Selectivity studies in the analytical separation of oligonucleotides using anion exchange chromatography

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Introduction

- Synthetic oligonucleotides are becoming increasingly popular as biotherapeutic agents.
- When purification moves from analytical to manufacturing scale, having the same bonding chemistry is helpful, since the selectivity is expected to remain the same.
- TSKgel SuperQ-5PW analytical columns have the same backbone chemistry and selectivity as the bulk process scale resins *viz.* TSKgel SuperQ-5PW and Toyopearl SuperQ-650.
- Here we report a one-step analysis of a 20-mer DNA-based oligonucleotide, using a TSKgel SuperQ-5PW,10 µm, 7.5 mm ID × 7.5 cm column.

Introduction

- The effect of pH, salt concentration, temperature, and sample load on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, are discussed.
- Two other columns: one packed with 5 µm hydrophilic non-porous resin particles of which the surface consists of an open access network of multi-layered anion exchange groups (TSKgel DNA-STAT) and the other column packed with 2.5 µm non-porous, high efficiency, hydrophilic polymer beads which are surface modified with a weak anion exchanger (TSKgel DNA-NPR) are used for reference.

Objective

To show the usefulness of the TSKgel SuperQ-5PW analytical column in the separation of oligonucleotides, particularly in reference to the fact that the TSKgel SuperQ-5PW bulk resin and the TSKgel SuperQ-5PW analytical column both have the same selectivity.

Characteristics of TSKgel Ion Exchange Columns Used in this Study – Product Attributes

	TSKgel DNA-NPR	TSKgel SuperQ-5PW	TSKgel DNA-STAT
Matrix	hydroxylated methacrylic polymer	polymethacrylate	hydrophilic polymer
Particle size (mean):	2.5 µm	10 µm & 13 µm	5 µm
Pore size (mean):	non-porous	1000 Å	non-porous
Functional group:	proprietary	trimethylamino	quarternary ammonium
Counter ion:	ClO_4^-	Cl^-	Cl
pH range:	2-12	2-12	3-10
Capacity (mg BSA/mL):	5 mg	100 mg	
Static binding capacity (mg BSA/g dry gel):			ca. 35 (5 µm)
Small ion capacity:	>0.1 meq/mL	>0.13 meq/mL	270 µeg/g dry gel
pKa:	11.2	12.2	10.5
Hardware:			stainless steel tubing & fittings, PEEK frits

Material and Methods

HPLC System: Analyses were carried out using an Agilent-1200 HPLC system running Chemstation (ver B.04.02).

Columns: TSKgel SuperQ-5PW, 10 µm, 7.5 mm ID × 7.5 cm (S0082-84NM)

TSKgel DNA STAT, 5 µm, 4.6 mm ID × 10 cm (R0036-502N)

TSKgel DNA-NPR, 2.5 µm, 4.6 mm ID × 7.5 cm (R0037-87M)

Sample: Phosphodiester deoxyoligonucleotide (20-mer) EcoRI sequence (Trilink Biotechnology, San Diego, CA): Lot# T34-C01A

5' - GAA TTC ATC GGT TCA GAG AC – 3’

- purchased unpurified.
- the extinction coefficient was 199.9 OD units/µmol
- the molecular weight of the free acid – 6140.9 Da
- this sequence was chosen to minimize the amount of secondary structure effects during the purification experiments.
- reconstitution of oligonucleotide: For all of the experiments performed, the crude oligonucleotide was diluted into the equilibration buffer (buffer A) before loading onto the column.
- stock concentration: 26.6 mg/mL, final dilution: (1:100) in mobile phase A
- final concentration: 0.266 mg/mL = 0.266 µg/µL

Chromatographic Conditions

Mobile phase: TSKgel SuperQ-5PW

- buffer A: 20 mmol/L Tris, pH 9.0
- buffer B: 20 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
- 40-80% B over 30 minutes – an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide

Mobile phase: TSKgel DNA-STAT

- buffer A: 100 mmol/L Tris, pH 9.0
- buffer B: 100 mmol/L Tris, pH 9.0 + 1 mol/L NaCl
- 20-100% B over 30 minutes – an optimum gradient found to separate both N+1 and N-1 peaks of oligonucleotide

Mobile phase: TSKgel DNA-NPR

- buffer A: 20 mmol/L Tris, pH 9.0
- buffer B: 20 mmol/L Tris, 1 mol/L NaCl, pH 9.0
- 0-100% buffer B (15 minute gradient)
- 100% buffer B (3 minutes)
- 0% buffer B (5 minutes)

Chromatographic Conditions

Flow rate:

- 0.9 mL/min (TSKgel Super Q-5PW)
- 0.5 mL/min (TSKgel DNA-STAT)
- 0.7 mL/min (TSKgel DNA-NPR)

Detection: UV @ 260 nm

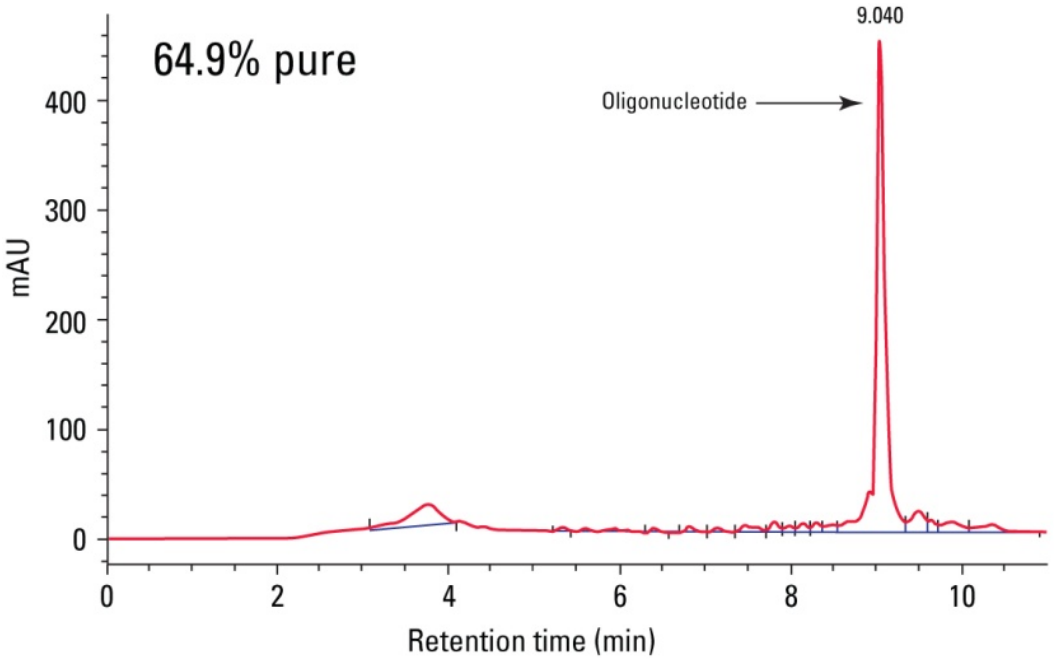
Temperature:

- TSKgel Super Q-5PW - Ambient & 60 °C
- TSKgel DNA-STAT - 60 °C
- TSKgel DNA-NPR - 40 °C

Injection vol.:

- 15 µL (TSKgel SuperQ-5PW)
- 10 µL (TSKgel DNA-STAT)
- 15 µL (TSKgel DNA-NPR)

