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Analysis of Synthetic Deoxyoligonucleotides by Anion Exchange HPLC and Capillary Gel Electrophoresis

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Introduction

Synthetic deoxyoligonucleotides are becoming increasingly important in a wide variety of applications ranging from use as hybridization probes, primers for DNA sequencing and the polymerase chain reaction, to utilization as potential therapeutics in antisense and related technology investigations. Although improved chemistries and automated instrumentation have significantly simplified the synthesis of these molecules, the actual amount of desired full length product varies depending upon coupling efficiencies as well as on the length of the desired DNA product. The analysis of DNA synthesis reaction mixtures is therefore often desired to determine the quality of synthesized material prior to use or to ascertain the degree of product homogeneity resulting from post-synthesis purification.

This poster will describe the principles and utilization of capillary gel electrophoresis (CGE) and high resolution, anion exchange HPLC for the analysis of synthetic deoxyoligonucleotides. Synthetic DNA of various chain length and sequence will be used to demonstrate the relative resolving power of each analytical technique. In addition, the migration / retention time as well as the quantitation reproducibility of each methodology will be shown. Finally, the comparative strengths / weaknesses of each technique will also be described.

Principles of Separation Techniques

Capillary Gel Electrophoresis:

Capillary gel electrophoresis (CGE) has been cited as an efficient technique for the analysis of synthetic deoxyoligonucleotides (1, 2 and 3). The basis of the separation involves the molecular sieving of the various size DNA species as they are electrophoretically driven through the gel matrix (e.g. cross-linked polyacrylamide). A variety of oligonucleotide structural characteristics (e.g. length, charge to mass ratio and Stokes radius) influence the gel's ability to separate the DNA molecules.

Compared to slab gels, the small internal diameter (e.g. 0.75μm) of gel filled device enables excellent heat dissipation from the gel matrix during the separation. Therefore, CGE separations can be performed at higher field strengths (250 Volts/cm) than are possible using conventional slab gels. This results in very efficient separations in relatively short run times. In addition, current instrument design uses on-line UV detection for real-time monitoring of the separated DNA species as they migrate through the capillary. The actual detector flow cell is comprised of a discrete section of the capillary in which the protective polyamide coating has been removed. This design allows for the detection of nanogram to picogram quantities oligonucleotides.

Anion Exchange HPLC:

Anion exchange HPLC has been described as an effective technique for the analysis of synthetic oligonucleotides (4, 5). Separations rely primarily upon the interaction of the negatively charged phosphate groups on the DNA backbone with the positively charged cations contained on the column packing. Elution of the bound deoxyoligonucleotides, in order of increasing chain length, occurs using a gradient of increasing ionic strength. The degree of component resolution depends upon the HPLC column packing and conditions used well as the overall length of the oligonucleotides analyzed. Use of an appropriate high performance anion exchange column, can provide N from N-1 chain length resolution particularly for samples less than 30 bases in length.

Compared to conventional slab gel electrophoretic techniques, HPLC instrument design enables real-time, on-line monitoring of the DNA species as they elute from the column and pass through the UV detector. Staining or post-run visualization techniques are not required for the detection of nanogram amounts of sample.

Materials and Methods

Sample Preparation:

Phosphodeoxythymidine [pd(T) 18-24] and phosphodeoxyadenine [pd(A) 25-30 and pd(A) 40-60] standards were purchased from Pharmacia LKB Biotechnology.

Deoxyoligonucleotides (i.e. 3mer, 12mer, 13mer, 14mer ,15mer, 16mer) were synthesized at the 200 nM scale using either a Millipore Cyclone Plus or 8750 DNA instrument with ß-cyanoethylphosphoramidite chemistry. When required, final sample detritylation was performed on the instrument. Product cleavage from the controlled pore glass support and phosphate / exocyclic nitrogen deprotection were performed off-line by incubation overnight at room temperature with 30% ammonium hydroxide. A detritylated 76mer deoxyoligonucleotide was obtained from Ms. JoAnn Iwasa, Scios, Inc. Mountain View, CA.

Prior to CGE or HPLC, the samples were filtered through a Millipore 0.45 µm Millex-HV membrane. The concentration of the deoxyoligonucleotides was determined by measuring the optical density (OD) at 260nm for each sample using a Milton Roy Spectronic 1001 Split-Beam Spectrophotometer. An OD of 1.0 corresponds to 33 µg/ml of deoxyoligonucleotide.

Instrumentation:

The following were used for the anion exchange HPLC investigations:

- Waters Gen-Pak™ FAX Anion Exchanger, 2.5µm, Non-porous (4.6 x 100mm)
- Waters 600 Gradient Solvent Delivery System
- Waters 717 Autosampler
- Waters Temperature Control System
- Waters 486 Tunable Absorbance Detector
- Waters Expert Ease™ Chromatography Workstation

The following were used for the gel filled capillary investigations:

- J&W Scientific µPAGE-5™Gel Filled Capillary (5%T/5%C) 75µmx60cm
- Waters Quanta™ 4000 Capillary Electrophoresis System with Negative Power Supply
- Waters Expert Ease™ Chromatography Workstation

Effect of Sample Load on Resolution (Figures 1A and 1B)

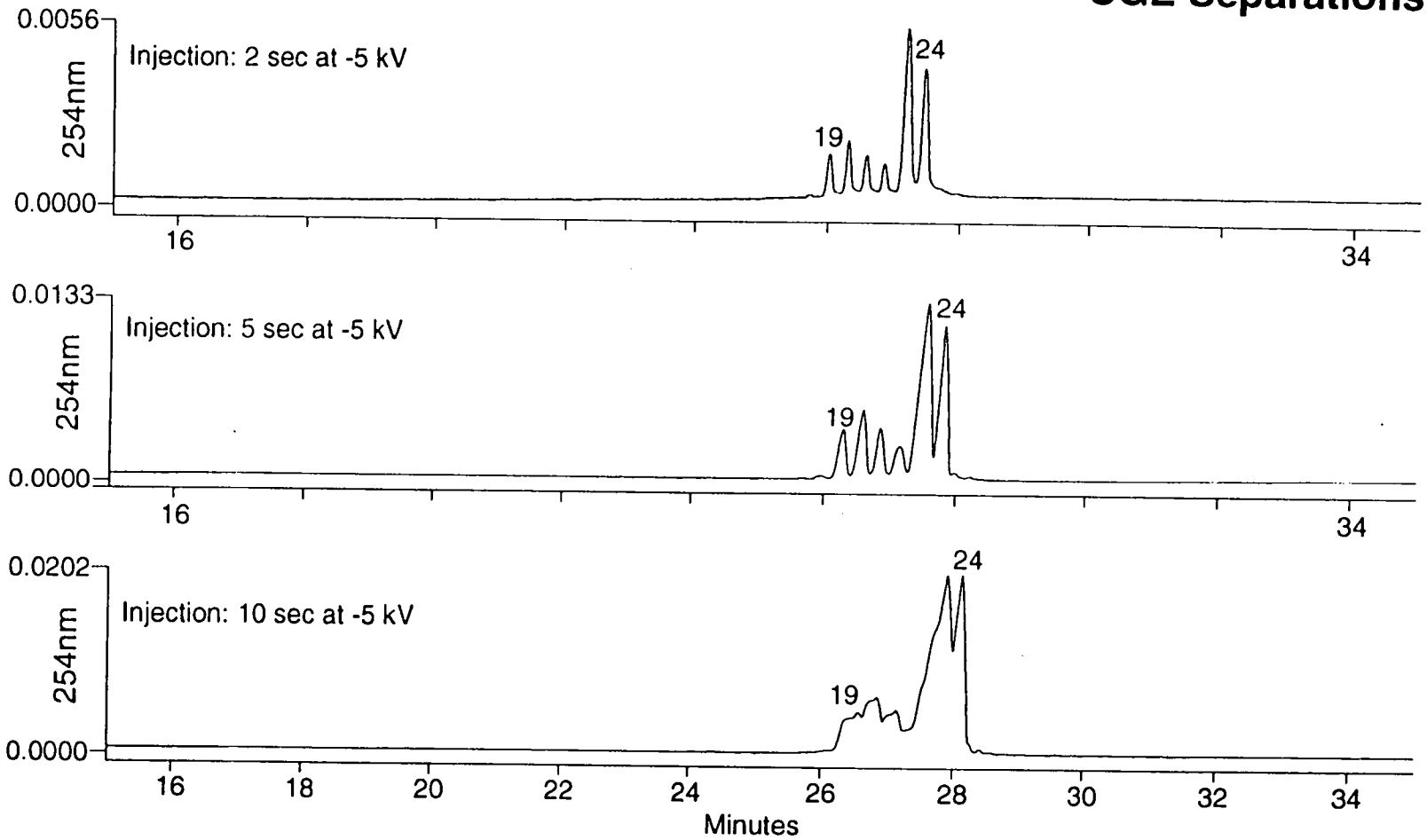
Results:

