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Isoelectric focusing field-flow fractionation

II. Experimental study of focusing of methyl red in the trapezoidal cross-section channel

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ABSTRACT

Isoelectric focusing field-flow fractionation is a technique for the separation of ampholytes using in addition to the electric field and pH gradient the flow of the carrier ampholyte solution through the fractionation channel as the third active separating factor. Flow action permits a decrease in the channel dimension in the direction of the electric field and thereby also a decrease in the absolute values of the voltage while keeping a high field strength, which results in lower Joule heat production and a shorter time required for focusing. Focusing of methyl red in the trapezoidal cross-section channel was investigated as a function of the applied voltage and flow-rate of the carrier ampholyte solution. On the basis of the results obtained, it is obvious that methyl red is readily focused under suitable conditions inside the channel. Focusing is more efficient at higher electric field strengths and lower flow-rates of carrier ampholyte solution.

INTRODUCTION

Isoelectric focusing (IEF) is an electrophoretic technique that gives a very high resolution of amphoteric compounds [1]. The separation is carried out in a pH gradient which is established between two electrodes. In this technique, amphoteric compounds migrate until they align themselves at their isoelectric positions, where these compounds possess no net overall charge and therefore they concentrate at these points as migration ceases. IEF is an equilibrium technique in which diffusion works in the opposite direction to the driving forces, concentrating amphoteric compounds around the equilibrium position. As soon as dynamic equilibrium between the concentrating and dispersive processes has been established, focused zones of amphoteric compounds are formed.

In addition to the electric field and pH gradient used in IEF, isoelectric focusing field-flow fractionation (IEF_4) employs a third active separation-affecting factor, *viz.*, the flow of the liquid through the separation channel with the direction of the flow perpendicular to that of the electric field [2]. The shape of the flow velocity profile formed is influenced by the geometry of the fractionation channel [3]. Amphoteric

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solutes are transported via isoelectric focusing to the equilibrium positions and narrow focused solute zones with nearly Gaussian concentration distribution are formed. Provided that different solutes exhibit different isoelectric points, they are focused in different positions across the fractionation channel where the local values of pH are the same as the isoelectric points of the solutes. The velocity profile formed in the liquid flow causes the migration of focused zones along the channel at different velocities, so that the solutes are longitudinally separated.

The flow acting as the separation factor makes it possible to reduce the channel dimension in the direction of the electric field, which enables absolute voltage values to be decreased with the maintenance of a high field strength. This results in a decrease in the Joule heat production and the time required for focusing. Further, the laminar flow of the carrier liquid stabilizes the pH gradient against convection and the elution character of the technique enables liquid chromatographic detectors to be used for the detection of solute zones.

The technique was applied in practice by Chmelík *et al.* [4] in the trapezoidal cross-section channel and by Thormann *et al.* [5] in the rectangular cross-section channel. The latter group named this technique electrical hyperlayer field-flow fractionation.

The generation of pH gradient in the IEF_4 channel was studied in previous work [6]. It was found that the pH gradient generation was sufficiently fast and reproducible for IEF_4. The present work was aimed at investigating focusing of a low-molecular-weight amphoteric dye (methyl red) in the trapezoidal cross-section channel and at studying the influences of some experimental parameters on the methyl red zone width. A number of theoretical and experimental studies characterizing the focusing of amphoteric compounds in different techniques has been published (for a review, see ref. 7). However, this work is the first experimental investigation of focusing of a low-molecular-weight amphoteric compound in the IEF_4 channel.

THEORETICAL

Svensson [1,8] and Vesterberg and Svensson [9] derived an equation to express the zone width of a focused amphoteric compound:

$$\sigma_\Phi = \sqrt{\frac{-D}{E(\mathrm{d}u/\mathrm{dpH})(\mathrm{dpH}/\mathrm{d}x)}} \tag{1}$$

where σ_Φ is the standard deviation of the zone width in the direction of the applied electric field, D is the diffusion coefficient of an amphoteric compound, E is the electric field strength around the isoelectric point of an amphoteric compound, $\mathrm{d}u/\mathrm{dpH}$ is the mobility slope of an amphoteric compound at its isoelectric point and $\mathrm{dpH}/\mathrm{d}x$ is the pH gradient at the zone location.

