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Phase system selectivity and two-dimensional separations in liquid column chromatography

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Abstract

Correlations between the separation selectivity in aqueous and non-aqueous reversed-phase systems and in normal-phase LC systems were investigated for samples containing different numbers of two repeat structural elements. Such samples are best separated in "orthogonal" two-dimensional chromatographic systems, showing selectivity for one type of the repeat structural element only in the first dimension and for the other structural element only in the second dimension. The number of resolved compounds improves as the degree of orthogonality of the separation systems increases with decreasing correlation between the selectivities for the sample structural distribution in the two dimensions. Orthogonal systems with non-correlated selectivities for each repeat structural element provide the highest number of separated peaks and regular arrangement of the peaks over the two-dimensional retention space according to the individual structural element distribution and the best use of the available peak capacity. Fully orthogonal systems are difficult to find in practice. Partially orthogonal system with correlated selectivities for one structural type distribution, but with one system non-distinguishing the distribution for the other structural element are still useful for the two-dimensional separations. The correlations between the selectivities for repeat regular structural increments were employed to evaluate the suitability of phase systems for two-dimensional HPLC separations. The selectivity correlation in various reversed-phase and normal-phase systems was evaluated for two sample types:

(1) Various RP columns show significantly inversely correlated selectivities for acyl lengths and numbers of double bonds distribution, but the differences in the double bond selectivity can be used for practical separations of triacylglycerols with the same equivalent carbon numbers.
(2) Synthetic EO–PO block (co)oligomers with two-dimensional distribution of oxyethylene and oxypropylene monomer units were separated according to the two distribution types using on-line two-dimensional reversed-phase–normal-phase LC with a C18 column in the first dimension and an aminopropyl silica column in the second dimension.

Keywords: Column liquid chromatography; Two-dimensional separations; Phase system selectivity; Peak capacity; Ethylene oxide–propylene oxide (co)oligomers; Triacylglycerols

1. Introduction

Although two-dimensional gas chromatography is much more frequently used and more elaborated technique for separation of complex sample mixtures [1,2], two-dimensional liquid chromatographic methods, whose development is more difficult, are gaining increasing attention in the recent years (often in connection with mass spectrometry), because of the demands on high-resolution LC separations raised by fast developing research in proteomics and metabolomics [3,4]. In two-dimensional (2D) systems, different chromatographic modes suiting particular separation problems are combined in each dimension to increase the number of separated compounds in complex samples [5].

The selectivity of separation in the individual modes is principally based on the differences in the size, polarity and shape of the sample molecules, on the acidity/basicity and on the specific charge of ionic compounds. Because of a mixed character of the forces controlling the retention mechanism in

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real separation systems, the selectivity in various systems can be more or less correlated. Both reversed-phase and normal-phase separations are based on the differences in the polarities of the analytes, even though various selective interactions such as dipole–dipole or proton-donor/acceptor interactions may cause major selectivity differences for various isomers or homologous compounds in these phase systems. Molecular size is the basis of separation in size-exclusion chromatography, but it often contributes also to the retention and separation selectivity in reversed-phase or ion-exchange LC systems by hydrophobic, polar, or ionic interactions of the structural elements in large molecules [6,7].

Most often, synthetic polymers [8], peptide fragments or other biopolymers [3,4,9,10] are separated using size-exclusion chromatography for the separation according to the molar mass distribution in one dimension and reversed-phase [8–10] or ion-exchange [4] LC for the separation of species with different functionalities in the second one [2,4]. For separation of biopolymers, bioaffinity chromatography in the first dimension can be combined with reversed-phase LC in the second dimension [3]. Recently, applications of two-dimensional reversed-phase chromatography employing different stationary phases in each dimension (such as combination of silica- and zirconia-based columns) were reported [11–15]. "Pseudo-multidimensional" reversed-phase chromatography on a single column using sequential application of two or more selective gradient elution modes designed to elute only a certain class of compounds also increase the total peak capacity [16]. In so-called "comprehensive" two-dimensional liquid chromatography, every fraction from the first dimension is transferred on-line to the second dimension [17–20].

In multidimensional chromatography the number of resolved compounds considerably increases. However, based on the assumption that the retention of the sample components is a random process, the probability theory predicts that the number of peaks occurring in a chromatogram is much lower than the maximum peak capacity of a multi-dimensional separation system [21–25]. The number of singlets, overlapping doublets and triplets in poorly resolved chromatograms can be predicted by statistical overlap theory [26] and can be decoded to some extent using chemometric procedures [27,28].

Both the practical objective of the analysis and the sample type very often imply certain degree of structural relationship among the analytes of interest, for example, molar mass or end-group differences in polymers, different number of repeat units in homologues or homo-oligomers, equal structural units at different positions in the molecules in positional isomers, different numbers and annelation of aromatic rings in polyaromatic hydrocarbons, or different number and polarities of functional groups in various sample types of environmental pollutants, naturally occurring compounds, drugs and metabolites, etc. Giddings [9] called the regularity in the structural distribution of sample components "sample dimensionality". A two-dimensional sample exhibits a disordered peak distribution in one-dimensional separation systems, but shows a more or less ordered distribution of peaks in two-dimensional separation systems [28,29]. The overlapping peak distribution in one-dimensional separation systems usually originates from dual distribution of the number of one or more repeat structural elements in the sample, both of which contribute more or less to the retention, depending on the sample character.

The best separation of "multidimensional samples" is accomplished in "orthogonal" separation systems with complementary techniques [30]. Each dimension should provide maximum separation selectivity for one type of distribution, but should be as far as possible insensitive to the differences in the other type of distribution [31].

Separation selectivity for repeat structural elements is a convenient measure of the extent to which structural differences in sample molecules affect the retention behaviour. It has important impact on the selection of suitable conditions for a particular separation problem, depends on both the stationary and the mobile phases and can be used for general characterization of separation systems to resolve sample compounds on the basis of various structural differences, such as size, polarity, shape, etc. [3,4,31].

In this work, correlation of selectivity for molecules differing in polarities (lipophilities) was investigated in aqueous and non-aqueous reversed-phase LC and in normal-phase LC. The approach was applied to triacyl glycerols in plant oil samples and to ethylene glycol–propylene glycol (EO–PO) (co)oligomers to develop orthogonal 2D LC separation methods by combination of reversed-phase and normal-phase systems.

Two-block (co)polymers are two-dimensional samples characterized by the distributions of the number of each monomer unit. EO–PO block (co)polymers are frequently used surfactants in washing machines, emulsifiers and solubilizers of flavors and fragrancies in cosmetic products. The oxypropylene chain $-[CH_2-CH(CH_3)-O]_m-$, i.e., $(PO)_m$, is significantly less polar than the oxyethylene chain $-[CH_2-CH_2-O]_n-$, i.e., $(EO)_n$. Two types of block copolymers were studied: (1) $(EO)_n$–$(PO)_m$–$(EO)_n$ (Slovanik) and (2) $(PO)_m$–$(EO)_n$–$(PO)_m$ (Novanik). Increasing average molecular mass of the industrial products enhances gelation and with increasing number of PO units, the wetting properties are improved. Consequently, molar mass distribution, chemical composition distribution and block sequence distribution are all important for the characterization of the industrial products of this type. Because of different polarities of EO and PO units, reversed-phase and normal-phase LC systems differ significantly in selectivities for the individual blocks [6,32].

