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SEPARATION OF NUCLEOSIDES ON POLYACRYLAMIDE GEL COLUMNS: FURTHER DEVELOPMENTS

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SUMMARY

Some new developments in the method of Carrara and Bernardi for the separation of nucleosides on polyacrylamide gel columns are described.

The four nucleosides of each series (ribo- and deoxyribo-) can be separated on a single column. The method has been scaled down to permit the routine analysis of as little as 0.5 μg of DNA.

Two additional applications of the method are reported. The first one concerns an improved simultaneous determination of chain length and terminal nucleosides in oligonucleotides; the second one, the separation of the eight nucleosides from the two series.

INTRODUCTION

A technique for the separation of nucleosides on polyacrylamide gel columns (Bio-Gel P-2, 200–400 mesh; Bio-Rad, Richmond, Calif.) was described in 1968 by CARRARA AND BERNARDI[1] and has been extensively used in our laboratory for the analysis of DNA and RNA[2–5]. This technique has several interesting features: nucleosides are eluted in small volumes and show very clean spectra, little material is needed for analysis (2–4 $A_{260\ m\mu}$ units of nucleic acid digests) and quantitative recoveries of nucleosides from the columns are obtained. Its only drawback is that, two chromatograms, run at alkaline and neutral pH, respectively, are necessary in order to perform a nucleic acid analysis. In fact guanosine and thimidine (or uridine), and cytidine and thymidine (or uridine) are not separated from each other at alkaline and neutral pH, respectively, and have therefore to be re-run at the other pH.

We wish to report here conditions for the analysis of nucleosides on columns of fractionated Bio-Gel P-2 (< 400 mesh) particles. The major advantages of the improved procedures we have developed, are the use of a single column and the possibility of scaling down the technique to permit the analysis of as little as 0.5 μg of DNA, namely less than 1 % of the material needed in the original method. Conditions found in the early part of this work have been used to analyse yeast mitochondrial DNA[6].

Two additional applications of the modified technique concern an improved simultaneous determination of chain length and terminal nucleosides in oligonucleotides and the separation of the eight nucleosides from the two series (ribo- and deoxyribo-) on a single column.

MATERIALS AND METHODS

Fractionation of the gel

Dry Bio-Gel P-2 (< 400 mesh) was suspended in water and allowed to swell under stirring overnight at room temperature. A first fractionation of the swollen gel was obtained in the following way: 470 ml of gel (obtained from 100 g of dry beads) were suspended in 2 l of water in a 8 cm × 42 cm cylinder; the homogeneous suspension was allowed to sediment for 15 min, and then the top 100 ml were syphoned off; this operation was repeated 11 times at 15-min intervals. The remaining 800 ml of suspension were brought to 2 000 ml, and the whole procedure just described was repeated 8 times. After complete settling the heavy fraction of the gel (Fraction A) was collected. The light fraction of the gel (Fraction B), removed by syphoning, was also collected after settling. The pinkish, non-sedimenting material left in the supernatant was discarded. Fraction B represented about 10 % (v/v) of the starting material.

Fraction B was further fractionated by the back-washing technique of HAMILTON[7], using deionized water pumped with a peristaltic pump through a conical glass funnel (height, 35 cm; base diameter, 14 cm; inlet diameter, 0.2 cm) containing the gel and some glass beads to reduce axial streaming. The particles collected on a sintered glass filter between flow rates of 200 and 800 ml/h (Fraction C) represented about 10 % (v/v) of Fraction B and, therefore, of the order of 1 % (v/v) of the starting material. Fig. 1 shows the distribution of the sizes found for the starting material and Fraction C, respectively. It should be noted that HAMILTON's procedure cannot be used effectively on unfractionated gel.

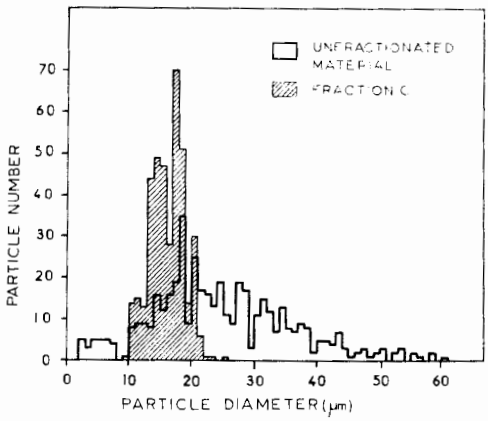

Fig. 1. Size distribution of Bio-Gel P-2 beads (< 400 mesh) swollen in water before and after fractionation (see text). Diameter measurements were made on micro-photographs of the gel particles (final enlargement × 1700).

