High sensitivity purity analysis of peptide - oligonucleotide conjugates

Oligonucleotide-based therapeutics demonstrate growing clinical relevance and strong application potential. Several oligonucleotide-based therapeutics have already obtained approval, while others are advancing in clinical trials. But effective delivery to the target still poses a major challenge as both oligonucleotides and the cellular membrane are negatively charged. Therefore, it is difficult for therapeutical oligonucleotides, e.g. siRNA (small interfering RNAs) or ASOs (antisense oligonucleotides), to cross the membrane. Conjugating oligonucleotides with membrane-permeable peptides such as oligoarginine significantly enhances cellular delivery. These peptide-oligonucleotide conjugates (POCs) provide an improved intracellular delivery, leading to an enhanced therapeutical potential. Most established synthesis workflows rely on post-synthetic coupling with separate preparation steps for oligonucleotides and peptides. Incomplete conjugation will reduce the intracellular delivery. Therefore, accurate quality control is required. However, exact differentiation of structurally similar impurities remains technically demanding and requires analytical precision.
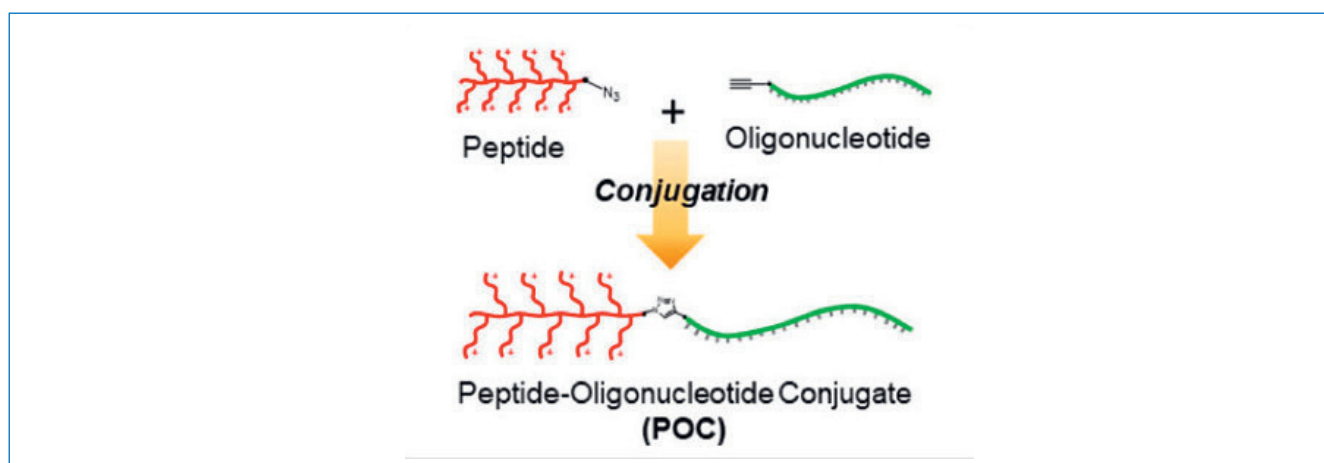

Figure 1: Synthesis of a peptide-oligonucleotide conjugate [1].

