How to influence oligonucleotide analysis by IP-RP

The natural structure of siRNA with an electron-rich anionic phosphate backbone hampers its cellular uptake and is responsible for a poor pharmacokinetic profile. The additional resistance towards nuclease-mediated degradation led to the development of chemical modifications of the nucleotides structure. A common option is the introduction of synthetic artificial linkages such as phosphorothioate linkages (PS) instead of the natural phosphodiester linkages (PO). The exchange of the oxygen atom with sulphur in PS linkages decreases the polarity of the nucleotides and sensitivity to enzymatic degradation. As a result, PS oligonucleotides are more commonly synthesized and used for in vivo and in vitro applications. The chemical synthesis of oligonucleotides is a multistep process, where the nucleotide chain is extended successively. During synthesis, impurities with only minor structural differences such as shortmers (n-x) and longmers (n+x) can accumulate. Therefore, the purity and quality of oligonucleotides needs to be controlled closely since impurities can affect their efficacy. The analysis of oligonucleotides is highly challenging due to their negatively charged phosphate groups in the backbone. Ion pair reversed phase liquid chromatography (IP-RP) is considered the gold standard for oligonucleotide analysis. The selection of several parameters such as pH, gradient and column temperature highly influences the performance. Also the choice of ion pair reagent, pore size and hardware plays an important role. This technical note demonstrates the most important factors that need to be considered regarding the analysis of oligonucleotides by IP-RP.
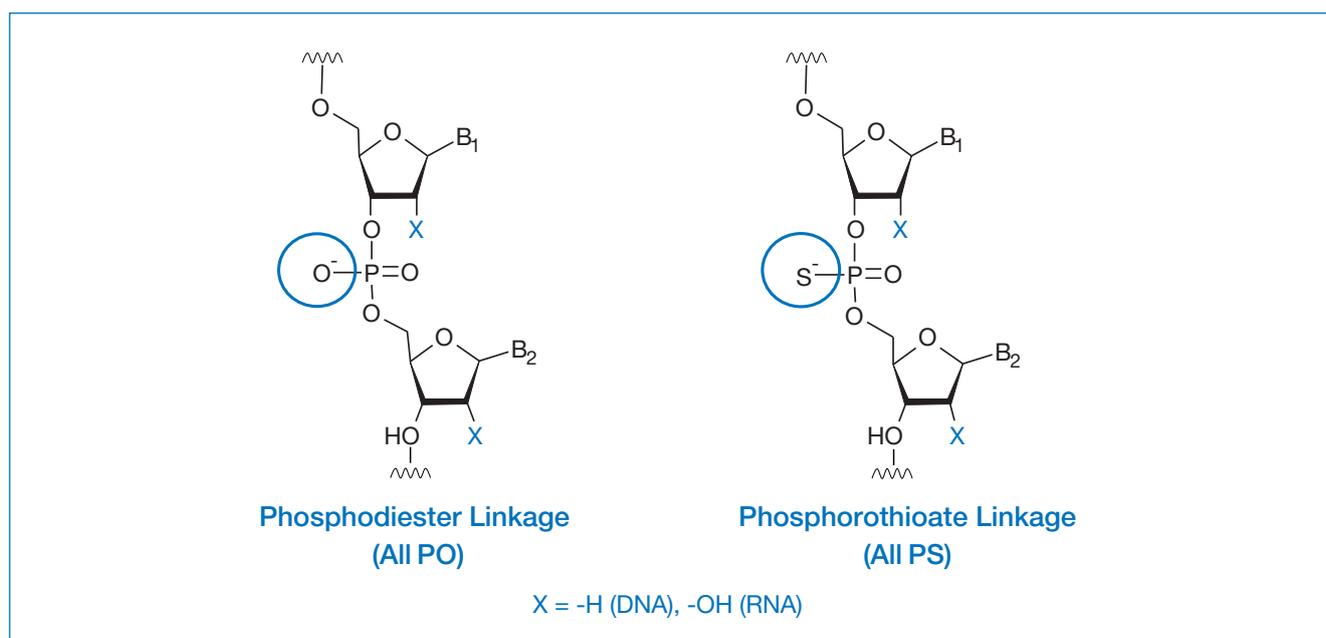
Figure 1: RNAs with phosphodiester linkage (All PO) and with sulphur modified phosphorothioate linkage (All PS).

1. Effect of ion pair reagents and pH on oligonucleotide separation

Chromatographic separation of charged biomolecules by RP is challenging because they are not retained sufficiently on the column. Since oligonucleotides possess an ionic structure, ion pairing reagents with an opposite charge need to be added to the mobile phase. Their hydrophobic part of the agent bonds to the hydrophobic stationary phase while the charged part pairs with the analytes from the mobile phase. This results in the oligonucleotides being retained on the stationary phase and separated by length, type and presence of modifications. Triethyl-amine (TEA) is frequently used as the ion pair agent and is often combined with the acidic counterion 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and methanol as organic modifier. The advantage of this mobile phase composition is its compatibility with electrospray ionisation mass spectrometry (ESI-MS). This is based on the higher volatility of HFIP, which results in faster evaporation of HFIP compared to TEA during ESI. The pH in the droplets increases and the TEA-paired oligonucleotide dissociates in the source so that the detectability is increased.

The parameters of the mobile phase were evaluated by use of four RNAs with phosphodiester linkage (PO RNAs) and two RNAs with phosphothioate linkage (PS RNAs) as samples (table 1).

Table 1: RNA samples and internal standard.

