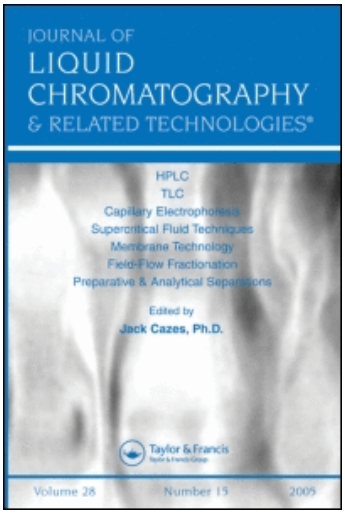

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PECULIARITIES OF THE RETENTION MECHANISM OF CIRCULAR AND LINEAR DNA FRAGMENTS USING NON-EQUILIBRIUM CHROMATOGRAPHY

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PECULIARITIES OF THE RETENTION MECHANISM OF CIRCULAR AND LINEAR DNA FRAGMENTS USING NON-EQUILIBRIUM CHROMATOGRAPHY

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ABSTRACT

Two different methods have been used to investigate the retention mechanism of a series of DNA fragments in non equilibrium chromatography (NEC) over a range of column temperatures (T) and with different mobile phase flow-rates (F). The first approach was the separate study of each factor affecting the retention mechanism; the second method was the simultaneous variation of all these factors. The apparent relative retention time of each DNA fragment was used as a retention marker.

The data obtained showed that the retention mechanism of the DNA fragment was dependent on the F and T values, and also on the DNA form, i.e., linear or circular form. For low F values, the retention mechanism was based on the classical hydrodynamic

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regime (HDC) specific to the circular form. On the contrary, for the linear forms, their retention mechanism was based on a slalom chromatographic process (SC) specifically for high F values at low column temperature. This confirms that the SC and HDC modes are interconnected and that the HDC⇆SC transition exists and was clearly visualized.

In addition, using a simplex process connected to the chemometric methodology, the optimal conditions for F and T were calculated to obtain the more efficient separation of the linear DNA forms in a minimum analysis time.

INTRODUCTION

The separation of biopolymers such as DNA by conventional HPLC modes is usually based on an equilibrium phenomenon between mobile and stationary phases. In the case of exchange ion chromatography, the retention mechanism is dependent on the electrostatic interaction between the phosphate groups of the DNA molecules and the cationic groups of the stationary phase. For hydrophobic-interaction chromatography, an additional contribution to the macromolecule retention is encountered via the hydrophobic effect between the stationary phase and DNA bases. Finally, the gel permeation chromatography is dominated by the capability of the DNA molecules to penetrate into the stationary phase pores in relation to their size.

Alternative chromatographic procedures are available for the separation of flexible biological or synthetic polymers. These two techniques, slalom chromatography (SC) and hydrodynamic chromatography (HDC), are based on the use of the laminar flow which occurs in the interstitial spaces created between the particles packed in the column.[1–12]

The separation process depends on the flow rate and the particle size of the column packing and not on their pore size or chemical nature. The HDC has been principally developed and applied to the separation of synthetic polymers such as polystyrenes.[4–6] The elution order in HDC is the same as in gel permeation chromatography due to the exclusion for the large polymers from the low velocity regions near the particle wall.[4] The separation is only valid when the polymer is in a random coil form.[4]

The separation in SC has been reported for double stranded DNA molecules.[6–11] The elution order for the DNA molecule is the opposite to that expected for a HDC mechanism, the larger strands are eluted after the smaller ones.[6] When the DNA chain is applied to a chromatographic system, it frequently turns around the spherical obstacles; the larger the fragments the more difficulty it has to travel across the interstitial spaces created inside the column.

These two techniques, HDC and SC, cannot be explained in terms of an equilibrium constant between the mobile and the stationary phase and form a global separation mode based on a non equilibrium principle; called non equilibrium chromatography or NEC. This paper describes a study, both by chemometric methods and traditional methodology, for a separate study of each individual factor of the effect of the mobile phase flow-rate and column temperature on the apparent relative retention time of DNA fragments. A study was then conducted to elucidate the retention mechanism variation with F and T and for circular and linear DNA fragments.

