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Separation and characterization of oligomers by reversed-phase high-performance liquid chromatography; a study on well-defined oligothiophenes

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) was used for the separation of 3-hexylthiophene oligomers in the range of 3 to 30 monomeric units, while systematically varying stationary and mobile phases. A set of different columns was chosen, covering a broad range of silica types, pore sizes and bonding chemistry. Mobile phases of tetrahydrofuran (THF) combined with water, acetonitrile (ACN) or methanol (MeOH) were used. Although differences between columns were small, a higher selectivity correlated with a lower hydrophobicity parameter from the Galushko column test. The model of Jandera, based on the linear solvent strength model of Snyder, was used to describe the retention of the oligomers in gradient mode. This gave information about selectivities on different stationary phases similar to the hydrophobicity parameter. Contrary to the stationary phase, the mobile phase had a major influence on the selectivity. The THF–water combination gave much higher selectivities compared to THF combined with MeOH or ACN. Using the aqueous mobile phase even enabled separation of different isomers. Determination of thermodynamic parameters for the model compounds showed that retention of the different isomers was mainly determined by the orientation of the side chains at both ends of the chain. An additional repeating unit in the middle of the polymer backbone gave a similar contribution to retention, irrespective of the orientation of its side chain. Three model isomers were separated by preparative RP-HPLC and identified by proton nuclear magnetic resonance spectroscopy. The combination of subsequent preparative size-exclusion chromatography, RP-HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectrometry enabled the identification of the two major oligomeric series in the sample as the regioregular product with one bromine end group and, in smaller amounts, a regioirregular product with two bromine end groups.

Keywords: Oligomers; Nuclear magnetic resonance spectroscopy; Matrix-assisted laser desorption ionization time-of-flight mass spectrometry; 3-Hexylthiopene; Oligothiopenes

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1. Introduction

The analysis and characterization of oligomers by gradient reversed-phase high-performance liquid chromatography (RP-HPLC) is often reported in literature. The separation mechanisms however are still not completely understood [1–3]. This results in the use of many different chromatographic systems. Typically C_8 or C_{18} modified silica stationary phases of different pore sizes, and mobile phases consisting of tetrahydrofuran (THF) or dichloromethane as strong solvent combined with non-solvents like methanol (MeOH), acetonitrile (ACN) or water are used [4–8].

For this study we selected oligomers of the 3-alkylthiophene type, which presently get a lot of attention for their electrical and optical properties. The final properties of these polythiophene materials in the solid state are determined to a great extent by the size and orientation of the alkyl side chains. Attention is therefore focused on the synthesis of well-defined chemical structures. Polymerization of 3-alkylthiophenes has recently been extensively reviewed and optimal properties are reported for *regioregular* polymers, in which predominantly head-to-tail (HT) linkages are present [9–11]. In Fig. 1 the basic structures of regioregular and regioirregular 3-alkylthiophenes are presented.

The regioregular poly(3-hexylthiophene)s, hereafter denoted ''(3HT)*n*'', investigated here were prepared according to the McCullough procedure [12,13]. In this procedure the thiophene monomer is polymerized using a catalytic amount of $Ni(dppp)Cl_2$ to give predominantly regioregular (3HT)*n*. The target oligomers were isolated from the crude reaction mixture by Soxhlet extraction with hexane. In some cases new end groups were introduced by modification of the terminal bromines [14–17].

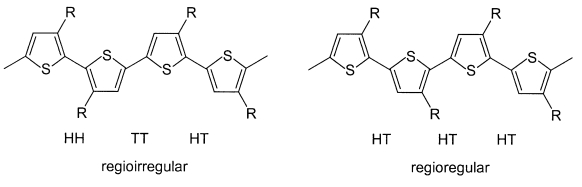

Fig. 1. Regioregular monosubstituted polythiophenes contain only head-to-tail (HT) linkages, whereas the regioirregular analogues contain also head-to-head (HH) and tail-to-tail (TT) linkages.

Investigations on these low-molecular-mass oligomers are highly interesting from both polymer and analytical point of view. Since many properties vary significantly with chain length at the lower mass range, the structure–property relationships can be studied relatively easy. As an example, the absorption maximum shifts from 360 nm for the smallest oligomer in the sample to 440 nm for the larger oligomers. Insight in these structure–property relationships can also be extrapolated to higher molecular masses. Furthermore, the well-defined structure of these oligomers enables detailed studies of their chromatographic behavior in RP-HPLC, contributing to a more fundamental understanding of oligomer separations in general.

To investigate the influence of the stationary phase on the separations of these oligomers a set of different columns was selected, covering a broad range of carbon loads, silica types and pore sizes. To study their influence quantitatively, these phases must be characterized. In the first instance we used the well-known Galushko test [18] for that purpose. In this model the stationary phase C_{18} layer is assumed to be a quasi-liquid with its own characteristics (i.e., surface tension and dielectric constant). Retention of a solute is determined by the difference of solvation energy in the mobile phase and in the surface layer. This test method uses a well-defined, isocratic mobile phase composition of MeOH–water (60:40) and a set of specific test solutes; uracil (as t_0 marker), aniline, phenol, benzene and toluene. From the retention data and the known molecular descriptors particular column parameters like stationary phase hydrophobicity and polarity are calculated.

Alternatively, we investigated the use of the retention model of Jandera for the evaluation of the different columns. This model is based on the linear solvent strength (LSS) model of Snyder, which derives a set of equations to describe the retention in gradient elution [19]. Jandera et al. successfully extended the LSS model to describe the retention of oligomers or homologues in RP-HPLC [20]. This model assumes a linear relation between the logarithm of the retention factor and the volume fraction of strong solvent (Eq. (1)):

$$\ln(k) = a - m\varphi \quad (1)$$

where φ is the volume fraction strong solvent and a

and m are the regression coefficients. For a linear gradient, where the volume fraction of strong solvent increases linearly with time, the retention time can be described by Eq. (2):

$$t_R = \frac{t_0}{b} \cdot \ln(k_0 b) + t_0 + t_D \quad (2)$$

where:

$$\ln(k_0) = a - m\varphi_0 \quad (3)$$

$$b = aBt_0 = \frac{a\Delta\varphi t_0}{t_G} \quad (4)$$

where t_0 is the retention time of a non retained solute, b is the gradient steepness parameter, k_0 is the retention factor of the solute for the initial eluent composition, t_D is the ''dwell'' time, i.e., the time it takes before the eluent composition at the column inlet first changes after the gradient start, φ_0 is the volume fraction strong solvent at the gradient start, B is the gradient steepness (1/min), $\Delta\varphi$ is the difference of volume fraction strong solvent between begin and end of the gradient and t_G is the gradient time. The analytes are supposed to have negligible migration in the column during the dwell time.

