YMC SEPARATION TECHNOLOGY

Development of Oligonucleotide Analysis Methodology with RP-HPLC Column Designed for Separation of Highly Polar Compounds

Noriko Shoji, Chie Yokoyama, and Naohiro Kuriyama
YMC Co., Ltd., Ishikawa, Japan

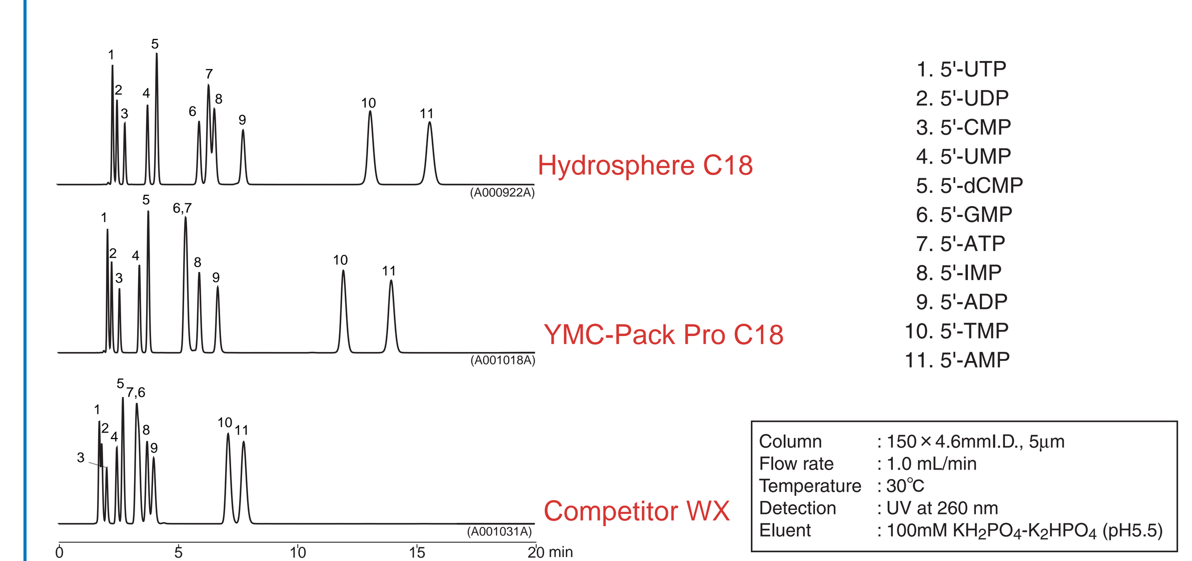
1. Introduction

Synthetic oligonucleotides have been used extensively as primers of DNA sequencing or PCR, hybridization probes, and antisense drugs, and separation methodology using reversed-phase HPLC has been widely applied also to analysis and purification of these oligonucleotides. Since it is difficult to retain and separate highly polar compounds like short oligonucleotides on the ordinary reversed-phase columns, an ion-pairing buffer containing triethylammonium acetate (TEAA) at a high concentration, e.g., 100-200mM, has been commonly used to improve the poor retention and resolution. However, in case of a buffer containing TEAA at a concentration higher than 50mM, the signal intensity decreases in electrospray ionization mass spectrometry (ESI-MS), which is one of the most important analytical methodologies for oligonucleotides.[1] Moreover, the high concentration of buffer has an adverse effect on column lifetime. In addition, peak tailing is often caused by basic functional groups residing in the oligonucleotide molecules.

We have a silica-based C18-bonded packing material named Hydrosphere C18, which has been specially designed for separation of highly polar compounds. It provides strong retention of polar compounds and an excellent peak shape even in case of basic compounds, and it can be used with a 100% aqueous mobile phase. Figure 1 compares nucleotide separation among Hydrosphere C18 and two C18 phases shown in Table 1. Hydrosphere C18 shows enhanced retention and improved resolution of highly polar compounds like nucleotides.

We are presenting here some example cases of analyzing 2-20mer Poly-d(pT) oligonucleotides by means of Hydrosphere C18 under different mobile phase conditions. We also compare the retention and elution achieved with Hydrosphere C18 and those achieved with other C18 columns.