From eqn. 1 it follows that the smaller is the compound to be separated (having a higher value of D) the larger is the zone width. The zone width is inversely proportional to the square root of the electric field strength. By increasing the field strength the zone width and also the duration of focusing are reduced. The zone width is also inversely proportional to the pH gradient and the mobility slope. Whereas the diffusion coefficient and the mobility slope are internal properties of the amphoteric

compound, the electric field strength and pH gradient can be created in such a way as to make the separation of amphoteric compounds as effective as possible.

Another important parameter is the time of focusing. Focusing consists of two phases, a relatively rapid separation phase and a slower stabilizing phase. During the former phase the amphoteric compounds migrate to their isoelectric positions, generating pure zones of individual components. During the latter, much slower, phase the ampholytes are assuming their final steady-state profiles [7]. For long focusing times the zone width approaches its steady-state value.

The efficiency of the field-flow separation system is characterized by the height equivalent to a theoretical plate, H. Directions of the applied field and carrier liquid flow and the system of coordinates of trapezoidal cross-section channel are shown schematically in Fig. 1. For focusing field-flow fractionation in the trapezoidal cross-section channel we derived [2,3,10] the equation

$$H = \frac{U_{av}}{D} \frac{24m^4\sigma_\Phi^4 \tan^2\alpha}{3 + \tan^2\alpha} + \frac{W^2(\Phi_{max})\bar{U}(\Phi_{max})}{105D} \tag{2}$$

where U_{av} is the average linear velocity of the carrier liquid flow at the considered coordinate ψ, m is a dimensionless parameter of the order of 2, α is the angle included by the adjacent longer walls of the channel, $W(\Phi_{max})$ is the width of the channel in the position of the zone maximum Φ_{max} and $\bar{U}(\Phi_{max})$ is the linear velocity of the carrier liquid averaged at a given position Φ_{max} over the whole ψ range (interval).

From the well known equation

$$H = \sigma_\tau^2 / L \tag{3}$$

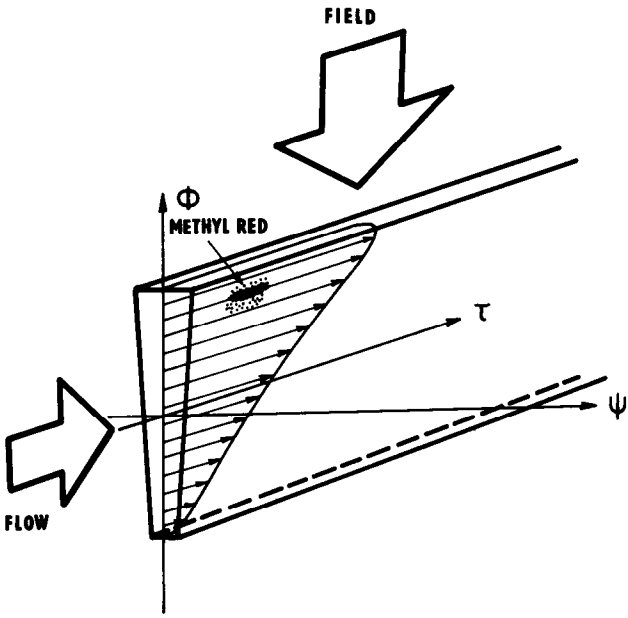


Fig. 1. Schematic representation and coordinate system of the trapezoidal cross-section channel.

the standard deviation of the zone of an amphoteric compound in the direction of the carrier liquid flow, σ_τ, can be expressed by the equation

$$\sigma_\tau = \sqrt{\frac{L}{D}\left[\frac{24U_{av}m^4\sigma_\Phi^4\tan^2\alpha}{3+\tan^2\alpha} + \frac{W^2(\Phi_{max})\bar{U}(\Phi_{max})}{105}\right]} \quad (4)$$

where L is the length of the channel.

