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Injection bias of DNA fragments in capillary electrophoresis with sieving

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Abstract

The relative amounts of DNA fragments in a mixture injected into the capillary by electromigration or hydrodynamically by pressure were compared. Even if the electrophoretic mobilities of DNA fragments with different sizes are the same in a free solution in the sample vial, the size bias is brought about by the different mobilities in a sieving medium and by the electroosmosis. The experiments were performed in capillaries filled with a solution of liquified agarose, a replaceable sieving medium. The experimental results were compared with a theoretical model.

1. Introduction

The most important aspect for a quantitative interpretation of electropherograms is the knowledge of a reliable method for the transformation of the peak parameters into the injected amount of a sample. The knowledge of the absolute amount of the sample introduced into the capillary in almost all known cases is eliminated by using the same techniques as those currently used in chromatography [1], namely (i) internal standard technique, (ii) internal normalization technique and (iii) calibration technique where the same amounts of the standards and samples are introduced in the same way regardless the fact that the actual amounts are not known.

By examining all these techniques in detail, one can find that there is always one prerequisite, namely that the sample introduced into the separation column is a real aliquot of the sample existing in the sample vial. The molar ratios of the solutes introduced into the column must be the same as those in the original sample. In capillary electrophoresis (CE), this condition is always fulfilled when the sample introduction is done by siphoning overpressure or underpressure [2]. There is, however, a very convenient system of sample introduction into the capillary, electromigration injection. Here, the solutes are driven into the capillary by an electric field and by electroosmotic flow simultaneously. It is well known that this technique does not introduce an aliquot; rather, a strong discrimination among solutes exists, depending on their mobilities in the sample solution [3,4]. Regardless of these drawbacks, the electromigration injection of a sample by a voltage applied at one electrode in a sample vial is one of the favourite techniques in CE and is the exclusive method of sample introduction into capillaries filled with cross-linked gels.

During recent experimental studies [5], we

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observed a bias between electromigration injection and overpressure injection of DNA samples, which could not be explained by existing physico-chemical models [3,4,6]. The objective of this paper is to examine this bias and develop a mathematical description of the situation where the samples of DNA fragments in free solutions are injected into a capillary filled with a sieving medium. Further, we would like to show that our theory brings a solution to a general problem of electromigration sampling in the case where a solute has a certain mobility in a sample vial and a different mobility inside the capillary.

2. Theory

Let us assume a sample containing n solutes i in a free solution, the concentration of these solutes being c_i. The molar ratio $\phi_i[m]$ of a species i in the sample is

$$\phi_i[m] = \frac{c_{i,1}}{\sum_{i=1}^{n} c_{i,1}} \tag{1}$$

When applying hydrodynamic or pressure injection, part of a sample is hydrodynamically introduced into a capillary. The total injected amount is

$$m_i = S_2 c_{i,1} v \, \Delta t \tag{2}$$

where v is the mean velocity determined by the flow-rate at which the sample is introduced into the capillary, S_2 is the capillary cross-section and Δt is the injection time. For the molar ratio of a species i in the sample injected,

$$\phi_i[m, p] = \frac{c_{i,1}}{\sum_{i=1}^{n} c_{i,1}} = \phi_i[m] \tag{3}$$

where $\phi_i[m]$ and $\phi_i[m, p]$ denote the molar ratio in the sample solution and the molar ratio injected by pressure, respectively. Eqs. 2 and 3 show that hydrodynamic injection is without any preference with respect to the components. Here, a real aliquot is injected, i.e., the molar ratio of a species i injected is equal to that in the sample vial.