Figure 1 : Comparison of nucleotide separation among Hydrosphere C18 and two ordinary C18 phases



2. Experiments

Table 1: Specifications of stationary phases used in this study

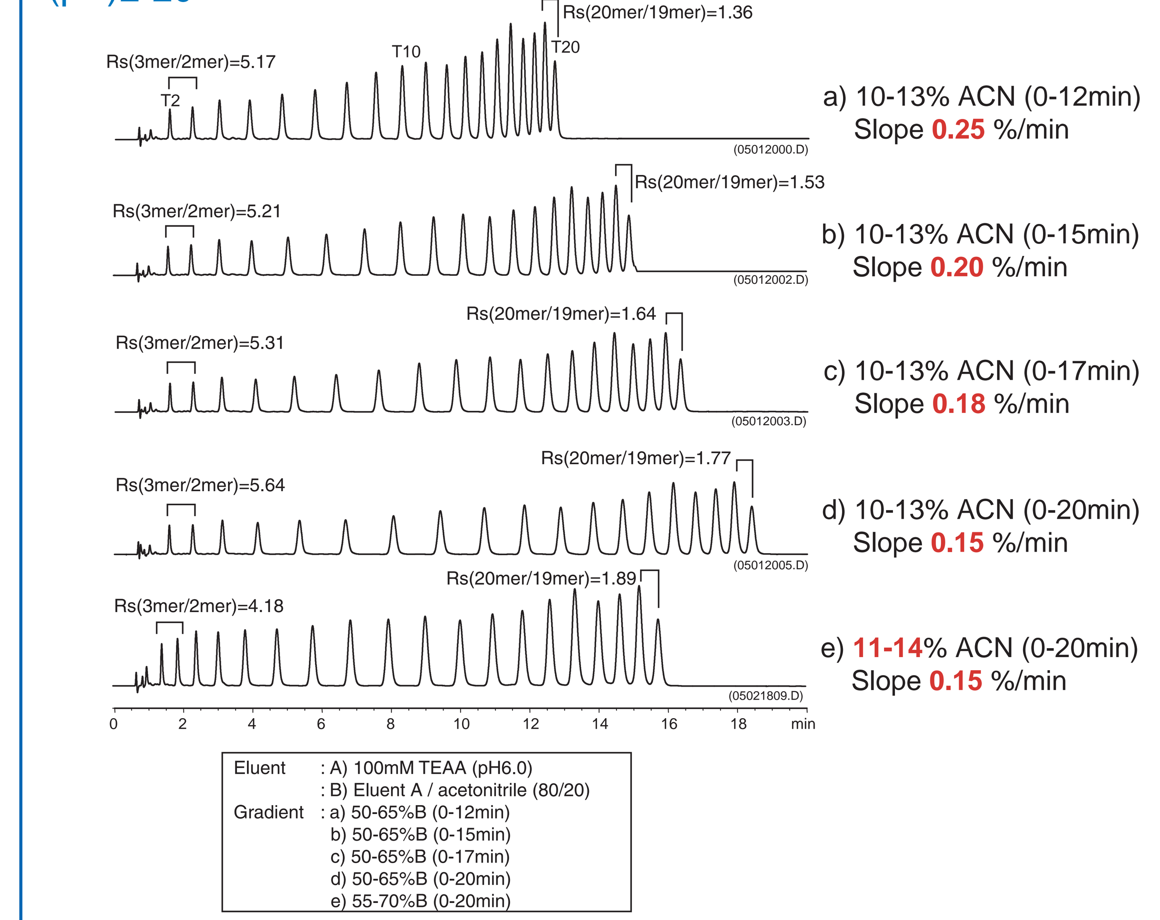
Brand name	Stationary phase	Particle size (μm)	Pore size (Å)	Carbon content (%)	Endcapping
Hydrosphere C18	C18	3, 5	120	12.0	yes
YMC-Pack Pro C18	C18	5	120	16.0	yes
Competitor WX	C18	5	125	15.5	yes

