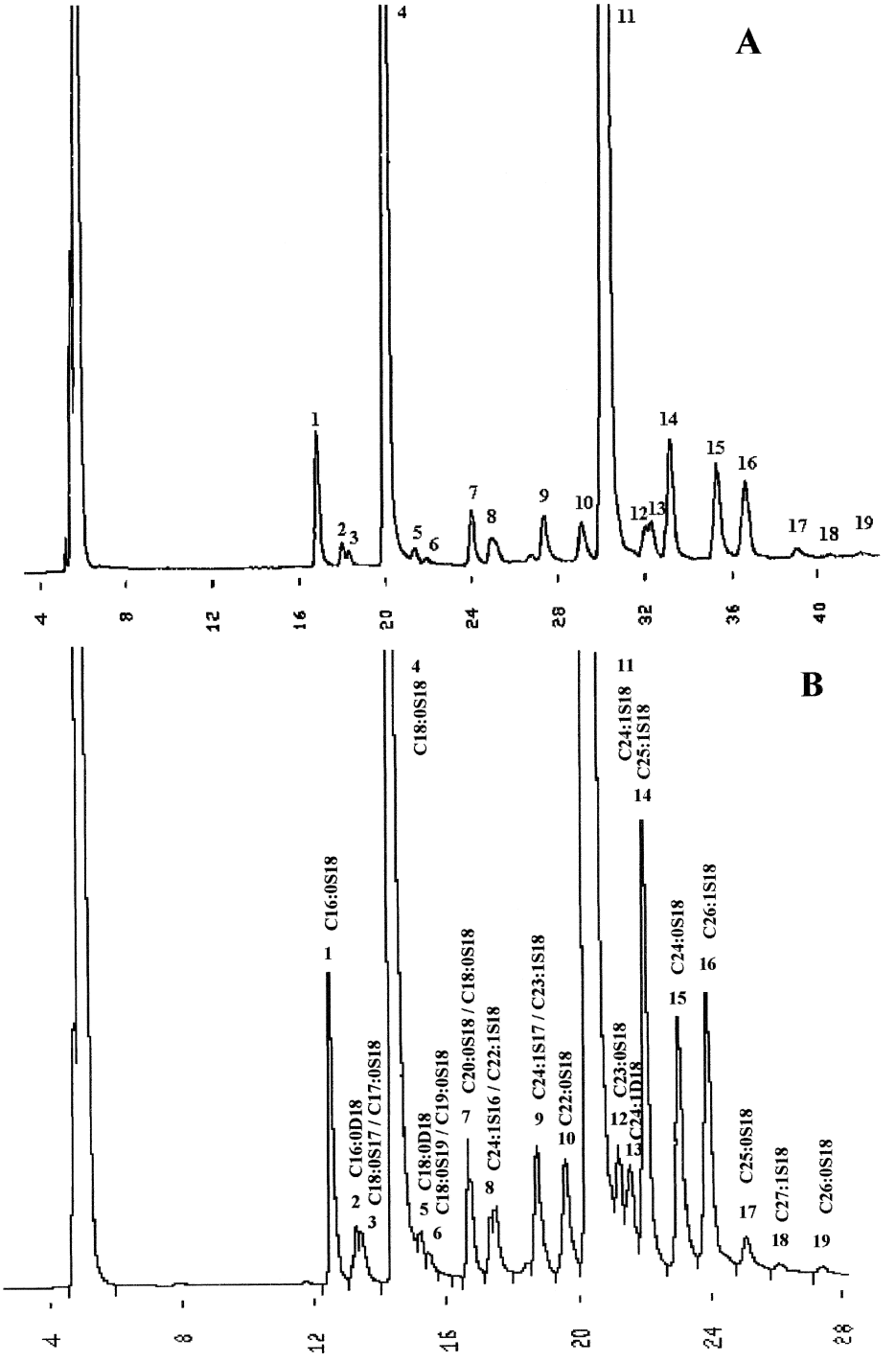

Fig. 3. Ceramide Type III (A) 25°C, 5% MeOH, 10 MPa, 3 ml·min^{-1}. (B) 31°C, 6% MeOH, 13 MPa, 3.2 ml·min^{-1}.