The a and m values in Eq. (1) that describe the retention behavior of a solute can be determined from the two unknown parameters in Eq. (2), b and k_0. This is most conveniently accomplished by performing two gradient runs with the same experimental conditions, except for the gradient steepness. In order to estimate the reliability of this approach in this study three gradient runs with different steepnesses were performed. This allowed calculations for two independent combinations, thus providing an estimation about errors in a and m values and an indication whether model assumptions were met.

When a and m values are calculated for a series of homologues or oligomers, linear relationships are often found between the values of a and m and the number of repeating units, n [21]. In that case the correlation coefficients a_0, a_1, m_0 and m_1, as defined by the model of Jandera (Eqs. (5) and (6)), describe the retention properties for a series of oligomers for that specific chromatographic system:

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

$$m = m_0 + m_1 n \quad (6)$$

The m_1 parameter is of special interest, since it describes the retention for different THF concentrations in the mobile phase as a function of the number of repeating units. Here we have investigated whether these parameters could be used for further column evaluation.

Besides column characterization and investigation of the potential of these parameters for oligomer separations, another goal of this study is to understand the thermodynamic backgrounds of these separations. Information about retention mechanisms in isocratic elution can be obtained when measurements are repeated at several different temperatures and Van 't Hoff plots are constructed. This approach has also been used for several oligomers and homologous series [22–25]. Since isocratic elution is difficult to perform for the original oligomeric mixture, we decided to use a set of thiophene model compounds of well-defined structure. The retention factor of a solute is given by:

$$\ln(k) = \frac{-\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln(\Phi) \quad (7)$$

where k is the retention factor of the solute, ΔH^0 and ΔS^0 are the standard enthalpy and standard entropy, respectively, of transfer of the solute from the mobile phase to the stationary phase, R is the gas constant, T is the absolute temperature and Φ is the phase ratio of the column. In principle the ΔH^0 and ΔS^0 values can be calculated from plots of $\ln(k)$ versus $1/T$ (the Van 't Hoff plot). However, for calculation of ΔS^0 the phase ratio must be known. Since this phase ratio is not known for many commercially available columns, differences between retention factors of the solute and some reference compound can be used, resulting in values for $\Delta\Delta H^0$ and $\Delta\Delta S^0$.

To identify the different oligomers separated by RP-HPLC we used matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). In recent years this technique has found many different applications in polymer and oligomer characterization [26–30]. Polymers up to very high molecular masses (>1 MDa) can be measured with very little or no fragmentation [31]. For lower molecular masses the high resolution

allows detection of copolymer composition or individual end groups [32–35]. MALDI-TOF-MS was used in this study for the determination of the number of monomeric units and type of end groups for individual peaks collected from RP-HPLC.

The intention of this study is twofold: (i) to develop an efficient analysis procedure for a specific type of oligomer, in particular to distinguish between the several possible side chain conformations and end groups, (ii) to contribute to a more fundamental understanding of the separation of oligomers in general. We particularly investigated the influence of the reversed-phase columns and mobile phase compositions on these separations.

In this paper we first present the investigations on the chromatographic behavior of (3HT)*n*. After discussing the influence of the stationary and mobile phases we study the separation mechanism for a set of thiophene model compounds by evaluation of thermodynamic parameters. The detailed characterization of the (3HT)*n* mixture by the combination of chromatographic techniques and MALDI-TOF-MS is discussed in the last part.

2. Experimental

2.1. Equipment

HPLC measurements were performed on a HP 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany), equipped with a diode-array detector and a HP Chemstation for process control and data handling. The detector was set at 400 nm.

Preparative HPLC was performed on a system consisting of a Waters DELTA PREP 4000 pump, an absorbance detector Model 486, a system controller Model 4000 (Waters, Milford, MA, USA) and a Rheodyne injection valve Model 7010 (Rheodyne, Cotati, CA, USA) equipped with a 50-μl injection loop. Data acquisition and handling were done by means of a Nelson Analytical interface Model 760 and Nelson 2600 chromatography software (Nelson Analytical, Cupertino, CA, USA).

The MALDI-TOF-MS measurements were performed with a Voyager-DE Pro (PerSeptive Biosystems, Framingham, MA, USA) in reflectron mode with delayed ion extraction. Spectra were averaged over 256 laser shots. Theoretically expected masses and isotope patterns of the oligomers were calculated with the shareware program IsoPro 3.0.

Proton nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-400 spectrometer (Bruker Analytik, Rheinstetten, Germany).

2.2. Materials

Solvents used for HPLC were methanol, acetonitrile and tetrahydrofuran, all supra-gradient grade, obtained from Biosolve (Bio-Lab, Jerusalem, Israel) and were filtered prior to use. Water was prepared with a Milli-Q purification system (Millipore, Milford, MA, USA). Mixtures and gradients were made by volumetric mixing by the HPLC pump.

Uracil (Fluka, Buchs, Switzerland) was used to measure column dead volume. Test solutes were aniline, phenol, both from Merck (Darmstadt, Germany) and benzene and toluene, both from Aldrich (Milwaukee, WI, USA).

The preparative HPLC column was packed with Zorbax C_{18} 10 μm stationary phase using acetone (Merck) as a slurry solvent.

The matrix used for the MALDI-TOF-MS measurements was α-cyano 4-hydroxy-cinnamic acid from Aldrich.

2.3. Columns

The analytical columns used were a Nova-Pak C_{18} column (150×3.9 mm, d_p=4 μm, pore size 6 nm), a Nova-Pak C_8 column (150×3.9 mm, d_p=4 μm, pore size 6 nm), a Symmetry C_{18} column (150×3.9 mm, d_p=5 μm, pore size 10 nm), a Symmetry300 C_{18} column (150×3.9 mm, d_p=5 μm, pore size 30 nm), all from Waters, a Zorbax SB300 C_{18} column (250×4.6 mm, d_p=5 μm, pore size 30 nm) and a Zorbax Extend C_{18} column (150×4.6 mm, d_p=5 μm, pore size 8 nm), both from Hewlett-Packard (Agilent Technologies, Newport, DE, USA), and an Alltima C_{18} column (150×4.6 mm, d_p=5 μm, pore size 11 nm) from Alltech (Deerfield, IL, USA).

