Size-exclusion electrochromatography with controlled pore flow

Remco Stol, Wim Th. Kok*, Hans Poppe

Polymer Analysis Group, Department of Chemical Engineering, University of Amsterdam, Nieuwe Achtergracht 166,
1018 WV Amsterdam, The Netherlands

Abstract

In size-exclusion electrochromatography (SEEC) there exists an optimum in pore-to-interstitial flow ratio with respect to the resolution. Two methods for finding and controlling the optimal pore-to-interstitial flow ratio in SEEC have been studied: (i) varying the ionic strength of the mobile phase and (ii) the application of a hydrodynamic flow in addition to the electrco-driven flow. Both methodologies have been evaluated in terms of efficiency and applicability with columns packed with silica particles containing pores of either 10 or 50 nm in diameter, and with different ionic strength mobile phases. Using the first method with the 10-nm pore particles, the flow ratio could be adjusted within an appropriate range. However, with the wide-pore (50 nm) particles it appeared that the pore-to-interstitial flow ratio was too high at all conditions tested to obtain proper selectivity. In the second approach, the desired pore flow was generated by the electric field and the pore-to-interstitial flow ratio could then be adjusted by an applied pressure over the column. This method was applicable with both particle types studied. The application of a (low) voltage gradient in addition to a pressure-driven flow resulted in a sharply improved separation efficiency as a result of a strongly improved mass transfer due to intra-particle electroosmotic flow. When optimized, pressurized SEEC generates identical separation efficiencies for polystyrene standards as are obtained with pure SEEC, while the reduction in selectivity, in comparison to pressure-driven SEC, is kept minimal. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Size exclusion electrochromatography; Electrochromatography; Pore flow; Mass transfer; Polystyrene

1. Introduction

In recent years capillary electrochromatography (CEC) has rapidly evolved from a highly experimental technique [1–3] into a valuable analytical tool suitable for routine application. The advantages claimed for CEC over conventional LC are 2-fold. First, smaller particles can be used as stationary phase material, since the velocity of an electro-driven liquid flow through the column is virtually independent of the particle size [4,5]. Secondly, as has amply been shown in practice [1–8], a particular capillary column provides a higher separation efficiency when operated in the CEC mode than in pressure-driven LC mode. This increase of the efficiency has been attributed to improved homogeneity of the mobile phase flow across the column. Moreover, for columns packed with porous particles an improvement of the efficiency can also be obtained as the result of a substantial electroosmotic liquid flow through the pores of the particles [6].

So far, CEC has been performed almost exclusively in the reversed-phase mode. However, in previous work in our laboratory it was shown that it is also

*Corresponding author. Tel.: +31-20-525-6539; fax: +31-20-525-6638.

E-mail address: wkok@its.chem.uva.nl (W.T. Kok).

possible to do size-exclusion chromatography (SEC) with an electro-driven mobile phase flow, with columns packed with porous silica particles in so-called size-exclusion electrochromatography (SEEC) [7,8]. In these reports it was argued that an optimum exists between the efficiency enhancing and the selectivity reducing effects of pore flow in SEEC [8].

The beneficial effect of pore flow on the separation efficiency in CEC can be attributed to an enhancement of the stationary phase mass transfer processes. It is to be expected that this enhancement is most manifest for the macromolecular compounds typically separated by size-exclusion techniques. Since polymers have naturally low diffusion coefficients, slow mass transfer seriously limits the separation efficiency and speed in SEC, so that analysis times of well over 1 h are not uncommon [9]. It has been argued that even in conventional pressure-driven SEC of high mass polymers a (small) liquid flow through the pores may be significant for the efficiency [10,11]. For (bio)polymers speciality phases have been designed that supposingly exploit this effect, the so-called giga-porous particles for perfusion chromatography [12].

In perfusion chromatography the relative velocity of the flow through the particles, the pore-to-interstitial flow ratio, is determined by the ratio of the flow resistances of the pores to the interstitial channels in the packed bed. Control over the relative pore flow velocity may therefore be exerted by the choice of the (average) pore diameter and the particle size. However, in practice limitations are set on these choices by the limited stability and rigidity of the giga-porous materials available [13]. In electrochromatography it has been shown to be much easier to obtain a relatively high pore flow velocity [6].

