Optimising the IP-RP analysis of protected and unprotected oligonucleotides

Short oligonucleotides are usually produced via solid phase synthesis. To achieve a controlled sequence the 5' terminus is protected with a protecting group such as dimethoxytrityl (DMT). After the successful synthesis, specifically after every synthetic step, the protecting group is removed.

This technical note describes the optimisation of the analysis of two single stranded DNA samples (20mer) using ion pair reversed-phase chromatography (IP-RP). Both samples consist of the same sequence (see Table 1) but differ in that the DMT protecting group is still present (Trityl-ON) and the DMT protecting group has been removed (Trityl-OFF). Parameters such as ion pairing agents and their concentration, gradient, column hardware and temperature were optimised for both samples. An overview of the general chromatographic conditions is shown in Table 2.

Table 1: Sample sequence.

Trityl-ON (20mer)	5'-ATACCGATTAAGCGAAGTTT-3'
Trityl-OFF (20mer)	

Table 2: Chromatographic conditions.

Columns:	YMC-Triart C18 (12 nm, 1.9 µm) 100 x 2.0 mm ID (standard hardware) YMC Accura Triart C18 (12 nm, 1.9 µm) 100 x 2.1 mm ID (bioinert hardware)
Part Nos.:	TA12SP9-1002PT TA12SP9-10Q1PTC
Eluents:	A) TEAA, DBAA, TEA–HFIP* at different conc. B) Methanol or acetonitrile
Gradient:	15–35%B for first screening Optimised: 0.33%B/min–0.58%B/min slope
Flow rate:	0.2 mL/min 0.4 mL/min for temperature screening
Temperature:	25 °C 15–90 °C for temperature screening
Injection:	5 µL
Detection:	UV at 260 nm
Sample:	20mer single stranded DNA Trityl-ON and Trityl-OFF (1 mg/mL)

** Dibutyl ammonium acetate (DBAA), triethyl ammonium acetate (TEA) and triethylamine (TEA) with 1,1,1,3,3,3-hexafluoride-2-propanol (HFIP)*

Influence of ion pairing agents

Due to the electron rich phosphate backbone of DNA the retention on common hydrophobic stationary phases is too low. Ion pairing agents such as *n*-alkylamines can help to overcome this challenge. The ion pairs formed between *N*-alkylamine and oligonucleotide can be separated according to their hydrophobicity using a reversed phase column.

Dibutyl ammonium acetate (DBAA), triethyl ammonium acetate (TEAA) and triethylamine (TEA) with 1,1,1,3,3,3-hexafluoride-2-propanol (HFIP) were used for the initial screening at concentrations of 10 mM and 100 mM for HFIP (pH not adjusted). Acetonitrile was chosen as eluent B and a gradient from 15–35%B was applied (shown in Figure 1).