Natural fat and oil samples contain saturated and unsaturated fatty acids, their esters or other derivatives and mono-, di- and tri-acylglycerols. These are typical multi-dimensional samples with acyl of different lengths (numbers of carbon atoms) in the first sample dimension, the number of double bonds in the second sample dimension and the positions of

the double bonds in the third dimension. Occurrence of *cis*-/*trans*- and *sn*-1/*sn*-2/*sn*-3 isomers adds additional sample dimensionalities [29,33–35]. Non-aqueous reversed-phase LC shows high selectivity for compounds differing in acyl lengths and number of double bonds, whereas silver-ion (argentation) chromatography is much more selective for the separation of positional isomers and off-line coupling of these methods has been used for the characterization of triacylglycerol composition of vegetable oils [35].

2. Experimental

2.1. Samples and materials

Technical samples of EO–PO block (co)polymer, Novanik 600/20, Novanik 1010 and Slovanik 1010 were obtained from Sloveca (Nováky, Slovak Republic) and a sample of *Dracocephalum moldavica* oil from Galena, Opava, Czech Republic.

Columns: Separon SGX C18, 5 μm, 150 mm × 3.3 mm i.d., Separon SGX Amine, 5 μm, 150 mm × 1 mm i.d. (both from Tessek, Prague, Czech Republic), Zorbax 300 Extend C_{18}, 5 μm, 150 mm × 4.6 mm i.d., Zorbax Rx-C_{18}, 5 μm, 250 mm × 4.6 mm i.d. (both from Agilent, Palo Alto, CA, USA), NovaPak C_{18}8, 4 μm, 150 mm × 3.9 mm i.d (Waters, Milford, MA, USA), Alltima C_{18}, 5 μm, 250 mm × 4.6 mm i.d. (Alltech, Deerfield, IL, USA), Aqua C_{18}125A, 5 μm, 150 mm × 3.0 mm i.d. (Phenomenex, Torrance, CA, USA), Purospher RP-18e, 5 μm, 250 mm × 4.0 mm i.d. (Merck, Darmstadt, Germany), Polymer C_{18}, 5 μm, 150 mm × 4.6 mm i.d. (Astec, Whippany, NJ, USA).

Ethanol, 2-propanol and *n*-heptane, all of HPLC grade (Baker, Deventer, The Netherlands), were dried and kept in tightly closed dark bottles over molecular sieve beads Dusimo 5 A (Lachema, Brno, Czech Republic), previously activated at 300 °C (ca. 30–40 g/l). Acetonitrile (Lichrosolv grade, Merck, Darmstadt, Germany) was used as obtained. Water was double distilled in glass. All solvents were filtered using a 0.45 μm filter (Millipore, Bedford, MA, USA) and degassed in ultrasonic bath immediately before the use. Mobile phases were either pre-mixed in required volume ratios, or prepared by mixing in appropriate volume ratios directly in the HP 1090M instrument from the components continuously stripped by a stream of helium.

2.2. Instrumentation

An HP 1090M liquid chromatograph equipped with an automatic sample injector, a 3DR solvent delivery system, a thermostated column compartment and a Series 7994A workstation (Agilent, Palo Alto, CA, USA) was used for all measurements. It was operated in connection with a UV diode-array detector at 210 nm for the analysis of triacylglycerols. For the analysis of oxyethylene–oxypropylene (EO–PO) co-oligomers, a Sedex 75 evaporative light-scattering detector (ELSD, Sedere, Alfortville, France) equipped with a micro nebulizer was employed (detection conditions: 60 °C nitrogen pressure 2.4 bar, sensitivity setting 3).

In the two-dimensional setup, a reversed-phase Zorbax Extend 300, 5 μm column, 4.6 mm × 150 mm i.d. (Agilent), was employed in the first dimension and was connected on-line to an aminopropyl silica Separon SGX Amine, 5 μm column, 150 mm × 1 mm i.d. (Tessek, Prague, Czech Republic), in the second dimension via a six-port two-position switching valve with a 3 μl injection loop. The columns and the switching valve were kept in a thermostatted column compartment at 40 °C. The mobile phase was delivered to the aminopropyl silica column in the second dimension using a self-standing HPP 5001 pump (Laboratory Instruments, Prague, Czech Republic). The data from the ELSD were evaluated using a CSW Chromatography Station for Windows, version 1.7 (DATA APEX, Prague).

2.3. Methods

2.3.1. One-dimensional column liquid chromatography

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-microlitre sample volumes were injected in each experiment. The column hold-up volumes, V_M, were determined using a non-retained marker compound, uracil in reversed-phase systems and trichloroethylene in normal-phase systems and were corrected for the volume of the connecting tubing. In gradient experiments, the gradient was started at the time of injection. After the end of each gradient run, the composition of the mobile phase was gradually set back to the starting values and 15 column volumes were pumped through the column for equilibration before the start of the next analysis. The column temperature was kept at 40 °C and the flow rate at 0.5 ml/min in all experiments.

2.3.2. Two-dimensional liquid column chromatography

Fifty to hundred microlitres Novanik and Slovanik samples were injected onto the first-dimension C18 column, and a linear gradient of 50–100% acetonitrile in water was run in 110 min at 0.2 ml/min. The second-dimension aminopropyl silica column was run under isocratic conditions with 5% propanol in 95% hexane as the mobile phase. The fractions from the first dimension were cut at the times corresponding to the band maxima recorded in an independent single-dimension RP experiment.

3. Results and discussion

3.1. Sample dimensionality and repeat group selectivity in one-dimensional chromatographic systems

The knowledge of the distribution of the structural elements in various samples usually provides useful informa-

tion on the sample properties and often is a primary target of the sample characterization. Giddings [29] introduced the term "sample dimensionality" to characterize ordered peak appearance often observed in chromatograms of samples with structural regularities. In various LC modes, except for size-exclusion chromatography (SEC), repeat structural elements such as methylene groups or monomer units in homopolymers and block (co)polymers, often show a constant contribution to the Gibbs free energy of retention, $-\Delta G$, which causes a regular increase in $\log k$ of analytes (Martin rule [36]). Assuming (a) a constant contribution of the repeat elements to the energy of retention over the whole structure distribution range, (b) a low entropic contribution to the retention so that size-exclusion can be neglected to first approximation, the effect of a dual distribution of structural elements A and B on the retention factors, k, can be characterized, to first approximation, by Eq. (1):

$$\log k = \log \beta + n_A \log \alpha_A + n_B \log \alpha_B; \quad (k = \beta {\alpha_A}^{n_A} {\alpha_B}^{n_B}) \tag{1}$$

Here, α_A is the relative retention between the samples differing by one repeat structural unit A, whereas α_B is the relative retention between the samples differing by one repeat structural unit B. These quantities characterize the separation selectivity for the individual distribution types A and B, respectively. β is a measure of the contribution of non-repeat structural elements to the retention and n_A, n_B are the numbers of the repeat structural elements A and B, respectively, in the individual sample components. In a separation system with constant separation selectivities for each structural element, α_A and α_B are correlated with each other:

$$\log \alpha_A = C' \log \alpha_B \tag{2}$$

The correlation constant C' depends on the type of the structural element and on the stationary and mobile phases. This means that individual solutes with some combinations of the numbers of structural elements A and B, n_A and n_B, coelute in a common peak characterized by the retention factor k:

$$n_A \log \alpha_A + n_B \log \alpha_B = (n_A + C n_B) \log \alpha_A = \log \left(\frac{k}{\beta} \right) \tag{3}$$

The selectivity constants α, β depend on the composition of the mobile phase, e.g., decrease in linear manner in reversed-phase systems as the concentration of the organic solvent increases in aqueous-organic mobile phases [37]. $\log \alpha_A$ and $\log \alpha_B$ can be either positive or negative and the retention may increase or decrease with increasing number of structural elements A and B both in reversed-phase and in normal-phase LC. For example, the retention of ethylene oxide–propylene oxide (EO–PO) block (co)oligomers in reversed-phase systems increases with increasing number of PO units, but either is independent of or decreases with increasing number of EO units ($\alpha_{PO} > 1$, $\alpha_{EO} < 1$,or $\alpha_{EO} = 1$); on the other hand the retention in various normal-phase systems increases with increasing number of EO units, but decreases with increasing number of PO units (inverse correlation, $\alpha_{PO} < 1$, $\alpha_{EO} > 1$) [6,32].

