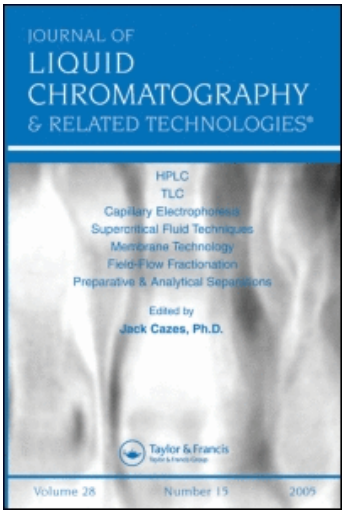

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K. M. S. Sundaram[a]; L. Sloane[a]
[a] Natural Resources Canada, Canadian Forest Service Forest Pest Management Institute, Sault Ste. Marie Ontario, Canada

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LIQUID CHROMATOGRAPHIC ASSAY FOR THE SEPARATION OF SINGLE- AND DOUBLE-STRANDED DNA BY USING UV AND UV DIODE-ARRAY DETECTORS AND HYDROXYLAPATITE COLUMN

K. M. S. SUNDARAM AND L. SLOANE
Natural Resources Canada, Canadian Forest Service
Forest Pest Management Institute
1219 Queen Street East, Box 490
Sault Ste. Marie, Ontario, Canada P6A 5M7

ABSTRACT

A high-performance liquid chromatographic (HPLC) method, using UV and UV diode-array (DA) detection, is reported for the separation of single-stranded (s.s.) and double-stranded (d.s.) DNA molecules. Commercially available calf thymus DNA was used as the standard, to develop and optimize necessary analytical procedures and chromatographic parameters. Bio-Gel® hydroxylapatite was used as the column packing and the sorbed polynucleotides on the column matrix were separated by using an ionic strength gradient system consisting of phosphate buffer at pH 6.8. The stationary phase was stable and proved sufficiently reliable in the separation and resolution of s.s. and d.s. DNA molecules in the standard. Pointedly, the DA detector was more sensitive to the analytes than the UV detector. The response of both detectors was higher for the s.s. DNA compared to the d.s. DNA. Minimum quantification limits (MQL) for the s.s. DNA molecules by the DA and UV detectors were, respectively, 0.10 and 0.50 μg in 10 μL injections. The corresponding value for the d.s. DNA, using both detectors, was 1.0 μg. The plot log (μg of DNA) *vs* absorbance (mAU) was linear for the d.s. DNA. The MQL, using both detectors, was 0.10 μg in 10 μL injection volume. Extension of the method to separate the viral DNA molecules showed some promise. However, problems associated with sample purity and homogeneity, peak characterization, quantification of the analytes etc. were encountered and these drawbacks are discussed.

INTRODUCTION

The use of high-performance liquid chromatography (HPLC) is gaining importance in the separation and analysis of nucleic acids over the conventional electrophoresis method because of its reproducibility and quantitative accuracy (1,2). The introduction of several novel column packing materials enhanced the isolation and analysis of nucleic acids and their fragments with excellent resolution (3-9). In normal phase chromatography, organic resins used previously have been replaced by microparticle silica gels, improving resolution, sensitivity and speed (2). On the reverse phase columns, the introduction of alkylated supports, such as C_8 or C_{18} bonded nonporous silica gel and silica gel-based weak anion and cation exchangers, is finding extensive use in the separation process of different types of biopolymers (2).

The use of microcrystalline hydroxylapatite $[Ca_{10}(PO_4)_6(OH)_2]$ as stationary phase in HPLC has been documented earlier and used for the rapid and quantitative separation and purification of nucleic acids (10-12). The analyte molecules bind to hydroxylapatite by electrostatic interactions between the Ca^{2+} ions in the stationary phase and PO_4^{3-} ions in the polynucleotides (13). Elution of the analyte is achieved by the competition of PO_4^{3-} ions in the eluate buffer, for hydroxylapatite binding sites (14).

Recently, we investigated the separation patterns of s.s. and d.s. nucleic acid components of a denatured nuclear polyhedrosis virus (NPV), isolated from the insect pest, gypsy moth (*Lymantria dispar* L.) (GM), by using a high-resolution hydroxylapatite column linked separately to two liquid chromatographs, one containing an ordinary UV detector and the other a UV diode-array (DA) detector. Prior to the injection of GM-NPV solutions, we used commercially available denatured calf thymus DNA, containing both single-stranded

(s.s.) and double-stranded (d.s.) DNA, as standard (15) to establish the necessary experimental conditions and instrumental parameters required for the successful completion of the experiment. Results of this study are reported in this paper.