Phosphodiester linkage RNA (PO RNA)	5'-CACUGAAUACCAAU-3'	14mer
	5'-UCACACUGAAUACCAAU-3'	17mer
	5'-UCAUCACACUGAAUACCAAU-3'	20mer
	5'-GUCAUCACACUGAAUACCAAU-3'	21mer
Internal standard (I.S.)	Caffeine	
Phosphorothioate linkage RNA (PS RNA)	5'-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3'	20mer
	5'-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3'	21mer

* phosphorothioated

At first, the changes in concentration of TEA (1–30 mM) and as a consequence alteration in pH (7.4–8.9) were evaluated. The concentration of HFIP was kept constant at 100 mM. With increasing concentration of TEA up to 8 mM (pH 8.3), the retention time of both RNA types, PS and PO, also increased (figure 2). A concentration of 15 mM TEA (pH 8.6) or higher resulted in a decrease of retention and of resolution. This behaviour is common for oligonucleotides with PO and PS ligands. Higher pH leads to a reduced amount of ionised TEA since the ionisation is suppressed. Therefore, at higher pH, the retention of the RNAs is decreased (figure 3). The highest retention for the oligonucleotides tested was at around pH 8. For further optimisation, conditions were tested at this pH.

Table 2: Overall chromatographic conditions unless otherwise stated.

Column:	YMC-Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PT
Eluent:	A) 15 mM TEA - 400 mM HFIP (see figure 2)
	B) methanol
Gradient:	1%B /min (initial%B = 8%)
Flow rate:	0.42 mL/min
Detection:	UV at 260 nm
Temperature:	65 °C
Injection:	1.0 µL
Sample:	PO RNA, PS RNA
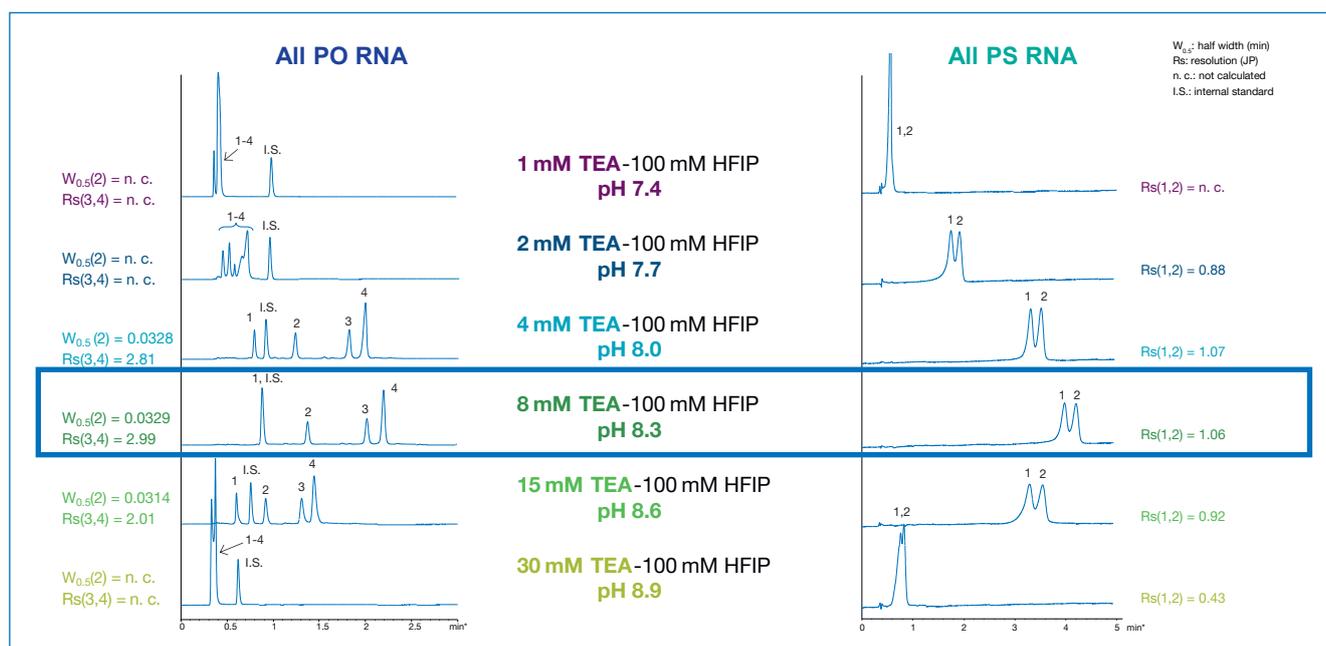
Figure 2: Influence of different TEA concentrations and resulting pH on PO RNA and PS RNA separation.

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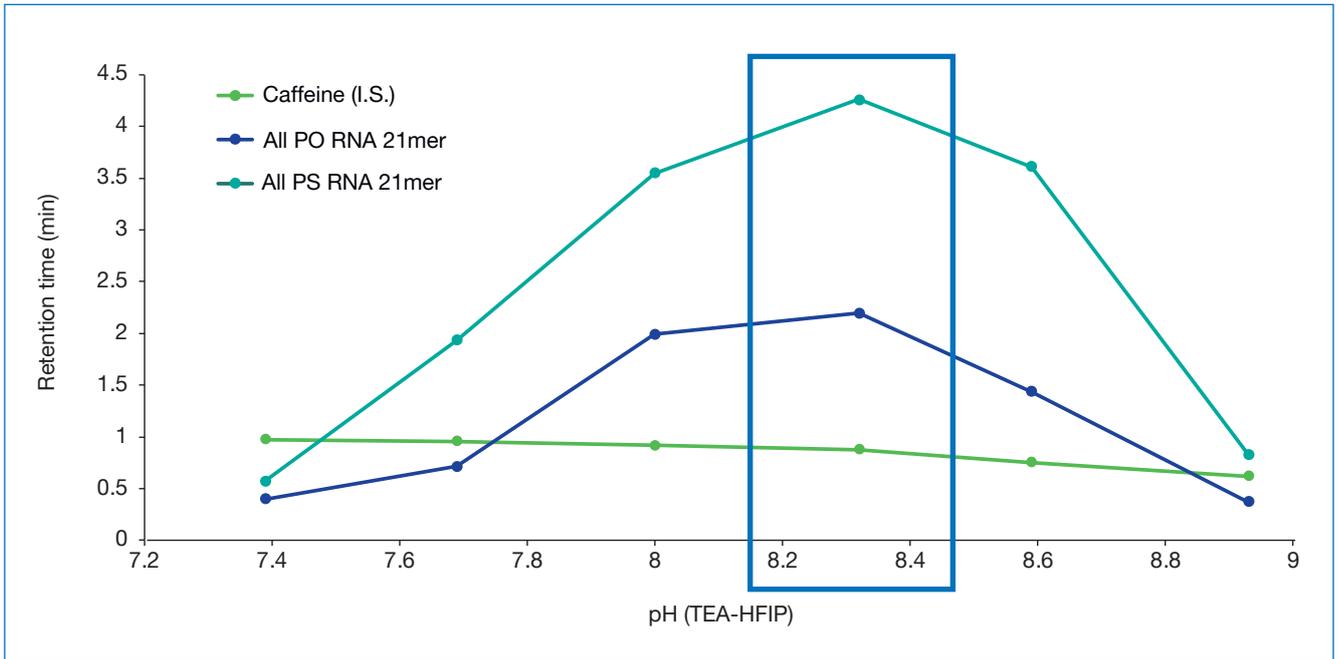
Figure 3: Impact of pH on retention and resolution of oligonucleotides.

