How to optimise your oligonucleotide analysis

Nucleic acid therapeutics such as silencing RNA duplexes (siRNA), messenger RNA (mRNA) and antisense oligonucleotides (ASOs) are extremely promising candidates for drug therapy in a wide range of diseases. Therapeutic oligonucleotides are often modified to enhance their stability or improve pharmacokinetics. They can be classified by modifications of the backbone, base or sugar moieties and conjugation of the structure *[1]*. In general, oligonucleotides have phosphodiester linkages (PO). One of the most common modifications contains the replacement of the oxygen atom by sulphur which leads to a phosphorothioate linkage (PS). This PS linkage results in an increase in stability towards nucleases and a prevention of nucleolytic degradation. PS oligonucleotides are often used for in vivo and in vitro techniques as they are more robust than PO.

An additional modification that is often made is a replacement of the nucleophilic hydroxyl moiety at the 2'-ribose position with an O-methyl (2'-OMe) group. This further increases the stability towards nuclease digestion.

Their monitoring during purification and in quality control analyses is crucial since impurities and metabolic products can affect their efficacy. Ion exchange (IEX) and ion-pairing reversed phase (IP-RP) chromatography enable the detection of small structural differences. Therefore, they are often used in oligonucleotide analysis. This technical note highlights the most important aspects of IEX- and IP-RP method development for the separation of oligonucleotides with slightly differing structures. Since the two modifications described above occur very frequently, they will be the focus of this note.

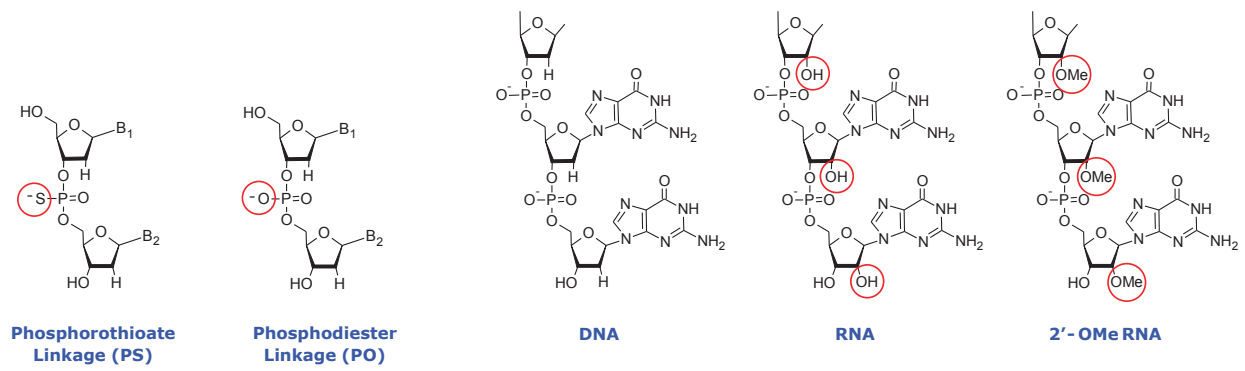


Fig. 1: Oligonucleotide modifications.

1. IEX analysis for oligonucleotides

Due to the negatively charged phosphate groups in the backbone of oligonucleotides their analysis is typically performed using anion exchange (AEX). YMC's non-porous anion exchange column BioPro IEX QF has a quaternary amine residue as the functional group. The non-porous particles of BioPro IEX QF provide high efficiency and offer high resolution separations.
In the following sections, different optimisation steps were made for single-stranded unmodified and modified DNA and RNA to show how important it is to have an efficient method. The first sample group covers single-stranded DNA and RNA with PO linkages differing in length (20 and 21 mers), since N-1 (shortmer) and N+1 (longmer) are the most common impurities during oligonucleotide purification. This sample group also contains single-stranded DNA with single base differences. The second group (section 1.2.) deals with PS linked single-stranded DNA and RNA.

1.1. Sample Group 1: Phosphodiester oligonucleotides; PO

Changes in the type and concentration of salt used for buffer solution or gradient modifications were made to achieve less peak tailing and carryover.

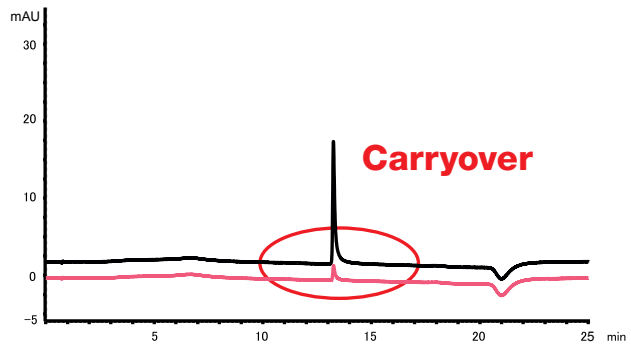
Sample group 1:

1	Single-stranded DNA	5'-TCATCACACTGAATACCAAT-3‘ (DNA 20 mer)
2		5'-GTCATCACACTGAATACCAAT-3' (DNA 21 mer)
3	Single-stranded RNA	5'-U(M)C(M)A(M)U(M)C(M)A(M)C(M)A(M)C(M)U(M)G(M)A(M)A(M)U(M) A(M)C(M)C(M)A(M)A(M) U(M)-3' (2'-OMe RNA 20 mer)
4		5'-G(M)U(M)C(M)A(M)U(M)C(M)A(M)C(M)A(M)C(M)U(M)G(M)A(M)A(M) U(M) A(M)C(M)C(M)A(M) A(M)U(M)-3' (2'-OMe RNA 21 mer)
5		5'-UCAUCACACUGAAUACCAAU-3' (RNA 20 mer)
6		5'-GUCAUCACACUGAAUACCAAU-3' (RNA 21 mer)
7	Single-stranded DNA with single base differences	5'-ATCATCACACTGAATACCAAT-3' (DNA 21 mer)
8		5'-CTCATCACACTGAATACCAAT-3' (DNA 21 mer)
9		5'-TTCATCACACTGAATACCAAT-3' (DNA 21 mer)

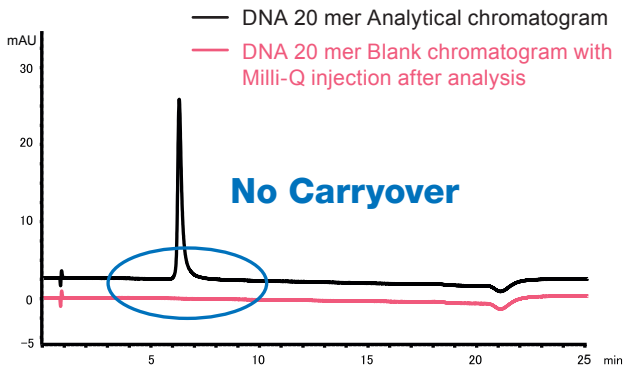
N(M)=2'-OMe RNA

1.1.1. Minimising carryover

When the initial gradient concentration of NaCl is low (e.g. 50 mM), carryover is observed. By increasing the initial gradient concentration of NaCl up to 400 mM carryover can be avoided with a corresponding increase in reproducibility.



