Analytical Study of the Separation Behaviour of Oligonucleotides on IP-RPLC

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Introduction

Ion-pair reversed phase liquid chromatography (IP-RPLC) using a mobile phase containing TEA (triethylamine) as IP reagent and HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) as acidic modifier has been widely used for oligonucleotide analyses. In this study, we investigate the effect of analytical conditions such as pH, concentrations of TEA and HFIP, column temperature, gradient slope, and the stationary phase on the retention and peak shape of oligonucleotides. We also compare the separation behaviour of 20 and 21mer oligo-RNAs which contain phosphorothioate modifications at every linkage (all PS RNAs) to phosphodiester RNAs (all PO RNAs) with the same nucleotide sequence.

In addition, we demonstrate the effect of column hardware on oligonucleotide separation. Standard column hardware is compared to the recently introduced YMC-Accura Triart columns with bioinert coated hardware.

Model Case Study

- **Analytical method development for phosphodiester RNA (PO RNA) and phosphorothioated RNA (PS RNA) with various lengths**
- **YMC-Triart Bio C18 (pore size: 30 nm) as stationary phase**
- **TEA-HFIP/methanol mobile phase system**

Samples

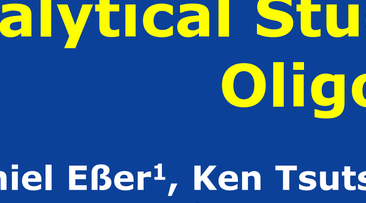
Phosphodiester linkage RNA (All PO RNA) + Internal Standard (I.S.)

1.	5′-CACUGAAUACCAAU-3′	(14mer)	1.8 nmol/mL
2.	5′-UCACACUGAAUACCAAU-3′	(17mer)	1.8 nmol/mL
3.	5′-UCAUCACACUGAAUACCAAU-3′	(20mer)	1.8 nmol/mL
4.	5′-GUCAUCACACUGAAUACCAAU-3′	(21mer)	3.6 nmol/mL
5.	I.S. (Caffeine)		10 µg/mL

Phosphorothioate RNA (All PS RNA)

1.	5′-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3′	(20mer)	1.0 nmol/mL
2.	5′-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3′	(21mer)	1.0 nmol/mL

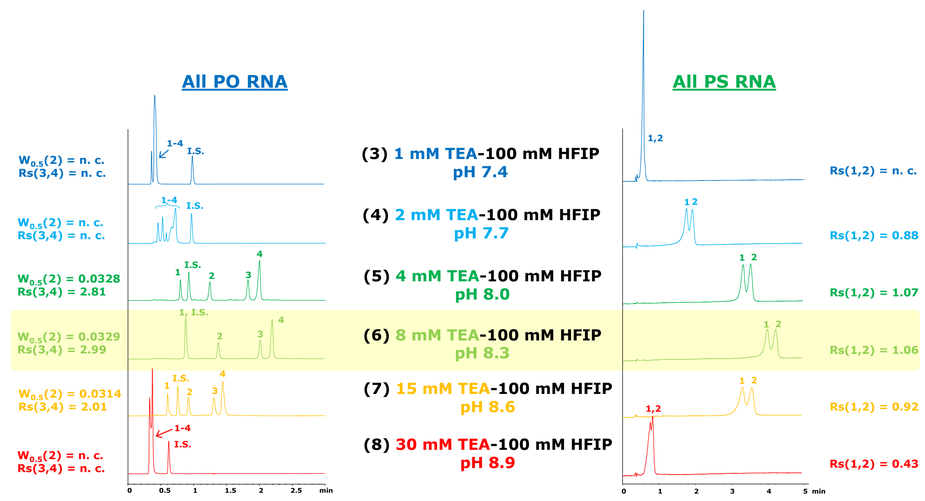
*: phosphorothioated



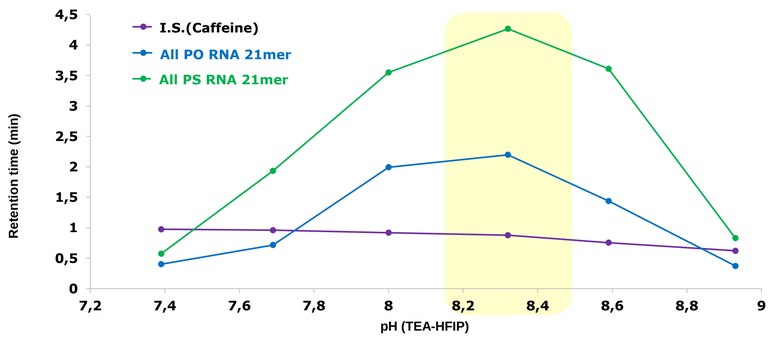
Phosphodiester Linkage (All PO) — **Phosphorothioate Linkage (All PS)**

