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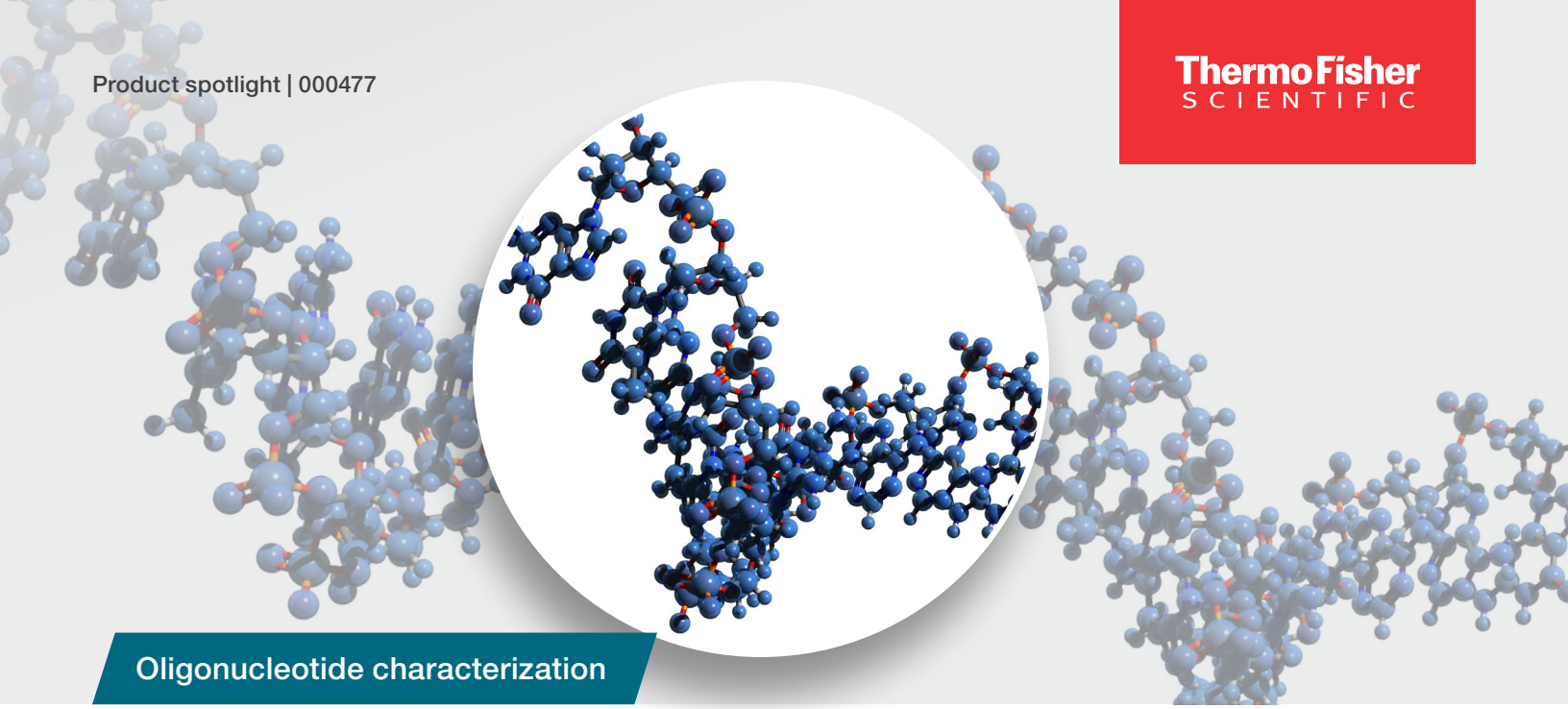

Oligonucleotide characterization

Oligonucleotide characterization using µPAC columns in ion-pairing reversed-phase high performance liquid chromatography mode

Keywords
Micro pillar array column, µPAC, antisense oligonucleotides, ASOs, low-flow chromatography, low-flow LC, IPRP-HPLC

Abstract

Single stranded oligonucleotides such as antisense oligonucleotides (ASOs) represent a broad-spectrum therapeutic platform. Ion-pairing reversed-phase high performance liquid chromatography (IPRP-HPLC) is well-suited for the separation of native oligonucleotides. However, to improve their stability, safety, and potency, therapeutic oligonucleotides are chemically modified. Typically, phosphorothioate (PS) linkages are incorporated in the backbone, which result in the creation of chiral centers. Depending on the number of PS linkages, the complexity increases as $2^{(n-1)}$ isomers are formed, resulting in heterogeneous retention behavior in IPRP-HPLC causing IPRP to frequently fail because of substantial peak broadening. This is problematic for assessing the purity of the synthesized molecule in a pool of closely related, in particular N-1 truncated, fragments. Furthermore, the various diastereomers have different pharmacological and physicochemical properties, thus a detailed characterization of the isomers is crucial as well. In this product spotlight, the resolving power of the Thermo Scientific™ µPAC™ HPLC Column is demonstrated and compared to packed bed columns. Excellent peak capacity and separation efficiency of shortmers and impurities can be achieved using a strong ion-pairing buffer system. When diastereomeric selection of oligonucleotides is required, a weak ion-pairing buffer system can be used and the µPAC columns will offer an unique diastereomeric selectivity compared to packed bed columns. Both ion-pairing systems have been proven to be MS-compatible. Finally, a model sequence closely resembling therapeutic RNA oligonucleotides with 4 PS

linkages was used to confirm the superior resolution of both the 50 cm and the 200 cm µPAC columns at nano LC flow rates. The highest peak capacity can be obtained with the 200 cm µPAC column at a cost of extended run times (>600 for a 10 hr gradient) whereas the highest flexibility and throughput can be achieved with the 50 cm µPAC column with a proven 4-fold increase in separation efficiency compared to a packed bed column in 15 minute runs at 1,500 nL/min runs.

