

## 1. Introduction

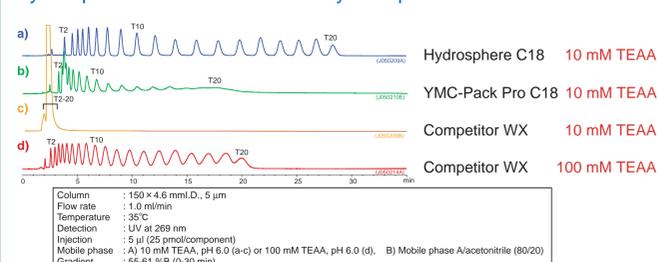
Reversed-phase HPLC has been widely applied to analysis and purification of synthetic oligonucleotides, such as primers of DNA sequencing or PCR, hybridization probes, and antisense drugs. Because it is difficult to retain and separate highly polar compounds like short oligonucleotides on ordinary reversed-phase columns, an ion-pairing buffer containing triethylammonium acetate (TEAA) at a high concentration, e.g., 100-200 mM, has been commonly used to improve poor retention and resolution. However, for a buffer containing TEAA at a concentration higher than 50 mM, the signal intensity decreases in electrospray ionization mass spectrometry (ESI-MS), which is one of the most important analytical methodology for oligonucleotides. Though it has been reported that the mobile phase of 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-TEA buffer/methanol gives an advantage in MS sensitivity, it is necessary to add HFIP, which is not so commonly used for RP-HPLC, with a relatively high concentration such as 400 mM to achieve good LC/MS analysis.<sup>1</sup>

We have developed 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 for basic compounds, and it can be used with a 100% aqueous mobile phase.

In our previous study,<sup>2</sup> we applied Hydrosphere C18 to separation of short oligonucleotides up to 20 mer under various mobile phase conditions. As shown in Fig. 1, Hydrosphere C18 showed enhanced retention and improved resolution at a low concentration of TEAA buffer, such as 10 mM. Ordinary C18 phases could not achieve any acceptable separation in identical conditions.

This study developed an efficient analytical method for short oligonucleotides using Hydrosphere C18 with an ion-pairing buffer containing di-n-butylammonium acetate (DBAA). It provides both good HPLC separation and high-sensitivity in ESI-MS.

**Figure 1: Comparison of d(pT)2-20 separation among Hydrosphere C18 and two ordinary C18 phases**



## 2. Experimental

### HPLC conditions for separation of d(pT)2-20 in Fig. 2

Column : Hydrosphere C18, 50 × 4.6 mm I.D., 3 μm  
Flow rate : 1.0 ml/min  
Temperature : 35°C  
Detection : UV at 269 nm  
Injection : 5 μl (25 pmol/component)  
Mobile phase : Four kinds of gradient systems are used  
10 mM TEAA (pH 6.0)/acetonitrile (a)  
5 or 10 mM DBAA (pH 6.0)/acetonitrile (b, c)  
10 mM TEAA (pH 6.0)/methanol (d)  
5 or 10 mM DBAA (pH 6.0)/methanol (e, f)  
Gradient conditions are shown in Fig. 2

### LC-MS analyses conditions in Fig. 3 and 4

Column : 50 × 2.0 mm I.D., 3 μm  
Flow rate : 0.2 ml/min  
Column temperature : 35°C  
Injection : 5 μl (25 pmol/component) (Fig. 3)  
1 μl (10 pmol/component) (Fig. 4)  
Mobile phase : Mobile phase conditions are shown in the figures  
Ionization mode : ESI negative-mode  
Capillary : 2.50 kV  
Cone : 35 V (Fig. 3)  
140 V (Fig. 4)  
Source temperature : 100°C  
Desolvation temperature : 350°C