For determination of the best combination of TEA and HFIP, the ratio of both components was varied. A concentration of TEA of 2–15 mM was tested in combination with a concentration of HFIP of 50–400 mM. By use of 8 mM TEA and 200 mM HFIP the retention of the PO RNAs strongly increases. Also, the resolution of peak shape of the PS RNAs was improved at this ratio. However, the best results were obtained with 15 mM TEA and 400 mM HFIP for both RNA types. These results indicate that TEA and HFIP concentrations have a drastic impact on retention and resolution of oligonucleotides. Small changes can improve the separation notably. The performance may vary with MS application since HFIP tends to form adducts when applied in high concentrations, which leads to a lower resolution.
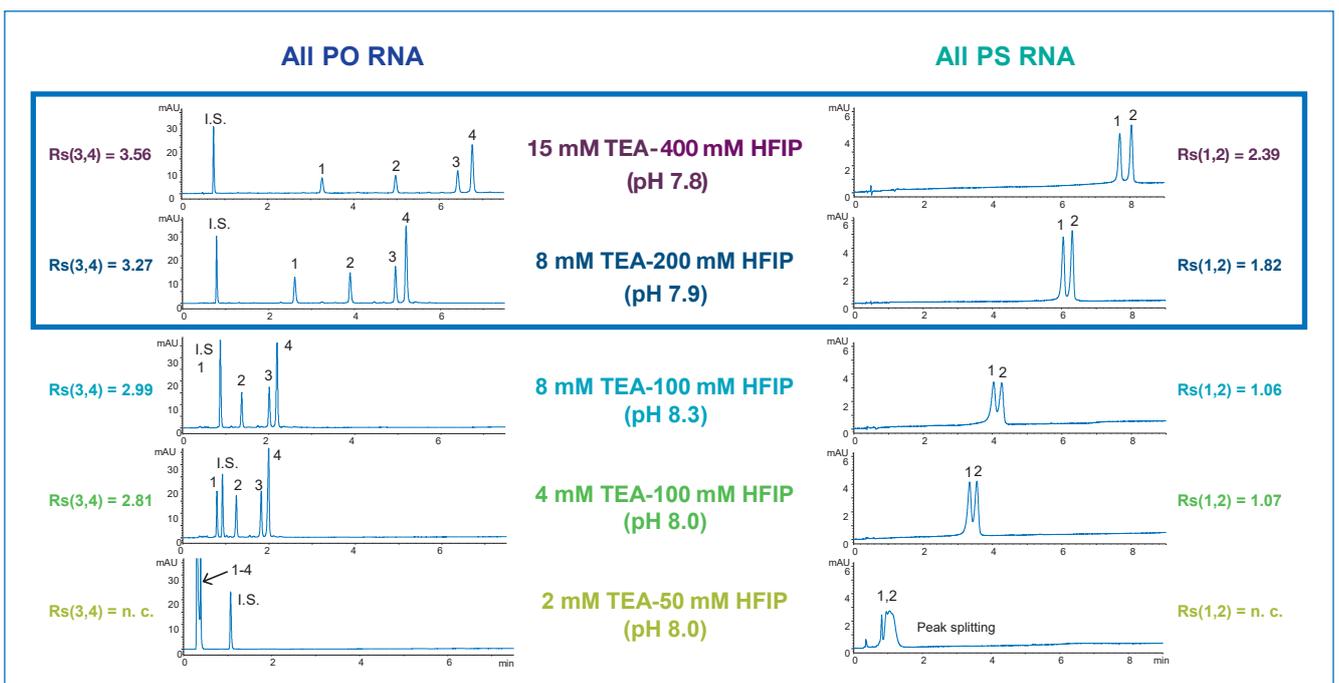
Figure 4: Effect of different concentrations of the ion pair reagent TEA and HFIP on the separation of PO RNA and PS RNA.

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To show the impact of various ion pair reagents on the separation of oligonucleotides, TEA was substituted for other alkylamines in different concentrations and evaluated in combination with different concentrations of HFIP (table 3, 4). A YMC-Accura Triart Bio C18 column was used and the gradient was adjusted individually (figure 5,6). The resolution of the oligonucleotides was sufficient with all the concentrations tested with TEA. However, an improvement in resolution could be observed for PO RNAs using 15 mM dibutylamine (DBA) or hexylamine (HA) in combination with 400 mM HFIP (figure 5). Good resolution was also achieved by use of 2 mM n-butylamine (BA) with only 50 mM HFIP. Similar results were observed for PS RNAs. Low concentrations of DBA and HA with low concentrations of HFIP gave the best resolution (figure 6). The various types of oligonucleotides are influenced differently by the ion pairs used. Therefore, change of ion pair reagent and concentration might be useful to improve the separation of challenging oligonucleotides. Since HFIP is costly, the use of alternative ion pair reagents in order to reduce the required amount of HFIP, might also be more cost-efficient. In analysis of PS RNA using DBA and HA, the amount of HFIP was reduced to one-eighth of its initial concentration. This would result in a high reduction of costs, especially when high-throughput analyses are performed.