Column preparations

Fractions A or B were loaded under stirring using a funnel mounted on the top of the column. Fraction C was loaded as a thick suspension (1 vol. of settled gel : 1 vol. of solvent). The dead volume from the column outlet to the recording cell was about 50 μl for columns containing Fractions A or B and less than 10 μl for those

containing Fraction C. Fraction C columns have been kept in operation for many months with no change in their chromatographic properties.

As a rule, columns are kept under a constant flow of eluent.

Chromatographic procedure

Chromatographic runs were performed at room temperature using a Technicon (Chauncey, N.J.) peristaltic pump. In the experiments done with Fraction C (see below), a Labotron (Gelting, Germany) piston pump was used. In both cases, pumps were used to keep constant the flow rate; the pressure on the columns was very slight.

Elution was accomplished with 2 mM $(NH_4)_2CO_3$ solutions adjusted to pH 10.3 with NH_4OH; 0.1 mM $Na_2B_4O_7$ was present in the eluent used for the separation of ribo- and deoxyribonucleosides. Columns were equilibrated with the eluents before use.

Effluents from the columns containing Fractions A or B were monitored for their ultraviolet absorption at 253.7 mμ using a Uvicord (LKB, Stockholm, Sweden) equipped with a flow cell having an optical path of 0.3 cm and a capacity of about 150 μl.

Effluents from the columns containing Fraction C were monitored for their ultraviolet absorption at 271 mμ using a PMQ II Zeiss spectrophotometer (Oberkochen, West Germany) equipped with a Zeiss transmission–absorbance converter and a Zeiss MR 1 D microcell having an optical path of 1 cm and a capacity of about 10 μl. Absorbance was recorded on a Multiriter (Texas Instruments, Houston, Texas) recorder.

Samples were applied on the drained top of the columns; one volume of solvent was used to wash the sample.

Sample preparation

Artificial mixtures of nucleotides and nucleosides were prepared by dissolving commercial products (Calbiochem, Los Angeles, Calif.) in water.

DNA hydrolysates were prepared by digesting DNA solutions in 0.05 M ammonium acetate (pH 5.5) with spleen acid deoxyribonuclease[8], spleen acid exonuclease[9] and spleen acid phosphomonoesterase B[10]. Deoxyribonuclease and exonuclease solutions were in 0.05 M ammonium acetate, pH 5.5, phosphatase in 0.05 M ammonium acetate (pH 4.0).

DNA solutions having an $A_{260\ m\mu} = 0.5$ were digested with 0.05 unit of acid deoxyribonuclease, 0.025 unit of exonuclease and 0.15 unit of acid phosphatase per ml of substrate solution (for the definition of units see the references given above). A digestion time of 3 h at room temperature was enough to obtain a complete hydrolysis. DNA solutions having an $A_{260\ m\mu} = 0.1$ were digested with 0.025 units of deoxyribonuclease, 0.01 unit of exonuclease, 0.15 unit of phosphatase per ml of substrate solution; in this case digestion was accomplished overnight at room temperature. It should be noted that dephosphorylation of nucleotides is never complete because of the competitive inhibition of the enzyme by released phosphate. Nucleosides are obtained, however, in yields higher than 95 %; in addition no significant preferential hydrolysis toward one or more nucleotides was found at the end of the digestion.

Before loading, samples were dried under reduced pressure, and dry ma-

terials were dissolved in the eluting solvent. The actual pH of the samples to be loaded can vary over a wide range (5 to 10) without any adverse effect on the chromatographic separation.

Determination of the extinction coefficients of nucleosides at 271 mμ and pH 10.3

9-ml samples of nucleoside solutions in 2 mM $(NH_4)_2CO_3$ (pH 10.3) having a known absorbance at 271 mμ (close to 0.5) were acidified with 1-ml vol. of 1 M HCl. The absorbance of these solutions was determined at 271 mμ and at λ_{max}. The values obtained were corrected for dilution.

