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Transfer of gradient chromatographic methods for protein separation to Convective Interaction Media monolithic columns

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Abstract

Convective Interaction Media (CIM) columns are monolithic columns optimized for the separation of macromolecules. Some of them operate in the axial mode while others operate in the radial mode depending on the column size. In this work we tested the approach suggested by Yamamoto [*Biotechnol. Bioeng*., 48 (1995) 444] for transfer of gradient methods between columns of different size. A simplified equation for transfer was derived together with a criterion for its application. Separation was evaluated for a standard protein mixture and peroxidase enzymes present in fermentation broth. Salt and pH gradients were applied. Similar resolutions were obtained for each sample on all columns which demonstrates that the proposed approach can be successfully used for method scale-up on this type of column.

Keywords: Gradient elution; Monolithic columns; Proteins

1. Introduction

Chromatography is one of the most important techniques for the analysis and purification of various compounds. Since most of the chromatographic methods are developed on low-volume columns, transfer of the chromatographic method from a small to a large column is of the utmost importance to obtain the required quality of the final product. To avoid any changes, separation conditions should be the same regardless the size of the column. Unfortunately, this is not a trivial task since usage of large columns inevitably changes the column properties, such as dimensions and mobile phase distribution and also commonly affects other parameters like, e.g., type of chromatographic resin. Because of that, different scale-up criteria were suggested such as a constant column height, a constant ratio between column length and a particle diameter, a constant residence time, a constant Biot number, etc. [1]. The above criteria are especially important for analyzes, where multiple interactions with stationary phase are required for separation. In the case of large molecules, this is however commonly not the case. Because of the extremely steep adsorption isotherms, large molecules like, e.g., proteins or DNA are retained almost irreversibly on the resin under loading conditions. To elute them, a change of the mobile phase is required, typically in the form of a linear or step gradient. For gradient elution, a model for reversed-phase separation of proteins indicated that column length does not significantly influence the

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separation [2]. In the ion-exchange mode, more detailed models taking into consideration zone spreading and zone compression effects, suggest that for steady state conditions, resolution is proportional to the square root of column length when linear mobile phase velocity, the particle diameter and gradient slope normalized on the column length are kept constant. Based on this model Yamamoto introduced a different scale-up approach which predicts a gradient slope according to the column dimensions and the characteristics of the applied resin [3]. This approach gave similar resolution for proteins on axial columns with different volumes and chromatographic resins for all but very short columns [4]. No explanation for the latter observation was given in the paper but one can speculate that this is due to unreached steady state or problems related to column packing or extra column band spreading [5].

Yamamoto's model is based on the assumption that steady state is achieved. However, the model gives no information when such a situation is obtained. Estimation of the critical distance at which the quasi-steady state is attained was derived by Dubinina et al. [6]. They showed that critical distance depends on the concentration at which the protein elutes, linear velocity, steepness of the gradient and strength of binding of the protein to the matrix. To achieve a steady state at the column exit, the physical column length should be equal to or longer than the critical distance calculated according to the mathematical model. On this basis it is possible to calculate the gradient slope for a given column where Yamamoto's scale-up criteria can be applied.

While the scale-up approach was already successfully tested on axial columns filled with particles [3], no application with the monolithic column was reported so far. Monoliths consist of a single piece of highly porous material. The pores are interconnected forming a network of transitional channels. Since the mobile phase flows through the channels, the molecules to be separated are transported by convection. This results in flow-unaffected resolution and dynamic binding capacity [7,8] which is especially advantageous for large molecules with low mobility. This fact was the main reason for the introduction of the methacrylate-based monoliths [9], which are currently marketed under the trademark CIM (Convective Interaction Media). Columns are available in various sizes [10] but they all have a short bed particularly designed and optimized for separation and purification of large molecules [11]. While small columns operate in an axial mode, large columns are constructed in a tubular shape [12–14] applying a radial operation mode [10].

In this work we tested a scale-up approach proposed by Yamamoto [3] for the transfer of chromatographic method to different CIM monolithic columns. A mixture of standard proteins as well as a real sample of a fungal fermentation broth was used to estimate the suitability of this approach.