Table 3
Influence of the modifier nature: 10% of modifier, 10 MPa, 30°C

MeOH–ACN	Number of peak	Analysis time (min)	α (C=C)	α (CH_2)
100-0	18	21.5	1.176	0.620
90-10	18	22.5	1.182	0.622
70-30	15	22.4	1.187	0.615
50-50	14	23.6	1.200	0.622
20-80	10	27.8	1.232	0.627

Table 4
Identification of the studied selectivities[a]

Selectivity	Number of peak	Corresponding structures
α_1	11/12	C_{24}:$1S_{18}$/C_{23}:$0S_{18}$
α_2	12/13	C_{23}:$0S_{18}$/C_{24}:$1D_{18}$
α_3	13/14	C_{24}:$1D_{18}$/C_{25}:$1S_{18}$
α_4	14/15	C_{25}:$1S_{18}$/C_{24}:$0S_{18}$
α_5	15/16	C_{24}:$0S_{18}$/C_{26}:$1S_{18}$

[a] Peak numbers correspond to the numbers in Fig. 3

keep ceramides soluble in the mobile phases, because ACN reduced the ceramide solubility.

Under a percentage of 50% of ACN in modifier the chromatographic system seemed to be isoeluotropic, since methylene selectivity was not significantly altered. Beyond 50% of ACN content in modifier, the eluotropic strength of the fluid decreased which led to a decrease of the chromatographic efficiency (peak broadening) due to the decrease of the solubility of ceramides in ACN. This influence was also reported for other classes of compounds [9,11,21].

However, the selectivity between two ceramides which differ by an unsaturation increased when the percentage of ACN in MeOH increased. The retention of saturated ceramides increased faster than unsaturated relatively to the increase of ACN percentage. The first consequence of this relative retention variation was underlined by an increase of the separation of ceramides which differ by an unsaturation (e.g. C_{22}:$1S_{18}$/C_{22}:$0S_{18}$). Unfortunately, a second consequence was a loss of separation between ceramides differing by an unsaturation and two methylene groups (e.g. C_{22}:$0S_{18}$/C_{24}:$1S_{18}$) because of their close retention times. Therefore, ACN was no further tested as modifier.

3.2. Optimisation of the chromatographic parameters

To select the chromatographic conditions in SubFC, the first criterion was to obtained a value of the adjusted retention time (t'_R) of the compound C_{25}:$0S_{18}$ (peak 17 on chromatogram Fig. 3A), equal to 19 min. This value of t'_R was obtained for this compound in our separation in NARP–LC [8]. The other criteria were the greatest peak number and the best selectivity between the poorest resolved pairs of peaks.

Table 4 summarises the pairs of peaks which appeared critical during the study of the number of columns and the nature of the modifier. Selectivities were assessed for each chromatographic parameters: temperature, pressure and percentage of organic.

First, the selectivity was studied in regard to the temperature. In Fig. 4A, α_2 of the poorest resolved pair of peak increased when the temperature was increased whereas the other selectivities remained higher than α_2. Therefore, the highest tested temperature of 31°C was selected to improve α_2 and to keep the fluid in subcritical condition.

Second, the percentage of MeOH was studied at 31°C and 10 MPa. Percentages were tested up to 20% of MeOH in supercritical carbon dioxide. At higher percentages, high pressure values were obtained which prevented to work with five columns. In the tested percentage range, the retention factors decreased when the percentages of MeOH increased. This behaviour is generally observed with the addition of this polar modifier [9,11,21]. α_2 decreased to one very quickly since 15% of MeOH was used (Fig. 4B). Consequently small percentage of MeOH should be used to stay at the best α_2 value. However, under 5% of modifier, the detection response decreased because of a decrease of ceramide solubility and an increase of the background noise. Therefore, a minimum of 6% of MeOH was then used.