The preparative column was packed in our laboratory using the Self Packing Preparative Column System (Merck) consisting of a stainless steel column (250×25 mm) and a hydraulic pump for the axial compression of the packed bed by a piston.

2.4. Procedures

The study of the different columns were performed with the Nova-Pak C_{18} column as a reference. Measurements on all other columns were performed with adjusted flow-rate (to preserve an identical linear flow velocity through the column) and gradient time (to obtain an identical gradient steepness parameter *b*, see Eq. (5)).

In gradient elution mode the gradient was started at the time of injection. The dwell volume of 0.95 ml was determined from the (extrapolated) intersection point of the stable baseline before the start of the gradient and the increasing baseline during the gradient. Before the analysis of samples a blank gradient was run. At the end of each gradient the eluent composition was gradually set back to the starting values and 20 column volumes were pumped through the column for equilibration prior to the next analysis. Samples were dissolved in THF and small volumes were injected (typically less than 5 μl).

The results of the Galushko test were obtained using the software program ''Chromlife'' (Merck).

The preparative column was packed using a standard packing procedure for reversed-phase stationary phases, using acetone as a slurry solvent [36].

Preparative size-exclusion chromatography (SEC) was performed using a 50×5 cm glass column under gravitation. The column was packed with Bio-Beads S X1 stationary phase from Bio-Rad (Hercules, CA, USA). This stationary phase material allows separations in the range of 600 to 14 000 Da. Dichloromethane (Biosolve) was used as mobile phase.

Samples for the MALDI-TOF-MS measurements were collected at the RP-HPLC outlet and, without further sample preparation, mixed with the α-cyano 4-hydroxy-cinnamic acid solution (10 mg/ml in THF) in sample–matrix ratios of 2:1, 1:1 and 1:2. A 0.5-μl aliquot of these solutions was pipetted on the target plate and dried at room temperature. Best results were obtained for one sample–matrix ratio, depending on the sample concentration after RP-HPLC.

Proton NMR spectra were recorded in deuterated chloroform (CIL, Andover, MA, USA). Chemical shifts are reported in ppm downfield from trimethylsilyl (TMS).

3. Results and discussion

3.1. Stationary phases

To investigate the influence of the stationary phase on the separation of oligomeric samples by RP-HPLC a set of columns was selected. This selection covered a sufficient range of silica types, pore sizes and bonding chemistry of the columns. More specifically, the set of columns comprised of C_8 and C_{18} ligands, mono-, di- and polyfunctionally attached to the substrates and endcapped as well as non-endcapped after synthesis. At first all columns were tested using the Galushko test and the hydrophobicity, polarity, NH_2 interactions and the hydrophobic selectivity were calculated. This enables the quantitative comparison of column properties. In Fig. 2 the results for all columns are summarized, together with the plate numbers.

The results of the Galushko test show that hydrophobicity and NH_2 interactions vary significantly (from 1.6 to 11.0 and from 0.12 to 0.96, respectively) within the set of columns, while other parameters show much less variation within the investigated column set (less than 40%). The low variation for the polarity and hydrophobic selectivity are remark-

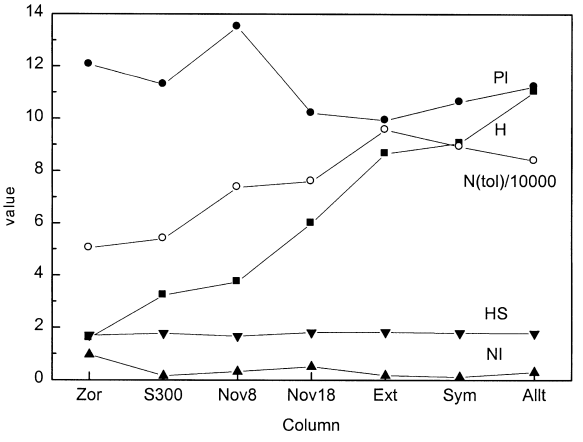

Fig. 2. Galushko parameters for the different columns. Abbreviations Pl, polarity; H, hydrophobicity; HS, hydrophobic selectivity and NI, NH_2 interactions. $N_{(tol)}$ is the plate numbers calculated from the toluene peak (plates per meter/10 000). Column abbreviations: Zor, Zorbax SB C_{18} 300; S300, Symmetry C_{18} 300; Nov8, Nova-Pak C_8; Nov18, Nova-Pak C_{18}; Ext, Extend C_{18}; Sym, Symmetry C_{18} and Allt, Alltima C_{18}.

able results when considering the different columns in this set, ranging from more classical stationary phases like the Nova-Pak to more recent generations of columns like the Alltima and Extend.

The separation of the (3HT)*n* requires gradient elution, and to compare the columns, all flow-rates and gradient times were adjusted in order to obtain identical linear flow-rates through the column and the same gradient steepness parameter *b* (see Eq. (4)). From the resulting chromatograms now peak positions can be easily expressed as alternative retention factors (*k**). These latter values can be considered as standardized retention volumes of the several analytes. Contrary to isocratic measurements, where *ratios* are used to calculate selectivity, we decided to use the *differences* in *k** values to characterize separation performance under gradient conditions. Consequently, absolute retention will not affect these selectivities, which seems a reasonable assumption in gradient elution mode.

In order to describe in detail the separation potential of the investigated columns and eluents for these particular (3HT)*n* we decided to use several different selectivities. These selectivities were calculated from *k** values of selected peaks (indicated peaks A to G). This way parameters like size selectivity for low-molecular-mass oligomers and isomer selectivity for intermediate molecular mass were defined. Several separation windows were also defined, representing a range in which all oligomers of a certain length will elute. A list of applied selectivities is presented in Table 1. For THF (solvent) and water (non-solvent) the resulting chromatograms for the (3HT)*n* separation obtained on the different columns are presented in Fig. 3, which also include the peaks A to G. Fig. 4 shows the calculated selectivities obtained from the data of Fig. 3, numerical values are also given in Table 2.