While pore flow has a positive effect on the efficiency in SEEC, it will affect the mass selectivity of the separation negatively, since the separation of molecules of different sizes is based on the difference in flow velocity of the mobile phase inside and in between the particles. Therefore, the pore-to-interstitial flow velocity ratio has to be optimized to obtain the highest resolution in a SEEC system. As has been shown in a previous study [14], the ionic strength of the mobile phase is an important parameter to control the pore flow velocity. It was shown that for particles with narrow pores and for a mobile phase with a relatively high ionic strength the pore flow velocity can be adequately predicted by a classical double-layer overlap model [15]. For wide-pore materials the performance of this model and the effectiveness of the pore flow control through the ionic strength still has to be studied.

An alternative method to control the relative pore flow velocity is by applying an electric field and a pressure gradient over the column simultaneously. In this approach the electric field may be utilized to create a desired pore flow velocity, while with the pressure the interstitial flow can be regulated. These two methods to control the pore flow will be compared in their effectiveness in the optimization of the separation according to efficiency, mass selectivity and speed.

2. Experimental

2.1. Chemicals and materials

N,*N*-Dimethylformamide (DMF), methanol and toluene were obtained from Across (Geel, Belgium). Lithium chloride came from Merck (Darmstadt, Germany). The narrow polystyrene (PS) standards were purchased from Merck and Polymer Labs. (Church Stretton, UK) and all of these had a polydispersity <1.2, as was specified by the manufacturers. Of each of the PS standards a stock solution was prepared in DMF at a concentration of 10 mg ml^{-1}. Sample mixtures were prepared by mixing appropriate volumes of the stock solutions and pure DMF to obtain polymer concentrations of approximately 1.0 mg ml^{-1}. Toluene served as the totally permeating marker and was added to all samples in a concentration of 10 µl ml^{-1}.

The mobile phases consisted of DMF containing LiCl at various concentrations and were degassed prior to use by ultra-sonification for 10 min. The non-modified silica stationary phases employed in this study, Nucleosil 100-10 and Nucleosil 500-10, were obtained from Machery–Nagel (Düren, Germany). Both have a nominal particle diameter of 10 µm and nominal pore diameters of 10 and 50 nm, respectively.

2.2. Apparatus

All experiments were performed on a HP^{3D}CE system (Hewlett-Packard, Waldbronn, Germany). A nitrogen gas bottle delivered the external high pressure which was applied as a driving force in either the pure pressure- or the combined-elution mode. Under pure electro-drive conditions, a high pressure (10 bars) was applied at both ends of the column in order to prevent gas bubble formation. The use of the CEC system for the pressure drive experiments allows simple switching between the several elution modes, without the need for a separate HPLC system. Furthermore, it reduces column handling, thereby limiting the risk of column damage considerably. The CEC system is capable of applying a maximum pressure of 12 bar onto the column inlet, so that a relatively large particle diameter had to be used in order to obtain reasonable analysis times when using pressure-driven flow. When the ionic strength of the mobile phase was changed between experiments, the column was pneumatically flushed with the mobile phase for 20 min at an inlet pressure of 12 bar and successively flushed electrokinetically for 10 min at a voltage of 10 kV.

UV detection was performed at 260 nm. Injections were performed electrokinetically in all three elution modes by the application of 5 kV for 10 s. All experiments were performed in duplicate and the mean values were used for further calculations. Migration times and plate numbers were calculated using the standard Chemstation software (Hewlett-Packard).

2.3. Column preparation

At one end of a piece of capillary (375 μm O.D.×100 μm I.D., Polymicro Technologies, Phoenix, AZ, USA) a temporary frit was prepared by tapping it into a pile of dry silica particles of 5 μm in diameter (Nucleosil 100-5, Macherey–Nagel) and sintering the particles in place with a small gas flame.

A slurry containing 10 mg ml^{-1} of the respective stationary phase was prepared in methanol. A 20-cm piece of stainless steal tubing served as the slurry chamber and was filled with the slurry and then connected to a high pressure membrane pump using standard capillary LC connectors. High pressure was used to drive the particles into the capillary, using methanol as the displacement liquid. The slurry chamber was mechanically shaken until the capillary was completely filled with the particles. When columns were prepared from the particles with 10-nm pores, the maximum applied packing pressure was 400 bar, while for the 50-nm pore particles the maximum pressure used for packing was only 250 bar, to prevent the fracture of the particles [6]. Complete packing of the capillaries took approximately 5 min but high pressure was maintained on the column for at least 30 min before the pressure was allowed to decrease slowly.