2. Experimental

2.1. Gradient calculation

For gradient separation Yamamoto [3] introduced a dimensionless parameter O defined as:

$$O = \frac{LI_a}{G\mathrm{HETP}_{\mathrm{LGE}}} \tag{1}$$

where L is the column length, G the gradient slope normalized with respect to column void volume, I_a the dimensional constant having a value of 1 and $\mathrm{HETP}_{\mathrm{LGE}}$ the plate height in a linear gradient elution mode.

In order to obtain an equal resolution for two columns, the dimensionless parameters O should also be equal. Since for the same stationary phase HETP is equal, this criterion is simplified to:

$$\frac{L_1}{L_2} = \frac{G_1}{G_2} \tag{2}$$

where G is the gradient slope normalized with respect to the column void volume (V_v) which can be rewritten as:

$$G = gV_v = \frac{\Delta C}{V_g} \cdot V_v \tag{3}$$

where g is the gradient slope, ΔC the concentration difference between the beginning and the end of the gradient and V_g the gradient volume. V_g can further be expressed as:

$$V_g = Ft_g \quad (4)$$

where F is the flow-rate and t_g the gradient time.

Combining Eqs. (2), (3) and (4) the following expression is obtained:

$$\frac{L_1}{L_2} = \frac{\Delta C V_{v,1} F_2 t_{g,2}}{\Delta C V_{v,2} F_1 t_{g,1}} \quad (5)$$

Since the concentration difference is equal for both gradients the expression for gradient time finally becomes:

$$t_{g,2} = t_{g,1} \cdot \left(\frac{V_{v,2}}{V_{v,1}}\right) \cdot \left(\frac{F_1}{F_2}\right) \cdot \left(\frac{L_1}{L_2}\right) \quad (6)$$

In the case where the bed represents most of the column volume and the bed porosity is the same for differently sized columns, the column void volume can be substituted with the total column volume.

2.2. Calculation of resolution

For comparison of transferred methods the resolution, R_s, defined by Eq. (7) [15], was calculated between all adjacent peaks:

$$R_s = |V_{e1} - V_{e2}| / [(W_1 + W_2)/2] \quad (7)$$

where V_{e1} and V_{e2} are the peak elution volumes and W_1, W_2 are the peak elution widths measured at the baseline.

2.3. Calculation of critical distance

Critical distance at which a steady state is attained was calculated according the equation [6]:

$$X_0 = \frac{\lambda u C}{ZB} \quad (8)$$

where X_0 is the critical distance (cm), λ is an auxiliary parameter having a value 0.5 [16], u the linear velocity (cm/min), C the concentration of displacer salt at which protein elutes (M), the Z factor is the ratio of the protein charge and displacer charge (–) and B the gradient steepness (M/min).

Eq. (8) requires a Z factor value, which can be calculated according the following equation [17]:

$$\log k' = \log K - Z \log c \quad (9)$$

where K is the ion-exchange equilibrium distribution constant and k' is defined as:

$$k = \frac{t_p - t_s}{t_s} \quad (10)$$

where t_p is the protein retention time and t_s the delay time.

To obtain steady state at the column exit, the column length should be longer or equal to X_0. The following criterion should therefore be fulfilled:

$$\frac{L}{X_0} \geq 1 \quad (11)$$

2.4. Samples

The standard protein mixture consisted of myoglobin (Sigma, St. Louis, MO, USA), conalbumin (Sigma) and soybean trypsin inhibitor (Fluka, Buchs, Switzerland) was dissolved in 20 m*M* Tris–HCl, pH 7.4 (binding buffer) in the following concentrations: 2, 6 and 8 mg/ml. Lignin peroxidases (LiP enzymes) were produced by the fungus *Phanerochaete chrysosporium* MZKI B-223 (ATCC 24725) in a nitrogen-limited medium according to Ref. [18] while for production of manganese peroxidases (MnP enzymes) the medium contained in addition 1 m*M* Mn^{2+} [19]. Before application, the medium was filtered, frozen overnight, rethawed, centrifuged, concentrated and dialyzed overnight against 10 m*M* sodium acetate (pH 6). Concentration of LiP and MnP enzymes was not determined but samples of the same composition were used for all the experiments.