Non-aqueous reversed-phase liquid chromatography (NARP LC), which is widely used for the separation of complex samples of natural triacylglycerols (TAGs) [33–35], provides an example of the effect of repeat structural elements on retention, resulting in ordered chromatograms. The retention of TAGs in NARP LC systems generally increases with increasing number of carbon atoms, n_C, in the acyl chains and decreases with increasing number of double bonds, n_{DB}. In the systems with various columns and isocratic mobile phases or gradient programs one double bond in an unsaturated fatty acid acyl decreases the retention factors by approximately the same measure as two methylene groups in the acyls increase the retention. Fatty acids, their derivatives such as *p*-bromophenacyl and other esters, or acylglycerols with equal so-called "equivalent carbon numbers" (ECN) defined as the total carbon number in all acyl chains minus two times the number of double bonds, $ECN = n_C - 2n_{DB}$, show similar retention times in reversed-phase LC systems [33]. If we denote a methylene group as the structural element A and a double bond as the structural element B, the retention of TAGs, fatty acids and their derivatives with various numbers of carbon atoms, n_C, in the acyl chains and of double bonds, n_{DB}, can be described by empirical Eq. (4), to first approximation:

$$\log k = \log \beta + n_C \log \alpha_A - C n_{DB} \log \alpha_A \tag{4}$$

According to an empirical rule, the constant $C \approx -2$ in Eq. (4). However, in real NARP LC systems, some TAGs with equal ECNs but different numbers of double bonds can be more or less resolved on efficient columns [38]. This means that the constant C in Eq. (4) is not exactly equal to 2 and its exact value depends on the stationary phase properties. Nevertheless, strong inverse correlation between the double bond and acyl length selectivities complicates the possibilities of 2D separation according to the two sample distribution types. The NARP LC separation in the first dimension is usually combined with argentation (silver-ion) normal-phase chromatography in the second dimension, where the separation is based on the differences in π–π electron interactions of silver ions with unsaturated species differing in the number, position and substitution of double bonds [35].

Table 1 illustrates the validity of Eqs. (2)–(4) for non-aqueous reversed-phase HPLC on six different RP columns with linear gradients of ethanol in acetonitrile with gradient times normalized to 60 column hold-up volumes. It was earlier demonstrated that the differences in the retention times (volumes) in gradient LC are approximately directly proportional to $\log \alpha$ [31,38]. With all six columns, excellent linearity (with correlation coefficients 0.986–0.999) was found for the plots of the retention volumes V_R versus the

Table 1
Effect of the number of double bonds (n_{DB} = 0–9) and of number of carbon atoms (n_C = 50–54) in the acyls of fatty ester triacylglycerols on the retention volumes V_R in gradient NARP on various RP columns: $V_R = a - bn_{DB}$, $V_R = c - dn_C$; R = correlation coefficient; gradient 0–70% ethanol in acetonitrile in gradient times t_G corresponding to 60 column hold-up times (1 ml/min, 40 °C)

Column	t_G (min)	a	b	R	c	d	$-b/d$
Nova-PakC18	56	51.6	−4.03	0.9981	−61.0	1.88	2.14
Alltima C18	98	105.5	−6.55	0.9943	−57.0	2.66	2.45
Aqua C18125A	31	34.3	−2.39	0.9981	−25.5	0.98	2.44
Zorbax Rx C18	98	94.4	−6.46	0.9957	−48.8	2.28	2.83
Purospher RP-18e	64	72.9	−4.59	0.9987	−50.1	2.05	2.24
Polymer C18	65	42.1	−3.86	0.9857	−47.2	1.41	2.73

number of double bonds n_{DB} in 10 triacylglycerols with ECNs ranging from 36 to 54. The plots of V_R versus the total number of carbon atoms n_C in the TG acyls are also linear, demonstrating almost perfect inverse correlation between the methylene (lipophilic) selectivity, α_A, and double bond (polar) selectivity, α_{DB}, in NARP LC of TAGs. The ratios of the proportionality constants ($\log \alpha_{DB}/\log \alpha_A$) = b/d range in between −2.14 and −2.83 for different columns, close to the empirical value of the constant $C = -2$ in Eq. (4). The differences in the constant C characterize different selectivities of reversed-phase columns for the TAGs with equal ECNs, as illustrated in Fig. 1 for the resolution of TAGs with ECN = 40 (LLLn–OLnLn–LnLnP), ECN = 42 (LLL–OLLn–LLnP) and ECN = 44 (OLL–LLP–OLnP). In spite of generally strong co-elution, this behaviour opens some possibilities for adjusting the separation of the vegetable oil samples by selection of the stationary phase for NARP LC. Columns with great differences in the values of the constant C are likely to provide separation of different particular TAGs, even though many TAGs co-elute on any column under NARP conditions. For example, this is the case with the column Aqua C18, where the TAGs pairs OLnLn/LnLnP with ECN = 40 and OLLn/LLnP with ECN = 42 co-elute, hence the resolution cannot be determined and is not plotted in Fig. 1.