The column was then flushed with water at 100 bar for 30 min and at this pressure the permanent in- and outlet frits were prepared from the packed section itself at a distance of 25 cm from each other by heating locally with a resistively heated metal strip device. After the permanent frits were prepared, the pressure was released and the column was flushed with water from the inlet side in order to remove the excess of the particles. A detection window was prepared immediately adjacent to the outlet frit by burning off the protective polyimide coating, using the same hot filament device used to prepare the permanent frits. Next, the column was installed in the CEC system and flushed with the mobile phase as described above.

3. Results and discussion

3.1. Repeatability of the inlet pressure

The CEC system is equipped with a pressure system capable of delivering a maximum of 12 bar of pressure on one side of the column. The repeatability of the pressure-induced flow was tested by measuring the migration time of a totally permeating solute (toluene) through the column for 5 times at three different inlet pressures (2, 7 and 12 bar). The results of this test are shown in Table 1.

The performance of the CEC system with respect to the high-pressure application is acceptable (RSD≤ 12%), although a higher precision is desirable when more elaborate or detailed studies are performed. Also options for applying higher pressures would be

Table 1
The repeatability of the migration time of toluene, tested at three
different column inlet pressures[a]

Inlet pressure (bar)	Mean migration time (min)	RSD
2	28.9	12.3
7	6.88	7.4
12	3.91	5.1

[a] Column: $L = 33$ cm(25 cm effective) Nucleosil 100-10. Mobile
phase, DMF containing 1.0 mmol l^{-1} LiCl.

required if smaller particles are to be used with this
system.

3.2. Pressure- versus electro-driven SEC: mass calibration

First the retention behavior of the polystyrene
standards on the two packing materials was studied
in SEEC and in pressure-driven elution mode. Mass
calibration curves were constructed by measuring the
retention times of narrow PS standards relative to the
migration time of toluene. In the pressure-driven
(PD) mode an inlet pressure of 10 bar was applied.
In the SEEC mode a separation voltage of 15 kV was
used with three different ionic strengths mobile
phases. The retention ratios of the PS relative to
toluene are shown in Fig. 1 for both packing
materials.

With the material containing the 10-nm pores, the
pore-to-interstitial flow ratio can be easily controlled
by changing the ionic strength of the mobile phase.
At an ionic strength of 1.0 mmol l^{-1} no noticeable
pore flow can be observed from the mass calibration
plots. At higher ionic strengths, electrical double
layer overlap within the pores is reduced, resulting in
a smaller retention window as expected. This can be
seen from the shift towards higher retention ratios (τ)
in the mass calibration plots of the PS standards.
However, for the 50-nm pore particles, pore flow
cannot be fully suppressed using this method, even
when the ionic strength of the mobile phase is as low
as 0.1 mmol l^{-1}. At lower ionic strengths, mass
calibration with PSs may become problematic, due to
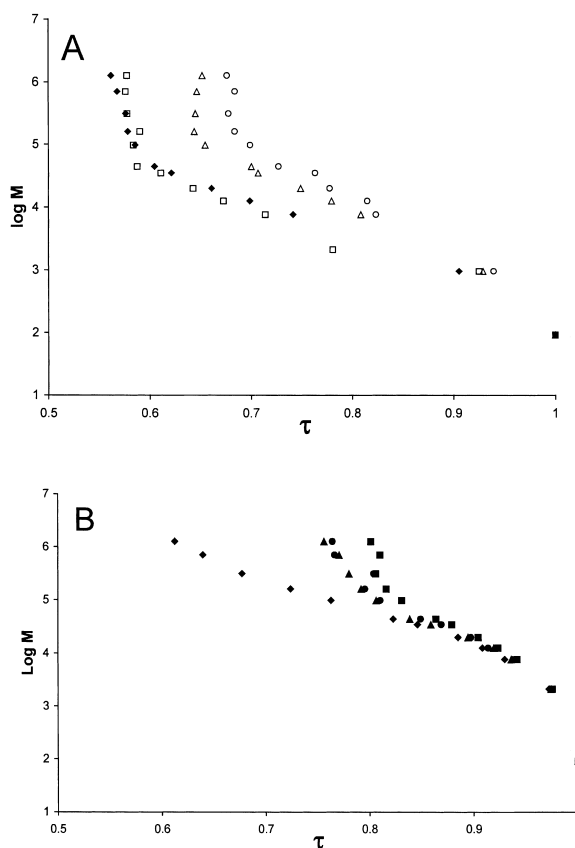secondary interactions between the silica and the PSs
[8].