2.5. Buffers

For the separation of the standard protein mixture 20 m*M* Tris–HCl, pH 7.4 was used as loading buffer and 20 m*M* Tris–HCl + 1 *M* NaCl, pH 7.4 as an eluting buffer. LiP isoenzymes were separated according the method proposed by Podgornik et al. [20] using 10 m*M* sodium acetate, pH 6 as a binding buffer and 1 *M* sodium acetate, pH 6 as the eluting buffer. Separation of MnP isoforms was performed applying pH gradient, using sodium acetate, pH 5 (10 m*M*, binding buffer) and sodium acetate, pH 3.5 (10 m*M*, eluting buffer) [21]. All chemicals were of analytical grade from Sigma.

2.6. Columns

Weak ion-exchange monolithic columns bearing diethylaminoethyl (DEAE) groups were used throughout this work (BIA Separations, Ljubljana, Slovenia). The columns had the following dimensions: (1) CIM disk monolithic columns operating in the axial mode had a diameter of 12 mm and various volumes of 0.34, 0.68, 1.02 and 1.36 ml of lengths 3, 6, 9 and 12 mm, respectively. They were constructed by placing from one up to four CIM disks in a single CIM housing. (2) CIM tube monolithic columns operating in radial mode were of three different volumes: 8 ml with the bed thickness of 6.5 mm, 80 ml with a thickness of 16 mm and 800 ml with a thickness of 21 mm.

2.7. Determination of the column void volume

For all monolithic columns except for the 800-ml monolithic column it was assumed that the entire void volume is represented by the porosity of the monolithic bed since no significant differences between pulse response experiments under non-binding conditions and values predicted from the porosity of the monolith itself were found. Therefore, for scale-up criteria (Eq. (6)) the total volume of the monolith was considered. For the 800-ml column the column void volume is larger due to construction of the column, which has some void volume in the middle of the cylindrical monolith. The void volume was calculated according the following procedure. Empty column housing was dried and weighted. Then the housing was filled with distilled water, sealed from the both sides with the end fittings and weighted again. From a weight difference the total void volume of the housing was calculated. Since the total monolith volume is 800 ml and the porosity is around 62%, the skeleton volume is approximately 300 ml. By subtracting this value from the total housing volume a void column volume was calculated and found to be approximately 750 ml. This value was in good agreement with the value obtained from the pulse response experiment.

2.8. High-performance liquid chromatography (HPLC) system

Experiments on the CIM disk monolithic columns and the 8-ml CIM monolithic tube column were performed on a gradient HPLC system consisting of two Pumps 64, an injection valve with a stainless steel sample loop, a variable-wavelength monitor with a 10-mm optical path set to 280 nm (409 nm for peroxidase runs) and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany). A Knauer mixing chamber with its relatively large dead volume was replaced by a polyether ether ketone (PEEK) mixing tee 9000-0665 with an extra low-dead volume (Jour Research, Uppsala, Sweden). In the case of MnP isoforms separation, the pH value of the outflow was measured with an on-line pH meter (MA 5740, Metrel, Slovenia) connected to the HPLC system.

Experiments on the 80- and 800-ml CIM monolithic columns were carried out on a preparative gradient HPLC system comprising two preparative pumps K-1800 allowing for flow-rates up to 1000 ml/min, an injection valve with a 1-ml stainless steel sample loop, a preparative UV detector K-2500 set to 280 nm, a preparative mixing chamber, all connected by means of 1.5 mm I.D. PEEK capillary tubes and HPLC hardware/software (data acquisition and control station), all from Knauer.

3. Results and discussion

To obtain similar resolution on differently sized monolithic columns, a gradient slope should be carefully adjusted. If a gradient is simply transferred from a small to a large column normally lower resolution on a larger column is obtained as shown in Fig. 1. Resolution also varies significantly when a different gradient slope is applied on the same column (Fig. 1). Therefore, a general and simple procedure to calculate a gradient slope for keeping constant resolution on different monolithic columns is preferred.

