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DNA fragment analysis by capillary polymer sieving electrophoresis using poly(acryloylaminoethoxyethanol)-coated capillaries

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Abstract

Capillary polymer sieving electrophoresis (CPSE) using dynamic sieving polymer solutions provides a reliable technique for the accurate size identification of doublestranded (ds) DNA fragments. The polymer sieving systems described here resolve dsDNA fragments in the 100–4000 base pair size range. The effect of temperature, capillary length and sieving polymer concentration were examined. Best performance has been achieved with capillaries coated with a new monomer, poly acrylolyaminoethoxyethanol (AAEE). The poly(AAEE) coating exhibits superior stability at high pH as compared to linear polyacrylamide (LPA). This coating provides the necessary stability to ensure reliable analyses over many runs.

Keywords: Capillary columns; Coated capillaries; DNA

1. Introduction

Agarose slab gel electrophoresis has been the traditional method to analyze DNA samples, however this method is time consuming and difficult to automate. In the last 7 years numerous reports have appeared dealing with DNA analysis by capillary electrophoresis [1–3]. This method offers an ideal approach for nucleic acid separations, providing rapid analysis, quantitative information, minimal consumption of sample and direct detection of resolved fragments.

Adaptation of polyacrylamide gels to the high-performance capillary electrophoresis format has been limited by difficulties encountered in preparing and using gels in capillaries. Capillary electrophoresis in aqueous solutions of dissolved polymers [4] provides separations of DNA and proteins substantially equivalent to polyacrylamide and agarose gel electrophoresis. This consists of dissolving in the appropriate buffer, linear hydrophilic polymers which exert a dynamic sieving effect on macromolecular analytes. The effective size range for a particular separation will depend upon the nature of the sieving polymer as well as its concentration and molecular size. This method of capillary polymer sieving electrophoresis (CPSE) is increasingly becoming the method of choice for the separation of a wide range of single-stranded oligonucleotides such as synthetic oligonucleotides [5–7], as well as the separation of dsDNA molecules in polymerase chain reaction (PCR) products [8–10] and restriction digest analysis [10,11].

In this study a proprietary sieving polymer (dsDNA Run Buffer) was utilized in the separation of dsDNA molecules ranging in size from 100 base pair (bp) to 4000 bp. The analysis was performed in capillaries coated with the hydrophilic, hydrolytically

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stable, polyacryloylaminoethoxyethanol [poly-(AAEE)] on the internal surface [12].

2. Materials and methods

For all experiments a BioFocus 3000 capillary electrophoresis instrument (Bio-Rad Laboratories, Hercules, CA, USA) was used. Temperature of the sample and buffer carousels was maintained at 20°C. Separations were carried out in capillaries installed in User Assembled Cartridges (UAC) from Bio-Rad Laboratories. Separations of DNA fragments were monitored on-line using UV detection at 260 nm.

Capillaries coated internally with poly(AAEE) and linear polyacrylamide (LPA), Tris, boric acid, EDTA and the 100 base pair ladder were obtained from Bio-Rad Laboratories (Hercules, CA, USA). The 200 base pair and 500 base pair DNA standards were obtained for GenSura (Del Mar, CA, USA) and the ΦX174 RF DNA/Hae III digest was obtained for Gibco BRL (Gaithersburg, MD, USA). Fused-silica capillaries were obtained for Polymicro Technologies (Phoenix, AZ, USA). The dsDNA sieving buffer (available as dsDNA Run Buffer, Bio-Rad Laboratories, Hercules, CA, USA) consisted of a proprietary sieving polymer in 2 X TBE (178 m*M* Tris, 178 m*M* boric acid and 4 m*M* EDTA, pH 8.3) and dilution buffer was 2 X TBE. The 100 base pair ladder was a mixture of 10 dsDNA fragments ranging in length from 100 to 1000 bp in exact 100 bp increments. The DNA mixture contained approximately 10 μg/ml of each fragment in TE buffer (25 m*M* Tris, and 2 m*M* EDTA, pH 8.0).

Samples were loaded electrophoretically and generally run at 2.0–5.0 kV at 40°C. The dsDNA sieving buffer was degassed by centrifuging the vials for at least 4 min in a microcentrifuge immediately before inserting them into the BioFocus automatic sampler carousels. The dsDNA sieving buffer was replenished before each run using a 60 s high pressure (100 p.s.i.; 1 p.s.i.=6894.76 Pa) purge cycle. The electrode buffer was the same as the sieving buffer used for filling the capillary. Two water rinse cycles were used to remove residual buffer from the capillary and electrode surfaces to prevent buffer carryover into the sample vial.

After electrophoresis, resolution was calculated based on the auto integration data the instrument provided:

$$R_s = 1.18 \cdot \frac{T_2 - T_1}{W_2 + W_1}$$

R_s is the resolution of peak 1 and peak 2, T is the migration time of the peaks and W is the peak width at half height.

Capillaries were coated using a new monomer N-acryloylaminoethoxyethanol (AAEE) first described by Chiari et al. [5]. The procedure used to coat the capillaries with AAEE as well as acrylamide was similar to the method described by Hjerten [13].