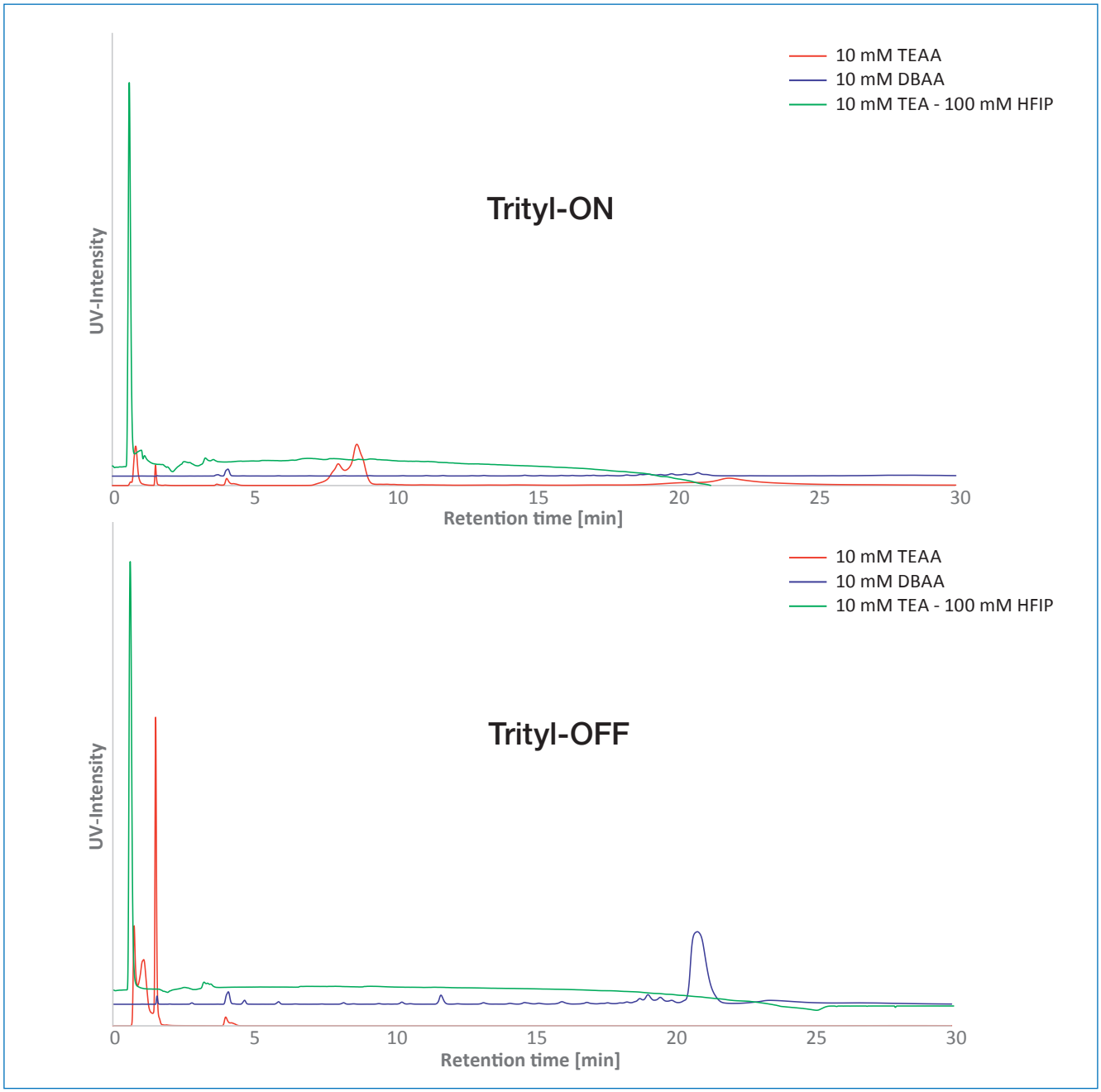


Figure 1: Chromatograms of the first eluent screening for Trityl-ON (top) and Trityl-OFF (bottom).

When using TEAA, the retention time of the sample is very short. In addition, multiple peaks elute indicating that the ion pairing is incomplete. The chromatogram of the Trityl-ON sample shows that there is no elution when using DBAA. The gradient slope needs to be adjusted. A peak is obtained for Trityl-OFF but its resolution and peak shape are not sufficient. When using TEA-HFIP, both samples elute directly from the column, indicating that the initial conditions are too strong. The gradient and starting conditions were adjusted for further testing.

The chromatograms with optimised conditions can be seen in Figure 2. For TEAA and DBAA, the gradient slope was reduced, while for DBAA the initial gradient ratio was also increased to 20%B. Conversely for TEA-HFIP, a reduction in the initial gradient ratio was necessary to attain retention. High resolutions were obtained using gradient slopes between 0.33 %/min and 0.67 %/min. Shallower gradients resulted in greater resolution, while steeper gradients allow faster analyses with higher sensitivities. The organic solvent was changed from acetonitrile (ACN) to methanol (MeOH) using TEA-HFIP as eluent A, as HFIP is not soluble in acetonitrile. Methanol was also used for the separation using TEAA as ion pairing agent. TEA-HFIP provides the highest resolution and best separation for both samples.

