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CAPILLARY ZONE ELECTROPHORESIS

QUANTITATIVE STUDY OF THE EFFECTS OF SOME DISPERSIVE PROCESSES ON THE SEPARATION EFFICIENCY

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SUMMARY

The contributions of diffusion, Joule heating, sampling and electroosmotic flow to the dispersion of zones separated by capillary zone electrophoresis were evaluated theoretically and verified experimentally. The theoretical model predicts that an optimum electric field strength, E , exists at which the number of theoretical plates, N , is a maximum. Both open and closed capillaries were considered and a simple formula was derived for the calculation of a maximum with a given experimental arrangement. The theoretical efficiency curves, N vs. E , were calculated and compared with those obtained experimentally in capillaries of various I.D.'s and filled with background electrolytes of various conductivities. The possibilities of minimization of some dispersion factors are discussed.

INTRODUCTION

Capillary zone electrophoresis (CZE) is one of the most rapidly developing electrophoretic techniques. Its most attractive feature is the simplicity of the experimental arrangement and the speed and efficiency of the separation. For the quantitative description of the separation efficiency, the concept of the number of theoretical plates has been proposed¹ and is currently used in a similar way as in chromatography. The number of theoretical plates, N , depends on the working conditions, the electric field strength, E , being of key importance since it controls the migration velocity. Jorgenson and Lukacs² regarded diffusion as the exclusive dispersion factor and obtained a linear dependence of N on the voltage, V , across the capillary. For low values of E the theoretical and experimental data are in good agreement. Several authors^{3–6}, however, have reported the existence of a maximum in the experimental dependence of N vs. V and tried to explain it in a qualitative way by means of the Joule heat effects.

In gel electrophoresis, the theory concerning the dispersion due to temperature gradients and the prediction of the existence of such maxima has been published and

verified experimentally⁷. In CZE, the theory concerning the Joule heat effects was published many years ago⁸, however no comparison of the theoretical and experimental curves, $N = f(E)$, has been published till now.

The theory and discussion given here accounts for the dispersion caused by diffusion, the initial sample pulse width, the Joule heat and the electroosmotic flow in both open and closed capillaries of various I.D.'s. Moreover, a new relationship has been derived which describes the maximum in the curve of $N = f(E)$ and thus the optimum working conditions for a separation.

THEORETICAL

Plate numbers

The electrophoretic separation of ionic substances is based on their different speeds of migration in an electrolyte column across which an electric voltage is applied. During this process the zones of the various substances are not only separated but they are also continually broadened due to a series of dispersive factors, e.g., diffusion, convection, etc.

To obtain good separation of two substances with similar mobilities, minimization of the dispersion is of key importance. For the description of the separation efficiency the number of theoretical plates was introduced¹ as

$$N = L^2/\sigma^2 \quad (1)$$

where L is the migration distance and σ^2 is the variance of the concentration distribution in the zone.

The number of theoretical plates required for the complete separation (resolution, $R = 1$) of two separands is¹

$$N_{\text{req}} = 16 \cdot \frac{1}{(\Delta v/\bar{v})} \quad (2a)$$

where Δv is the difference in their migration velocities and \bar{v} is their average velocity. If the movement of a separand is caused only by electromigration, then eqn. 2a can be written in the form

$$N_{\text{req}} = 16 \cdot \frac{1}{(\Delta u/\bar{u})} \quad (2b)$$

where Δu is the difference in their effective mobilities and \bar{u} is their average mobility.

The variance (total) can be assumed to be the sum of the variances due to particular sources of dispersion

$$\sigma^2 = \sigma_{\text{I}}^2 + \sigma_{\text{D}}^2 + \sigma_{\text{E}}^2 + \sigma_{\text{T}}^2 + \sigma_{\text{E}_0}^2 + \sigma_0^2 \quad (3)$$

where the right-hand terms represent the contributions of injection, diffusion, electromigration, temperature profiles due to Joule heat, electroosmosis and other effects, respectively.

Except for σ_1^2 , the variances are directly proportional to the analysis time, the constants of proportionality being the respective dispersion coefficients, D_i . The total variance of the sample distribution is expressed by the relationship

$$\sigma^2 = \sigma_1^2 + 2t \sum_i D_i \quad (4)$$

where t is the migration time. It can be expressed as

$$t = L/v \quad (5)$$

where v is the velocity of the respective migrating zone.