This Application Note presents an optimised IP-RP method for a model POC, providing a reliable foundation for quality control workflows. The model POC (R9-ER(dec)-R) is using a 21mer DNA sequence (ER(dec)-R) conjugated to a nona-arginine peptide (R9) conjugated through a click reaction (Figure 2).
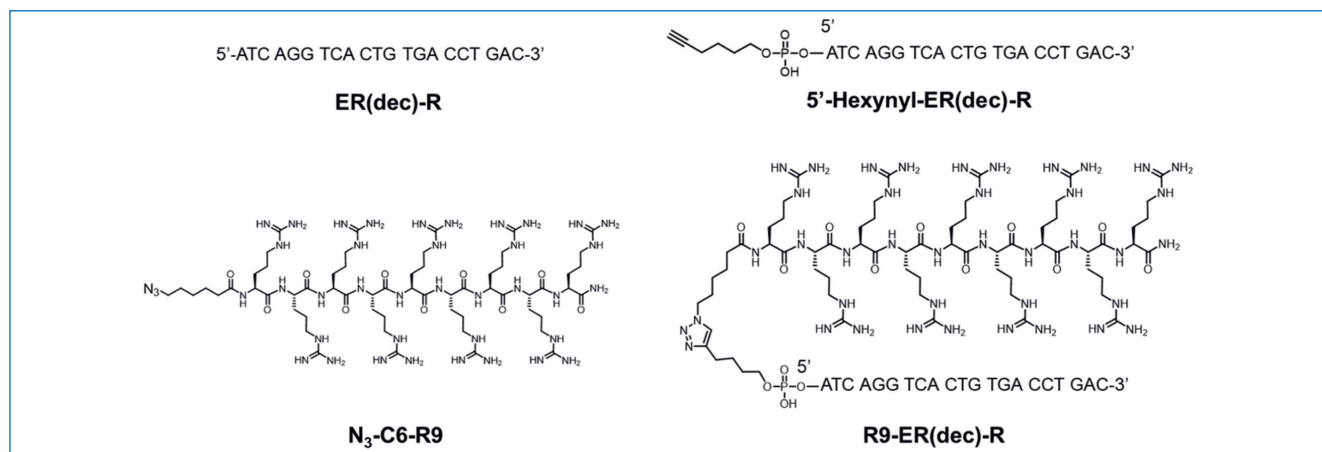
5'-ATC AGG TCA CTG TGA CCT GAC-3'

ER(dec)-R

5'-Hexynyl-ER(dec)-R

N₃-C6-R9

R9-ER(dec)-R
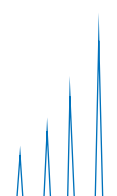
Figure 2: Model POC and its impurities [1].

Influence of the ion-pairing agent

Table 1: Chromatographic screening conditions [1,2].