Table 3: Optimised gradient conditions.

	a)	b)	c)	d)
Eluent A	10 mM TEAA	10 mM DBAA	10 mM TEA - 100 mM HFIP	10 mM TEA - 100 mM HFIP
Eluent B	methanol	acetonitrile	methanol	methanol
Gradient	15–45%B (0–60 min)	20–40%B (0–30 min)	0–35%B (0–60 min)	0–25%B (0–60 min)
Gradient slope	0.5%B/min	0.67%B/min	0.58%B/min	0.42%B/min

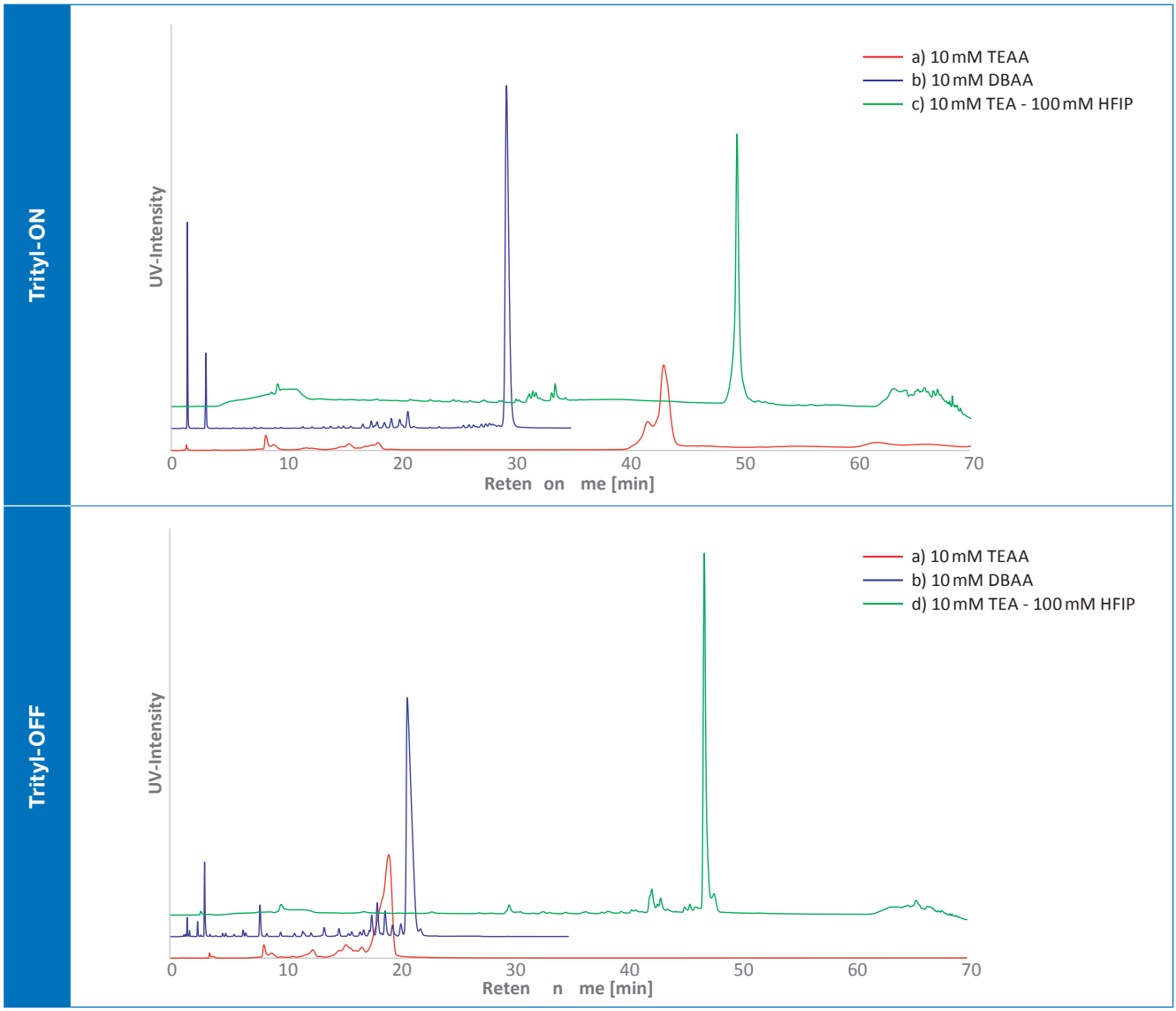


Figure 2: Chromatograms for Trityl-ON and Trityl-OFF using different eluents with optimised gradients.

Optimising the ion pairing agent concentration

The optimum concentration of ion pairing agent depends largely on the sample. Therefore, both lower and higher concentrations were tested. The exception was TEAA, which was only tested at a higher concentration of 100 mM, as the sample was not fully paired at a concentration of 10 mM. In addition, different TEA concentrations were tested in combination with 100 mM as well as 200 mM HFIP.