The pH stability of the linear polyacrylamide (LPA) and poly(AAEE) coated capillaries were evaluated by washing capillaries for 3 days with the following buffers: 100 m*M* sodium acetate, pH 5.5; 100 m*M* sodium borate, pH 8.5 and 20 m*M* sodium carbonate, pH 10.2. Three capillaries (64 cm×50 μm I.D.×375 μm O.D.) of each coating were purged with each of these buffers and then the ends placed in two test tubes containing the respective buffer. The reservoirs were adjusted to give a 20–22 cm height difference in the liquid levels so as to produce gravity flow through each capillary. All capillaries were checked at the beginning of the experiment to ensure flow. Following the three day wash at ambient temperature the capillaries were purged with water before testing for electroendosmosis (EOF). The EOF voltage test was performed by filling the capillaries with a low conductivity buffer (1 m*M* sodium phosphate, pH 7.0) and placing them in electrode reservoirs containing a high conductivity buffer (10 m*M* sodium phosphate, pH 7.0). Power supply was set at 15 kV and the voltage reading measured on each capillary after 1 min and a final reading taken after 60 min. Data are expressed as percentage voltage change between the two values.

3. Results

Capillaries were coated using a new monomer N-acryloylaminoethoxyethanol (AAEE) first described by Chiari et al. [5]. This monomer is more hydrophilic than acrylamide and yields more hydrolytically stable polymers than polyacrylamide [12,14]. Capillaries were coated using a procedure

similar to that described by Hjerten [13]. A comparison of the pH stability of capillaries coated with poly(AAEE) and linear polyacrylamide is shown in Fig. 1. Sets of capillaries with these two coatings were washed using gravity flow for 3 days at pH 5.5, 8.5 and 10. After this treatment the capillaries were purged with water and the relative EOF values were determined for each of the capillaries. The two coatings exhibited very similar EOF values after the pH 5.5 treatment, while after pH 8.5 the LPA coated capillaries exhibited about twice the EOF values as the poly(AAEE) capillaries. However the pH 10.2 wash resulted in very high EOF values in the LPA capillaries while the poly(AAEE) capillaries were only increased slightly. These results clearly demonstrate the stability of the poly(AAEE) coated capillaries to high pH. Fig. 2 compares the separation of the 100 bp ladder at 40°C in 75 μm×375 μm×24 cm uncoated poly(AAEE) and LPA coated capillaries. This standard is a mixture of 10 DNA fragments ranging in length from 100 to 1000 bp in exact 100 bp increments. Using the dsDNA sieving buffer baseline separation was achieved between all fragments on both coated capillaries with an analysis time of less than 15 min. The uncoated capillary was unable to completely separate the higher molecular mass fragments.

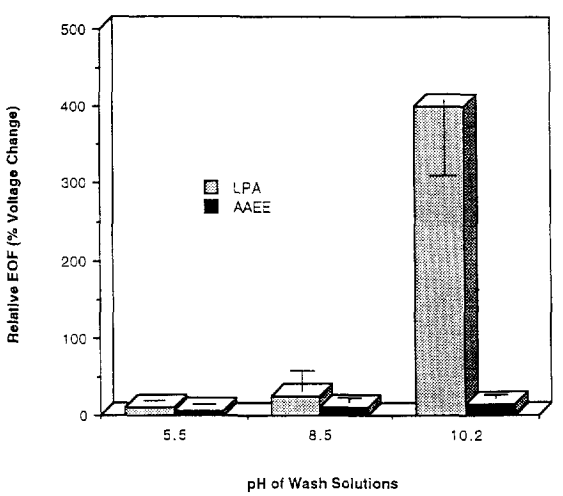

Fig. 1. Effect of wash pH on the EOF of polyacrylamide and poly(AAEE) coated capillaries. Capillaries coated with polyacrylamide and poly(AAEE) were subjected to washes at the indicated pH values and the relative EOF determined as described in Section 2. The values and error bars represent the average and standard deviations from three capillaries.

Fig. 3 examines the lifetime of the poly(AAEE) and LPA coated capillaries. The 100 bp ladder was analyzed over 60 consecutive runs and the performance evaluated by plotting the efficiency of the largest fragment. For the poly(AAEE) coated capillary the efficiency of the 1000 bp fragment remained fairly constant between 310–340 K over the 60 runs. In contrast the efficiencies from the LPA coated capillary started at 300 K and steadily decreased to 250 K. In data not presented, up to 250 injections have been performed on poly(AAEE) capillaries with no significant decrease in performance.

Fig. 4 depicts the effect of capillary length on the separation ΦX174/Hae III digest on poly(AAEE) coated capillaries at 40°C. This digest produces 11 fragments from 72 to 1353 bp. Particularly difficult to separate are the 271 and 281 bp fragments. The separations in Fig. 4 were carried out at constant field strength (0.9 kV/cm) using the dsDNA sieving buffer in coated capillaries with effective lengths of 20, 30 and 40 cm. On the 20 cm capillary there was very poor resolution of the 271 and 281 bp fragments. The best separation was achieved on the 40 cm effective length capillary where all eleven fragments were resolved within 35 min.