Column:	YMC Accura Triart Bio C18 (1.9 µm, 30 nm), 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PTC
Eluents 1:	A: 15 mM TEA-400 mM HFIP
	B: methanol
Gradient 1:	10–80%B (0–5 min), 80%B (5–10 min)
Eluents 2:	A: 100 mM TEAA (pH 7.0), 100 mM DEAA (pH 7.0) or 100 mM BAA (pH 7.0)
	B: acetonitrile
Gradient 2:	0–50%B (0–5 min), 50%B (5–10 min)
Flow rate:	0.4 mL/min
Temperature:	80°C
Injection:	1 µL (50 µM)
Detection:	UV at 260 nm
Sample:	crude model peptide-oligonucleotide conjugate
System:	Nexera X2 (SHIMADZU)
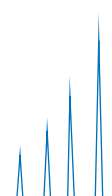
In a first step a mobile phase screening using different ion-pairing agents was performed (Figure 3). Although a triethylamine - 1,1,1,3,3,3-hexafluoro-2-propanol system (TEA/HFIP) generally provides better retention and separation, the peak area was not reproducible in this case. Diethylammonium acetate (DEAA) also failed to deliver stable results under the given conditions. Triethylammonium acetate (TEAA) produced reproducible chromatograms with satisfactory peak shape. The most promising results were provided by butylammonium acetate (BAA). Follow-up concentration studies confirmed that 100 mM BAA ensures optimal peak symmetry and recovery performance.
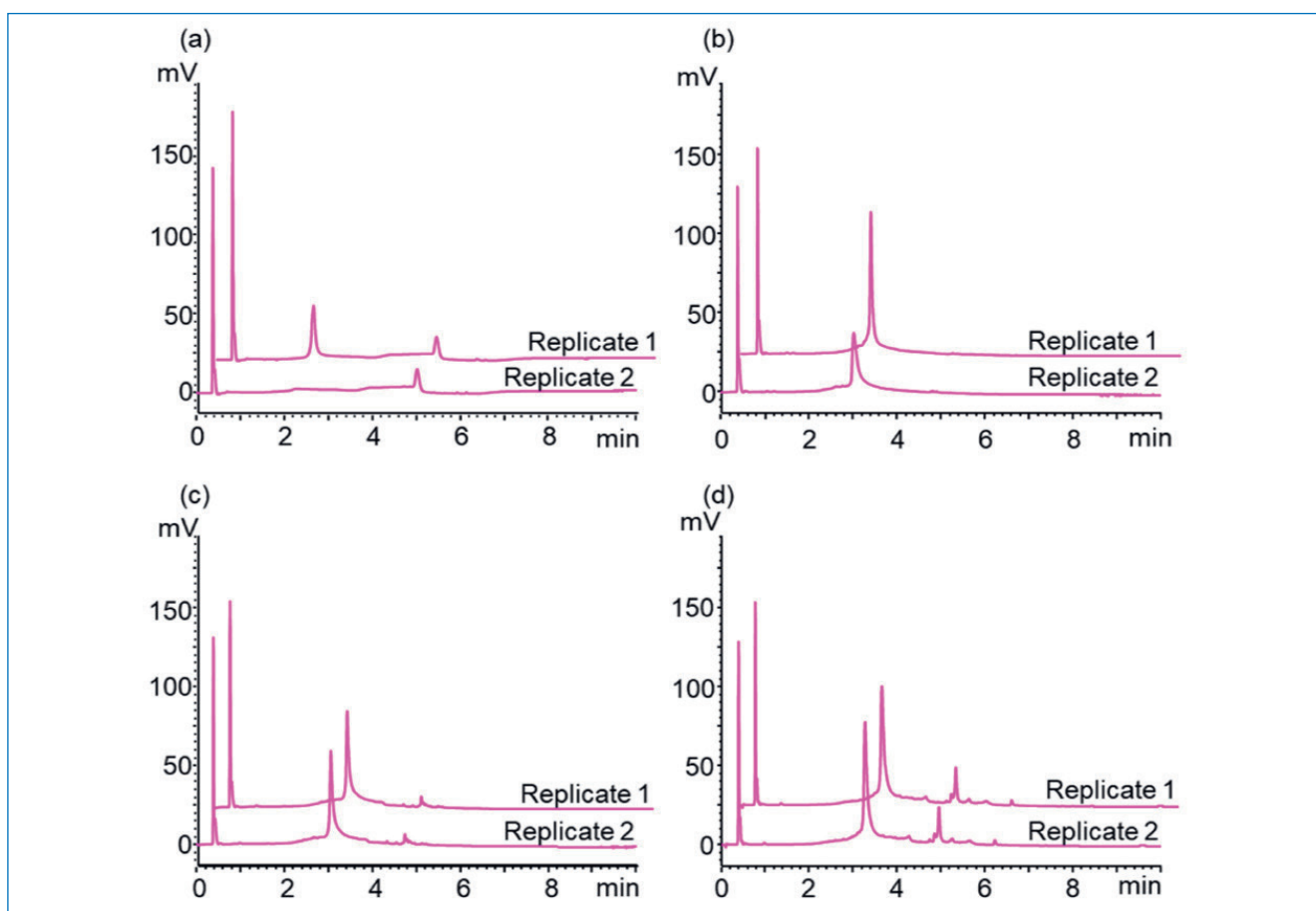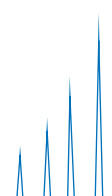

Figure 3: Eluent screening results for the crude POC using the following ion-pairing agents: (a) 15 mM TEA, 400 mM HFIP, (b) 100 mM DEAA (pH 7.0), (c) 100 mM TEAA (pH 7.0) and (d) 100 mM BAA (pH 7.0) [1].

