Determination of optimum method parameters for the analysis of oligonucleotides via HILIC

Hydrophilic interaction liquid chromatography (HILIC) is an interesting alternative to anion exchange chromatography (AEX) and ion pair reversed phase liquid chromatography (IP-RP) for robust and highly sensitive UHPLC methods of oligonucleotides. Due to the highly polar nature of oligonucleotides, HILIC is ideally suited for their characterisation. In addition, it can be an advantage in some cases to employ an ion pair-free method.

In this technical note, the separation of mixtures of short DNA oligonucleotides (dT10, dT15 and dT20) and short RNA oligonucleotides (14, 17, 20 and 21mer) was used to examine important parameters for method optimisation in HILIC mode.

Table: Chromatographic conditions.

Columns:	YMC-Triart Diol-HILIC (3 µm, 12 nm) 150 x 2.1 mm ID (regular hardware)
	YMC-Accura Triart Diol-HILIC (3 µm, 12 nm) 150 x 2.1 mm ID (bioinert coated hardware)
Part Nos.:	TDH12S03-15Q1PTH
	TDH12S03-15Q1PTC
Eluent:	A) 50 mM $HCOONH_4$ (pH 3.6, 6.5 or 9.5)/acetonitrile (30/70)
	B) 50 mM $HCOONH_4$ (pH 3.6, 6.5 or 9.5)
Gradient:	7.1–21.4%B (0–20 min), 21.4%B (20–25 min)
	(65–55% acetonitrile (0–20 min), 55% acetonitrile (20–25 min))
Flow rate:	0.21 mL/min
Temperature:	40 °C, 50 °C or 60 °C
Detection:	UV at 260 nm
Sample:	DNA oligonucleotides (dT10, dT15, dT20)
	RNA oligonucleotides (all PO RNA; 14, 17, 20, 21mer)
	Phosphorothioated RNA oligonucleotide (all PS RNA; 20mer)
Injection:	DNA: 2 µL (each 2.0 nmol/mL)
	PO RNA: 1 µL (1.8 nmol/mL–3.6 nmol/mL)
	PS RNA: 1 µL (1.8 nmol/mL)

Influence of hardware

As observed for IP-RP mode, oligonucleotides also tend to interact with metallic surfaces of regular hardware in HILIC mode. Due to their electron-rich backbone, oligonucleotides can be irreversibly adsorbed onto the metallic surface of the column hardware.

The use of bioinert column hardware such as bioinert coated YMC-Accura columns can mitigate this phenomenon. Consequently, the use of YMC-Accura Triart Diol-HILIC column shows better peak shapes and higher sensitivity due to virtually no adsorption compared to the corresponding stainless steel column (see figure 1).