Fig. 5 shows the effect of temperature on the separation of the ΦX174/Hae III digest. These separations were performed in coated capillaries of 44 cm total length (40 cm effective length). In the figure only the first seven fragments (71 to 310 bp) of the digest are shown. Particularly noteworthy was the influence of temperature on the separation of the 271 and 281 bp fragments which were not separated at 20°C, much better resolved at 30°C and almost baseline resolved at 40°C. This effect of temperature on the separation of these two fragments was not entirely consistent with the overall effects of temperature on the other fragments which exhibited 10–30% decreases in efficiency as the temperature was increased. For example, the efficiencies for the 310 bp fragment at 20, 30 and 40°C were 790, 750 and 710 K, respectively. This suggests there may be some sequence specific interactions causing anomalous migration. To examine this, a plot of the log migration time against fragment size is shown in Fig. 6. This should give a concave, smooth curve indicat-

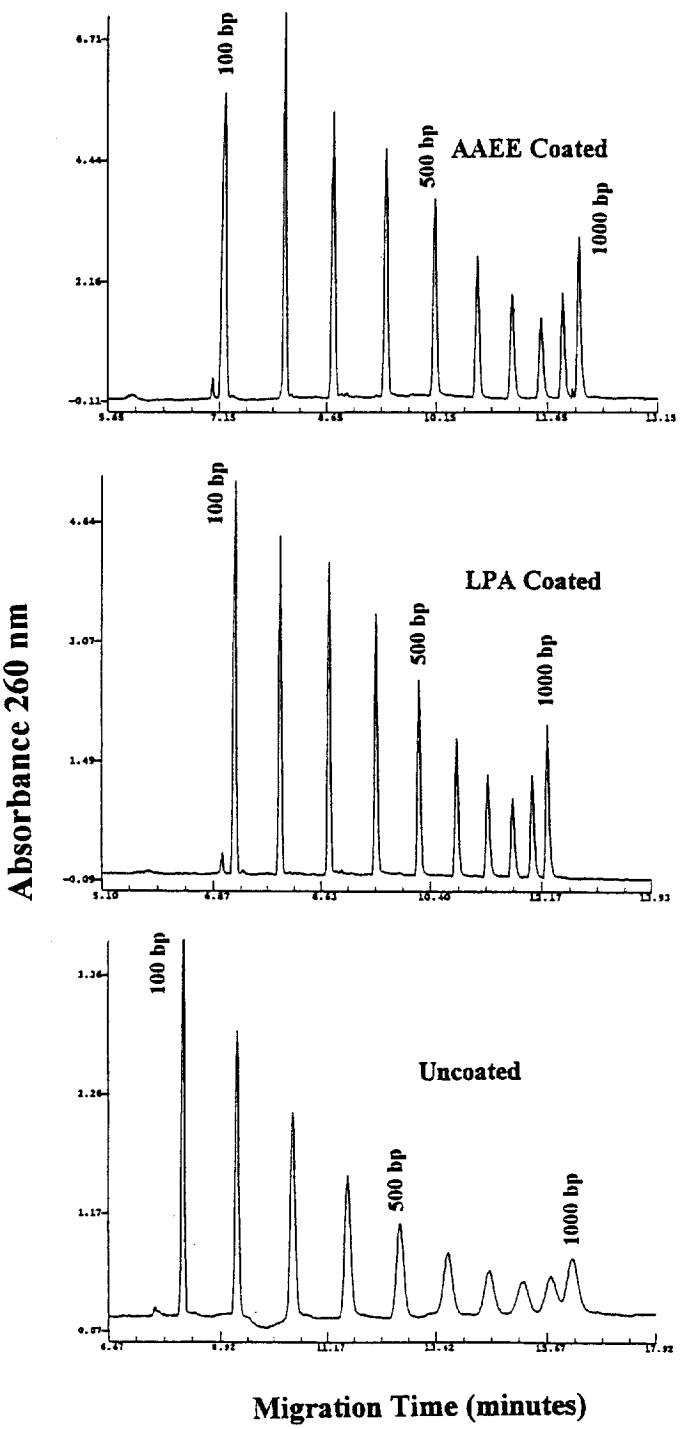

Fig. 2. Comparison of dsDNA separations on uncoated and polyacrylamide and poly(AAEE) coated capillaries. The 100 bp dsDNA ladder was analyzed on the indicated capillaries (75 μm I.D.×375 μm O.D.×24 cm) using the dsDNA sieving buffer. The sample was injected electrophoretically at 10 kV for 2 s and separations were performed at 40°C with a 4 kV run voltage.

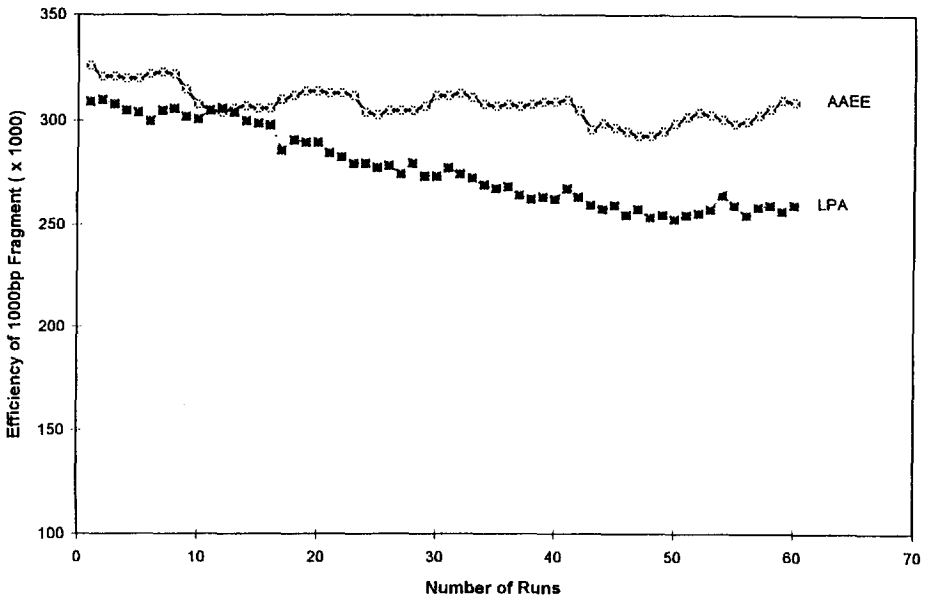

Fig. 3. Lifetime on poly(AAEE) and LPA coated capillaries. The 100 bp ladder was analyzed for 60 consecutive runs on poly(AAEE) and LPA coated capillaries using the conditions described in Fig. 2. The dsDNA run buffer was changed after 30 runs. The results are expressed as the efficiency of the last peak (1000 bp fragment).