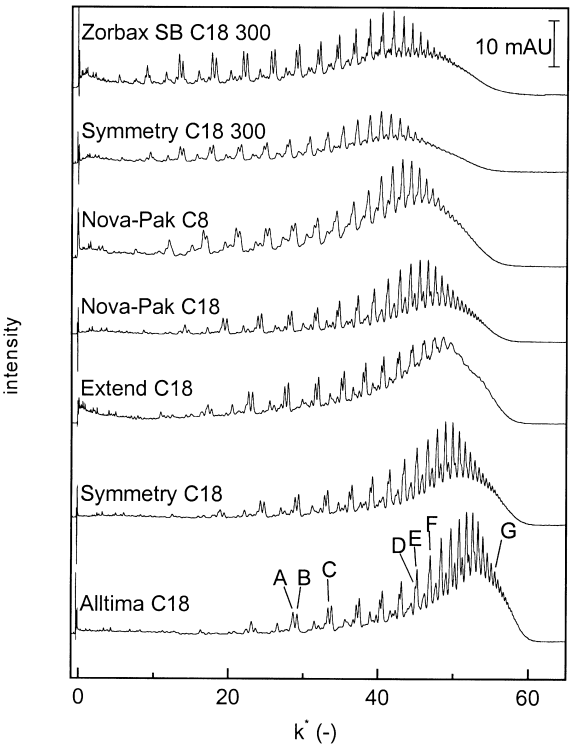

Fig. 3. RP-HPLC of (3HT)*n* with thiophene end groups on the investigated columns, gradient starts at THF–water (60:40) (equiv. 0.5 %/min), temperature 30°C, UV detection at 400 nm. Flow-rates and gradient times adjusted to obtain identical linear flow velocity through the column and identical gradient steepness parameter *b*.

Visual inspection of the chromatograms for the different columns reveals that all columns separate the oligomers in a similar fashion; the first, smallest, oligomers are separated very well, showing a lot of detail, while with increasing length the peaks get closer until no individual peaks can be distinguished. However, more variation between the different columns can be observed for the separation of larger

Table 1
Calculated differences in *k** values, peaks are indicated in Fig. 3

$k^*_B - k^*_A$	Isomer selectivity at low molecular mass ($n=4$)
$k^*_C - k^*_A$	Size selectivity at low molecular mass ($n=5-n=4$)
$k^*_D - k^*_A$	Separation window for low-molecular-mass oligomers (range $n=4$ to 10)
$k^*_G - k^*_A$	''Total'' separation window (range $n=4$ to 20)
$k^*_E - k^*_D$	Isomer selectivity at intermediate molecular mass ($n=9$)
$k^*_F - k^*_E$	Size selectivity at intermediate molecular mass ($n=10-n=9$)
$k^*_G - k^*_D$	Separation window for high-molecular-mass oligomers (range $n=9$ to 20)

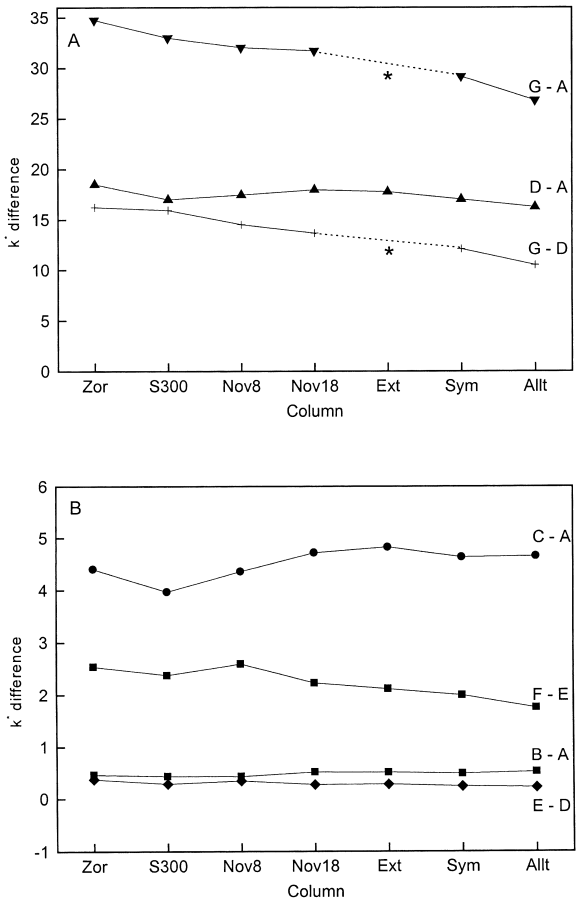

Fig. 4. (A) and (B) Selectivities as defined in Table 1 for the different columns. Columns ordered with decreasing total separation window. Column abbreviations: Zor, Zorbax SB C_{18} 300; S300, Symmetry C_{18} 300; Nov8, Nova-Pak C_8; Nov18, Nova-Pak C_{18}; Ext, Extend C_{18}; Sym, Symmetry C_{18} and Allt, Alltima C_{18}. *Data not available.

oligomers as compared to smaller oligomers. For example, the oligomers of higher molecular mass elute in a much smaller range on the Alltima C_{18} column then on the Zorbax SB C_{18} 300 column.

Closer inspection of the selectivity data in Table 2 reveals that differences between columns are more pronounced for longer oligomers. For example, the size selectivity at low and intermediate molecular mass (C–A and F–E) vary 22 and 48%, respectively within the investigated column set. The same can be observed for the isomer selectivity and the separation window. In general however, the column type has limited influence on the separation of these oligomers.

Although differences between columns are small, a remarkable phenomenon appears in Fig. 4B. Here the size selectivity for low and intermediate molecular mass (C–A and F–E, respectively) are plotted for all columns, and it shows that for low molecular mass the Nov18, Ext, Sym and Allt columns have the highest selectivity, whereas at intermediate molecular mass these columns have the lowest selectivity. In other words, columns best suited for separations of low-molecular-mass analytes, are not necessarily optimal for separations of higher-molecular-mass analytes. The same can be concluded from the isomer selectivity at low and intermediate molecular mass (B–A and E–D, respectively). However, in this case the differences are smaller.