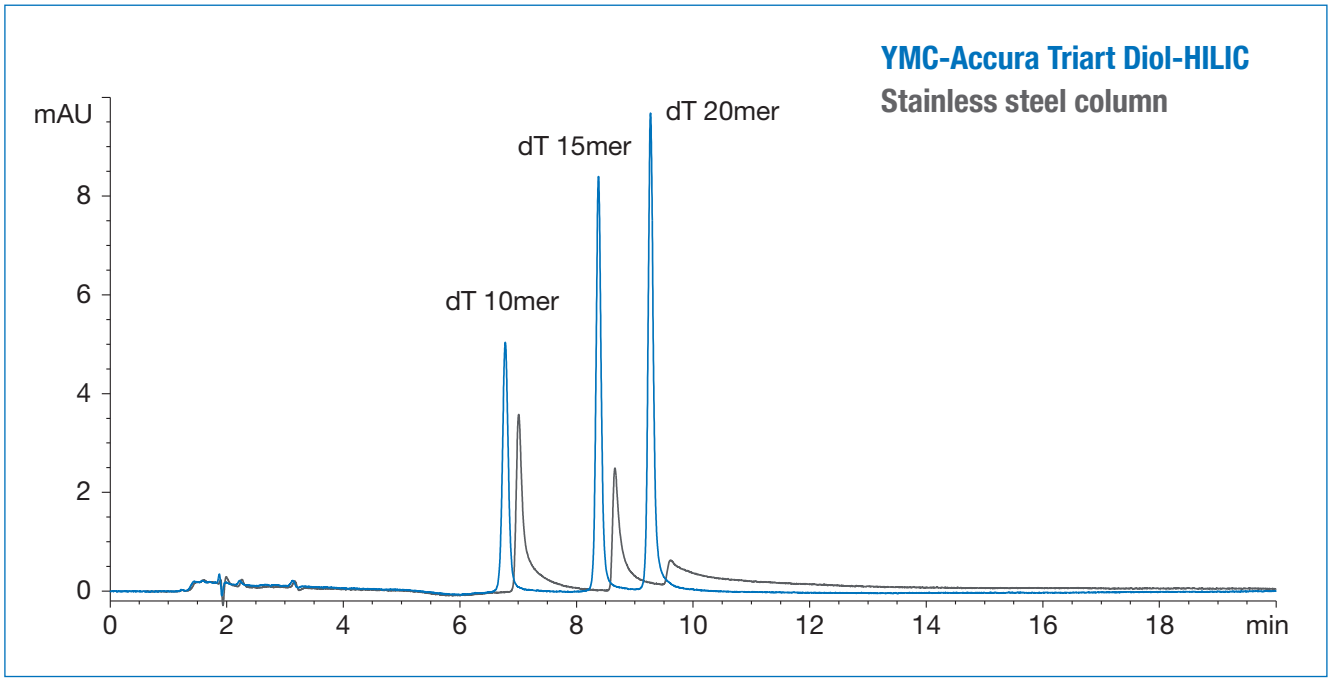

Figure 1: Analysis of DNA oligonucleotides using the bioinert coated YMC-Accura Triart Diol-HILIC (blue) and the corresponding stainless steel column (grey).

Influence of sample solvent

The influence of the sample solvent on peak shape especially for early eluting peaks is significant. As water is the strong eluent in HILIC mode, the ratio of organic solvent must be the same or higher than the initial gradient composition. Figure 2 shows the analysis of the DNA oligonucleotide mixture diluted with a low organic ratio (40%) compared to a sample solvent with high organic ratio (80%).

By using a sample solvent with low organic ratio, a higher injection peak as well as early eluting additional peaks occurs. Furthermore, the oligonucleotide peaks show broadening and the peak of dT10 even shows peak splitting. In comparison, by using a high organic ratio the peaks appear sharper and with higher sensitivity.