The first four terms on the right-hand side of eqn. 3 represent the effects inherent to the principle of the method and can never be suppressed to zero; however, their influence upon the separation efficiency can be controlled by appropriate design of the instrument and selection of the working conditions.

Dispersion effects

(i) The injection term is related to the shape of the initial sample pulse. In an ideal case, the sample is introduced into the separation capillary as a rectangular pulse. For a sample pulse of width l , the input variance is constant^{8,9}:

$$\sigma_1^2 = l^2/12 \quad (6)$$

In order to obtain highly efficient separation, in practice it is necessary to keep the width of the initial sample pulse smaller than 1% of the length of the separation capillary.

(ii) The diffusion term in eqn. 3 can be calculated from the Einstein equation

$$\sigma_D^2 = 2D_D t \quad (7)$$

where D_D is the diffusion coefficient.

(iii) Electromigration dispersion is related to changes in the local electric field strength in the migrating zone with respect to that in the background electrolyte¹⁰. It can be minimized by decreasing the difference between the mobility of the separand and that of the background electrolyte constituent of the same sign, or by decreasing the concentration in the separated zones. Usually the electromigration dispersion is negligible when the concentration of the separated ions is more than two orders of magnitude lower than that of the background electrolyte¹⁰. In such a case the use of extremely sensitive detectors is necessary.

(iv) The Joule heat affects the dispersion of zones via the effects of the temperature gradients formed. The capillary is cooled only on its outer surface and the temperature difference between the solution in the centre and the inner wall can be several degrees even in very narrow capillaries. Since the mobility of most ions increases by 2% per 1 K, ions located near the wall of the capillary move slower than those in the centre and the migrating zone broadens. The dispersion caused by this

temperature effect increases with increasing Joule heat and, thus, with the voltage applied across the capillary. The exact temperature profile in the tubular column can be described by Bessel functions¹¹, however, it can be closely approximated by simple parabolic functions^{12,13}.

For such a case the dispersion coefficient due to the Joule heat can be expressed in the form⁸

$$D_T = \frac{\kappa^2 \delta^2 u^2 E^6 R^6}{3072 D \lambda_T^2} \quad (8)$$

where κ is the conductivity of the background electrolyte, δ the temperature coefficient of the mobility of the separand, R the radius of the separation capillary, D the diffusion coefficient of the separand and λ_T the thermal conductivity of the background electrolyte.

(v) The effects of electroosmosis on the performance of CZE can be described as follows. Between the inner wall of the capillary and the electrolyte solution an electric double layer is always formed which can be characterized by its zeta potential and which is manifested by electroosmotic movement of the liquid in the capillary when an electric field is applied. The velocity of this flow is proportional to the zeta potential according to the Helmholtz–Smoluchowski equation

$$v = -\frac{\varepsilon \zeta E}{\eta} \quad (9)$$

where ε is the permittivity of the solvent, ζ the zeta potential and η the viscosity of the background electrolyte. With respect to the effects of electroosmosis upon the dispersion and migration of zones, two cases must be distinguished:

Open capillary. In the case of a capillary with open ends and a constant zeta potential along its axis, the electroosmotic flow has almost a piston character and its contribution to the dispersion of the migrating zone is small^{14–16}. Thus, especially for low electroosmotic velocities, $D_{E_0} \rightarrow 0$. However, the analysis time is strongly influenced by electroosmosis in this case.

The migration velocity of a zone is given by the sum of the electrophoretic migration and the electroosmotic flow

$$v = (u + u_{E_0})E \quad (10)$$

where u_{E_0} represents the constants on the right-hand side of eqn. 9, *i.e.*

$$u_{E_0} = -\frac{\varepsilon \zeta}{\eta} \quad (11)$$

Hence, in open capillaries, the electroosmosis can contribute only to the transport of separands by either increasing or decreasing the overall migration velocity according to the sign of the zeta potential. However, in the case of non-uniformly charged walls of the capillary, local turbulences can occur causing irreproducible dispersion¹⁷.

Closed capillary. In the case of a closed capillary, e.g., by a semipermeable membrane, the electroosmotic flow near the wall of the capillary is balanced by the opposite flow in the centre, causing continual dispersion of the sample zone during the migration¹⁸. The dispersion coefficient due to the electroosmotic flow in a closed capillary can be expressed in the form⁸:

$$D_{E_0} = 1/48 \cdot \frac{R^2 \varepsilon^2 \zeta^2 E^2}{D \eta^2} \quad (12)$$

Separation efficiency vs. electric field strength

As mentioned above, the cases of open and closed capillaries must be clearly distinguished.