Finally, the influence of the pressure was assessed from 8 to 15 MPa at 31°C and 6% of MeOH (Fig. 4C). Increasing the pressure reduced α_2. This decrease was more pronounced between 13 and 15

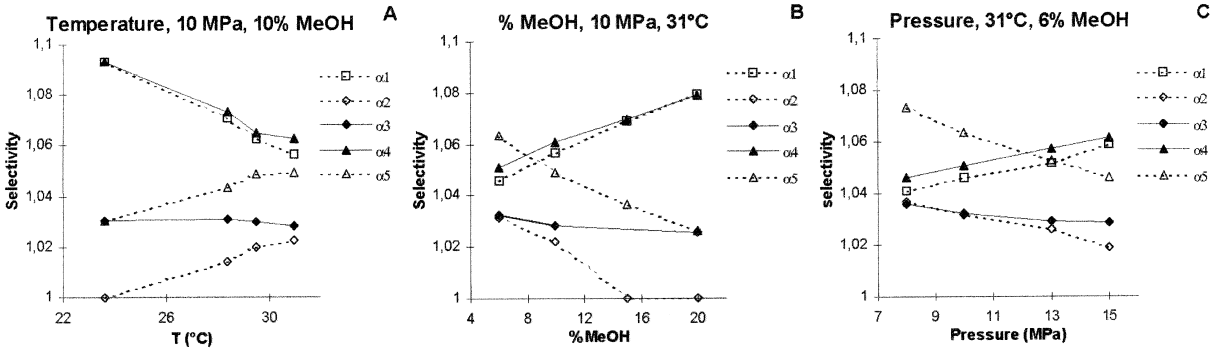

Fig. 4. Influence of the temperature (A), the percentage of modifier (B) and the pressure (C) on the selectivity of the critical pair of peaks. See Table 4 for details about selectivities.

MPa. However, in the studied pressure range, the values of selectivities remained higher than one. Consequently this parameter was mainly used to modulate the retention in order to establish the t'_R equal to 19 min for peak 17. The pressure was set at 13 MPa which represents the more reasonable compromise between the selectivity and the target t'_R. However, the flow-rate was increased from 3 to 3.2 ml. min^{-1} to reach this t'_R.

The final conditions for the analysis of ceramides in SubFC were 31°C, 6% of MeOH and 13 MPa (see chromatogram B in Fig. 3). The obtained chromatogram contained nineteen peaks with an analysis time about 28 min in isocratic conditions. The optimisation of the parameters had enabled to increase the separation of the compounds 12 and 13 and to reduce the analysis time.

From the graphs in Fig. 4, the selectivities α_1 and α_4 exhibited the same behaviour in regard to the modifications of three chromatographic parameters: temperature, percentage of MeOH and pressure. At this stage, the identification of the peaks was performed for the final conditions, and showed that these two selected pairs of peaks corresponded to the same structural difference: the addition of a further unsaturation and one methylene group. Unlike these selectivities, the selectivity α_5 followed an opposite behaviour. This selectivity corresponds to the structural difference of the addition of a further unsaturation and two methylene groups. As in this case, the order of elution between the unsaturated and the saturated ceramide was inverted, the selectivity calculation resulted of describing the reverse behaviour.

3.3. Retention behaviour

The influence of temperature, pressure and percentage of MeOH were studied with three different selectivities (Fig. 5A, B and C respectively for each chromatographic parameters). The couple of ceramides: C_{16}:0S/C_{18}:0S provided the methylene selectivity. The couple of ceramides: C_{24}:1S/C_{24}:0S provided the selectivity between ceramides which differ by the presence of an unsaturation on the fatty acid. And the couple of ceramides: C_{24}:0S/C_{24}:0D provided the selectivity between ceramides which differ by the nature of the sphingoid base (sphingosine and dihydrosphingosine).