- The degree of deoxyoligonucleotide component resolution in CGE and anion exchange HPLC is affected by the amount of sample used in the analysis.

- A greater range of oligonucleotide sample loads can be satisfactorily analyzed via anion exchange HPLC than CGE.

Figure 1A

CGE Separations

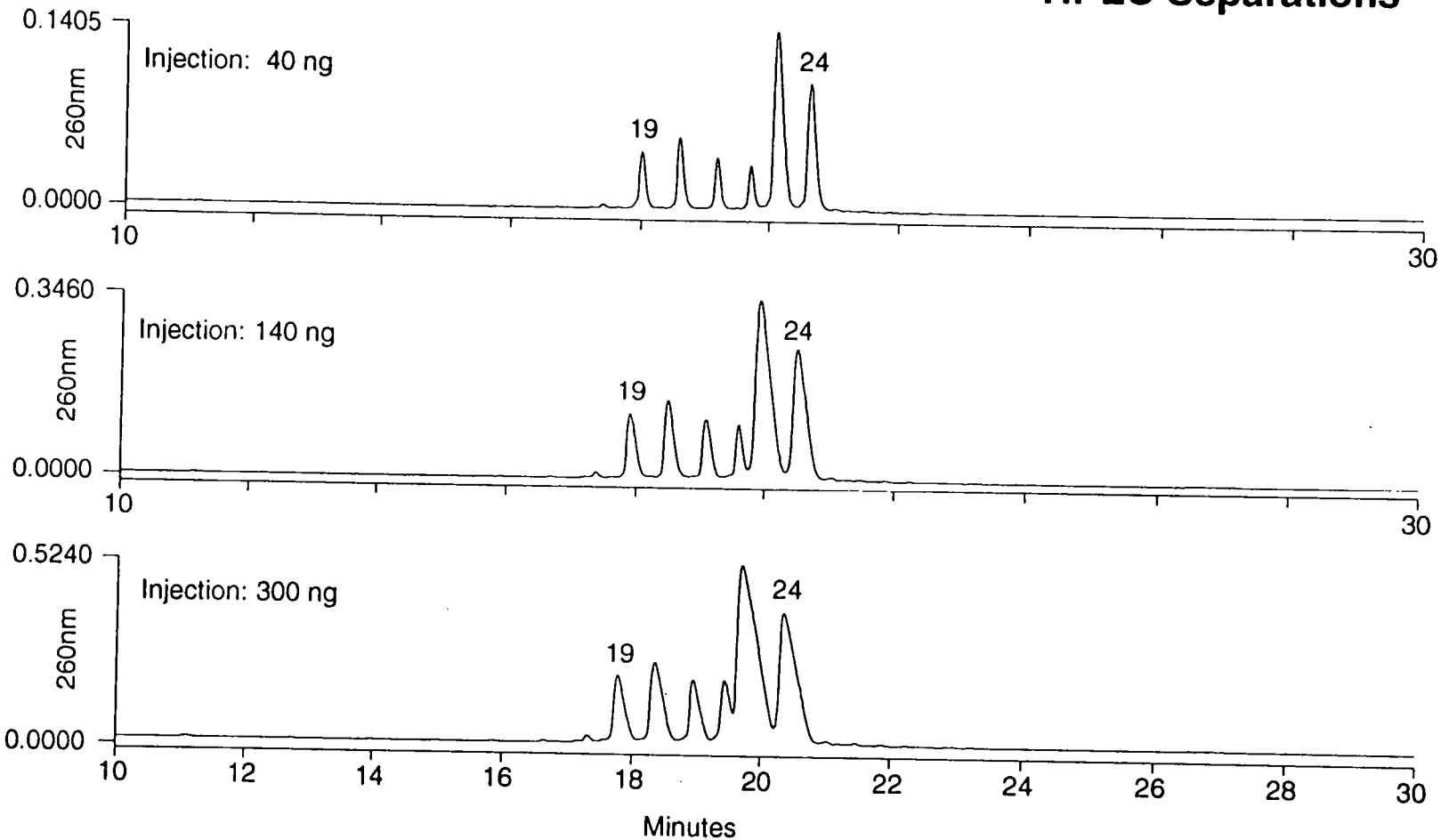


Sample: pd(T) 19-24 (660 ng/µl)
Capillary: µPAGE-5 gel filled
Electrolyte: 100mM Tris-Borate, pH 8.3 with 7M Urea

Injection: Electrokinetic
Run: -13 kv
Temp: Ambient

Figure 1B

HPLC Separations



Sample: pd(T) 19-24 (20 ng/µl)
Column: Gen-Pak FAX
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% Acetonitrile
Buffer B: A + 1.0M NaCl

Gradient: 25-60%B in 30 min
Flow: 0.75 ml/min
Temp: 30°C

Analysis of Self-Complementary Oligonucleotide Sequences (Figures 2A and 2B)

Results:

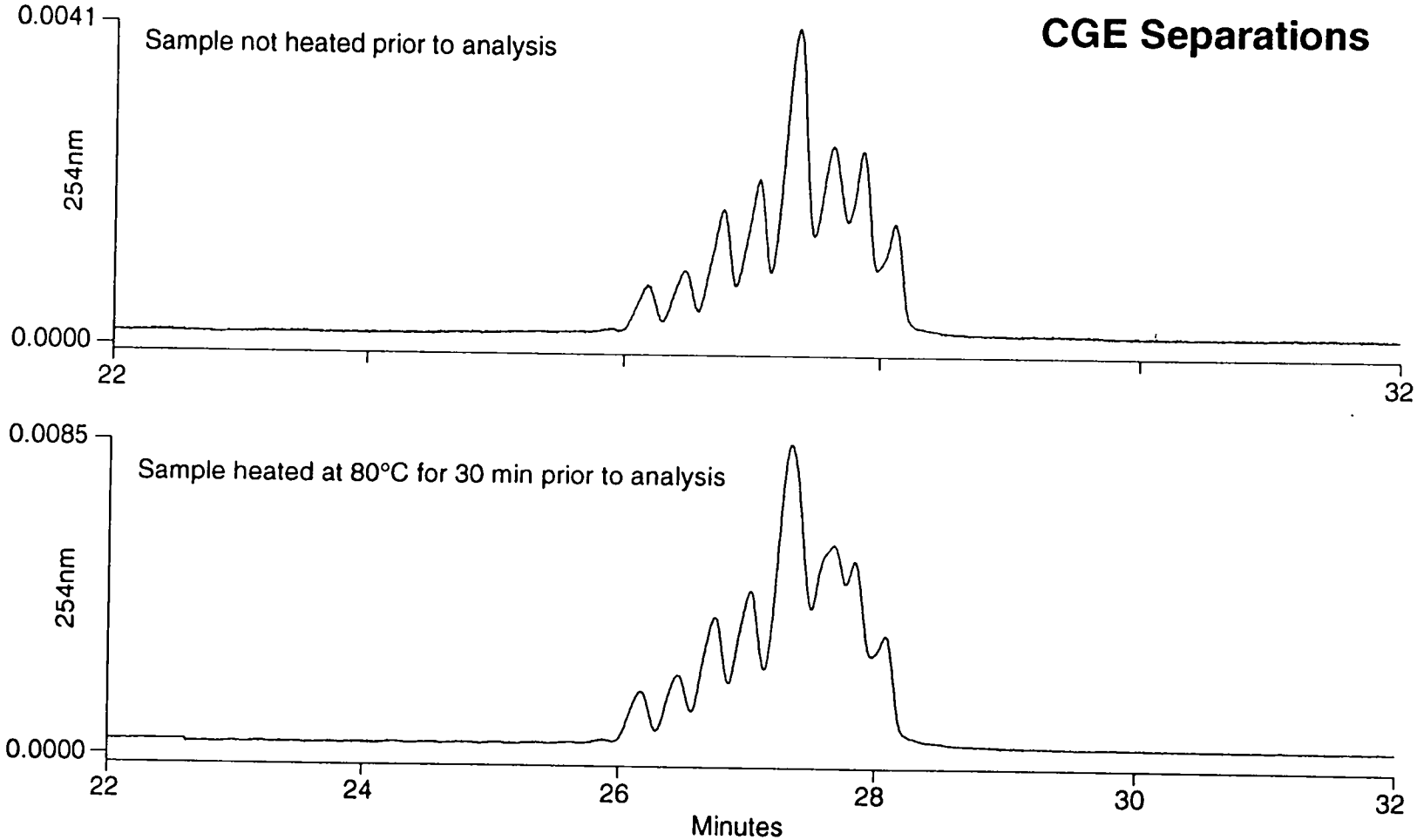
- Although CGE separations are performed in electrolyte containing denaturant (i.e. 7M urea), these conditions could not completely resolve a mixture of self-complementary oligonucleotides.

- Because of accelerated gel matrix degradation at elevated temperatures, CGE separations were only performed at ambient temperature.

- Anion exchange HPLC, when performed at denaturing temperatures (e.g. 80°C), results in baseline resolution of the same mixture of self-complementary DNA.

- The anion exchange HPLC column tested is stable when used at oligonucleotide denaturing temperatures.

Figure 2A

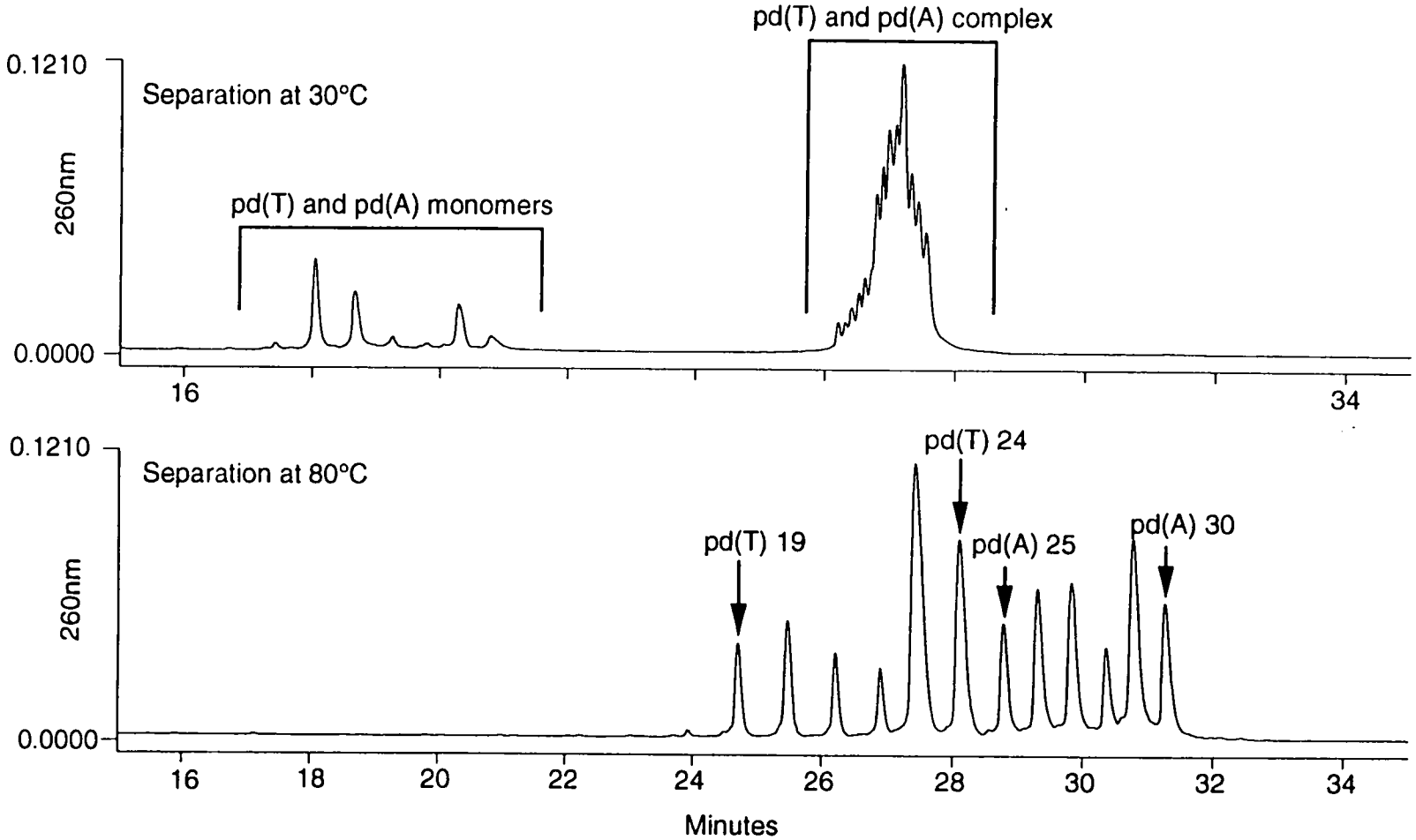


Sample: pd(T) 19-24 and pd(A) 25-30 at 330 ng/ul
Capillary: µPAGE-5 gel filled
Electrolyte: 100mM Tris-Borate, pH 8.3 with 7M Urea