Furthermore, the effect of the pore size of the stationary phase can be illustrated by comparing the Symmetry C_{18} 300 (S300, pore size 30 nm) and Symmetry C_{18} (Sym, pore size 10 nm). These columns consist of the same stationary phase, except

Table 2
Pore sizes, column dead volume, V_0, and selectivities as defined in Table 1 for the different columns[a]

Column	Pore size (nm)	V_0 (ml)	$k^*_B - k^*_A$	$k^*_C - k^*_A$	$k^*_D - k^*_A$	$k^*_G - k^*_A$	$k^*_E - k^*_D$	$k^*_F - k^*_E$	$k^*_G - k^*_D$
Zorbax SB C_{18} 300	30	2.58	0.46	4.41	18.52	34.77	0.38	2.54	16.25
Symmetry C_{18} 300	30	1.31	0.44	3.97	17.02	32.97	0.29	2.38	15.95
Nova-Pak C_8	6	1.08	0.43	4.36	17.48	32.01	0.35	2.59	14.54
Nova-Pak C_{18}	6	1.05	0.52	4.72	17.99	31.68	0.28	2.23	13.69
Extend C_{18}	8	1.37	0.52	4.83	17.79	n.a.[b]	0.29	2.12	n.a.[b]
Symmetry C_{18}	10	1.07	0.49	4.64	17.05	29.17	0.25	2.00	12.12
Alltima C_{18}	11	1.49	0.53	4.66	16.27	26.79	0.23	1.76	10.52

[a] Columns are ordered with decreasing total separation window ($k^*_G - k^*_A$).
[b] Data not available.

for the pore size. Comparing the separation window for low- and high-molecular-mass oligomers, the S300 separation window is 30% larger for high molecular mass, while for low-molecular-mass oligomers their separation windows are identical. Not surprisingly, pore size plays a role in the separation of oligomers, since larger oligomers are excluded from the smaller pores and therefore have a lower retention. Of course this effect will be more evident for smaller pore size stationary phases.

Correlation between the several oligomer selectivities used in this study and the Galushko parameters is obvious when considering hydrophobicity; the higher the hydrophobicity, the lower the separation window. For example, the Zorbax SB300 C_{18} column has the largest separation window (34.8) and the lowest hydrophobicity (1.59) and the Alltima C_{18} column has the smallest separation window (26.8) and the highest hydrophobicity (11.0).

It is noteworthy that the Galushko hydrophobicity parameter gives useful information about the total separation window of these oligomers. Although this hydrophobicity is determined by measurements of small analytes, it still appears to be applicable for the separation of larger molecules as well. The correlation of other Galushko parameters with the separation of oligomers might improve if larger test analytes are used. Unfortunately, such larger test analytes with known molecular descriptors are not (yet) available.

Alternatively, we compared the column properties by describing the retention behavior of the major series of oligomers in the sample using the model of Jandera [20]. A linear relationship between the logarithm of the retention factor and the volume fraction strong solvent in the mobile phase is assumed in this model. The calculated regression coefficients, a and m, differed only slightly for two independent combinations of gradient runs with different gradient steepness (typically less than 4%), which was taken as proof that the assumption of linearity was correct. Similar to results reported for other types of oligomers [20,21], we found a linear dependence of the a or m values and n, the number of repeating units (results not shown).

In Table 3 the fit parameters a_0, a_1, m_0 and m_1 from the model Jandera, derived from the a and m data, are summarized. The m_1 parameter is of special interest, since it describes the retention for different THF concentrations in the mobile phase as a function of the number of repeating units, n. For the two large pore columns (Zor and S300), and the C_8 column (Nov8), the m_1 values are lowest, indicating the smallest decrease of retention factor for oligomers of increasing length with increasing THF concentration in the mobile phase. Thus these columns show the highest selectivity in gradient elution. This is in agreement with the results presented in Fig. 4, where the Zor, S300 and Nov8 columns have the largest separation window. Ordering the columns according to their m_1 values gives a slightly different result than the ordering according to their total separation window shown in Table 2, but both methods of describing length selectivity for these oligomers in gradient elution give comparable results.

Table 3
Regression coefficients for the equation $\ln(k) = a - m\varphi$, for (3HT)n oligomers with thiophene end groups ($n > 6$), with $a = a_0 + a_1 n$ and $m = m_0 + m_1 n$[a]

Column	a_0	a_1	m_0	m_1
Zorbax SB C_{18} 300	6.55	1.53	9.69	1.62
Symmetry C_{18} 300	8.31	1.26	11.72	1.32
Nova-Pak C_8	7.11	1.53	9.94	1.64
Nova-Pak C_{18}	5.91	1.76	8.29	1.89
Extend C_{18}	4.88	2.10	7.10	2.25
Symmetry C_{18}	6.19	1.79	8.01	1.95
Alltima C_{18}	4.59	2.21	5.90	2.38

[a] See text for details about calculations. Error of determination 3%, correlation coefficients >0.999.

It can be concluded that the nature of the (RP) stationary phase nature has only a limited effect on the separation of these oligomers and that from the Galushko column parameters only the hydrophobicity gives relevant information about the suitability of a stationary phase for these separations. The m_1 parameter from the model of Jandera correlates fairly well with the Galushko hydrophobicity parameter.

3.2. Mobile phases

So far it has been shown that the stationary phase has limited influence on the separation of the thiophene oligomers in RP-HPLC. Further investigations were performed to study the influence of the mobile phase on these separations. Because of

the low solubility of the (3HT)*n* we always used THF as a strong solvent, and only varied the non-solvent of the gradient. Water, ACN and MeOH were investigated as non-solvents. The Nova-Pak C_{18} column was used to illustrate the influence of the mobile phase on oligomer separations. The THF–ACN and THF–MeOH gradients were adjusted arbitrarily to obtain oligomer retention times similar to those obtained for the THF–water gradient. The resulting chromatograms are shown in Fig. 5. This figure clearly demonstrates the major influence of eluent nature and composition on these separations.

Unfortunately, quantitative comparison between these measurements is difficult, because the gradient steepnesses for different solvent combinations cannot be directly compared. The use of Hildebrand's solubility parameters, δ, could in principle be used to compare eluent strength [37]. However, from Fig. 5 it can be observed that MeOH is a stronger solvent than ACN, while using the δ values ($cal^{1/2}\ cm^{-3/2}$; 1 cal = 4.1868 J) of 9.9 for THF, 13.1 for ACN, 15.9 for MeOH and 25.5 for water, we would expect ACN to be a stronger solvent than MeOH. Apparently we cannot use these solubility parameters to describe solvent strength in this case. However, it is clear, from visual inspection alone, that the selectivity of these separations is much more influenced by the mobile phase than the stationary phase.

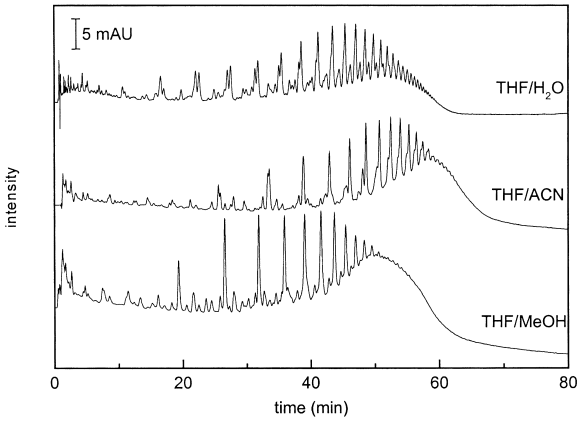

Fig. 5. RP-HPLC of (3HT)*n* with thiophene end groups, Nova-Pak C_{18} column, temperature 30°C, UV detection at 400 nm. Gradients: THF–MeOH (0:100–80:20) (80 min), THF–ACN (0:100–80:20) (80 min) and THF–water (60:40–100:0) (80 min).