THEORY

In the proposed hydrodynamic model, the column packing was treated as a three dimensional network of pores with an average diameter r. This diameter is linked to the average particle diameter d_p and the interparticle porosity n by:[12]

$$r = 0.42\ d_p\ (n/(n-1)) \quad (1)$$

The Gaussian chain model with a persistence length was used to describe the semi-rigid conformation of the DNA chains.[13–15] The progression of the DNA fragments through the closed column packing depended on the respective values of the pore diameter r and the DNA gyration radius Rg. For Rg <<< r, the DNA could maintain its random coil conformation and the DNA fragments were separated on the basis of hydrodynamic chromatography (HDC).[4–6] For Rg >>> r, the DNA molecule progressed through the closed column packing as a snake edging its way into long grass (Fig 1).

In this case, the DNA fragments were separated on the basis of slalom chromatography (SC).[6–11] Using the retention time t_R of the various DNA species and the void time t_o defined by KI, the apparent relative retention time RRT equal to t_R/t_o was used as a DNA retention marker.

Traditional Methodology (T)

This method was used to separately study the influence of mobile phase flow-rate F (mL/min) and the column temperature T, on the relative retention time RRT. For a flow-rate equal to 0.03 mL/min, the RRT of each DNA-molecule was measured at 270 K, 273 K, 278 K, 283 K, 288 K, 293 K, 298 K, 303 K, 308 K, 313 K, 318 K, 323 K, 328 K, 333 K. Experiments were performed at each of these temperature with F equal to 0.1, 0.2, 0.5, 1.4 mL/min. A total of 56 experiments were performed.

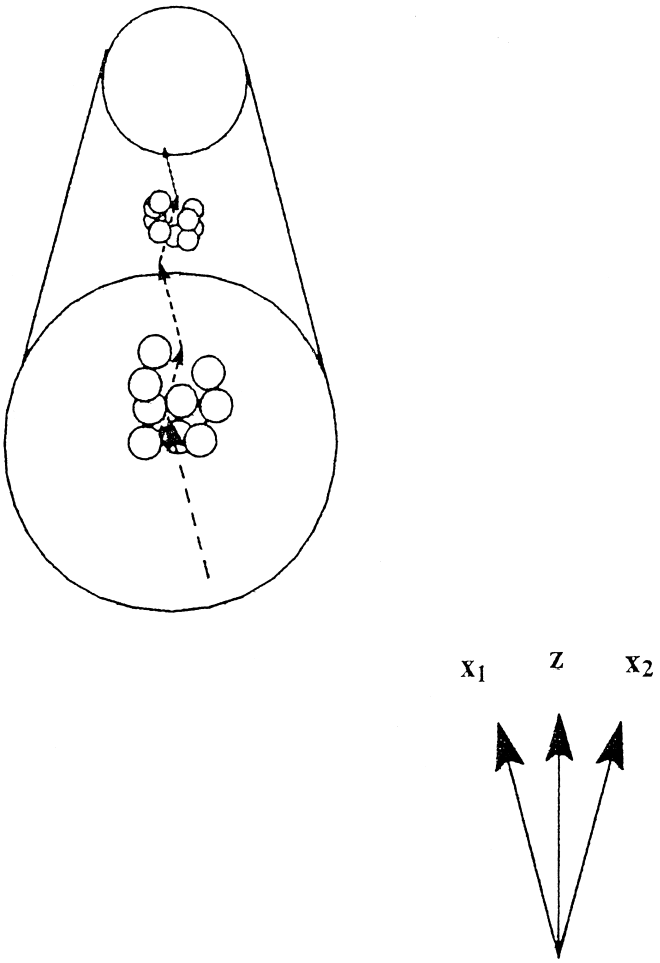

Figure 1. Representation of the DNA chains (arrows) through the closed column packing particles.