A) 20 mM Tris-HCl (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1.0 M NaCl
5–70%B (0–15 min), 74%B (15–18 min), 5%B (18–33 min)
Initial : 50 mM NaCl



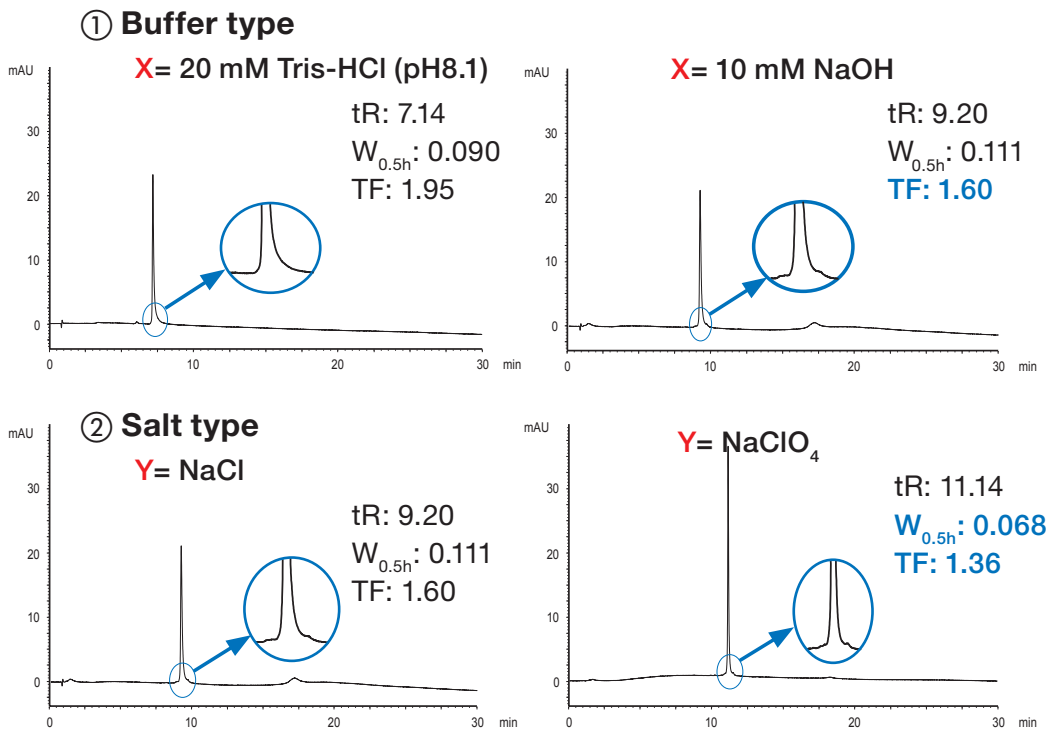
A) 20 mM Tris-HCl (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1.0 M NaCl
40–70%B (0–15 min), 74%B (15–18 min), 40%B (18–33 min)
Initial : 400 mM NaCl

Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.: QF00S05-1046WP
Flow rate: 1.0 mL/min
Temperature: 25°C
Detection: UV at 260 nm
Injection: 2 µL (10 nmol/mL)
Sample: DNA 20 mer (sample 1)

However, increasing the initial concentration is not always successful since a too high salt concentration can decrease the solubility of the analyte. Please consider this in your specific analysis.

1.1.2. Improvement of peak tailing

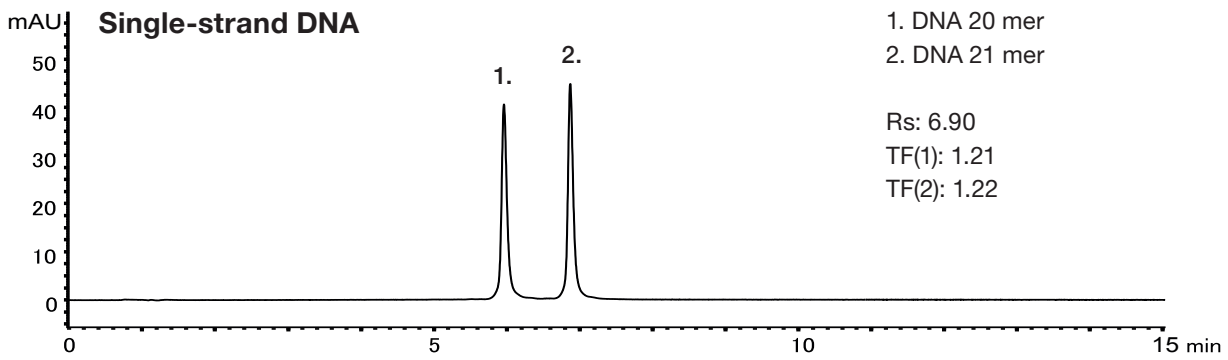
Using different buffers can be helpful if peak tailing occurs during the analysis. By changing the buffer from 20 mM Tris-HCl (pH 8.1) to 10 mM NaOH, the tailing factor for an oligonucleotide is reduced. In addition, peak tailing can be further suppressed when changing the counter ion from Cl^- to ClO_4^-. The gradient profile for $NaClO_4$ was adjusted because the elution strength of $NaClO_4$ is two to three times greater than that of NaCl in ion exchange chromatography.

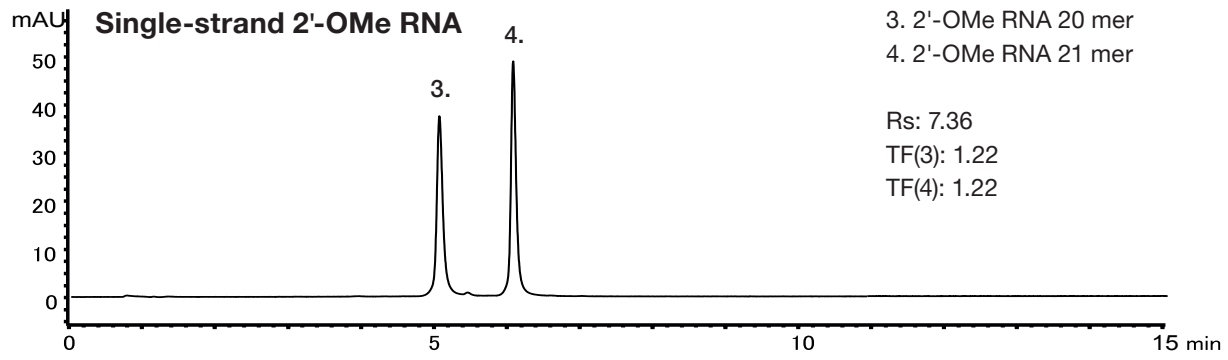


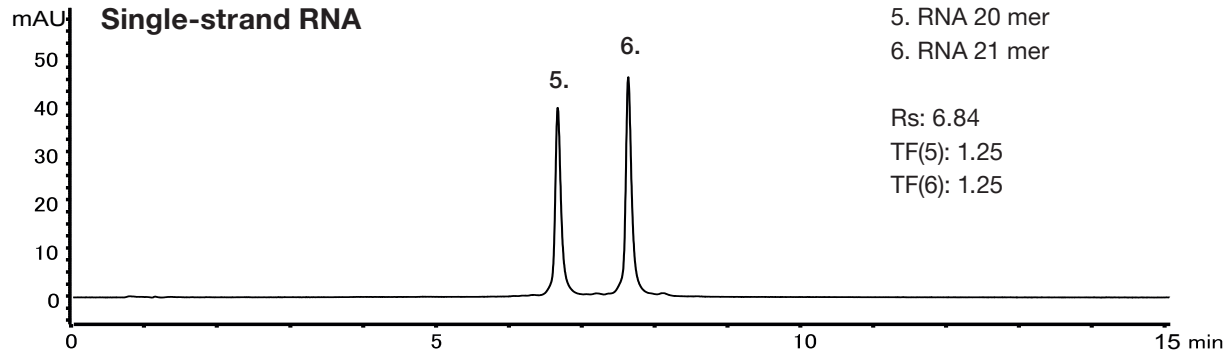
Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 20 mM Tris-HCl (pH 8.1) or 10 mM NaOH
	B) 20 mM Tris HCl (pH 8.1) or 10 mM NaOH containing 2.0 M NaCl or $NaClO_4$
Gradient:	15–100%B (0–30 min) for NaCl
	5–50%B (0–30 min) for $NaClO_4$
Flow rate:	1.0 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	2 µL (10 nmol/mL)
Sample:	RNA 20 mer (sample 5)

1.1.3. Separation examples under optimised conditions

Single-stranded DNA, RNA and 2'OMe RNA, all with a single-base difference in length, show well resolved chromatograms. The conditions used are NaOH (10 mM, as buffer solution), $NaClO_4$ (as salt) and a higher initial gradient concentration of salt in order to suppress carryover and peak tailing. Each single-stranded DNA (samples 1 and 2), single-stranded RNA (samples 5 and 6) and 2'-OMe single-stranded RNA (samples 3 and 4) with single-base difference in length can be separated under these optimised conditions.