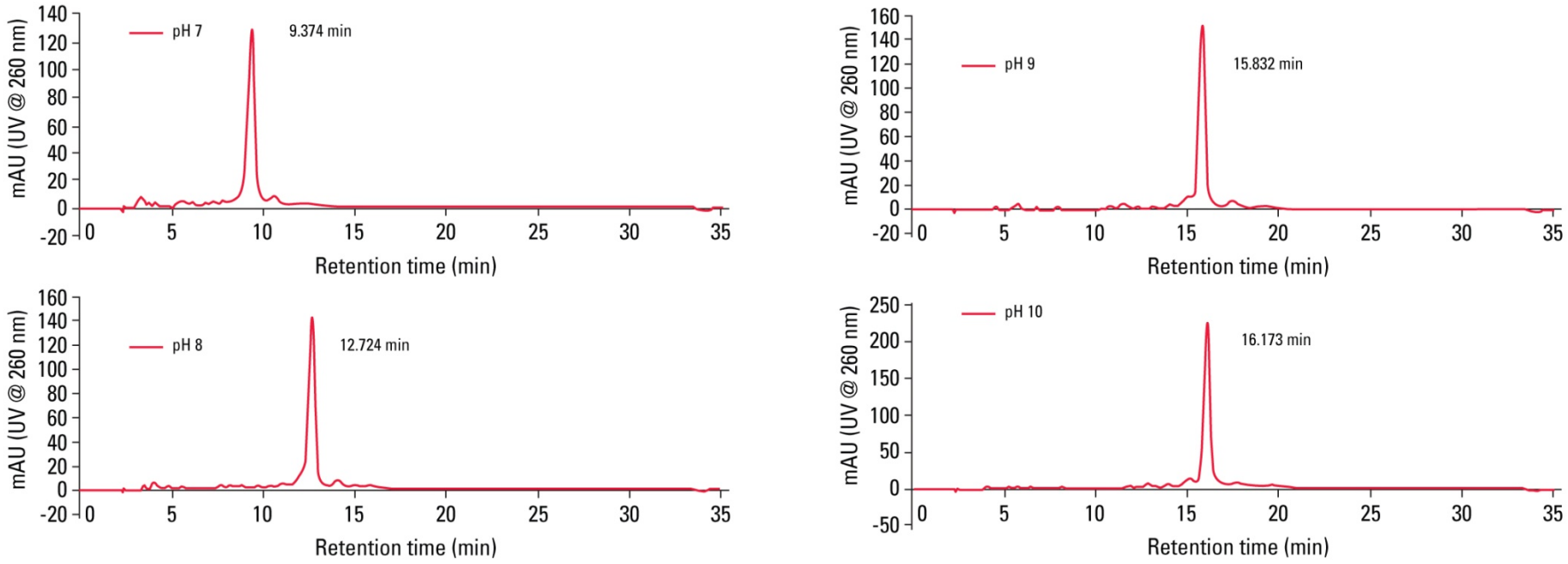
Figure 1: Analysis of Crude Oligonucleotide Using TSKgel DNA-NPR Column



TSKgel DNA-NPR:
buffer A: 20 mmol/L Tris, pH 9.0
buffer B: 20 mmol/L Tris, 1 mol/L NaCl, pH 9.0
15 µL sample injection
0-100% buffer B (15 minute gradient)
100% buffer B (3 minutes)
0% buffer B (5 minutes)

The oligonucleotide was estimated at 64.9% purity by HPLC in lyophilized form.

Figure 2: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different pH



- **The effect of pH on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, was studied. (Please see the reference 1 for the method of characterization of the N-1 and N+1 peaks by gel electrophoresis analysis of purified oligonucleotides.)**
- **The best pH value was found to be pH 9.0.**

Figure 3: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different pH (zoomed in view)