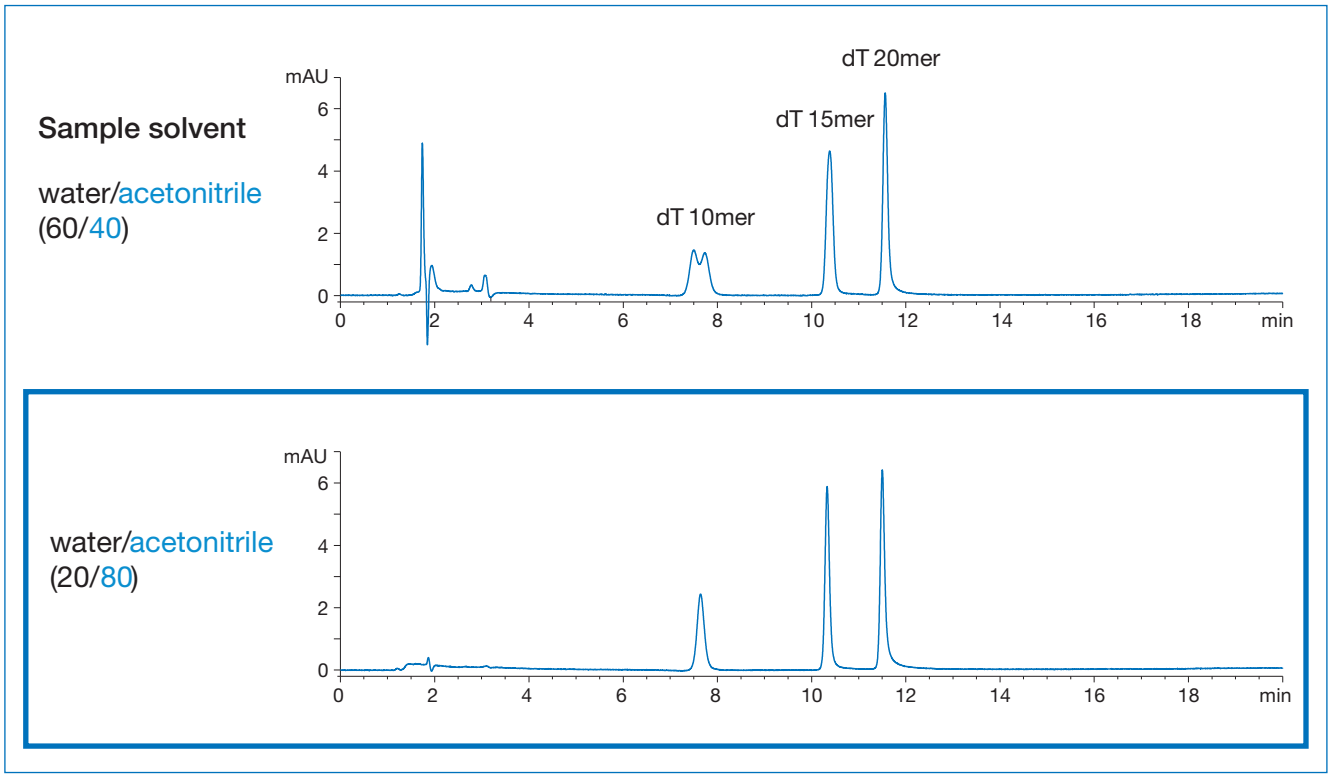

Figure 2: Optimisation of the sample solvent of a DNA mixture.

Influence of mobile phase pH

The mobile phase pH has a significant effect on retention and peak shape. Figure 3 shows the analysis of an all PO RNA mixture (14, 17, 20, 21mer) using a basic (9.5), a neutral (6.5) and an acidic mobile phase pH (3.6). The oligonucleotides are more strongly retained when the pH becomes lower. But with an acidic pH massive adsorption of the oligonucleotides occurs. For the 14mer the peak area is reduced by more than half compared to basic or neutral pH (see figure 4), while the internal standard is not affected.

Using the basic mobile phase, the retention is the lowest but the resolution is visibly reduced, so that the neutral mobile phase pH shows the best results for this mixture. In general, a neutral to basic mobile phase pH is recommendable for the HILIC analysis of oligonucleotides, depending on type and mixture.