## 3. Results and Discussion

**Figure 2: Comparison of d(pT)2-20 separation under different ion-pairing buffers and organic solvents**

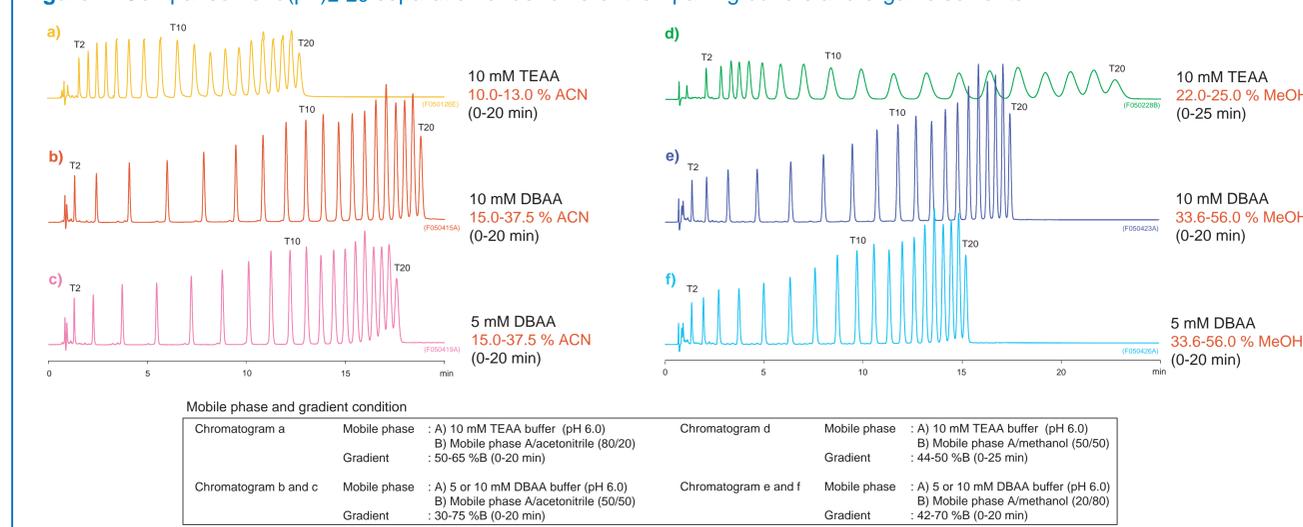
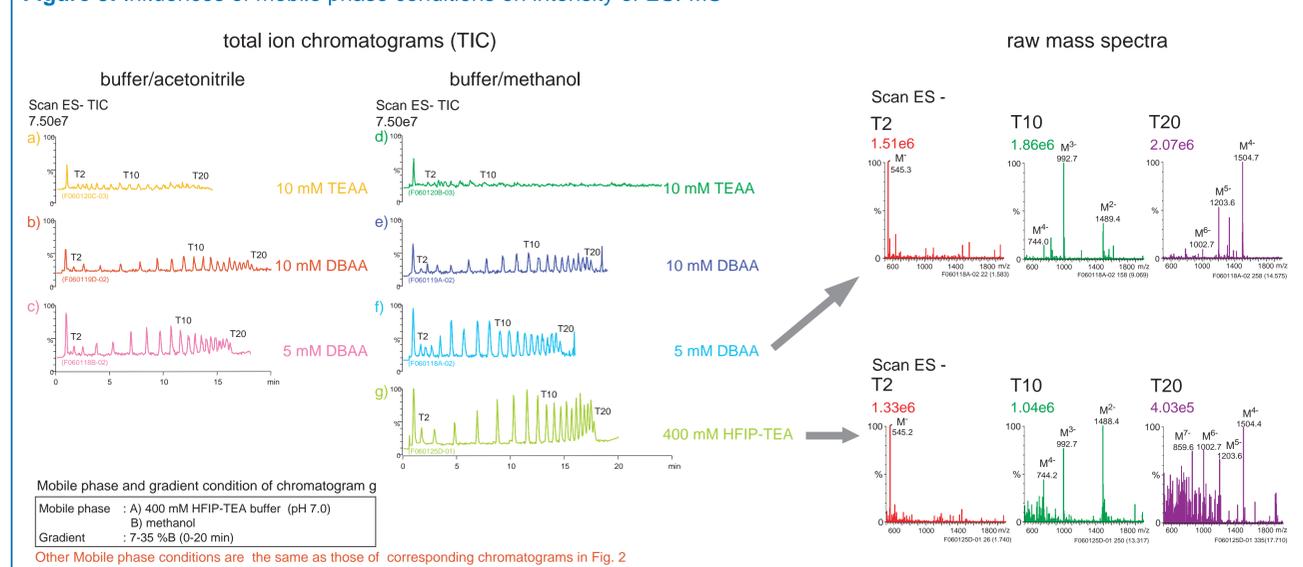


Figure 2 compares separation of polydeoxythymidylic acid, d(pT)2-20, among four different gradient systems of TEAA buffer/acetonitrile (a), DBAA buffer/acetonitrile (b, c), TEAA buffer/methanol (d) and DBAA buffer/methanol (e, f), with the gradient slope which has been optimized at 10 mM buffer concentration. In case of the DBAA buffer, d(pT)2-20 can be separated favorably with higher initial concentration and higher gradient slope of organic solvent with both acetonitrile and methanol than in case of the TEAA buffer. This result indicates that the ion-pairing interaction between di-n-butylamine and oligonucleotides is stronger than that between triethylamine and oligonucleotides. Even at a lower concentration of DBAA, such as 5 mM, the acceptable separation and good peak shape was maintained under the same gradient condition as 10 mM DBAA buffer, as shown in Chromatograms 2c and 2f.