Common Conditions
Column: YMC-Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Eluent: A) **X** mM TEA - **Y** mM HFIP B) methanol
Gradient: 1%B/min (initial %B = 8 %)
Flow rate: 0.42 mL/min
Detection: UV at 260 nm
Temperature: 65 ºC
Injection: 1.0 µL

Influence of IP-Reagent (TEA) Concentration and pH of Eluent



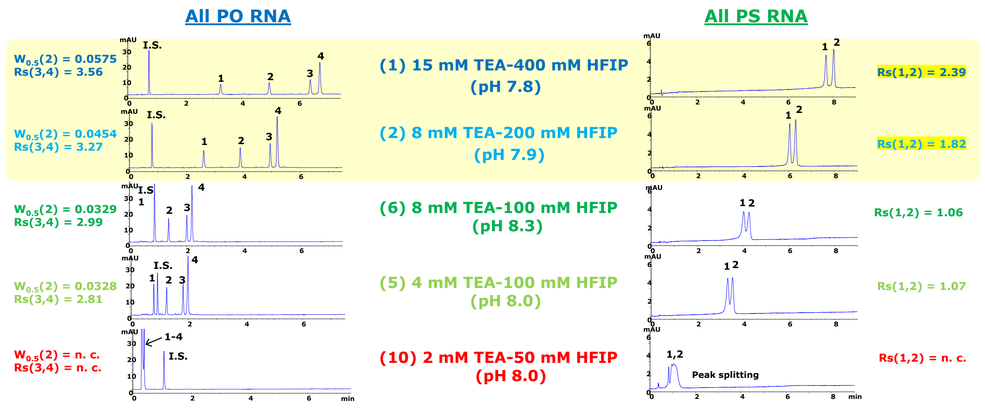
As the concentration of TEA increases (1 mM - 8 mM), the retention time of the oligonucleotides increases. At higher TEA concentrations (greater than 8 mM), the retention time of the oligonucleotides decreases. This trend is common for PO and PS oligonucleotides.



Retention times of PO and PS RNA are affected by mobile phase pH, and therefore TEA concentration. The pKa of TEA is 10.7. Retention of RNAs increases as TEA concentration increases. However, at a higher pH, the ionisation of TEA is suppressed and the content of ionised TEA decreases. As result, retention of RNAs decreases.

→ Optimal pH of oligonucleotides analysis using TEA-HFIP system is about 8.

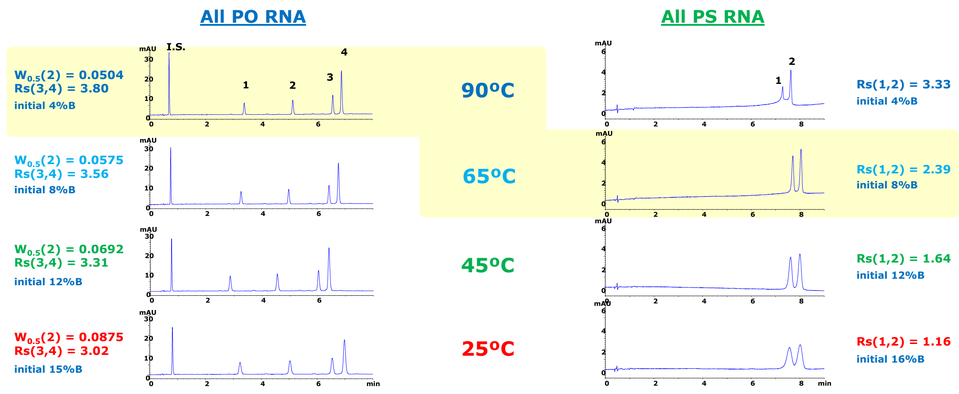
Optimal Combination of TEA and HFIP Concentrations at About pH 8



Higher TEA-HFIP concentrations provide better resolution and higher retention times. Especially for PS RNA, peak shape improves and baseline resolution is achieved with 8 mM TEA-200 mM HFIP or higher concentrations. While the best resolution is obtained using 15 mM TEA-400 mM HFIP, acceptable high resolution and retention time is still achieved with 8 mM TEA-200 mM HFIP (pH 7.9) and is the best compromise for these oligonucleotide samples.

NOTE: The argument here is based on UV detection. A lower concentration may be more suitable for MS detection from the standpoint of ion suppression.