Open capillary. After the substitution of the respective dispersion coefficients ($D_{E_0} = 0$) and rearrangement of eqn. 1, one obtains

$$N = \frac{A_1 E}{B_1 E^6 + F_1 E + H_1} \quad (13)$$

where $A_1 = 1536(u + u_{E_0})LD\lambda_T^2$, $B_1 = \delta^2 \kappa^2 u^2 R^6$, $F_1 = 128 D\lambda_T^2 l^2 (u + u_{E_0})/L$ and $H_1 = 3072 D^2 \lambda_T^2$.

The electric field strength at the maximum of efficiency ($\partial N/\partial E = 0$) is:

$$E_{\max} \approx 2.92 \sqrt[3]{\frac{D\lambda_T}{\delta \kappa u}} \cdot \frac{1}{R} \quad (14)$$

The maximum efficiency for $l \Rightarrow 0$ is then

$$N_{\max} \approx 0.82(u + u_{E_0}) \frac{L}{R} \sqrt[3]{\frac{\lambda_T}{D^2 \delta \kappa u}} \quad (15)$$

which represents the maximum theoretical efficiency accessible for a given capillary, background electrolyte and separand mobility. Obviously, the higher is u_{E_0} the higher is N_{\max} . However, in this case, N_{req} is given by

$$N_{\text{req}} = 16 \cdot \frac{u + u_{E_0}}{\Delta u} \quad (16)$$

and the ratio N_{\max}/N_{req} is independent of u_{E_0} . Hence, in open capillaries, the electroosmotic flow will displace the efficiency curves upwards, but the higher plate numbers thus obtained are not effective for the separation, since all migrating zones, together with the background electrolyte, are transported only along the capillary¹⁹. The mutual separation of zones is based on the relative difference in effective mobilities.

Closed capillary. Closed capillaries prevent hydrodynamic movement of the solutions inside, and, thus, no care need be taken about the levels of the solutions in the electrolyte chambers. As the electroosmotic flow does not contribute to the transport of the separand, the migrating time is determined only by the electrophoretic mobility of the separand. On the other hand, electroosmosis contributes to the dispersion of the moving zone with the dispersion coefficient expressed by eqn. 12. The resulting expression for the separation efficiency in a closed capillary is

$$N = \frac{A_2 E}{B_2 E^6 + C_2 E^2 + F_2 E + H_2} \quad (17)$$

where $A_2 = 1536 u L D \eta^2 \lambda_T^2$, $F_2 = 128 D \lambda_T^2 \eta^2 l^2 u/L$, $C_2 = 64 R^2 \varepsilon^2 \zeta^2 \lambda_T^2$, $B_2 = \eta^2 \delta^2 \kappa^2 u^2 R^6$ and $H_2 = 3072 D^2 \eta^2 \lambda_T^2$.

To derive the expression for the optimum E corresponding to the maximum N , the procedure based on $\partial N/\partial E = 0$ can again be employed. However, in contrast with the case of the open capillary, the relationship for the optimum E has implicit form and can be solved only numerically.

The dependence $N = F(E)$ for the closed capillary is shown in Fig. 1. Here, for a given capillary, background electrolyte and separand mobility, the value of the zeta potential is chosen as a parameter. The curve at zero zeta potential is identical for both a closed and an open capillary with completely deactivated walls. It is seen that very high plate numbers can be reached, but in closed capillaries it is necessary to decrease the zeta potential to less than 2 mV. This can usually be done either by deactivation of the capillary surface or by use of additives to the background electrolyte as in capillary isotachophoresis²⁰.

EXPERIMENTAL

Equipment

The experiments were carried out in laboratory made equipment, see Fig. 2, consisting of Perspex electrolyte chambers with electrodes (E1, E2) connected to

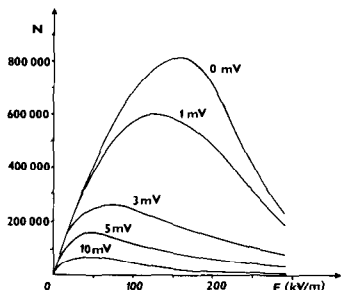


Fig. 1. Calculated dependences of the separation efficiency, N , on the intensity of the electric field, E , with parametrically chosen values of the zeta potential in mV. Closed capillary: 0.5 m \times 0.1 mm I.D. Conductivity of the background electrolyte: 0.05 S/m. Other parameters used for the calculation are listed in Table I.