When applying electromigration injection, the injection end of the capillary and an appropriate electrode is dipped into the sample solution and high voltage is applied for a certain period of time. Let us imagine this situation as a transport of ions through the gel–sample solution interface. For the theoretical treatment of the present problem, we assume steady-state conditions at the interface during the whole period of the injection. The steady-state conditions imply no changes in the concentrations during the injection at both sides of the interface with time. However, adjustment of concentrations when crossing the boundary proceeds. Further, we neglect electroosmosis; the case with electroosmosis is treated later. It follows from the continuity equation [7] that the mass fluxes J_i in the sample solution (subscript 1) and in the capillary (subscript 2) of injected component i with molar concentrations c_i and its effective electrophoretic mobilities u_i are equal:

$$J_{i,1}[e] = S_1 E_1 u_{i,1} c_{i,1} = J_{i,2}[e] = S_2 E_2 u_{i,2} c_{i,2} \tag{4}$$

where $[e]$ denotes the conditions with electromigration injection and S and E are the capillary cross-sections and electric field strengths during the injection at sides 1 and 2 of the boundary, respectively. The complex geometry of the injection vial is represented here by effective cross-section S_1 and electric field strength E_1 for the sake of simplicity. It is convenient to express the electric field strength by electric current density ι and specific conductivities κ [8], which are related by the equation $E = \iota/\kappa$. The cross-sections on both sides of Eq. 4 can be eliminated by a further substitution of electric current I for current density $\iota = I/S$. The electric current I must be equal through the whole system and hence it holds that

$$c_{i,1} \cdot \frac{u_{i,1}}{\kappa_1} = c_{i,2} \cdot \frac{u_{i,2}}{\kappa_2} \tag{5}$$

The total amount of a component i injected into the capillary with known geometry and electric field strength can be expressed as the mass flux

$J_{i,2}$ in Eq. 4 multiplied by the injection time Δt. After the substitution of the unknown concentration $c_{i,2}$ in Eq. 4 with the help of Eq. 5, we have

$$m_i = c_{i,1} \cdot \frac{u_{i,1}}{\kappa_1} I \Delta t \quad (6)$$

Realizing that $c_{i,1}u_{i,1}/\kappa_1$ is $T_{i,1}/F$, where T is the transference number and F the Faraday constant, and $I\Delta t = Q$, where Q is number of coulombs, Eq. 6 is identical with relationship already derived by Boček and Chrambach [9]. The equation predicts an injection bias, which is determined by the differences in electrophoretic mobilities of various substances $u_{i,1}$ inside the sample vial and is independent of the mobilities $u_{i,2}$ inside the capillary. It further follows that the information needed for the calculation of the absolute amount of substance i injected cannot be obtained from a single experiment. The specific conductance κ_1 and mobility in the sample solution $u_{i,1}$ must be determined independently. For the molar ratio $\phi_i[m, e]$ of an injected solute, it holds that

$$\phi_i[m, e] = \frac{c_{i,1}u_{i,1}}{\sum_{i=1}^{n} c_{i,1}u_{i,1}} \quad (7)$$

Obviously, the molar ratio $\phi_i[m, e]$ is independent of all operational parameters $(S, I, \Delta t)$; however, it is dependent on $u_{i,1}$ and suffers with bias with respect to $\phi_i[m, p]$.

Let us now consider the analysis of DNA fragments where the sample vial contains their free solution. In free solution, the mobilities of various fragments are the same [10], i.e., $u_{i,1}$ is constant for all of them. In practice, at 25°C, it amounts ca. $36.7 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}.

Thus, according to Eq. 7, the molar ratio of a fragment reaching the gel interface is controlled by its concentration in the sample reservoir. Assuming size-independent mobility, the solution to Eq. 7 is $\phi_i[m] = c_{i,1}/\Sigma_i\, c_{i,1}$. This means that the amount injected is given by the molar ratio of component i in the original sample. It holds that

$$\phi_{\mathrm{DNA}}[m, e] = \phi_i[m] \quad (8)$$

regardless of fact that the mobilities of DNA inside the capillary may be mutually different.

However, the situation may be dramatically changed by the presence of electroosmosis. Eqs. 7 and 8 hold true if there is no electroosmotic flow inside the capillary and the ions move under the action of electrophoresis only. In the case of the separation system for DNA we are usually working at slightly alkaline conditions (pH 8.3) and electroendoosmosis must be taken into account. The sample is injected from free solution of DNA where all fragments have the same mobility and the capillary is filled with a sieving medium where the mobilities are reduced proportionally to the fragment sizes, or number of base pairs (bp).