MATERIALS AND METHODS

Chemicals and Reagents

DNA Standard

Native, heat-denatured calf thymus DNA (50.0 mg/mL), containing both s.s. and d.s. DNA, was obtained from Boehringer-Mannheim (201 Boulevard Armand Frappier, Laval, Quebec, Canada, H7V 4A2). A stock solution containing 5.0 mg/mL was prepared in 0.50 M sodium phosphate buffer (pH 6.80 ± 0.05) containing 0.02 % sodium azide and 0.01 mM calcium chloride. The standard solutions, ranging in concentration from 10 μg/mL to 500 μg/mL, were prepared by the serial dilution of the stock solution using deionized Milli-Q® purified water.

GM-NPV DNA

Two samples (labeled as X and Y) of denatured and purified DNA isolated from the NPV of GM, containing 0.043 mg/mL (X) and 0.066 mg/mL (Y) in 0.4 M sodium phosphate buffer, were used in this study after filtering through Millipore® 0.20 μm filters.

Mobile Phase

The mobile phase solvents, A and B, used in HPLC were phosphate buffers (pH 6.80 ± 0.05) containing HPO_4^{2-} and $H_2PO_4^-$. The 0.5 M buffer (B) was prepared according to the procedure of Efiok (16) by using equimolar quantities of ACS grade Na_2HPO_4 and NaH_2PO_4,

and deionized Milli-Q® purified water. The 10 mM buffer (A) was prepared by diluting aliquots of buffer B fifty times with Milli-Q®purified water. The pH of the buffers were checked with a pH meter and if slight adjustments were required, they were done by adding, dropwise, concentrated HCl or NaOH. Each buffer also contained 0.02% NaN_3 and 0.01 mM $CaCl_2$. They were filtered (Millipore® 0.20 μm filter) and degassed prior to use and refrigerated immediately afterwards. No detectable deterioration in DNA resolution of the standards occurred with monthly aged buffers; nevertheless, fresh preparations were made every two weeks as a precaution.

HPLC Instrumentation

Two liquid chromatographs, Hewlett-Packard (HP) Model 1080 fitted with a UV variable wavelength detector and HP Model 1090 fitted with a DA detector, were used in the study. Both instruments had Rheodyne® injectors equipped with 10 to 100 μL loops and computing facilities to extract the necessary analytical data from the chromatograms generated. Full descriptions of these two instruments are published elsewhere (17, 18). The parameters used for the two instruments were similar. A flow rate of 1.0 mL/min was used and the adsorbed DNA was eluted and separated by the stepwise use of a binary gradient system consisting of two phosphate buffers, one with low (buffer B, 10 mM) and the other with high (buffer A, 0.5 M) ionic strength (14). The UV and DA detectors were set at 260 nm (4 nm bandwidth) for the sample wavelength and 400 nm (50 nm bandwidth) for the reference wavelength. The column used was a Bio-Gel® HPHT column (100 × 7.8 mm, 4.8 mL bed volume) packed with Bio-Rad's hydroxylapatite, preceded by a guard column (50 × 4.0 mm) (both columns from BioRad Labs Canada Ltd., Mississauga, Ontario, L4W 2A6) containing inert hydrophilic polymer particles of 10 μm size. The column was thoroughly equilibrated

in the starting buffer prior to sample injection. The oven temperature and column pressure were maintained at 50°C and approx. 1.4×10^4 kPa, respectively, and the total run time for each injection was 20 min.

The ionic strength of the buffer was increased, stepwise, by starting initially at 40% of buffer B for 3 min, increasing it to 45% for the next 4 min and then switching completely to 100% B for the remaining 13 min. Most of the instrumental parameters and operating conditions listed in this study were painstakingly arrived at by trial-and-error, and eventually optimized to attain precision and accuracy in measurements.