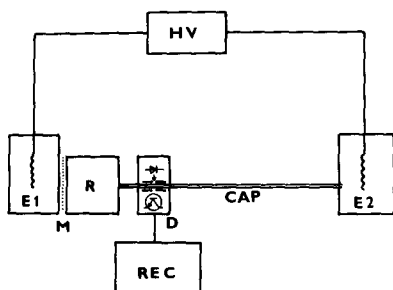


Fig. 2. The experimental equipment used. For details see text.

a high-voltage power supply (HV) and the reflushing block (R) to which capillaries (CAP) of various lengths and diameters can be connected. A cellophane membrane (M) placed between the reflushing block and the electrolyte chamber prevented the hydrodynamic flow inside the capillary. A laboratory-made optical on-column VIS absorbance detector (D) was employed where a light emitting diode (LED) and a phototransistor were used as the source and detector, respectively. The detector signal was recorded by a line recorder (REC). An high-voltage power supply (16 kV, 500 μ A), developed originally for capillary isotachopheresis²¹ and enabling simultaneous measurement of the voltage and current, was used. Soft glass capillaries of desired diameter were kindly provided by Drs. K. Tesařík and K. Janák from this Institute. To obtain high electric field strengths with the high voltage power supply used, the separation capillaries had to be a bit shorter than those currently used. The length of each capillary tested was 160 mm, with the detector situated 118 mm from the injection point in all experiments. The capillary was cooled by an air fan at room temperature.

Chemicals and electrolytes

Triethylamine and caproic acid were of analytical reagent grade (Lachema, Brno, Czechoslovakia). Night blue and hydroxypropylcellulose (HPC) were from Lachema and EGA-Chemie (Steinheim/Albuch, F.R.G.), respectively. Methyl green for microscopy (Fluka, Buchs, Switzerland) was used as a sample. The stock solution of HPC was purified on a mixed-bed ion exchanger; other chemicals were used without further purification.

The background electrolytes consisting of 0.002 or 0.008 *M* triethylamine + caproic acid to pH 4.9 with the additives 0.1% HPC and $5 \cdot 10^{-5}$ *M* night blue were used in all experiments. The sample solution was $2 \cdot 10^{-4}$ *M* methyl green in the background electrolyte diluted with water 1:5. The sample was introduced into the capillary by the electromigration technique², where the respective electrolyte chamber *E*₂ was replaced by a beaker containing the sample solution and a small current (1–5 μ A) was applied for 10 s. Thus, since the capillary was closed at the opposite end by the membrane M, the sample was introduced by electromigration only. Due to the adjustment of concentrations when crossing the stationary concentration boundary between the background electrolyte and the sample solution, methyl green was concentrated into a sharp initial pulse in the capillary, the length of which was 1 mm. The capillary was rinsed and filled with fresh background electrolyte for each new experiment.

RESULTS AND DISCUSSION

Preliminary isotachophoretic experiments were done to find the sample having the same effective mobility as the ion of the background electrolyte with the same sign, to avoid electromigration dispersion, and, the buffering counter ion having low mobility to minimize the electrolytic conductivity and thus the excessive production of Joule heat. Methyl green (ionic mobility $ca. 34 \cdot 10^{-5} \text{ cm}^2/\text{V s}$) and triethylamine (ionic mobility $ca. 33 \cdot 10^{-5} \text{ cm}^2/\text{V s}$) were finally selected as the separand and the background electrolyte constituent, respectively. The pH of the background electrolyte was set to 4.90 using caproic acid (ionic mobility $30.2 \cdot 10^{-5} \text{ cm}^2/\text{V s}$, $pK_a = 4.86$)²². For the suppression of the electroosmotic flow, 0.1% HPC was added to the background electrolyte.

In the first experiments, severe zone tailing due to the adsorption of the methyl green zone onto the capillary walls was observed. Neither the use of fused-silica capillaries nor silylation of the capillary walls brought about substantial improvement. Then the competitive adsorption²³ of a substance which binds to the capillary walls more strongly than does the separand was examined. The addition of $ca. 10^{-5} \text{ M}$ of night blue to the background electrolyte was found to eliminate the tailing completely, see Fig. 3, and this system was adopted for further measurements.