Fig. 1. Mass calibration curves for polystyrene in DMF for the
two particle types in pressure-driven ($\blacklozenge$) and in SEEC modes at
different two ionic strengths. Columns: $L = 33$ cm (25 cm effec-
tive) Nucleosil 100-10 (A) and Nucleosil 500-10 (B). Mobile
phase PD: DMF containing 1 mmol l^{-1} LiCl. Mobile phases
SEEC: DMF containing: ($\square$) 1 mmol l^{-1}; ($\triangle$) 10 mmol l^{-1}; (O)
20 mmol l^{-1}; ($\blacktriangle$) 0.1 mmol l^{-1}; ($\bullet$) 0.5 mmol l^{-1}; ($\blacksquare$) 1 mmol
L^{-1} LiCl.

3.3. Pressure- versus electro-driven SEC: efficiency

Before the optimal pore-to-interstitial flow ratio
can be determined, the relative importance of the
efficiency augmenting effects in CEC mode has to be
determined. Due to the narrow molecular mass
distribution (MMD) of the polystyrene standards
used, the separation efficiency can be described
using a simplified plate height equation [16]:

$$H = A + Cu \qquad (1)$$

where H is the height equivalent to a theoretical

plate, A is the flow inhomogeneity contribution, C is the mass transfer coefficient and u is the linear velocity of the mobile phase. With this simplification evaluation of the A and C terms from the $H-u$ plots under pressure- and electro-driven conditions is possible using simple linear regression.

$H-u$ curves for PSs in PD-SEC were measured through variation of the inlet pressure to up to 12 bar. In the SEEC mode, for each particle type different ionic strength mobile phases were used and a voltage gradient up to 30 kV was applied. Typical $H-u$ curves for selected PS standards are shown in Figs. 2 and 3, for the 10- and 50-nm pores particles, respectively. The A and C terms calculated from these data by linear regression from these data are reported in Table 2.

For the 10-nm pore containing material switching from the PD to the SEEC mode at low ionic strength results in a relatively small improvement of the flow homogeneity (A term). The main gain in efficiency originates from an improved mass transfer. However, at higher ionic strengths both terms are effectively reduced in comparison to the PD mode. The lower A term in SEEC at higher ionic strength may originate from pore flow, which results in a more homogeneous flow across the column cross-section.

With the wide-pore material the C term strongly diminishes in SEEC even at low-ionic strength. With this material the pore flow velocity is already quite
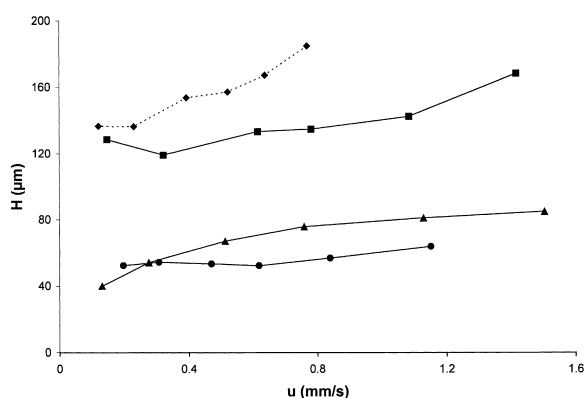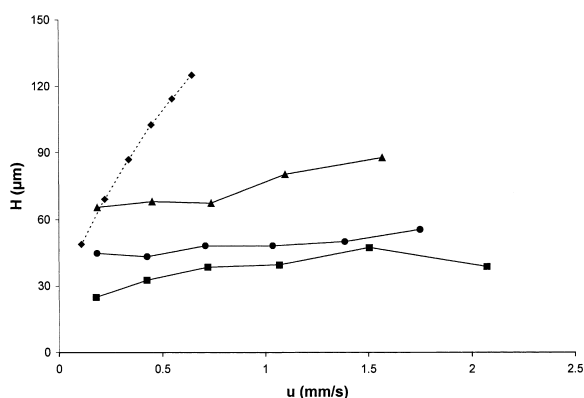

Fig. 3. The $H-u$ curves for the polystyrene standard with mass 160 kDa obtained on the 50-nm pores containing stationary phase in pressure-driven mode (dotted line) and in SEEC mode (solid lines) at an LiCl concentration of (▲) 0.1 mmol l^{-1}; (●) 0.5 mmol l^{-1} and (■) 1 mmol l^{-1}.

high with low-ionic-strength buffers. At higher ionic strengths both the A and C terms are further improved. It is noted that both the flow homogeneity and the mass transfer in electrochromatography appear to be related to the ionic strength, probably as a result of flow through the particles [6,8].

