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Stationary-phase effects in gradient high-performance liquid chromatography

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Abstract

The type of the stationary phase for reversed-phase liquid chromatography significantly affects the sample polarity range that can be covered using gradients of organic solvents in water. The polarity range available for gradient separations of samples containing compounds differing in the lipophilic parts of the molecules can be characterized by "gradient lipophilic capacity", Pl, based on the retention of standard compounds with a repeat lipophilic structural unit, such as a methylene group. The gradient lipophilic capacity is also suitable to characterize the separation possibilities of the columns in non-aqueous reversed-phase gradient elution of strongly non-polar compounds, such as triacylglycerols. In the same way, the suitability of various columns for reversed-phase gradient separations of oligomers can be characterized by "gradient oligomer capacity", as demonstrated in the example of oligo(ethylene glycols). To enable a comparison of the properties of stationary phases independent of column efficiency and dimensions, the gradient lipophilic capacity or the gradient oligomer capacity should be normalized for a "standard" column plate number, gradient range and volume (in column hold-up volume units). The gradient lipophilic capacity or the gradient oligomer capacity and the number of compounds that can be resolved during a gradient run decrease as the initial concentration of the strong solvent in the mobile phase increases and (or) the gradient time decreases. These quantities can be used to select a suitable column and to adjust the optimum gradient profile (the initial composition of the mobile phase and the gradient steepness) with respect to the time of analysis and the number of oligomers or other compounds with regular repeat structural groups that can be resolved during the gradient run.
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1. Introduction

With ever increasing requirements on the analysis of complex samples and on generic methods for rapid characterization of a large number of samples as the products of automated synthesis in the pharmaceutical industry [1], gradient elution HPLC techniques are becoming increasingly important. The development of a new gradient method or a transfer of an established one between the instruments and columns is less straightforward than in isocratic HPLC, because of the synergistic effects of the gradient program and the other operation parameters, such as column geometry and mobile phase flow-rate on the retention and resolution. With some knowledge of the theory, the effects of the operation conditions on the retention in gradient elution can be predicted.

The net retention volumes, $V'_R = V_R - V_m$, in reversed-phase gradient-elution chromatography can be calculated from Eq. (1) [2,3]:

$$V'_R = \frac{1}{mB} \log\{2.3\, mB[V_m 10^{(a-mA)} - V_D] + 1\} + V_D \qquad (1)$$

Here, the parameter $B = (\varphi_e - A)/V_G$ characterizes the steepness of a linear gradient of an organic solvent in water, $\varphi = A + BtF_m$, between the initial concentration, A, and a final concentration, φ_e (in volume fractions), accomplished in a gradient time, t_G, corresponding to the gradient volume, $V_G = t_G \times F_m$, at a mobile phase flow-rate, F_m. V_D is the gradient "dwell volume", corresponding to the delay in the transport of the mobile phase between the gradient mixer and the top of the column. a and m are the constants of the reversed-phase retention Eq. (2) relating the isocratic retention factors, k, of sample solutes to the concentration of the organic solvent in aqueous–organic mobile phases [2,4–6]:

$$\log k = a - m\varphi \qquad (2)$$

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Unlike to the isocratic conditions, bandwidths in gradient-elution HPLC, w_g, are—to the first approximation—equal to the isocratic bandwidth in the mobile phase at the time of elution of the band maximum and are approximately constant for all sample compounds eluting before the end of the gradient program [2,7–10]. A convenient measure of the gradient-elution column performance is the peak capacity, P, which is defined as the number of peaks that can be separated with the resolution $R_S = 1$ between the elution times of the first and of the last peaks in the chromatogram, $t_{R,1}$ and $t_{R,Z}$, respectively. P can be—to the first approximation—calculated from Eq. (3), as suggested by Giddings [7]:

$$P = \frac{\sqrt{N}}{4}\left(\frac{t_{R,Z}}{t_{R,1}} - 1\right) \quad (3)$$

Another definition, introduced by Neue et al. [10] and Neue and Mazzeo [11], understands the peak capacity in reversed-phase gradient-elution HPLC as the maximum number of peaks of an approximately constant average bandwidth, w_g, that can be separated on a column with N theoretical plates with the resolution $R_S = 1$ during the whole gradient run time, t_G, which is directly proportional to the gradient volume, V_G, at a constant mobile phase flow-rate, F_m.

In recent years, great attention was focused on developing new LC stationary phases with increased stability over extended pH range and with suppressed residual silanol activity, aimed at improving the separation of basic or acidic compounds [12–16]. Further, stationary phases with embeded polar groups were designed to improve the wetting properties of the bonded alkyl chains and consequently the column performance in highly aqueous mobile phases [17] and new monolithic stationary phases improve the flow-rate through the column [18]. Such modifications of the stationary phases affect the column selectivity for both polar and lipophilic compounds [19].