In this study seven different CIM monolithic columns were used, four operating in the axial mode and three in the radial mode. Axial operating columns were constructed by placing from one up to four CIM disks in the same CIM housing [8]. It was shown that columns constructed in this way have the same efficiency as a single monolithic rod of the corresponding length [22]. Therefore, it was assumed

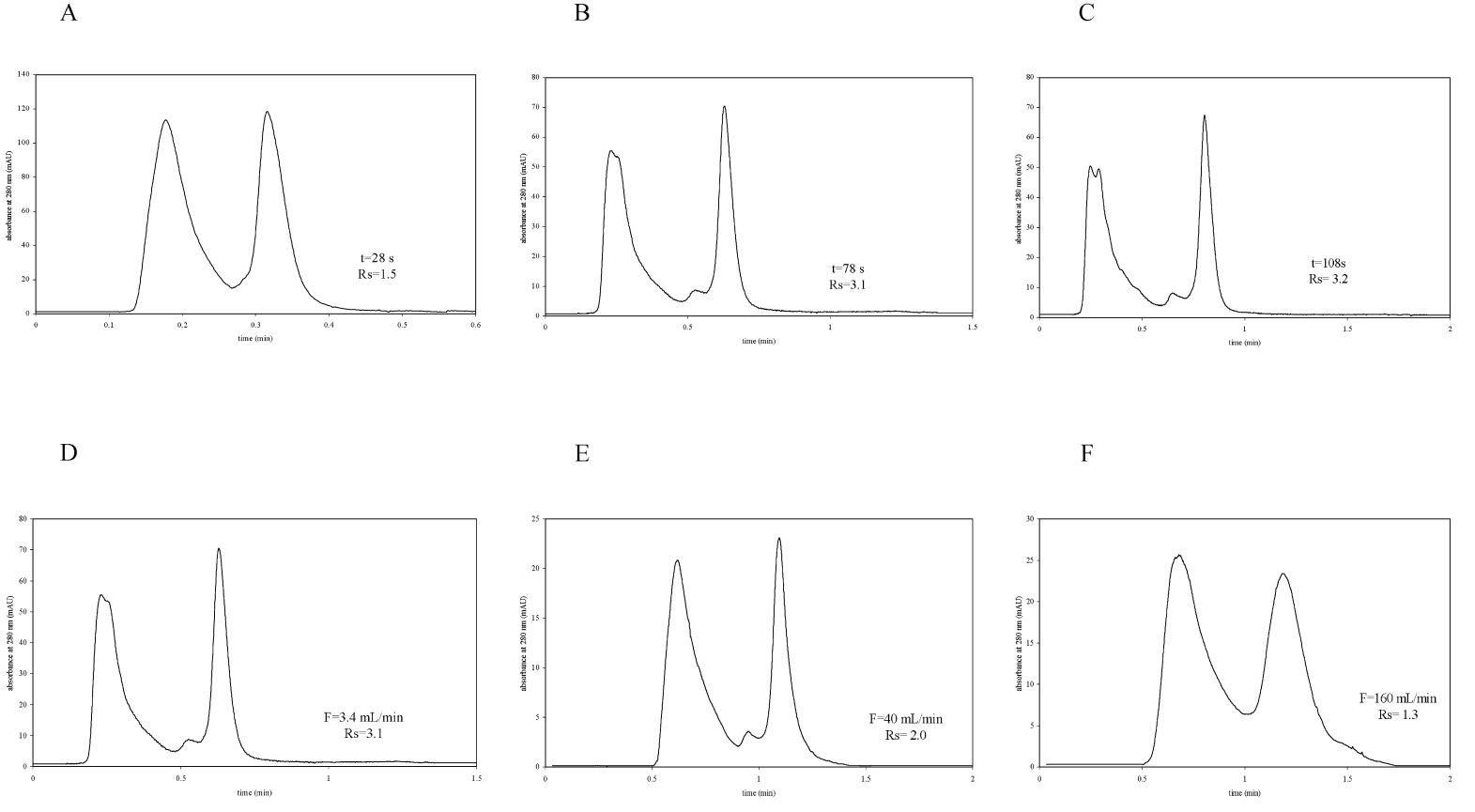

Fig. 1. Chromatograms of standard protein mixture varying gradient on a single monolithic column and keeping constant gradient on different monolithic columns. Conditions: Sample: conalbumin (first peak), trypsin inhibitor (second peak); mobile phase: buffer A: 20 m*M* Tris–HCl buffer, pH 7.4; buffer B: 20 m*M* Tris–HCl buffer + 0.7 mol/l NaCl, pH 7.4; columns: for A, B, C and D columns with the monolith volume of 0.34 ml, for E of 8 ml and F of 80 ml; flow-rate: for A, B and C of 3.4 ml/min and for D, E and F as shown in figures; gradient times: for D, E and F of 78 s and for A, B, C as shown in figures. Detailed description of the columns are given in Table 1. Spectrophotometric detection was done at 280 nm. Resolutions are shown in all figures.