3.3. Model compounds

In order to study the separation of thiophene oligomers in more detail, we constructed Van 't Hoff plots and determined the enthalpic and entropic contribution to retention. For this purpose isocratic retention data at different temperatures were required. Since isocratic elution is difficult to perform for the original (3HT)*n*, we decided to use model compounds. For this purpose, a set of thiophene oligomers of well-defined structure were synthesized. The chemical structures of these model compounds are comparable to the original (3HT)*n*. The structures are presented in Fig. 6. We used the monomer [denoted $T_3(C_{12})$, structure A], the dimer [$T_6(C_{12})_2$, structure B] and the trimer [$T_9(C_{12})_3$, structure C]. Dimerization of the asymmetric monomeric unit

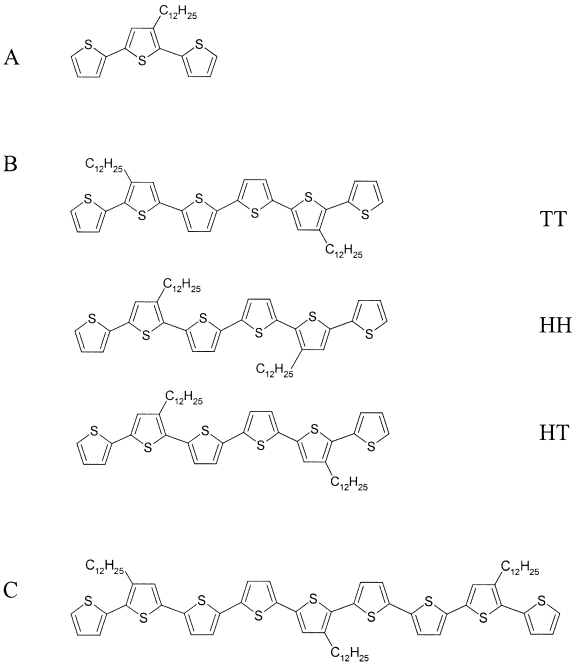

Fig. 6. (A) Structure of repeating unit of the model compounds. (B) Structures of $T_6(C_{12})_2$ that are formed by coupling of two molecules shown in (A). Three structures differ in the orientation of the side chains: both orientated outwards (TT, tail-to-tail), inwards (HH, head-to-head) or in the same direction (HT, head-to-tail). (C) One of the possible structures of $T_9(C_{12})_3$. Three other isomers possess other orientation of the dodecyl side chains, similar to (B).

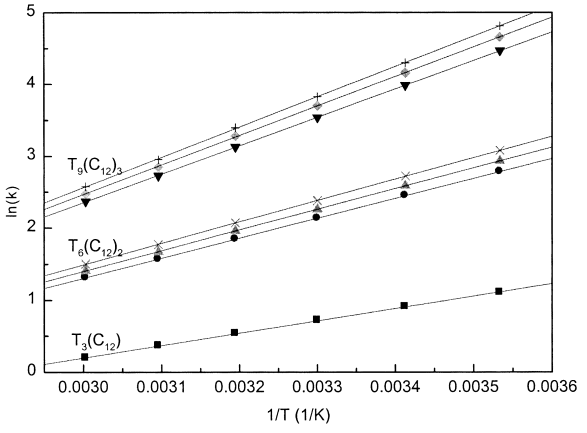

Fig. 7. Van 't Hoff plots for model compounds $T_3(C_{12})$, $T_6(C_{12})_2$ and $T_9(C_{12})_3$ on the Nova-Pak C_{18} column, mobile phase: THF–water (67:33), temperature ranging from 10 to 60°C.

results in three isomers; head-to-head (HH), head-to-tail (HT) and tail-to-tail (TT) coupling. If coupling is completely random, a ratio HH–HT–TT of 1:2:1 is expected. Similarly four isomers in the ratio 1:1:1:1 are expected for the trimer.

To further investigate the retention mechanism we also studied the influence of the THF concentration in the mobile phase on the thermodynamic parameters for the Nova-Pak C_{18} column using THF–water mobile phases. For every mobile phase composition three baseline separated peaks, representing the three isomers, were observed for $T_6(C_{12})_2$. $T_9(C_{12})_3$ also showed three baseline separated peaks, while four isomers were expected. Since from statistics four isomers are expected in equal amounts, the middle, largest, peak probably contains two isomers. An typical example of the constructed Van 't Hoff plots is presented in Fig. 7. The data nicely fit a linear correlation ($R>0.999$), indicating a similar retention mechanism over the whole range of investigated temperatures.

The thermodynamic parameters were calculated from the regression coefficients of the linear fits; the enthalpy (ΔH^0) from the slope of the line, the entropy (ΔS^0) from the intercept. For the latter the phase ratio of the column must be determined, which is not straightforward. To circumvent this problem a reference compound was defined, namely the $T_3(C_{12})$ model compound. Thus the calculated $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values give the extra enthalpic and entropic contributions to retention, relative to this $T_3(C_{12})$ model compound. The results for different THF concentrations in the mobile phase are presented in Table 4. The ratio of enthalpic over entropic contribution at 40°C is also given to facilitate comparison between measurements.

Table 4 shows that both enthalpic and entropic contributions to the retention decrease (become more negative) with longer oligomers. As the entropy changes most, the enthalpy–entropy ratio decreases.