Table 3: Alternative alkylamine reagents and their properties.

	Structural formula	MW	Solubility in water	pKa	log K_{ow}	Bp (°C)
Triethylamine (TEA)	H_3C—N(CH_3)(CH_3)	101.19	170 g/L (25 °C)	10.78 (25 °C)	1.45	89
n-Butylamine (BA)	H_3C—NH_2	73.14	Soluble	10.78 (20 °C)	0.97	78
Dibutylamine (DBA)	H_3C—NH—CH_3	129.25	3.5 g/L (25 °C)	11.25 (2 °C)	2.83	159
Hexylamine (HA)	H_3C—NH_2	101.19	12 g/L (25 °C)	10.64 (25 °C)	2.06	132
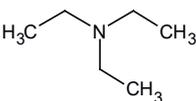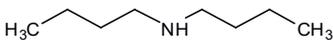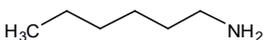
Table 4: Concentrations of alkylamines and HFIP used with resulting pH values.

		Alkylamine concentration (mM)					
		15		8		2	
HFIP concentration (mM)	400	BA TEA DBA HA	7.8 7.8 7.8 7.9				
	100			BA TEA DBA HA	8.3 8.2 8.3 8.3		
	50					BA TEA DBA HA	8.0 8.0 7.9 8.0

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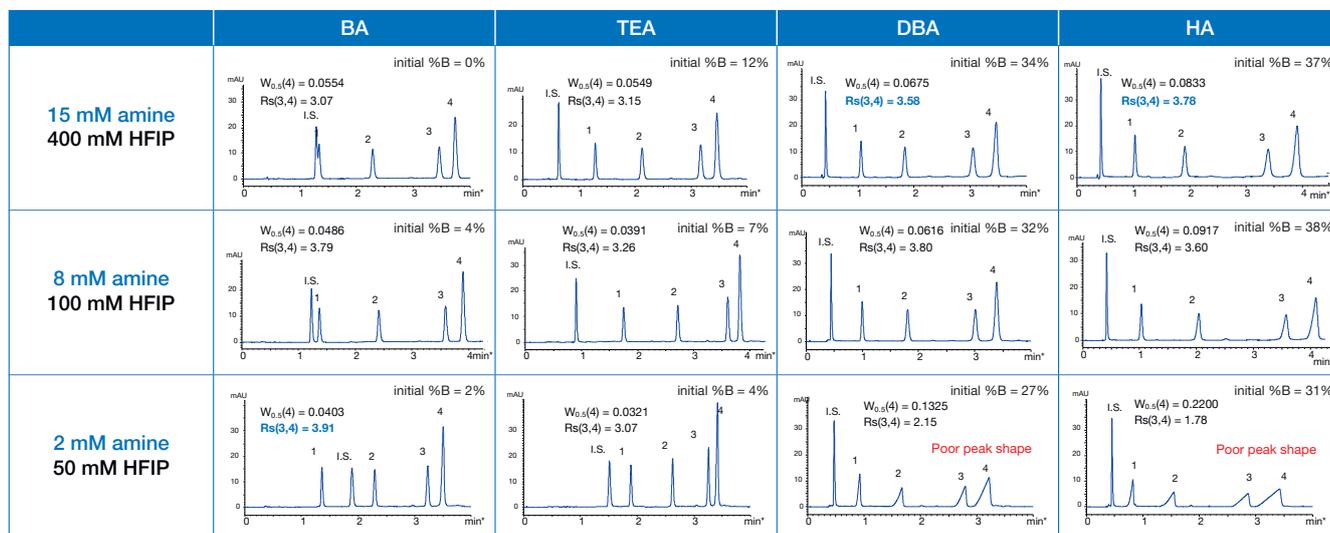
Figure 5: Comparison of four alkylamine ion pair reagents in different concentrations regarding their separation efficiency of PO RNAs.
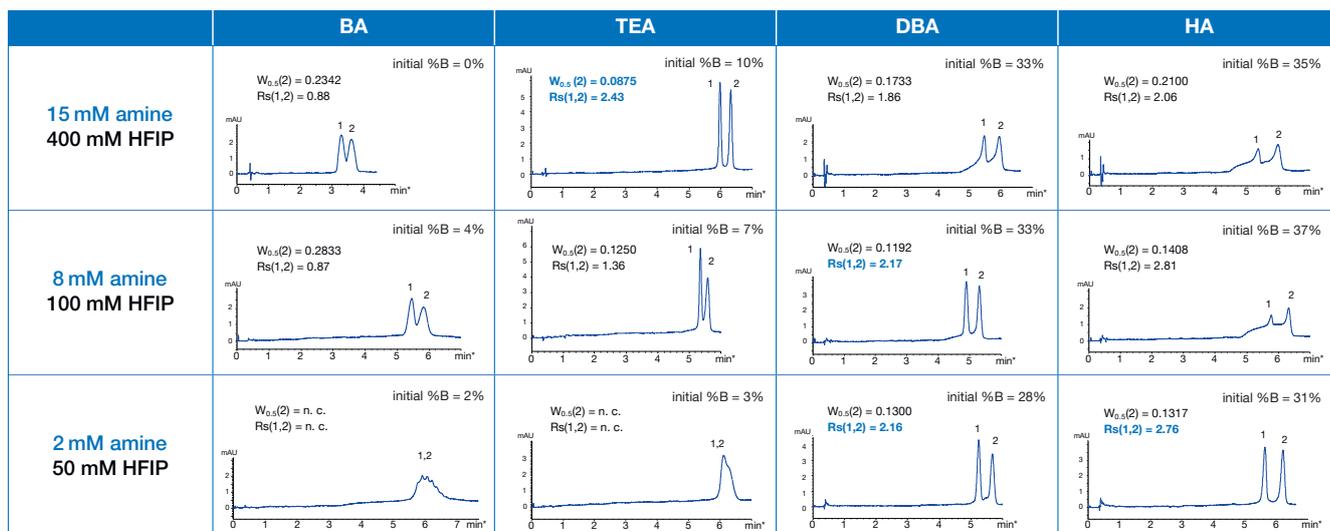
Figure 6: Comparison of four alkylamine ion pair reagents in different concentrations regarding their separation efficiency of PS RNAs.