Figure 3 shows the screening at different ion pairing concentrations. The same gradients as described in Table 3 were used.

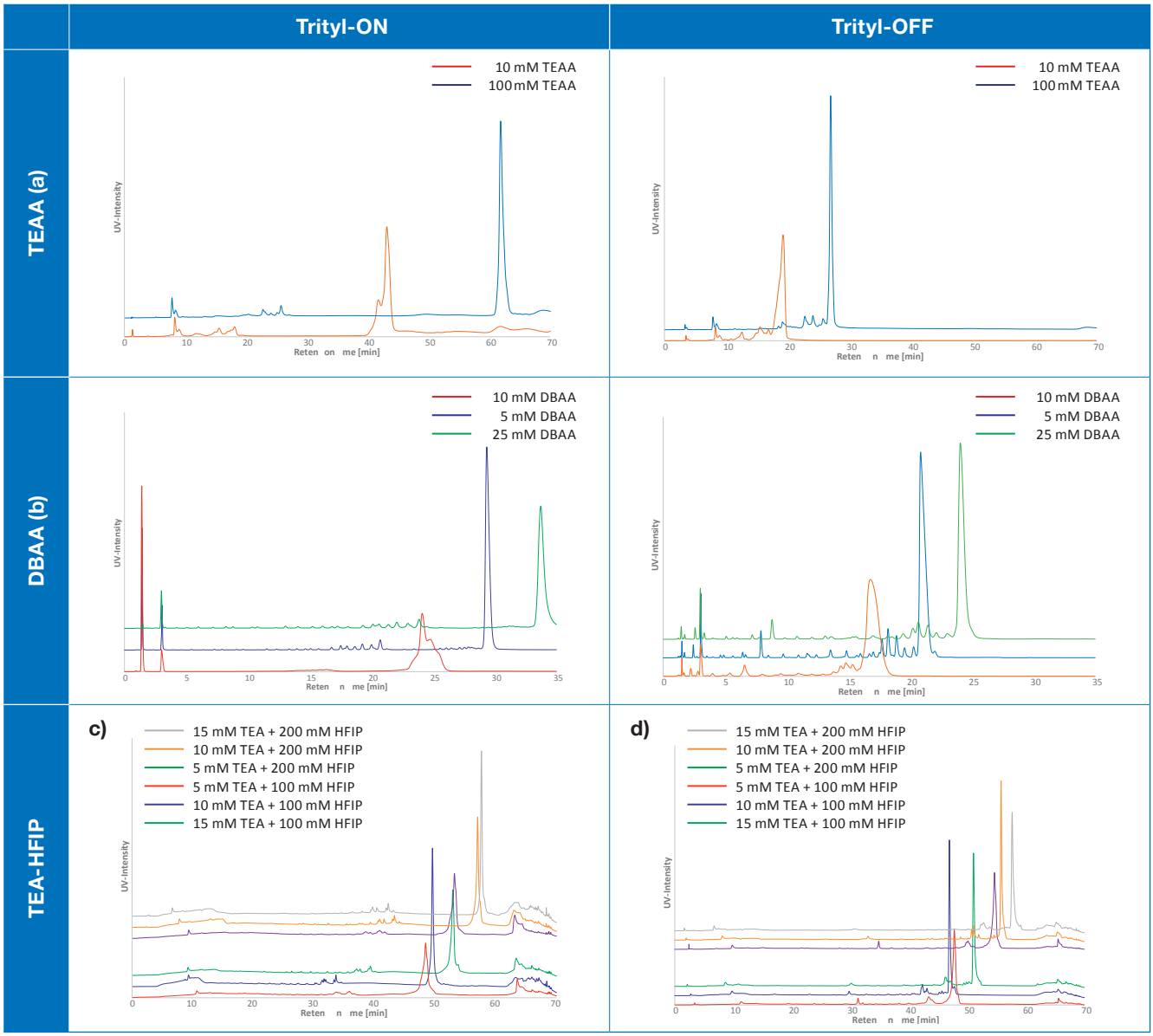


Figure 3: Screening of the optimum ion pairing agent concentration.