Table 4
Enthalpic and entropic contribution to retention on the Nova-Pak C_{18} column, as a function of φ, volume fraction of THF in water[a]

Compound	$\varphi=0.61$			$\varphi=0.67$			$\varphi=0.73$		
	$-\Delta\Delta H^0$	$-\Delta\Delta S^0$	Ratio	$-\Delta\Delta H^0$	$-\Delta\Delta S^0$	Ratio	$-\Delta\Delta H^0$	$-\Delta\Delta S^0$	Ratio
$T_6(C_{12})_2$									
HH	13.1	28.1	1.49	8.8	16.9	1.66	5.3	8.4	2.02
HT	14.3	30.9	1.48	9.6	18.8	1.63	5.8	9.5	1.95
TT	15.6	33.9	1.47	10.5	20.5	1.64	6.4	10.8	1.89
$T_9(C_{12})_3$	27.7	61.6	1.44	18.6	37.9	1.57	11.3	19.6	1.84
	29.3	65.0	1.44	19.9	40.8	1.56	11.9	20.8	1.83
	30.7	68.3	1.44	20.8	42.6	1.56	12.5	22.1	1.81
$T_9(C_{12})_3$–$T_6(C_{12})_2$	14.6	33.5	1.39	9.8	21.0	1.49	6.0	11.2	1.71
	15.0	34.1	1.41	10.3	22.0	1.50	6.1	11.3	1.72
	15.1	34.4	1.40	10.3	22.1	1.49	6.1	11.3	1.72

[a] $T_3(C_{12})$ used as a reference compound, $\Delta\Delta H^0$ in kJ/mol, $\Delta\Delta S^0$ in J/mol K, the ratio $\Delta\Delta H^0/T\Delta\Delta S^0$ at 40°C. $T_9(C_{12})_3$–$T_6(C_{12})_2$ gives the extra contribution to retention of the middle monomeric unit for isomers with the same orientation of the side chains of the first and last monomeric unit.

With increasing THF concentration in the mobile phase both $\Delta\Delta H^0$ and $\Delta\Delta S^0$ increase (become less negative), and the enthalpy–entropy ratio increases. These results are in agreement with data earlier reported for polystyrene and polyester oligomers [22]. Obviously, increasing the THF concentration in the mobile phase will increase the affinity of the solute to the mobile phase, thus increasing $\Delta\Delta H^0$. The increase of $\Delta\Delta S^0$ with increasing THF concentration is more difficult to explain. A higher THF concentration in the mobile phase will cause a higher entropy of the solutes in the mobile phase, and thus a decrease of $\Delta\Delta S^0$. The entropy of the solute in the stationary phase must consequently increase to compensate for this effect. A possible explanation for a decrease of ordering in the stationary phase with higher THF concentration is the absorption of THF in the bonded phase layer. This will increase the swelling of the stationary phase, causing the C_{18} chains to become more flexible and thus less ordering of the solutes (partly) present in this bonded layer.

The three well-separated isomers of $T_6(C_{12})_2$ (HH, HT and TT) show significantly different $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values. The interaction between a neutral solute and stationary and mobile phase in RP-HPLC is mainly determined by the analyte's molar surface [24]. Hence, the decrease in $\Delta\Delta H^0$ values when going from HH to TT might be explained by an increase of molar surface, caused by the more ''outward'' orientation of the dodecyl side chains. The decrease of $\Delta\Delta S^0$ values for this HH, HT and TT series can be the result of a increasing entropy of the solute in the mobile phase (less hindering between the two side chains), or a decreasing entropy in the stationary phase (more restricted by the spreading of the dodecyl side chains).

For the $T_9(C_{12})_3$ four isomers were expected, while only three peaks were detected. The middle, largest, peak probably contains two isomers. Similar to the $T_6(C_{12})_2$ isomers, the isomer with lowest retention is assumed to be the isomer with both side chains of the first and last monomeric unit orientated inwards, the one with highest retention the isomer with both side chains orientated outwards. The two remaining isomers, both having one side chain orientated inwards and one outwards at both ends of the chain, co-elute in between the other isomers. Retention seems to be mainly determined by the orientation of the two dodecyl side chains of the first and last monomeric unit of the chain. Consequently, the extra monomeric unit of the $T_9(C_{12})_3$ isomers when compared to the $T_6(C_{12})_2$ isomers should have the same contribution to retention for all three pairs of isomers having the same side chain orientation. The extra enthalpic and entropic contributions to the retention for the extra monomeric unit, given in the bottom rows of Table 4, show that indeed $\Delta\Delta H^0$ and $\Delta\Delta S^0$ values are reasonably constant at each investigated mobile phase composition. It can therefore be concluded that the isomers can be separated when using a THF–water mobile phase and the retention is mainly determined by the orientation of the dodecyl side chains of the first and last monomeric units.

In an attempt to compare different stationary and mobile phases by the evaluation of thermodynamic parameters, we repeated the measurements of the model compounds on two other columns (Zorbax SB C_{18} 300 and Symmetry C_{18}). Besides THF–water also THF–MeOH and THF–ACN mobile phases were used on the selected columns (results not shown). Separation of isomers occurred on all selected columns and was more pronounced if a THF–water mobile phase was used.

3.4. Characterization

In an attempt to identify in detail the main compounds in the (3HT)*n* mixture, a combination of SEC, RP-HPLC and MALDI-TOF-MS was used. First the original (3HT)*n* mixture was fractionated by preparative SEC. Several fractions of different molecular masses were selected which typically contained between 4 and 10 different oligomers (differing in chain length and end groups) of similar molecular mass. These fractions were further separated using RP-HPLC in the isocratic mode and individual peaks were collected. Sufficient amounts of material for the MALDI-TOF-MS measurements were provided in a single run. The fractions were mixed with the matrix solution (α-cyano 4-hydroxycinnamic acid, 10 mg/ml in THF) without further sample pretreatment.

In Fig. 8 a chromatogram of the original oligomer mixture is shown. It was obtained by using a Nova-Pak C_{18} column with a THF–water gradient. As an