EXPERIMENTAL

The scheme of the experimental arrangement is shown in Fig. 2. An M 122 doser (Mikrotechna, Prague, Czechoslovakia) with two injection syringes was used to pump solutions of electrode electrolytes, *i.e.*, solutions of 0.2 *M* acetic acid (pump A) and 0.2 *M* sodium hydroxide (pump B) into the electrode reservoirs. The flow-rates were 500 μl/min in both instances. An LD 2 linear feeder (Development Workshops of Czechoslovak Academy of Sciences, Prague, Czechoslovakia) was used to pump 1% ampholyte solution (Servalyt 4-9T; Serva, Heidelberg, Germany) into the channel in the flow-rate range 124–380 μl/min. Another LD 2 device with the same ampholyte solution was used to pump the sample, 0.02 *M* methyl red (Chemapol, Prague, Czechoslovakia) in ampholyte solution, into the channel with the aid of a six-port valve with a 30-μl sample loop through an injection capillary situated at a distance of 20 mm from the channel inlet where the carrier ampholyte solution is pumped. The results of focusing were recorded by an HP 1040 A diode-array detector in the range 400–600 nm. A stabilized power supply unit (Aritma, Prague, Czechoslovakia) was used in the range up to 60 V and the maximum current was 80 μA.

The fractionation channel was composed of three parts of Perspex clamped together with screws. The main part of the channel was a block comprising the slit of trapezoidal cross-section. The block was assembled by joining the two symmetrical parts into which side walls of the channel were milled. The block was provided with three capillaries, at the inlet and outlet of the channel and at the sample injection port. The upper and lower parts of the channel were identical and served for fixing wire

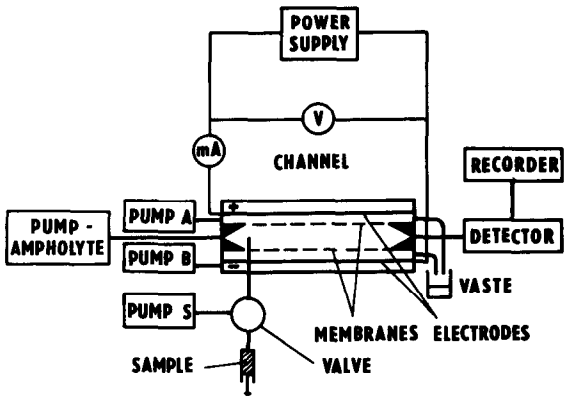


Fig. 2. Block diagram of the experimental arrangement.

platinum electrodes and comprising electrode reservoirs. They were situated on the opposite sides of the channel and were separated from its internal space with PGCL ultrafiltration membranes (Millipore, Bedford, MA, U.S.A.) with a nominal molecular weight cut-off 10 000. A diagram of the channel is shown in Fig. 3. The dimensions of the trapezoidal cross-section channel were length 250 nm, height 5 mm and widths of opposite walls of the trapezoid 0.45 and 0.95 mm.