ing the size-dependence on electrophoretic mobility. At 20°C a definite deviation in the mobility is apparent for the 271 bp fragment. This deviation was decreased at 40°C yielding a smooth curve showing that for these dsDNA fragments this polymer sieving buffer can yield accurate size determination.

Table 1 examines the separation of the 100 bp ladder in 24 cm coated capillaries with different internal diameters (I.D.). The resolution of the 900/1000 bp fragments increased somewhat in going from the 50 μm to the 100 μm I.D. capillary. The peak height of the 1000 bp fragment was 3 fold higher in the 100 μm capillary as compared to the 50 μm capillary, while the current was almost 4 times higher.

The effective size separating range of the polymer sieving buffer can be extended by dilution of the polymer in the sieving buffer. Fig. 7 depicts the separation of a 500 bp DNA ladder using three concentrations of the dsDNA sieving buffer. Undiluted buffer is depicted in Panel A showing poor separation above 1.5 kb. The 2-fold dilution in Panel B shows some improvement while the best conditions are presented in Panel C which uses a 4-fold dilution of the DNA sieving buffer. Fig. 8 depicts the separation of a 200 bp dsDNA standard as well as the 500 bp standard using the 4-fold diluted DNA sieving buffer. These two ladders were analyzed at a lower run voltage (2.0 kV) which resulted in an improvement in the separation of the higher fragments. For the 200 bp ladder, 200 bp resolution was obtained out to 4000 bp.

4. Discussion

Acrylamide has been widely used in capillary electrophoresis in gel filled capillaries for the separation of DNA [15]. However, cross-linked gel filled capillaries have a number of drawbacks such as shrinkage of the gel, formation of air bubbles and overall short life span. Gel filled capillaries are now being replaced by capillaries filled with polymer solutions, a technique first described by Zhu et al. [4]. In this technique incorporation of a hydrophilic, uncross-linked polymer in the electrophoresis buffer causes a molecular sieving effect in which the nucleic acids are separated. Barron et al. [16,17] have examined the mechanism of DNA separation, by investigating the effects of polymer properties on