that the properties of the resin were equal regardless of the column length. Another point of consideration is a transfer of the chromatographic method from the axial to radial operation mode. In the radial operation mode the linear velocity increases from the outer part of the bed toward the inner part [13,14]. This increase can be more than an order of magnitude [13] which might influence the chromatographic efficiency of the resin [23]. However, for the methacrylate monolithic resins the column efficiency was found to be unaffected by flow [24]. Such a conclusion was also confirmed for the radial columns showing that the resolution and the dynamic binding capacity are unaffected by flow [13]. Therefore, from this point of view, a change of the linear velocity does not influence the column efficiency and thus a monolithic radial column should behave like an axial one.

For all experiments the chromatograms were analyzed in terms of resolution calculated according to Eq. (7). As a rule, the initial chromatographic method was optimized on a single disk monolithic column having a volume of 0.34 ml and adjusted to other columns according to Eq. (6).

For the optimized chromatographic method on the smallest monolithic column it should be verified whether it fulfills the criterion for the steady state conditions, which is required for the validity of Yamamoto's scale-up criteria. The first step was calculation of the Z factor. We calculated the Z factor only for soybean trypsin inhibitor since we did not have pure peroxidases while conalbumin consists of different isoforms, which start to separate under isocratic conditions and determination of k' becomes questionable. Seven isocratic experiments were performed injecting 80 μg of soybean trypsin inhibitor into 20 mM Tris buffer, pH 7.4 containing following NaCl concentrations: 0.4, 0.45, 0.5, 0.55, 0.6, 0.65 and 0.7 M. Experiments with the mobile phases containing 0.9 and 1 M NaCl gave the same retention time which was taken as t_s in the calculation of k' (Eq. (10)). Logarithms of calculated k' were related to the corresponding logarithms of NaCl concentration. Data were successfully fitted with a straight line using Eq. (9) giving the value of Z factor 4.76 with the correlation index of 0.992.

From the data in Table 1, taking into account that a diameter of the monolith is 12 mm, the calculated linear velocity u through the smallest monolithic column is 3 cm/min and the gradient steepness B is 0.538 M/min. From Fig. 1 one can estimate the critical elution concentration for trypsin inhibitor to be approximately 0.5 M and according to Eq. (8) the critical distance X_0 is calculated to be 0.29 cm. Since the length of the smallest column is 0.3 cm (Table 1) the ratio between X_0 and L is larger then 1 and steady state is attained (see Eq. (11)), justifying the application of Yamamoto's scale-up criterion.

Table 1
Chromatographic methods applied on different monolithic columns calculated according to Eq. (6)

Sample	Column type	Monolith volume, V (ml)	Column length, L (cm)	Flow-rate, F (ml/min)	Gradient time, t_g (s)	Injected volume (μl)	Sample concentration (mg/ml) (protein mixture)
Standard proteins	Axial	0.34	0.3	3.4	78	20	4
	Axial	0.68	0.6	6.8	39	20	4
	Axial	1.02	0.9	5.1	52	20	4
	Axial	1.36	1.2	2.7	97	20	4
	Radial	8	0.65	16	180	100	4
	Radial	80	1.6	160	73	500	24
	Radial	800	2.1	800	168	1000	50
LiP	Axial	0.34	0.3	6	120	500	
	Axial	0.68	0.6	6	120	500	–
	Axial	1.02	0.9	3	240	500	–
	Radial	8	0.65	45	174	3000	–
	Radial	80	1.6	215	149	10 000	–

Conditions: standard proteins: linear gradient of 0–0.7 mol/l sodium chloride in a Tris–HCl buffer solutions, pH 7.4 within a specific gradient time (t_g); LiP isoenzymes: linear gradient of 10 mM–1 M acetate buffer, pH 6 within a specific gradient time.