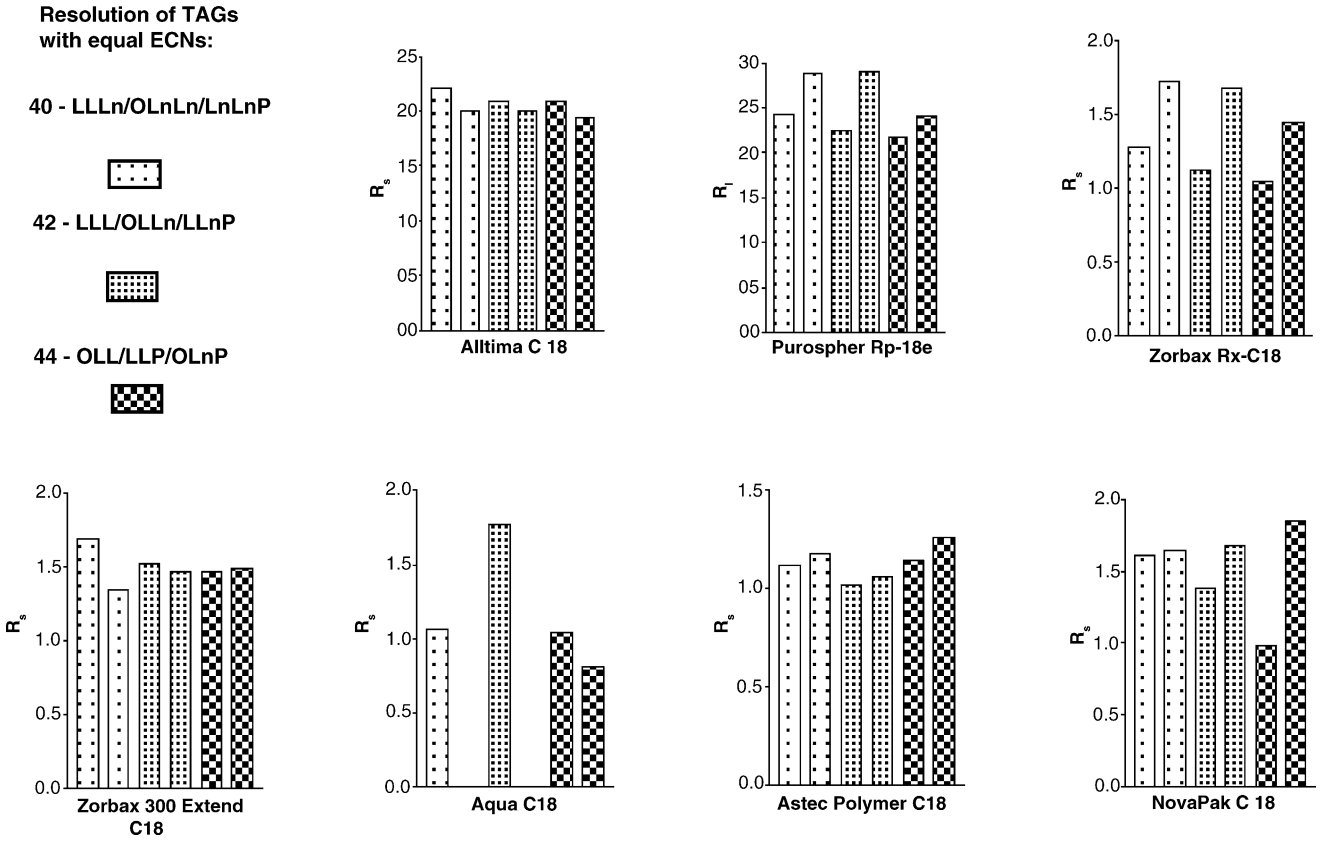

Fig. 1. Effect of stationary phase on NARP resolution of triacylglycerols with ECN = 40, 42, 44. Gradients of ethanol in acetonitrile with gradient time normalized to 60× column hold-up volume. For each triplet of TAGs with equal ECN, the first value of R_S in the diagram shows the resolution of the peaks 1 and 2; the second value the resolution of peaks 2 and 3 TAG notation: L, linoleic; Ln, linolenic; O, oleic; P, palmitic acid acyls.

3.2. The impact of repeat group selectivity on two-dimensional chromatographic separations

Eq. (1) can be adapted to describe the effect of the dual-type distribution of repeat structural elements A and B on the retention selectivity in two LC systems, 1 and 2, as follows:

- System 1:

$$\log k_{1,A} = \log \beta_1 + n_A \log \alpha_{1,A} + n_B \log \alpha_{1,B} \quad (5)$$

- System 2:

$$\log k_{2,A} = \log \beta_2 + n_A \log \alpha_{2,A} + n_B \log \alpha_{2,B} \quad (6)$$

When $\alpha_{1,A} > 1$, $\alpha_{2,A} > 1$, $\alpha_{1,B} > 1$ and $\alpha_{2,B} > 1$, the retention in both systems increases as the number of the two structural elements increases. However, any of the repeat structural elements may decrease the retention in some systems ($\alpha_{1,A} < 1$, or $\alpha_{2,A} < 1$, or $\alpha_{1,B} < 1$, or $\alpha_{2,B} < 1$), as in previously discussed example the retention of fatty acid derivatives in reversed-phase systems decreases with increasing number of double bonds. If the systems 1 and 2 differentiate species with various numbers of the two repeat structural elements A and B, they show correlated selectivities with respect to the distribution of both structural elements in each dimension, i.e., the 2D systems are "non-orthogonal". In a non-orthogonal 2D system, the fractions transferred from the first to the second dimension contain individual compounds with mixed type of distribution, which makes necessary the identity confirmation using an independent method (most often MS). Furthermore, there is a strong probability that some species with different combinations of the numbers of elements A and B are not separated in such 2D systems. Finally, the total 2D system peak capacity is not completely utilized, depending on the correlation between the selectivities for A and B in the dimensions 1 and 2.

The coverage of the separation space in 2D systems with correlated selectivities α_A for structural elements A can be evaluated from the plots of the retention factors, k_2, in the second dimension versus k_1 of the species with the same numbers of the repeat structural elements, n_A, n_B in the first dimension. The equation describing these plots can be obtained after re-arranging Eqs. (5) and (6):

$$\log k_2 = \log k_1 \frac{\log \alpha_{A,2}}{\log \alpha_{A,1}} + \log \beta_2 - \log \beta_1 \frac{\log \alpha_{A,2}}{\log \alpha_{A,1}} + n_B \left(\log \alpha_{B,2} - \log \alpha_{B,1} \frac{\log \alpha_{A,2}}{\log \alpha_{A,1}} \right) \quad (7)$$

Eq. (7) describes a set of plots with correlated selectivities for the structural elements A and B in both dimensions 1 and 2. Such plots have a form of "bananagrams" [8] covering incompletely the two-dimensional retention space, as illustrated schematically in Fig. 2. The individual plots for sets of compounds with different numbers of the elements B, n_B, described by Eq. (7), are shifted along both the k_1 and the k_2 axes in this diagram. The number of co-eluted

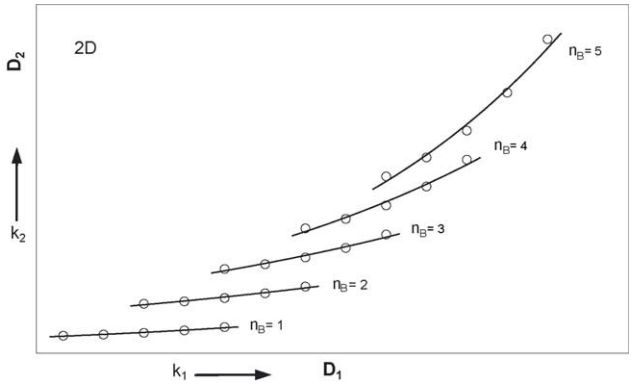

Fig. 2. The retention space coverage in two-dimensional separation of samples showing correlated selectivities for the distribution of structural elements A and B. The lines connect the points corresponding to increasing number of repeat units of the structural element A, n_A at a constant number of repeat units of the structural element B, n_B. k_1 and k_2 are the retention factors of the species with different combinations of the numbers of repeat structural elements A and B in the first and in the second dimension, respectively. As the graph represents only a schematic illustration, no axes scales are given.

compounds increases and the utilization of the maximum 2D peak capacity decreases as the correlation between the selectivities in the two systems, $\alpha_{1,A}$ and $\alpha_{2,A}$; $\alpha_{1,B}$ and $\alpha_{2,B}$, increase. For completely correlated (identical) systems with equal selectivities in the two dimensions, $\alpha_{1,A} = \alpha_{2,A}$ and $\alpha_{1,B} = \alpha_{2,B}$; $\log \beta_1 = \log \beta_2$ and the retention factors of the individual species are equal in the two systems, $k_1 = k_2$. In such systems, the utilization of the maximum peak capacity allowed by the column efficiency in a 2D system, P_{2D}, is minimum, as in the system with two columns of the same type and peak capacities P_1, P_2, connected in series [31]:

$$P_{2D} = \sqrt{P_1^2 + P_2^2}. \quad (8)$$

On the other hand, for completely orthogonal systems the separation selectivities for both structural elements A and B are not correlated,, $\alpha_{A,1} \neq 1 \neq \alpha_{A,2}$ and $\alpha_{B,1} \neq 1 \neq \alpha_{B,2}$. Here, the total 2D peak capacity is fully utilized and is equal to the product of the peak capacities in the systems 1 and 2:

$$P_{2D} = P_1 P_2 \quad (9)$$

In partially correlated systems $\alpha_{A,1} \neq 1$, $\alpha_{A,2} = 1$ and $\alpha_{B,1} = 1$, $\alpha_{B,2} \neq 1$ and the increase in the peak capacity is in between the two limiting cases described by Eqs. (8) and (9) [31].