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2. How the eluent gradient slope can affect oligonucleotide analysis

Finding a suitable gradient slope can optimise analysis time and resolution. Different gradients were tested with 0.4%–2.0% methanol/min and a starting condition of 8% methanol (figure 7). All oligonucleotides tested were separated with sufficient resolution at all conditions. A shallower gradient slope led to higher resolution; however, the sensitivity declined and the run time was prolonged. Adjustment of starting conditions with a higher percentage of methanol can also decrease run time as required.
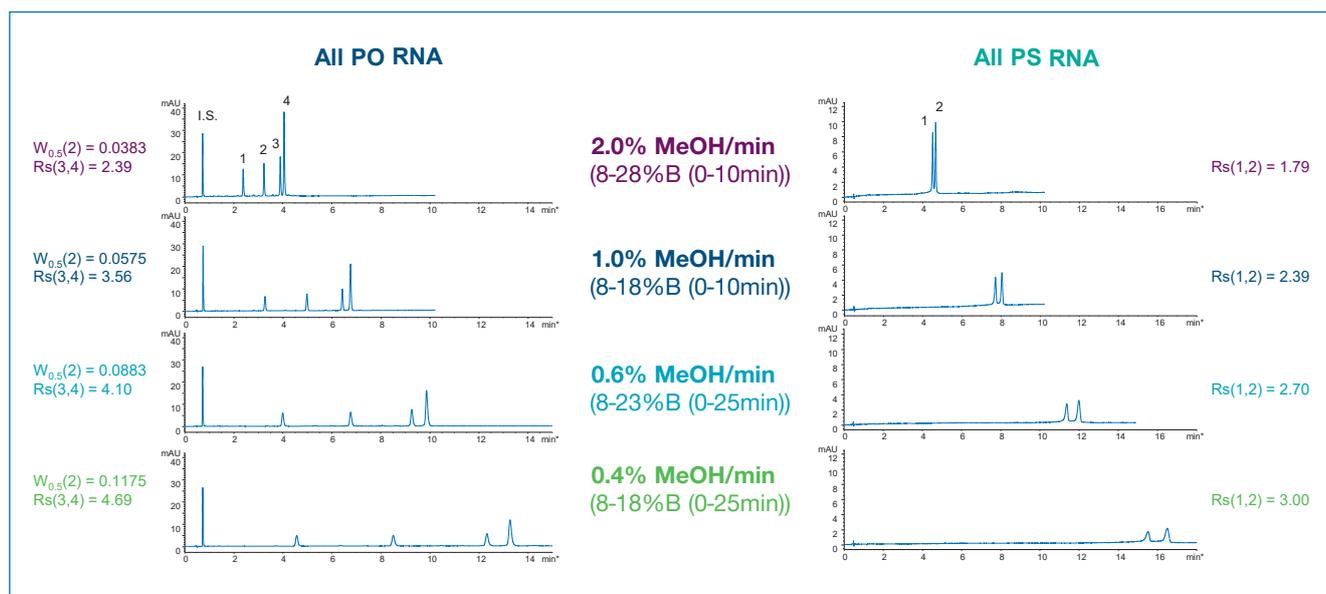
Figure 7: Effect of different methanol gradient slopes on PO RNA and PS RNA separation.

3. The importance of column temperature on oligonucleotide separation

The column temperature has a great influence on the chromatographic performance. An increased temperature improves the diffusion during chromatographic separation, which results in backpressure being reduced, retention time decreased and peak shapes improved. High temperatures above 60 °C are often applied in oligonucleotide analysis. They prevent secondary structure formation, which affects the retention of oligonucleotides. However, the required temperature depends on the oligonucleotide composition such as guanine-cytosine content and tendency for secondary structure formation. To determine the best column temperature for analysis of the PO RNAs and PS RNAs, four different column temperatures from 25 °C–90 °C were evaluated (figure 8). The gradient was adjusted individually. Good separation of PO RNAs was achieved at all temperatures; however, increased resolution and sharper peak shapes were observed at elevated temperatures. The best separation of PO RNAs was obtained at 90 °C. For PS RNAs acceptable resolution and peak shape were achieved even at 45 °C. Best results were obtained at 65 °C. At 90 °C peak deterioration was observed for PS RNAs, possibly due to structural changes of the RNA. The use of neutral buffers with ion pairing reagents and high temperatures is more suitable for RP-HPLC analysis of oligonucleotides. However, silica-based columns often cannot withstand these extreme conditions due to their lack of stability. YMC-Triart columns such as the YMC-Triart Bio C18 column used provide excellent stability even at high temperature and pH. This makes YMC-Triart columns an ideal choice for oligonucleotide analysis.