Optimisation of the gradient slope

The initial screening gradient (0–50% B in 5min) was systematically optimised to enhance peak resolution (see Figure 4). A shallow gradient from 7% to 17% acetonitrile over 10 minutes delivered the most effective separation of main and minor components.
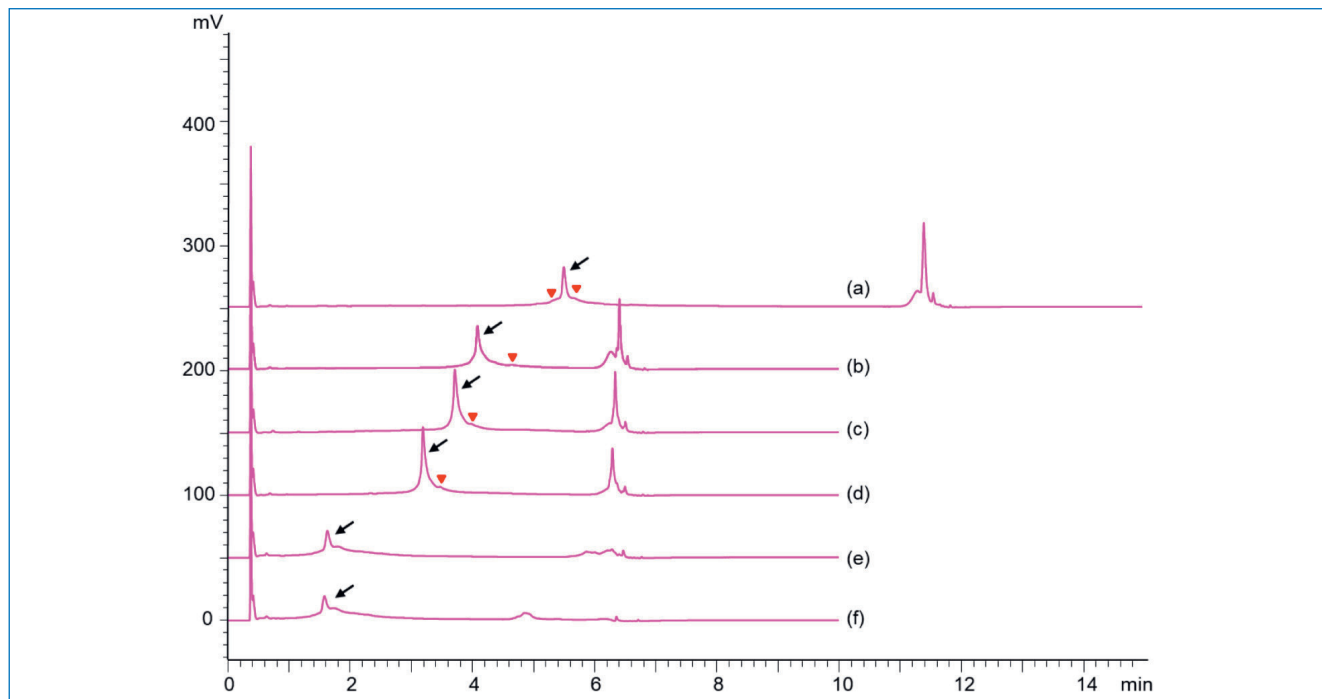

Figure 4: Gradient optimisation using the following slopes: (a) 7–17% B (0–10 min), 100% B (10–15 min); (b) 7–17% B (0–5 min), 100% B (5–10 min); (c) 5–25% B (0–5 min), 100% B (5–10 min); (d) 7–27% B (0–5 min), 100% B (5–10 min); (e) 10–30% B (0–5 min), 100% B (5–10 min); and (f) 10–40% B (0–5 min), 100% B (5–10 min). The black arrows indicate the POC and red triangles point out impurities [1].