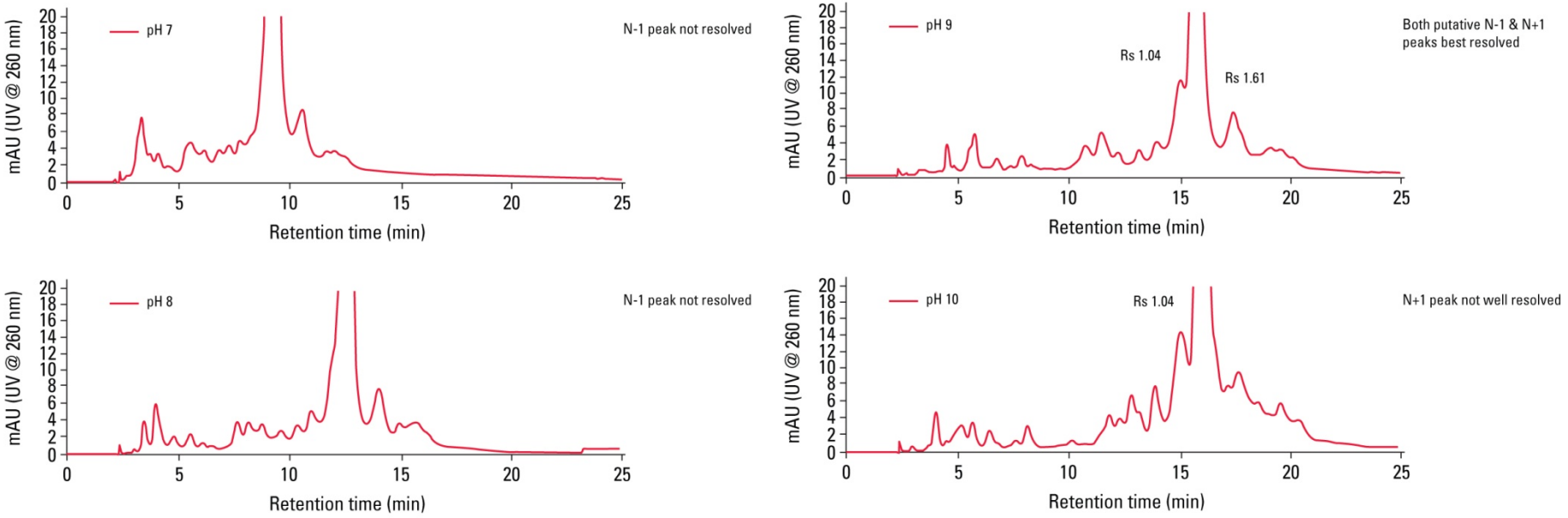
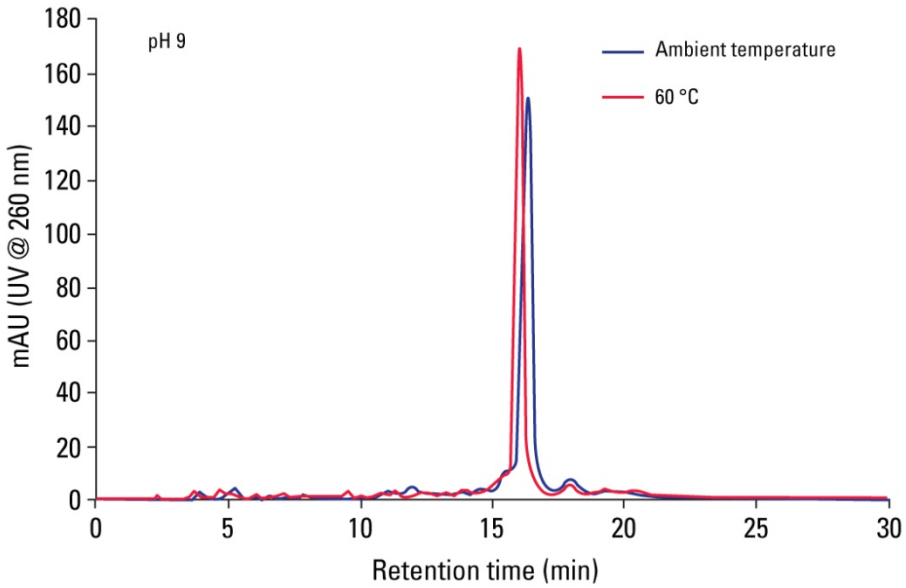
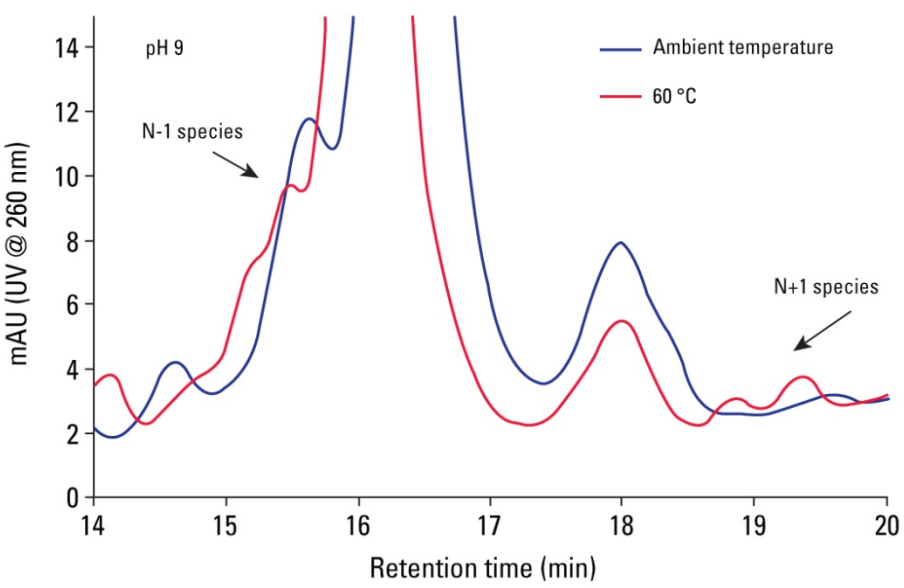