Using 100 mM TEAA instead of 10 mM increased the retention time for both samples. The peak for unpaired DNA is reduced showing complete pairing had not been achieved. With the lowest DBAA concentration of 5 mM the samples are not fully paired. The main peak broadens. At the highest concentration of 25 mM DBAA, the retention time increases but the peaks become broader, too.

TEA concentrations of 5 mM, 10 mM and 15 mM were tested in combination with 100 mM and 200 mM HFIP, respectively. Increasing the TEA concentration lead to a significant increase in separation efficiency. Double the HFIP concentration had only a slight effect when using a concentration of 5 mM TEA or 10 mM TEA.

One reason could be that the thermodynamic equilibrium for the protonation of TEA has been already reached with 100 mM HFIP. At a concentration of 15 mM TEA, increasing the HFIP concentration improves resolution and separation efficiency more significantly.
The following concentrations provided the best resolution and peak shape and were therefore selected for further testing:

100 mM TEAA
10 mM DBAA
15 mM TEA + 200 mM HFIP

Influence of column hardware

Oligonucleotides can be irreversibly adsorbed on metal surfaces including the wetted parts of conventional (U)HPLC columns. This nonspecific adsorption significantly disrupts recovery and peak shape. The effect is even greater when working at low to neutral pH, as metals are more electropositive under these conditions. To solve this problem, HPLC columns and systems can be passivated with strong acids or pre-conditioned with a similar sample. However, these procedures are time-consuming and a recurring task. In addition, non-specific adsorption can occur again when the sample is changed.

A simpler and much more robust solution is to use fully bioinert YMC Accura Triart columns. These are equipped with a bioinert coating on the stainless-steel column body and frits which ensures full inertness towards sensitive compounds.

A direct comparison between regular stainless-steel hardware and YMC Accura column hardware shows the clear advantages of the bioinert column: improved resolution and recovery. The previously optimised ion pairing agent concentrations and gradients were applied. For the further investigations, only the YMC Accura Triart C18 was used.