**Figure 3: Influences of mobile phase conditions on intensity of ESI-MS**



In Fig. 3, the influences of mobile phase conditions on signal intensity in HPLC-ESI-MS analyses of d(pT)2-20 are compared among five different gradient systems of TEAA buffer/acetonitrile (a), DBAA buffer/acetonitrile (b, c), TEAA buffer/methanol (d), DBAA buffer/methanol (e, f) and HFIP-TEA buffer/methanol (g). The intensity of TIC in ESI-MS obtained with the 10 mM DBAA buffer is much superior to that obtained with the 10 mM TEAA buffer with both acetonitrile and methanol (Chromatograms 3a, 3b, 3d and 3e). Furthermore, the 5 mM decrease of DBAA concentration in mobile phase resulted in approximately 1.5-3 times increasing of the intensity of the peaks with sufficient column performance (Chromatograms 3b, 3c, 3e and 3f). The intensity obtained with 5 mM DBAA buffer/acetonitrile or 5 mM DBAA buffer/methanol can be comparable to that obtained with 400 mM HFIP-TEA buffer/methanol. Also, the multiply-charged ions of d(pT)2-20,  $[M-nH]^n$ , were obtained with good intensity in the raw mass spectra.

**Figure 4: LC-MS analysis of synthetic 27-30 mer oligonucleotides**

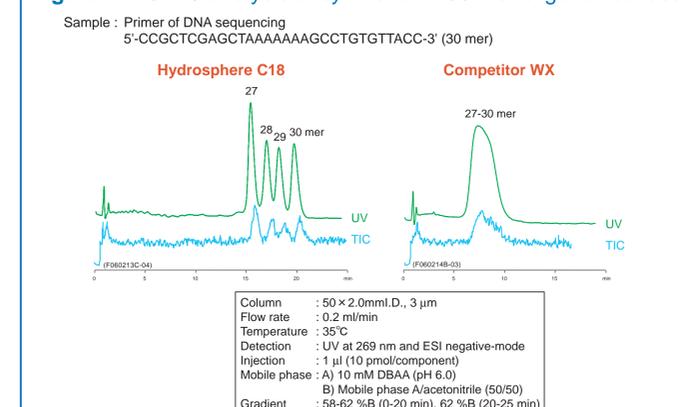


Figure 4 shows the LC-MS analyses of a mixture of synthetic 27-30 mer oligonucleotides using Hydrosphere C18 and the ordinary C18 phase, competitor WX. The mobile phase consisting of the 10 mM DBAA buffer and acetonitrile was suitable for separation of these oligonucleotides. Hydrosphere C18 can achieve excellent separation by one-nucleotide difference and sufficient intensity in ESI-MS. The separation and intensity with Competitor WX were less favorable as compared with Hydrosphere C18 in identical conditions.

## 4. Conclusions

- Oligonucleotide separation was compared under mobile phase conditions containing two different ion-pairing buffers, TEAA and DBAA, using Hydrosphere C18. It is possible to reduce the salt concentration and increase the organic solvent concentration with the DBAA buffer than with the TEAA buffer.
- Hydrosphere C18 showed strong retention and good resolution of short oligonucleotides at a relatively low concentration of DBAA, e.g. 5-10 mM, compared to the ordinary C18 phase.
- There was sufficient intensity of ESI-MS when 5-10 mM DBAA buffer was used to analyze oligonucleotides with both acetonitrile and methanol. The intensity obtained with 5 mM DBAA buffer was equal to or greater than that obtained with 400 mM HFIP-TEA buffer.
- This simple method using Hydrosphere C18 with a low concentration of DBAA buffer provides a great potential for various chromatography of oligonucleotides, such as analytical and preparative HPLC or HPLC-MS.

### References

- Alex Apffel, John A. Chakel, Steven Fischer, Kay Lichtenwalter, William s. Hancock, *Anal. Chem.* **1997**, *69*, 1320-1325
- Noriko Shoji, Chie Yokoyama, Naohiro Kuriyama, *Pitcon 2005*, 760-12P