HPLC Analysis

During calibration of the instruments using calf thymus DNA as standard, 10 μL volumes, containing 0.1 to 5.0 μg of the analyte, were injected (in triplicate) in each instrument and the corresponding detector response was recorded. Calibration curves were prepared for the s.s. and d.s. DNA by plotting the average peak area (y-axis) against the mass of DNA injected (x-axis). The standard deviation (SD) of the peak area for each concentration at 1.0 μg in 10 μL and above was found to be roughly proportional to its mean, however this was not the case for concentrations below the 1.0 μg/10 μL level, wherein the SD was high. The average retention times (RTs) for the s.s. and d.s. calf thymus DNA were found to be 8.1 (range 8.0 to 8.2) and 11.9 (range 11.7 to 12.1) min, respectively.

Aliquots of the two test solutions, X and Y, containing the GM-NPV DNA were then injected and the peak areas were computed from the chromatograms obtained. Unfortunately, none of the RTs of the peaks obtained corresponded to those of the standards. The peaks with RTs 10.1 and 15.3 min in sample X and 10.8 and 15.9 min in

sample Y, of the GM-NPV, were assumed to correspond to the s.s and d.s. DNAs, respectively. Their relative concentrations in the two samples were computed using the calibration curves generated for the calf thymus standard.

RESULTS AND DISCUSSION

Response of UV and DA Detectors to DNA Standard

Under the HPLC parameters used and from the calibration curves (Figure 1), it is apparent that both the UV and DA detectors responded satisfactorily to the s.s. and d.s. DNA molecules. It is evident that the response of the DA detector to the s.s. DNA was linear over the concentration range of 0.1 to 5.0 μg in 10 μL injection volume. However, the response of the UV detector to the s.s. DNA was generally low and was linear only from 0.5 to 5.0 μg. Below 0.5 μg, the detector response was rather erratic. Within these ranges, the reproducibility of peak area measurements in both detectors was 93.5% as determined by 10 repeat injections. The linear regression equations for the two calibration curves for the s.s. DNA, from the DA and UV detectors, and the correlation coefficients (R^2) for them are:

DA detector: s.s. DNA $y = 1053 x - 14.70$, $R^2 = 0.999$

UV detector: s.s. DNA $y = 692.7 x - 141.6$, $R^2 = 0.989$

From the standard curves in Figure 1, it is apparent that the DA detector is more sensitive (higher slope) to the s.s. DNA than the UV detector. The minimum quantification limits (MQL) for the s.s. DNA standard in the DA and UV detectors, as assessed from the respective standard curves, were 0.10 and 0.50 μg, respectively.

The standard curves obtained (Figure 1) for the d.s. DNA using the DA and UV

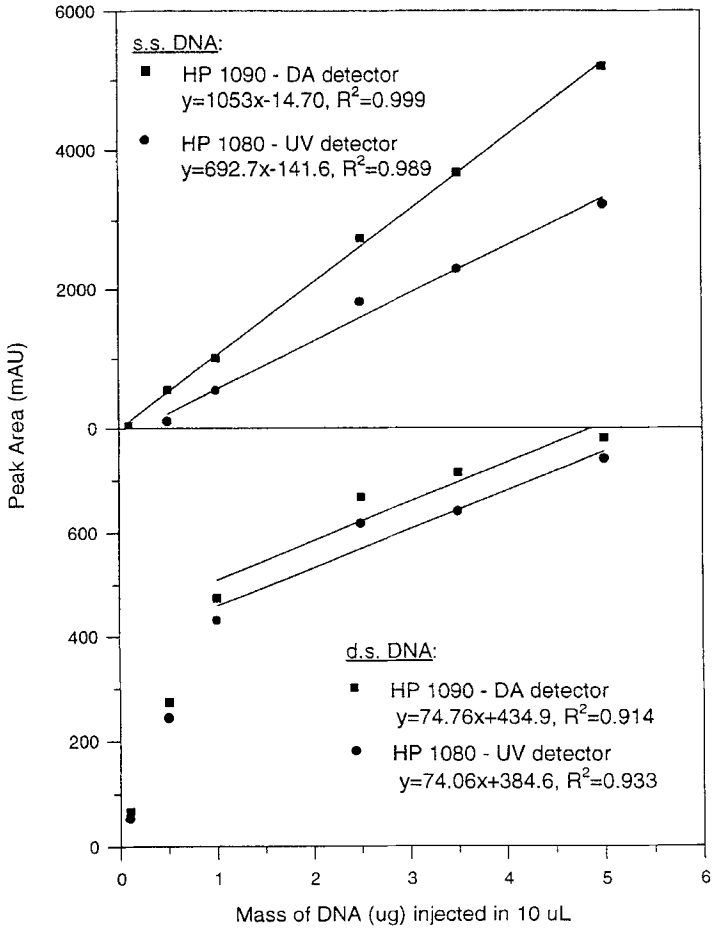

FIGURE 1. Calibration of HP 1080 and HP 1090 using calf thymus DNA - single and double strands.