HPLC conditions for separation of d(pT)2-20 in Figures 2-4

Column : Hydrosphere C18 (3μm) 50 × 4.6 mmI.D.
Flow rate : 1.0 mL/min
Temperature : 35 ℃
Detection : UV at 269 nm
Injection : 5μL (25pmol/component)
Eluent : Three kinds of gradient systems are used in buffer concentration range of 10-100mM at pH6.0
Eluent 1 : triethylammonium acetate (TEAA) buffer/acetonitrile
Eluent 2 : ammonium acetate buffer/acetonitrile
Eluent 3 : potassium phosphate buffer/acetonitrile
Gradient conditions are shown in the figures

3. Results and Discussion

Figure 2 : Influences of gradient conditions on separation of d(pT)2-20



The influences of gradient conditions on separation of polydeoxythymidylic acids, d(pT)2-20, are shown in Figure 2. The retention and resolution were greatly influenced by both gradient slope and initial composition of mobile phase. As shown in Chromatograms a-d, a slight change in slope from 0.25% ACN/min to 0.15% ACN/min resulted in enhanced retention and improved resolution especially in case of longer oligonucleotides, and it enabled baseline separation of d(pT)2-20 even by one-nucleotide difference. Furthermore, even with the same slope, only a 1% increase in initial acetonitrile concentration could shorten each run time maintaining the favorable separation, as shown in Chromatograms d and e. It seems therefore important to choose the appropriate gradient slope and initial composition of mobile phase carefully for achieving better separation of oligonucleotides.

Figure 3 : Comparison of d(pT)2-20 separation among TEAA, acetate and phosphate buffers