Introduction

Single stranded oligonucleotides represent a broad-spectrum therapeutic platform that continue to evolve with innovations in applications, chemistry, and tissues.[1] ASOs, as an example of a class of therapeutic DNA or RNA oligonucleotides, are used to modify the expression of specific genes as they possess specific sequences that bind with high specificity to target RNA through complementary base pairing, resulting in inhibition of the target RNA. Ion-pairing RP-HPLC is well-suited for the separation of native oligonucleotides. However, decades of research have resulted in a variety of chemical modifications that can be applied to increase resistance to degradation and simultaneously activate the RNase H-degradation pathway without disrupting normal Watson-Crick base pairing.[2] The most widely used modification is phosphorothioation of ASOs, where non-bridging oxygen atoms of the phosphate group are replaced by a sulfur atom. This introduces a chiral center resulting in many diastereomers. Depending on the number of PS linkages, and if all phosphate linkages in an n-long oligonucleotide are PS modified, the complexity increases due to the formation of $2^{(n-1)}$ isomers. For example, a 15-nucleotide-long PS-ASO, can have 16,384 possible diastereomers.

Figure 1 shows the possible diastereomers (Rp and Sp) that are introduced at each phosphorothiate linkage. In addition to the complexity which is introduced through phosphorothioation, many impurities can be generated during oligonucleotide synthesis, such as shortmers, longmers, as well as depurination and deamination products.[2] This extensive isomerisation results in heterogeneous retention behaviour in IPRP-HPLC causing IPRP to fail frequently because of substantial peak broadening, which is problematic for assessing the purity of the synthesized molecule in a pool of closely related, in particular N-1 truncated, fragments. In addition, because the various diastereomers have different pharmacological and physicochemical properties, a detailed characterization of the isomers is crucial as well.

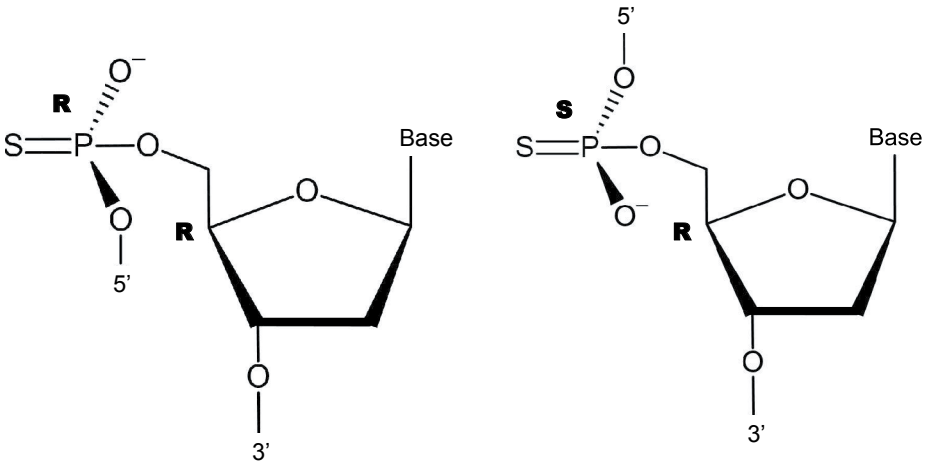

Figure 1. Phosphorothioate internucleotide linkages. Sugar moieties in oligonucleotides modified with sulfurizing reagent are linked by phosphorothioate (includes a non-bridging sulfur). The random R and S configuration at the stereogenic α-phosphorus internucleotide linkage results in diastereomers.

Alternative chromatographic methods such as ion exchange, hydrophilic interaction and size exclusion chromatography have also been used for this purpose, but the relative incompatibility of mobile phase solvents with mass spectrometry has hindered their elaborate use in practice. Recent developments in 2D-LC have however been described where the hyphenation of IPRP-HPLC and anion exchange chromatography (AEX) to hydrophilic interaction liquid chromatography (HILIC) can be used to overcome mass spectrometry (MS) incompatibility.[3] Even though the use of triethylammonium acetate (TEAA) as ion-pairing agent significantly reduces ionization efficiency in liquid chromatography-mass spectrometry (LC-MS) analyses, it has been used historically for the characterization of oligonucleotide synthesis impurities by IPRP.

In 1997, Apfel et al. proposed the use of triethylamine (TEA) combined with hexafluoroisopropanol (HFIP) to increase MS sensitivity without compromising the chromatographic separation.[4] The use of HFIP with TEA limits hydrophobic interactions, which are detrimental for the separation of impurities in the presence of diastereomers and results in size-based separations. It can be used to obtain maximum resolving power for samples containing oligonucleotides with different lengths, synthesis related impurities, or oligonucleotide metabolites but diastereomer selectivity is impaired to a large extent.

Both silica and polymer based HPLC columns (both in packed bed and monolithic format) have been successfully implemented for the analysis of oligonucleotides by IPRP. Whereas polymer based columns typically have greater tolerability to elevated pH and temperature, silica based columns can offer alternative selectivity and higher mass loadability. In contrast to these conventional reversed-phase liquid chromatography (RPLC) column types, the µPAC micro pillar array columns represent a separation format which can be used to obtain extremely high resolution LC separations. Whereas conventional LC columns contain randomly packed beads as their stationary phase, micro-chip based pillar array chromatography columns have a separation bed of perfectly ordered and freestanding pillars obtained by lithographic etching of a silicon wafer. The regular mobile phase flow pattern through these micro-chip pillar array columns adds very little dispersion to the overall separation, resulting in better peak resolution, sharper elution and increased sensitivity.[5] The freedom of design also leads to much lower back pressure build-up and makes it possible to operate longer columns.[6] The stationary phase morphology of the µPAC columns (porous shell pillars—100-300 Å mesopores—C18 functionalized) makes them ideally suited to perform IPRP separation of oligonucleotides, which is illustrated based on of several sample sets and buffer systems. A TEA/HFIP ion-pairing system (referred to as Strong ion-pairing system[3]—was used to separate a ladder of T5-T30 oligos and compares the intrinsic chromatographic performance of a 50 cm and a 200 cm µPAC column. In order to obtain diasteromer selectivity, the ion-pairing buffer strength was reduced,[7] yielding a weak ion-pairing system comprising of TEAA without HFIP. The effect of ion-pairing system strength and the benefits of using µPAC technology for the characterization of diastereomers will be discussed based on separations obtained for thioate modified T5 and T15 oligonucleotide standards as well as for some model sequences closely resembling therapeutic oligonucleotides.