Using ε values at λ_{max}, pH 1 (Calbiochem Catalogue): deoxyadenosine, 14.1; thymidine, 9.65; deoxyguanosine, 12.3; deoxycytidine, 13.2; the following ε at 271 mμ, pH 10.3 values were calculated: deoxyadenosine, 8.93; thymidine, 7.71; deoxyguanosine, 10.7; deoxycytidine, 9.10.

RESULTS

Chromatographic separation of the four deoxyribonucleosides on a single column

Fig. 2a shows the results obtained with a 0.8 cm × 120 cm column of Fraction A obtained from Bio-Gel P-2 < 400 mesh. The separation of the four nucleosides is

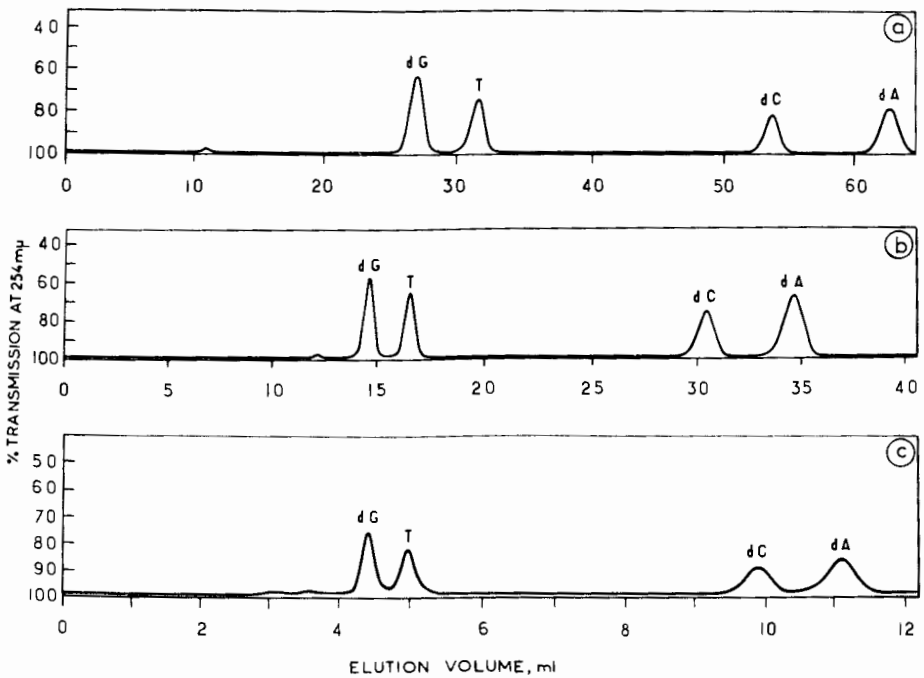

Fig. 2. Chromatography on Bio-Gel P-2 (< 400 mesh) columns of nearly equimolar artificial mixtures of deoxyribonucleosides. (a) Fraction A column (0.8 cm × 120 cm). Load was about 3 $A_{260\ m\mu}$ (pH 7) units in 100 μl. Flow rate was 5.1 ml h. (b) Fraction B column (0.8 cm × 64 cm). Load was about 2.5 $A_{260\ m\mu}$ (pH 7) units in 100 μl. Flow rate was 3.2 ml/h. (c) Fraction B column (0.8 cm × 20 cm). Load was 0.5 $A_{260\ m\mu}$ (pH 7) unit in 50 μl. Flow rate was 6 ml/h.

complete and the first two peaks show spectra identical with those of deoxyguanosine and thymidine, respectively. This method has been successfully used for the analysis of mitochondrial DNA from yeast[6]. Its main disadvantage is that it is time-consuming: the analysis requires 12 h since the flow rate of the buffer cannot be increased above 6 ml/h with the columns and tubing connections used.

Using a shorter (0.8 cm × 64 cm) column along with the finer Fraction B gave similar results as far as duration of analysis is concerned since a flow rate of only 3 ml/h was the highest usable (Fig. 2b). Nucleosides were eluted from this column in smaller volumes than from the previous one. It appeared feasible, therefore, to achieve a shorter duration of analysis by decreasing the length of the column and, at the same time, the load. Fig. 2c shows a chromatogram obtained by loading only 0.5 $A_{260\ m\mu}$ unit of nucleosides on a 0.8 cm × 20 cm column of Fraction B; the flow rate was 6 ml/h and the analysis time was only 2 h.