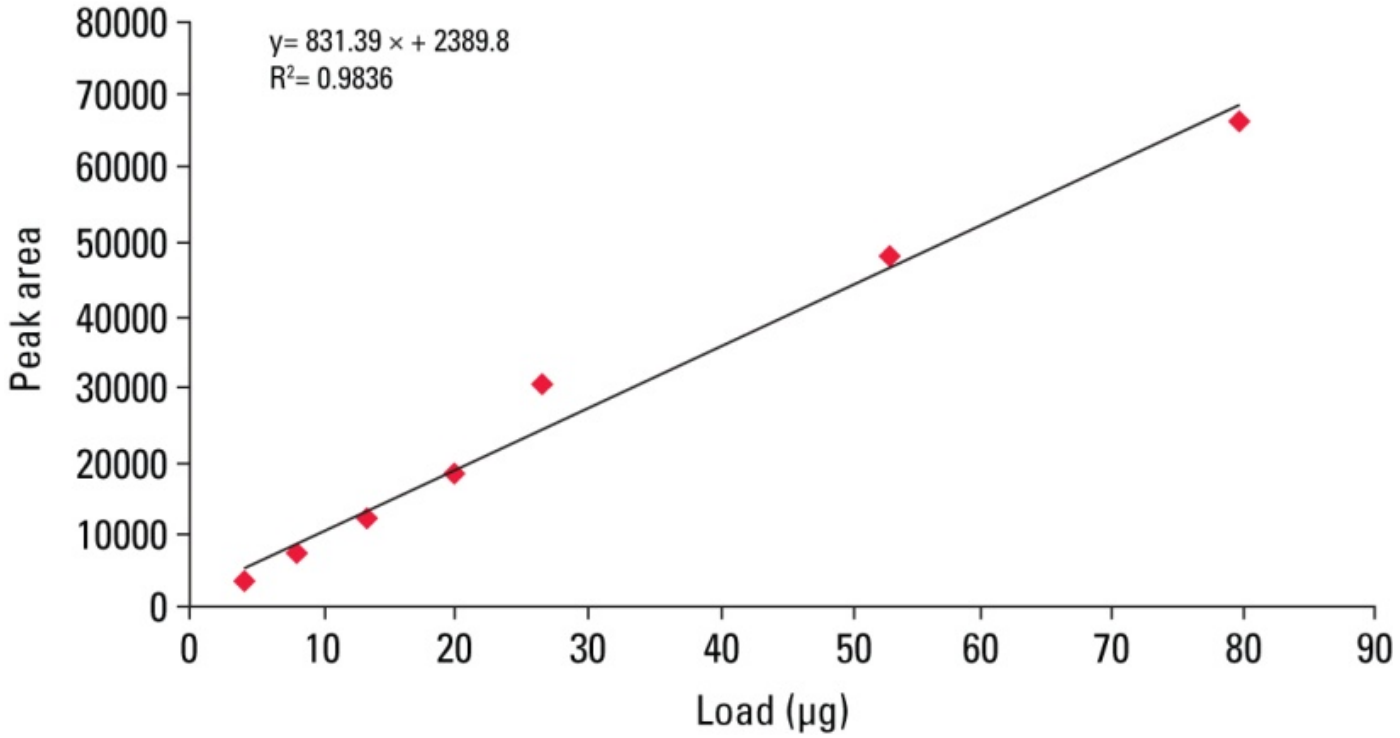
Figure 4: Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column at Different Temperature