Unusual behavior was observed for peaks of totally excluded polymers, which showed a severe tailing which became stronger at higher field strengths. Possibly, the pore flow creates a hydrodynamic pressure on these polymers, forcing them to squeeze into pores in which they normally would not fit. It has been suggested by De Gennes and co-workers [17,18] to make use of such reptational behavior for separations according to "stiffness of the chain or branching".

The rather small reduction of the A term found in SEEC is in contrast with most published work on CEC that indicate that this term is strongly reduced using electro-driven flow. This might be due to the relatively large particles used in this study so that mass transfer resistance is completely dominating the observed plate height. It is observed that in the present SEEC system the largest improvement of the separation efficiency over conventional SEC can be attributed to improved mass transfer. Slow mass transfer set limits on the performance of conventional SEC in relation to the upper mass limit and separation speed. With SEEC this condition is

Fig. 2. The $H-u$ curves for the polystyrene standard with mass 7.6 kDa obtained on the 10-nm pores containing stationary phase in pressure-drive mode (dotted line) and in SEEC mode (solid lines) at a LiCl concentration of (■) 1 mmol l^{-1}; (▲) 10 mmol l^{-1} and (●) 20 mmol l^{-1}.

Table 2
Comparison of the flow inhomogeneity (A term) and mass transfer factors (C term) for selected polystyrene standards under pressure (PD) and electro-driven (ED) conditions

Elution mode	Ionic strength (mmol L^{-1})	*A* term (μm)	*C* term (μm)
Nucleosil 100-10/PS (M_r 7600)			
PD	1	123	74
ED	1	114	32
ED	10	45	30
ED	20	49	11
Nucleosil 500-10/PS (M_r 160 000)			
PD	0.1	37	141
ED	0.1	43	5.7
ED	0.5	42	6.8
ED	1.0	44	0.9

strongly relaxed providing possibilities for high-speed operation and extension of the mass range that can be handled by exclusion chromatography.

3.4. Combined elution mode: flow development and retention window

The second method of regulating the pore-to-interstitial flow ratio is by the combined application of a hydrodynamic and an electrokinetically driven flow. In this methodology the electrokinetic flow is used mainly to generate the desired pore flow velocity, while the pressure-driven flow is used to regulate the interstitial flow velocity. According to the definitions of Knox [19], this elution mode would be appropriately termed *pressurized* capillary size-exclusion electrochromatography.

The effect of the combined application of a hydrodynamic and an electrokinetic force on total flow development through both the column and the particles was studied on both materials. To cover a wide range of flow conditions, the inlet pressure was varied between 2 and 12 bar, while the applied electric field was varied to up to 25 kV. The intra- and inter-particle flow regimes were measured using a totally permeating marker and an excluded PS standard. The migration velocities of these marker solutes through the columns at the various conditions studied are shown in Fig. 4.

The observed non-linearity in the velocities of both the totally permeating and the excluded marker solutes in relation to the applied field strength (E)
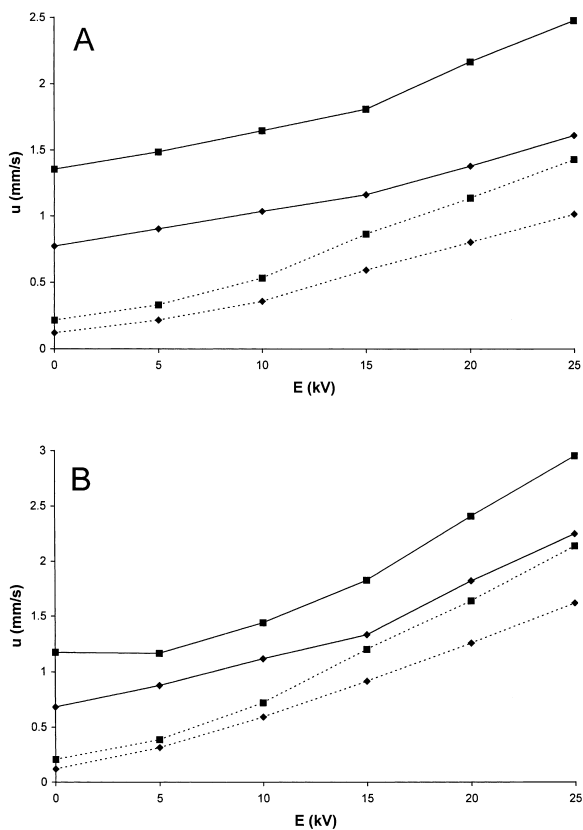