Increasing temperature, percentage of MeOH and pressure, decreased the methylene selectivity (C_{16}:0S/C_{18}:0S). This decrease of methylene selectivity was due to the rise of the eluotropic strength of the mobile phase when the three parameters increased. This behaviour was clearly established in SubFC [21].

Increasing all these parameters decreased the C_{24}:0S/C_{24}:0D selectivity. The structural difference is the presence of an unsaturation on the sphingosine base next to the second hydroxyl group. This selectivity behaviour was similar to methylene selectivity (C_{16}:0S/C_{18}:0S). Thus further unsaturation modified the polarity of the ceramide polar head, but does not seemed to be directly involved in specific interaction.

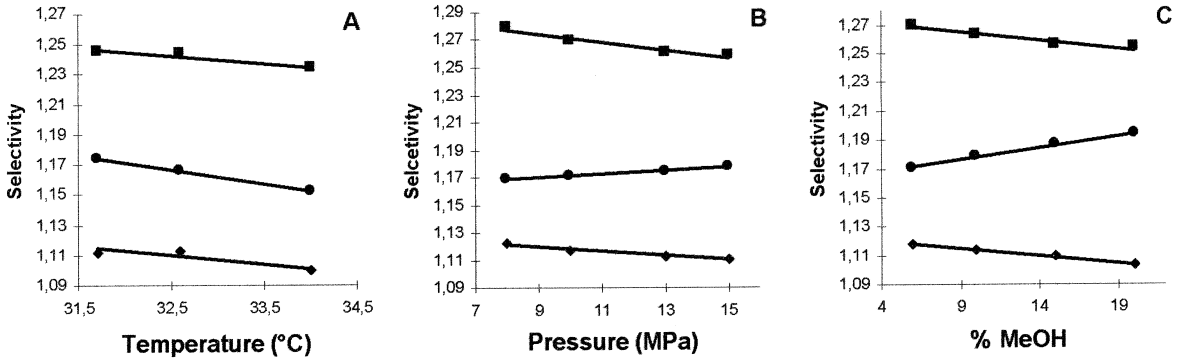

Fig. 5. (A) Influence of the temperature on selectivity at 15 MPa and 5%MeOH. (B) Influence of pressure on selectivity at 6% MeOH and 31°C. (C) Influence of percentage of MeOH on selectivity at 10 MPa and 31°C. ■ represents the methylene selectivity (C_{16}:0S_{18}/C_{18}:0S_{18}); ♦ the selectivity between ceramides with sphingosine and dihydrosphingosine (C_{24}:0S_{18}/C_{24}:0D_{18}); ● the selectivity between ceramides which differ by an unsaturation (C_{24}:1S_{18}/C_{24}:0S_{18}).

From the structural difference due to the addition of an unsaturation on the fatty acid chain length, the chromatographic parameters acted differently. Whereas, increase of temperature decreased the selectivity (C_{24}:1S/C_{24}:0S), increasing MeOH percentage or pressure increased it.

These two previous results underlines that the position of the unsaturation either on the ceramide polar head or on the fatty acid chain, plays an important role on the retention.

The main difference in ceramide retention between pSubFC and NARP–LC is the elution order of ceramides which differ by the presence of an unsaturation on the fatty acid chain length. In NARP–LC, ceramides with same amine base led to the following order of elution: first C_{n+2}:1$S_{n'}$ and then C_n:0$S_{n'}$ (e.g. α C_{24}:1S_{18}/C_{22}:0S_{18}) whereas in SubFC: first C_n:0$S_{n'}$ and after C_{n+2}:1$S_{n'}$ (e.g. α C_{22}:0S_{18}/C_{24}:1S_{18}). This difference of behaviour has been already observed comparing the C_{16}:0 and the C_{18}:1 chains of triglycerides [9,23]. In ceramide Type III sample, this inversion of retention had allowed to obtain the separation of C_{22}:0S_{18} with C_{24}:1S_{18} which was not obtained in NARP–LC because C_{22}:0S_{18} was eluted in the peak tail of a major peak (C_{24}:1S_{18}) [8].