Chemometric Methodology (CH)

With this method only 9 experiments were needed. They were selected using a composite central design[16,17] as explained elsewhere.[18–20] This method determined a second order experimental design which linked the RRT of each DNA molecule to F and T.

$$\ln RRT = a_0 + a_1 \ln F + a_2 \ln T + a_{11} (\ln F)^2 + a_{22} (\ln T)^2 + a_{12} \ln F.\ln T$$

where a_0, a_1, a_2, a_{11}, a_{22}, a_{12}, were constants. (2)

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Shimadzu pump LC 10 AT VP (Touzart et Matignon, Courtaboeuf, France), an Interchim Rheodyne injection model 7125 (Montluçon, France) fitted with a 20 μL sample loop, and a Merck L 4500 diode array detector. A C1 Kromasil column (particle size: 5 μm, column size: 150 mm × 4.6 mm) supplied by Interchim, was used with controlled temperature in a TMN°701 Interchim oven.

Reagents

Circular doule-stranded DNA (P_3, P_5, P_{10}) with λ DNA (48.5kb) and restriction enzyme KpnI were supplied by New England Biolabs (Gagny, France). Ethanol, EDTA, acetonitrile, sodium hydrogen phosphate, and sodium dihydrogen phosphate were purchased from Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France), fitted with a reverse osmosis cartridge.

Digestion of λ DNA

A restriction enzyme KpnI was used for cleavage of the lambda DNA into three fragments of different sizes: 29.95 kb, 17.05 kb, and 1.50 kb. The λ DNA (2 μg) was treated with 3U of 15 KpnI in 15 μL of the reaction mixture at 37°C for 3 hours, precipitated by ethanol, dissolved in 20 μL of water and stored at –20°C until use.

Chromatographic Conditions

The mobile phase consisted of a sodium phosphate salt, 0.010 M-EDTA 0.001 M mixture at pH = 6.8. The column temperature varied from −3 to 60°C. 20 μL of DNA solution were injected and the retention times were measured at different flow-rate values varying from 0.03 to 1.40 mL/min.

RESULTS AND DISCUSSION

Hirabayashi and Kasai[21] have shown that columns developed for reversed-phase chromatography (such as the C1 used in this study) are useful for slalom

chromatography. In order to eliminate a possible hydrophobic interaction, which could interfere with the hydrodynamic principle, they used an aqueous mobile phase containing 5 to 20% of organic modifier such as acetonitrile.[21] It was found that the hydrophobic interaction was negligible in such conditions. Thus, all the experiments were carried out with a bigger proportion of acetonitrile in the mobile phase (20%).

The RRT values were calculated using both traditional methods and the chemometric method. Each experiment was repeated three times. The coefficient of variation of the RRT values was less than 0.05%, indicating the high reproducibility and stability of the chromatographic system. The results given by the experimental design were processed by computer, and the parameters of Eq.2 were obtained.

The fitting of the model to the results was good (96% was the worst value obtained for the circular DNA fragment P_3). With only the terms lnF, lnT in Eq. 2, the worst fit of the model to the results was 17%. On adding the interaction term lnF.lnT, the fit improved slightly (45%). Finally, the addition of the second order terms a_{11} $(lnF)^2$ and a_{22} $(lnT)^2$ resulted in an excellent fit, 96%.

Thus, Eq. 2 was statistically meaningful. The correlation between predicted and experimental RRT is presented in Figure 2. The slope 1.03 (ideal is 1.00) and r^2 (0.997) is indicative of excellent correlation between predicted and experimental relative retention time. The surfaces corresponding to Eq. 2 was plotted as a three dimensional diagram for linear DNA fragments (Figure 3). When F increased or T decreased, the steady state extension of the linear DNA fragment[22] increased producing an increase in the RRT values. This result confirmed that the hydrodynamic force generated by the mobile phase constituted the main parameter which governed the retention behavior of DNA in SC.