Experimental

Sample information

An overview of all oligonucleotides discussed in this product spotlight are listed in Table 1 A-C. They were custom synthesized by Integrated DNA Technologies (Leuven). The lyophilized oligonucleotides from Table 1 A-C were dissolved by the addition of 1× TE buffer which was also used to prepare appropriate dilutions. To determine peak capacity, a reference standard mixture ranging from 5 to 30 thymidine-oligonucleotides was prepared in house at a final concentration of 75 ng/µL each. The oligonucleotides from Table 1C were dissolved in water (1 µg/µL) and further diluted in water to a final concentration of 250 ng/µL.

Table 1. Overview of oligonucleotide sequences used for evaluation column performance and diastereomer selectivity. A) 5-30mer deoxythymidines (dTs). B) T5 and T15 oligonucleotides with their phosphorothioate linkages indicated and the theoretical number of diastereomers calculated. C) Test oligonucleotide sequences with their phosphorothioate linkages indicated and the theoretical number of diastereomers calculated.

A

Name of oligonucleotide	Sequence 5'-3'	# nt
T5	T5 TTTTT	5
T6	T6 TTTTTT	6
T7	T7 TTTTTTT	7
T8	T8 TTTTTTTT	8
T9	T9 TTTTTTTTT	9
T10	T10 TTTTTTTTTT	10
T11	T11 TTTTTTTTTTT	11
T12	T12 TTTTTTTTTTTT	12
T13	T13 TTTTTTTTTTTTT	13
T14	T14 TTTTTTTTTTTTTT	14
T15	T15 TTTTTTTTTTTTTTT	15
T16	T16 TTTTTTTTTTTTTTTT	16
T17	T17 TTTTTTTTTTTTTTTTT	17
T18	T18 TTTTTTTTTTTTTTTTTT	18
T19	T19 TTTTTTTTTTTTTTTTTTT	19
T20	T20 TTTTTTTTTTTTTTTTTTTT	20
T21	T21 TTTTTTTTTTTTTTTTTTTTT	21
T22	T22 TTTTTTTTTTTTTTTTTTTTTT	22
T23	T23 TTTTTTTTTTTTTTTTTTTTTTT	23
T24	T24 TTTTTTTTTTTTTTTTTTTTTTTT	24
T25	T25 TTTTTTTTTTTTTTTTTTTTTTTTT	25
T26	T26 TTTTTTTTTTTTTTTTTTTTTTTTTT	26
T27	T27 TTTTTTTTTTTTTTTTTTTTTTTTTTT	27
T28	T28 TTTTTTTTTTTTTTTTTTTTTTTTTTTT	28
T29	T29 TTTTTTTTTTTTTTTTTTTTTTTTTTTTT	29
T30	T30 TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	30

B

Name of T5 oligonucleotide	Sequence	# Thiobonds	# Diastereomers
T5-1S	T*TTTT	1	2
T5-2S	TT*TTT	1	2
T5-3S	TTT*TT	1	2
T5-4S	TTTT*T	1	2
T5-1-2S	T*T*TTT	2	4
T5-1-4S	T*T*T*T*T	4	16

Name of T15 oligonucleotide	Sequence	# Thiobonds	# Diastereomers
T15-1S	T*TTTTTTTTTTTTTT	1	2
T15-7S	TTTTTTT*TTTTTTTT	1	2
T15-14S	TTTTTTTTTTTTT*T	1	2
T15-1-2S	T*T*TTTTTTTTTTTTT	2	4

C

Number of oligonucleotide	Sequence 5'-3'	# PS bonds
5	As-Ts-C-T-A-T-A-C-A-A-G-C-T-G-Ts-C	3
8	Ts-C-T-A-T-A-C-A-A-G-C-T-G-Ts-C	2
9	C-T-A-T-A-C-A-A-G-C-T-G-Ts-C	1
13	As-Afs-Cs-Af-U-Uf-G-A-G-Cf-G-Af-U-Af-U-Cf-C-As-C	5

Table 2. Analytical columns

Thermo Scientific µPAC HPLC	50 cm bed length, 18 µm pillar length
Thermo Scientific µPAC HPLC	200 cm bed length, 18 µm pillar length
Packed bed A	1.6 µm particles, 75 µm × 25 cm
Packed bed B	1.7 µm particles, 75 µm × 15 cm

Results and discussion

Strong ion-pairing system

A strong ion-pairing system containing 16.3 mM TEA with 400 mM HFIP at a value of pH 7.9 was first used to determine the intrinsic chromatographic performance which can be achieved for both the 50 cm and the 200 cm µPAC column. This ion-pairing system

has been documented to suppress diastereomer separation and maximize resolution for synthesis impurity detection and size based separation of oligonucleotides. Typical UV chromatograms obtained for the separation of 5-30mer deoxythymidines (dTs) are shown in Figures 2 A-B.