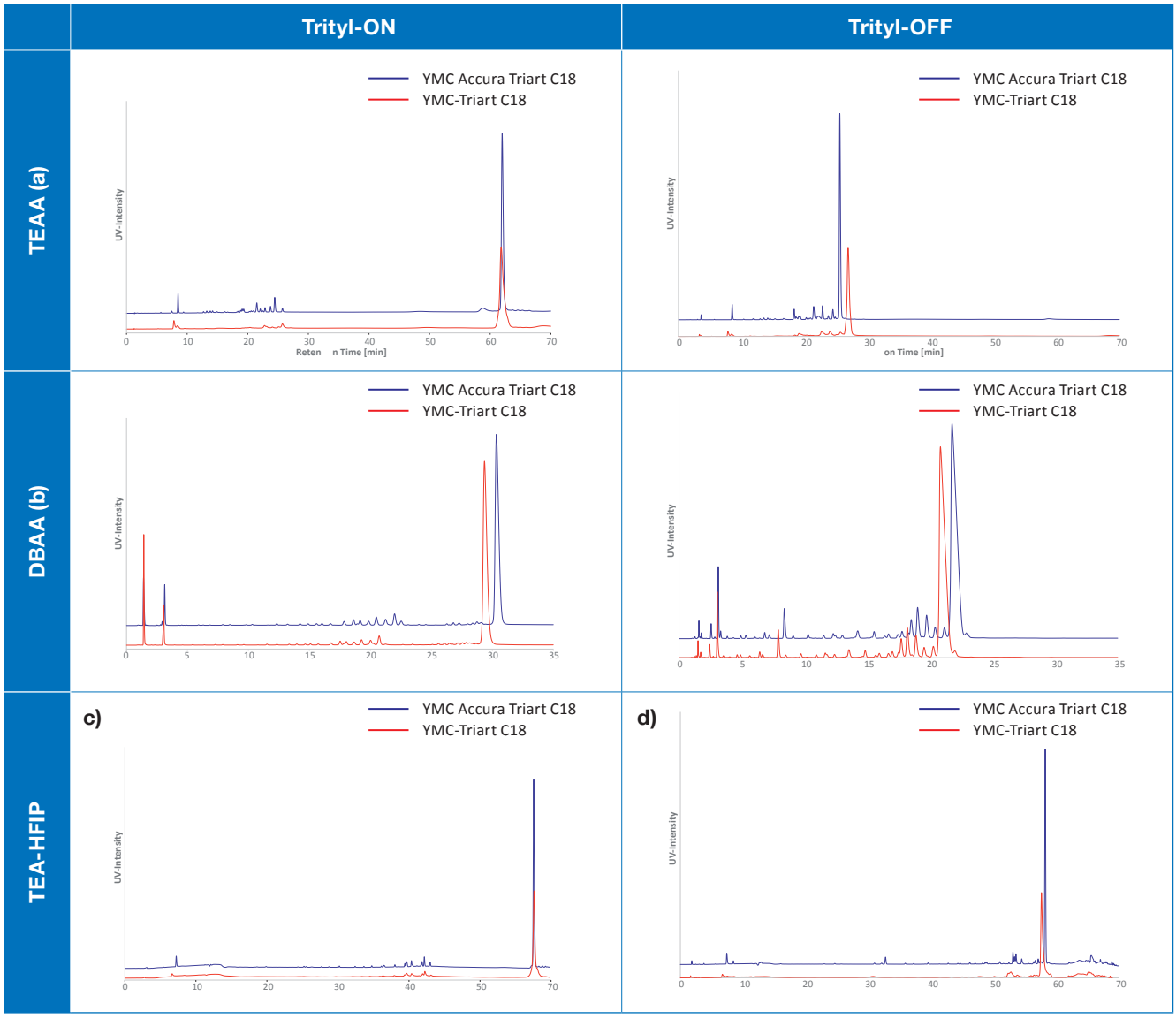


Figure 4: Comparison of stainless-steel and bioinert coated YMC Accura column used for Trityl-ON and Trityl-OFF.

Optimisation of column temperature

Temperature can have a significant impact on the retention in IP-RP because the electrostatic interactions with the ion pairing agents are usually enhanced as the temperature is increased. Meanwhile, the hydrophobic adsorption strength of the oligonucleotides and ion pairing agents on the stationary phase decreases. For this reason, the temperature optima are highly dependent on the ion pairing agents, organic solvents and the samples themselves and require experimental determination of the temperature optimum.

To determine the optimal temperatures, samples were analysed at different temperatures between 15 °C (25 °C) and 90 °C. In order to shorten the run time for the different methods, the flow rate was previously doubled to 0.4 mL/min. In all cases, doubling the flow rate resulted in a lower separation efficiency than for the previous flow rate. The effect of temperature on the separation of Trityl-ON and Trityl-OFF DNA samples is shown in Figure 5.