detectors were not overly influenced by the analyte concentration. Concomitantly, the response of both detectors was very low, indicating their low sensitivity to the d.s. DNA. Linearity of the detectors, by plotting μg of DNA *vs* absorbance (mAU), was observed only over the concentration range from 1.0 to 5.0 μg in 10 μL injection volume. Moreover, the standard curves did not pass through the origin and their slopes were low. The reproducibility of peak area measurements was about 91% as determined by 15 repeat injections. The linear regression equations for the two calibration curves for the d.s. DNA, from the DA and UV detectors, and the corresponding correlation coefficients are:

DA detector: d.s. DNA $y = 74.76\ x + 434.9$, $R^2 = 0.914$

UV detector: d.s. DNA $y = 74.06\ x + 384.6$, $R^2 = 0.933$

The MQL established for the d.s. DNA standard, from the calibration curves of both detectors, was 1.0 μg.

The plot of log (μg of DNA) *vs* absorbance (mAU) was linear for the d.s. DNA, for both detectors over the concentration range of 0.10 to 5.0 μg in 10 μL injection volume. The linear regression equations and corresponding correlation coefficients are:

DA detector: d.s. DNA $y = 438.1\ x + 471.3$, $R^2 = 0.985$

UV detector: d.s. DNA $y = 412.4\ x + 430.6$, $R^2 = 0.982$

The MQL for the d.s. DNA standard, using linear regression equations from plotting log (μg of DNA) *vs* absorbance (mAU), was 0.10 μg in 10 μL injection volume for both detectors.

HPLC Chromatogram of the DNA Standard

A typical chromatogram of the calf thymus DNA standard, obtained by injecting (in triplicate) 2.5 μg in 10 μL onto the hydroxylapatite column connected to the HP 1080 and

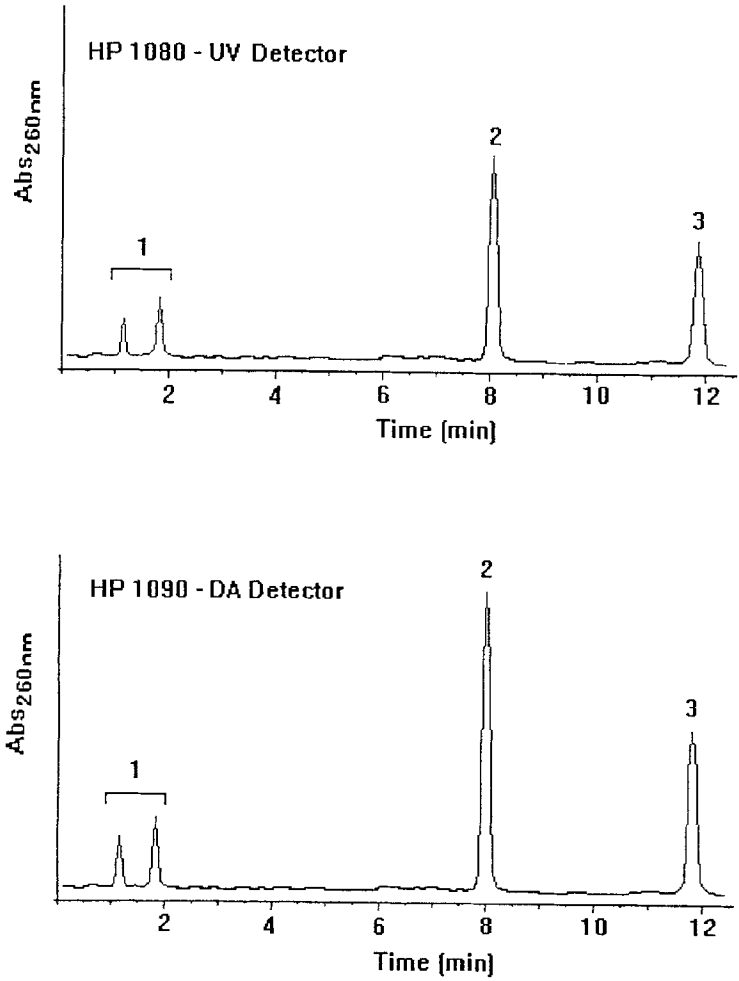

FIGURE 2. Chromatographic trace of calf thymus DNA standard, 2.5 μg and 10 μL injection. Peak 1: low molecular weight materials; peak 2: s.s. DNA (RT, 8.1 min); peak 3: d.s. DNA (RT, 11.9 min). Chromatographic conditions are given in the text.