The column peak capacity can be defined in a slightly different way to allow a better characterisation of the stationary phase effects on gradient-elution behaviour of compounds differing in a constant lipophilic structural increment, such as a methylene group. This criterion, "gradient lipophilic capacity", Pl, is defined as the number of peaks that can be resolved in between the gradient elution times of two homologues differing by one non-polar repeat unit, such as a methylene group:

$$\mathrm{Pl} = \frac{V'_{R,(n+1)} - V'_{R,(n)}}{w_g} - 1 = \frac{\sqrt{N}}{4}\frac{1}{V_m}\frac{\Delta V_R}{1+k_e} - 1 = \frac{\sqrt{N}}{4}\frac{\Delta V_R}{V_m}\frac{1}{1+10^{[a-mA-mBV'_{R,(n+1)}]}} - 1 \quad (4)$$

Here, k_e is the retention factor at the time of elution of the band maximum (Eq. (2)), V_m is the column hold-up volume measured as the elution volume of a non-retained standard, $V_{R(n)}{}'$, $V_{R(n+1)}{}'$ are the net retention volumes of two homologues differing by one repeat group [19], such as two alkylbenzenes differing by one methylene group (noted by the subscripts n and $n+1$, respectively), ΔV_R is the difference in their retention volumes. In a homologous series, the retention factors usually change regularly with increasing number of repeat monomer units, n, contributing regularly to the energy of retention. This contribution generally decreases as the concentration of the organic solvent (methanol) in the mobile phase increases, in agreement with Eq. (2) [19–26]. Assuming a constant contribution of a repeat oligomer unit to the retention energy in an oligomer series, $-\Delta\Delta G$, the constants a and m of Eq. (2) depend in a linear manner on n [20,21]:

$$a = a_0 + a_1 n \quad (5a)$$

$$m = m_0 + m_1 n \quad (5b)$$

Eqs. (5a) and (5b) can suitably describe the retention of various homologous and oligomeric series both in reversed-phase and in normal-phase systems. Using the experimentally determined constants a_0, a_1, m_0, m_1, Pl can be calculated by combining Eqs. (1), (2), (4), (5a) and (5b).

To allow a comparison of the effects of the stationary phase chemistry and of the character of the support material, such as porosity, regardless of the column geometry and efficiency, the gradient lipophilic capacity should be normalized by selecting a standard column efficiency, e.g., 10,000 theoretical plates and a standard gradient range and volume (e.g., 0–100% organic solvent in water in the gradient volume equal to 10 column hold-up volumes). [19]. The gradient oligomer capacity, $P(o)$, is defined by Eq. (4) in the same way as Pl, but for compounds with repeat monomer units other than a methylene group, e.g., a repeat $–CH_2–CH_2–O–$ group for polyethylene oxides.

In the present work, the gradient lipophilic capacity approach was extended to the characterization of suitability of HPLC columns for the separation of a broader range of compounds in aqueous–organic and non-aqueous gradient reversed-phase LC. This is illustrated on the gradient separation of moderately polar oligo(ethylene glycol)s (OEGs) and of very lipophilic triacylglycerols (TAGs).

2. Experimental

2.1. Equipment

An HP 1090 M liquid chromatograph equipped with a UV diode-array detector, an automatic sample injector, a 3DR solvent delivery system, a thermostated column compartment and a Series 7994 A workstation (Hewlett-Packard, Palo Alto, CA, USA) was used for all measurements. The gradient dwell volume of the instrument was 0.40 ml. The flow-rate of the mobile phases was kept at 1 ml min^{-1} and the temperature at 40 °C. The detection wavelength was set

Table 1
Properties of the columns tested

No.	Trade name, dimensions, ($L \times$ i.d. (mm)), particle size	V_m (ml)	%C	S ($m^2 g^{-1}$)	Manufacturer
1	Alltima C_{18}, 250 × 4.6, 5 μm	2.53	16.1	311	Alltech, Deerfield, IL, USA
2	Purospher RP-18e, 250 × 4, 5 μm	1.65	N.A.	N.A.	Merck, Darmstadt, Germany
3	Zorbax Rx C_{18}, 250 × 4.6, 5 μm	2.26	12	180	Agilent, Palo Alto, CA, USA
4	Zorbax SB-Aq, 250 × 4.6, 3.5 μm	2.81	N.A.	180	Agilent, Palo Alto, CA, USA
5	Zorbax 300 Extend C_{18}, 150 × 4.6, 5 μm	1.99	N.A.	50	Agilent, Palo Alto, CA, USA
6	Inertsil ODS 2, 250 × 4.6, 5 μm	2.57	18.5	320	GL Sciences, Tokyo, Japan
7	Aqua C_{18} 125A, 150 × 3.0, 5 μm	0.8	N.A.	N.A.	Phenomenex, Torrance, CA, USA
8	Atlantis C_{18}, 150 × 3.9, 3 μm	1.37	N.A.	N.A.	Waters, Milford, MA, USA
9	Polymer C_{18}, 150 × 4.6, 5 μm	1.69	–	N.A.	Astec, Whippany, NJ, USA
10	Nova-PakC_{18}, 150 × 3.9, 4 μm	1.45	N.A.	N.A.	Waters, Milford, MA, USA

L: Column length, i.d.: inner diameter, V_m: hold-up volume, S: specific surface area, %C: percent of bonded carbon, N.A.: data not available.

at 205 nm for non-aqueous HPLC of TAGs. For OEGs that do not absorb the UV radiation, a Sedex 75 evaporative light scattering detection (ELSD) system was used (Sedere, Alfortville, France), the mobile phase flow-rate was kept at 0.75 ml, the pressure of nitrogen in the detector was 3.4 bar and the detector temperature was set at 60 °C. The suppliers, types and characteristics of the columns used in this work are listed in Table 1. Some columns were purchased, other were obtained as a gift or as a loan.

2.2. Mobile phases and samples

Methanol, ethanol (99.9%) and acetonitrile (all LiChrosolv grade, Merck, Darmstadt, Germany) were used as obtained. Water was double distilled in glass with addition of potassium permanganate. All solvents were filtered using a Millipore 0.45 μm filter and degassed in an ultrasonic bath immediately before the use. Mobile phases were prepared by mixing in appropriate volume ratios directly in the HP 1090 M instrument from the components continuously stripped by a stream of helium.