The surfaces (Figure 3) showed that: (I) the RRT values varied strongly with the column temperature in the opposite direction to the mobile phase flow rate which was previously defined as the main parameter of this chromatographic mode;[22] (II) above a critical F value ≈ 0.30 mL/min; the linear DNA fragment was close to being fully elongated and the RRT values remained relatively constant.[22]

Figure 4 represents the response surface of a plot ln(RRT) values against F (mL/min) and the DNA fragments (circular and linear forms). Two zones on the graph can be differentiated. At the left of this surface, corresponding to the first zone containing the DNA circular forms, the ln(RRT) variations with F values are very weak. In this region, the separation of the circular forms P_3, P_5, P_{10} appeared only at the lowest mobile phase flow-rate. For these lowest mobile phase flow-rates, the circular DNA fragment maintained its random coil conformation during its progression in this pore network.

In this case, the elution order of the DNA fragments through the column was the same as in gel permeation chromatography due to the exclusion for the

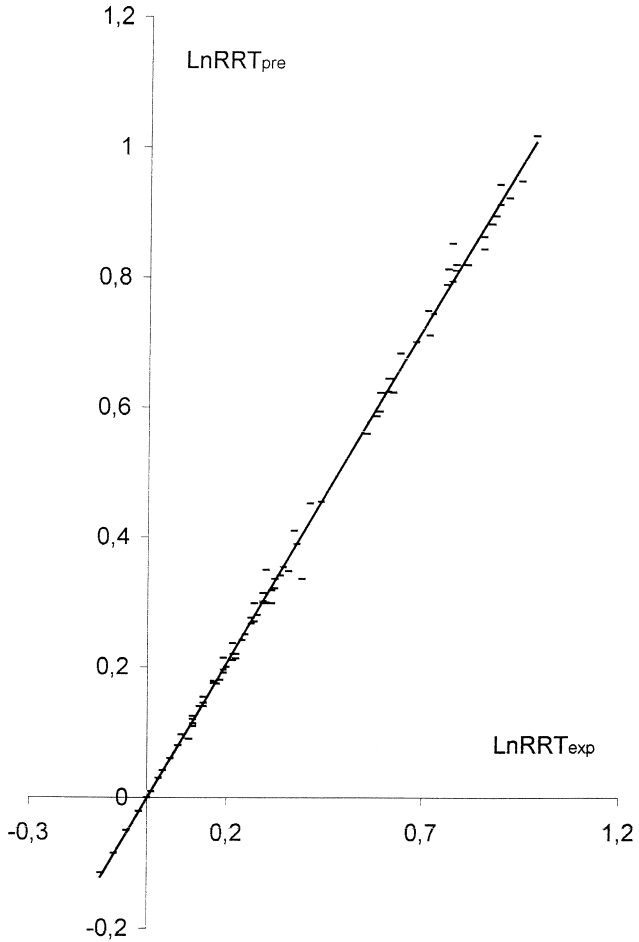

Figure 2. Correlation between predicted (Eq.2) and experimental apparent relative retention time for the DNA fragments. The slope is 1.03 with a r^2 coefficient of 0.997 as determined by linear regression.

large circular DNA fragment from the low velocity regions near the particule well. The circular DNA fragment in this region can therefore be separated via the hydrodynamic chromatographic (HDC) mechanism HDC.[4–6] At the right of this surface, corresponding to the second zone containing only the DNA linearized forms, a strong increase in ln (RRT) values was observed. In this region, the DNA linearized forms, which have a highly flexible structure contrary to the circular forms, can progress through the closed column packing like a snake.