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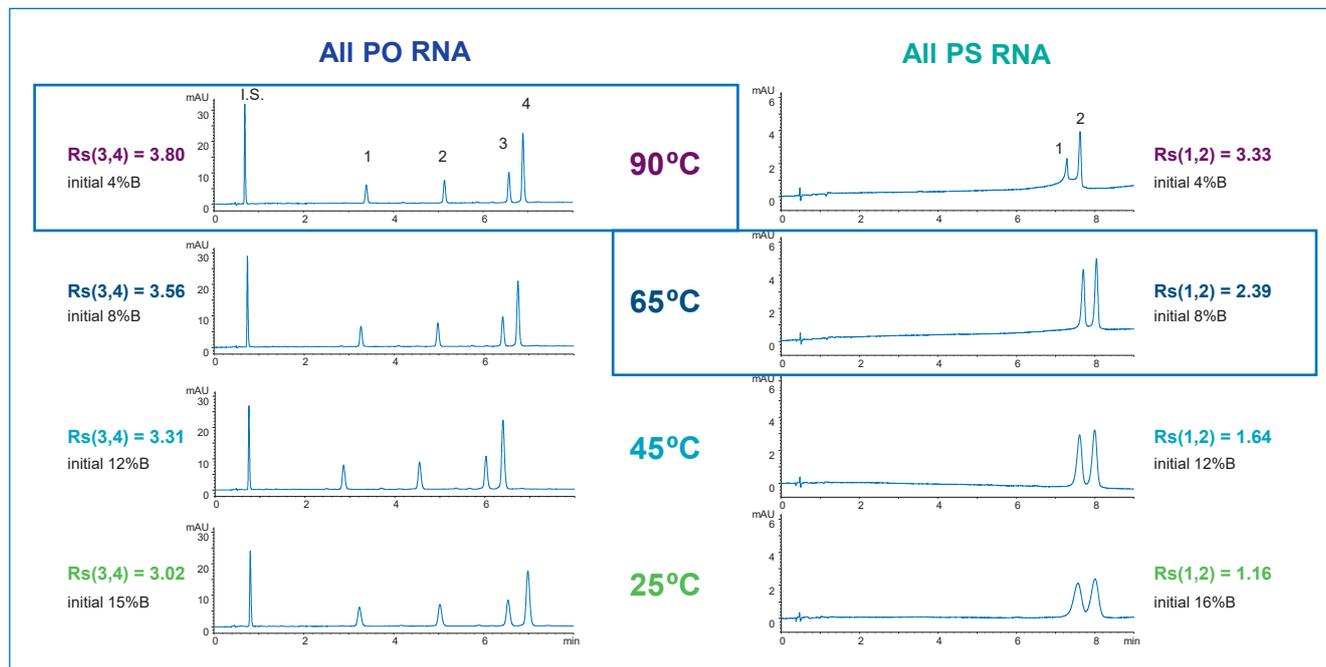
Figure 8: Influence of four different column temperatures on separation efficiency of PO RNAs and PS RNAs.

4. Influence of linkage modification on oligonucleotide separation

Oligonucleotides are often modified, which influence their chemical characteristics. Two oligonucleotides with the same sequence and length (both 20/21mer) but different linkages were compared under various chromatographic conditions (figure 9). The use of two different concentrations of TEA (4 mM, 15 mM) and HFIP (100 mM, 400 mM) in combination with two different temperatures (65 °C, 90 °C) were evaluated. The gradient was adjusted individually. The PO RNAs showed a good resolution at all conditions, even with lower concentration of TEA. Improved peak shapes were observed at elevated temperatures (90 °C).

In contrast, PS RNAs were retained longer on the stationary phase and resolution was clearly improved with higher TEA concentration at 65 °C. In oligonucleotides, the modification of a linkage from oxygen to sulphur does affect their configuration. Due to the sulphur atom, a stereogenic α-phosphorous is generated. The emerging diastereomers possess slightly different retention times. With rising numbers of sulphur atoms being incorporated, the hydrophobicity of oligonucleotides increases, which leads to a longer retention on the stationary phase.
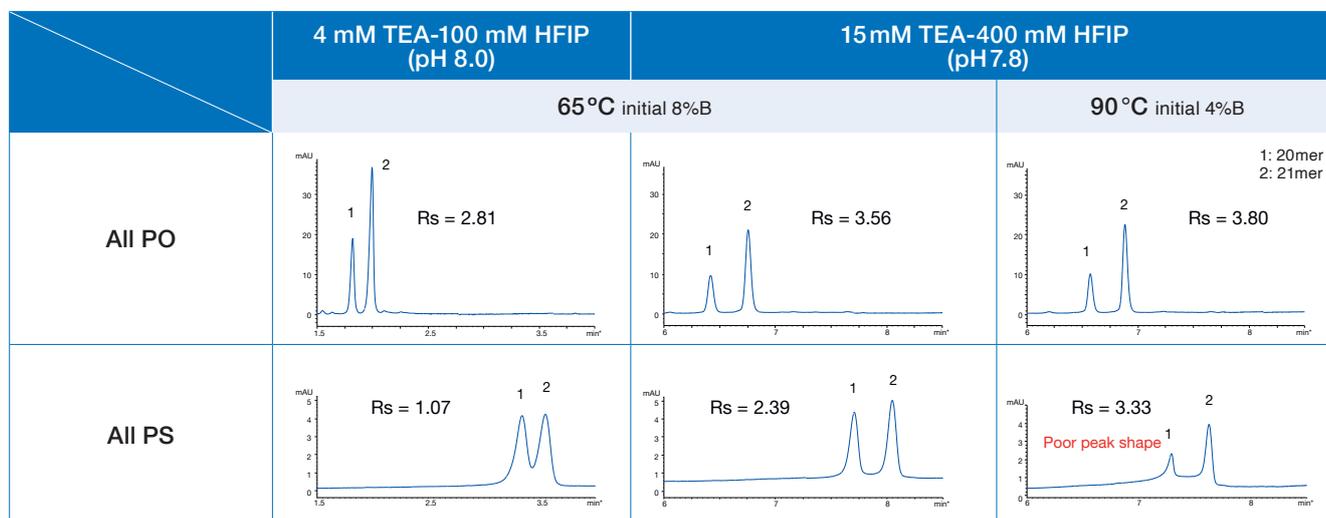
Figure 9: Impact of oligonucleotide linkage on retention and resolution of two PO RNAs and PS RNAs.

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5. The selectivity of particle pore size in oligonucleotide analysis

In oligonucleotide analysis, C18 stationary phases are the most popular ones. They are usually able to separate oligonucleotides with variation in only one base. Three different C18 pore sizes (8 nm, 12 nm, 30 nm) were tested to analyse their influence on oligonucleotide analysis (figure 10). 8 mM TEA and 200 mM HFIP and a gradient of 10%–20% methanol were used. The shortest retention time is obtained with particles with a pore size of 30 nm.