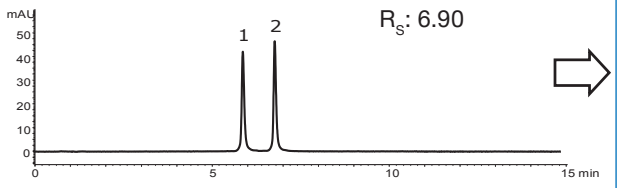
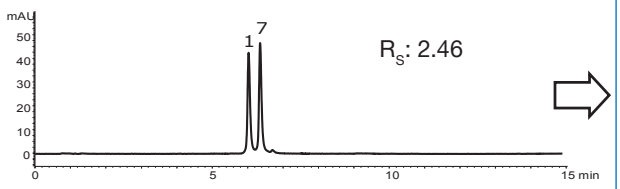
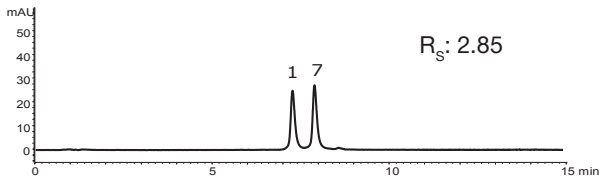
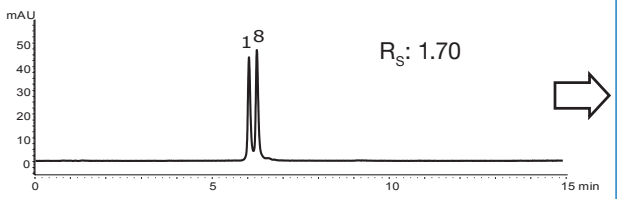
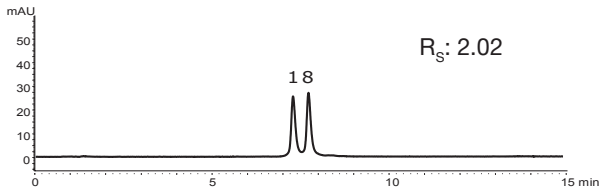
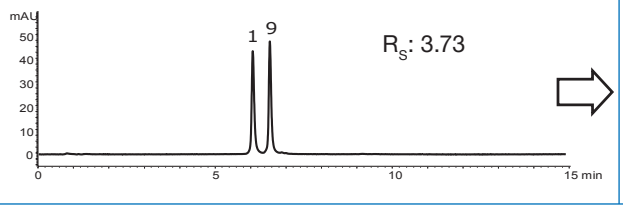
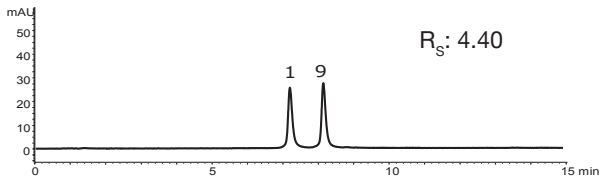




Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID	Detection:	UV at 260 nm
Part No.:	QF00S05-1046WP	Injection:	4 µL (5 nmol/mL each)
Eluent:	A) 10 mM NaOH	Samples:	DNA 20 and 21 mer (samples 1 and 2),
	B) 10 mM NaOH containing 1.0 M $NaClO_4$		RNA 20 and 21 mer (samples 5 and 6),
Gradient:	25–55%B (0–15 min), 100%B (15–20 min)		2'-OMe RNA 20 and 21 mer (samples 3 and 4)
Flow rate:	1.0 mL/min		
Temperature:	25 °C		

1.1.4. Separation of single-stranded DNAs with single-base differences in the type of base of 5' terminal

In the example presented in section 1.1.3., separations of single-stranded DNAs and RNAs with single-base differences in length were shown, whereas in this section separations with differences in the type of base of 5' terminal are presented. Applying the separation conditions described above, all peak separations were well separated using the shallower gradient.

	25–55%B (0–15 min) Δ=2%B/min	25–40%B (0–15 min) Δ=1%B/min
G*		
A*		
C*		
T*		

Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 10 mM NaOH
	B) 10 mM NaOH containing 1.0 M $NaClO_4$
Flow rate:	1.0 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	4 µL (5 nmol/mL each)
Sample:	DNA 20 and 21 mer (samples 1, 2, 7, 8 and 9)

*base of 5'end of DNA 21 mer

1.2. Sample Group 2: Phosphorothioate oligonucleotides; PS

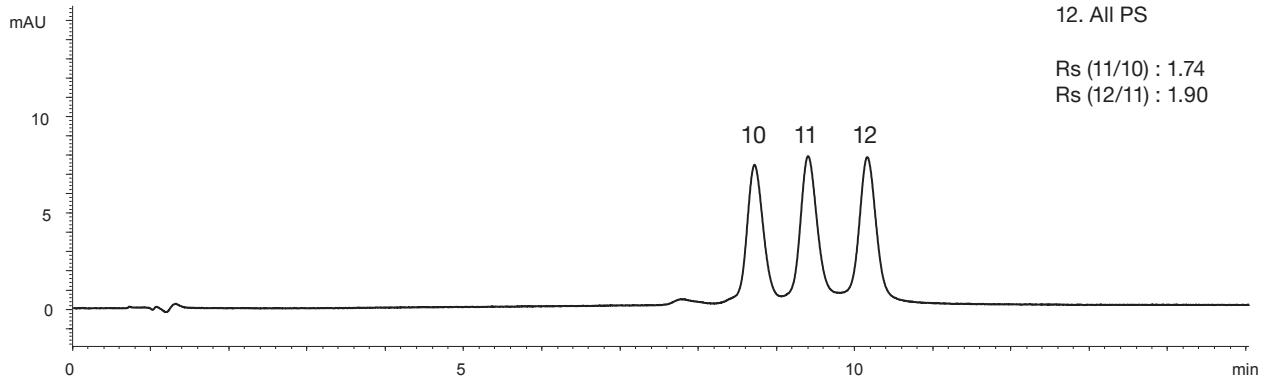
10	Single-stranded DNA	5'-~T~T~T~T~T~T~T~T~T~T~TTT-3' (DNA 15 mer 12PS, 2PO)
11		5'-T~T~T~T~T~T~T~T~T~T~T~T~TT-3' (DNA 15 mer 13PS, 1PO)
12		5'-T~T~T~T~T~T~T~T~T~T~T~T~T~T-3' (DNA 15 mer All PS)
13	Single-stranded RNA	5'-U~C~A~U~C~A~C~A~C~U~G~A~A~U~A~C~C~A~A~U-3' (RNA 20 mer All PS)
14		5'-G~U~C~A~U~C~A~C~A~C~U~G~A~A~U~A~C~C~A~A~U-3' (RNA 21 mer All PS)

~=Phosphorothioated

1.2.1. Resolution of phosphorothioate oligonucleotides with different degrees of thiolation

Using the optimised conditions described above, [all PS], [13PS, 1PO] and [12PS, 2PO] of DNA 15 mer (samples 10–12) were clearly separated by ion exchange chromatography.

DNA 15 mer group (sample 10–12)



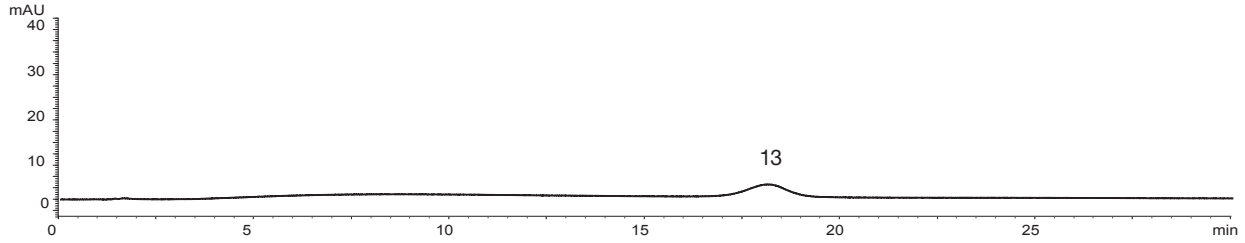
Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 10 mM NaOH
	B) 10 mM NaOH containing 1.0 M $NaClO_4$
Gradient:	40–70%B (0–15 min), 100%B (15–20 min)
Flow rate:	1.0 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	6 µL (each 3.3 nmol/mL)
Sample:	DNA 15 mer (samples 10, 11 and 12)

1.2.2. Difference in salt concentrations required for eluting modified RNA (all PS) and normal RNA (all PO)

Usually, PS products show broader peaks than PO products due to their higher acidity, so that a higher salt concentration is required for elution. The separation of RNA 20 mer all PS is compared to that for RNA 20 mer all PO under the same conditions. The peak of all PS material is much broader because it is thought that the all PS sample may contain as many as 2^{19} (524,288) stereoisomers.