Influence of Column Temperature

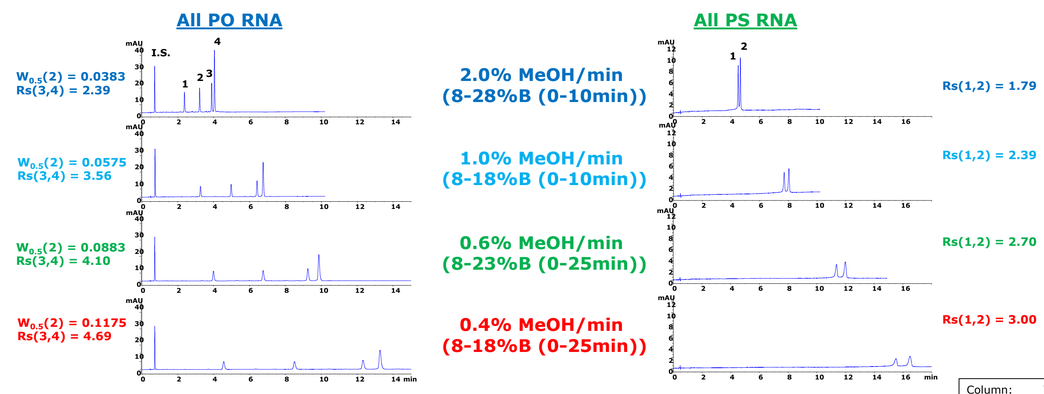


Column: YMC-Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Eluent: A) 15 mM TEA-400 mM HFIP B) methanol
Gradient: 1%B/min initial %B as stated in chromatograms

All PO RNA: Better resolution and sharper peaks are obtained at a higher temperature, although good separation is achieved even at a lower temperature.

All PS RNA: The best result is achieved at 65ºC, but acceptable results are obtained at 45ºC. However, at 90ºC peak deterioration is observed (probably due to a structural change of RNA).

Influence of Gradient Slope

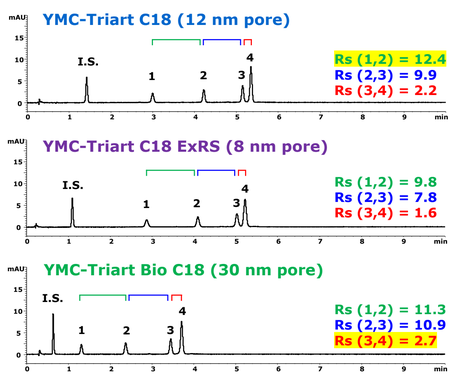


All gradient slopes ranging from 2.0% methanol/min to 0.4% methanol/min provide sufficient resolution. Shallower gradient slopes provide improved resolution value(s), but sensitivity is lost and the runtime is increased.

Column: YMC-Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Eluent: A) 15 mM TEA-400 mM HFIP B) methanol
Gradient: as stated in chromatograms
Temperature: 65ºC

Influence of Stationary Phase – C18 with Different Pore Sizes

All PO RNA

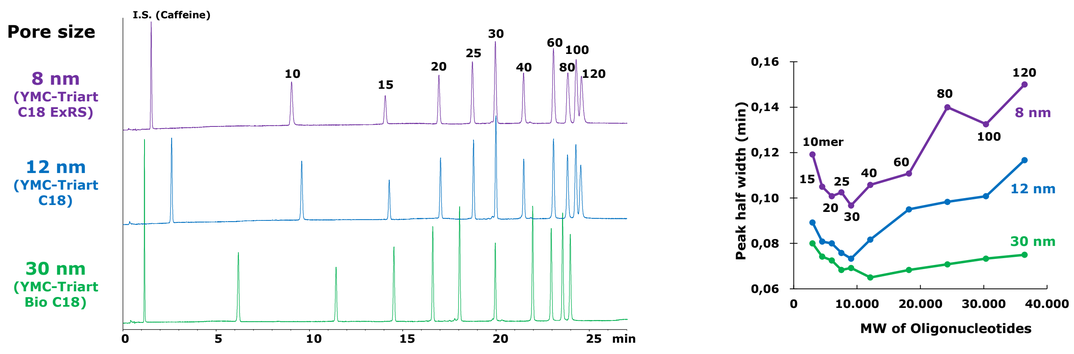


Columns: 3 µm, 50 x 2.1 mm ID
Eluent: A) 8 mM TEA-200 mM HFIP B) methanol
Gradient: 10-20%B (0-10 min)
Flow rate: 0.42 mL/min
Temperature: 65ºC
Detection: UV at 260 nm
Injection: 1 µL

YMC-Triart Bio C18 with 30 nm pore shows shorter retention times than with YMC-Triart C18 and YMC-Triart C18 ExRS. YMC-Triart C18 (12 nm pore) showed better resolution for the separation of shorter oligonucleotides. On the other hand, the best resolution was obtained with YMC-Triart Bio C18 for the separation of 20mer and 21mer oligonucleotides.