Influence of pH on peak separation

The pH value of the mobile phase was screened at pH 6.0, 7.0, and 8.0 to assess its influence on separation performance (Figure 5). While retention remained relatively stable, resolution was noticeably reduced at pH 6.0. At pH 8.0, the method suffered from increased carry-over. Optimal results were achieved at pH 7.0, which delivered sharp peak resolution and minimal carry-over, establishing it as the preferred condition.
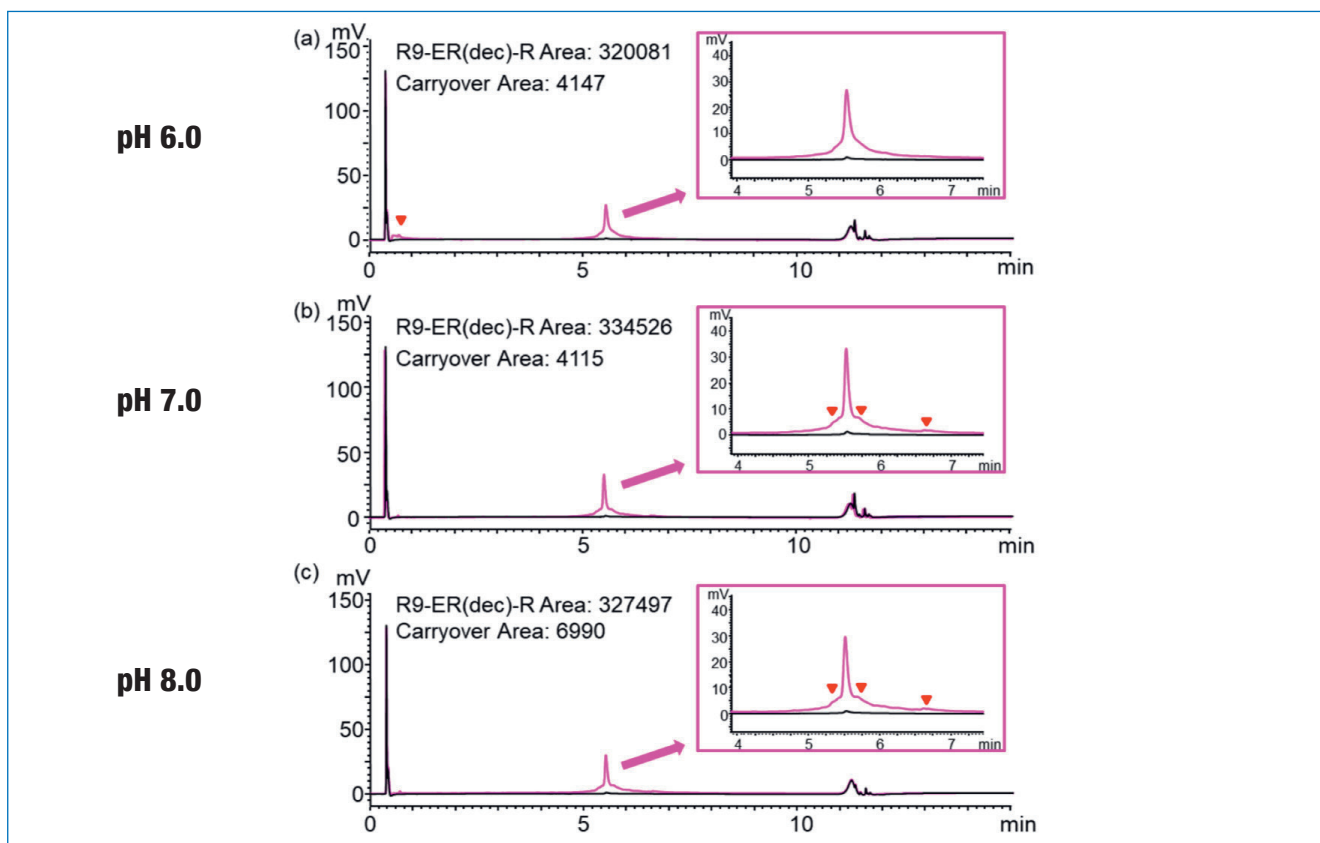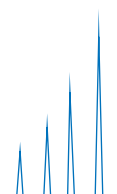

Figure 5: Influence of the buffer pH on the analysis of crude POC using BAA buffer at pH (a) 6.0, (b) 7.0, and (c) 8.0 (pink). After every injection a blank run was performed (black) [1].