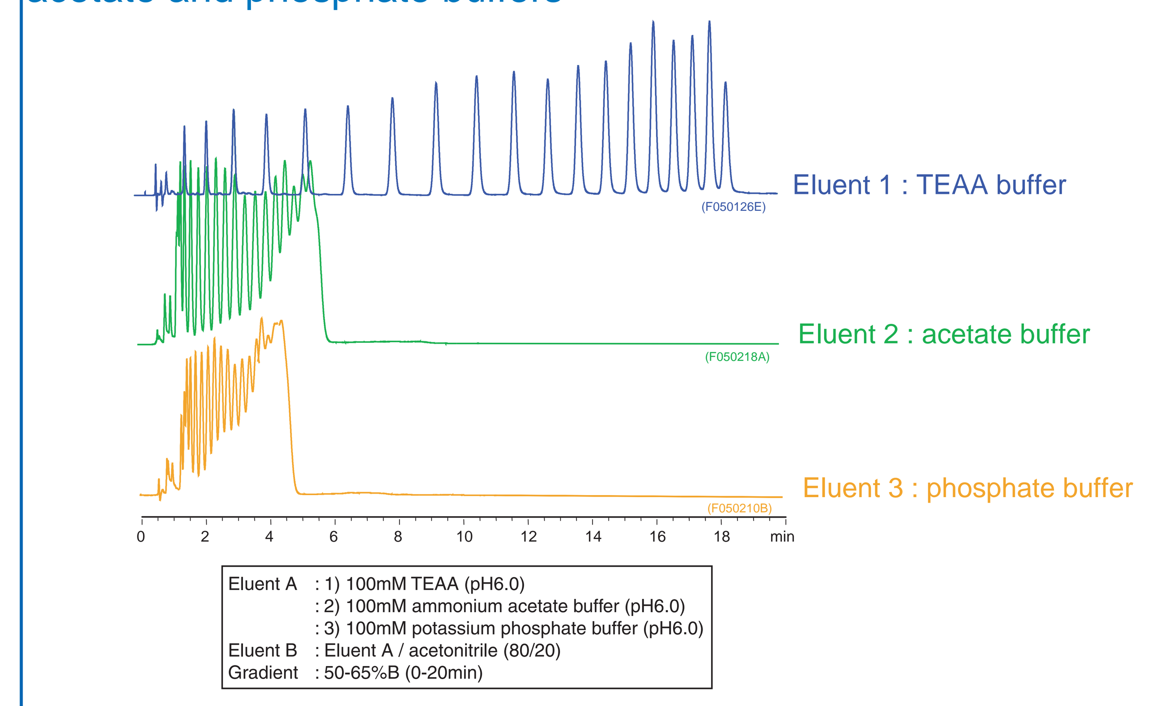
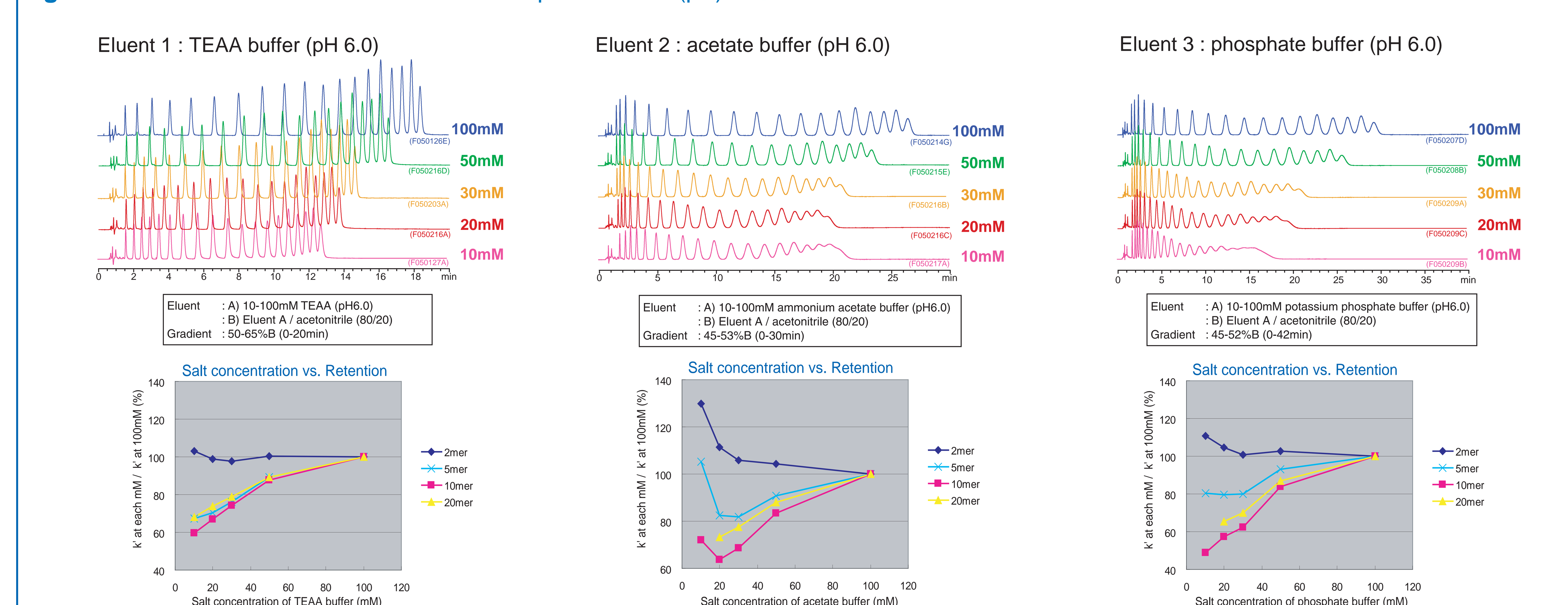


Figure 3 compares d(pT)2-20 separation among the TEAA, ammonium acetate and potassium phosphate buffers under the same gradient condition. The retention achieved with the TEAA buffer was stronger than that achieved with the acetate or phosphate buffer. This indicates that the retention/elution mechanism in using a mobile phase containing triethylamine is mainly based on the ion-paring interaction between the highly hydrophilic phosphodiester groups of oligonucleotides and the somewhat lipophilic triethylammonium ions in the buffer, and the ion-paring interaction produces some interaction between the hydrophilic oligonucleotides and the lipophilic sorbent on the stationary phase, resulting in stronger retention of oligonucleotides.