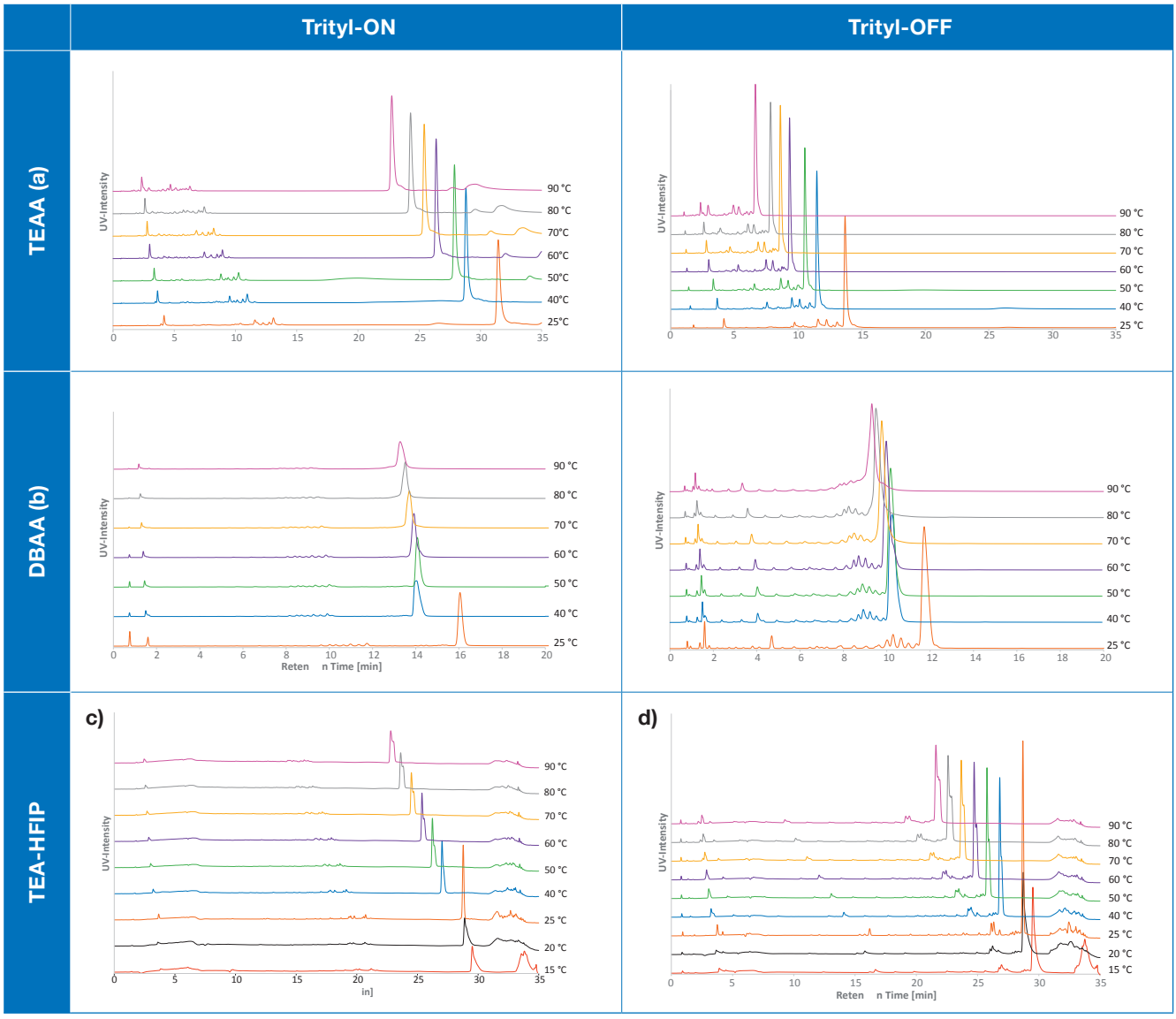


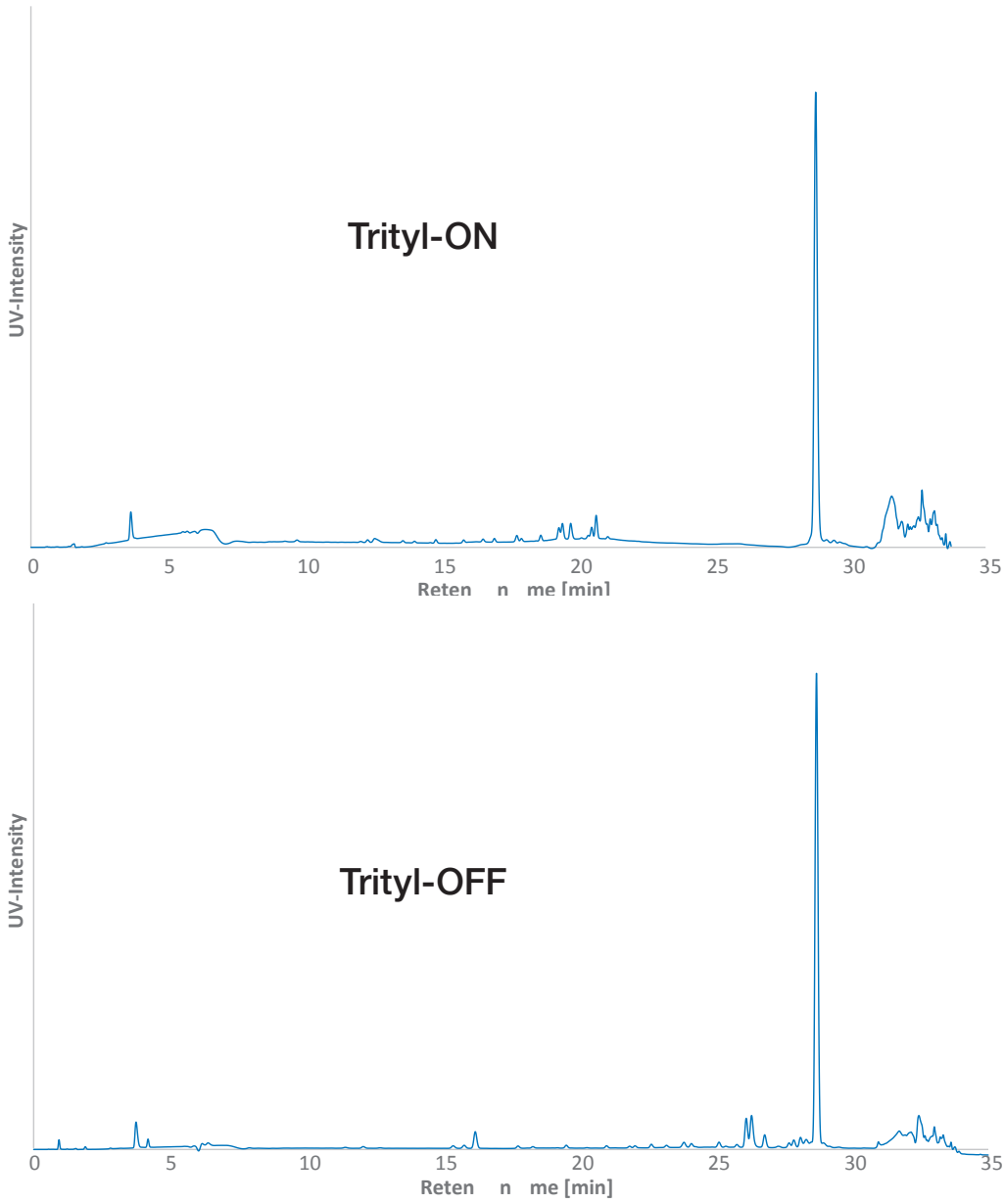
Figure 5: Temperature screening using a flow rate of 0.4 mL/min and a YMC Accura Triart C18 column.