Optimising peak shape by temperature adjustments

A temperature screening (40-80°C, Figure 6) showed that increasing the column temperature leads to sharper peaks. At 60°C, the main peak remained broad, while the peaks became sharper at 70°C. 80°C was chosen as optimal temperature because the peaks are sharp and minor peaks can be separated. No elution was observed at 40°C, although a minor UV increase between 7 and 9 minutes indicated partial interaction.
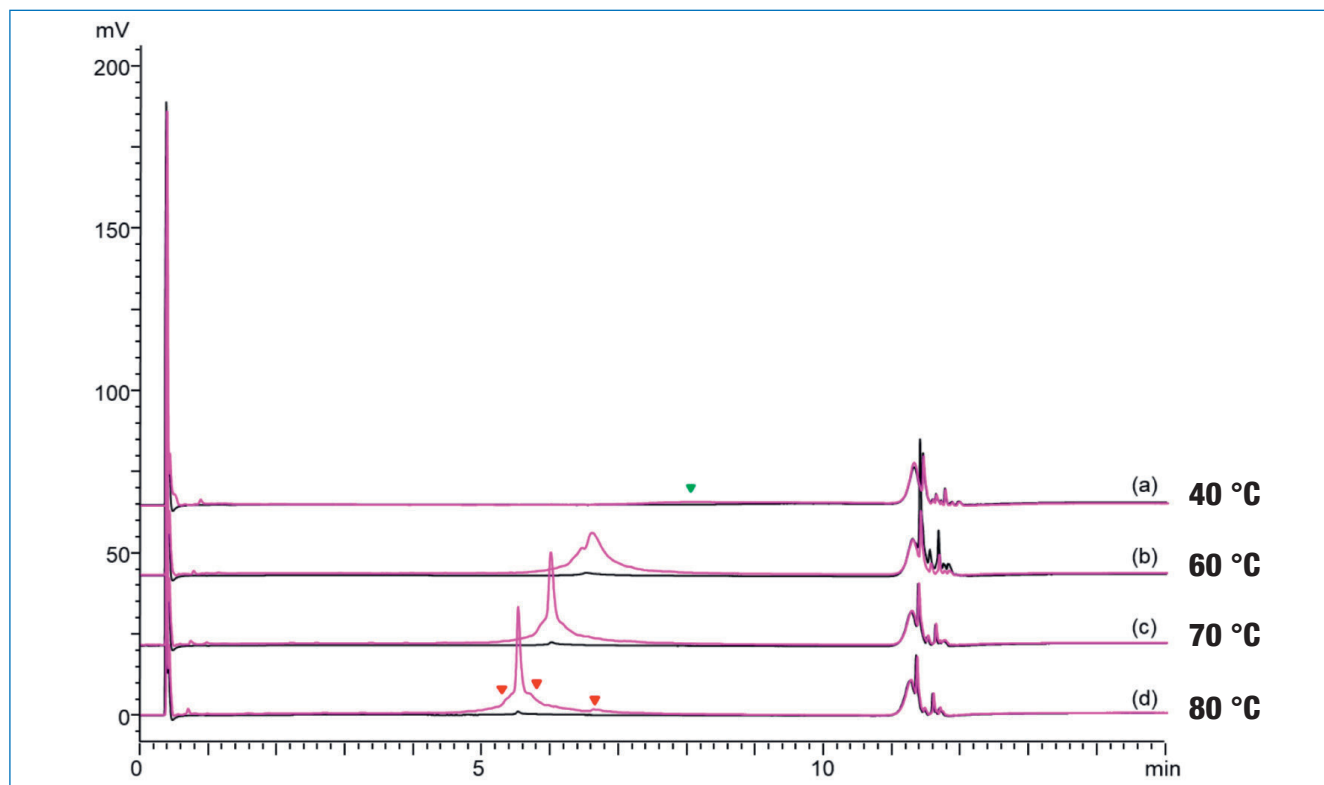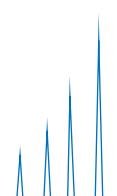

Figure 6: Chromatograms obtained at different temperatures of (a) 40, (b) 60, (c) 70, and (d) 80 °C (pink). After every injection a blank run was performed (black) [1].