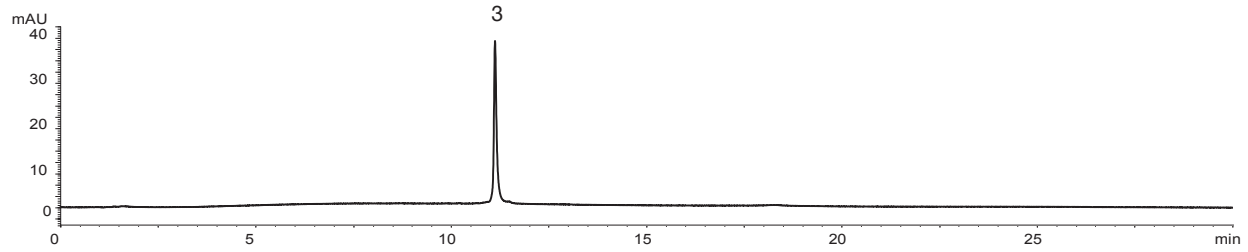
Sample 13; RNA 20 mer All PS

tR : 18.12



Sample 5; RNA 20 mer All PO

tR : 11.14

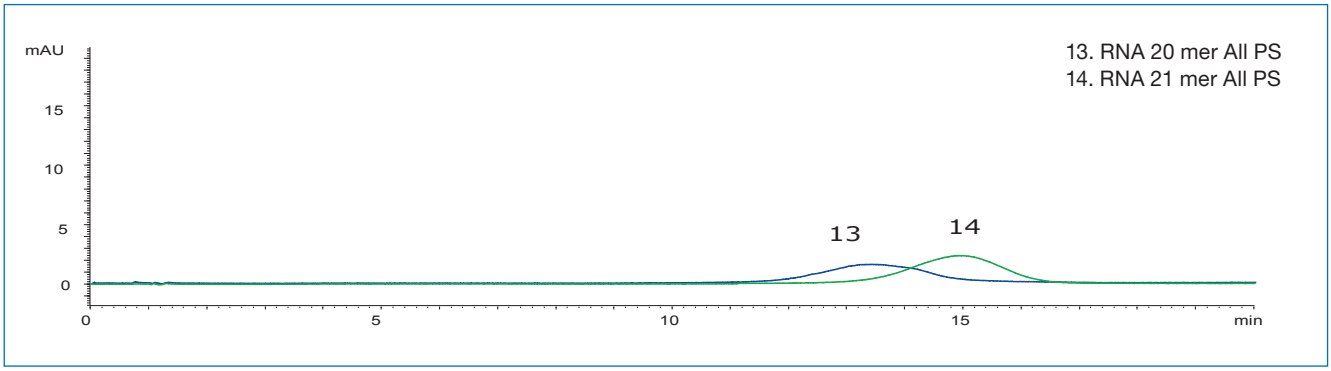


Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 10 mM NaOH
	B) 10 mM NaOH containing 1.0 M $NaClO_4$
Gradient:	10–100%B (0–30 min)
Flow rate:	1.0 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	2 µL (10 nmol/mL)

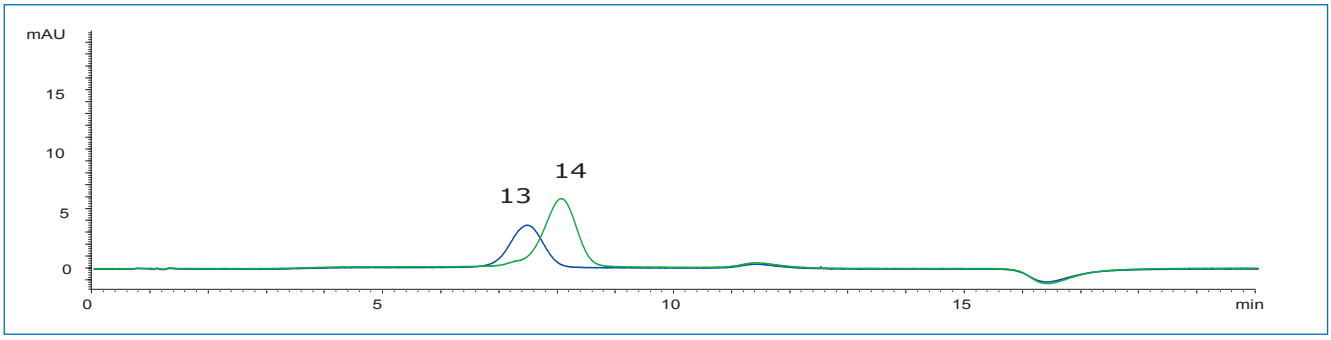
1.2.3. Separation of optimisation trial of phosphorothioate oligonucleotides with single-base differences in length

The following chromatograms show how to achieve narrow peaks and high sensitivity by optimising the separation of PS oligonucleotides in only 5 steps using the strong anion exchanger BioPro IEX QF.

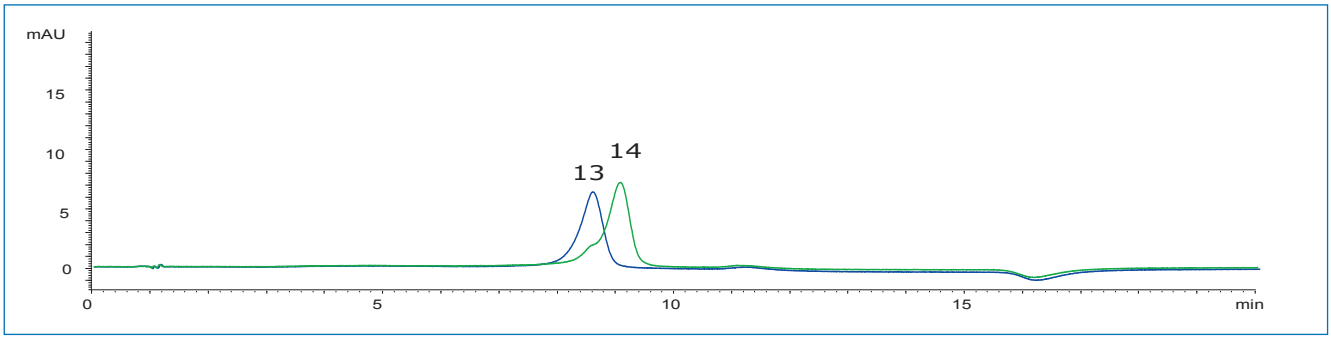
As a first step the PS RNA is analysed without using an organic modifier with a gradient of 32–80% B in 24 min at 25 °C. Broad peaks and low sensitivity were obtained.



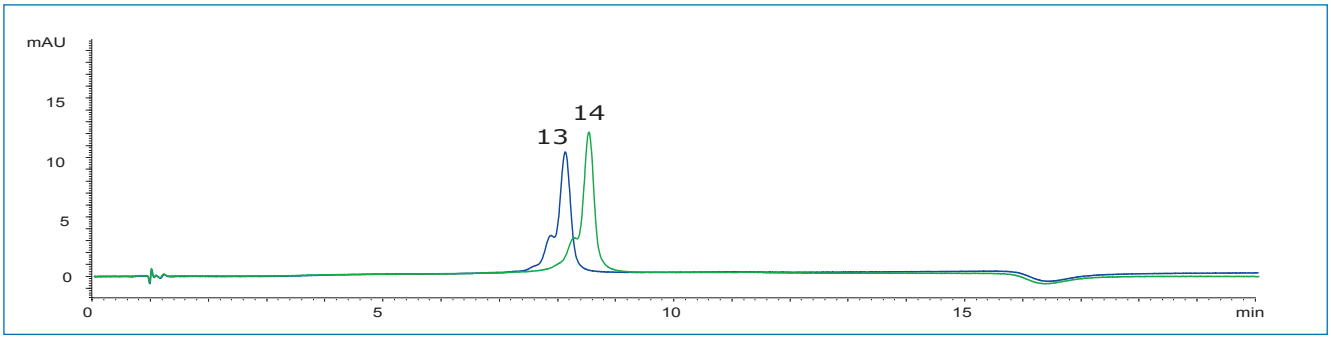
Using a steeper gradient of 32–80%B in 8 min the peaks became a little bit narrower, because the salt concentration is also rising.