Di(ethylene glycol), 99%, and oligo(ethylene glycol)s, number-average molecular masses (M_n) = 200, 300 and 1000, were obtained from Sigma–Aldrich, Prague, Czech Republic. Samples of *Silybum arianum* and *Dracocephalum moldavica* oils were obtained from Galena, Opava, Czech Republic or were prepared from plant seeds as described elsewhere [27]. The samples were dissolved in the isocratic mobile phase or in the mobile phase used at the start of gradient elution to provide adequate response of the UV detector (approximately 10–20 μg ml^{-1}). Five microliter sample volumes were injected in each experiment.

2.3. Methods

The columns were first equilibrated with approximately 20 column hold-up volumes (V_m) of the mobile phase and then the retention volumes, V_R, of OEGs were measured under isocratic conditions in mobile phases containing different concentrations of methanol in water and with different gradient programs. V_R of TAGs were measured with non-aqueous gradients of ethanol in acetonitrile. The flow-rate of the mobile phases was kept at 1 ml/min in isocratic and non-aqueous gradient experiments and at 0.75 ml min^{-1} in aqueous–organic gradient experiments, the temperature was kept at 40 °C. All retention data were measured in triplicate and mean values were used in calculations. The parameters of the retention Eq. (2) of OEGs were determined from the isocratic retention factors, $k = (V_R/V_m - 1)$, in 30–50% aqueous methanol using linear regression and were employed in the calculations of the gradient oligomer capacity using Eq. (4) after introducing the Eqs. (1), (5a) and (5b) for V_R', m and a to describe the dependence of the gradient-elution volumes on the number of EO units, n [22–25].

In gradient-elution experiments, a 5 min reversed gradient (to speed-up the column re-equilibration) and a 5 min isocratic equilibration time with the starting mobile phase were used after the end of each experiment to re-equilibrate the column. Using this procedure, the reproducibility of the retention times of the individual EO oligomers or triacylglycerols in different samples was 1.5% or better. The repeatability of the differences in the retention times of the adjacent peaks was <0.05 min, which corresponds to the error of 0.1 units or less in the resolution and in the oligomer or lipophilic gradient capacity. The column hold-up volumes, V_m, were determined using uracil as a non-retained marker compound. The retention times of di(ethylene glycol) and poly(ethylene glycol)s (M_n = 200 and 300) were used to assign the correct oligomer unit number to the peaks of the poly(ethylene glycol) 1000. The peak assignment was verified in independent HPLC–MS experiments, as described elsewhere for OEGs [26] and for TAGs [27,28]. The gradient lipophilic capacities for TAGs were calculated directly from the experimental gradient elution volumes and bandwidths using Eq. (4) and were normalized to standard column dimensions and efficiency. All calculations were performed in the spreadsheet format using the Quattro Pro 5.0 table editor.

3. Results and discussion

3.1. Gradient lipophilic capacity in non-aqueous reversed-phase chromatography of triacylglycerols

Non-aqueous reversed-phase (NARP) chromatography is often necessary to separate very non-polar compounds, such as triacylglycerols (TAGs) in animal fat and plant oil samples. The individual TAGs generally elute in the order of increasing "equivalent carbon number" (ECN), which is equal to the number of carbon atoms (NC) minus twice the number of double bonds (DB) in the acyl substituents to the glycerol moiety, ECN = NC − 2DB. As a great majority of naturally occurring TAGs have even ECNs, it is theoretically possible to achieve baseline resolution of 2×Pl peaks in between the TAGs differing by two ECNs. Usually, fewer TAGs with equal ECNs but different alkyl lengths and numbers of double bonds (iso-ECN TAGs) can be resolved in practice.

In the first part of this work, we used the gradient lipophilic capacity approach suggested earlier for aqueous–organic reversed-phase chromatography [19] to compare seven different stationary phases for the separation of TAGs with 36–52 ECNs in *Dracocephalum moldavica*, *Silybum arianum* oil and of other 14 plant oils using non-aqueous gradients of ethanol in acetonitrile, which enables relatively fast separations of the TAGs with different ECNs [27]. The standard non-aqueous gradient lipophilic capacity for triacylglycerols, Pl, was calculated using Eq. (4) from the experimental gradient elution volumes and bandwidths and was normalized for "standard" conditions (N = 10,000, 0–100% ethanol in acetonitrile in gradient volumes $V_G = 60V_m$). Pl generally linearly decreases as ECN increases with all columns tested, except the Astec Polymer C_{18} column, showing the lowest non-aqueous gradient lipophilic capacity from among all the columns tested. For this column, the Pl versus the ECN plots can be described by a second order polynomial equation (Fig. 1). Significant differences are found between the values of Pl and the slopes of the plots measured for the individual columns tested. The highest Pl were found for the Alltima C_{18} and Purospher C_{18}e columns.

On a Zorbax Extend C_{18} and on a Nova-Pak C_{18} column, the main TAGs in the *Dracocephalum moldavica* oil with ECNs 36–46 can be resolved in 30 min, the TAGs in *Silybum arianum* oil with ECNs 40–52 in 40 min under comparable standardized conditions. Fig. 2 shows chromatograms of triacylglycerols in *Silybum arianum* oil with a broader elution volumes interval on a Zorbax Extend C_{18} column, corresponding to a higher Pl in comparison to the Nova-Pak C_{18} column.