For the quantitative determination of each nucleoside, peaks obtained from chromatograms like those shown in Figs. 2a and 2b were collected in 10-ml cylinders, and spectra were taken after acidification to pH 1. In the third case (Fig. 2c), nucleosides were eluted in less than 1-ml volumes; these were estimated by weight, and silica cells of reduced capacity were used for spectral analysis.

DNA base composition at the nmole level

The results obtained with Fraction B columns, particularly the short duration of analysis and the low amount of material needed, indicated that the technique could be further improved by using a finer and more homogeneous gel fraction. In fact experimental conditions were found permitting the separation in 4 h of nmole amounts of nucleosides on Fraction C columns (about 0.4 nmole of each nucleoside can be routinely analyzed). Since, under these conditions, nucleosides were eluted in 150–300 μl of solvent, a volume comparable to the capacity of the 0.3 cm Uvicord cell used, a different flow cell and monitoring system had to be used (see MATERIALS AND METHODS).

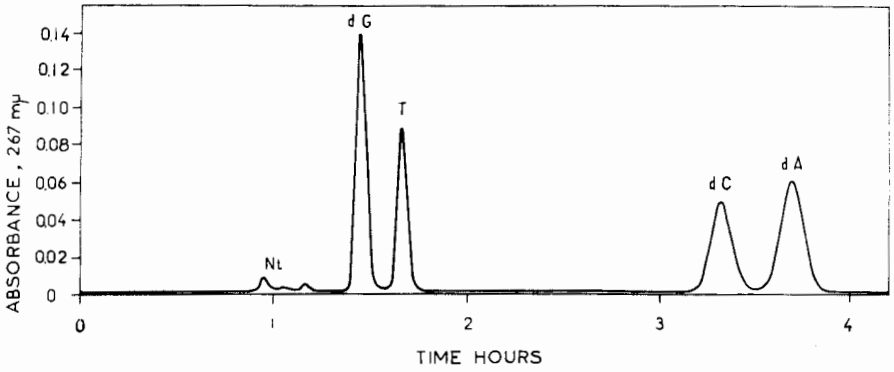

Fig. 3. Chromatography of about 0.02 $A_{260\ m\mu}$ unit of an enzymatic hydrolysate of *E. coli* DNA on a Fraction C column (0.4 cm × 20 cm). Load volume was 10 μl in 1 M ammonium acetate. Flow rate was 0.75 ml/h. In this particular case recording was made at 267 mμ. The two first small peaks are formed by residual nucleotides which in this case are slightly subfractionated. The third small peak is formed by unidentified ultraviolet-absorbing material; this peak is also found when running artificial mixtures of nucleosides.

Fig. 3 shows a typical chromatogram obtained by loading about 0.02 $A_{260\ m\mu}$ unit of an *Escherichia coli* DNA hydrolysate on a 0.4 cm × 20 cm Fraction C column. Generally, the column effluent was monitored at 271 mμ using a 0–0.2 absorbance scale. The quantitative estimation of nucleosides was done by integration of the areas under the peaks, using the ε values at 271 mμ, pH 10.3, of nucleosides. Integrations were done by the cut-and-weight method, by planimetry or, more recently, using Simpson's method; in the latter case a program developed in our laboratory by EHRLICH AND THIÉRY[11] for the Olivetti Programma 102 with data storage attachment was used (this program is available upon request). Since the transmission to absorbance conversion using the 0–0.2 expanded scale deviated slightly from linearity, the recorded values were corrected. This was also done using a program and the Olivetti computer[11].

The reproducibility of the results obtained using the procedure just described was investigated. It was found that the deviation from the mean values obtained in two experiments performed on *E. coli* and yeast mitochondrial DNA (each analysis was made in quadruplicate), was lower than 2 % at the 0.8 nmole level and increased up to 5 % when the nucleoside level decreased to 0.2 nmole.

It is evident from the chromatogram shown in Fig. 3 that the separation between nucleotides and nucleosides makes unnecessary the preliminary DEAE-cellulose chromatography which we used in order to remove the small amounts of residual nucleotides (undigested by phosphatase) and enzymes present in DNA hydrolysates. It should be noted that the enzymes present in the hydrolysate are either eluted within the excluded volume (in this case their contribution to the ultraviolet absorption of the effluent is negligible) or denatured by the alkaline solvent used (and remain absorbed on the column.) In the chromatography of nucleosides obtained from DNA, we were interested to load solutions having a high ammonium acetate concentration since this allowed us to analyze also dilute DNA solutions. We observed that the 0.4 cm × 20 cm column of Fraction C could be loaded with 10 μl of a DNA hydrolysate in 1 M ammonium acetate (see Fig. 3) and still give an excellent separation. Similarly, the 0.8 cm × 20 cm columns of Fraction B can be loaded with 50 μl of a DNA hydrolysate in 2 M ammonium acetate.