→ Further study on influence of pore size shown below.

DNA (Poly dT) 10-120mer

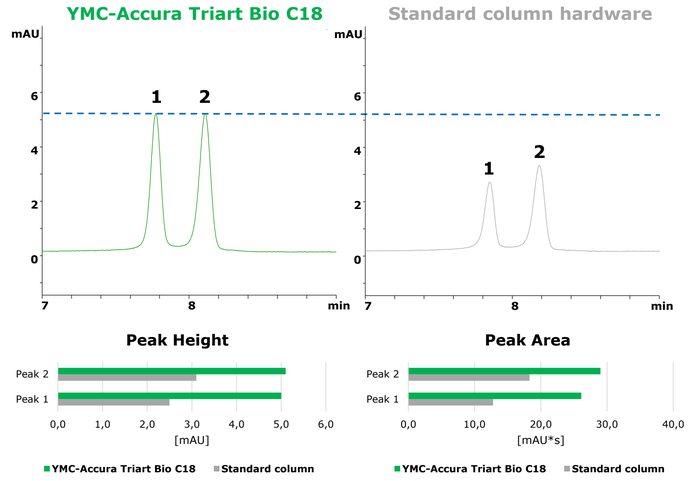


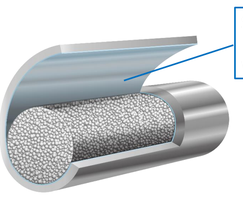
It is considered that medium sized oligonucleotides can fully permeate the pore of YMC-Triart Bio C18 and be well interacted with C18 bonded phase. YMC-Triart C18 with 12 nm pore may have some advantages on smaller oligonucleotides (up to 30mer or MW of 10 kDa). Due to short retention time of smaller nucleotides, YMC-Triart Bio C18 even shows sharper peak shape. YMC-Triart C18 ExRS with 8 nm pore is not suitable for oligonucleotide separations in general.

Columns: 3 µm, 50 x 2.1 mm ID
Eluent: A) 4 mM TEA-100 mM HFIP B) methanol
Gradient: 5-20%B (0-30 min)
Flow rate: 0.42 mL/min
Temperature: 65ºC
Detection: UV at 260 nm
Injection: 1 µL (0.2 – 2.0 µM)
Sample: poly dT (10-120mer)

Influence of Column Hardware – Bioinert YMC-Accura Triart

NEW





Columns: **YMC-Accura Triart Bio C18** and standard YMC-Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Eluent: A) 15 mM TEA-400 mM HFIP B) methanol
Gradient: 10-20%B (0-10 min)
Flow rate: 0.42 mL/min
Temperature: 65ºC
Detection: UV at 260 nm
Injection: 1 µL
Sample: 1. All PS RNA 20mer 2. All PS RNA 21mer

The YMC-Accura Triart Bio C18 column provides significantly improved peak heights and peak areas for the target oligonucleotides compared to the results for regular stainless-steel columns. YMC-Accura Triart columns enhance the sensitivity significantly and help to save precious samples without any loss due to the higher recovery.
As the HPLC system configuration also affects the sensitivity, it is recommended that in order to maximise the performance of YMC-Accura columns a bioinert/metal free HPLC system should be used.

Conclusions

- **Mobile phase:** Good resolution and retention times are obtained when the ion-pairing effect is enhanced (high TEA concentration, at about pH 8).
- **Column temperature:** Temperature is one of the key factors. Temperatures near 60ºC is the first choice. However, temperature dependency varies due to the target oligonucleotide samples
- **Gradient slope:** 2.0 - 0.4% MeOH/min works in most cases. It should be optimised by checking resolution and peak height (sensitivity).
- **Stationary phase:** YMC-Triart Bio C18 with 30 nm pore size is the first choice, based on the applicable MW range and chemical stability. YMC-Triart C18 also works for smaller oligonucleotides. A phase screening is helpful when the ideal separation is not obtained with these columns.
- **Column hardware:** YMC-Accura Triart with its bioinert coated hardware ensures high recovery and reproducibility – ideally when combined with a bioinert HPLC system.

List of abbreviations: $W_{0.5}$: half width (min); Rs: resolution; n. c.: not calculated