3.4. Modelisation of the relationship retention–structure

Retention data of ceramides in SubFC were associated to their structures under the form of a chart. The interest of this correlation was to obtain a model of the relationship retention–structure of ceramide in order to allow their identification. We have already developed this approach in NARP–LC on ODS with gradient elution [8]. In this case, the retention data were t'_R and the structural differences were expressed in equivalent fatty acid chain length where the number of carbon on the fatty acid and base moiety were considered independently. However, using this retention chart for the identification of structures contained in a commercial mixture (ceramides Type III, Sigma), showed the same assignment for ceramides containing the same number of methylene unit. The presented retention charts herein are in total number of carbons of the two alkyl chains (Fig. 6). Thereby, the number of straight lines decreased which were plotted with more points. As our SubFC method is isocratic, the retention data used for the modelisation were the logarithm of retention factor (ln k).

In order to plot retention chart, increments (Δ) were calculated for the main structural differences. Table 5 reports the results of increment calculation. The increments combined with structural differences determined by GC–MS [26] had enabled us to assign a structure for each peak of the samples. Thus, with ceramides Type III and IV mixtures, further structures can be used to refine the retention chart. (see structure attribution of the ceramide Type III peaks in Fig. 3B).

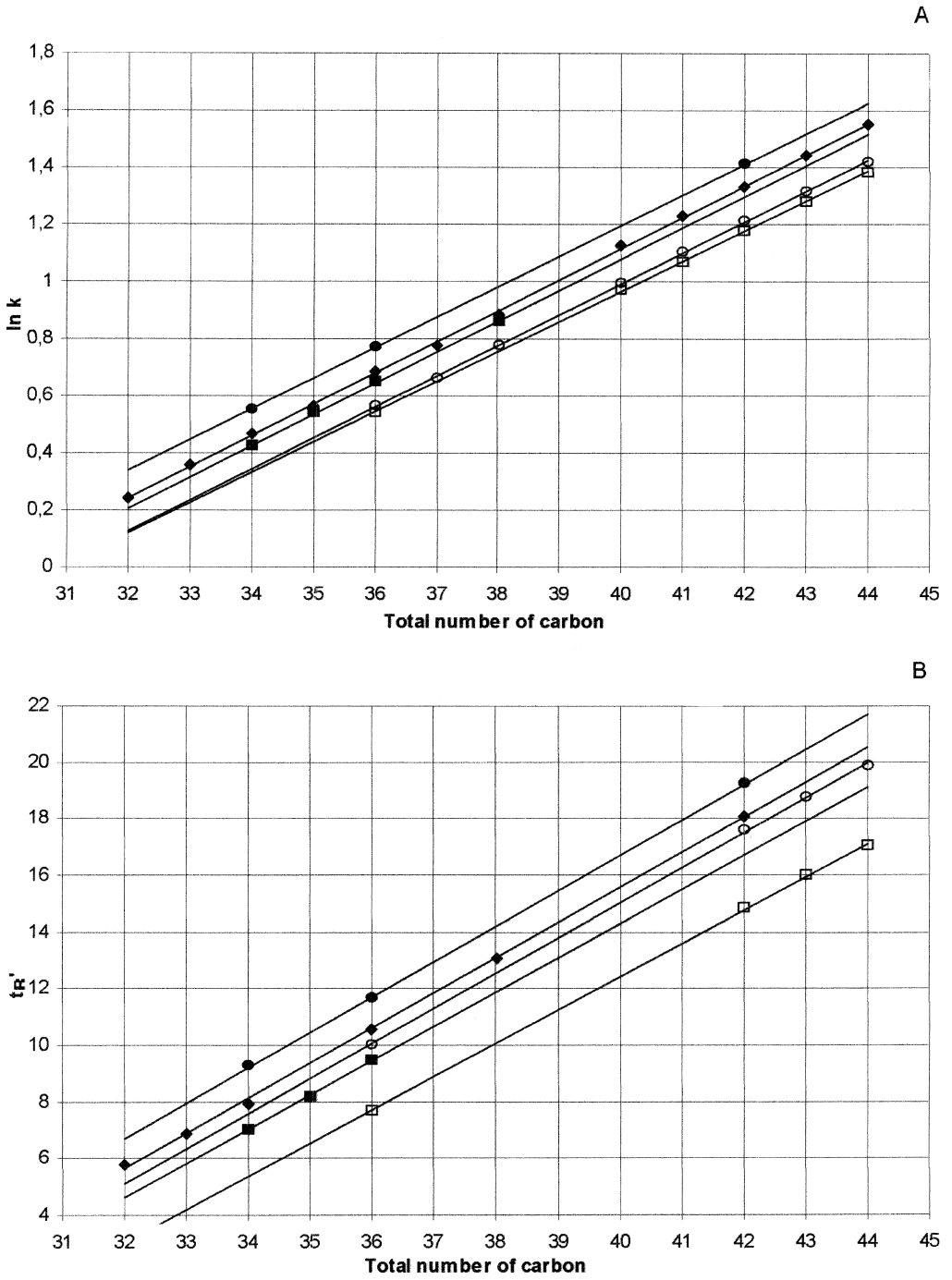

Fig. 6. Retention chart in SubFC (A) (6% MeOH, 13 MPa, 31°C, 3.2 ml·ml^{-1}); Retention chart in NARP–LC (B) (gradient from ACN–THF 95:5 to ACN–THF–propanol 35:5:60 in 30 min, 0.4 ml·ml^{-1}); data of abacus ● for ceramide with dihydrosphingosine and saturated fatty acid (curve 1); ♦ for ceramide with sphingosine and saturated fatty acid (curve 2); ■ for ceramide with phytosphingosine and saturated fatty acid (curve 3); ○ for ceramide with sphingosine and α-hydroxy fatty acid (curve 4); □ for ceramide with sphingosine and unsaturated fatty acid (curve 5).