HP 1090 liquid chromatographs, is shown in Figure 2. As afore-mentioned, the DA detector in HP 1090 HPLC was more sensitive to the s.s. DNA (peak area 2740 ± 45 mAU, n=3) than the UV detector in HP 1080 instrument (peak area 1812 ± 50 mAU, n=3). In contrast, the response of both detectors to the d.s. DNA was nearly the same, yielding a similar degree of resolution and sensitivity. This is further corroborated by comparing the peak areas obtained in both instruments. The peak area in HP 1090 for the standard (d.s. DNA) was 667 ± 40 mAU, whereas in HP 1080, it was 622 ± 32 mAU.

The chromatographic peaks corresponding to the s.s. (RT = 8.1 min) and d.s. (RT = 11.9 min) DNA molecules in the standard are well resolved and symmetrical. The low RT of the s.s. DNA is presumably the consequence of weak interactions between the Ca^{2+} ions in the stationary phase (hydroxylapatite) and shielded PO_4^{3-} ions in the flexible, disordered and sterically hindered s.s. DNA molecules (19). On the contrary, the higher RT of the d.s. DNA (11.9 min) is most likely due to the unfolding and derotating of the rigid and ordered double helix, which resulted in exposing more PO_4^{3-} ions for strong interaction and binding with Ca^{2+} ions on the sorbent surface. Because of the resultant strong electrostatic interaction, the d.s. DNA molecules are eluted later, giving a higher RT (19). The two small peaks appearing close to the solvent front could be due to low molecular weight nucleotides.

Chromatograms of the Viral DNA

Initial injections of samples X and Y, at 40 to 70 μL range, in both HPLCs, did not produce consistent detector responses and the peak area measurements varied considerably. Increasing the injection volume to 100 μL gave reduced fluctuations and reasonably consistent detector responses. Typical chromatograms obtained in HP 1090 are

shown in Figure 3. The chromatograms contained three large peaks and a number of small peaks of different sizes and shapes, indicating the heterogeneity of DNA moieties and the presence of UV-absorbing impurities in the two samples. The distinct separation of the large peaks indicated that the instrumental and operational parameters used to separate the DNA and other components in the samples were reliable and could be pursued in the future, with necessary modifications, for the purposes of isolation, separation and purification of DNA-type biopolymers.

Examination of Figures 2 and 3 show that none of the peak RTs in Figure 3 matched with the peak RTs of DNA standard in Figure 2. Furthermore, the RTs of the peaks and their shapes (narrow *vs.* broad) in sample X and Y (Figure 3) are different from one another and none of them matched, indicating that the composition of the DNA moieties in the two samples are probably different. The two large peaks (RTs, 2.5 and 2.9 min in Figure 3) near the solvent front, could be due to low molecular weight DNA fragments (20). The next two smaller peaks are likely caused by the partly denatured and hybrid nucleic acid molecules, which displayed low affinities for hydroxylapatite (19). Comparing the chromatograms of the DNA standard (Figure 2), we assume that the peaks with RT of 10.1 min (sample X) and 10.8 min (sample Y) (Figure 3), could correspond to the s.s. viral DNA molecules. We are making this assumption, of course, with very little experimental evidence, aside from the fact that elution behavior is: (1) controlled by the interaction between the Ca^{2+} and PO_4^{3-} ions of the adsorbent and adsorbate, respectively; and (2) separation, as aforementioned, is size- and strand-dependent. Similarly, the peaks with RTs of 15.3 min (sample X) and 15.9 min (sample Y) (Figure 3) are assumed to belong to the d.s. viral DNA molecules, although as pointed out above, we have very little experimental basis for these