As the detected peaks were symmetrical, the number of theoretical plates was calculated using the formula $N = 5.545 (l/w_{1/2})^2$, where l is the distance of the peak from the start on the record and $w_{1/2}$ is the width at half height. Each measurement was repeated five times and average values were plotted. The relative standard deviation was less than 10%. A typical example of the detector trace is shown in Fig. 4.

The theoretical and experimental dependences of N vs. E in capillaries of various inside diameters are shown in Fig. 5a,b. The concentration of the background electrolyte with respect to triethylamine was 0.008 M and its conductivity 0.04 S/m in

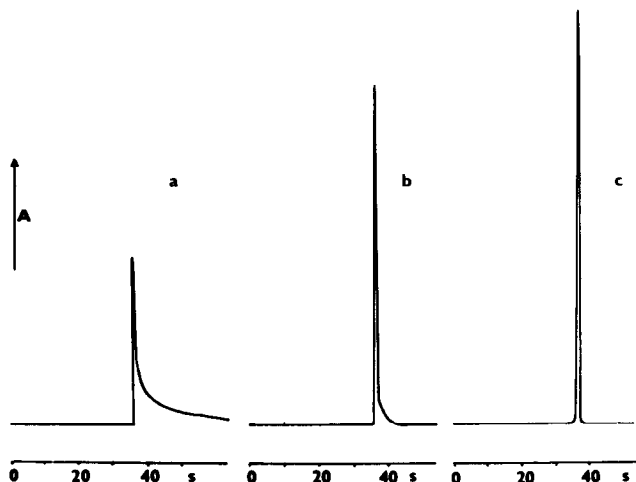


Fig. 3. The elimination of zone tailing by competitive adsorption. (a) Without additive; (b) 10^{-5} M night blue added to the background electrolyte; (c) $2 \cdot 10^{-5} \text{ M}$ night blue added to the background electrolyte. A = Absorbance at 640 nm.

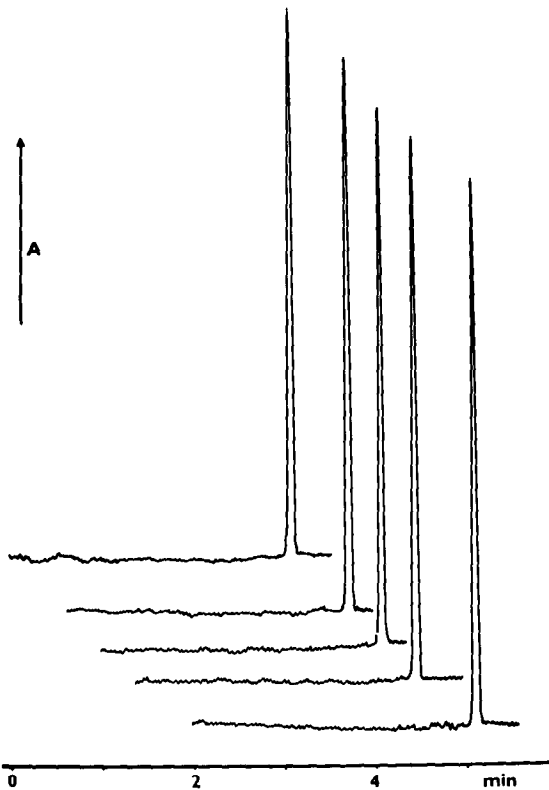


Fig. 4. An example of a set of measurements used for the calculation of N . Capillary: 0.2 mm I.D. Conductivity of the background electrolyte: $400 \mu\text{S}/\text{cm}$, $18 \text{ kV}/\text{m}$.

the case of Fig. 5a, and 0.002 M and $0.01 \text{ S}/\text{m}$ in the case of Fig. 5b. The numerical values of the constants used for the calculation are listed in Table I. In accord with experiments published by Lukacs and Jorgenson²⁴, it is clearly demonstrated that the smaller is the inside diameter of the capillary the higher are the plate numbers. An additional increase in the separation efficiency can be achieved by lowering the conductivity of the background electrolyte, *e.g.*, by decreasing its concentration. A decrease in the background electrolyte concentration and the use of very narrow capillaries makes higher demands on the detection system. In our case the limited sensitivity of the detector prevented measurements with capillaries narrower than 0.1 mm since the reproducibility of the plate number reading drastically decreased due to detector noise. For the same reason, experimental data measured in an 0.125 mm I.D. capillary filled with a low conductivity electrolyte are not included.