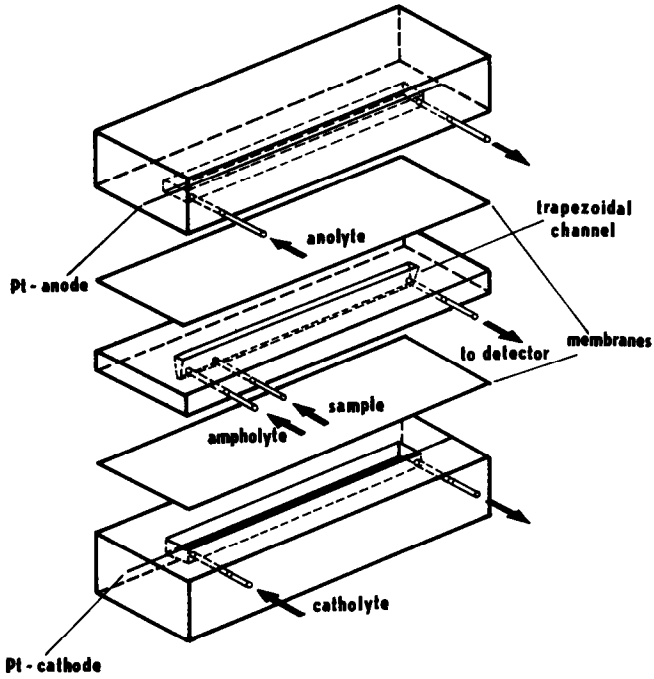


Fig. 3. Schematic representation of the channel for IEF_4.

Although measurements were performed at different flow-rates, the samples of methyl red were injected into the channel in the same way. The starting flow-rate of ampholyte solution at the inlet of the channel was 124 μl/min (pump-ampholyte in Fig. 2). The flow-rate of ampholyte solution at the sample injection port was 80 μl/min (pump S in Fig. 2) for 1 min. After this period the pump was stopped and the flow-rate of pump-ampholyte was changed to the required value. This procedure was used to ensure the same shape of the injected methyl red zone.

Suitable concentrations of ampholyte, sample and electrode solutions were chosen on the basis of previous measurements [6].

RESULTS AND DISCUSSION

From the theoretical considerations mentioned above, it follows that the standard deviation of a solute zone at the outlet of the channel, σ_τ, is approximately

proportional to the second power of the standard deviation of the solute zone in the direction of the applied electric field, σ_Φ, and σ_Φ is inversely proportional to the square root of the electric field strength. This means that σ_τ is inversely proportional to the electric field strength, *i.e.*, the higher the electric field strength used, the smaller is the width of the solute zone at the outlet of the channel. The width of the solute zone in the direction of the electric field is exponentially (with a negative sign) proportional to the focusing time and approaches its steady-state (minimum) value for long focusing times, and the same holds for the width of the solute zone at the outlet of the channel. For these reasons its dependences on the flow-rate of the carrier ampholyte solution and the applied electric field were investigated. A change of the methyl red colour from yellow to red occurs in the pH range between the neutral medium of the ampholyte solution (pH 6.2) and its isoelectric point (p*I* 3.9). This change aids both visual investigation and spectrophotometric detection of the processes in the channel. For this reason the fractograms were recorded at four different wavelengths, 400, 450, 500 and 550 nm. The fractograms at 400 nm presumably show the yellow neutral form of methyl red and the fractograms at 550 nm presumably correspond to the red acidic (isoelectric) form.

Orientations of pH gradient and electric field lead in these experiments to focusing of acidic solutes in wider section of the channel where solutes elute at higher velocities. Location of the methyl red zone inside the trapezoidal cross-section channel is shown schematically in Fig. 1. From visual investigation it is obvious that only a small part of the channel is required for transferring methyl red from the site of injection to the position corresponding to its isoelectric point.