This wide pore size also gave the best separation of longer PO RNAs of 20 and 21 nucleotides length. In contrast, the best resolution for shorter oligonucleotides of 14 and 17 nucleotides length was observed with a standard pore size of 12 nm. The particle with only 8 nm pore size showed sufficient separation but had not advantage over the particles with other pore sizes regarding retention and resolution.
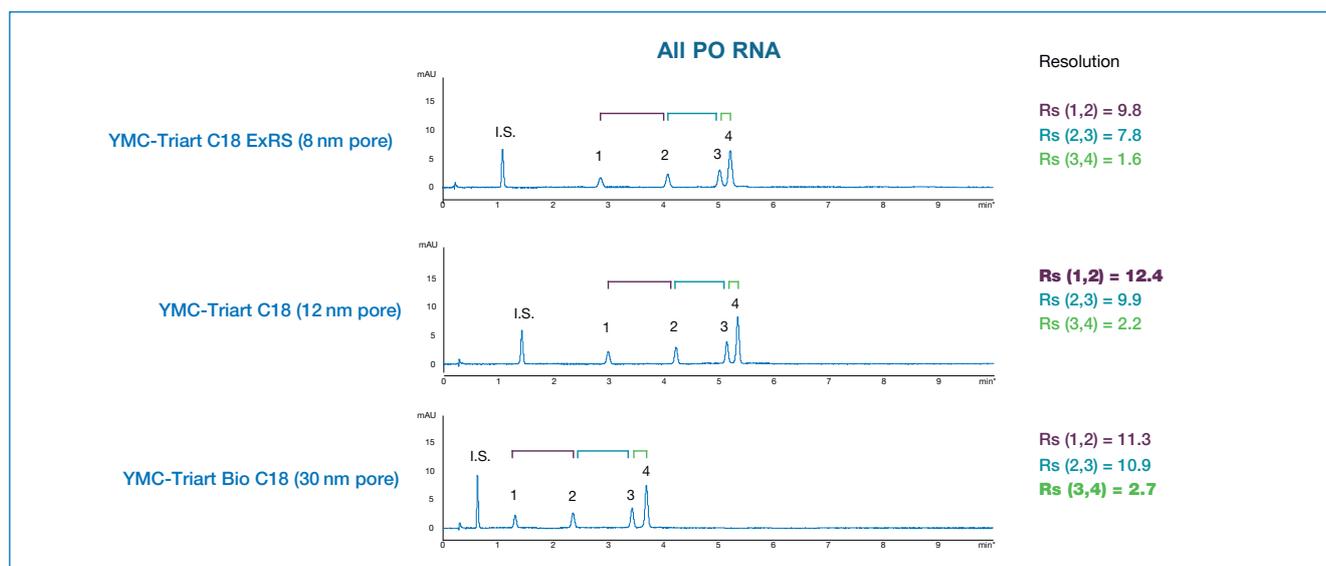
Figure 10: Effect of three different C18 columns with distinct pore sizes on PO RNAs separation.

To analyse the influence of oligonucleotide length over a larger range, DNA fragments of 10–120 nucleotides were tested with the same particle pore sizes mentioned previously (figure 11). A concentration of 4 mM TEA and 100 mM HFIP with a gradient of 5%–20% methanol was used. The particles with a wider pore of 30 nm also showed good resolution also for longer oligonucleotides due to their ability to penetrate the large pores.

Additionally, the shorter retention time of the smaller oligonucleotides produced sharper peak shapes. The smaller pore size of 12 nm also showed good separation of shorter oligonucleotides with length up to 20 nucleotides. A particle pore size of 8 nm was less suitable for the separation of oligonucleotides. The results indicate that a particle pore size of 30 nm is the best option for analysis of oligonucleotides of various sizes.
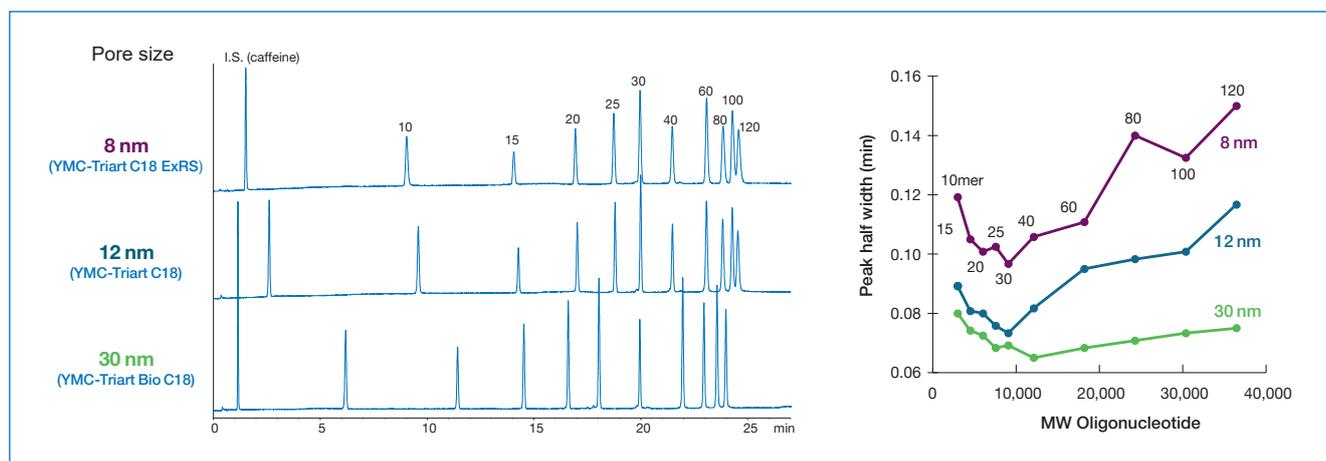
Figure 11: Influence of three different C18 columns with distinct pore sizes on separation of DNA fragments of 10 to 120 nucleotides length.