Table 5
Calculated values of increments from retention factors of ceramide standards

Structural variations	Increment k (min)	Standard deviation	Relative standard deviation (%)	Number of data
Δ CH2	0.1093	0.007	6.2	11
Δ C=C	−0.1331	0.008	5.9	6
Δ α-OH	−0.1311	–	–	2
Δ S/P	−0.0361	0.006	15.4	8
Δ S/D	0.0883	–	–	1

Each straight line represents one class of ceramides characterised by the same base with different length of the alkyl chains (Fig. 6A). Parallel straight lines were obtained except for ceramides with unsaturated fatty acid and sphingosine base where the slope was slightly different of the others (Table 6) which underlines an heterogeneous behaviour. The set of unsaturated ceramides was divided into three groups: one with C_{18}:1$S_{n'}$ (n'=16, 17, 18, 19, 20) from the trace homologues of *N*-Oleoyl-D-sphingosine, another one with C_{24}:1$S_{n'}$ (n'=16, 17, 18, 19, 20) from the trace homologues of *N*-Nervonoyl-D-sphingosine and the last one with C_n:1S_{18} (n=18, 22, 23, 24, 25, 26) from the trace homologues of ceramide Type III. Fig. 7 represents the retention chart obtained respectively for these ceramides which cannot be represented by a unique straight line. The two ceramide groups (C_{18}:1$S_{n'}$ and C_{24}:1$S_{n'}$) possess an unsaturation on the position 9 of the fatty acid chain from the opposite extremity of the polar head. Consequently, the number of methylene groups of the fatty acid chain between the polar head and the cis unsaturation of unsaturated ceramide changed from 7 (for C_{18}:1$S_{n'}$) to 13 (for C_{24}:1$S_{n'}$). The position of the cis unsaturation on the hydrocarbon chain length seems to act as a break in the chain and thus plays an important role on the retention. From this assumption, the couples in ceramide Type III: C_{22}:1S_{18}–C_{24}:1S_{16} (assigned to peak 8), and C_{23}:1S_{18}–C_{24}:1S_{18} (assigned to peak 9) should be separated into two peaks. The peak 8 appears to be like two coeluted compounds. In the ascent of the peak 9, a little hump can be observed. (Fig. 3B)