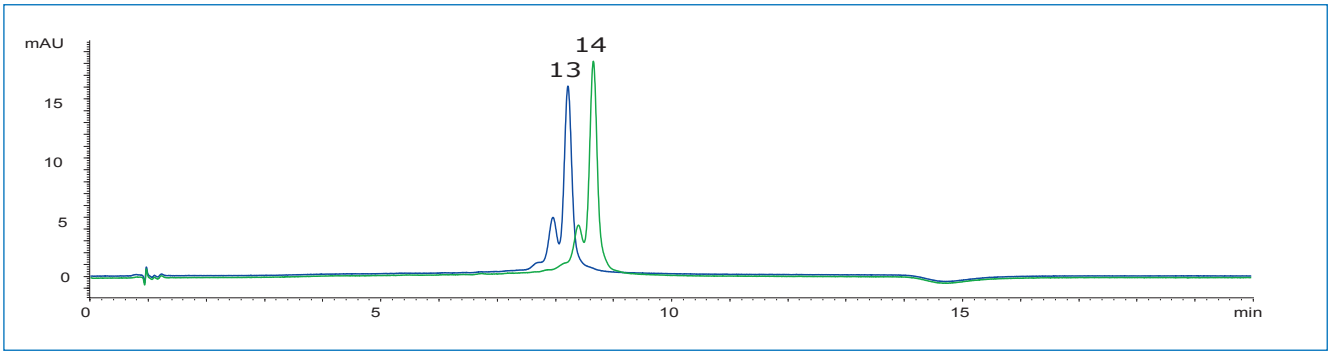
Increasing the column temperature to 60 °C led to narrower peaks and improved resolution.



With the addition of methanol as organic modifier (Eluent A and B: 20% MeOH) the resolution and sensitivity improved. To achieve the same salt concentration the gradient was adjusted to 40–100%B in 8 min.



By increasing the ratio of organic modifier (30% MeOH in A and B) narrow peaks with partly resolved variants and higher sensitivity were obtained. The gradient is adjusted to 40–100%B in 6.3 min.



Final chromatographic conditions were:

Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	A) 10 mM NaOH/methanol (70/30)
	B) 10 mM NaOH containing 1.0 M $NaClO_4$/methanol (70/30)
Gradient:	40–100%B in 6.3 min
Flow rate:	1.0 mL/min
Temperature:	60°C
Detection:	UV at 260 nm
Injection:	2 µL (10 nmol/mL)
Sample:	RNA 20 and 21 mer All PS (samples 13 and 14)

As shown above, varying the different parameters in your oligonucleotide analysis can have a very large influence on the separation. To sum this up, for AEX the most important influencing factors are:

- **Type of salt used for elution**
- **Type of buffer**
- **Initial concentration of salt**

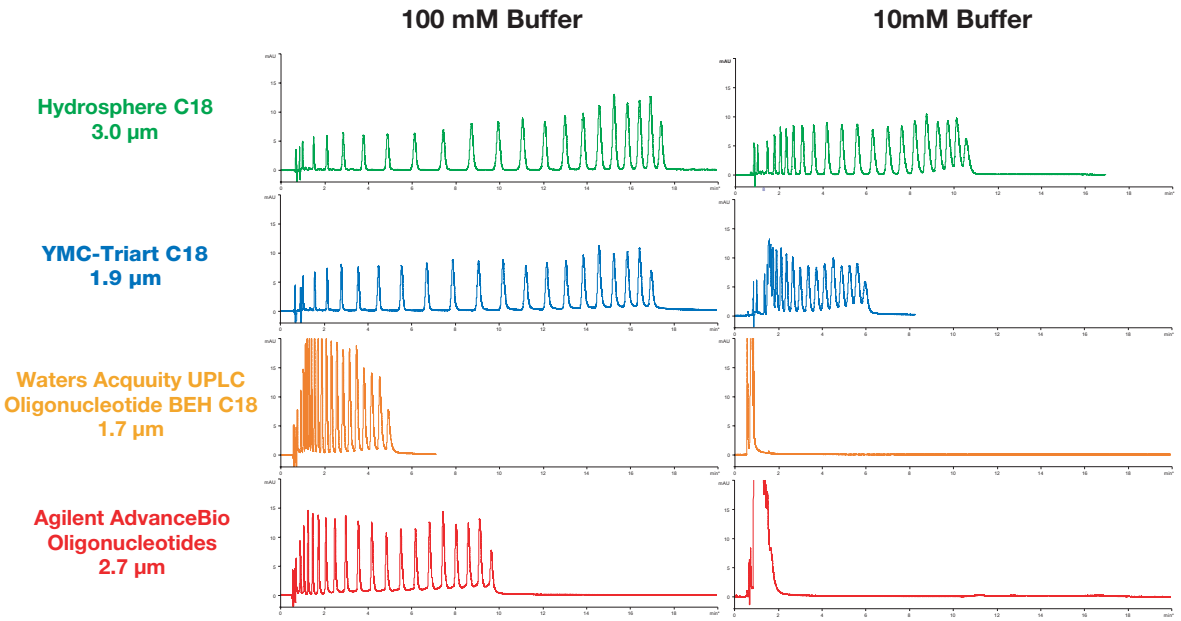
2. IP-RP analysis for oligonucleotides

Reversed phase HPLC has been widely used for the analysis and purification of synthetic oligonucleotides. Because it is difficult to retain and separate highly polar compounds such as short oligonucleotides on ordinary reversed phase columns, columns used for these separations have to meet specific requirements. These requirements include stability in 100 % aqueous mobile phases, temperature stability and larger pore sizes. YMC offers a range of columns for oligonucleotide analysis: YMC-Triart C18, YMC-Triart Bio C18, YMC-Triart C8 and Hydrosphere C18. YMC-Triart columns provide full flexibility in method development due to their robust hybrid particle, whereas the silica-based Hydrosphere C18 provides strong retention of polar compounds. The different pore sizes available for the different stationary phases are an important factor, since the pore size has an influence on the diffusivity of the oligonucleotide and therefore on the peak shape.

In the following sections, various suggestions are presented that can be shown to be helpful in improving oligonucleotide analysis by ion-pairing reversed phase chromatography (IP-RP). For a successful separation, ion-pairing agents need to be present in the mobile phase due to the ionic structure of oligonucleotides.

2.1. Selecting an appropriate stationary phase

The separation of oligo(deoxythymidylic acids), $d(pT)_{2\text{-}20}$, was compared using 100 mM or 10 mM triethylammonium acetate (TEAA) buffer, under the same gradient conditions. Both Hydrosphere C18 and YMC-Triart C18 showed enhanced retention and resolution compared to other commercially available C18 phases designed for oligonucleotide analysis, even at the low buffer concentration such as 10 mM. The higher concentration provides stronger retention and superior resolution of oligonucleotides, although a lower concentration has the advantages of increasing the signal intensity and reducing system contamination in HPLC-MS analysis.