Stationary phase screening

A wide-pore YMC Accura Triart Bio C18 column (30 nm) was used for prior investigations, because this column is known for its suitability in oligonucleotide analysis. In order to assess potential alternatives, YMC Accura Triart Bio C4 (30 nm pore) and YMC Accura Triart C18 (12 nm pore) columns were also tested under the already optimised conditions. All columns were equipped with the bioinert YMC Accura coating to mitigate interactions of the POC with metal surfaces. Figure 7 shows that a more hydrophobic stationary phase is beneficial for the resolution, simultaneously a larger pore of 30 nm is required to achieve optimum recovery and low carry-over. This screening confirms YMC Accura Triart Bio C18 as ideal column for the analysis of the POC.
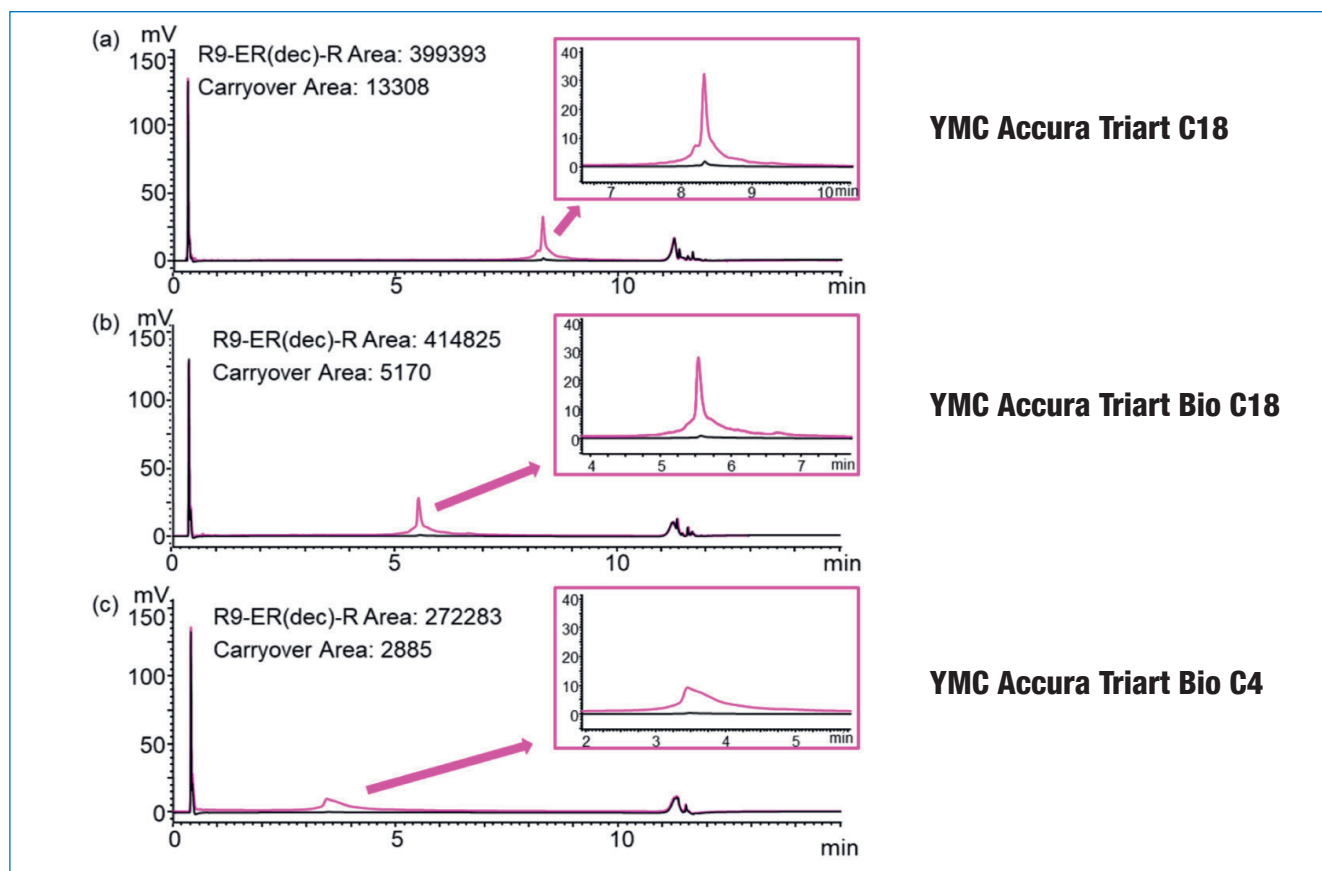

Figure 7: Results of the stationary phase screening (pink) using (a) YMC Accura Triart C18 (pore size: 12 nm), (b) YMC Accura Triart Bio C18 (pore size: 30 nm), and (c) YMC Accura Triart Bio C4 (pore size: 30 nm). After every injection a blank run was performed (black) [1].