There are some differences between the retention of iso-ECN TAGs, which can be utilized for their separation. The separation selectivity for the iso-ECN triacylglycerols strongly depends on the properties of the stationary phase and generally improves on columns with higher Pl, providing a larger space in between the peaks of TAGs with different ECNs—see for example the resolution R_s of iso-ECN triacylglycerols LLLn/OLnLn/LnLnP with ECN = 40, LLL/OLLn/LLnP with ECN = 42, or OLL/LLP/OLnP with ECN = 44 under normalized non-aqueous gradient conditions in Table 2 (L means linoleic, Ln linolenic, O oleic and P palmitic acyls). On the other hand, the band spacing is significantly affected by selective polar interactions of the individual acyls with residual silanol groups and (or) other polar groups in the stationary phases. For example, the resolution of some iso-ECN TAGs (such as OlnP/LLP

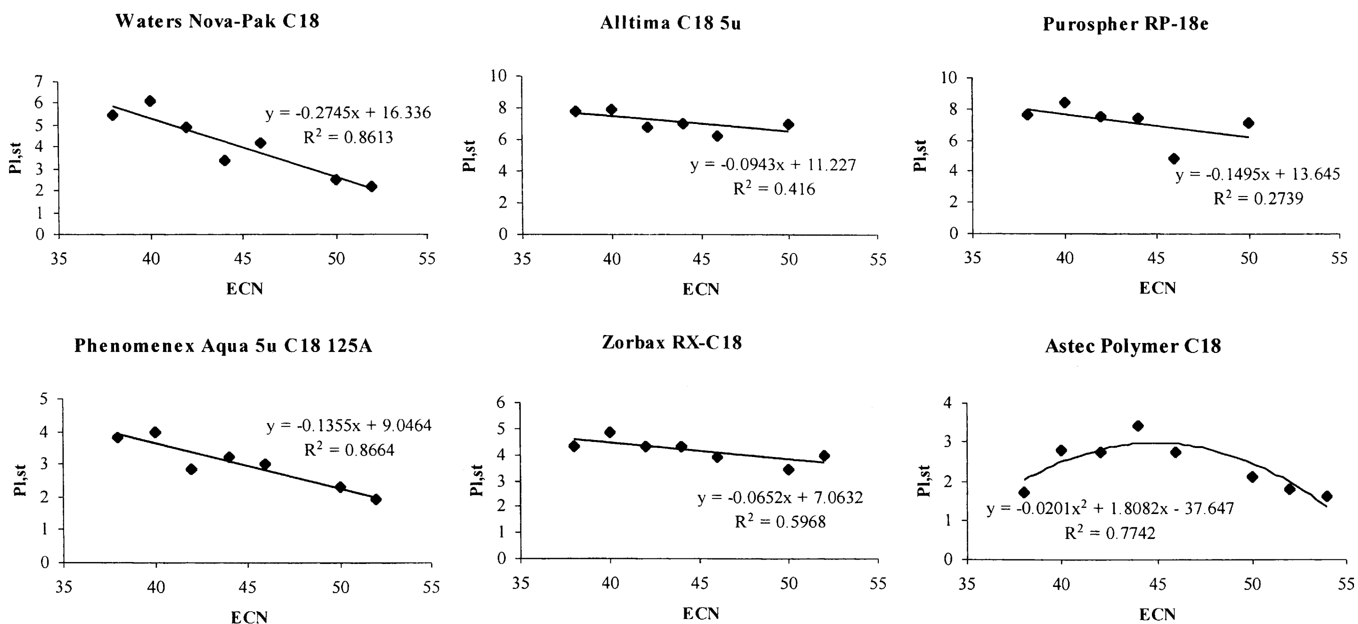

Fig. 1. Standard gradient lipophilic capacity, Pl, st, for triacylglycerols. Non-aqueous reversed-phase chromatography, gradients 0–100% ethanol in acetonitrile, $V_G = 60V_m$, 1 ml/min, N = 10,000. Equivalent carbon number (ECN).