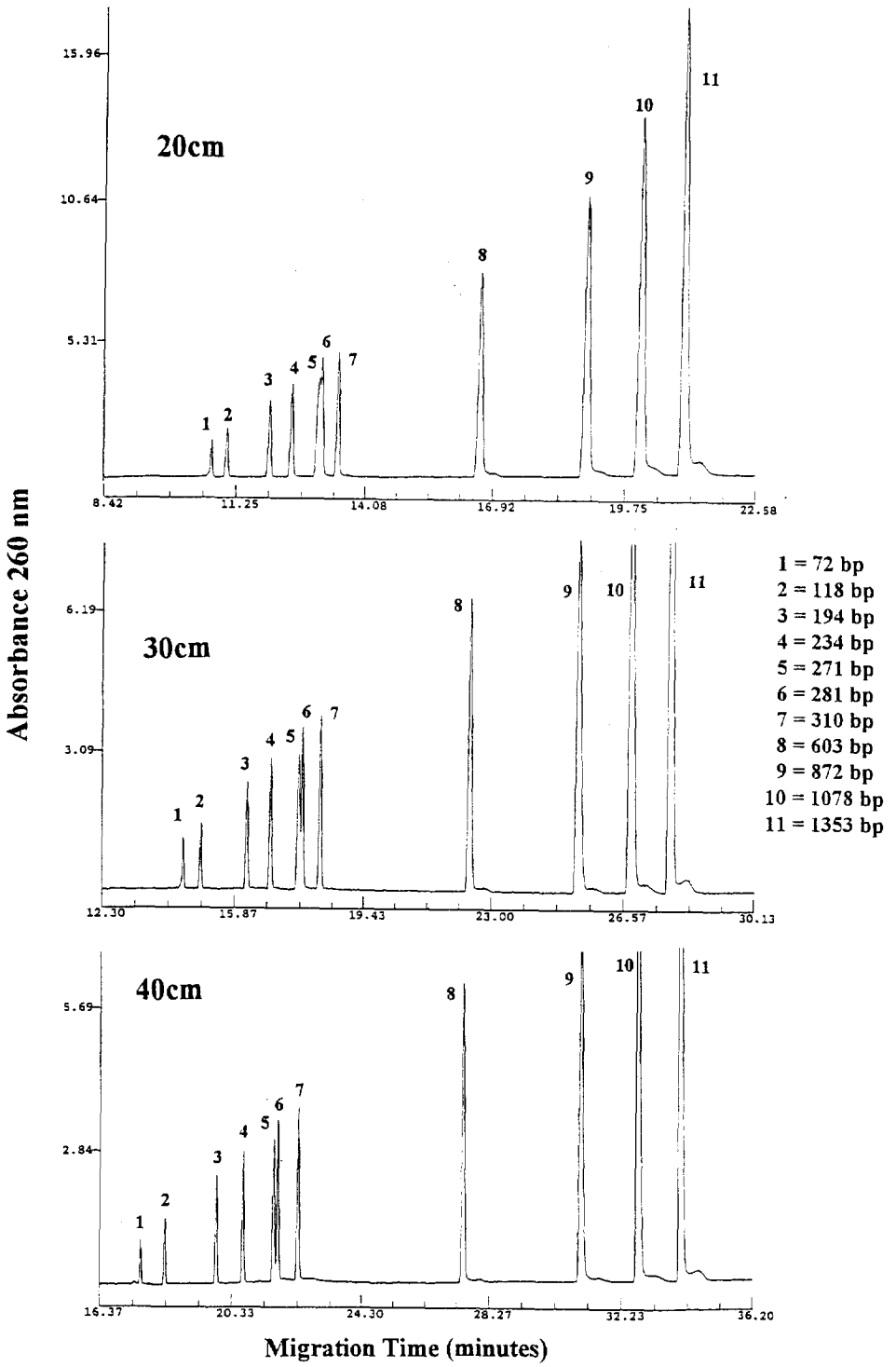

Fig. 4. Effect of capillary length on the dsDNA separation. The ΦX174/Hae III fragments were separated on poly(AAEE) coated capillaries (75 μm I.D.×375 μm O.D.) of the indicated effective lengths using the dsDNA sieving buffer. The sample was injected electrophoretically at 10 kV for 2 s and separated at constant field strength (0.90 kV/cm) on the three different lengths of capillary.

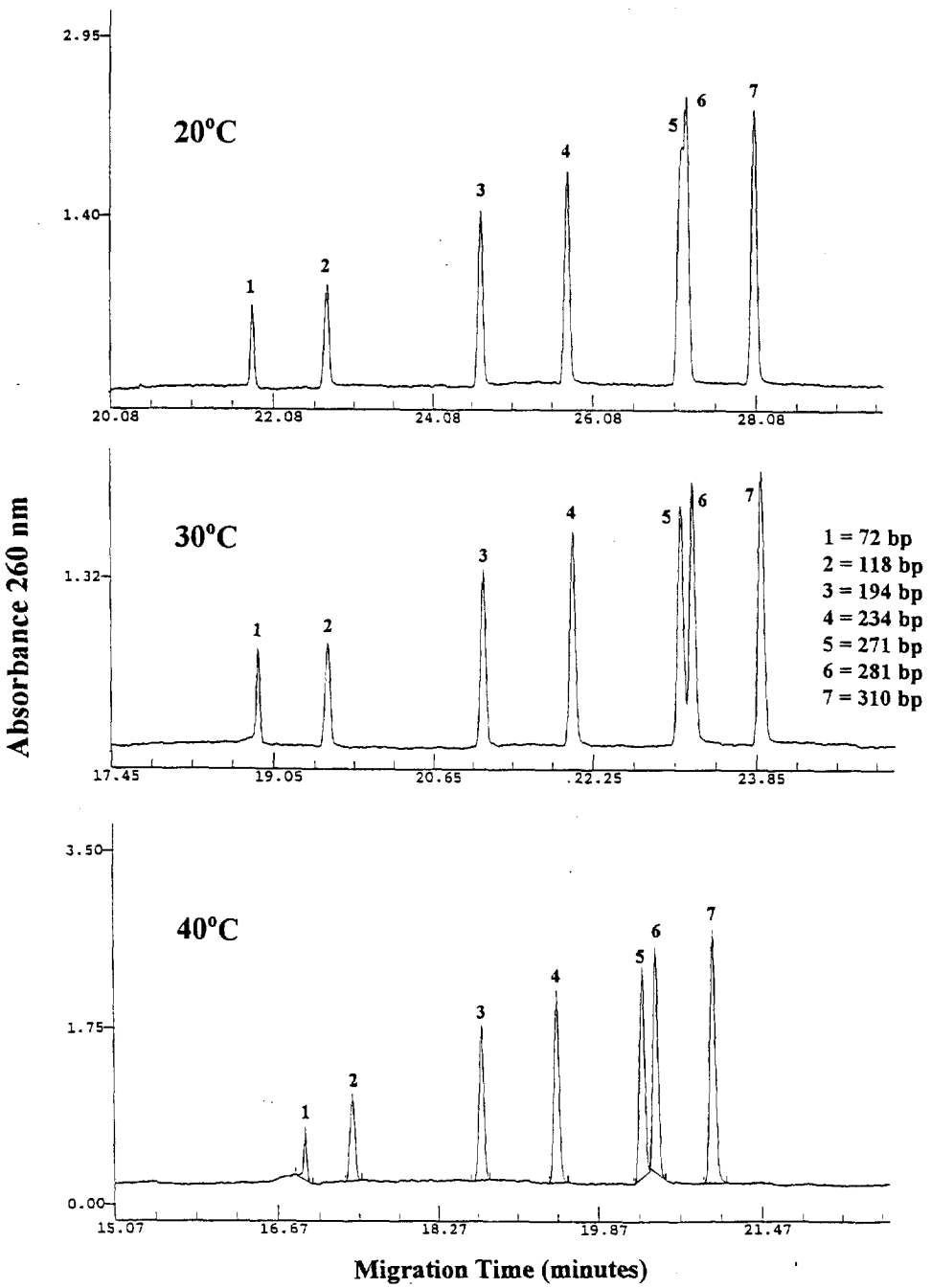

Fig. 5. Effect of temperature on the dsDNA separation. The ΦX174/Hae III fragments were separated on poly(AAEE) coated capillaries (75 μm I.D.×375 μm O.D.×44 cm) using the dsDNA sieving buffer. The sample was injected electrophoretically at 10 kV for 3 s and the separations were performed at 5 kV at the indicated temperatures. Only the first seven fragments are shown.