Injection: 5 sec at -5 kV
Run: -13 kV
Temp: Ambient

Figure 2B

HPLC Separations



Sample: pd(T) 19-24 and pd(A) 25-30 (66 ng/µl)
Column: Gen-Pak FAX
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% Acetonitrile
Buffer B: A + 1.0M NaCL

Injection: 13.2 µg
Gradient: 25-60%B in 30 min
Flow: 0.75 ml/min

Factors Affecting Resolution
(Figures 3A and 3B)

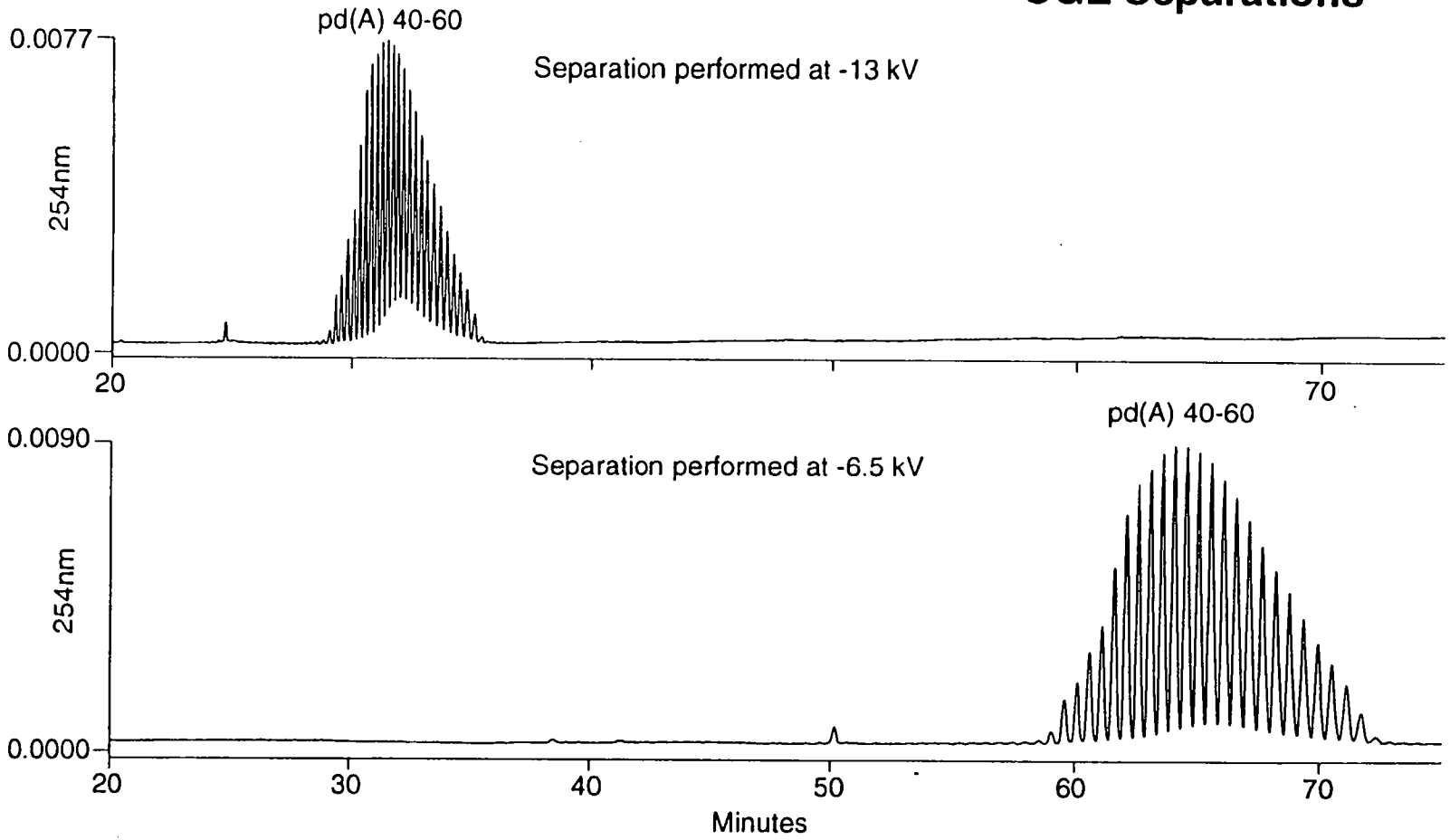
Results:

- Applied voltage during CGE is one factor which influences oligonucleotide separations. Lower running voltages result in marginal improvements in component resolution while they significantly increase the total analysis time.

- Gradient conditions used during anion exchange HPLC of oligonucleotides is a significant factor influencing their separation. Shallow gradients dramatically improve component resolution while only slightly increasing analysis times.