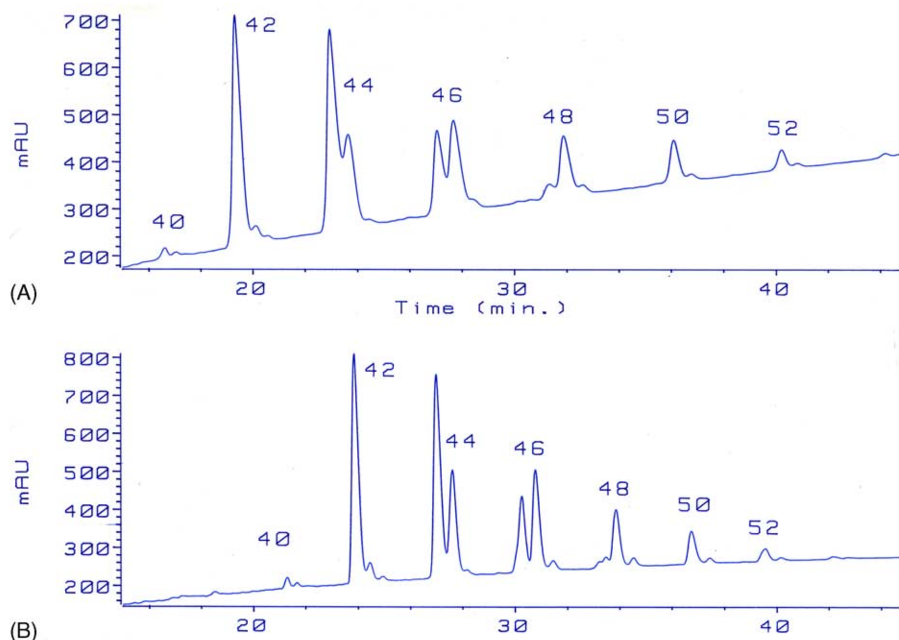

Fig. 2. Non-aqueous gradient separations of triacylglycerols. Sample: 5 μl of 10% solution of *Silybum arianum* oil in acetonitrile on: (A) a Zorbax Extend C_{18} column (No. 1) and (B) on a Novapak C_{18} column (No. 10); Gradient 0–70% ethanol in acetonitrile in 58 min ($V_G = 40V_m$), 0.75 ml/min, UV detection at 205 nm.

with ECN = 44 or PLP/OLP/OOL with ECN = 46) in a *Silybium arianum* oil sample is better on a non-endcapped Novapak C_{18} column with a relatively high silanol activity than on a Zorbax Extend C_{18} column with bidentate bonded alkyl ligands shielding a significant proportion of silanol groups (Fig. 2). Nevertheless, the lipophilic gradient peak capacity is useful for the selection of a column generally suitable for best separation of iso-ECN TAGs over a broad ECN range (such as Purospher RP 18 and Alltima C_{18} columns).

Table 2
Characteristics of non-aqueous reversed-phase (NARP) gradient-elution separation of triacylglycerols (TAGs)

ECN	TAG	R_s						
		1	2	3	5	7	9	10
40	OLn/LLLn	2.21	2.43	1.28	1.69	1.06	1.11	1.61
	LnLnP/OLnLn	2.00	2.89	1.72	1.35	≅0	1.18	1.65
42	OLLn/LLL	2.08	2.24	1.12	1.53	1.77	1.02	1.38
	LLnP/OLLn	2.00	2.91	1.68	1.47	≅0	1.06	1.68
44	LLP/OLL	2.09	2.16	1.05	1.47	1.04	1.14	0.98
	OLnP/LLP	1.94	2.42	1.45	1.49	0.81	1.26	1.85
	Pl, st (40)	7.9	8.4	4.8	5.5	4.0	2.8	6.2
	Pl, st (50)	7.1	7.1	3.5	4.1	2.3	2.1	2.5
	$V_R(50)/V_m$	36.9	38.8	36.1	24.9	37.2	20.2	30.3

Samples: *Silybum arianum*, *Dracocephalum moldavica* oils. Standard gradient, 0–100% ethanol in acetonitrile in 60 V_m, $N = 10{,}000$, 1 ml/min. R_S = NARP resolution of iso-ECN TAGs (with equal equivalent carbon numbers). Pl, st (40), Pl, st (50): standard lipophilic gradient capacity (Eq. (4)) for TAGs with ECN = 40 and ECN = 50, respectively. $V_R(50)/V_m$: elution volume of SOO (ECN = 50), in multiples of column hold-up volumes, V_m. Columns as shown in Table 1. TAG notation: Ln: linolenic acid, L: linoleic acid, O: oleic acid, P: palmitic acid, S: stearic acid acyls.

3.2. Oligomeric gradient capacity for oligoethylene glycols

Eq. (4) can be used to define the oligomer gradient capacity, $P(o)$, for various oligomers with different more or less polar repeat structural units, in similar way as the lipophilic gradient capacity, Pl, for homologous compounds with different numbers of methylene groups. With the polyethylene glycol (PEGs) series tested in the second part of this work, $P(o)$ characterizes the maximum number of peaks that can be separated with resolution $R_s = 1$ in between the peaks of the oligomers differing by one ethylene oxide (EO) repeat unit, which is bulkier, but more polar than a methylene group.

We tested several samples containing oligomers with various numbers of EO units (PEG 200, 300, 500 and 1000) and we found good validity of Eqs. (1), (2), (5a), (5b) for the oligomers containing up to at least 30 EO units on all the columns tested. The experimental constants a_0, a_1, m_0 and m_1 of Eqs. (5a) and (5b) for the OEGs on the individual columns listed in Table 3 reflect the effects of the amount of bonded carbon, of the chemistry of the support and bonded moieties and of the end-capping or other silanol-shielding procedures on the interactions with the EO units and with the end groups. The physical meaning of these constants can be interpreted as follows: a_0 characterizes the contribution of the end groups and a_1 of the repeat EO units

Table 3
Characteristics of gradient-elution separation of oligo(ethylene glycols)

	Column								
	1	2	3	4	5	6	7	8	9
a_0	0.26	0.38	1.29	−0.6	−1.8	0	−0.4	−0.4	−1.69
a_1	0.24	0.21	0.2	0.26	0.32	0.2	0.28	0.26	0.24
R	0.99	0.99	1	1	1	1	1	1	0.994
m_0	2.41	2.91	5.95	1.45	−1.2	2.56	1.61	1.6	−0.52
m_1	0.34	0.29	0.24	0.32	0.6	0.27	0.42	0.37	0.39
R	0.98	0.98	0.99	0.99	1	0.99	0.99	1	0.993
$P(o)$, $n = 5$	1.67	1.39	1.08	1.64	0.2	1	1.5	1.47	−0.32
$P(o)$, $n = 10$	1.2	1.19	0.97	2.32	1.43	1.38	1.6	1.78	0.79
$P(o)$, $n = 15$	0.47	0.62	0.62	1.37	0.79	0.94	0.7	0.93	1.2
$P(o)$, $n = 20$	0	0.17	0.32	0.59	0	0.44	0.1	0.28	0.64
n(last)	19	22	27	26	5–20 coel.	26	20	23	7–26 coel.
V_R'(last)	6.3	6.5	6.3	8.4	4.21 coel.	6.83	5.8	6.7	4.98 coel.