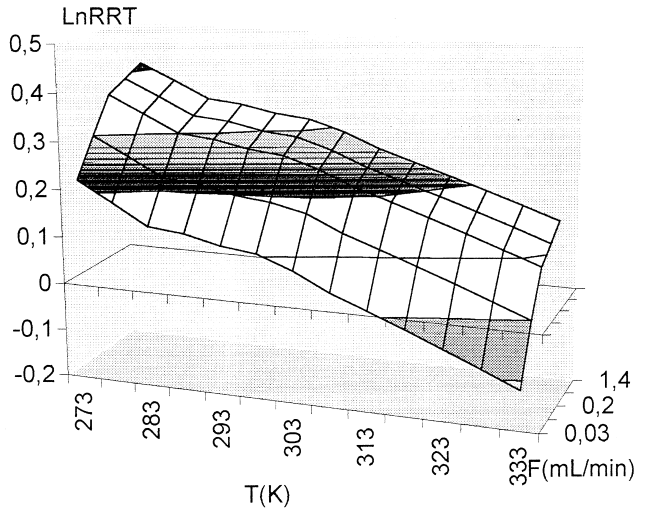

Figure 3. Response surface of ln (RRT) values against F and T for linear DNA fragments.

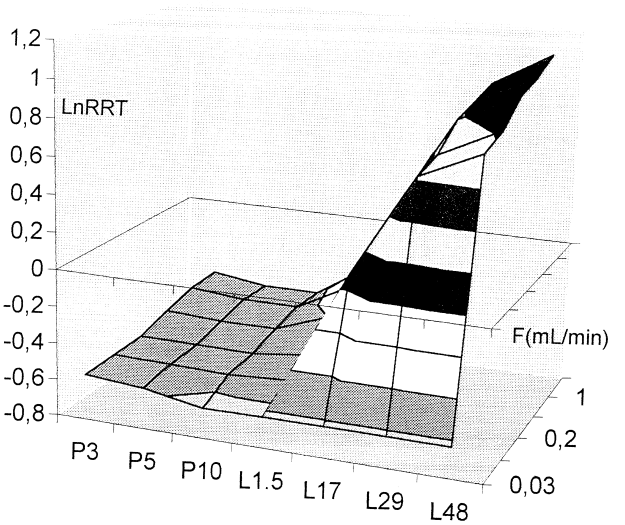

Figure 4. Response surface of ln (RRT) vs mobile phase flow rate/circular and linear DNA fragments. T = 25°C.

The elution order is obviously the opposite of the one obtained in HDC. In this region, the linearized forms can be separated on the basis of the SC mechanism. The retardation rate, i.e., $\lambda = \partial(\ln RRT)/\partial F$ is much higher than in the region containing the DNA circular forms (first region) due to the very high flexibility of the DNA linear forms. In the second region, the λ values were the lowest for the highest mobile phase flow rates because the DNA linearized forms were close to being fully elongated. The middle of the surface (~ $L_{1.5kb}$ fragment) corresponded to the HDC ⇆ SC transition. The HDC and SC mechanism were thus, interconnected and formed the global mode of separation based on a non equilibrium principle called non equilibrium chromatography or NEC.

An example of separation optimization of the four linear DNA fragments was studied. The selectivity α_{app} between two DNA fragments defined as the ratio of their apparent relative retention time was used as an image of the role of both column temperature T and flow-rate F on the separation quality. The separation quality of the linear DNA fragments was assessed by means of a response function ξ defined as:[18,23]

$$\xi = \text{Min}\,(\alpha_{app}) \text{ if Min } (\alpha_{app}) \le \alpha_{app,l}$$
$$\xi = \alpha_{app,l} + \frac{1}{t_a} \text{ if Min } (\alpha_{app}) \ge \alpha_{app,l} \quad (3)$$

where $\text{Min}(\alpha_{app})$ is the selectivity for the worst separated pair of peaks on the chromatogram. $\alpha_{app,l}$ called the apparent limit selectivity, is the minimum value of the accepted selectivity.