Figure 3A

CGE Separations

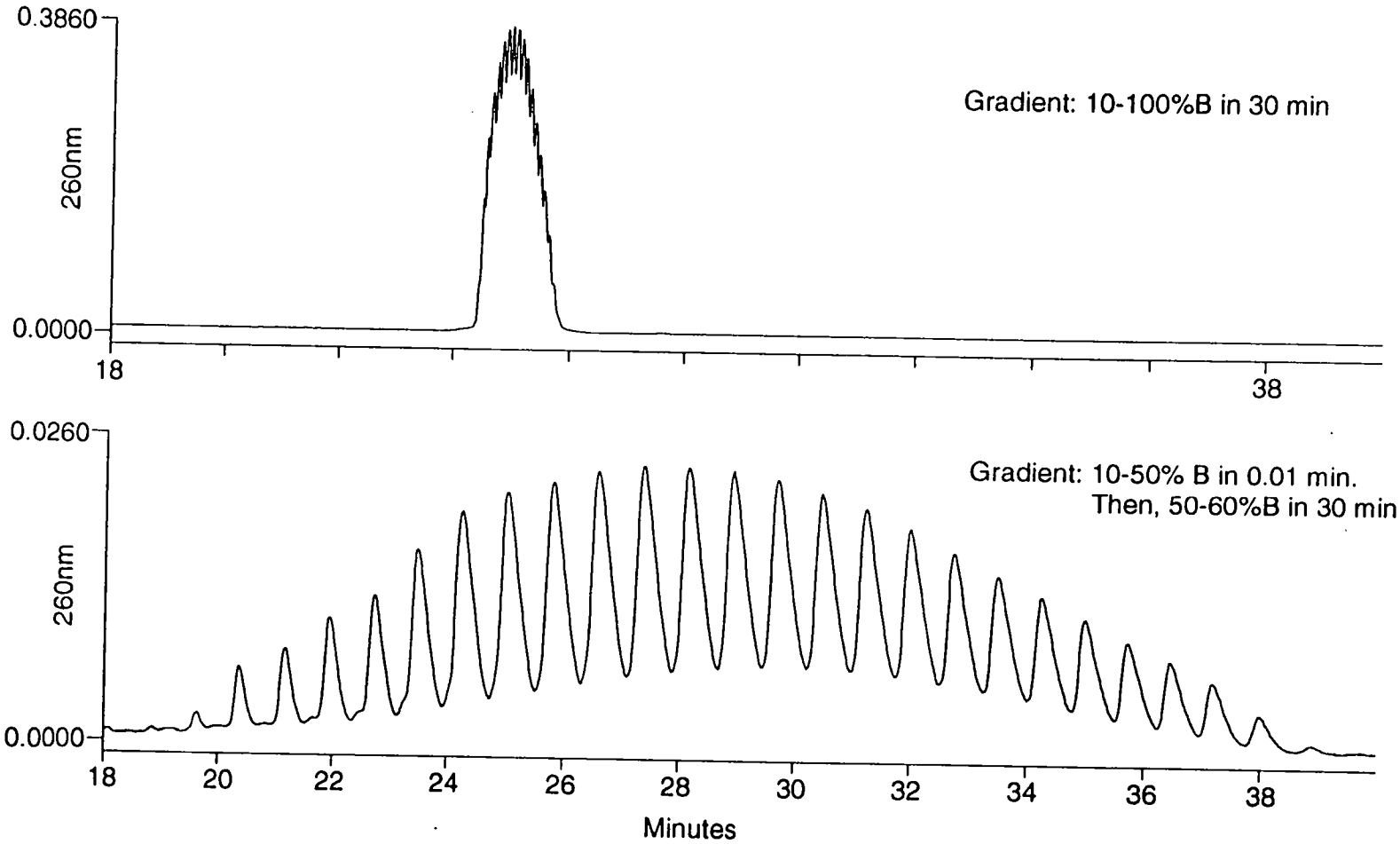


Sample: pd(A) 40-60 (660 ng/ul)
Capillary: µPAGE-5 gel filled
Electrolyte: 100mM Tris-Borate, pH 8.3 with 7M Urea

Injection: 5 sec at -5 kV
Temp: Ambient

Figure 3B

HPLC Separations



Sample: pd(A) 40-60 (660 ng/µl)
Column: Gen-Pak FAX
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% Acetonitrile
Buffer B: A + 1.0M NaCl

Injection: 3.3 µg
Flow: 0.75 ml/min
Temp: 80°C

Ruggedness and Reproducibility of Separations

Results:

- The gel filled capillary tested performed well up to 40 - 50 analyses before significant reduction in component resolution was observed.
- The anion exchange HPLC column tested provided high resolution separations for hundreds of analyses.
- Table1 summarizes the reproducibility of each technique.

Table 1: Reproducibility of CGE and Anion Exchange HPLC Analysis of a pd(A) 40-60 Standard Mixture(N=6)

Technique	Migration / Retention Time %RSD	Quantitation %RSD
µPAGE-5 CGE*	0.79%	2.0%
Gen-Pak FAX HPLC	0.07%	0.9%

* Changes in electrolyte composition occur during each CGE separation. In this study, fresh sample electrolyte was used for each analysis in order to obtain maximum migration time reproducibility.

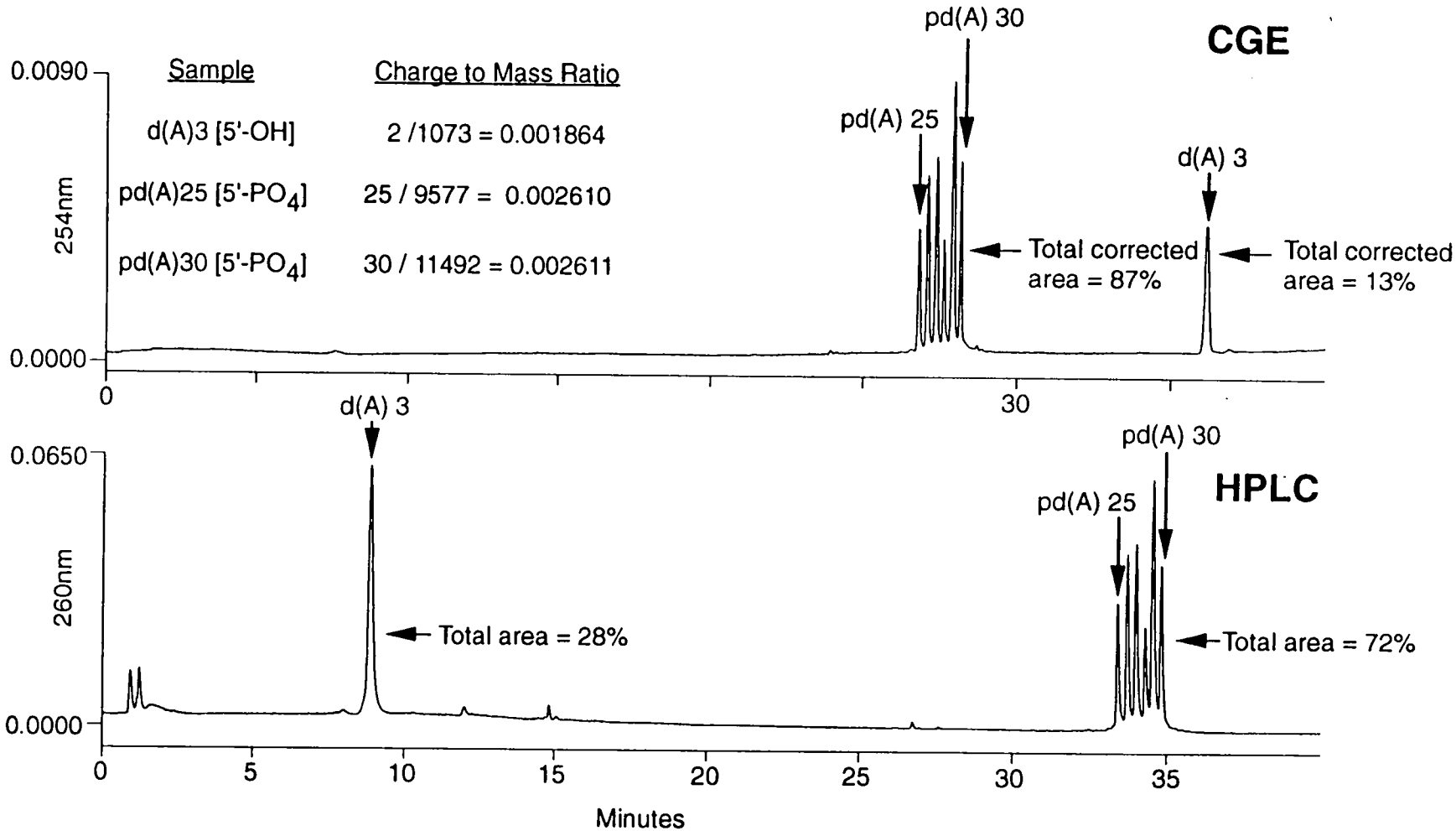
Effect of Charge to Mass Ratio on Separations (Figure 4)

Results:

- Compared to anion exchange HPLC, the separation of oligonucleotides in CGE is significantly affected by their charge to mass ratio.