a_0, a_1, m_0 and m_1 are constants of Eqs. (5a) and (5b) and corresponding correlation coefficients, R; n(last): number of EO units in the last resolved oligomer and its retention volume, V_R'(last) (ml) under fast gradient conditions (30–100% methanol in the gradient volume equal to six column hold-up volumes, $V_G = 6V_m$; $N = 10{,}000$). Column numbers as shown in Table 1. coel: coelution of low oligomers.

to the energy of retention in pure water; m_0 and m_1 are a measure of the contributions of the end groups and of the EO units, respectively, to a decrease in retention caused by increasing concentration of the organic solvent (methanol). Both hydrophobic interactions with bonded alkyls and polar interactions with residual silanol groups or polar groups in the bonded moieties can contribute to the retention. Because of a strong polarity of the end groups in the PEG, high a_0 and m_0 may indicate relatively strong contribution of the polar interactions of the stationary phase with the end groups to the retention. Hence, relatively high a_1 and m_1 and low a_0 and m_0 are usually found for very lipophilic columns with low concentrations of residual silanols and other polar groups, such as a Zorbax Extend C_{18} (column 5), double end capped with bidentate bonded ligands or an Astec C_{18} column with C_{18} alkyls covalently bonded to a vinyl polymer support. On the other hand, high a_0 and m_0 constants were found for Zorbax Rx C_{18}, Alltima C_{18}, Purospher RP-18e and Inertsil ODS2 columns. These constants are lower on Zorbax Sb-Aq C_{18}, Phenomenex Aqua C_{18} and Atlantis C_{18} columns, designed to retain hydrophilic compounds in highly aqueous mobile phases, possibly due to a good solvation yielding strong interactions of the polar end groups with water solvating the stationary phases.

The retention, the band spacing in the chromatograms and the number of well resolved oligomers eluting before the end of the gradient can be conveniently characterized by the gradient oligomer capacity, $P(o)$, defined by Eq. (4), as discussed above. $P(o)$ was calculated from Eq. (4) using the experimental constants of Eqs. (5a) and (5b) listed in Table 3. $P(o) = 0$ when the resolution between the oligomers differing by one repeat monomer unit $R_s = 1$. $P(o) < 0$ means that the oligomer resolution is insufficient, whereas $P(o) > 1$ indicates some space between the neighboring oligomer peaks available for separation of other compounds, e.g., isomers or oligomers with different end groups (traded for increased separation time).

Fig. 3 shows two chromatograms of a PEG 1000 sample obtained using steep gradients with 30–50% methanol in water in $V_G = 9V_m$. Poor resolution for early eluting oligomers on a strongly lipophilic Zorbax Extend C_{18} column 5 (Fig. 3A) or on an Astec organic polymer C_{18} column (not shown) was predicted from low a_0 and m_0 constants. The reason is a weak retention of low oligomers over a broad mobile phase composition range, which cannot be significantly improved by adjusting the gradient program. Better separation of lower OEGs can be achieved on other columns, such as a Phenomenex Aqua C_{18} column 7 (Fig. 3B) or an Atlantis C_{18} column (not shown). However, decreasing $-\Delta\Delta G$ in mobile phases with higher concentrations of methanol impairs the resolution of later eluting higher oligomers on these columns more significantly than on the columns providing inferior resolution for low oligomers (Fig. 3B).

To allow a meaningful comparison of the effects of the stationary phase on the separation of oligomers, the gradient oligomer capacity, $P(o)$, was normalized at the following "standard" conditions: column plate number $N = 10{,}000$, hold-up volume $V_m = 2.5$ ml, relatively steep gradients from 30 to 100% methanol in 15 ml gradient volume ($V_G = 6V_m$) and an instrumental gradient dwell volume of 0.4 ml. Under these conditions, $P(o)$ changes in an oligomer series and the number of oligomers well resolved before the end of the gradient is limited. The retention of the last well-resolved compound containing n(last) repeat units can be used for characterization of the column suitability to separate the lipophilic compounds. The standard $P(o)$ depends on the number of the repeat monomer units, n—it first increases to a maximum for oligomers containing 6–10 EO units and then decreases again for higher oligomers (Fig. 4). The $P(o)$ values and the maximum number of