Fig. 3A shows an example of band spacing for a sample of EO–PO (co)oligomers in a "non-orthogonal" system using an aminopropyl silica column both in the first and in the second dimensions. Both in the normal-phase system NP1 with propanol–hexane mobile phase and in a hydrophilic interaction (HILIC) normal-phase system NP2 with mobile phases containing acetonitrile, dichloromethane and water, the retention increases with increasing number of EO units, but decreases with increasing number of PO units. Hence, this

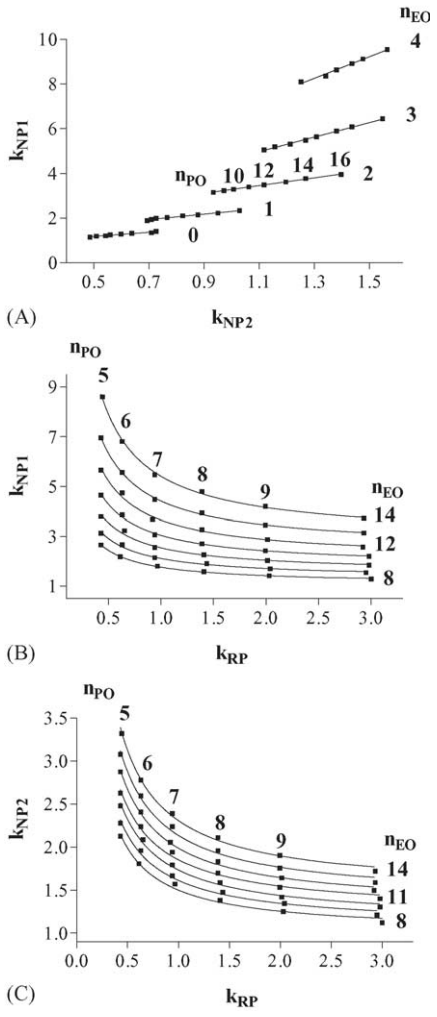

Fig. 3. The retention space coverage in two-dimensional separation of EO–PO (co)oligomers with different numbers of EO (n_{EO}) and PO (n_{PO}) monomer units. k_{RP}: the retention factors in a reversed-phase system (Separon SGX C18 column, 50% acetonitrile in water), k_{NP1}: the retention factors in normal-phase system NP1 (Separon SGX Amine column, 5% 2-propanol in hexane); k_{NP2}: the retention factors in normal-phase system NP2 (Separon SGX Amine column, acetonitrile/water/dichloromethane 39.6:0.4:60). (A) Normal-phase systems with strongly correlated selectivities for both EO and PO units and (B and C) 2D reversed-phase–normal-phase systems with correlated selectivities for PO units, but non-correlated selectivities for EO units (the RP system does not distinguish EO distribution).

2D LC system has strongly correlated selectivities with respect to both the EO and the PO unit distribution (Table 2, high correlation constants b_{EO} and b_{PO}) and the 2D retention space coverage shows the same pattern as predicted using Eq. (7) (Fig. 2).

Two-dimensional samples with dual distribution of structural elements, A and B, are best separated in two-dimensional chromatographic systems where the separation selectivity for repeat structural elements A and B in the first dimension system is completely independent of the separation selectivity in the second dimension system. In such "orthogonal" 2D systems, the separation selectivities in the two dimensions are non-correlated, $\alpha_{A,1} \neq 1$, $\alpha_{A,2} = 1$, $\alpha_{B,1} = 1$, $\alpha_{B,2} \neq 1$. In an appropriately designed orthogonal 2D system, every fraction transferred from the first dimension contains only species with equal numbers of A units, n_A, and second dimension chromatograms show regular coverage of the available retention space by the peaks corresponding to species with a single n_A, but different numbers of B units, n_B.

It is difficult to find fully orthogonal 2D LC systems with respect to each structural element distribution in two-dimensional samples. More often, some 2D LC systems can be "partially orthogonal", i.e., the first dimension does not distinguish one type of structural element distribution (B), $\alpha_{1,A} \neq 1$ and $\alpha_{1,B} = 1$, but the second dimension is more or less selective with respect to the distribution of the two structural elements A and B, $\alpha_{2,A} \neq 1$ and $\alpha_{2,B} \neq 1$. Here, only species with equal n_A are transferred in each fraction to the second dimension, but the second dimension distinguishes between the compounds containing not only different n_B, but also different n_A. This means that the retention times of the first peaks in consecutive fractions gradually shift, as the number of the elements A increases in the fractions transferred from the first dimension. All compounds can be theoretically separated in the second dimension, but the separation time in the second dimension is longer than with the elements B regularly distributed in the first dimension fractions (with $\alpha_A \neq 1$ and $\alpha_B = 1$). Two examples illustrating the coverage of two-dimensional retention space in a "partially orthogonal" 2D system are shown in Fig. 3B and C, again for EO–PO (co)oligomers, but with reversed-phase system in the first dimension (a Separon SGX C18 column and acetonitrile–water 1:1 as the mobile phase) and with normal-phase systems NP1 and NP2 in the second dimension. Whereas in the NP1 and NP2 systems the retention increases as the number of EO groups increases and as the number of PO groups decreases (see Fig. 3A), in the present RP system the retention increases as the number of PO units increases, but is almost independent of the number of EO units. Hence, both 2D RP–NP1 (Fig. 3B) and RP–NP2 (Fig. 3C) systems are orthogonal with respect to the EO unit distribution, which is demonstrated by the correlation constants b_{EO} close to zero between the RP system on one side and either NP1 or NP2 systems on the other (Table 2). The consequence is almost perfectly vertical arrangement of the data for the same number of PO units, but different numbers of EO units in the 2D retention space in both Fig. 3B and C. When the RP system is used in the first dimension, all fractions transferred to the second, normal-phase, dimension contain only species with equal numbers of PO units and the fractions are separated in the second dimension according to the normal-phase distribution of EO units only.