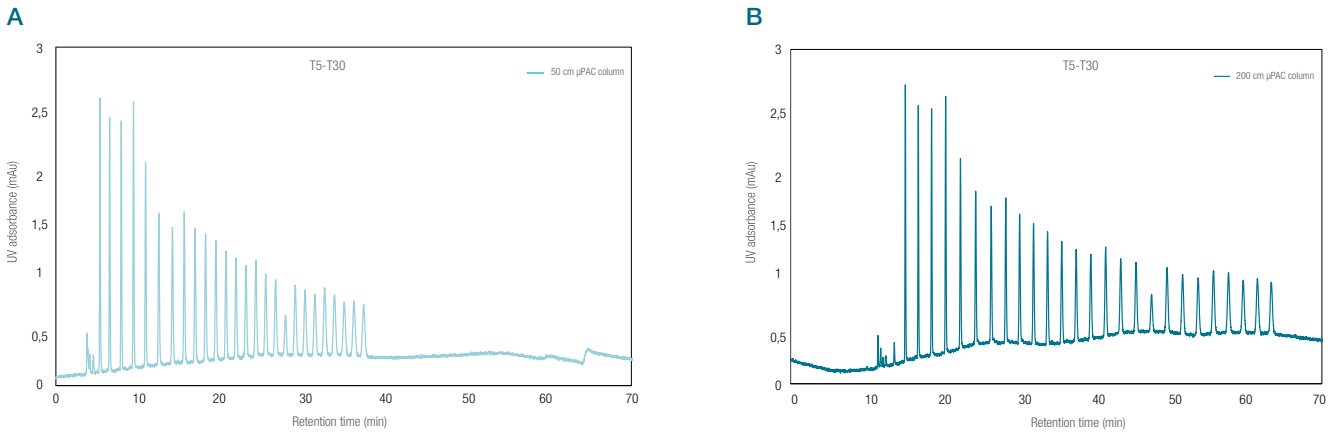

Figure 2. UV chromatograms obtained for the separation of 5-30mer deoxythymidines (dTs) on either A) the 50 cm µPAC column or B) 200 cm µPAC column. Flow rate 750 nL/min, 60 min gradient 20 to 70%B_C2 curve, A: 100% H_2O, B: 40% MeOH, UV 260 nm.

To obtain near baseline separation of all oligonucleotides, a non-linear gradient ranging from 20 to 70% solvent B was used. Both columns were evaluated over a range of flow rates and gradient times to assess the chromatographic performance which can be achieved within a given analysis time. The full width half maximum (FWHM) based peak capacity was calculated according to Neue where the elution window (t_E=t_n-t_1; t_n referring to the retention of the last eluting peak and t_1 to that of the first one) rather than the total gradient time (t_G) was used to give a more accurate representation of the true separation power achievable.[8]

When plotting the peak capacity as a function of t_E/t_0 (Figure 3A), it becomes clear that the maximum peak capacity that can be achieved for the different columns scales with column length according to the square root of the number of theoretical plates (N). At a t_E/t_0 value of 40 and at the optimal flow rate of 250 nL/min, peak capacity values of 734 and 348 were obtained for the 200 cm and 50 cm column.

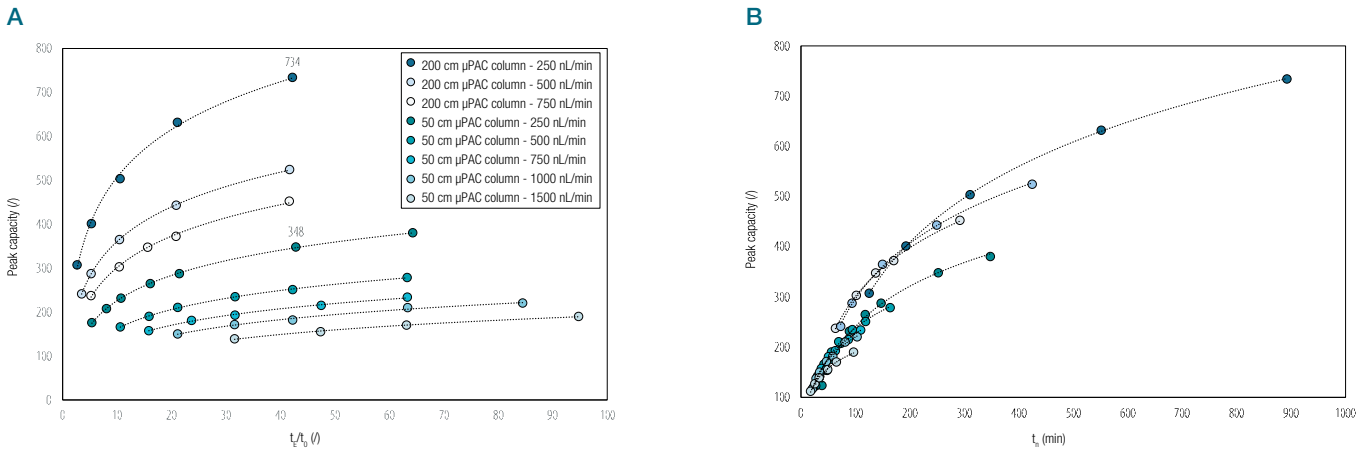

Figure 3. FWHM based peak capacity obtained for an oligonucleotide reference standard mixture (T5-T30). The performance obtained on a 200 cm and a 50 cm µPAC column is compared at different flow rates (250–1500 nL/min). A) Peak capacity plotted as a function of t_E/t_0. t_E= t_n-t_1. B) Peak capacity plotted as a function of t_n, retention time of the last eluting oligonucleotide (T30).

A different and more practical perspective is obtained by plotting the peak capacity values as a function of the retention time obtained for the last eluting analyte (t_n) (Figure 3B). The use of longer columns results in increased peak capacity, but at the cost of extended run times. For short analyses (t_G≤30 min), better performance will be achieved at higher flow rates. Whereas separation at the optimal flow rate (250 nL/min) will produce better performance for more complex separations at extended gradient lengths (t_G≥60 min).

Strong versus weak ion-pairing system

Even though maximum size based resolution is obtained by using a strong ion-pairing system, several studies have documented the suppression of diastereomer selectivity when using these ion-pairing systems. To investigate the potential of µPAC column technology for the separation of phosphorothioate diastereomers, a weak ion-pairing system consisting of 15 mM TEAA at pH 7 was evaluated. The effect of reducing the ion-pairing strength on diastereomer selectivity is clearly illustrated in Figure 4. Oligonucleotides containing either 1, 2 or 3 phosphorothioate linkages have been injected and separated on a 200 cm µPAC column using either the strong or the weak ion-pairing system. The use of a strong ion-pairing system consistently resulted in the elution of a single peak that exhibits increased retention according to the number of phosporothioate linkages. Further decrease of the solvent gradient slope or extending the solvent gradient length did not result in diastereomer selectivity. However, when changing to the weak ion-pairing system, respectively 2, 4 and 8 baseline separated diastereomer peaks could be detected for the oligonucleotides containing 1, 2 and 3 phosphorothiate linkages. In accordance with earlier reports on this topic, the combination of using shallow solvent gradients with a weak ion-pairing system promotes diastereomer selectivity on silica and silicon based stationary phases.