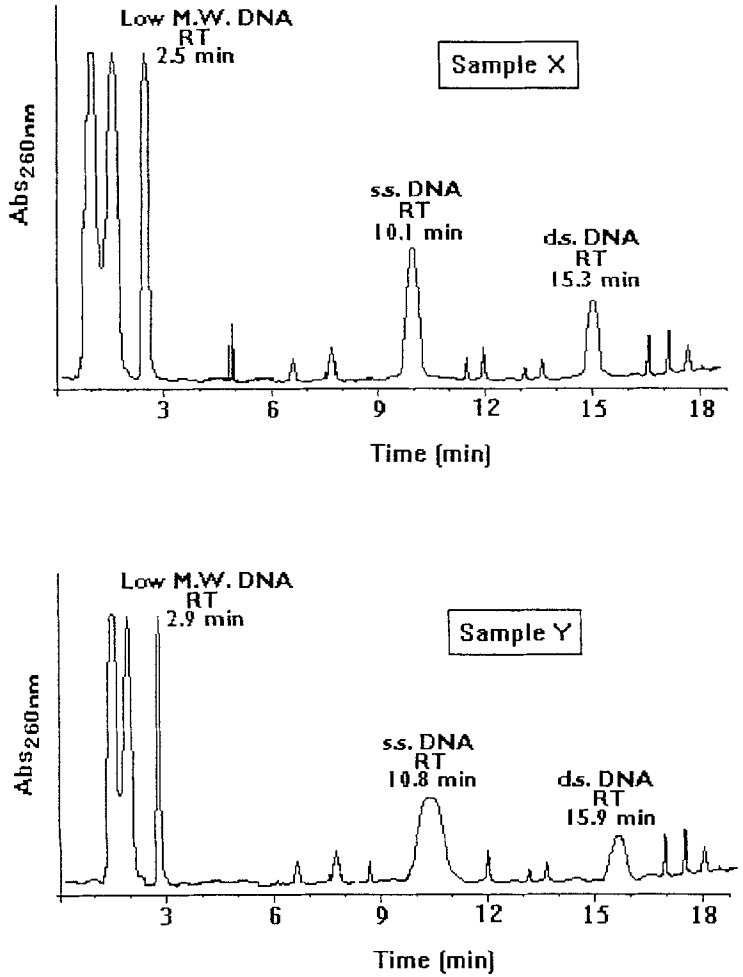

FIGURE 3. Chromatographic trace of viral DNA after injecting 100 μL sample, using the HP 1090 equipped with the DA detector.

assumptions, albeit the appearance of two well defined peaks (narrow in sample X and broad in sample Y) around the elution times of the standards, shown in Figure 2.

Using the calibration curves for the DNA standard in Figure 2 and assuming that the two pairs of peaks with RTs of 10.1 and 15.3 min (sample X), and 10.8 and 15.9 min (sample Y), shown in Figure 3, are due to the s.s. and d.s. viral DNA molecules, respectively, we calculated their concentrations in the two samples. The values obtained in HP 1090 were: s.s. viral DNA, 0.61 μg/100 μL and d.s. viral DNA, 0.70 μg/100 μL; or total viral DNA of 13.1 μg/mL in sample X. The values obtained for sample Y were: s.s. viral DNA, 1.29 μg/100 μL, and d.s. viral DNA 1.39 μg/100 μL; or total viral DNA of 26.8 μg/mL. These values are low and corresponded to only 30.5% and 40.6% of the total viral DNA concentrations expected to be present in the samples X and Y, respectively. Similar calculations, using the peak areas obtained for the analytes in HP 1080 HPLC (chromatograms not shown in figure), gave concentrations which were about 20% less than the HP 1090 values.

CONCLUSIONS

This paper describes a practical and sufficiently sensitive HPLC method to isolate and separate the s.s. and d.s. DNA molecules present in calf thymus DNA standard by using Bio-Gel hydroxylapatite column and phosphate buffer at pH 6.8 as the mobile phase. The column was stable and proved reliable in the separation of DNA molecules. Extension of the method to separate the s.s. and d.s. DNA molecules in two viral DNA preparations yielded chromatograms containing more than seven peaks with different RTs, peak shapes and peak areas for both samples. This indicated not only the heterogeneity in the DNA

composition, but also of the variability in their concentration. Quality maintenance and optimization of methods used in the isolation and preparative purification of the s.s. and d.s. DNA molecules from viral preparations could yield consistency in product excellence, consequently resulting in good chromatographic resolution and analyte separation. Nevertheless, the study succeeded in demonstrating the suitability of the method to isolate and separate nucleic acid moieties found in biological samples. Lack of authentic standards for the viral DNA molecules precluded the absolute quantification of the analytes in the two viral preparations.

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