Table 6
Comparison of the slopes of the curves, log k=f (nc) of the following sets of solutes: C_{18}:1$S_{n'}$, C_{24}:1$S_{n'}$, C_n:1S_{18} and C_n:0$S_{n'}$

	C_{24}:1$S_{n'}$	C_n:1S_{18}	C_n:0$S_{n'}$
C_{18}:1$S_{n'}$	4.212[a]	1.836	1.410
	31[b]	24	42
	SD	NSD	NSD
C_{24}:1$S_{n'}$		3.339	5.959
		39	57
		SD	SD
C_n:1S_{18}			5.077
			50
			SD

[a] Experimental student t value.
[b] Degree of freedom.
SD=significantly different at 5% risk.
NSD=not significantly different at 5% risk.

However, ceramides which contain the same number of total carbon cannot be separated (e.g. C_{18}:0S_{17} and C_{17}:0S_{18}) when the fatty acid is saturated. That explains why only 19 peaks were obtained after the optimisation of the separation although 25 different structures were present in ceramide Type III sample.

3.5. Comparison of the retention charts obtained in SubFC and NARP–LC

In Fig. 6A, only the straight line with C_n:1S_{18} structures was plotted for ceramides with unsaturated fatty acid and sphingosine base in order to compare with NARP–LC retention chart.

Higher number of data are present on the SubFC retention chart. In NARP–LC, more coelution occurred with Type III and IV samples which hindered the achievement of the retention measurements of several ceramide structures.

The distances between the straight lines cannot be directly compared between these two methods, since