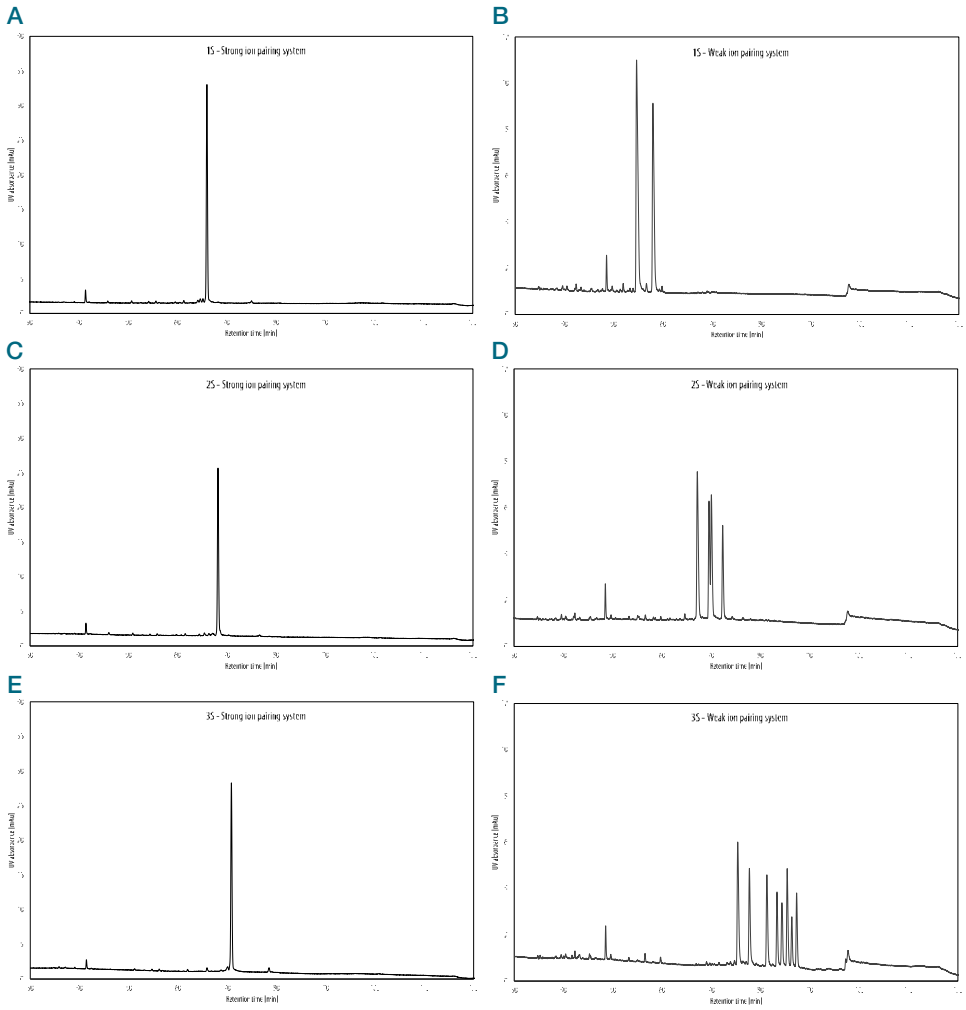

Figure 4. UV chromatograms obtained for the separation of phosphorothioate modified oligonucleotides using either a strong (left) or a weak (right) ion-pairing system as mobile phase. A-B test oligonucleotide #9 (Table 1C) with 1 phosporothioate linkage. C-D test oligonucleotide #8 (Table 1C) with 2 phosporothioate linkages. E-F test oligonucleotide #5 (Table 1C) with 3 phosporothioate linkages. Flow rate 250 nL/min, 60 min gradient 20 to 99% B for the strong ion-pairing system and 20–30% B for the weak ion-pairing system, A: 100% H_2O, B: 40% MeOH for the strong ion-pairing system and 40% acetonitrile for the weak ion-pairing system, UV 260 nm.

Weak ion-pairing system

To quantify the diastereomer selectivity for different column lengths and types, a set of T5 and T15 oligonucleotides with either 1 or 2 phosphorothiate linkage at different positions was injected and separated using the weak ion-pairing system. Sequences of the tested oligonucleotides and theoretical number of diastereomers have been listed in Table 1. It has to be noted that there can be a discrepancy between the number of diastereomers that can experimentally be observed and those present according to theory. Oligonucleotide synthesis conditions can have an effect on the number diastereomers and at the same time, diastereomers can be present over a wide concentration range. In order to compare different columns, the resolution ($R_s = 2(T_{r2}-T_{r1})/(W_1+W_{2n})$) obtained for baseline separated peaks can be calculated and used to compare diastereomer selectivity.

T5 and T15 oligonucleotides – 1 phosphorothioate linkage

An example of a diastereomer separation obtained for a T5 oligonucleotide containing 1 phosporothioate linkage (and thus 2 expected diastereomers) is shown in Figure 5. The resolution obtained between both oligonucleotide peaks was calculated for all columns and oligonucleotides tested and is plotted in Figure 6A.