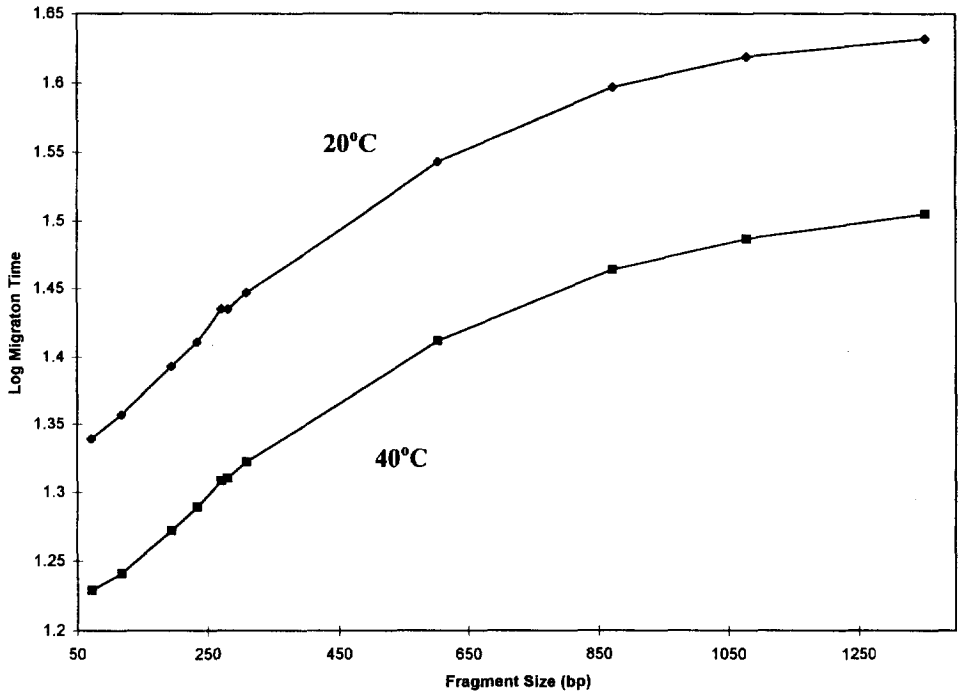

Fig. 6. Plots of log of migration versus fragment size. The plots were made from the data in Fig. 5. The plots include all 11 fragments of the digest.

the separation of a wide range of DNA masses. They propose that the separation of DNA occurs by a transient entanglement coupling mechanism, in which electrophoresing DNA molecules catch and drag the uncharged polymer chains, creating a size-dependent drag force [16].

The advantage of using polymer solution-filled capillaries is that sieving solutions can be forced out of the capillaries under pressure and then replaced allowing the capillaries to be easily re-used. In addition this means that electrophoretic runs can be easily aborted and restarted without the need to wait for all sample components to migrate out of the capillary. In the present study the polymer solution was replenished between each run which was important to maintain high efficiencies and ensure reproducibility.

In the present paper, a non-acrylamide water soluble polymer has been utilized for the rapid separation of double-stranded DNA in the 100–4000 bp range. Using the 100 bp ladder, the 10 double-stranded fragments ranging in length from 100 to 1000 bp were well separated in about 15 min using 24 cm poly(AAEE) coated capillaries (Fig. 2). The effect of capillary length and temperature were examined using the ΦX174/Hea III digest which

Table 1
Resolution and response with capillary diameters

	50 μm I.D.	75 μm I.D.	100 μm I.D.
Resolution of 900/1000 bp	3.1	3.3	3.7
Peak height of 1000 bp (mAU)	4.2	9.8	17.2
Current (μA) at 2.5 kV	5.2	10.4	19.7

The analyzes were performed at 2.5 kV field strength at 40°C on 24 cm (total length) LPA coated capillaries of the indicated internal diameters.