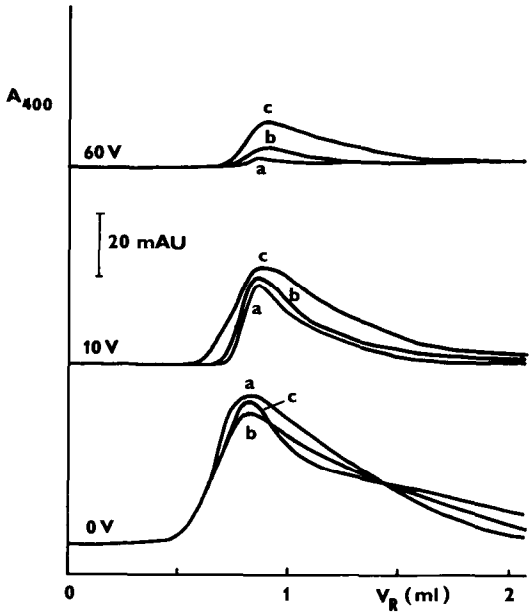


Fig. 4. Dependence of the width of the methyl red zone on the flow-rate of the carrier ampholyte solution at three voltages (0, 10 and 60 V). Flow-rates: (a) 124; (b) 190; (c) 380 μl/min. Fractograms were recorded at 400 nm. V_R = Retention volume.

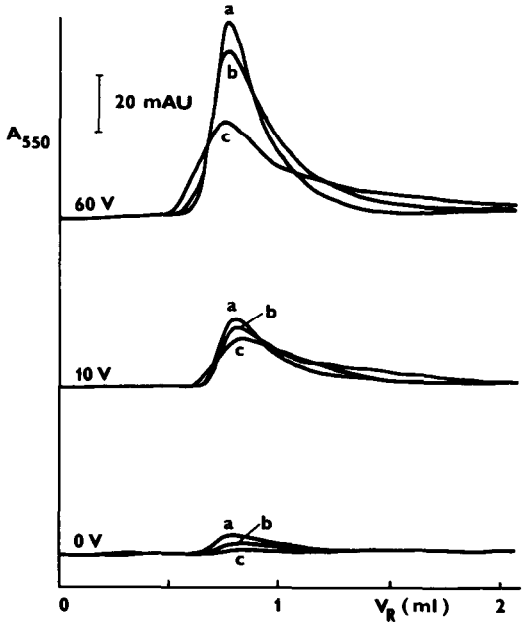


Fig. 5. As Fig. 4, with fractograms recorded at 550 nm.

The results of spectrophotometric detection show the increase in the content of the red form with increasing focusing time and electric field strength. The fractograms describing the dependence of the width of the methyl red zone on the flow-rate of the carrier ampholyte solution at three voltage values (0, 10 and 60 V) are shown in Figs. 4 (at 400 nm) and 5 (at 550 nm). It is apparent that with a decreasing flow-rate, *i.e.*, with increasing focusing time, zones become sharper and the extent of the change of the yellow neutral form of methyl red to its red isoelectric form is greater. Results of the investigation of the dependence of the methyl red zone width on the applied electric field at a flow-rate of 124 μl/min are shown in Figs. 6–9 for four wavelengths. The results at 400 nm (Fig. 6) show the decrease in the content of the neutral yellow form with increasing applied electric field. Because both methyl red forms absorb light at 450 and 500 nm (the spectra of yellow and red forms of methyl red in ampholyte solutions were published in a previous paper [6]), the observed changes in fractogram shapes provide information both on sharpening of zones and on the change in the content of yellow and red forms (Figs. 7 and 8). The increase of the red form and the decrease of the yellow form can be seen from the reversed ratios of peak heights at 450 and 500 nm at low and high voltages, respectively. The methyl red samples are found to be eluted as much sharper zones with increasing electric field strength, indicating the formation of much narrower zones in the direction of the electric field. The fractograms at 550 nm (Fig. 9) show the increase in concentration of the red isoelectric form with increasing electric field strength.