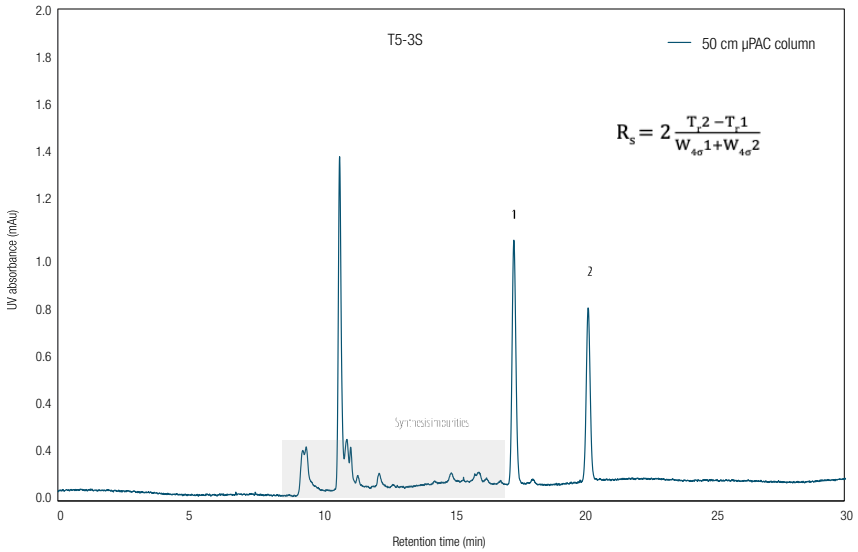

Figure 5. UV chromatogram obtained for the separation of a 5mer deoxythymidine oligonucleotide with a central phosphorothioate linkage (T5-3S). Diastereomer peaks have been labeled (1,2), several peaks originating from synthesis impurities are observed and elute prior to the full length oligonucleotide diastereomers. Formula for the calculation of resolution is given. Flow rate 250 nL/min, 30 min gradient 20 to 30%B, A: 100% H_2O, B: 40% acetonitrile with 15mM TEAA in both A-B, UV 260 nm.

Even though all columns show similar resolution for the T5 1S oligonucleotide (bond specific), substantially higher resolution is obtained for all other phosphorothiate positions when using the µPAC columns. For example, respective resolution values of 11.92 and 14.1 were obtained for a T5 2S oligonucleotide on the µPAC 50 cm and 200 cm column, whereas the resolution obtained on packed bed columns was 8.45 (packed bed column A) and 6.89 (packed bed column B). Similar differences in resolution are observed for 3S and 4S oligonucleotides, indicating the increased diastereomer selectivity for µPAC column stationary phases. These findings are confirmed when analyzing a set of T15 phosphorothiate modified oligonucleotides. Unfortunately, no results could be generated on packed bed column B due to a consistent increase in column backpressure, preventing its further use at the desired flow rate of 250 nL/min.

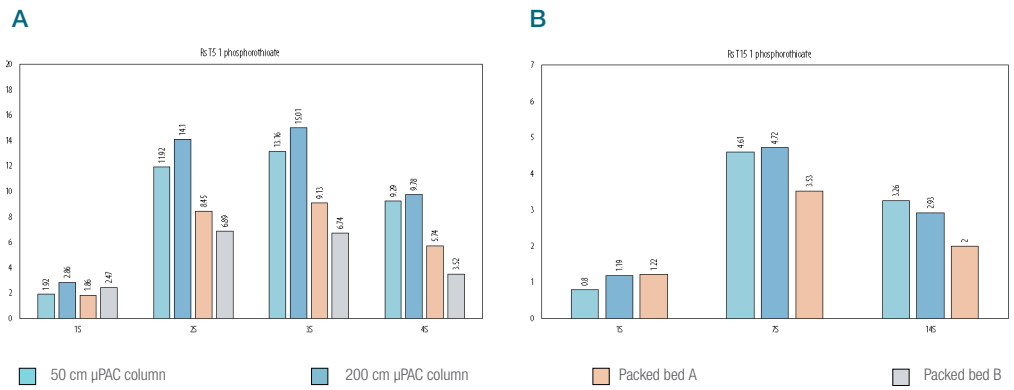

Figure 6. Resolution obtained between diastereomers of T5 and T15 oligonucleotides with a single phosphorothioate linkage, respectively on the left (A) and right (B). 50 cm and 200 cm µPAC columns have been compared to two silica based C18 packed bed columns (packed bed A and packed bed B). Flow rate 250 nL/min, 30 min gradient 20 to 30% B for 50 cm and 200 µPAC columns and packed bed B, 25–35% B for packed bed A for T5 oligos and for T15 oligos, 25–35% B was used for 50 cm and 200 µPAC columns and 30–40% B for packed bed A, A: 100%H_2O, B: 40% acetonitrile with 15mM TEAA in both A and B, UV 260 nm.

When comparing the results obtained on the µPAC columns and the packed bed column (Packed bed column A), an increase in separation resolution was observed for 2 out of 3 oligonucleotides (Figure 6B). Relatively low and similar resolution is obtained when the phosphorothiate linkage is at position 1, higher resolution and a more pronounced gain for µPAC stationary phases is observed at position 7 (T15 7S) and 14 (T15 14S). The observation that higher resolution is typically observed for oligonucleotides that have central phosphorothiate linkages has been described in literature. A possible explanation for this observation can be that the anionic sulfur is less shielded, i.e., more likely to interact with the ion-pairing reagent, if it is substituted at either end versus in the center of the oligonucleotide.[7, 9] Another consistent finding that is observed for all columns is an increase in diastereomer resolution when oligonucleotides are modified at the 3' versus at the 5' end, even though both modifications are located at the end and sulfur atoms should be equally accessible.

T5 and T15 oligonucleotides – 2 phosphorothioate linkages

Analogous to the evaluation for T5 and T15 oligonucleotides containing a single phosporothioate linkage, resolution values obtained on all 4 columns were calculated for baseline separated peaks produced from T5 and T15 oligonucleotides containing 2 phosphorothioate bonds (and thus yielding 4 potential diastereomers). As it was not possible to resolve all 4 diastereomers on all columns, 2 out 4 diastereomer peaks (peak 1 and 2) were considered for calculation of the resolution. Respective resolution values obtained for T5 and T15 oligonucleotides have been plotted in Figures 7A–B.