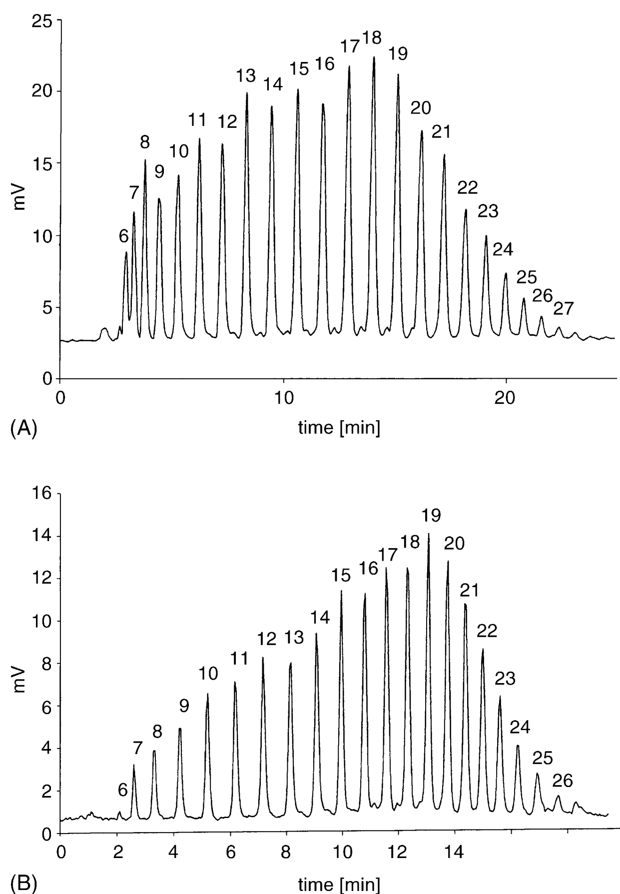

Fig. 3. Gradient elution separation of oligo(ethylene glycols) Columns: (A) Zorbax Extend C_{18}, 5 μm, 150 × 4.6 mm i.d.; (B) Phenomenex Aqua C_{18}, 5 μm, 150 × 3.0 mm i.d. Gradients 30–50% methanol in water, normalized for $V_G = 9V_m$; (A) $t_G = 24$ min and (B) 13 min; flow rate: 0.75 ml/min. Sample injected: PEG 1000, 10 μl, 10% solution in 30% methanol. ELSD, conditions in Section 2. R: detector signal.

the resolved oligomers, n(last), depend on the type of the stationary phase. In Table 3, n(last) and $P(o)$ are given for OEGs with 5, 10, 15 and 20 EO units for nine different columns with relatively fast gradients. At a constant gradient steepness, n(last) is practically independent of the initial

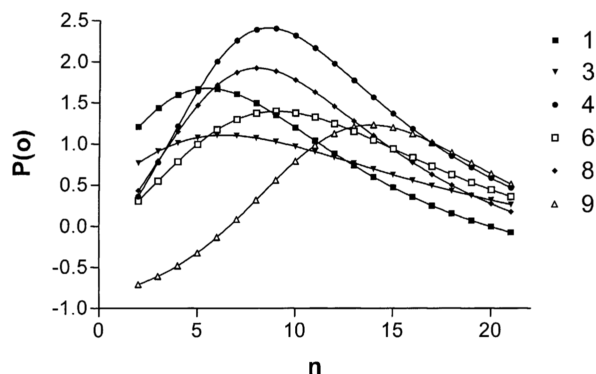

Fig. 4. Standardized gradient oligomer capacity, $P(o)$, for oligo(ethylene glycols) with n EO units. Column numbers as shown in Table 1. Standard gradient conditions: $N = 10{,}000$, 30–100% methanol in $V_G = 6V_m$.

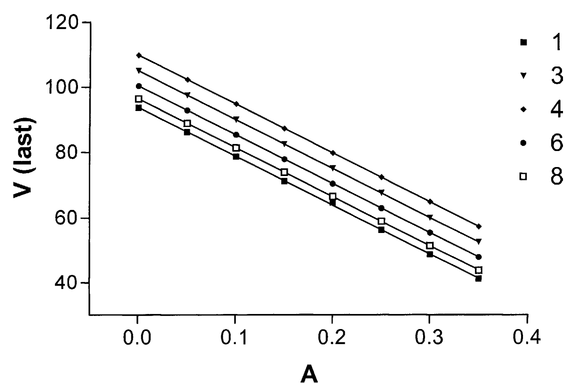

Fig. 5. Retention volumes, V last, of the last resolved oligo(ethylene glycol) oligomer. Column numbers as shown in Table 1. Gradient conditions: $100\times A-100\%$ methanol in water, constant gradient ramp 1.67% methanol per $V = V_m$, $N = 10{,}000$.

gradient concentration, A, of methanol, up to 40–45% for most columns (up to 35% for a Phenomenex Aqua C_{18} column), but n(last), $P(o)$ and resolution rapidly decrease with gradients starting at a higher methanol concentration. The retention times of the last resolved oligomer decrease in almost linear manner as the initial concentration of methanol increases, as illustrated in the examples in Fig. 5 for relatively shallow gradients with a constant steepness parameter corresponding to a ramp of 1.67% methanol in the volume of eluate equal to the column hold-up volume, V_m.

The retention of larger molecules is more strongly affected by a small change in the mobile phase composition than that of smaller ones and hence rather flat gradients should be used for the separation of OEGs to achieve good resolution [19]. At a constant initial gradient concentration of methanol, A, a decrease in the gradient volume, V_G (i.e., an increase in the gradient steepness parameter B in Eq. (1)) causes a rapid decrease in $P(o)$ and in the number of oligomers that can be resolved during a gradient run, n(last), see examples in Fig. 6A. The retention volumes of the last resolved oligomer, V_R' (last) also decrease as V_G decreases (Fig. 6B). Hence, for each gradient it is possible to separate only a limited number of oligomers at a desired resolution level, up to the last one, containing n(last) repeat monomer (EO) units. The n(last) depends on the properties of the stationary phase (the contents of the bonded carbon or silanol groups and on the gradient conditions, etc. see Fig. 3).