On the basis of the results obtained so far, it is apparent that methyl red (a low-molecular-weight amphoteric dye) is readily focused under suitable conditions

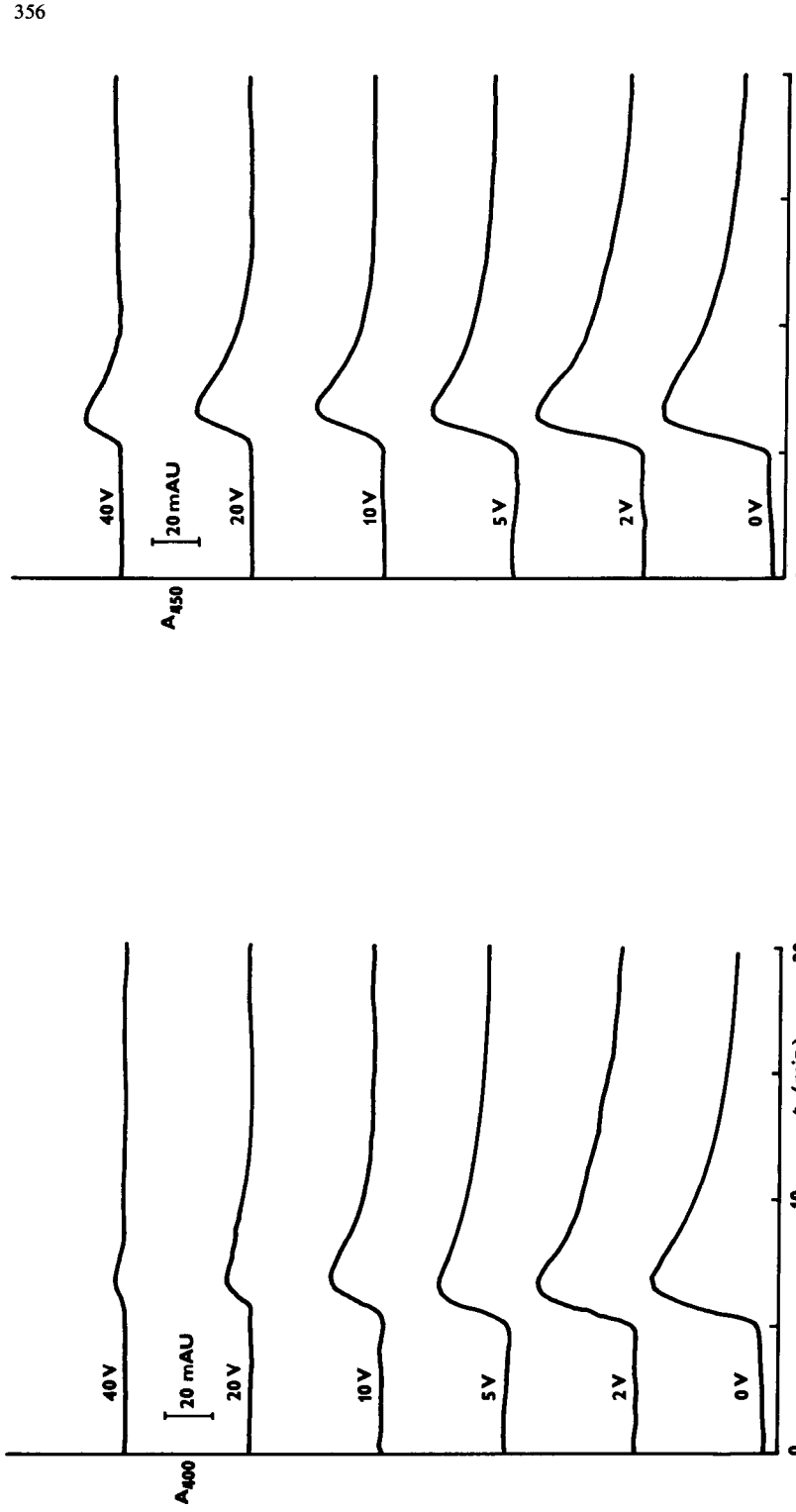
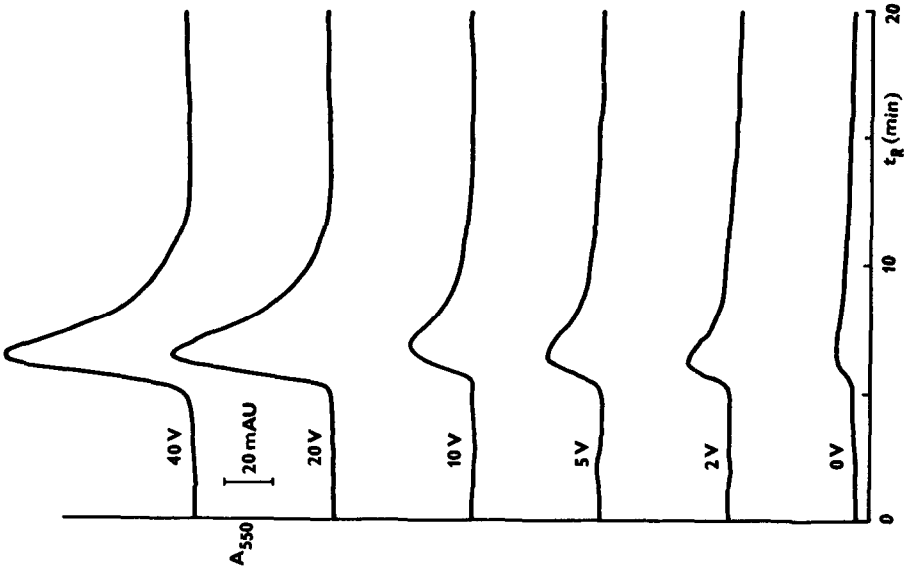