6. Influence of metal-free column hardware on sensitivity and recovery in oligonucleotide analysis

Due to the phosphate-containing backbone, oligonucleotides are susceptible to metal interactions. Therefore, they tend to adhere to metal components of the HPLC system such as stainless steel of column hardware and frits. This can impede chromatographic performance because of poor peak shape and recovery. Passivation and conditioning of the system with sample prior to analysis might lead to better results, but is time consuming and accompanied by loss of precious sample. This problem can be avoided by use of metal-free column hardware.

The YMC-Accura Triart columns provide a bioinert surface coated hardware, which leads to a significantly improved peak height and sensitivity in oligonucleotide analysis (figure 12). A gradient of 8%–18% methanol was used. The PS RNAs of 20 and 21 nucleotides length resulted in a doubled peak height and twofold increase in peak area compared to the regular YMC-Triart column. The use of bioinert hardware can prevent sample loss and improve sensitivity. To further maximize these effects, a completely "non-metal" HPLC system should be used.
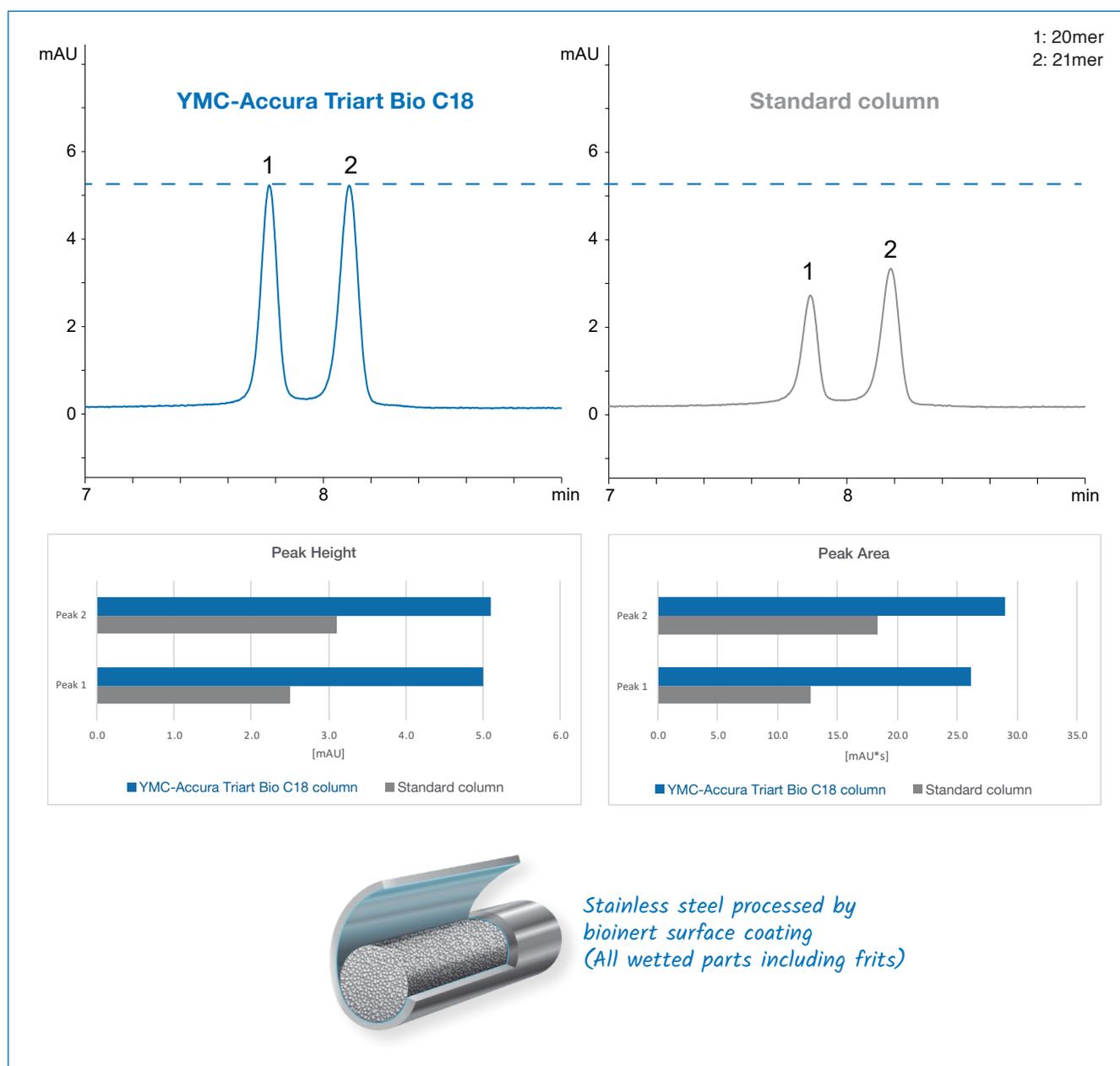
Figure 12: Impact of bioinert hardware on resolution, retention and sensitivity of a PS RNA 20mer and 21mer.

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Conclusions

Due to impurities which accumulate during oligonucleotide synthesis, chromatographic analysis is vital to monitor their quality and quantity. However, the characteristics of oligonucleotides are diverse because of variation in composition, length, modification and tendency for secondary structures. Therefore, oligonucleotide analysis often needs adjustment to improve separation and subsequent resolution. For example, the eluent composition, temperature and phase modification play a key role in method development. As described above, the following factors need to be considered in oligonucleotide analysis by IP-RP:

- **Ion pair reagent, concentration and the resulting pH**
- **gradient slope**
- **column temperature**
- **stationary phase with modification and pore size**
- **oligonucleotide linkage**
- **column hardware**

General recommendations for starting conditions are:

- **column with 30 nm pore-width**
- **eluent composition of 15 mM TEA and 400 mM HFIP**
- **buffer with neutral pH**
- **temperature of 65 °C**

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