- Because oligonucleotides of lower charge to mass ratio migrate slower in CGE than samples possessing higher ratios, disproportionate sample loading occurs. In addition, oligonucleotides of larger size and charge to mass ratio may actually migrate through the gel filled capillary faster than some smaller sequences.

Figure 4



Top: µPAGE-5 CGE separation
Sample: d(A) 3 and pd(A) 25-30 (330 ng/µl)
Electrolyte: 100mM Tris-Borate, pH 8.0 with 7M Urea
Injection: 5 sec at -5 kV
Run: -13 kV
Temp: Ambient

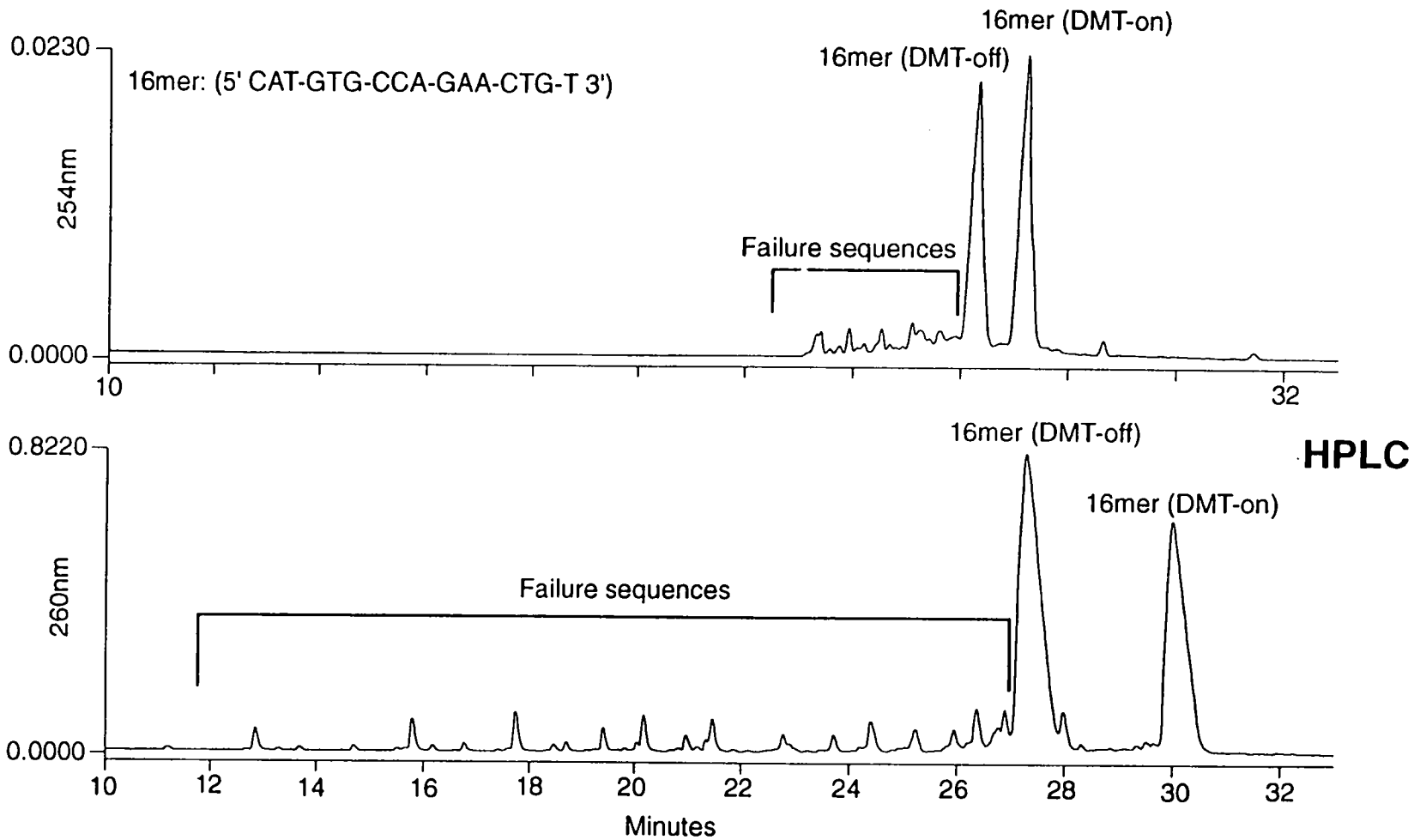
Bottom: Gen-Pak FAX HPLC separation
Sample: d(A) 3 and pd(A) 25-30 (66 ng/µl)
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% ACN
Buffer B: A + 1.0M NaCl
Injection: 660 ng. Flow: 0.75 ml/min. Temp: 80°C
Gradient: 0-60%B in 30 min. Then hold at 60%B for 10 min

Influence of 5' Dimethoxytrityl (DMT) Group (Figure 5)

Result:

- The presence of 5' DMT groups results in longer migration / elution times of oligonucleotide samples compared to detritylated species. The analyses of detritylated oligonucleotide samples is thus recommended for both CGE and anion exchange HPLC.

Figure 5



Top: µPAGE-5 CGE separation
Sample: Mixture of 16mer samples with and without DMT group at 1.5 µg/ul
Electrolyte: 100mM Tris-Borate, pH 8.0 with 7M Urea
Injection: 5 sec at -5 kV
Run: -13 kV
Temp: Ambient

Bottom: Gen-Pak FAX HPLC separation
Sample: Same as for CGE separation
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% ACN
Buffer B: A + 1.0M NaCl
Injection: 15 µg. Flow: 0.75 ml/min. Temp: 80°C
Gradient: 10-60%B in 30 min