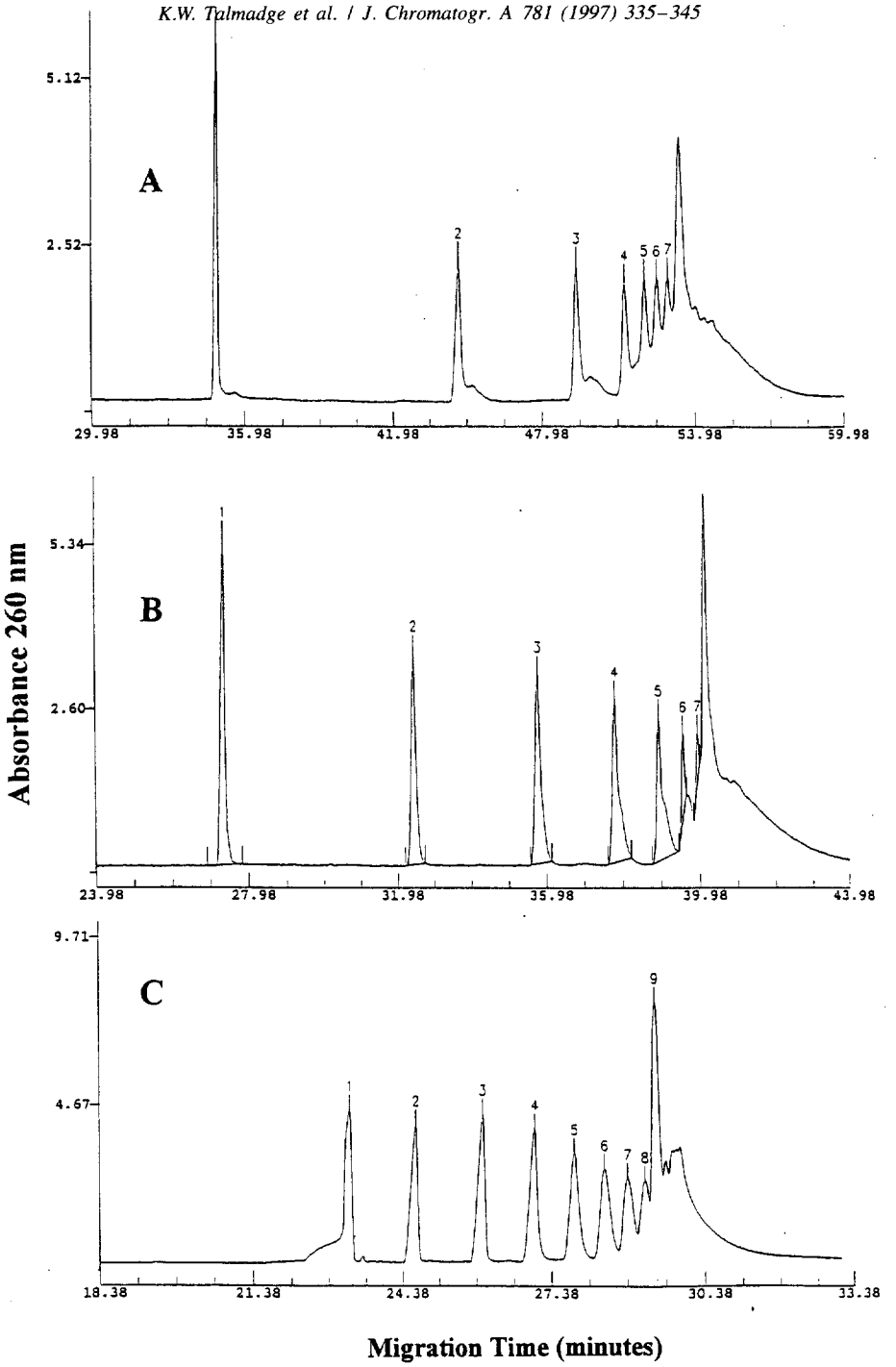

Fig. 7. Effect of polymer dilution on the DNA separating range of the dsDNA sieving buffer. The polymer in the dsDNA sieving buffer was diluted with 2 X TBE. The 500 bp ladder was separated in coated capillaries (44 cm×75 μm I.D.×375 μm O.D.) with the following dilution of the dsDNA sieving buffer: (A) undiluted; (B) 2-fold; (C) 4–12 fold. The sample was injected electrophoretically at 10 kV for 2 s and the separations were performed at 4 kV at 40°C. The peaks in the 500 bp ladder are identified as follows: 1–0.5 kb, 2–1.0 kb, 3–1.5 kb, 4–2.0 kb, 5–2.5 kb, 6–3.0 kb, 7–3.5 kb, 8–4.0 kb, 9–5.0 kb.