Figure 4: Influence of salt concentration on separation of d(pT)2-20



In Figure 4, the influence of salt concentration on separation and resolution of d(pT)2-20 is evaluated among the TEAA, ammonium acetate and potassium phosphate buffers. Regardless of salt type, the retention decreased and the peak broadened with decreasing salt concentration, except for the case of d(pT)2. The retention was unexpectedly influenced by salt concentration of the acetate or phosphate buffer, which has no ion-paring interaction, but this phenomenon might be associated with the charge status of oligonucleotides.

In case of the TEAA buffer, the favorable separation was maintained even at a low salt concentration, such as 10mM. The mobile phase consisting of TEAA and acetonitrile has been used commonly in RP-HPLC separation of oligonucleotides, but a high salt concentration is usually necessary to improve the poor retention and peak shape. Hydrosphere C18 can be used at a lower TEAA concentration than ordinary C18 phases.

Figure 5: Comparison of d(pT)2-20 separation among Hydrosphere C18 and two ordinary C18 phases

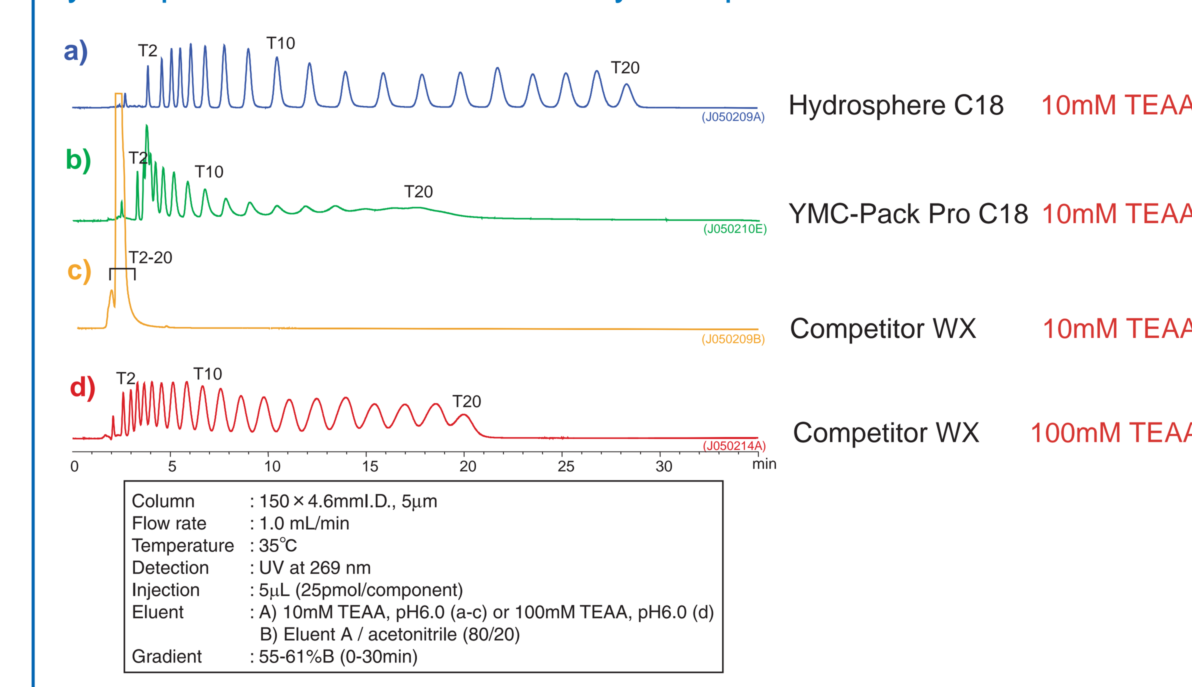
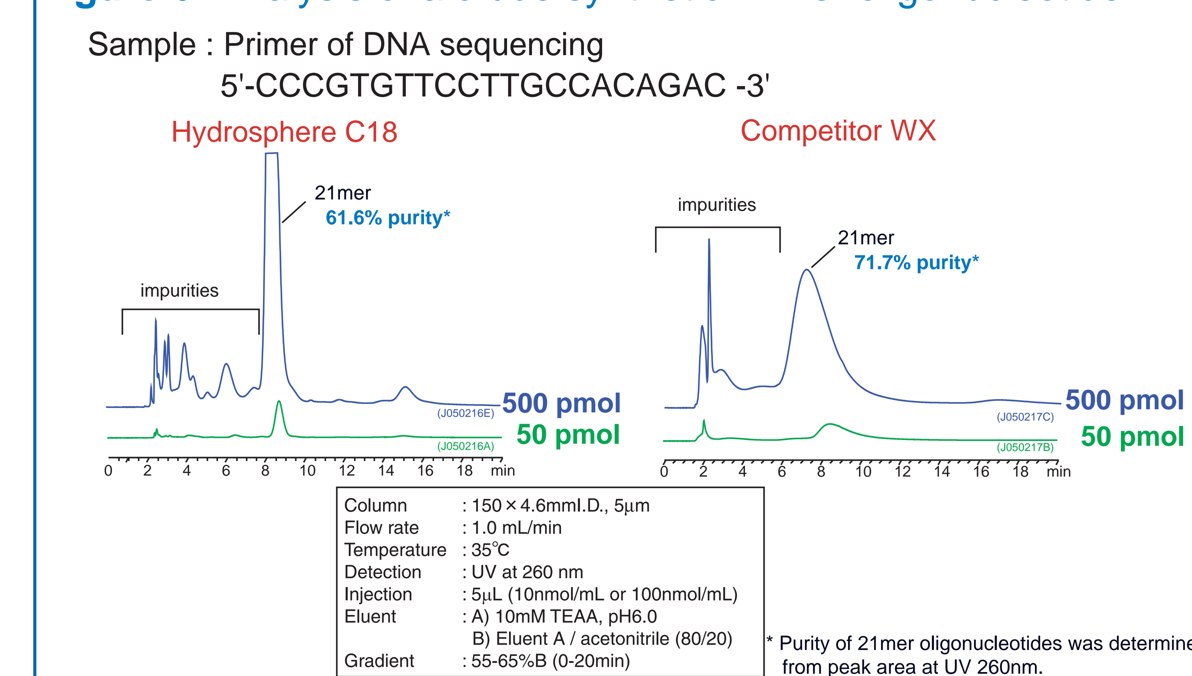