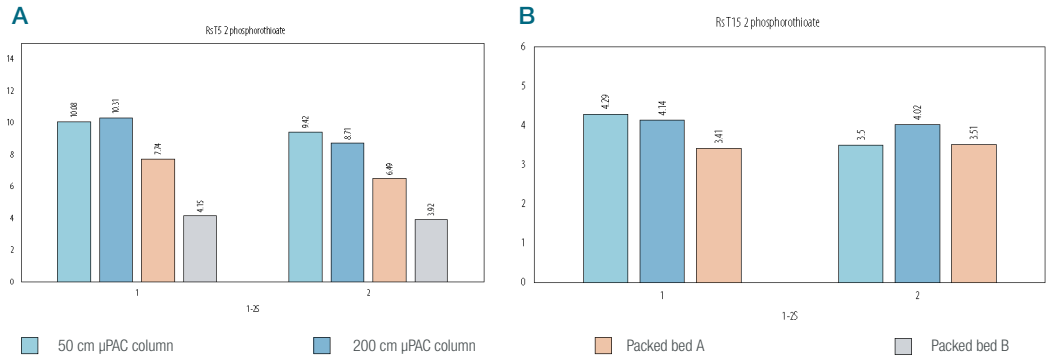

Figure 7. Resolution obtained between diastereomers of T5 and T15 oligonucleotides with 2 phosphorothioate linkages, respectively on the left (A) and right (B). 50 cm and 200 cm µPAC columns have been compared to two silica based C18 packed bed columns (packed bed A and packed bed B). Flow rate 250 nL/min, 30 min gradient 20 to 70% B, A: 100% H_2O, B:40% acetonitrile with 15mM TEAA in both A-B, UV 260 nm.

Again, similar observations were made with clearly higher resolution obtained with the µPAC columns as compared to packed bed columns. Under these conditions, similar resolution is obtained on both the 50 cm and 200 cm µPAC columns. In order to benefit from increased resolution that is typically observed for longer columns, gradient length should be adapted for the 200 cm column. With a gradient length of 120 minutes and longer, a significant increase in peak capacity (cfr. Figure 3) can be obtained with the 200 cm µPAC column. For the current sample set, the 50 cm µPAC columns already deliver adequate resolution.

4 phosphorothioate linkages

To evaluate the potential of µPAC column technology for the separation of diastereomers present in therapeutic RNA oligonucleotides, an oligonucleotide with a sequence closely resembling that of therapeutic RNA oligonucleotides was synthesized and injected on the 50 cm µPAC column using the 15 mM TEAA solvent.[10] When applying a 72 min gradient and operating the column at a flow rate of 250 nL/min, all 16 diastereomers could be resolved to a certain extent (Figure 8A). Following the early elution of a group of contaminants and synthesis byproducts, these diastereomers appear to elute in clusters, probably reflecting Rp and Sp pairs. When injecting and separating the same oligonucleotide sequence on a packed bed alternative (Figure 8B), similar elution behavior but with reduced resolution was obtained. Even though distinct peak maxima were observed for most of the diastereomers, extensive peak broadening resulted in poor separation.

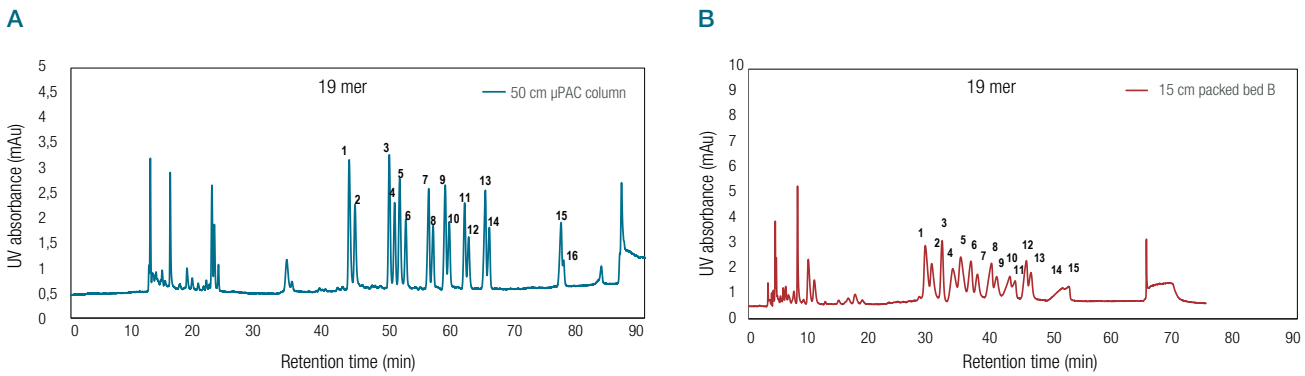

Figure 8. UV chromatogram obtained for the separation of a 19mer oligonucleotide sequence with a 4 phosphorothioate linkages (test oligonucleotide #13—Table 1C). Diastereomer peaks have been labeled (1–16), several peaks originating from synthesis impurities are observed and elute prior to the full length oligonucleotide diastereomers. A) the 50 cm µPAC column is compared to B) a 15 cm packed bed B. Flow rate 250 nL/min, 60 min gradient 28 to 38% B for 50 cm µPAC column and 33 to 43% B for packed bed A, A: 100%, B: 40% acetonitrile with 15 mM TEAA in both A-B, UV 260 nm.

Maximum separation resolution is typically achieved by operating the µPAC columns in the range of 200 to 300 nL/min and running relatively long gradient times. A significant increase in sample throughput can however be achieved by performing shorter analyses at increased flow rates. The high permeability (or low backpressure) associated with perfectly ordered stationary phase support backbones gives the additional advantage that flow rate and gradient time can be easily adjusted to the sample complexity or separation performance needs. To demonstrate this, an oligonucleotide containing 4 phosphorothioate linkages (T5-1-4S) was injected onto the 50 cm µPAC column and separated using a 10 min gradient at 1,500 nL/min. Out of the 16 expected diastereomers, 14 peaks could be distinguished within a total analysis time of 20 min (8 of which baseline separated and 6 co-eluting or poorly resolved).