The situation is depicted in Fig. 1. The DNA molecules migrate into the capillary at constant velocity from the free electrolyte but they are slowed at the interface between the free solution of the sample and the sieving medium inside the capillary. This interface may be considered to coincide with the plane of the capillary inlet opening. Behind this plane the fragments continue with selectively reduced mobilities.

However, the bulk electroosmotic flow, with an absolute mobility an order of magnitude lower than the electrophoretic mobility of DNA polyanions, acts against their movement and pushes the sample plug backwards. As a result, the small rear part of a sample is shifted out of

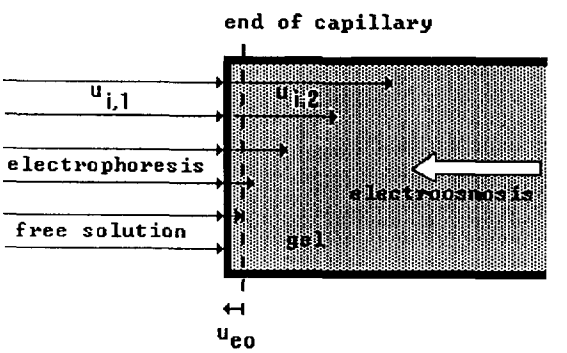

Fig. 1. Schematic representation of injection by electromigration. The electrophoretic mobility $u_{i,1}$ of the DNA fragments in free sample solution is independent of size. The size-selective mobility of the fragments in a sieving medium $u_{i,2}$ is affected by electroosmosis with mobility u_{eo}.

the capillary. The portion of sample molecules in this rear part of the sample plug is lost for the subsequent analysis, since it is washed out during the change of the sample vial to the electrode chamber before the start of analysis. The mass flux $J_{i,2}[e, eo]$ can be expressed similarly as in Eq. 4 and it holds that

$$J_{i,2}[e, eo] = S_2 E_2 (u_{i,2} \pm u_{eo}) c_{i,2} \quad (9)$$

The ± sign expresses the situation that electroosmosis may move in the same (+) or in the opposite (−) direction with respect to solute i. For the sake of simplicity, let us take an actual case with anodic migration of DNA fragments, where $u_{i,2}$ is positive, and cathodic electroosmosis, where u_{eo} is negative, and a minus (−) sign applies.

In our model, we assume that the concentration inside the capillary c_{i2} is adjusted as described by Eq. 5, as the bulk electroosmotic flow is a non-selective transport and does not affect the velocity at which the sample molecules enter the gel–sample interface. Under these assumptions, Eq. 5 can be used for the evaluation of $c_{i,2}$ and the amount injected can be expressed by

$$m_i = c_{i,1} \cdot \frac{u_{i,1}}{\kappa_1} \cdot I \, \Delta t \left(1 - \frac{u_{eo}}{u_{i,2}}\right) \quad (10)$$

This equation predicts an electromigration injection bias. As already stated, $u_{i,1}$ relates to the free solution in the sample vial and is the same for all DNA molecules, hence the respective molar ratio in the introduced sample is given by the relationship

$$\phi_i(e, eo) = \frac{c_{i,1}\left(1 - \frac{u_{eo}}{u_{i,2}}\right)}{\sum_{i=1}^{n} c_{i,1}\left(1 - \frac{u_{eo}}{u_{i,2}}\right)} \quad (11)$$

It follows that the amount injected by electromigration of a sample component is controlled by the ratio of the electroosmotic mobility to its effective mobility inside the medium and size-biased injection can be expected.

Let us look now at the records where UV absorbance detection is used and the peak area or peak height is followed as the quantity corresponding to the amount of solute present in a sample. The situation is similar to chromatography and the concept introduced by Novák [1] can be adopted. The theory declares a linear additivity of the analytical property, ε_i, to the concentration of a solute i in the detection window, $c_{i,2}$, and a linear response of the detector, f, to the analytical property. In the case of polynucleotides, the additivity of the analytical property of the molecule to the number of bp of the chain is also valid [11]. The analytical property of the monomer, or one bp, is denoted ε_{mi}. Then the instantaneous detector response, R_i, can be written as

$$R_i = f \varepsilon_{im} p_i c_{i,2} \quad (12)$$

where p_i is the number of bp in the fragment i.