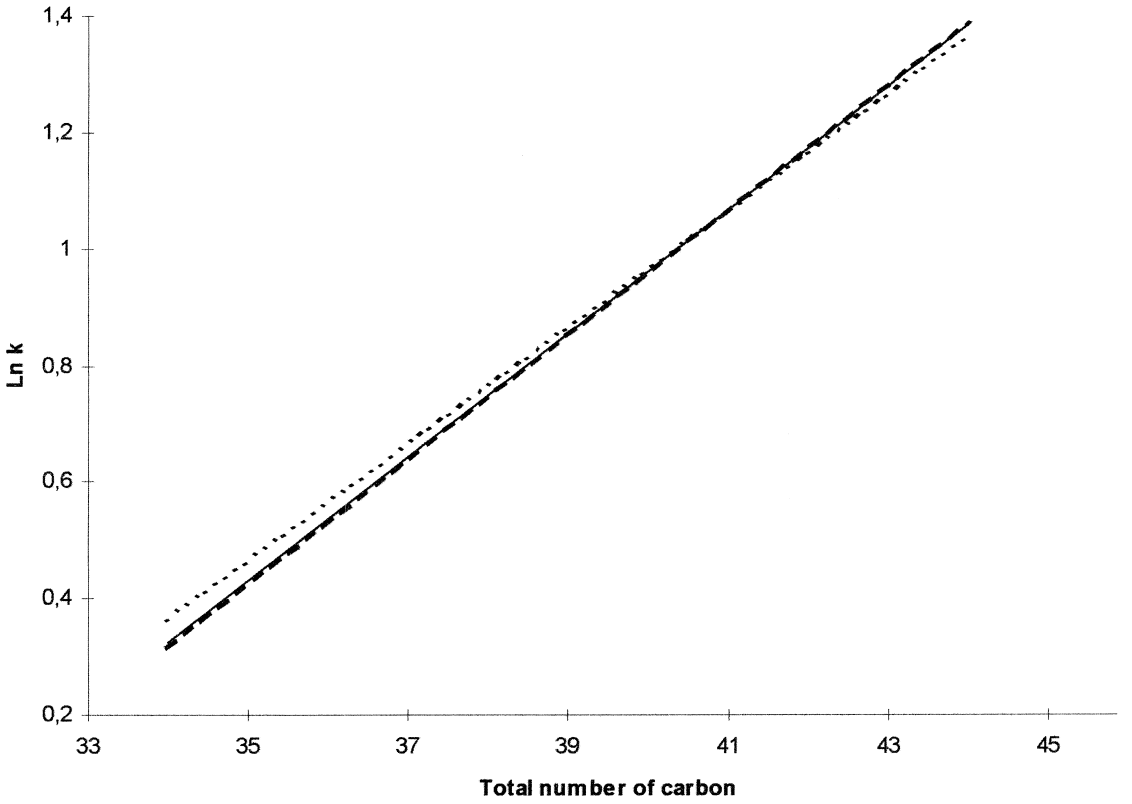

Fig. 7. Retention chart in SubFC only for ceramides with sphingosine base and unsaturated fatty acid. Dotted line for ceramides C_{24}:1$S_{n'}$ (n′=16, 17, 18, 19, 20), dashed line for C_{18}:1$S_{n'}$ (n′=16, 17, 18, 19, 20) and thin line for C_n:1S_{18} (n=18, 22, 23, 24, 25, 26). Corresponding to the numeration 2, 1 and 3 in Table 6.

different units were used due to the mode of elution, isocratic or gradient. However, the relative difference between straight lines can be discussed between Fig. 6A and 6B.

Qualitatively the elution order of ceramides with the same fatty acid and differing by their amine base is similar in the both methods (i.e. first phytosphingosine, sphingosine and dihydrosphingosine ceramides).

In NARP–LC, ceramides which differ by the presence of an α-hydroxyl on the fatty acid chain were little discriminating (curves 2 and 4), whereas a further hydroxyl group on the base moiety (phytosphingosine: curve 3), decreased significantly the retention (curves 2 and 3). In SubFC, the opposite behaviour can be noticed. The α-hydroxyl on the fatty acid decreased importantly the retention (curves 2 and 4), whereas a further hydroxyl group on the base moiety (curve 3) is almost ineffective on the retention variation. The position of the additional hydroxyl group (from ceramide with dihydrosphingosine base either to ceramide with phytosphingosine or to ceramide with sphingosine and an α-hydroxyl fatty acid) have a different effect in NARP–LC and in SubFC. However, comparing the curves 1, 2 and 5, where the difference in ceramides was an unsaturation on the base moiety (curve 1 and 2) or on the fatty acid chain (curves 2 and 5), the relative retention differences are nearly identical in both methods. An apparent lower selectivity appeared between ceramides with α-hydroxyl and ceramides with unsaturated fatty acid in SubFC than in NARP–LC. However, a better discrimination between ceramides with saturated fatty acid and ceramides with

α-hydroxy (or unsaturated) fatty acid is observed in SubFC.

4. Conclusion

From ceramide analysis, the advantage of pSubFC on NARP–LC was again demonstrated for separating hydrophobic molecules, even if the elution was isocratic in pSubFC and gradient elution in NARP–LC.

Classical retention behaviours were found for ceramides in pSubFC, i.e. the methylene selectivity decreases with eluotropic strength increase. Moreover, different order of elution occurred between ceramides with saturated and unsaturated fatty acid relatively in SubFC and NARP–LC.

Since more compounds were separated in pSubFC than in NARP–LC, an higher number of data was obtained for plotting retention chart in pSubFC. Therefore, more a accurate model was obtained in pSubFC than in NARP–LC. The higher power of separation of pSubFC was also emphasised by a particular behaviour observed with ceramide with unsaturated fatty acid. Some of them which differ only from the distribution of the carbon number on the two chains can be separated.

Retention charts from both the two methods had showed difference for the relative retention of ceramides, especially with α-hydroxy fatty acid. These differences can be useful for ceramide identification, either for selecting one of these in regard to the nature of ceramide sample, or either using these two models complementary.

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