The effects of the stationary phase on the band spacing and on the number of resolved OEGs, characterized by $P(o)$ and n(max) shown in Figs. 4–6 differ significantly from the results obtained earlier for alkylbenzenes [19], probably due to a more polar character of OEGs. Both the resolution and $P(o)$ improve, but the elution times increase in the following order on the stationary phases studied: Zorbax Rx C_{18} < Purospher RP-18e ~ Inertsil ODS 2 < Alltima C_{18} < Atlantis C_{18} ~ Phenomenex Aqua C_{18} < Zorbax SB-Aq C_{18}. The number of resolved oligomers, n(last), follows approximately the reversed order, with some exceptions. Zorbax Rx C_{18} and Inertsil ODS-2 stationary phases provide a

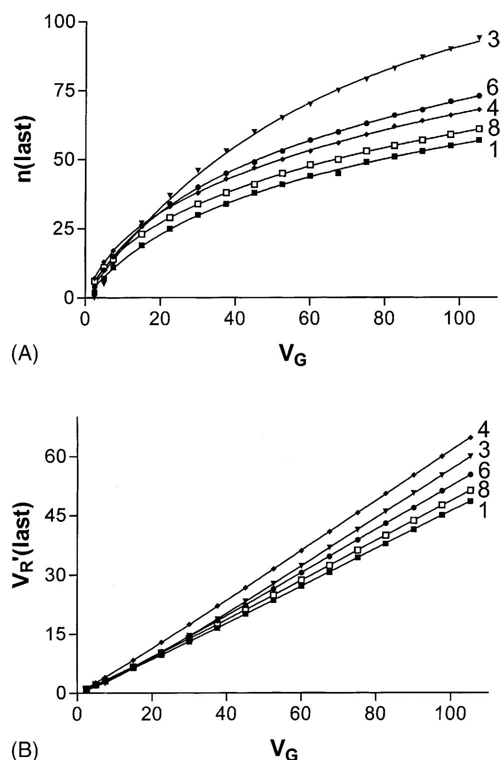

Fig. 6. Effect of the gradient volume, V_G (ml): (A) on the size of the last resolved OEG oligomer [in the number of EO units, n(last)] and (B) on the corresponding net retention volumes, V_R'(last) (ml). Column numbers as shown in Table 1. Gradient conditions: 30–100% methanol in water, gradient ramp 10% methanol in 15 min, 1 ml/min, $N = 10,000$.

higher number of separated oligomers, but a lower gradient oligomer capacity than the Zorbax Sb Aqua C_{18} column. Some stationary phases designed for the analyses of polar compounds in aqueous mobile phases (Alltima C_{18}, Atlantis C_{18} and Phenomenex Aqua C_{18}) provide faster analysis, but lower number of well resolved OEG oligomers. On the other hand, the bidentate ligand (more lipophilic) Zorbax Extend 300 C_{18} and Astec organic polymer C_{18} phases provide better separation of higher than of lower OEGs. Further experiments with other types of oligomers are in progress to allow for more general conclusions.

3.3. Optimization of oligomer separation using the gradient oligomer capacity

The gradient oligomer capacity can be used as a criterion for optimization of gradient separations of oligomers, with respect to the number of resolved oligomers, band spacing and the time of analysis. The "standard" gradient oligomer capacity, $P(o)$, gives useful hints for the selection of a suitable stationary phase. The initial gradient concentration of the organic solvent can be selected by setting it as high as allowed by the resolution of the lowest oligomers in the sample. Then, the gradient steepness (the gradient time at a maximum flow-rate allowed by the column impedance and efficiency) is to be adjusted as a compromise between the number of resolved oligomers and the analysis time.

Zorbax Rx C_{18} and, to a lesser extent, Inertsil ODS 2 offer the best separation of the lower OEGs studied in this work according to the molecular mass distribution, with the highest number of resolved oligomers. Thirty percent methanol was selected as the initial gradient mobile phase, as at lower starting methanol concentrations only the analysis time increases, but the number of resolved oligomers does not improve significantly. With gradients starting at this mobile phase composition, approximately 46 individual OEGs can be separated in 15 min on the first and 40 on the second column, whereas 30–35 oligomers on the other columns tested, except for Zorbax Extend C_{18} and Astec organic polymer C_{18} columns, which do not allow the separation of 5–7 lowest oligomers, even when using gradients starting in pure water (assuming the column efficiencies of 10,000 theoretical plates, and gradient ramp corresponding to an increase of 6% methanol per the volume of eluate corresponding to the column hold-up volume, V_m).

We believe that this approach can be useful for selecting optimum separation conditions for various oligomers not only in reversed-phase, but also in normal-phase systems, which will be subject of our future research.

4. Conclusions

The gradient lipophilic (or oligomer) capacity can be used to characterize the lipophilic and polar properties of various columns both in aqueous–organic and non-aqueous reversed-phase HPLC. The gradient lipophilic capacity, together with the polarity or the size of the last compound well resolved during a gradient run, can be used to evaluate the suitability of the column stationary phase (1) for the separation of solutes with small differences in the polarities and (2) for the separation of the samples containing compounds spread over a large polarity range. It can provide the information on the number of oligomers that can be separated in a single gradient run on a particular column. The "standard" gradient lipophilic and oligomer capacities are independent of the column size and efficiency and hence can characterize the retention properties of the stationary phases. The gradient oligomer capacity can be used to select the optimum column for separations of oligomers according to the molecular mass distribution. It also can give a hint how to adapt the gradient time and range when transferring separation methods between the columns with different stationary phases.

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