The peak area A_i, in a strip chart, can be expressed as the time integral of the $R_i(t)$ function multiplied by the recorder chart speed b [1]:

$$A_i = bf \varepsilon_{im} p_i \int_{t_1}^{t_2} c_{i,2}(t) \, dt \quad (13)$$

where f is the response factor including the apparatus and recorder constants and t_1 and t_2 are the passage times of the beginning and the end of the peak, respectively. The integral in Eq. 13 can be evaluated with the help of the amount injected. The total amount of a fragment i inside the capillary is given by the relationship

$$m_i = S_2 (u_{i,2} - u_{eo}) E \int_{t_1}^{t_2} c_{i,2}(t) \, dt \quad (14)$$

and is equal to the injected amount m_i in Eq. 2 or 10 for electromigration and pressure injections, respectively. Here, E represents the electric field strength in the capillary during the run of an analysis. By the combination of Eq. 14 with Eq. 2 or 10, the integral can be evaluated and used in Eq. 13. Then, the peak area of a solute i injected by electromigration is

$$A_i[e] = bf \varepsilon_{im} p_i c_{i,1} \cdot \frac{u_{i,1}}{\kappa_1} \cdot \frac{t_2}{u_{i,2} E} \cdot \Delta t \quad (15)$$

and by pressure injection is

$$A_i[p] = \frac{bf\varepsilon_{im} p_i c_{i,1} v\, \Delta t}{(u_{i,2} - u_{eo})E} \quad (16)$$

Eqs. 15 and 16 show the similarities and the differences in the character of the dependence of peak area on the size of molecule for both electromigration and pressure injections. Obviously, it holds for electromigration injection that

$$A_i[e] \sim k_e \cdot \frac{p_i}{u_{i,2}} \quad (17)$$

and for pressure injection that

$$A_i[p] \sim k_p \cdot \frac{p_i}{u_{i,2} - u_{eo}} \quad (18)$$

It is evident that the smaller is u_{eo}, the more similar are $A_i(e)$ and $A_i(p)$. Also, the appearance of the electropherograms for both injections should be similar. From both equations the relative peak areas, $\phi_i[A, e, eo]$ and $\phi_i[A, p, eo]$, with respect to the total area of an electropherogram can be derived as follows. For injection by electromigration, it holds that

$$\phi_i[A, e, eo] = \frac{c_{i,1} \cdot \dfrac{p_i}{u_{i,2}}}{\sum_{i=1}^{n} c_{i,1} \cdot \dfrac{p_i}{u_{i,2}}} \quad (19)$$

and, for pressure injection

$$\phi_i[A, p, eo] = \frac{c_{i,1} \cdot \dfrac{p_i}{(u_{i,2} - u_{eo})}}{\sum_{i=1}^{n} c_{i,1} \cdot \dfrac{p_i}{(u_{i,2} - u_{eo})}} \quad (20)$$

It is again obvious that for negligible electroosmosis both types of the sample introduction give identical results.

The peak height is another parameter for quantitative analysis. The relationship between the area under the curve A and its height h follows from the Gaussian curve theory: $A = h\sigma_x\sqrt{(2\pi)}$, where σ_x is the standard deviation expressed in length units. In reality, the peaks are not perfectly Gaussian and, therefore, the peak height can be used for quantitative evaluations only if the operating conditions are the same, i.e., the dispersions and the shapes of the peaks are approximately the same. The use of peak heights brings some special advantages, however, when the direct unambiguous comparison of the peaks by eye is very important. It is very important that the peak height is the quantity that is not directly affected by the migration velocity of a substance and by the recorder chart speed. The peak height is dependent on the maximum concentration c_i^*, the analytical property ε_i and the response factor of the detector, including the apparatus and recorder constant f. We can assume that the maximum concentration and the total amount injected (Eq. 10) are directly proportional with constant k. Then the maximum concentration for the injection by electromigration is

$$c_{i,2}^* = kI \cdot \frac{c_{i,1}}{\kappa_1} \cdot u_{i,1}\left(1 - \frac{u_{eo}}{u_{i,2}}\right) \Delta t \quad (21)$$

and for the related peak height, it holds that

$$h_i[e, eo] = f\varepsilon_{im} p_i kI \cdot \frac{c_{i,1}}{\kappa_1} \cdot u_{i,1}\left(1 - \frac{u_{eo}}{u_{i,2}}\right) \Delta t \quad (22)$$