Fig. 4. Migration velocities of the totally excluded (■) and completely permeating (♦) markers on the 10-nm pores (A) and 50-nm pores (B) containing materials in the combined elution mode. Mobile phase: DMF containing 20 mmol l^{-1} (A) or 1 mmol l^{-1} LiCl (B). Inlet pressures: 12 bar (solid lines) and 2 bar (dotted lines).

cannot be explained on basis of an increasing flow ratio with the electric field. Both the interstitial and the intra-particle flow regimes are expected to be linear with the applied field strength. The same non-linearity in electroosmotic flow was observed in pure electro-driven chromatography. During all the experiments the electrical current flowing through the column never exceeded 10 µA, so that Joule heating is unlikely to be of importance [2]. A possible explanation might be that the electric field results in a different orientation of the particles leading to a different permeability of the column. Also the combination of low ionic strengths, large particle diameters and the application of high electric fields may result in concentration polarization of the electrical double layer around the particles, leading to electroosmotic flow of the second kind [20,21].

The retention ratio between an totally excluded and a fully permeating marker defines the retention window in exclusion chromatography. The effect of combined electro- and pressure-driven flow on the retention window is shown in Fig. 5. The retention ratio is expected to have non-linear behavior, since the flow ratio increases with electric field strength towards the flow ratio as is observed in pure SEEC. From the results it can be concluded that the flow ratio can be controlled by varying the electrokinetic and hydrodynamic flow-rates.

3.5. Combined elution mode: efficiency

The separation efficiency of pressurized SEEC was studied on both particle types using PS standards. The inlet pressure was set at 2, 7 or 12 bar while the electric field was varied with applied voltages of 0, 1, 3, 5, 10 and 20 kV. The mobile phase velocity was calculated from the migration time of toluene. The theoretical plate height (H) for the selected PS standards is plotted as a function of the mobile phase velocity plots Figs. 6 and 7 for the 10- and 50-nm pore materials, respectively. The results for pure SEEC and PD modes are also included in these plots.

From these figures it can be seen that the application of an increasing voltage at constant pressure leads to lower plate heights, even though the mobile phase velocity is increased. Even the application of relatively low voltages (1–5 kV) in conjunction with
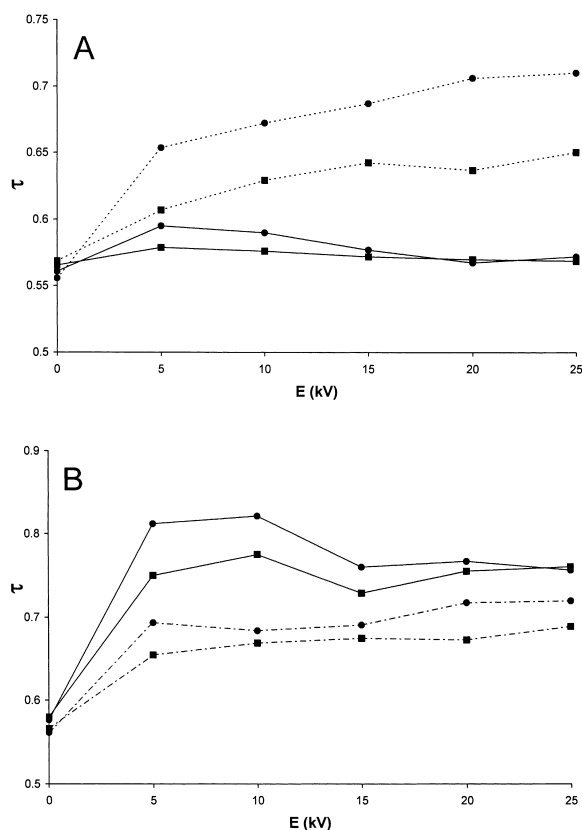

Fig. 5. The retention ratio between the excluded and the totally permeated marker on the 10-nm pores (A) and 50-nm pores (B) containing materials, respectively, in combined elution mode at different ionic strengths and column inlet pressures. Inlet pressures: (■) 12 bar and (●) 2 bar. Mobile phase: DMF containing 20 mmol l^{-1} (dotted lines); 1 mmol l^{-1} LiCl (solid lines) or 0.1 mmol l^{-1} LiCl (dashed–dotted lines).

a pressure gradient leads to separation efficiencies close to the efficiency observed in pure electro-driven exclusion chromatography. At higher field strengths the $H–u$ curves obtained in combined elution mode follow those of the pure electro-driven mode more closely.