Fig. 6. Dependence of the width of the methyl red zone on the applied electric field at a flow-rate of 124 μl/min. Fractograms were recorded at 400 nm. t_R = Retention time.

Fig. 7. As Fig. 6, with fractograms recorded at 450 nm.



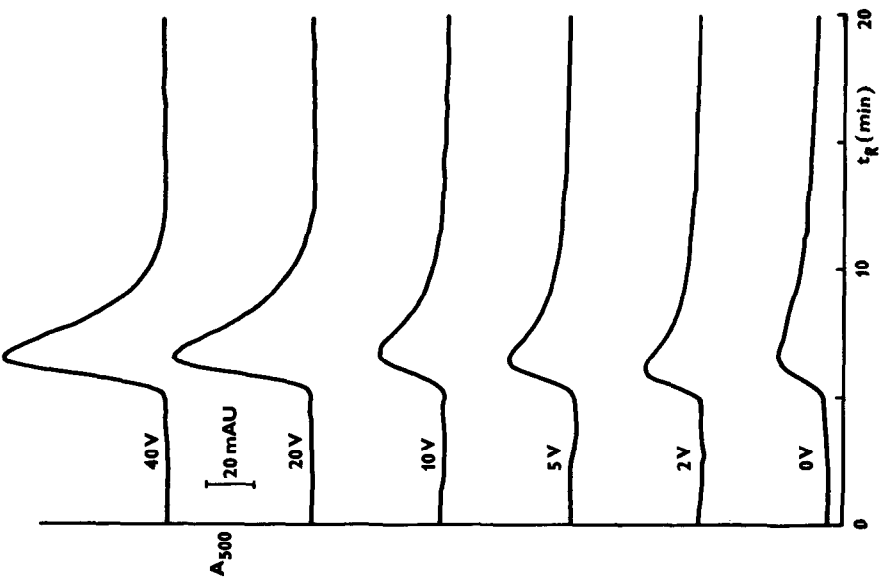


Fig. 8. As Fig. 6, with fractograms recorded at 500 nm.

Fig. 9. As Fig. 6, with fractograms recorded at 550 nm.

inside the trapezoidal cross-section IEF_4 channel. Focusing is more efficient at higher electric field strengths and at lower flow-rates of carrier ampholyte solutions. Further research is aimed at the investigation of focusing and separation of high-molecular-weight compounds.

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