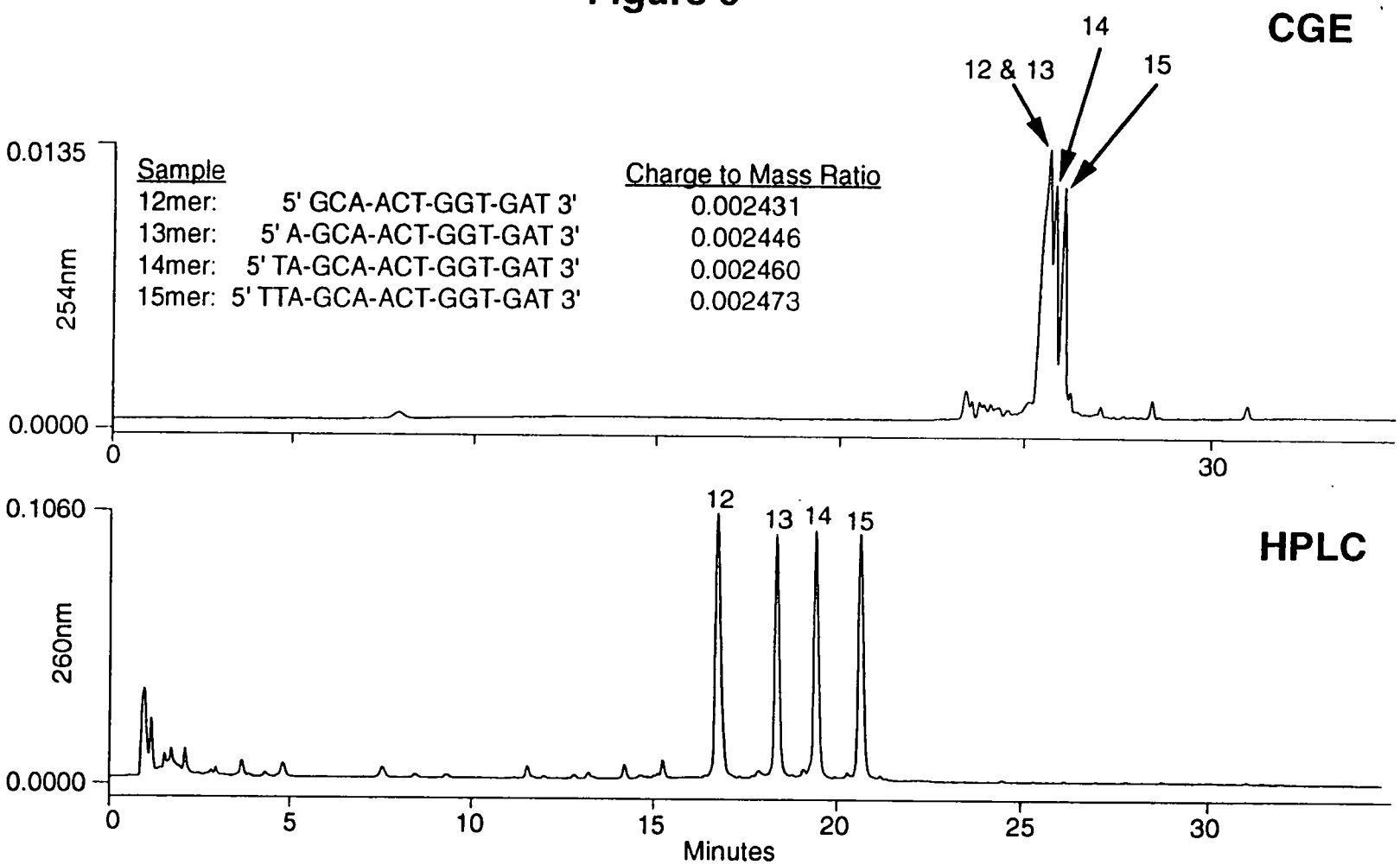
N From N-1 Resolution
(Figures 1, 3 and 6)

Results:

- CGE and anion exchange HPLC can provide N from N-1 degrees of component resolution of various size oligonucleotides (See Figures 1 and 3).

- Co-elution of <u>different</u> size oligonucleotides can occur in CGE if the separation, influenced by the gel matrix, is offset by differences in migration velocities due to charge to mass ratio differences (e.g. co-elution of 12 and 13mer in Figure 6).

Figure 6



Top: µPAGE-5 CGE separation
Sample: 12,13,14 and 15mer (DMT-off) at 110 ng/ul
Electrolyte: 100mM Tris-Borate, pH 8.0 with 7M Urea
Injection: 5 sec at -5 kV
Run: -13 kV
Temp: Ambient

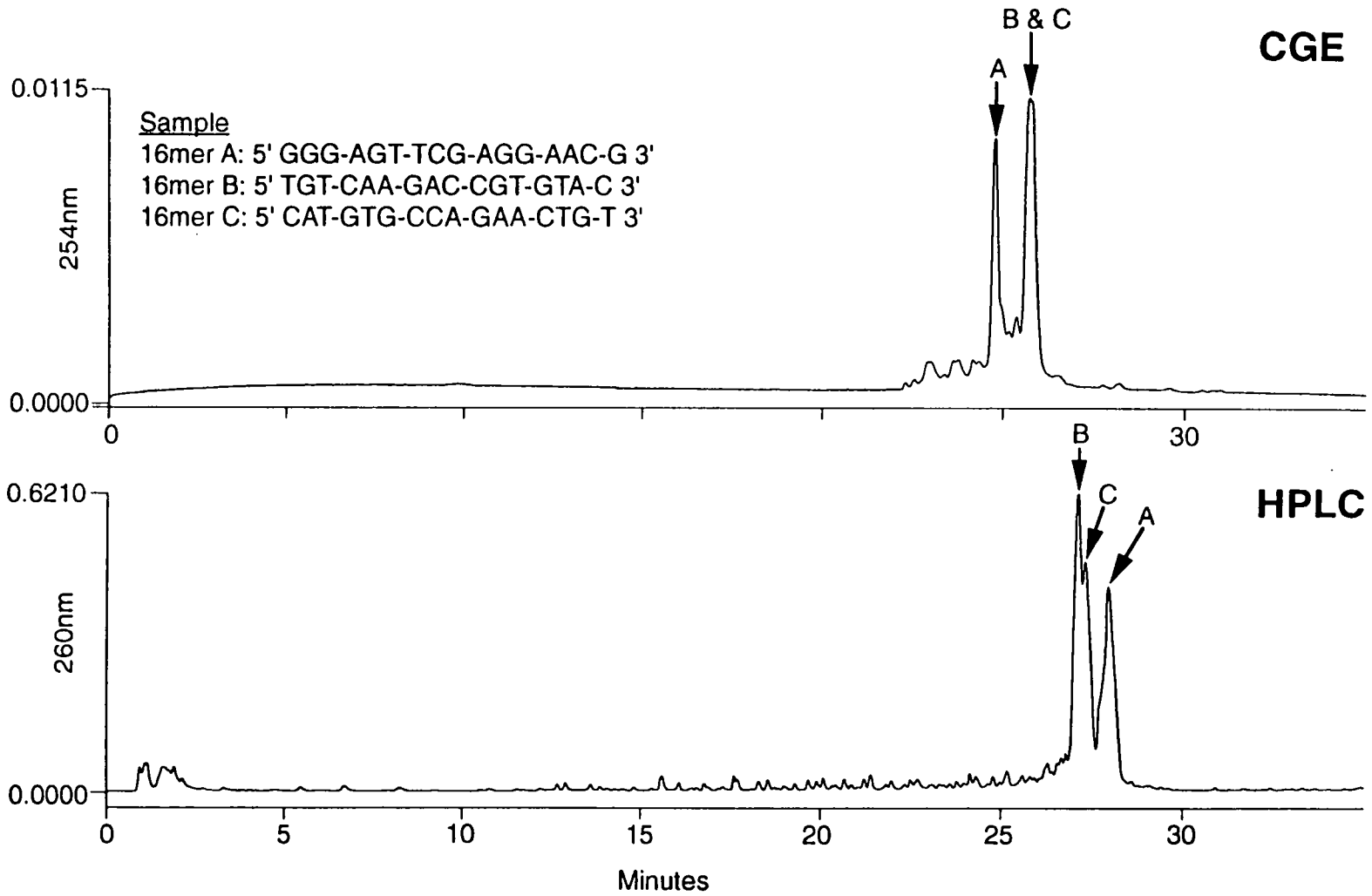
Bottom: Gen-Pak FAX HPLC separation
Sample: Same as for CGE separation
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% ACN
Buffer B: A + 1.0M NaCl
Injection: 550 ng. Flow: 0.75 ml/min. Temp: 80°C
Gradient: 25-60%B in 30 min

Analysis of Deoxyoligonucleotides of Equal Length but Different Sequence (Figure 7)

Results:

- Neither CGE or anion exchange HPLC can be used to determine the absolute length of an unknown oligonucleotide as shown in this example.