Apparently, at relatively low pore flow velocities the stationary phase mass transfer enhancement factor is already quite high and the separation efficiency is then limited by other factors, such as the mobile phase mass transfer resistance.

In Fig. 8 the efficiency enhancement factor when switching from pressure to electrochromatography mode is plotted as a function of the flow velocity
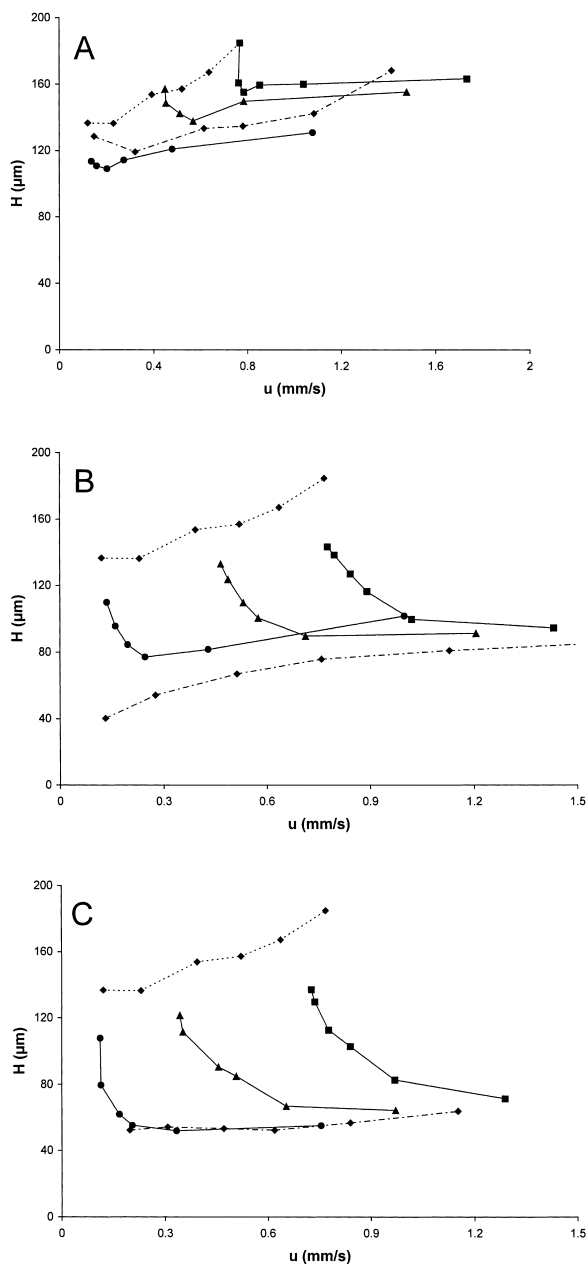
Fig. 6. The H–u curves for PS (M_r 7600) on the 10-nm pores containing material in pressure-driven mode (dotted lines), SEEC mode (dashed–dotted lines) and combined elution mode (solid lines) at different inlet pressures and mobile phase ionic strengths. Inlet pressure: (■) 12 bar; (▲) 7 bar and (●) 2 bar. Mobile phase: DMF containing (A) 1 mmol l^{-1}, (B) 10 mmol l^{-1} and (C) 20 mmol l^{-1} LiCl. Column: $L=33$ cm (25 cm effective) Nucleosil 100-10.
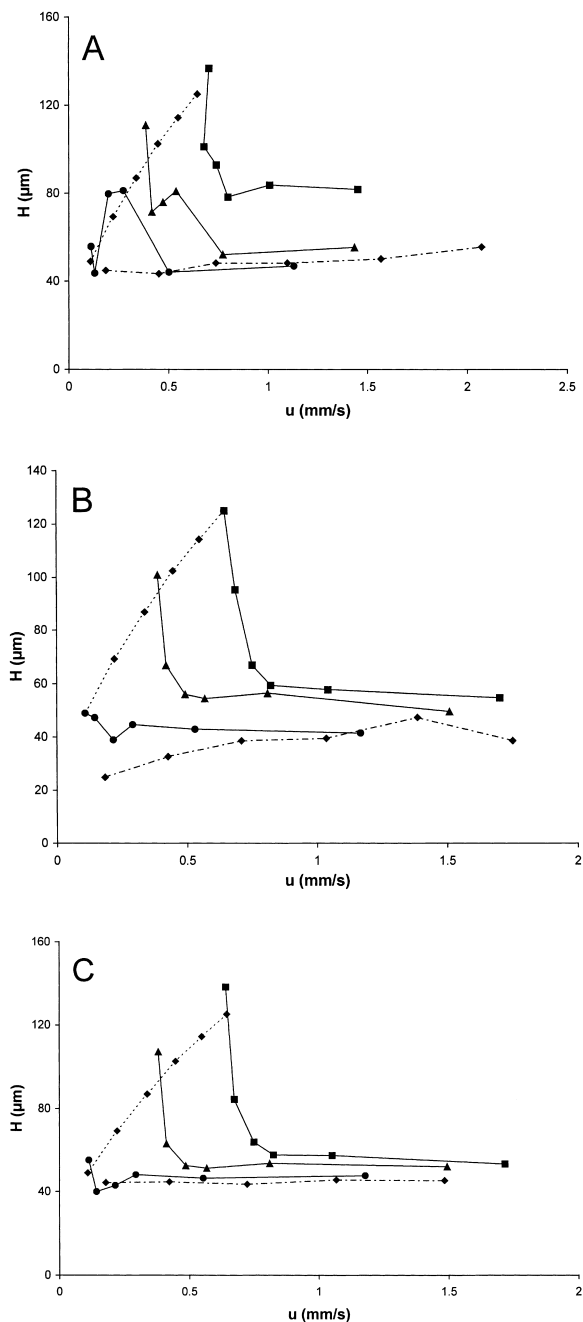
Fig. 7. The H–u curves for PS (M_r 160.000) on the 50-nm pores containing material in the pressure-driven mode (dotted lines), SEEC mode (dashed-dotted lines) and combined elution mode (solid lines) at different inlet pressures and mobile phase ionic strengths. Inlet pressure: (■) 12 bar; (▲) 7 bar; and (●) 2 bar. Mobile phase: DMF containing (A) 0.1 mmol l^{-1}, (B) 0.5 mmol l^{-1}, and (C) 1.0 mmol l^{-1} LiCl. Column: L=33 cm (25 cm effective) Nucleosil 500-10.
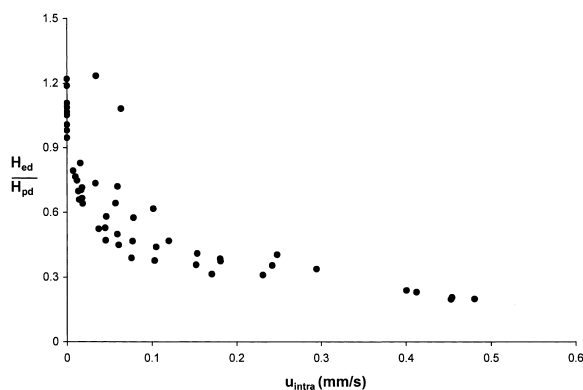
Fig. 8. The efficiency enhancement factor expressed as the plate height with an electro-driven flow over the plate height with an pressure-driven flow. Stationary phase: Nucleosil 500-10; Sample: PS (M_r 160 000).