The second dimension (NP1, NP2) systems show some selectivities not only for the EO distribution (structural element B), but also for the structural element A, PO units distribution, so that the NP systems and the RP system are "partially orthogonal" with respect to the PO selectivity. The peaks of the

Table 2
Correlation between the separation selectivities for EO units, α_{EO}, and PO units, α_{PO}, in EO–PO block (co)oligomers in a reversed-phase (RP) and two normal-phase (NP1 a NP2) systems

	2D LC system			
	RP	NP1	NP2	NP1–NP2
	Acetonitrile–water (50:50)[a]	2-Propanol–hexane (30:70)[a]	Acetonitrile–water–dichloromethane (39.6:0.4:60)[a]	
	Separon SGX C18[b]	Separon SGX NH_2[b]	Separon SGX NH_2[b]	
β	0.055	2.455	3.601	
$\alpha_{EO} = \alpha_B$	0.998	1.208	1.074	
$\alpha_{PO} = \alpha_A$	1.538	0.701	0.757	
b_{EO}	–	−0.011 (NP1/RP)	−0.028 (NP2/RP)	2.647 (NP2/NP1)
b_{PO}	–	−1.211 (NP1/RP)	−1.546 (NP2/RP)	1.276 (NP2/NP1)
fn (%)		24 (RP–NP1)	70 (RP–NP2)	–

$b_{EO} = \log(\alpha_{EO},RP)/\log(\alpha_{EO},NP)$ or $\log(\alpha_{EO},NP1)/\log(\alpha_{EO},NP2)$; $b_{PO} = \log(\alpha_{PO},RP)/\log(\alpha_{PO},NP)$ or $b_{PO} = \log(\alpha_{PO},NP1)/\log(\alpha_{PO},NP2)$, Eq. (7). fn: non-utilized part of the total separation space in the second (NP) dimension due to non-complete orthogonality with the RP system, i.e., the correlated PO selectivity. Eq. (11) was used for calculations, assuming maximum number of 5 PO and 14 EO units in the (co)oligomers. RP system: Separon SGX C18 column, 50% acetonitrile in water; NP1 system: Separon SGX Amine column, 5% 2-propanol in hexane; NP2 system (HILIC): Separon SGX Amine column, acetonitrile–water–dichloromethane 39.6:0.4:60.

[a] Mobile phase.

[b] Column.

solutes with equal numbers of EO units in the chromatograms of the individual fractions transferred to the NP systems will be more or less shifted to higher retention times, depending on the number of PO units in the transferred fractions. Hence, the full separation space and theoretical peak capacity available will not be fully utilized. Eq. (6) can be used to derive the formula (Eq. (10)) for estimating the non-utilized space (volume ΔV_f) in each fraction in the second dimension (NP) system, corresponding to the volume before the elution of the species with the lowest number of B units, i.e., with 1 EO unit:

$$\Delta V_f = V_{m,2}\beta_2\alpha_{2,A}^{(n_A)}\alpha_{2,B} \quad (10)$$

Here, $V_{m,2}$ is the hold-up volume of the second dimension column and n_A is the number of the repeat PO units in the fraction transferred from the first dimension. The sum of the contributions of the non-utilized separation space for all fractions $1 - Z$ transferred from the first to the second dimension can be expressed as a part of the total volume of all fractions in the second dimension necessary to elute the most strongly retained species, k_{max}, to yield Eq. (11) for the total non-utilized separation space in the second dimension fractions $1 - Z$, fn:

$$\text{fn} = \frac{\beta_2\alpha_{2,B}(1-(\alpha_{2,A})^z)/(1-\alpha_{2,A})}{Z(1+k_{max})} \quad (11)$$

The data in Fig. 3B and C show that the normal-phase system NP1 provides a better resolution and a more regular coverage of the retention space than the NP2 system, but at a cost of longer separation time. We used Eq. (11) to calculate the non-utilized separation space for the EO–PO (co)oligomers containing up to 5 PO and 14 EO units (see Fig. 3B and C). The results in Table 2 show that in propanol–hexane NP1 system approximately 24% of the total separation space is not utilized, whereas the non-utilized fraction of the separation space in the ACN–DCM–water NP2 system is considerably higher, approximately 70%. This is the consequence of a better orthogonality of the RP–NP12D system (a lower parameter b_{PO}) with respect to the orthogonality of the RP–NP2 system. Because of a relatively small non-utilized fraction

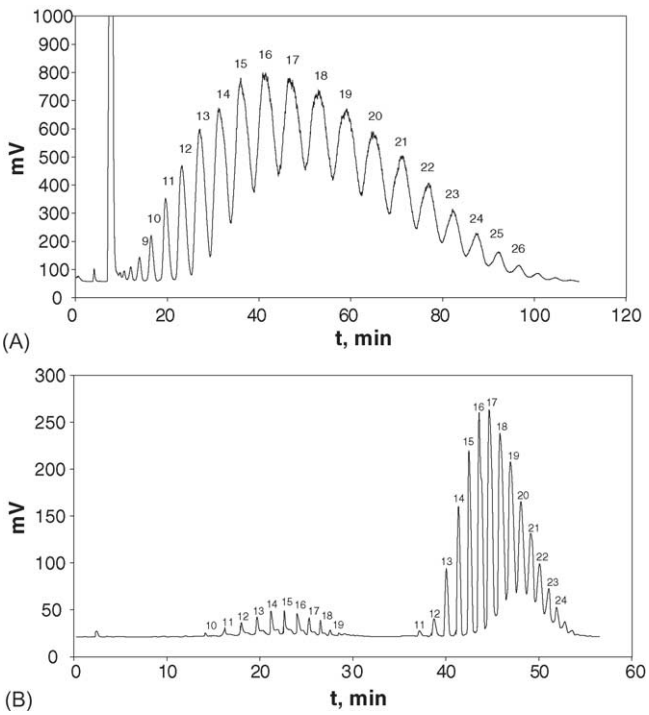

Fig. 4. Reversed-phase gradient separation of a Slovanik 1010 sample on Zorbax Extend C18 column, 5 (m, 150 mm × 3.3 mm with ELSD detection. Conditions: (A) linear gradient 50–100% ACN in 110 min at 0.2 ml/min, 40 °C, the peak numbers correspond to the numbers of PO units, each peak contains co-eluted species with 0–2 EO units; the peak of polyoxyethylene homopolymers at the column hold-up volume. (B) Segmented linear gradient, 5% ACN/0 min −20% ACN/25 min, 100% ACN/55 min, 1 ml/min, first group of peaks correspond to polyoxyethylene homopolymers, the second to EO–PO (co) oligomers with 0–2 EO units.

of the separation space, the NP1 system is useful in two-dimensional LC separations of EO–PO (co)oligomers, as shown below.

3.3. Two-dimensional LC NP-RP separation of EO–PO (co)oligomers

In reversed-phase LC on various C18 columns with acetonitrile–water or with methanol–water mobile phases, the EO oligomers can be separated according to the number of EO units only in mobile phases containing less than 50% organic solvent (not shown). In mobile phases more rich in organic solvents the (co)oligomers are separated only according to the distribution of PO units, and the species with different numbers of EO units co-elute. This applies also for gradient elution, as Fig. 4A and B illustrate for a Slovanik 1010 EO–PO sample containing (co)oligomers with 9–26 PO units and 0–2 EO units, together with some EO homopolymers. With a gradient starting at 50% acetonitrile, the homopolymer fraction elutes as non-retained peak at the column hold-up volume (Fig. 4A). However, with a two-segment linear gradient the homopolymer fraction can be separated according to the EO distribution in the early part (first segment) of the gradient at low acetonitrile concentration, whereas the EO–PO (co)oligomer fraction is resolved according to the number of PO units in the second part of the gradient with higher acetonitrile concentrations (Fig. 4B). Hence gradient elution starting at 50% acetonitrile can be conveniently used for the first-dimension RP separation of EO–PO (co)oligomers.