Figure 7



Top: µPAGE-5 CGE separation
Sample: (3) 16mers (DMT-off) at 550 ng/ul
Electrolyte: 100mM Tris-Borate, pH 8.0 with 7M Urea
Injection: 5 sec at -5 kV
Run: -13 kV
Temp: Ambient

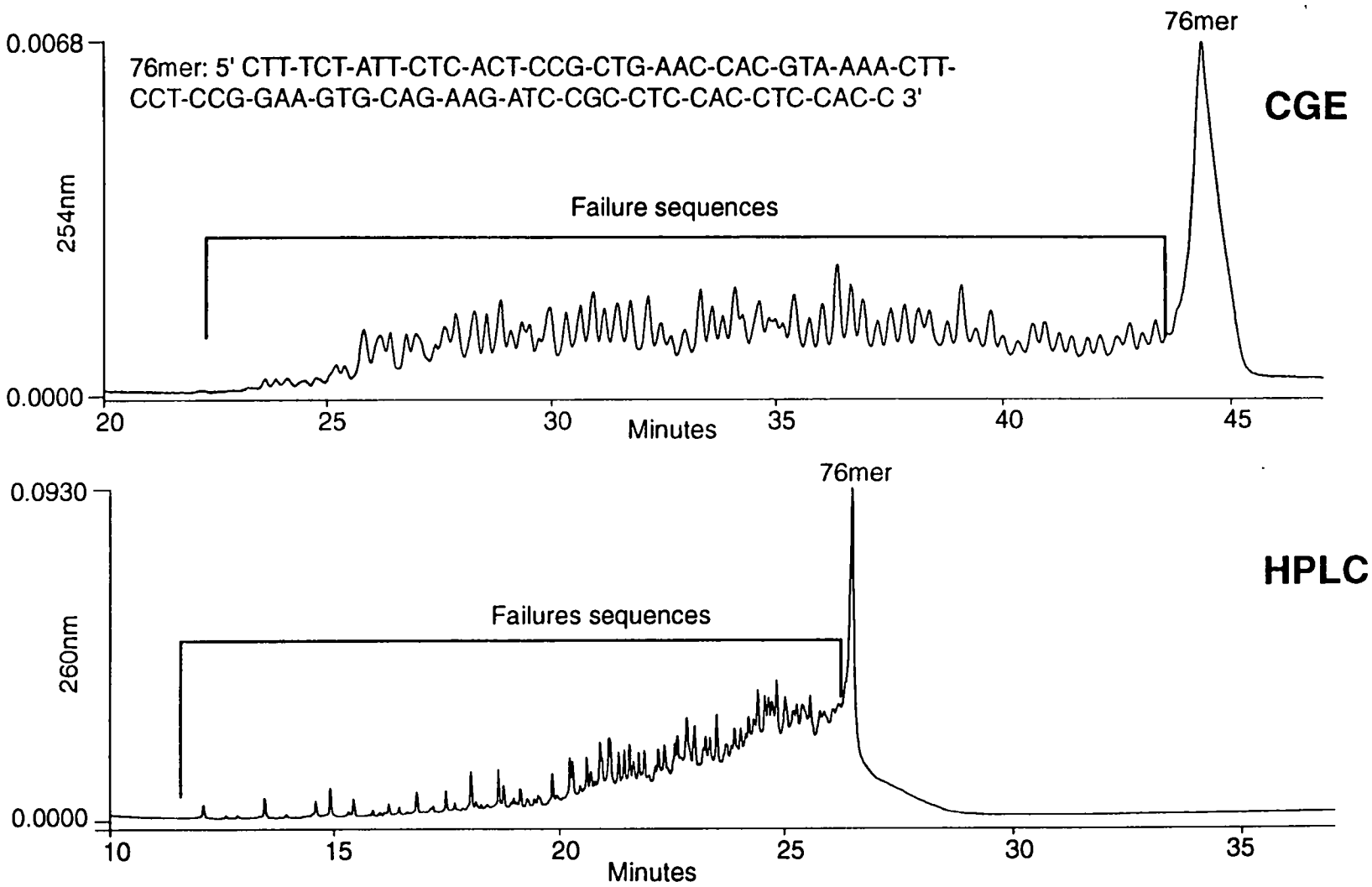
Bottom: Gen-Pak FAX HPLC Separation
Sample: Same as for CGE separation
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% ACN
Buffer B: A + 1.0M NaCl
Injection:19 µg Flow: 0.75 ml/min. Temp: 80°C
Gradient: 10-60%B in 30 min

Analysis of "Large" Deoxyoligonucleotide Sequences (Figure 8)

Results:

- The gel filled capillary tested provides better resolution of large oligonucleotides (e.g. 76mer) than the anion exchange HPLC column evaluated in this study.

Figure 8



Top: µPAGE-5 CGE separation
Sample: 76mer (DMT-off) at 330 ng/ul
Electrolyte: 100mM Tris-Borate, pH 8.0 with 7M Urea
Injection: 20 sec at -5 kV
Run: -13 kV
Temp: Ambient

Bottom: Gen-Pak FAX HPLC separation
Sample: Same as for CGE separation
Buffer A: 25mM Tris-HCl, pH 8.0 with 10% ACN
Buffer B: A + 1.0M NaCl
Injection: 3.3 µg. Flow: 0.75 ml/min. Temp: 80°C
Gradient: 10-100%B in 30 min

Conclusions

This study directly compared the analyses of several deoxyoligonucleotides using CGE (J&W Scientific's μPAGE-5 gel filled capillary) and high performance, anion exchange HPLC (Waters' Gen-Pak FAX column). Although improvements in both gel filled capillary devices as well as HPLC columns are anticipated, the present state of the art favors HPLC for the analyses of most deoxyoligonucleotide samples. Table 2 summarizes the results obtained in this study.

Table 2: Characteristics of μPAGE-5 and Gen-Pak FAX HPLC for the Analysis of DNA

Attribute	Characteristics of μPAGE-5 Capillary	Characteristics of Gen-Pak FAX Column
- Life of stored device.	3 to 4 months	> 1 year
- # of analyses per device.	40-50	200-500
- Run to run time.	Less than 45 min.	Less than 60 min.
- # of samples analyzed per run.	1	1
- Technique requires methods development.	No	Yes
- Resolution effected by sample load.	Yes	Yes
- Ability to resolve self-complementary DNA.	Good	Excellent
- Reproducibility using device.	Good	Excellent
- Disproportionate loading due to differing DNA charge to mass ratios.	Yes	No
- Capable of confirming length of oligonucleotide unknown.	No	No
- Ability to resolve "large" (e.g. 76mer) sequences.	Excellent	Good

References

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2) Guttman, A. and N. Cooke. 1991. Capillary Gel Affinity Electrophoresis of DNA Fragments. Anal. Chem. 63: 2038-2042.

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4) Drager, R.R. and F.E. Regnier. 1985. High-Performance Anion-Exchange Chromatography of Oligonucleotides. Anal. Biochem. 145: 47-56.

5) Kato, Y. T. Kitamura, A. Mitsui, Y. Yamasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara. 1988. Separation of Oligonucleotides by High-Performance Ion-Exchange Chromatography on a Non-Porous Ion Exchanger. J. Chrom. 447: 212-220.