- **Effect of temperature on the selectivity and resolution, particularly in reference to the separation of N-1 and N+1 peaks from the main peak, was studied.**
- **Better resolution could be obtained at 60 ºC column temperature compared to the experiment at ambient temperature.**
- **Both N-1 and N+1 peaks appear to be heterogeneous.**

Figure 5: Effect of Higher Loading on the HPLC Analysis of Crude Oligonucleotide Using TSKgel SuperQ-5PW Column



- **The peak area analysis was linear within the experimental range of 3.98 µg to 79.68 µg (based on single injection data).**
- **Higher loadings of crude oligonucleotide within this experimental range did not affect the peak purity percentage.**

Figure 6: Analysis of Crude Oligonucleotide Using TSKgel DNA-STAT Column

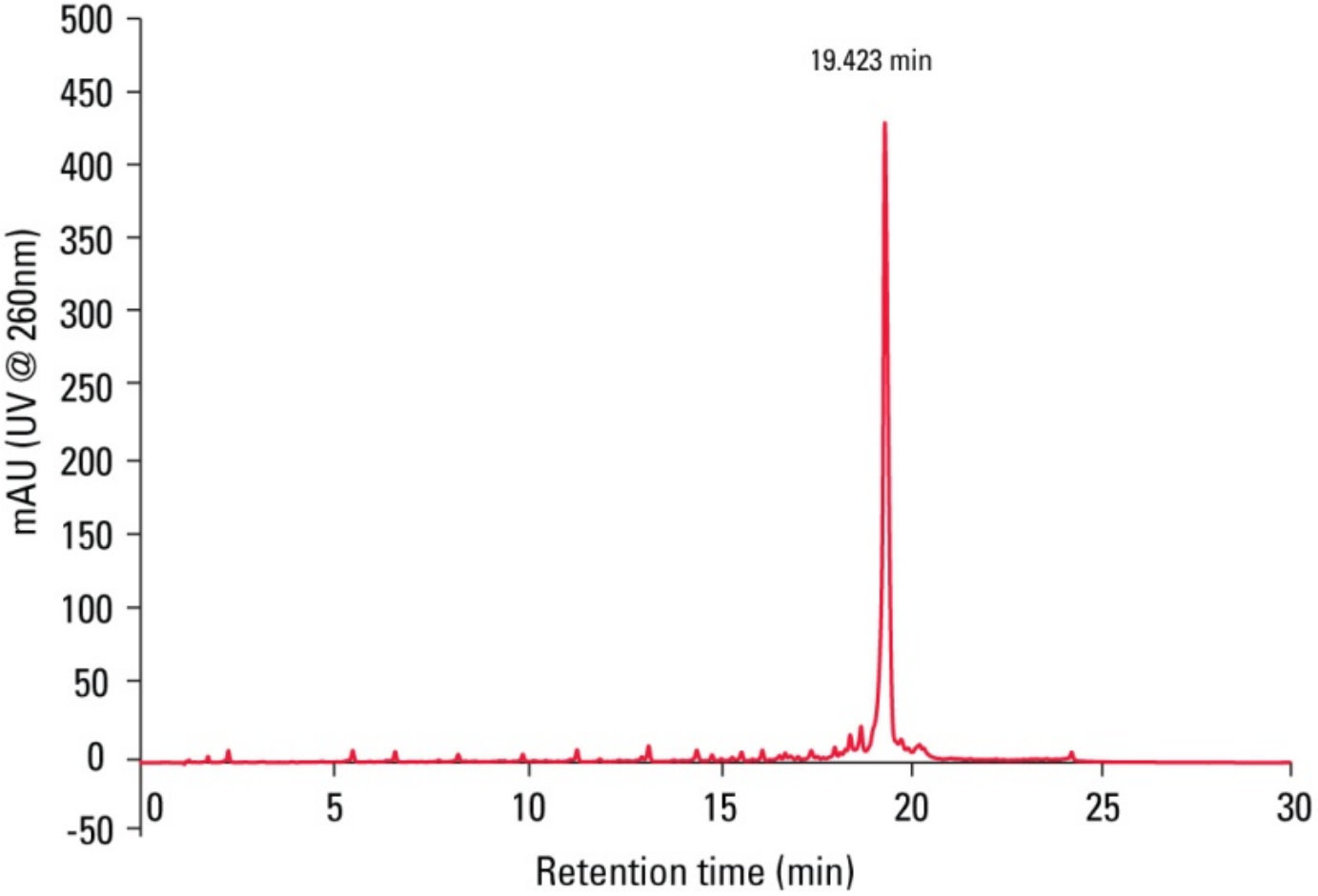
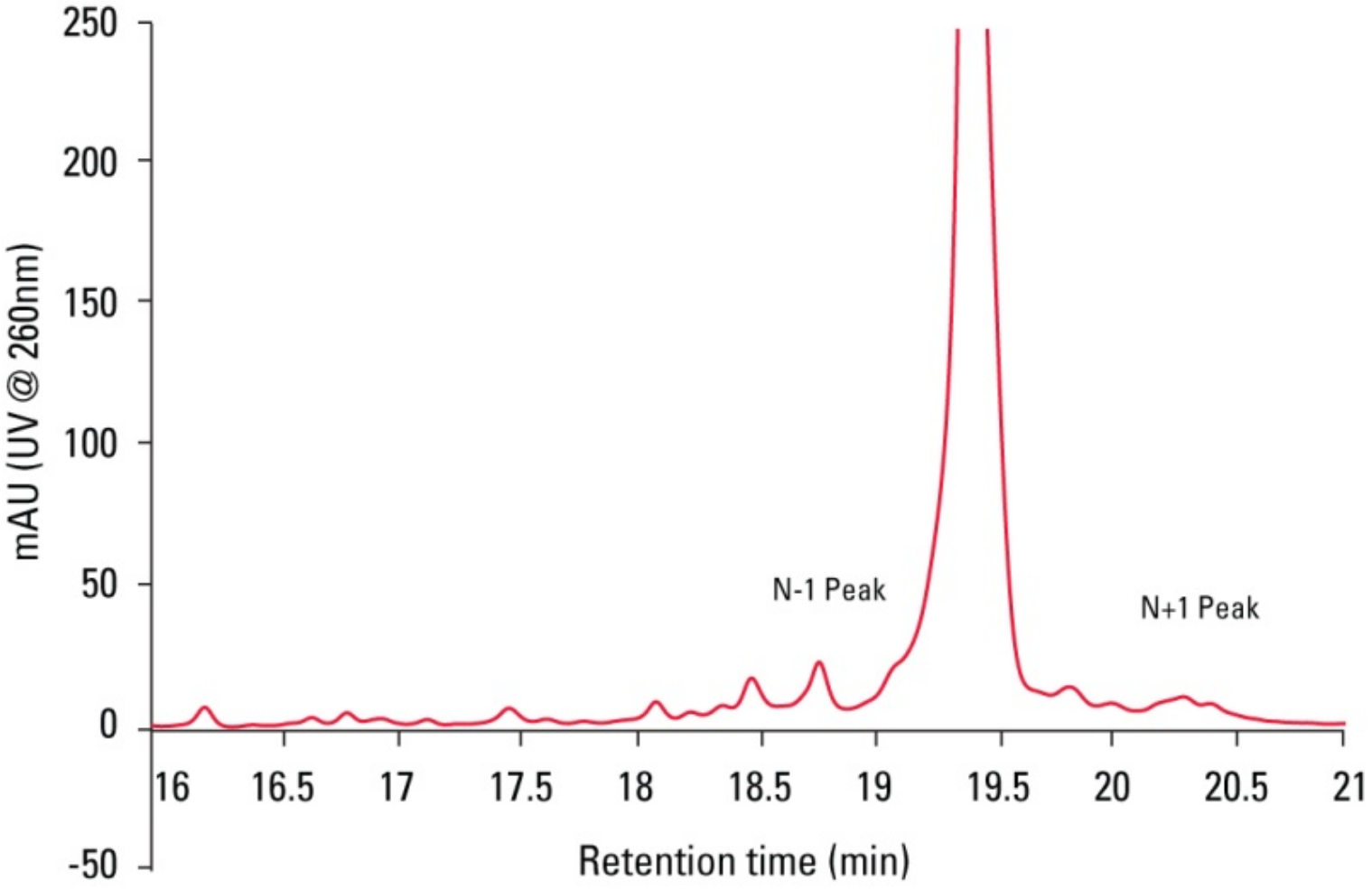