Simultaneous determination of terminal nucleotides and chain length of oligonucleotides

The average size of the oligonucleotides can be obtained by determining the total nucleotides: terminal nucleosides ratio after exonuclease digestion of the dephosphorylated oligonucleotides. Chromatography of dephosphorylated oligonucleotides digested with exonucleases on Bio-Gel P-2 columns permits the simultaneous determination of terminal nucleotides (which are separated as nucleosides on the column) and average oligonucleotide chain length since nucleotides are eluted before nucleosides. Originally this separation was performed at pH 7.0[1]. Such a separation can be made more convenient with the improved procedures developed in the present work because all four deoxyribonucleosides are separated and nucleotides are eluted in a small volume. Fig. 4 shows an example of such separations on a 0.8 cm × 20 cm column of Fraction B. A load of 0.5 $A_{260\ m\mu}$ unit of nucleosides and 15 $A_{260\ m\mu}$ units of nucleotides in 1 M ammonium acetate still gives a satisfactory resolution. In order to calculate the chain length of the starting oligonucleotides, readings are taken at 271 mμ at neutral pH for both nucleotides and nucleosides.

The amounts of nucleosides are estimated from their absorption readings at λ_{max}, pH 7.

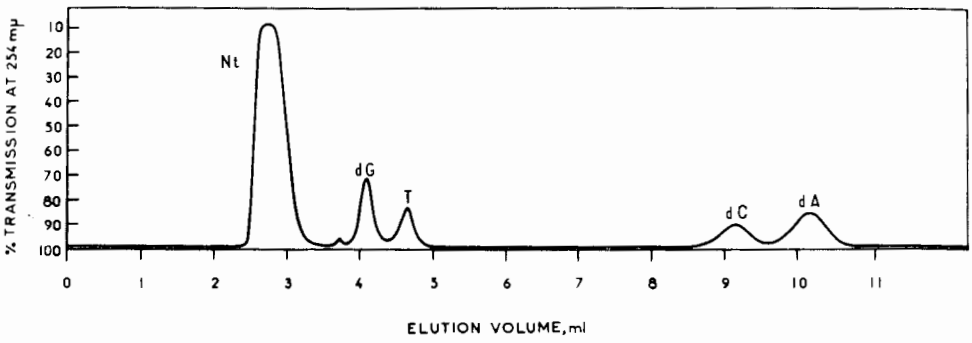

Fig. 4. Chromatography of a mixture of nucleotides (5 $A_{260m\mu}$ (pH 7) and nucleosides (0.5 $A_{260m\mu}$ (pH 7)) on a Fraction B column (0.8 cm × 20 cm). Load volume was 50 μl. Sample was 1 M in ammonium acetate. Flow rate was 6 ml/h.

Separation of ribonucleosides

This is also possible with a single column of P-2 (< 400 mesh). Using a 0.8 cm × 60 cm Fraction B column we obtained an excellent separation comparable with that shown in Fig. 2b for the deoxyribonucleosides.

In the case of the ribonucleosides the elution order is uridine, guanosine, cytidine, adenosine in agreement with VAN DEN BOS *et al.*[12]. It is interesting to notice the different chromatographic behavior of uridine and thymidine. In fact, uridine precedes guanosine, whereas thymidine follows deoxyguanosine; guanosine and deoxyguanosine have the same elution volumes.

Separation of ribo- and deoxyribonucleosides

In preliminary investigations on the chromatography of nucleosides on Bio-Gel P-2 columns, we succeeded in separating each ribonucleoside from its homologous deoxyribonucleoside on 0.8 cm × 50 cm Bio-Gel P-2 columns 200–400 mesh; in each case, the nucleosides of the ribo series were eluted first. The solvent used was 0.1 M $(NH_4)_2CO_3$, 1 mM $Na_2B_4O_7$ adjusted to pH 10.1 with NH_4OH.