In our application, $\alpha_{app,l}$ was 1.07. Therefore, if the selectivity for the worst separated pair of peaks on the chromatogram was lower than the chosen limit selectivity, then the ξ function would be equal to the selectivity. If not, separation conditions were obtained and then the analysis time t_a intervened in the form $1/t_a$. t_a depends on the RRT value of the last peak (RRT_l) on the chromatogram, i.e. the linear 48.5 kb DNA linear fragment.

$$t_a = t_o \times RRT_l \quad (4)$$

The t_o logarithm was modeled by Eq. 2 with an r^2 value equal to 0.999. Knowing the variation of the apparent selectivity values of the DNA linear fragments and the analysis time with F and T (Eq. 4), the ξ values (Eq. 3) can be given for different values of the two factors. By using a simplex process,[18,23] ξ reached its maximum for F = 1.20 mL/min and T = 20°C. The corresponding chromatogram is given in Figure 5.

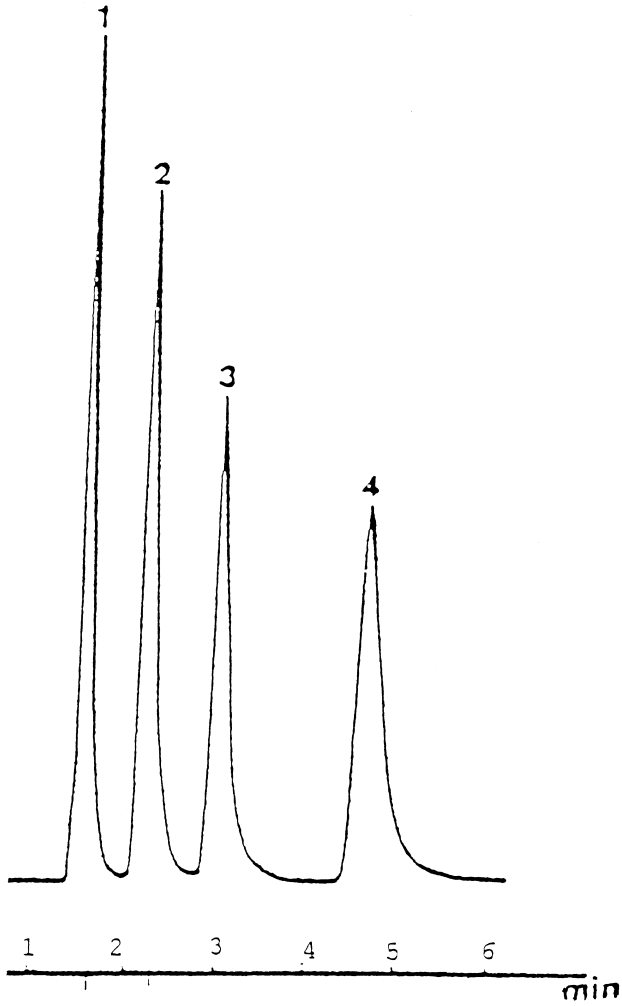

Figure 5. Chromatography of the digestion of the λ DNA (48.5 kb) (peak 4) into three fragments of different sizes: 29.95 kb (peak 3), 17.05 kb (peak 2), and 1.50 kb (peak 1). T =20°C, F =1.20 mL/min.

CONCLUSION

The retention mechanism of DNA fragments using non equilibrium chromatography (NEC) techniques was studied for linear and circular fragments by two different methods. From the apparent relative retention time of each frag-

ment, it was shown that the retention mechanism in NEC was strongly dependent on the polymer extension in the interstitial space created in the column. The slalom chromatography regime appeared specifically at high F values for low column temperature and for the more flexible DNA fragments, i.e., the linear forms. For the DNA circular forms (the lowest flexible forms), the separation mechanism was obtained particularly at low F values via a hydrodynamic chromatography (HDC) mode. The results showed that the HDC and SC mechanisms can be interconnected and the HDC ⇆ SC transition was clearly visualized. In addition, with the chemometric methodology associated with a simplex process, it was possible to calculate the optimal F and T values to obtain the best separation of the linear DNA fragments with a minimum analysis time.

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