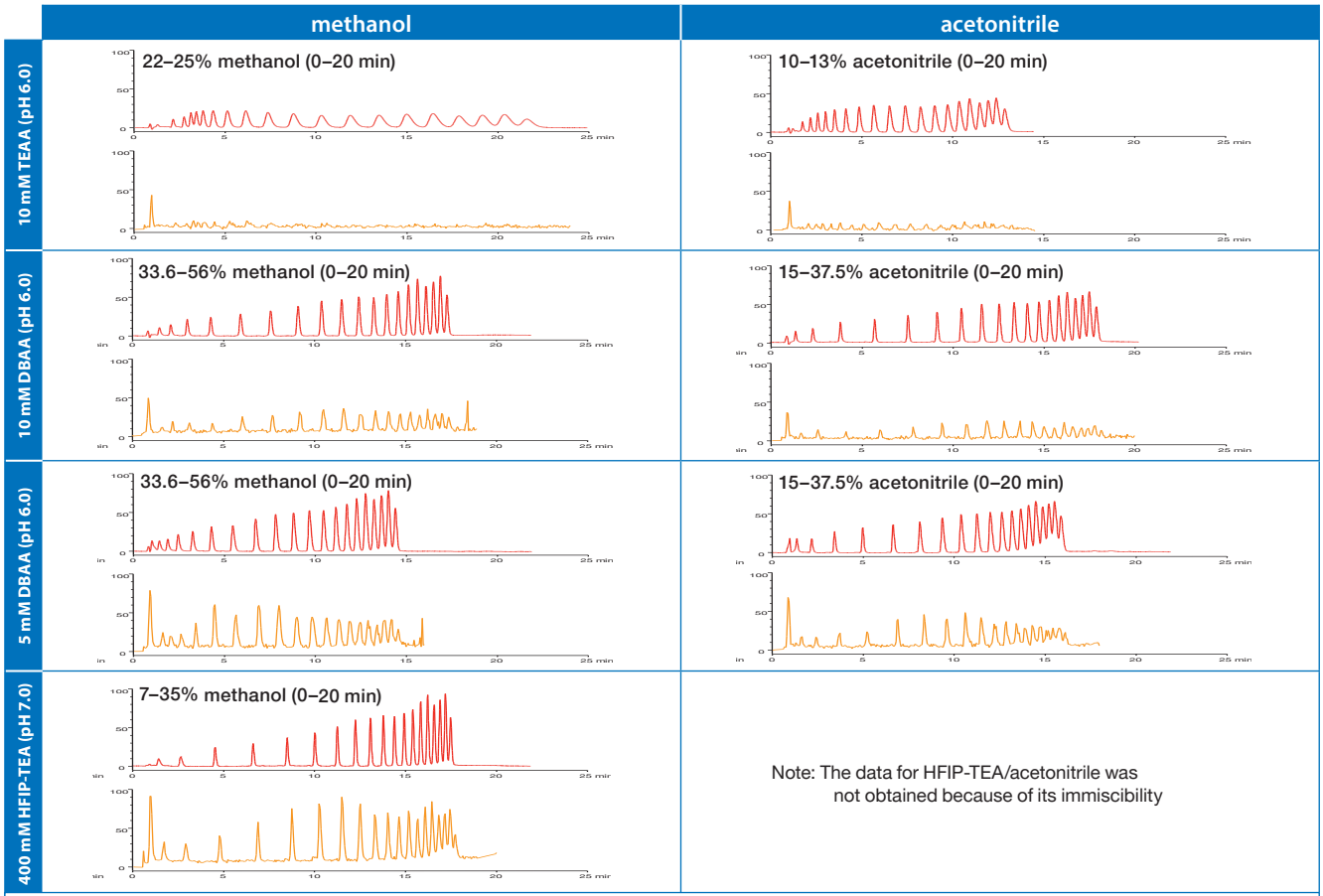
Column:	50 x 2.1 mm ID	Temperature:	35 °C
Eluent:	A) 100 mM or 10 mM triethylamine-acetic acid (pH 6)	Detection:	UV at 260 nm
	B) A/acetonitrile (80/20)	Injection:	2 µL (5 nmol/mL)
Gradient:	50–65%B (0–20 min)	Sample:	Oligodeoxythymidylic acid [$d(pT)_{2\text{-}20}$]
Flow rate:	0.21 mL/min		

2.2. Effect of salt concentration of ion-pairing mobile phase on the separation and signal intensity of oligonucleotides

Usually, IP-RP is coupled to electrospray ionisation mass spectrometry (ESI-MS) to characterise oligonucleotides. Due to the need for volatility of the mobile phase for ESI-MS not every buffer is suitable for this type of analysis. To evaluate the effects on the separation and signal intensity in ESI-MS, different volatile buffers were examined. The choice of which ion-pairing agent should be used is also one of the most important things to consider. Using different gradient conditions, acceptable retention and resolution can be achieved (UV-chromatograms, red trace) for each separation by optimising the gradient slope of the organic solvent regardless of the type of mobile phase.

The ESI-MS intensity is significantly influenced by the type and concentration of ion-pairing buffer as shown in the MS chromatograms (orange trace). 1,1,1,3,3,3-Hexafluoro-2-propanol-triethylamine (HFIP-TEA) buffer/methanol systems provide the maximum MS intensity. However, acetonitrile cannot be used with HFIP-TEA buffer due to its immiscibility. This is one reason why di-n-butylammonium acetate (DBAA) and triethylammonium acetate (TEAA) are also valuable buffer systems, even though they do not show the same high resolutions and intensities as HFIP-TEA. Ion-pairing reagents such as triethylamine (TEA) or dibutylamine (DBA) have both positive charge and hydrophobic moieties in molecule. Their hydrophobic interaction contributes to longer retention times and improvement in resolution.

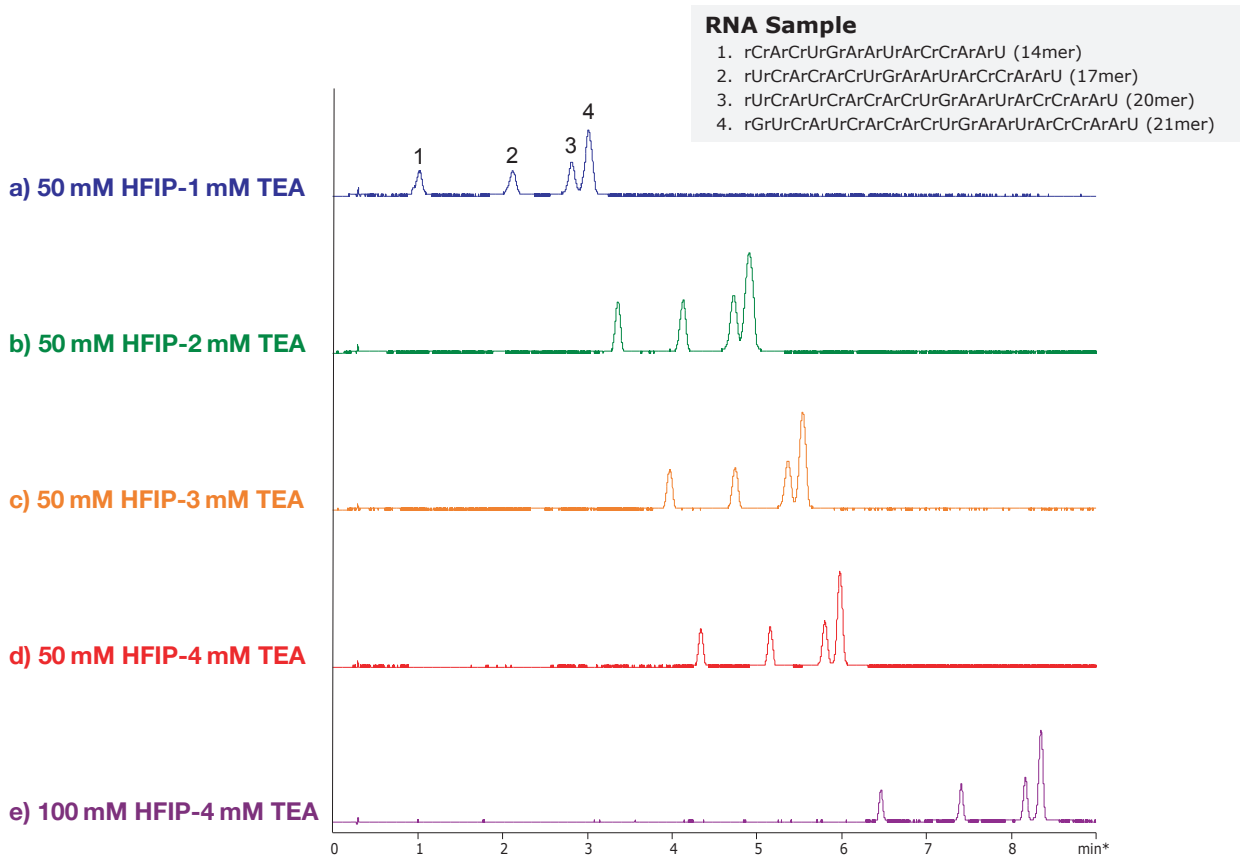
Using 10 mM DBAA enhanced retention and MS intensity are obtained compared to 10 mM TEAA buffer. The lower DBAA concentration of 5 mM results in approximately 1.5–3 times increase in the intensity without any change in the concentration of organic solvent. Another reason why DBAA and TEAA should be considered over HFIP-TEA as mobile phase is the cost effectiveness, especially for purification processes which demand high amounts of mobile phase.