Similarly, for pressure injection, with the help of Eq. 2 we can write

$$h_i[p, eo] = f\varepsilon_{im} p_i k c_{i,1} v S\, \Delta t \quad (23)$$

Obviously, the dependence of the peak height on the number of bp is linear for pressure injection and deviates from linearity for injection by electromigration. The relative peak height with respect to the sum of all peak heights in an electropherogram is, for injection by electromigration,

$$\phi_i[h, e, eo] = \frac{c_{i,1} p_i\left(1 - \dfrac{u_{eo}}{u_{i,2}}\right)}{\sum_{i=1}^{n} c_{i,1} p_i\left(1 - \dfrac{u_{eo}}{u_{i,2}}\right)} \quad (24)$$

and, for pressure injection,

$$\phi_i[h, p, eo] = \frac{p_i c_{i,1}}{\sum_{i=1}^{n} p_i c_{i,1}} \quad (25)$$

It is again obvious that electroosmosis brings bias of the electromigration injection to the pressure injection.

3. Experimental

3.1. Chemicals

A ΦX-174 DNA-Hae III digest (Gibco BRL No. 5613 SA) in the range 72–1353 bp was used as a model mixture. The solutions were stored overnight at 4°C. Agarose used as a sieving medium was SeaPrep (catalogue No. 50302; FMC Bioproducts, Rockland, MD, USA). A 2% solution was prepared gravimetrically in 89 m*M* Tris base–89 m*M* boric acid–2.5 m*M* Na_2EDTA (1 × TBE) at boiling temperature and stored at 60°C as described [9].

3.2. Capillary

A fused-silica capillary (100 μm I.D., 367 μm O.D.) was obtained from Polymicro Technologies (Phoenix, AZ, USA) and installed in a user-assembled cartridge (Bio-Rad, catalogue No. 148-3050). The capillary inner wall was coated with linear polyacrylamide by a procedure described elsewhere [9,12]. The total length of the capillary was 54 cm and the length from the end to the window was 50 cm. The window was cut off with a blade under a microscope. The electroosmotic mobility was determined by measuring the migration time of a diluted sample of neutral mesityl oxide placed in the anodic electrode vial. The electroosmotic mobility coefficient, u_{eo}, was found to be $1.83 \cdot 10^{-9}$ m^2 V^{-1} s^{-1}.

3.3. Apparatus

The experiments were performed with a BioFocus 3000 system (Bio-Rad, Hercules, CA, USA) at 30°C and a voltage of 10 kV, i.e., at an electric field strength of 295 V cm^{-1}. The capillary was kept at constant temperature with distilled water as a thermostating medium. The separations were detected by measuring the absorbance at 260 nm. After each run of the analysis the capillary was rinsed with fresh agarose solution under pressure. Both the cathodic and anodic chambers, with a volume of 1.6 ml, were filled with agarose solution. Samples were injected by pressure (20 p.s.i.; 1 p.s.i. = 6894.76 Pa) and by electromigration at 7 kV for 8 s. These values provided approximately equal peaks heights for both injection types.

4. Results and discussion

The model separations were performed with ΦX-174 DNA-Hae III digest, which consists of an equimolar mixture of eleven fragments in the size range 72–1353 bp [13]. The results of their separation after injections by electromigration and pressure are shown in Fig. 2A and B, respectively. There is no remarkable difference in the appearance of the electropherograms. This conclusion has already been expressed by Eqs. 15, 16, 22 and 23.