With a temperature change, the separation efficiency for TEAA and DBAA was improved, which in turn exceeds the separation efficiency at a flow of 0.2 mL/min. The optimum temperature for both samples is 60 °C for TEAA and DBAA. For TEA-HFIP the separation efficiency and resolution decrease with increasing temperature.

Therefore, lower temperatures of 15 °C and 20 °C were also investigated. At lower temperatures significant tailing is seen for both samples. Therefore, the optimum temperature for both samples was 25 °C.

Optimum conditions

For both single stranded DNA samples, Trityl-ON and Trityl-OFF, the ion pairing agent 15 mM TEA – 200 mM HFIP and a lower temperature of 25 °C provided the best resolution and sensitivity.



Column:	YMC Accura Triart C18 (12 nm, 1.9 µm) 100 x 2.1 mm ID
Part No.:	TA12SP9-10Q1PTC
Eluents:	A) 15 mM TEA – 200 mM HFIP
	B) Methanol
Gradient:	Trityl-ON: 0–35%B (0–30 min)
	Trityl-OFF: 0–25%B (0–30 min)
Flow rate:	0.4 mL/min
Temperature:	25 °C
Injection:	5 µL
Detection:	UV at 260 nm
Sample:	Single stranded DNA Trityl-ON and Trityl-OFF (1 mg/mL)

Figure 6: Optimum chromatographic results and conditions for Trityl-ON (top) and Trityl-OFF (bottom).

Conclusions

The following parameters are important for optimising an LC analysis of single-stranded protected/unprotected DNA (Trityl-ON/OFF):

- Choice of ion pairing agent
- Column temperature
- Column hardware

In this case, the best results were achieved with the ion pairing agent TEA-HFIP. Another advantage of these conditions is MS compatibility. However, the high costs of HFIP are a disadvantage. A TEAA buffer is also MS-compatible and offers very good resolution as cost-effective alternative, especially for high-throughput analyses. Additionally, it enables smooth and cost-efficient scale-up. Nevertheless, the analysis of DNA using IP-RP must always be individually optimised to the properties of the respective DNA.

It is also important that bioinert column hardware is used when analysing oligonucleotides. Ideally in combination with a bioinert (U)HPLC system. This boosts recoveries and ideal peak shapes are achieved.