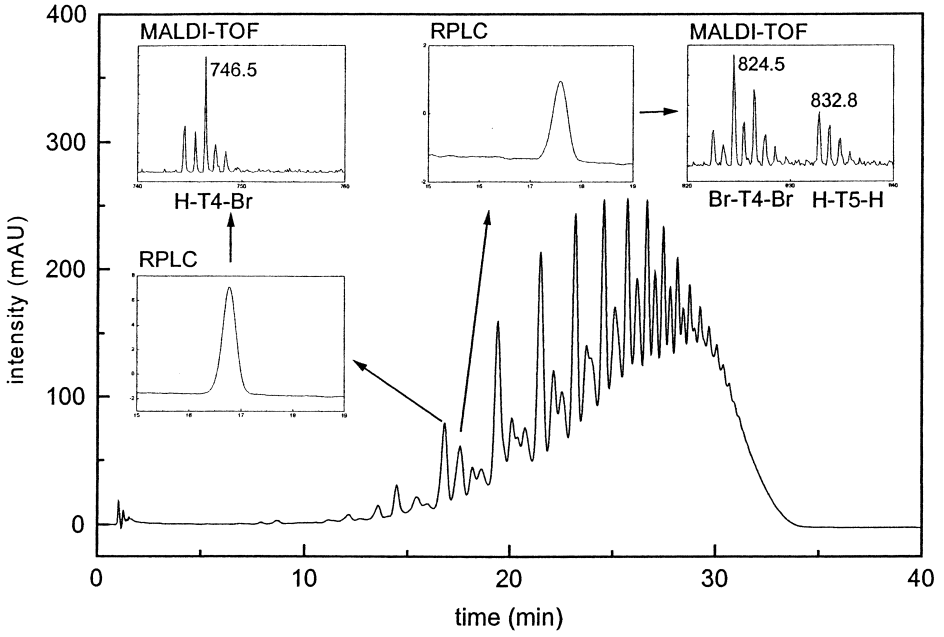

Fig. 8. HPLC of (3HT)*n*, with RP-HPLC of two collected peaks representing the two major oligomeric series and their MALDI-TOF spectra. Nova-Pak C_{18} column, gradient THF–water (60:40) (1%/min), temperature 30°C, UV detection at 400 nm.

example, inserts show the chromatogram and the mass spectra of two collected peaks, typical for the two main oligomer distributions present in the sample. Comparison of the measured masses and isotope patterns with the theoretically calculated ones show that the main products are oligomers with one bromine end group. This corresponds with the expected main reaction products, oligomers with monomers coupled in a regioregular head-to-tail fashion, with one bromine end group [12,13].

The other oligomeric series were identified to be oligomers with two bromine end groups. From the polymerization mechanism this can be explained by the activation of the catalyst, resulting in a regioirregular coupling, see Ref. [15]. Some small quantities of chains with two hydrogen end groups were also found. These were formed by de-bromination during polymerization.

Two possible explanations for the difference in retention between the two oligomers of the same length, but with one and two bromine end groups can be given. The extra retention could be caused by either the presence of an extra bromine end group (instead of a hydrogen), or by the orientation of the side chains. When an oligomer contains two bromine end groups, there must be a regioirregular coupling in the polymer backbone [15]. Consequently, the side chains are orientated outwards, increasing the size or molar surface of the molecule, and thus increasing retention. As was discussed above for the measurements of thermodynamic parameters using the model compounds, the retention is thought to be mainly determined by the orientation of the side chains of the first and last monomeric unit. Therefore the position of the regioirregular coupling will have a limited influence on retention, as, for all isomers, both side chains of the first and last monomeric unit are orientated outwards. The fact that the peak width for the oligomer with two bromine end groups is similar to the peak width of the regioregular oligomer with one bromine is in agreement with this assumption. If, on the contrary, retention would also be determined by the position of the regioirregular coupling, different isomers would have slightly different retention times, resulting in a broad, overlapping peak.

To confirm the influence of the orientation of the side chains on retention the three isomers of the

model compound $T_6(C_{12})_2$ were collected and analyzed by ^{1}H-NMR. Preparative HPLC using a 25 mm inner diameter column, packed in our laboratory with Zorbax C_{18}, 10 μm stationary phase was used to collect sufficient amounts of the three peaks on which to perform ^{1}H-NMR. The proton signals of the two middle thiophene rings (see Fig. 6) were used for identification. The first peak was identified as the HH isomer (symmetrical molecule, both protons at $\delta = 7.07$ ppm), the last one as the TT isomer (symmetrical molecule, protons at $\delta = 7.04$ and 7.13 ppm). The second peak, with the highest intensity, was the HT isomer (non symmetrical molecule, protons at $\delta = 7.04$, 7.07 and 7.13 ppm). This is in agreement with the idea of side chains ''spreading out'' and increasing the molecule size and thus retention. However, differences in volume are too small to be detected by SEC (results not shown).

It should be noted that the effects caused by the orientation of the side chains on retention might be different for the model compounds and the original oligomers. The first difference between the model compounds and original oligomer mixture is that the model compounds have only one side chain for every three thiophene rings, whereas the (3HT)*n* carries a side chain on each thiophene ring. A stronger effect of the side chains on retention might be expected for the (3HT)*n*, since the first thiophene ring carries a side chain, instead of the second one for the model compounds. However, the length of the side chains on the model compounds is longer (dodecyl) than on the original oligomers (hexyl). This might result in a larger influence of the side chains for the model compounds. Although it is hard to predict the net influence of the orientation of the side chains in the oligomer mixture when compared to the model compounds, at least some influence of the orientation of the side chains, and thus the presence of a regioirregular coupling, is to be expected.

4. Conclusions

First of all it can be concluded that RP-HPLC is a suitable technique for the separation of the investigated oligothiophenes.

The investigations presented in this paper show a limited influence of the stationary phase on the separation of the thiophene oligomers. Separation on a broad range of different modified silica columns gave similar results, although for the higher-molecular-mass oligomers, some influence of the hydrophobicity is found. With decreasing hydrophobicity, the oligomers were eluted with better selectivity. The m_1 value calculated from the model of Jandera gave similar information about selectivities.

Contrary to the stationary phase, the mobile phase had a major influence on the selectivity, especially on the selectivity for different isomers. Best selectivities were obtained with the THF–water mobile phases, whereas both acetonitrile and methanol combined with THF resulted in much lower selectivities.

Determination of thermodynamic parameters for model compounds separated using THF–water mobile phases showed negative values for both $\Delta\Delta H^0$ and $\Delta\Delta S^0$, the enthalpic and entropic contributions to retention relative to a reference compound. Increasing chain length caused a decrease in $\Delta\Delta H^0$, $\Delta\Delta S^0$ and the enthalpy–entropy ratio. Both $\Delta\Delta H^0$ and $\Delta\Delta S^0$ increased with increasing THF concentration in the mobile phase.

The determination of thermodynamic parameters for the different isomers of the $T_6(C_{12})_2$ and $T_9(C_{12})_3$ model compounds show that retention is mainly determined by the orientation of the side chains at both ends of the chain. An additional repeating unit in the middle of the polymer backbone gave a similar contribution to retention, irrespective of the orientation of its side chain.

The three isomers of the $T_6(C_{12})_2$ model compound were separated by preparative RP-HPLC and identified by proton NMR spectroscopy.

Fractionation of the (3HT)*n* mixture by subsequent preparative SEC and RP-HPLC provided fractions containing an individual peak. Identification of these fractions by MALDI-TOF-MS enabled the detailed characterization of the oligomeric samples. The (3HT)*n* mixture contained two major oligomeric series, the regioregular product with one bromine end group, and a product with a regioirregular coupling and two bromine end groups.

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