inside the pores. The intra-particle mobile phase velocities were calculated using a previously described model and the migration time of toluene [14]. Although the efficiency enhancement factor is not entirely representing the gain in mass transfer efficiency, the curve shape is in good correlation to what may be expected from theory [12,13].

It is seen that it suffices to generate a relatively low pore flow velocity (0.05 mm/s) for efficiency enhancement so that the loss in mass selectivity of the system can be kept relatively small. With DMF as the mobile phase and an appropriate pore diameter and ionic strength, the application of less than 5 kV over a 25-cm (effective) long column will generally suffice to produce such pore flow velocities. The observation that mass transfer enhancement can be exploited at relatively low pore flow velocities means that complications due to Joule heating are easier circumvented and that the pressurized-SEEC methodology may be applied to columns with larger bores. Moreover, the low pore flow velocities required may be obtained with solvents with low dielectric constants, which are more generally applied in size-exclusion chromatography of synthetic polymers [9] and with SEEC one does not rely completely on electroosmosis for flow development, so that higher mobile phase velocities as compared to pure SEEC may be used.

Secondly, the ability to improve mass transfer may

be used to enhance the resolving power of SEC, especially in the upper molecular mass range of SEC ($\gg 1 \times 10^6$) where the efficiency is limited by slow mass transfer.

Acknowledgements

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