Though the experimental values plotted in Fig. 5 do not exactly fit the theoretical curves, the agreement between the theory and experiment is good, especially in the case of low conductive background electrolyte. The theoretical and experimental values of plate numbers and electric field strengths in the maxima of the efficiency curves are compared in Table II. It should be noted that the shape of the theoretical curves strongly depends on the value of zeta potential chosen for computing (see Fig. 1). Its

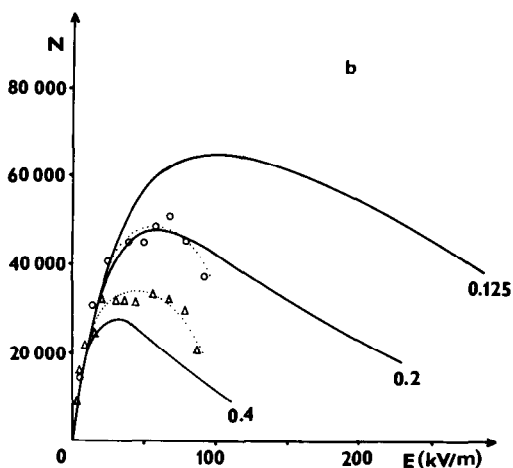
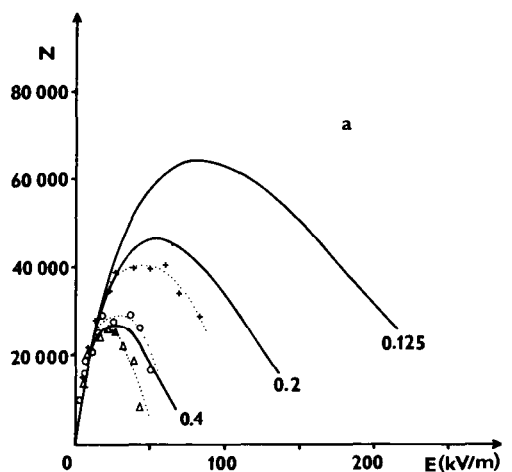


Fig. 5. The dependences of N versus E for background electrolyte conductivities (a) 0.04 and (b) 0.01 S/m. The internal diameter of the separation capillary (in mm) is marked on each theoretical curve. The experimental points are: +, 0.125 mm; O, 0.2 mm and Δ , 0.4 mm I.D.

actual value is hardly accessible during the run and in our case a value of 1.5 mV was adopted as the result of comparing our preliminary measurements with literature data²⁰. Moreover, due to the elevation of the mean temperature inside the capillary most parameters which are assumed constant during the calculations, may show variability. However, we can say that in our case, when using closed capillaries, dispersion due to electroosmosis is dominant, dispersions due to sampling, temperature gradients, electromigration and residual adsorption representing only minor contributions.

In the present capillary electrophoretic practice, the electric field strengths rarely exceed 50 kV/m and, thus, usually the plate numbers lie in the linear part of the

TABLE I

NUMERICAL VALUES OF CONSTANTS USED FOR THE CALCULATION OF PLATE NUMBERS

<i>Constant</i>	<i>Numerical value</i>
λ_T	0.6 W/m K
δ	0.024
ϵ	$7.12 \cdot 10^{-10}$ C ² /J m
η	0.001 kg/m s
u	$34 \cdot 10^{-9}$ m ² /V s
L	0.118 m
D	$8.8 \cdot 10^{-10}$ m ² /s
l	0.001 m
κ	0.04, 0.01 S/m
R	0.625, 0.1 and $0.2 \cdot 10^{-3}$ m

efficiency curve. The efficiencies thus obtained reach several hundreds of thousands of plates per metre of the separation capillary. By appropriate selection of the background electrolyte with respect to the electromigration dispersion and Joule heat as discussed previously, even higher efficiencies should be reached using high electric field strengths. The experiments presented here confirm that, even in relatively wide-bore capillaries, efficiencies of hundreds of thousands of plates per metre of the separation capillary can be reached if the separation conditions are selected carefully^{9,25}.