Column:	Hydrosphere C18 (3 µm, 12 nm) 50 x 2.0 mm ID	Temperature:	35°C
Part. No.:	HS12S03-0502WT	Injection	5 µL (25 pmol/mL)
Flow rate:	0.2 mL/min	Sample:	Oligo(deoxythymidylic acid) $[d(pT)_{2\text{-}20}]$
Detection:	UV at 269 nm (red), ESI negative mode (orange)		

2.3. Effect of composition of ion-pairing reagent on the separation

To show the importance of choosing the correct ion-pairing reagent, the separation of 17–21 mer RNA was compared using mobile phases consisting of different ratios of HFIP and TEA. The retention time was approximately doubled when the TEA concentration was increased from 1 to 4 mM at a constant 50 mM HFIP concentration (chromatograms a-d), and increased approximately 1.5 times when the HFIP concentration was increased from 50 mM to 100 mM at a constant 4 mM TEA concentration (chromatograms d and e). These results indicate that small changes in the concentration of basic ion-pairing reagents such as TEA and DBA provide a large impact on oligonucleotide separations.



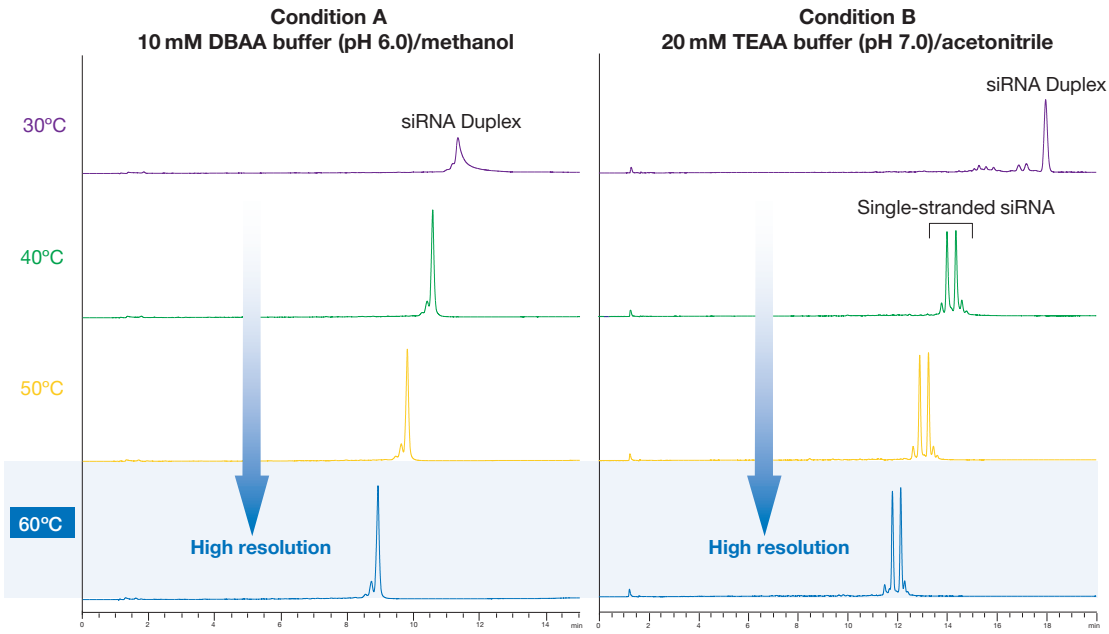
Column:	YMC-Triart C18 (1.9 µm, 12 nm) 50 x 2.1 mm ID	Flow rate:	0.42 mL/min
Part. No.:	TA12SP9-05Q1PT	Temperature:	65 °C
Eluent:	A) shown in figure	Detection:	UV at 260 nm
	B) methanol	Injection	1 µL (2–4 nmol/mL)
Gradient:	5–15%B (0–10 min)		

2.4. Effect of mobile phase and column temperature on separation of siRNA duplex

The separation of siRNA duplex was compared using different mobile phase conditions at various temperatures. Under both conditions A and B, peak shape and resolution between intermediate peaks was improved by increasing the temperature. As a result of enhanced dispersion and distribution velocity when increasing column temperature, biomolecules generally exhibit sharper peaks and improved resolution.

Under condition B at 40 °C or above, two peaks of single-stranded RNA, generated by the denaturation of the siRNA duplex, can be observed. This HPLC technique which uses high temperature to generate single-stranded RNA is called “Denaturing HPLC”, and is widely used in the field of gene mutation analysis. Denaturation of duplex DNA or RNA is also influenced by ionic strength (type and concentration), pH and polarity. It is recommended that these analytical conditions should be optimised depending on the characteristics of the target analyte and the purpose of the analysis.

The combination of neutral buffers containing amino ion-pairing agent and high temperature is useful for high-throughput analyses of oligonucleotides or for denaturing HPLC. However, silica-based reversed phase columns can rarely be used under such conditions due to their poor stability. YMC-Triart C18 columns offer excellent stability at elevated temperatures and pH and are therefore ideal for such oligonucleotide analysis.



Crude synthetic siRNA duplex (19bp): 5’-CGU ACG CGG AAU ACU UCG AdTdT-3’
3’-dTdTGCA UGC GCC UUA UGA AGC U-5’

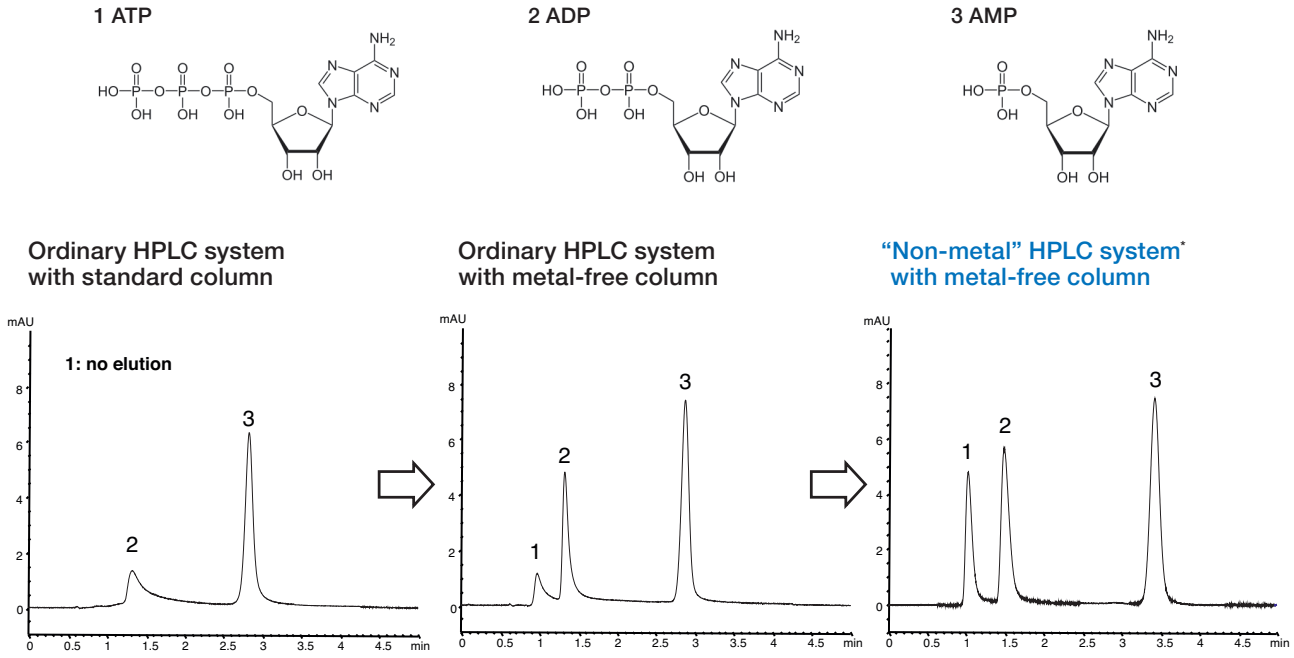
Column: YMC-Triart C18 (1.9 µm, 12 nm) 100 x 2.0 mm ID
Part. No.: TA12SP9-1002PT
Flow rate: 0.2 mL/min
Detection: UV at 269 nm
Injection 1 µL (5 nmol/mL)
System: Agilent 1290