In-line coupling of RP and NP LC systems is subject to major difficulties connected with the fraction transfer between the two dimensions, as aqueous–organic mobile phases in the first dimension RP systems usually show only limited miscibility with purely organic solvents used in NP LC (second dimension) and aqueous–organic mobile phases are incompatible with many normal-phase systems, where they strongly de-activate the stationary phase. Even 1 μl of an aqueous acetonitrile mobile phase fraction transferred from an RP column to a silica gel column completely destroys the normal-phase resolution. With an aminopropyl silica column, small volumes of the aqueous–organic solvent in the sample injected affect the retention to a lesser extent. This is demonstrated by chromatograms obtained for 3, 5, 10 and 25 μl samples containing Novanik EO–PO (co)oligomer in 50% aqueous acetonitrile, injected onto a 150 mm × 1 mm i.d. Separon SGX Amine column with 5% propanol in hexane as the mobile phase (Fig. 5). The separation of a 3 μl aqueous–organic sample was practically identical as for the same injected volume of the sample dissolved in 5% propanol–hexane. Up to 5 μl sample volume, the column still has acceptable selectivity and resolution for fractions containing 1–4 oligomer units.

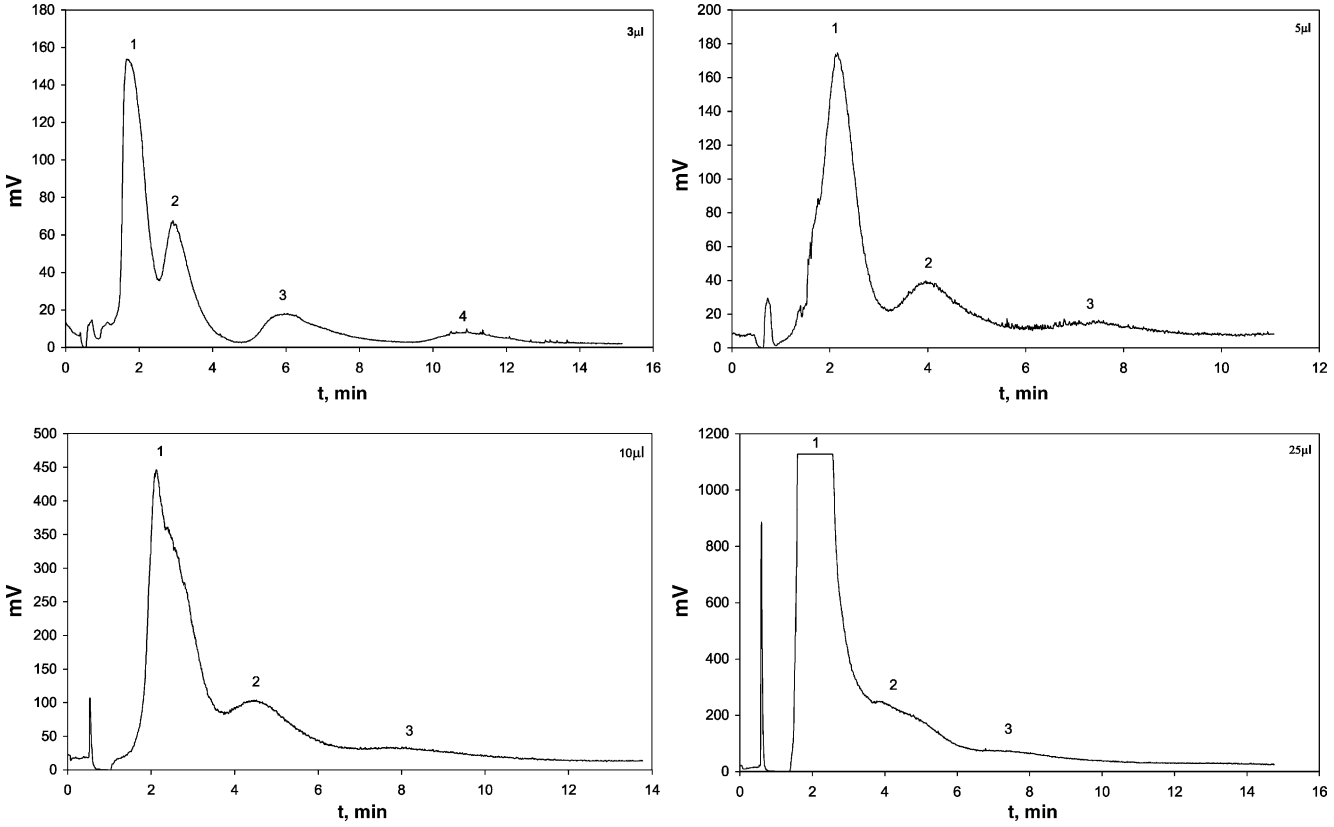

Fig. 5. Chromatograms of 3, 5, 10 and 25 μl samples of Slovanik 1010 in 50% aqueous acetonitrile solvent injected onto a Separon SGX Amine, 5 μm (150 mm × 1 mm i.d.) column. Mobile phase: 5% 2-propanol in hexane, 0.5 ml/min, 40 °C, ELSD detection.

Based on these results, we used a two-dimensional set-up for the separation of EO–PO (co)oligomers, consisting of a reversed-phase Zorbax 300 Extend, 5 μm, column, 150 mm × 4.6 mm i.d., in the first dimension, connected in-line via a six-port two-way switching valve with a 3 μl injection loop to an aminopropyl silica Separon SGX Amine, 5 μm, column, 150 mm × 1 mm i.d., in the second dimension. Onto the first-dimension column, 50–100 μl samples were injected and a linear gradent of 50–100% acetonitrile in water was run in 110 min at 0.2 ml/min (Fig. 4A and B). The second-dimension column was run under isocratic conditions with 5% propanol in 95% hexane as the mobile phase. Both columns and the switching valve were placed in a thermostated compartment and kept at 40 °C, with an evaporative light-scattering detector (ELSD) equipped with a micro-nebulizer connected to the outlet of the aminopropyl silica column. Three-microlitre fractions from the first dimension were cut at the times corresponding to the band maxima recorded in an independent single-dimension RP experiment. Fig. 6 shows the detector record during the on-line 2D separation of an EO–PO Slovanik sample, with an inset corresponding to a few normal-phase chromatograms of the fractions transferred from the first dimension onto the aminopropyl silica column. From the records, 2D RP–NP-chromatograms were constructed for the Slovanik sample (Fig. 7) and the numbers of the EO and PO units in the individual fractions were calculated (see Fig. 8).