However, in practice, when multicomponent samples are to be analysed, the optimum separation conditions for each of the analytes of interest will rarely be achieved simultaneously and a compromise must be looked for. Another practical problem is the selection of the capillary diameter. The use of wide-bore capillaries makes low demands on the detection sensitivity, but on the other hand it makes high demands on the cooling of the capillary since the Joule heat production is proportional to the square of the capillary diameter. As mentioned above, the temperature difference between the centre and the inner wall of the capillary can be several degrees. This temperature difference is determined mainly by the thermal conductivity of the

TABLE II

THEORETICAL AND EXPERIMENTAL NUMBERS OF THEORETICAL PLATES AND ELECTRIC FIELD STRENGTHS AT THE MAXIMUM OF THE EFFICIENCY CURVE

<i>Capillary I.D. (mm)</i>	$\kappa = 0.01$ S/m				$\kappa = 0.04$ S/m			
	<i>Theory</i>		<i>Experiment</i>		<i>Theory</i>		<i>Experiment</i>	
	<i>N</i>	<i>E (kV/m)</i>	<i>N</i>	<i>E (kV/m)</i>	<i>N</i>	<i>E (kV/m)</i>	<i>N</i>	<i>E (kV/m)</i>
0.4	27 600	29	34 000	56	27 100	26	26 000	26
0.2	47 400	58	50 000	67	46 700	53	29 000	38
0.125	65 000	92	—	—	64 000	58	40 000	40

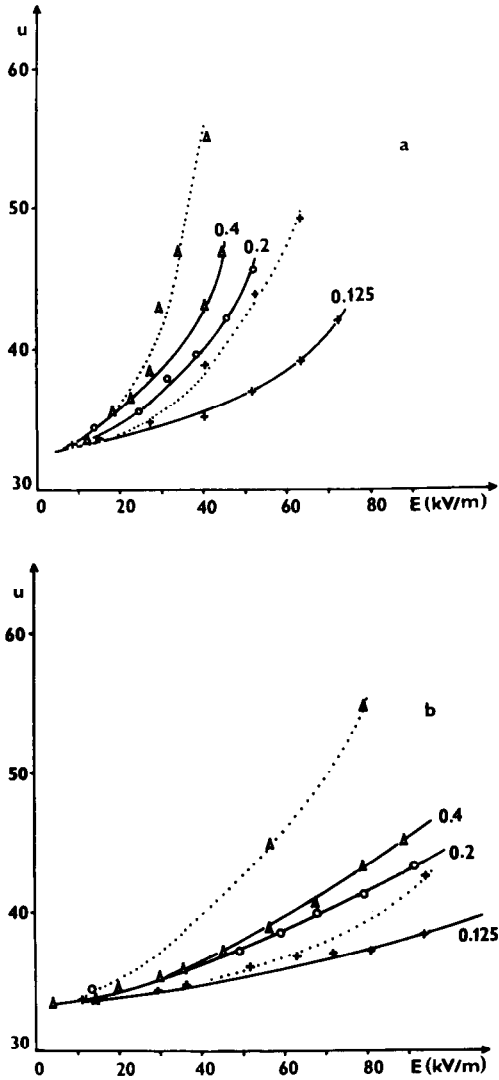


Fig. 6. The experimental dependences of the electrophoretic mobility of methyl green (in 10^{-5} $\text{cm}^2/\text{V s}$) on the intensity of the electric field used. The diameter of the separation capillary (in mm) is marked on each curve. The conductivity of the background electrolyte was (a) 400, (b) 100 $\mu\text{S}/\text{cm}$. The dotted lines represent the experiments in non-cooled capillaries.

background electrolyte and contributes significantly to the dispersion of zones as described in the Theoretical.

The diameter of the capillary also plays an important role in the elevation of the mean temperature of the background electrolyte. The mean temperature can be estimated by employing the experimental dependence of the separand mobility on the intensity of the electric field used. These dependences are shown in Fig. 6. Given the fact that the mobility increases by 2.4% per K, the mobility rise in Fig. 6 represents the

mean temperature elevation up to 50°C. This is in good agreement with data we obtained by thermocouple measurement of the temperature of the outside capillary wall and with published data²⁶. It is seen that the mobility increase and thus the temperature elevation is less pronounced in narrow-bore capillaries and when cooling is applied.

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