Condition A Eluent: A) 10 mM di-n-butylamine-acetic acid (pH 6.0)
B) methanol
Gradient: 35–60%B (0–15 min)

Condition B Eluent: A) 20 mM triethylamine-acetic acid (pH 7.0)
B) acetonitrile
Gradient: 5–12%B (0–20 min)

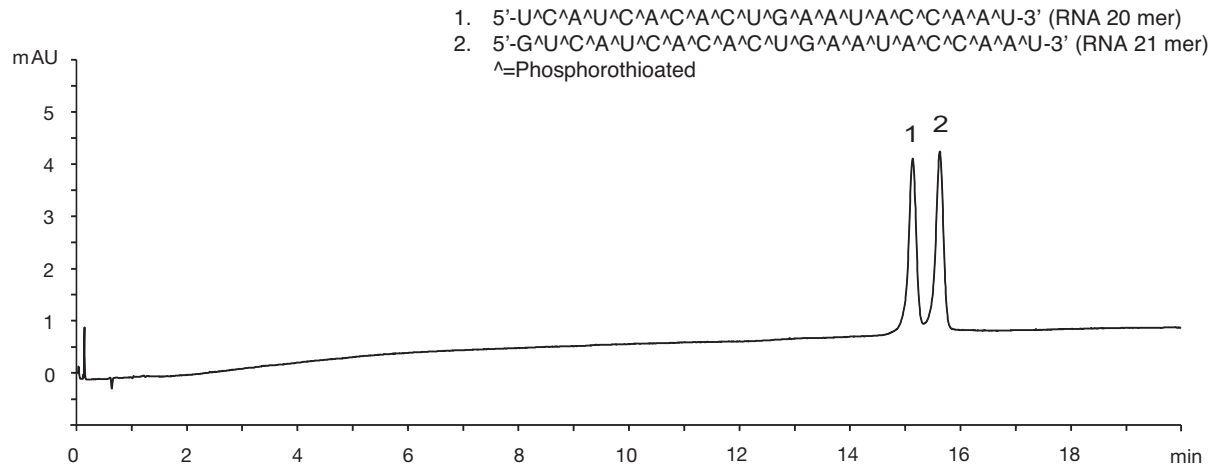
2.5. Metal-free column hardware

Metal coordinating compounds, which have a phosphate group in their structure such as oligonucleotides, tend to show poor peak shape due to interaction with metals, such as stainless steel in column bodies and frits. By using metal-free column hardware, better peak shapes can be expected. Nucleotides with phosphate groups show better peak shapes with metal-free column hardware when compared to regular column hardware. In a further step, the peak shape can be greatly improved by using a completely “non-metal” HPLC system.

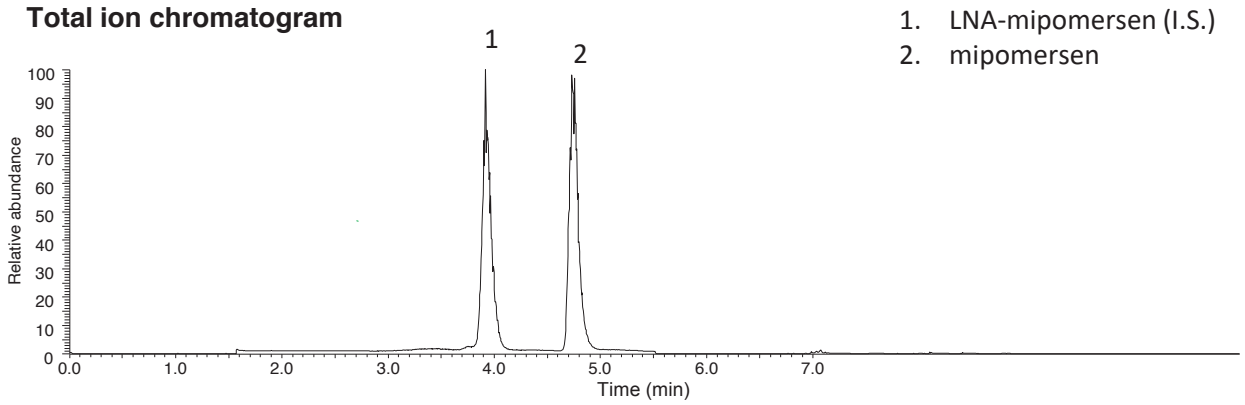


Column:	YMC-Triart C18 (3 µm, 12 nm) 50 x 2.1 mm ID
Part Nos:	TA12S03-05Q1PT (standard hardware)
	TA12S03-05Q1PTP (**metal-free** hardware)
Eluent:	5 mM $HCOONH_4$
Flow rate:	0.21 mL/min
Temperature:	25 °C
Detection:	UV at 265 nm
Injection:	1 µL (10 µg/mL)

Great peak shapes were also obtained for two oligonucleotide examples:



Column:	YMC-Triart C8 metal-free (1.9 µm, 12 nm) 100 x 2.1 mm ID
Part. No.:	TO12SP9-10Q1PTP
Eluent:	A) 15 mM triethylamine-400 mM HFIP
	B) methanol
Gradient:	10–20%B (0–20 min)
Flow rate:	0.42 mL/min
Temperature:	70 °C
Detection:	UV at 260 nm
Injection:	1 µL (each 1.25 nmol/mL)



Column:	YMC-Triart C8 metal-free (1.9 µm, 12 nm)* 100 x 2.1 mm ID
Part. No.:	TO12SP9-10Q1PTP
Eluent:	A) water/triethylamine/HFIP (100/0.4/2; triethylamine 28.0 mM, HFIP 135.8 mM)
	B) methanol/triethylamine/HFIP (100/0.4/2)
Gradient:	[Sample separation step]
	10-40%B (0-5 min)
	[Column wash steps]
	40–70%B (5.0–5.1 min), 70%B (5.1–7.0 min), 70–10%B (7.0–7.1 min),
	10%B (7.1–8.0 min), 10–90%B (8.0–8.1 min), 90%B (8.1–9.0 min),
	90–10%B (9.0–9.1 min), 10%B (9.1–10.0 min), 10–90%B (10.0–10.1 min),
	90%B (10.1–11.0 min), 90–10 %B (11.0–11.1 min)
Flow rate:	0.3 mL/min
Temperature:	50 °C
Injection:	10 µL (1,000 ng/mL)
System:	LC) Vanquish Binary Pump H system
	HRMS) Orbitrap HRMS Q Exactive Plus

*1 Prewash the column prior to the first use with water/methanol/phosphoric acid (70/30/0.1) for 1 hour

There are various factors that can influence your analysis using IP-RP as shown above. If your oligonucleotide separation by IP-RP does not work as required the following factors should be considered:

- Column selectivity
- Type of ion-pairing agent and its concentration
- Temperature
- Column hardware

Oligonucleotide LC-analysis is a complex field of interest. There are many factors that contribute to separation and consequently resolution. Due to the retention mechanism of AEX, the ionic strength of the eluent is the decisive factor. The type of salt, the initial concentration and the nature of buffer influence the chromatographic behaviour. Almost similar parameters influence IP-RP. Here, the nature and concentration of buffer are important factors. However, chemical modification of the stationary phase, column hardware and temperature provide an even greater impact on chromatographic results.

[1] A. Goyon et al., Characterization of therapeutic oligonucleotides by liquid chromatography, J. Pharm. Biomed. Anal., 2020 April 15, 182:113105 doi: 10.1016/j.jpba.2020.113105.