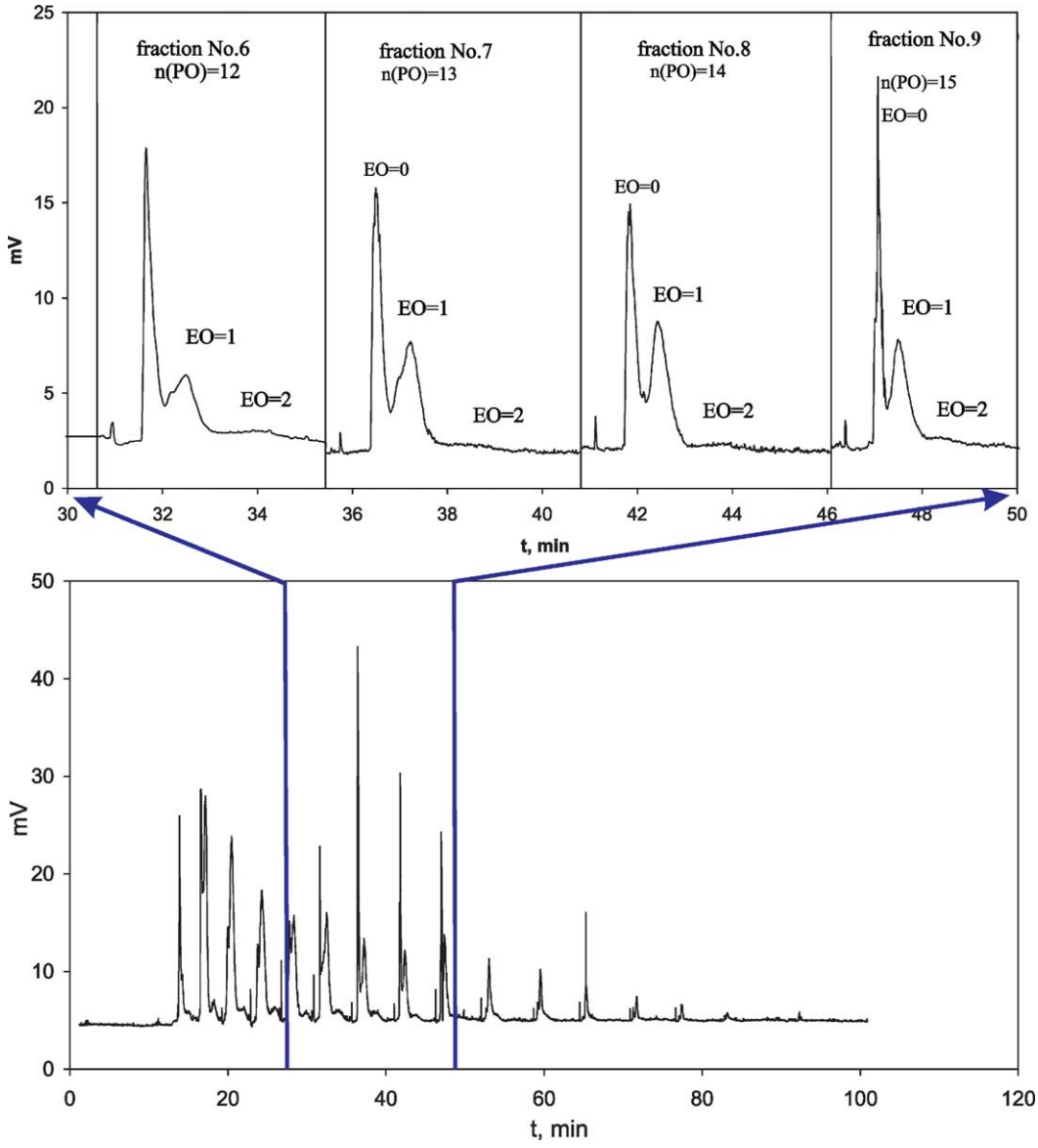

Fig. 6. Two-dimensional RP–NP in-line separation of a Slovanik 1010 sample. Dimension 1, RP: Zorbax Extend C18 column, 5 μm, 150 mm × 3.3 mm, linear gradient 50–100% ACN in 110 min at 0.2 ml/min, 40 °C. Dimension 2, NP: Separon SGX Amine, 5 μm, 150 mm × 1 mm i.d., 5% 2-propanol in hexane, 0.5 ml/min, 40 °C. Lower chromatogram: the ELSD record at the outlet from the second dimension NP column of consecutive 5 μl fractions heart-cut at the maxima of the first dimension RP column (details the separation of consecutive RP fractions 6–9 in the upper four chromatograms).

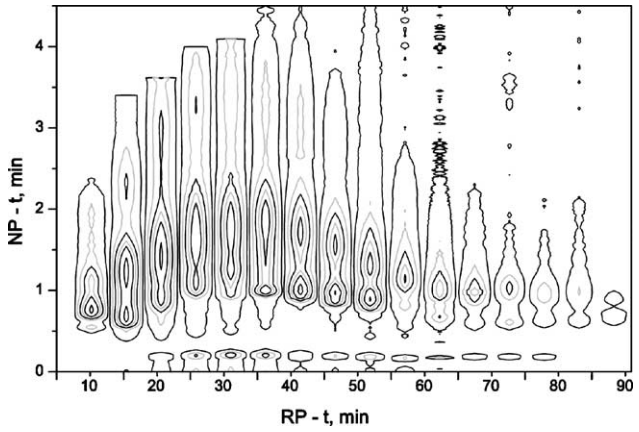

Fig. 7. 2D RP–NP chromatograms of a Slovanik 1010 sample. Conditions as in Fig. 6, *t*: time from the start of the analysis.

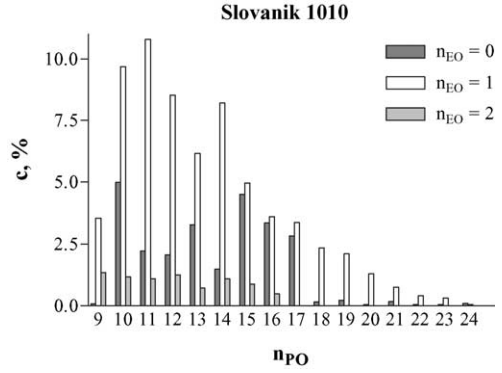

Fig. 8. Distribution of EO and PO units in a Slovanik 1010 samples. The concentrations, *c*, were evaluated from normalized peak areas in 2D in-line RP–NP chromatograms. n_{EO} and n_{PO} are the numbers of EO and PO monomer units, respectively.

4. Conclusions

Correlation between the selectivities for the individual repeat structural elements in two-dimensional samples can be used as a tool for selection of suitable separation systems. The selectivities for double bonds in unsaturated fatty acid triacylglycerols were found to strongly differ for various reversed-phase columns in non-aqueous reversed-phase gradient LC, providing significant differences in resolution of vegetable oil samples.

Further, correlations between the selectivity of various LC systems for regular repeat structural elements provide a useful tool for quantitative characterization of system orthogonality. Appropriate selection of suitable separation selectivity for an (at least partially) orthogonal 2D system is very helpful in the development of two-dimensional separations, as it provides more regular coverage of the retention space than non-orthogonal systems.

Gradient RP separation in the first dimension (90 min at 0.2 ml/min) and isocratic (micro-column) NP separation in the second dimension (5 min at 0.5 ml/min) enabled adequate 2D separation of EO–PO (co)oligomer samples. Due to the system orthogonality with respect to the EO unit distribution and an almost constant width of peaks in the first (RP gradient) dimension, the heart-cut fractions corresponding to the centres of the peaks from the first dimension contain representative oligomer distribution (see vertical spacing of (co)oligomers containing various numbers of EO groups in Fig. 3B). In a non-orthogonal system, reliable results could be obtained only using comprehensive 2D LC, where the whole eluate from the RP dimension 1 should be transferred in subsequent fractions to the NP dimension 2.

Real time comprehensive RP–NP 2D LC separations are principally difficult, as the speed and efficiency of separation in the second D should allow real time fraction collection and transfer from the first D. Further, the volume of transferred fractions should be sufficient for good detection sensitivity, but should also account for the mobile phase compatibility in the two dimensions. Column dimensions, flow-rate, sampling size and interval, mobile phase composition and (or) solvent (temperature) program should match the separation selectivity in the two dimensions.

Acknowledgements

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