Optimised conditions

Under optimised conditions, the method enables full separation of modified and unmodified oligonucleotides alongside the POC. All analytes elute with sharp peaks, delivering high resolution and analytical reliability suited for demanding quality control workflows.
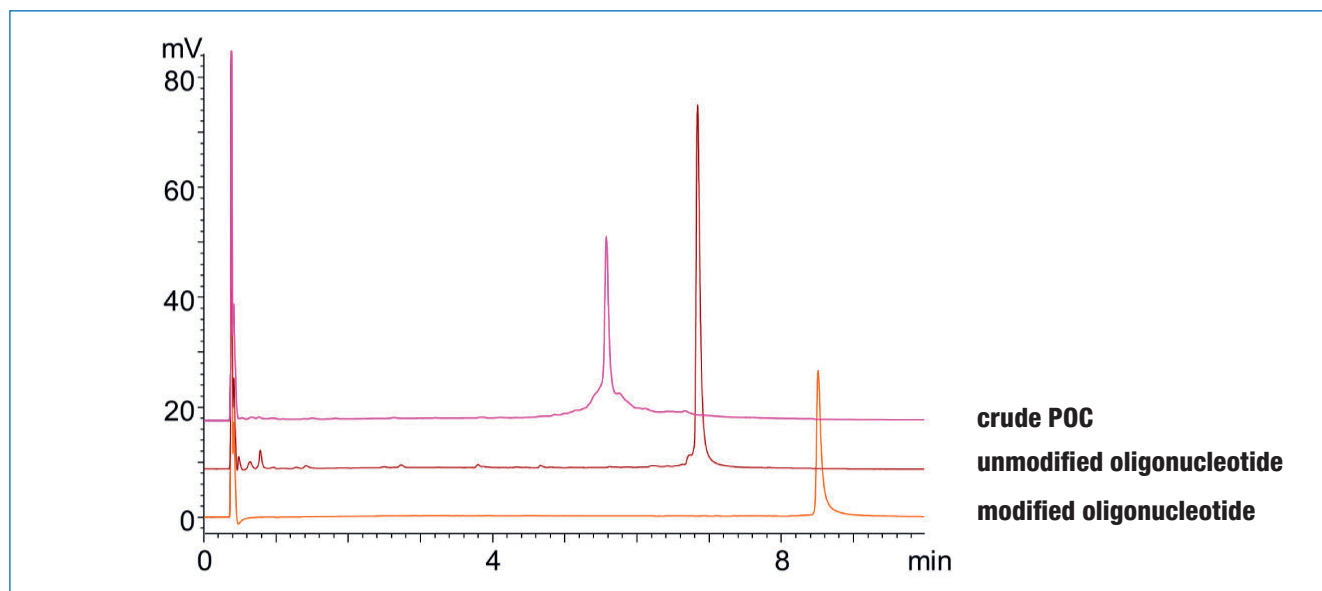

Figure 8: Analysis under optimised conditions of the crude POC (pink), the unmodified oligonucleotide (brown) and the modified oligo-nucleotide (orange) [1].

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Part No.:	TA30SP9-05Q1PTC
Eluents:	A: 100 mM BAA (pH 7.0)
	B: acetonitrile
Gradient:	7–17 %B (0–10 min), 100 %B (10–15 min)
Flow rate:	0.4 mL/min
Temperature:	80°C
Injection:	1 µL (50 µM)
Detection:	UV at 260 nm
Sample:	crude model peptide-oligonucleotide conjugate
	unmodified oligonucleotide
	modified oligonucleotide
System:	Nexera X2 (SHIMADZU)

Conclusion

This Application Note outlines a robust, reproducible IP-RP method to characterise POCs using a conjugate of DNA and oligoarginine as model compound. By optimising key parameters, it is demonstrated that a wide pore YMC Accura Triart Bio C18 with a BAA/acetonitrile eluent system provides excellent peak resolution and sensitivity for the analysis of POCs.

References

[1] ACS Omega 2025, 10, 20, 20578-20584

[2] Poster: Optimisation of HPLC Separation for Peptide-Oligonucleotide Conjugates: Enhancing Sensitivity in Purity Analysis Using Optimal Ion-Pairing Reagents, Daniel Eßer, Misato Amiya, Reira Hirai, Yuki Higuchi, Naoko Hata, Saoko Nozawa, Miyako Naganuma, Genichiro Tsuji, Yosuke Demizu, Taeko Nakajima presented at HPLC 2025