Figure 6: Analysis of a crude synthetic 21mer oligonucleotide



In Figure 5, the chromatographic performance was compared among Hydrosphere C18 and two ordinary C18 phases under the same condition which was found suitable to Hydrosphere C18 in Figure 4, i.e. use of the 10mM TEAA buffer. The ordinary C18 phases could not achieve acceptable retention and resolution with the 10mM TEAA buffer. Even in case of Competitor WX used at a salt concentration of 100mM, the separation was less favorable as compared with Hydrosphere C18 used at 10mM.

Figure 6 shows the analysis results of a crude synthetic 21mer oligonucleotide obtained with Hydrosphere C18 and Competitor WX. Even when the loading amount was increased from 50pmol to 500pmol, many impurities could be separated from the target product on Hydrosphere C18 with the resolution capacity maintained high as distinct from Competitor WX. Furthermore, a volatile buffer of a low concentration, like 10mM TEAA, is easily removed from the collected fractions by lyophilization or vaccum centrifugation as an additional advantage. Hydrosphere C18 may be suitable for purification of synthetic oligonucleotides.

4. Conclusions

- Oligonucleotide separation was compared under various mobile phase conditions using Hydrosphere C18. The retention and resolution of oligonucleotides were greatly influenced by gradient condition, salt type, and salt concentration.
- The ordinary C18 phases could not achieve any acceptable separation at a relatively low concentration of TEAA, e.g., 10mM. However, Hydrosphere C18 showed strong retention and good resolution of oligonucleotides under the same conditions.
- Hydrosphere C18 may be applicable to analysis and purification of various types of oligonucleotides.

Reference
1) Alex Apffel, John A. Chakel, Steven Fischer, Kay Lichtenwalter, William s. Hancock, *Anal. Chem.* 1997, 69, 1320-1325