The dependence of the relative peak areas with respect to the area of all the peaks in the electropherogram on the ratio $p_i/u_{i,2}$ is shown in Fig. 3. The linear dependence for electromigration injection is in accordance with Eq. 19. For the equimolar mixture the equation can be expressed as $\phi_i[A, e, eo] = p_i/u_{i,2} \cdot$ constant. The experimental points for this injection are shown as circles and the solid line represents the respective regression. The experimental points that belong to fragments introduced by pressure injection are shown as triangles. The dashed regression curve for pressure injection divides the line into two approximately equal parts. Fragments of size 603 bp and shorter were injected by electromigration in an amount higher than that for fragments injected by pressure, and vice versa for fragments longer than 603 bp. The electromigration injection bias is obvious.

Similar plots are shown in Fig. 4 for the relative peak heights with respect to the sum of the heights of all peaks in the electropherogram. The linear dependence of peak heights of fragments injected by pressure, depicted by the

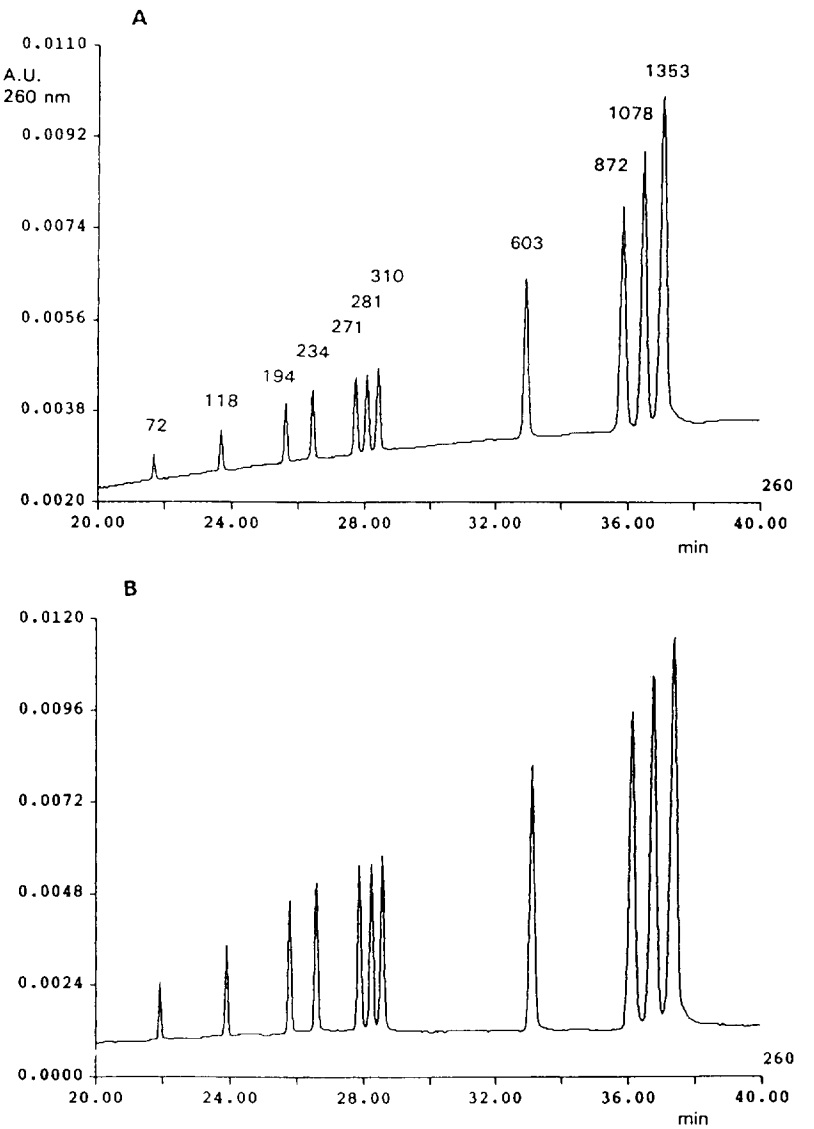

Fig. 2. Electropherograms of ΦX-174 DNA-Hae III digest obtained as a result of (A) pressure injection (20 p.s.i. s) and (B) electromigration injection (7 kV for 8 s). The other operating conditions were the same for (A) and (B): voltage 10 kV (295 V cm^{-1}); 2% solution of agarose SeaPrep; temperature 30°C; capillary, total length 54 cm, effective length 50 cm, I.D. μm. The numbers of bp of the fragments are given above the peaks.

dashed line and triangles, on the number of bp is in accordance with Eqs. 24 and 25. The slightly non-linear regression fit (solid line) of the dependence for electromigration injection intersects the line approximately at the same position as in the previous instance.