When we attempted the separation of the eight nucleosides on 0.8 cm × 60 cm columns of Fraction B using the same solvent, we were not successful since incom-

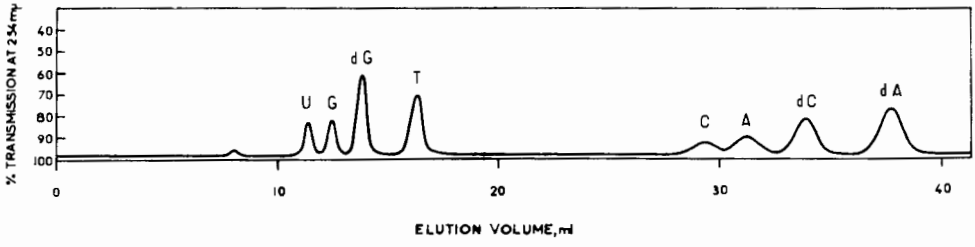

Fig. 5. Chromatography of a mixture of deoxyribonucleosides (2 $A_{260\ m\mu}$ (pH 7) units) and ribonucleosides (1 $A_{260\ m\mu}$ (pH 7) unit) on a Fraction B column (0.8 cm × 68 cm). Load volume was 50 μl. Flow rate was 2.8 ml/h.

plete separations were obtained. We tried then different borate concentrations along with 2 mM $(NH_4)_2CO_3$ (pH 10.2). The results obtained with 0.1 mM borate are shown in Fig. 5. In this series of experiments we observed that the separation was independent from the relative amounts of the nucleosides of the two series loaded on the columns. The separation of the eight nucleosides from the two series has been developed in view of its potential usefulness in investigations on DNA–RNA hybrids. It should be noted that a thin layer chromatographic method is also available for this seperation[13].

DISCUSSION

As already pointed out previously[1], the determination of the DNA base composition after enzymatic digestion to nucleosides has none of the disadvantages of other methods involving bases or nucleotides. Acid hydrolysis causes a selective destruction of the liberated bases; in addition some bases are selectively lost because of their poor solubility. On the other hand, nucleotides which can be obtained by enzymatic degradation may undergo partial dephosphorylation during concentration procedures[2]. Enzymatic digestion to nucleosides with the acid hydrolases from spleen prepared in our laboratory gives very satisfactory results; its only handicap, the incomplete dephosphorylation of nucleotides, is not serious since less than 5 % nucleotides are present in the hydrolysate at the end of digestion. Control experiments have shown that, although the initial rate of dephosphorylation is slightly higher for purine than for pyrimidine nucleotides, no preferred dephosphorylation can be detected at the end of digestion.

The mechanism underlying the separation of nucleosides on Bio-Gel P-2 columns has been investigated in several of its aspects[1,14]. Even if further studies are needed to clarify some particular problems, it is difficult to escape the conclusion that absorption phenomena play a very important if not an exclusive role in this type of chromatography, as shown by the fact that $K_{available}$ of nucleosides is equal to, or larger than, 1.0.

The improved resolving power of the polyacrylamide columns obtained with fractionated P-2 (< 400 mesh) seems to be due to two main factors: the increased surface available for nucleoside absorption, and therefore the increased number of theoretical plateaus in the columns, and the more uniform size distribution of the gel beads. The use of a single column is obviously less laborious than the previous two-column method. In the latter case the calculation of the molar fraction of nucleosides present in a digest was complicated by the necessary evaluation of the recovery obtained in the second chromatography. In fact, the total amount of nucleosides loaded on the second column could only be determined *via* the ε values at 271 mμ, pH 10.3, of the unresolved nucleoside solution, or by adding a third nucleoside to the mixture. It should be stressed that a presentation of several conditions of chromatography and determination of nucleosides was given here since they require quite different experimental set-ups.

Among the advantages of the present method over other existing techniques, the most important ones are the following: (1) the amount of DNA which can be easily analyzed is about 0.5 μg of DNA; it should be stressed that limitations to

further scaling down come rather from the detection system used than from chromatography itself; (2) the solvent contains a very small amount of a volatile salt; fractions may therefore be concentrated and desalted by rotary evaporation; the pH of the solvent is such that no chemical modifications of nucleosides can take place; (3) only commercially available instruments are used; experimental conditions are such that no high pressure nor high temperature is required.

Finally, it should be noted that analysis with Fraction C columns have already been extensively used in our laboratory with very satisfactory results. Analyses have mainly concerned terminal nucleotides[15] and yeast mitochondrial DNA.

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