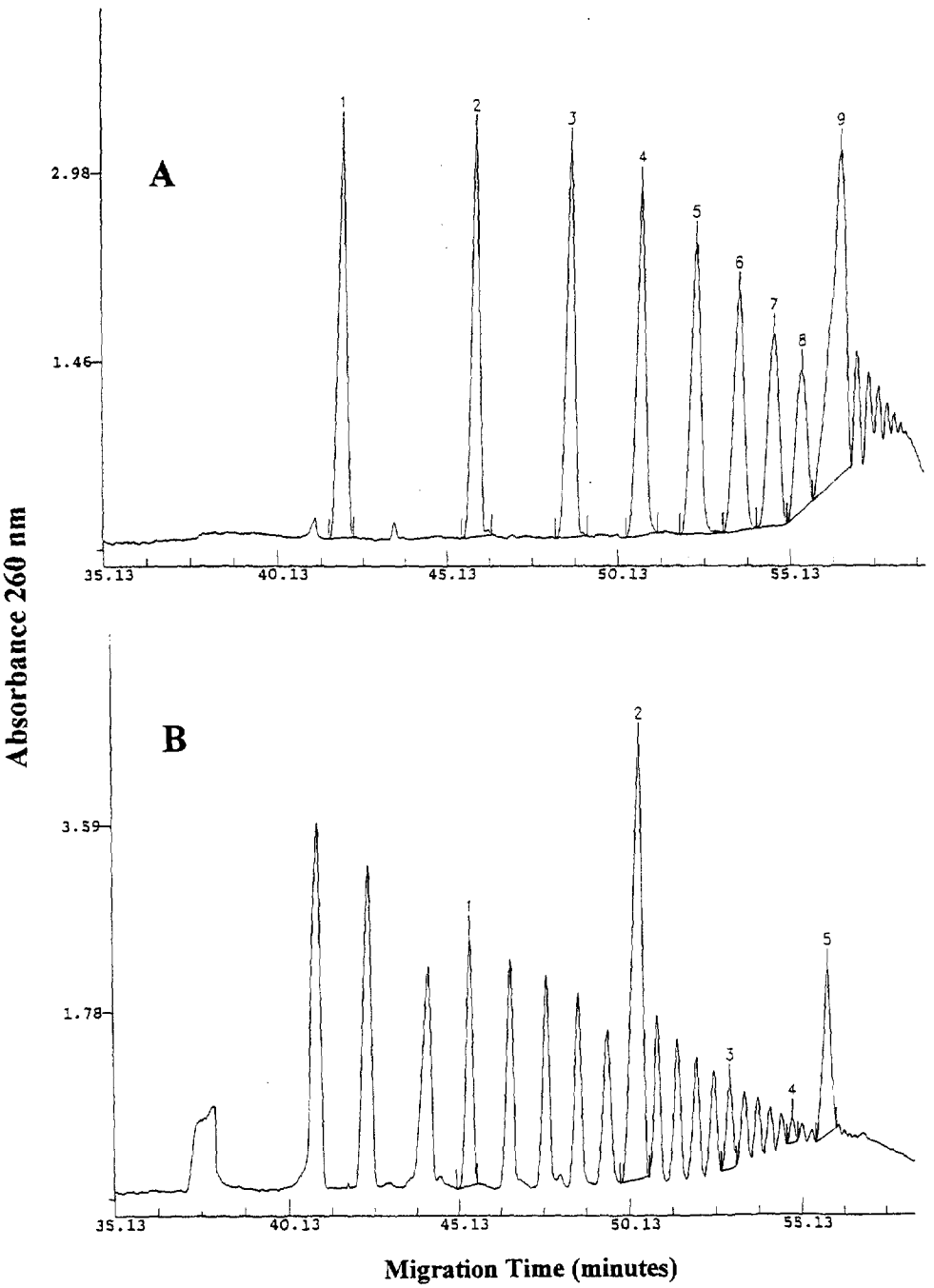

Fig. 8. Analysis of 200 bp and 500 bp ladders using 4-fold diluted sieving buffer. The polymer in the dsDNA sieving buffer was diluted 4-fold with 2 X TBE. The 500 bp (A) and 200 bp (B) ladders were separated in coated capillaries (44 cm×75 μm I.D.×375 μm O.D.). The samples were injected electrophoretically at 10 kV for 2 s and the separations were performed at 2 kV at 40°C. The peaks in the 500 bp ladder are identified as follows: 1–0.5 kb, 2–1.0 kb, 3–1.5 kb, 4–2.0 kb, 5–2.5 kb, 6–3.0 kb, 7–3.5 kb, 8–4.0 kb, 9–5.0 kb. The peaks in the 200 bp ladder are identified as follows: 1–1.0 kb, 2–2.0 kb, 3–3.0 kb, 4–4.0 kb, 5–5.0 kp.

produces 11 fragments from 72 to 1353 bp. Particularly difficult is the separation of the 271 and 281 bp fragments. A 24 cm capillary at 40°C was not able to separate these two fragments, while a 44 cm capillary fully resolved these using the same field strength. The effect of temperature was also significant in the separation of these two fragments separated by 10 base pairs. On a 44 cm capillary there was no separation at 20°C, some separation at 30°C and resolved peaks at 40°C. These results are somewhat in contrast to the efficiency values of some of the other fragments, which actually showed a 10–30% decrease in efficiency with increase in temperature. This discrepancy may be accounted for by sequence dependent anomalous mobility which has been noted for this digest by Berka et al. [18] in a linear polyacrylamide sieving matrix and by Wenz [19] in a proprietary sieving matrix. The anomalous migration noted in these two papers appeared to be more dramatic than seen in the present study, although in both of these studies, increasing the temperature aided in restoring molecular mass dependent separations.

The standard dsDNA sieving buffer will allow separations of dsDNA up to about 1 kb. The effective separating range was extended to 4 kb by lowering the concentration of the sieving polymer 4-fold in the buffer. Examples of separation of 200 and 500 bp ladders in 44 cm capillaries are shown in total analysis times less than 60 min (Fig. 8).

The DNA separations described here were performed in coated capillaries; uncoated capillaries gave very poor separation of dsDNA. The best overall performance was achieved with capillaries coated internally with a new polymer [poly(AAEE)]. This uses a novel acrylic monomer first described by Chiari et al. [5,12] which has the advantage of being more hydrophilic than acrylamide. The experiments performed here (Fig. 1) have clearly demonstrated that poly(AAEE) exhibits a higher resistance to hydrolysis at alkaline pH values than polyacrylamide. These results are in agreement with those of Chiari et al. [12] who have shown that poly(AAEE) is more stable to hydrolysis than polyacrylamide [12,14]. This is particularly important for nucleic acid analyses which are typically performed around pH 8.5. This could explain the steady decrease in performance of the polyacrylamide coated capillaries over time as compared to the poly(AAEE) coated capillary (Fig. 3). The use of poly(AAEE) coated capillaries is a crucial component for the long life performance.

In summary, the CE analysis system described permits the easy, rapid and quantitative analysis of dsDNA in the range of 100 to 4000 bp. In addition the polymer sieving buffer allows for the accurate size determination of DNA fragments. An important component for the reproducible analysis of dsDNA fragments was the use of capillaries coated with poly(AAEE).

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