5. Conclusions

The electrophoretic mobility of DNA fragments in free solution, which controls their transport to the sample solution–sieving medium interface during injection by electromigration, is

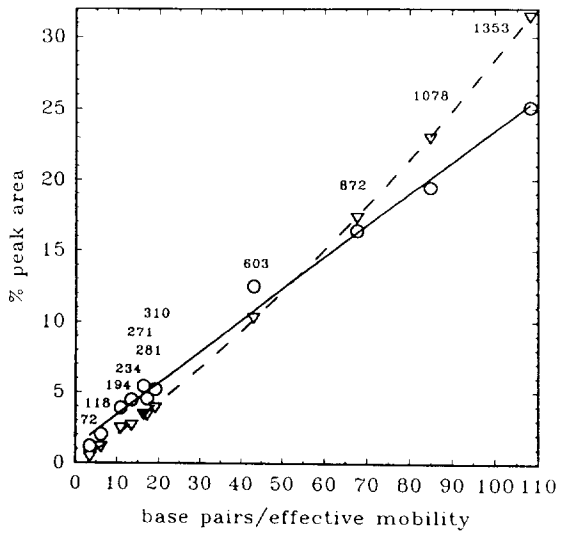

Fig. 3. Dependence of the peak-area percentage on the ratio of number of bp to the effective electrophoretic mobility $p_i/u_{i,2}$. Experimental points after the pressure (▽) and electromigration (○) injections, respectively, are given with the numbers of bp.

independent of size. However, size sampling bias of DNA fragments was observed when using this type of injection. In fact, electroosmosis removes part of molecules which are at the interface at the end of the injection. Under these conditions the amount injected is controlled by the size-selective mobility inside the sieving medium also (Eq. 10). Hence the electromigration sampling bias of DNA fragments must be taken into account whenever the electroosmosis affects the CE separation.

The molar ratios of the DNA fragments injected by pressure are the same as those in the original sample (Eq. 3). The lower the electroosmosis in a capillary, the closer are the molar ratios of the fragments injected by electromigration to those injected by pressure, i.e., to those in the original sample (Eqs. 7 and 11).

The apparatus constants and the detector response factors must be considered when the absolute amounts injected and molar ratios are to be acquired from the peak areas (Eqs. 15, 16, 19 and 20) and from the peak heights (Eqs. 22–25).

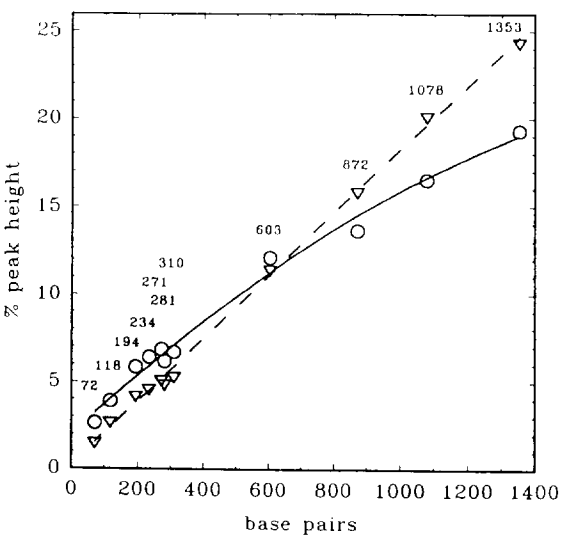

Fig. 4. Dependence of the peak-height percentage on the number of bp. Experimental points are marked as in Fig. 3.

Acknowledgements

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