Figure 7: Analysis of Crude Oligonucleotide Using TSKgel DNA-STAT Column (zoomed in view)



Reversed Phase Separation of Oligonucleotides

- The high throughput silica-based reversed phase UHPLC column, TSKgel ODS-140HTP, 2.3 µm, 2.1 mm ID × 5 cm, and one conventional HPLC column, TSKgel ODS-100V, 5 µm, 4.6 mm ID × 15 cm, are being compared for their use in orthogonal analysis of oligonucleotides.
- Preliminary study shows these two columns are useful for the separation of the oligonucleotides.
- Optimization of the best chromatographic conditions are in progress and will be published at a later date.

Conclusions

- TSKgel SuperQ-5PW analytical columns have the same backbone chemistry and selectivity as the TSKgel SuperQ-5PW bulk process scale resins.
- TSKgel SuperQ-5PW could separate impurities containing modified products from the pure product.
- Different chromatographic conditions (pH, salt, and temperature) were tried.
- The best result was obtained at pH 9.0 for the TSKgel SuperQ-5PW column.

Conclusions

- Each of the three main species of oligonucleotide (N-1, N=20 and N+1) were separated under the optimum chromatographic conditions using both TSKgel SuperQ-5PW (20) resin and the TSKgel SuperQ-5PW column.[1]
- Selectivity of the TSKgel SuperQ-5PW analytical column was found to be the same as that of the TSKgel SuperQ-5PW (20) resin in the separation of oligonucleotides.
- The study shows that the TSKgel SuperQ-5PW analytical column can be used in the separation of oligonucleotides and the method can be useful for scaling up using TSKgel SuperQ-5PW bulk resin, because of the same selectivity of both.

Reference

[1]"One-step oligonucleotide purifications using anion exchange chromatography resins" – Phu T. Duong, Shigeru Nakatani and J. Kevin O'Donnell – Poster presentation at ACS, August 2008, Philadelphia, PA