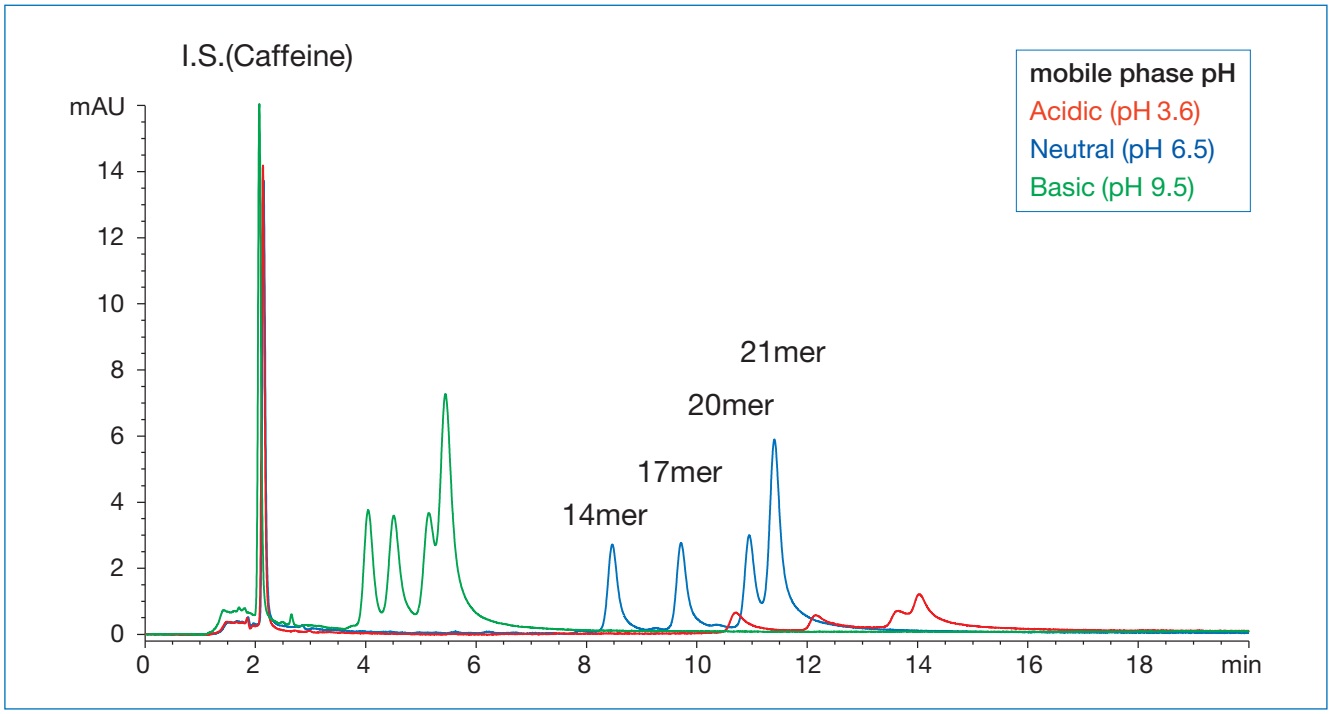

Figure 3: Influence of mobile phase pH on the separation of an all PO RNA mixture using an acidic (red), a neutral (blue) and basic pH (green).