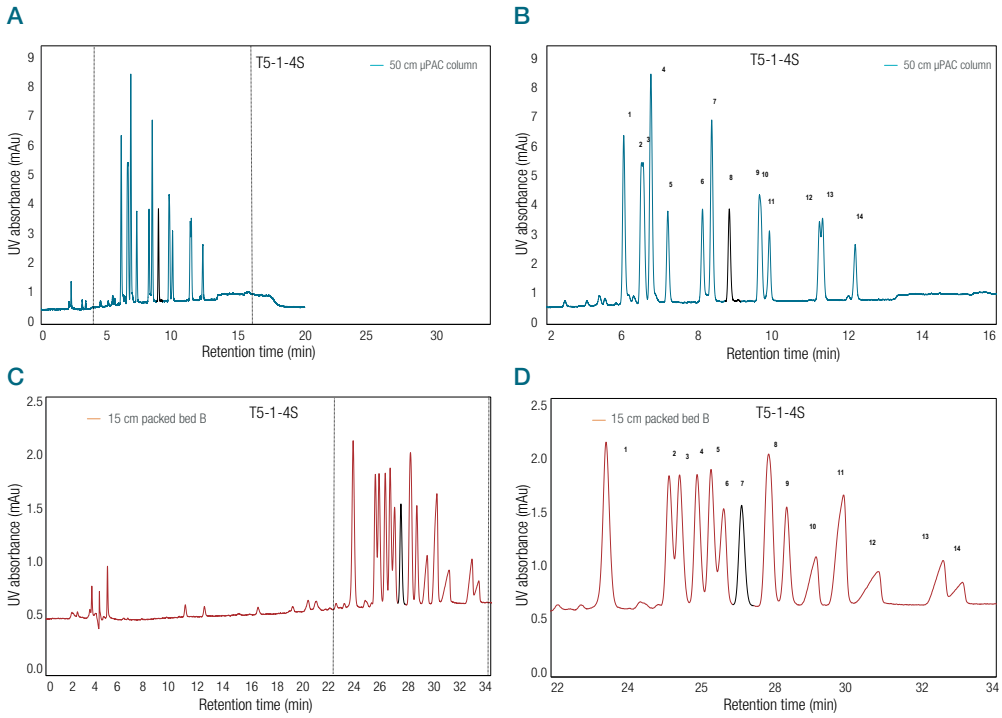

Figure 9. UV chromatogram obtained for the separation of a 5mer deoxythymidine oligonucleotide with 4 phosphorothioate linkages (T5-1-4S). Diastereomer peaks have been labeled (1–14), several peaks originating from synthesis impurities are observed and elute prior to the full length oligonucleotide diastereomers. A-B 10 min gradient at 1,500 nL/min on a 50 cm µPAC column, gradient profile 25 to 55% B, A: 100% H_2O 15 mM TEAA, B: 40% acetonitrile 15 mM TEAA, UV 260 nm. C-D 30 min gradient at 250 nL/min on a 15 cm packed bed column (packed bed B), gradient profile 20 to 40% B, A: 100% H_2O 15 mM TEAA , B: 40% acetonitrile 15 mM TEAA, UV 260 nm.

When comparing with a packed bed column, clear benefits for both sample throughput and diastereomer separation resolution are demonstrated. Based on the elution window in which oligonucleotide diastereomers elute (t_n-t_1) and the peak width of a baseline separated diastereomer, peak capacity values were calculated for both columns and plotted as a function of the time at which the last diastereomer elutes (t_n). In line with the peak capacity values obtained for a ladder of T oligonucleotides (Figure 3), a peak capacity value of 89 was achieved within 15 min when operating the 50 cm µPAC column at 1,500 nL/min (Figure 10). Producing a separation with 56% higher peak capacity within less than half the analysis time results in an 4-fold increase in separation efficiency. Using the current setup (which was limited in operating pressure to 400 bar because of the external nanovolume injector valve), no further flow rate optimization was possible for the packed bed column as it already gave approximately 300 bars of backpressure at 250 nL/min.

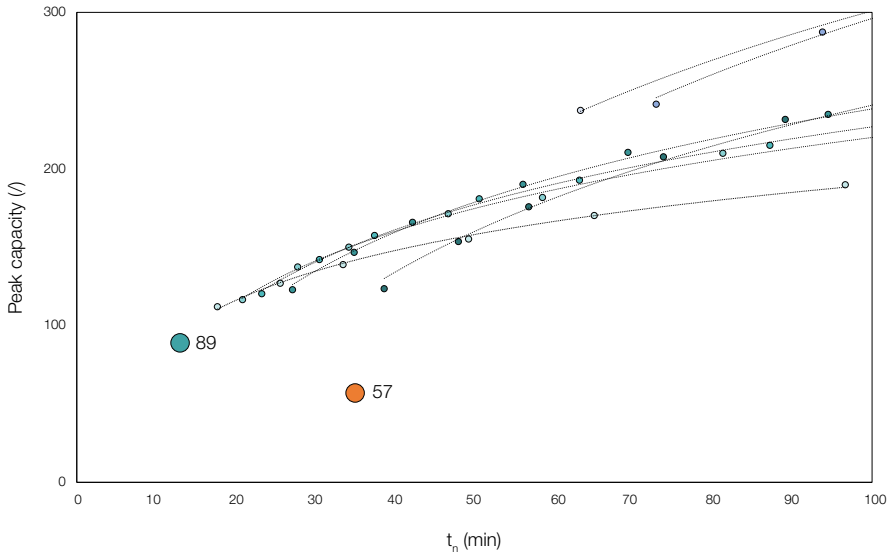

Figure 10. FWHM based peak capacity obtained for a 5mer deoxythymidine oligonucleotide with 4 phosphorothioate linkages (T5-1-4S) on either a 50 cm µPAC column and 15 cm packed bed A column. Peak capacity within the diastereomer elution window is calculated based on the peak width of a baseline separated peak and plotted as a function of t_n, retention time of the last eluting oligonucleotide diastereomer. Data points have been obtained from separations shown in Figure 9.

Conclusion

µPAC nano LC column technology is a versatile and high performing solution for the separation of oligonucleotides by ion-pairing reversed-phase chromatography.

- Excellent peak capacity and separation efficiency is demonstrated using a strong ion-pairing buffer system for the detection of oligonucleotide synthesis impurities and shortmers.
- Using a weak ion-pairing buffer system, unique diastereomer selectivity is achieved compared to packed bed alternatives, translating in improved detection of potential diastereomers.
- The unique format of µPAC columns allows versatile operation over a wide range of flow rates. Flow rate and gradient time can easily be optimized towards sample complexity and analysis throughput.
- For high-throughput screening and routine separation of oligonucleotides, the use of a 50 cm µPAC column is recommended.
- The 200 cm µPAC column is recommended when ultra-high resolution separation is needed.

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