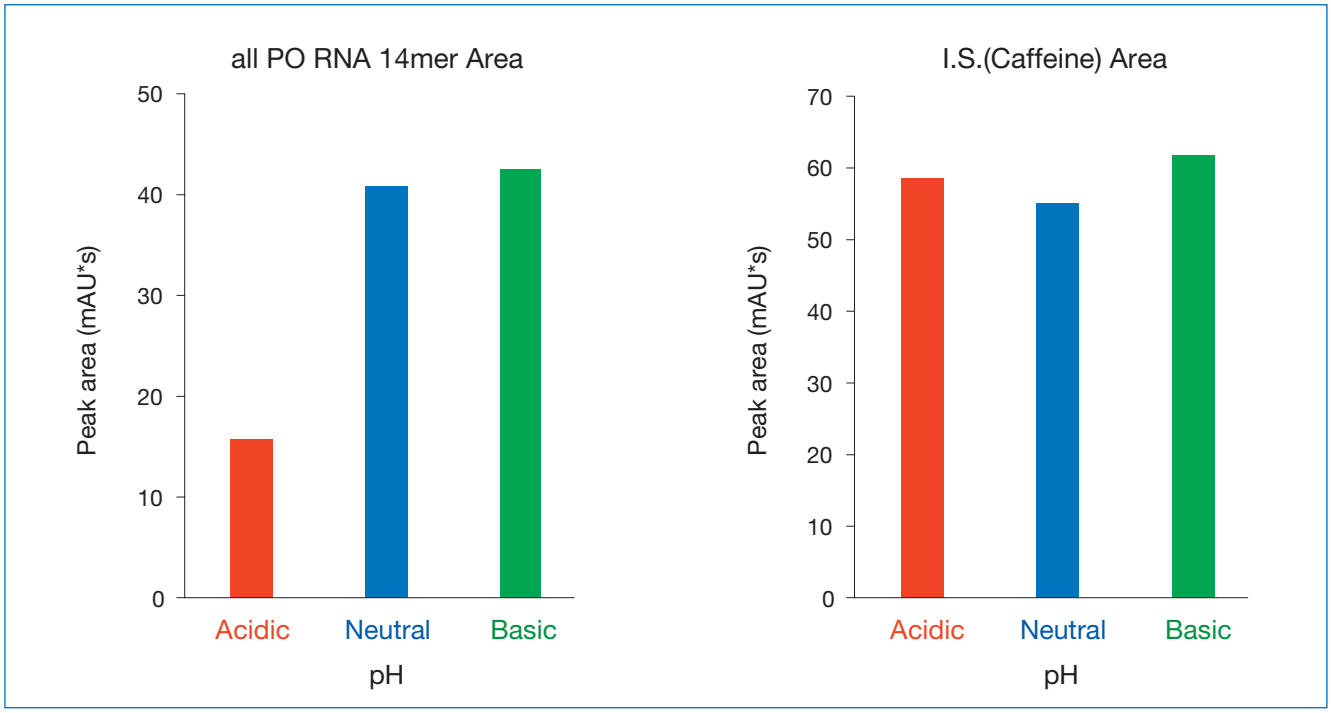

Figure 4: Peak areas at different mobile phase pH (3.6, 6.5 and 9.5) of the all PO RNA 14 mer (left) and the internal standard caffeine (right).

Influence of column temperature

The influence of column temperature on the separation of oligonucleotides was investigated by analysing the all PO RNA mixture using three temperatures of 40 °C, 50 °C and 60 °C (see figure 5). The retention is slightly reduced when using higher temperatures, but no influence on peak shape was observed.

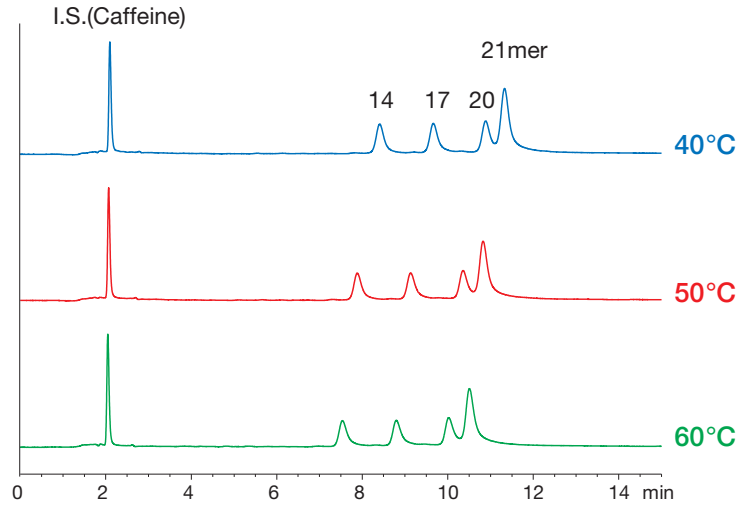

Figure 5: Influence of the column temperature on the analysis of an all PO RNA mixture.

The same observations were made when analysing an all phosphorothioated oligonucleotide (20mer, see figure 6).

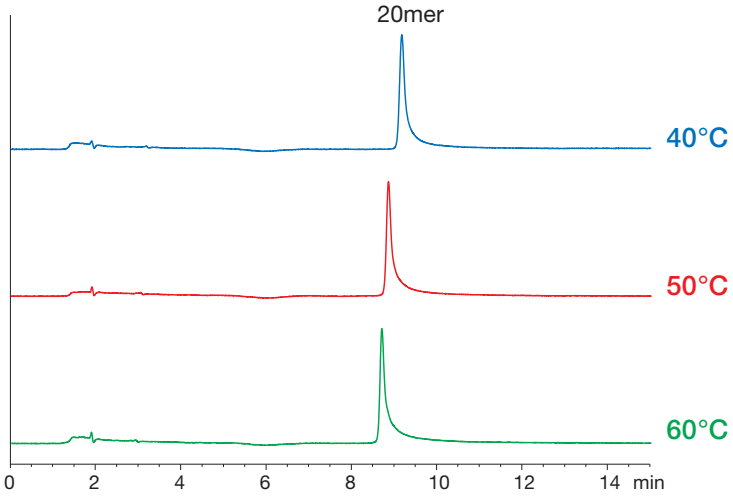

Figure 6: Influence of the column temperature on the analysis of all PS RNA (20mer).

Summary

These findings result in the following recommendations:

- **Bioinert hardware provides optimum peak shapes and high recovery**
- **High organic ratio of the sample solvent is important**
- **Neutral to basic mobile phase pH is recommended**
- **Influence of column temperature is insignificant**