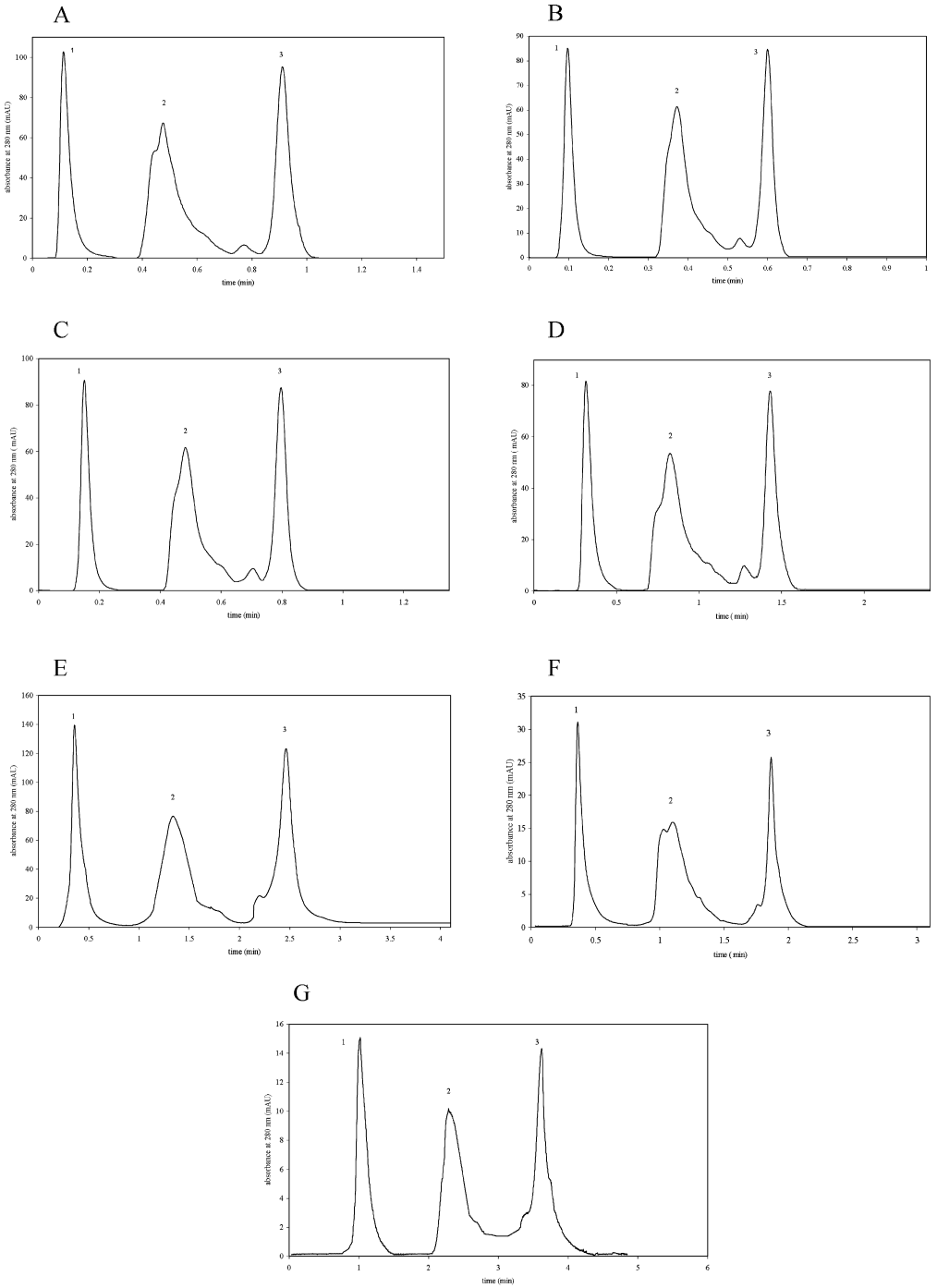

Fig. 2. Chromatograms of standard protein mixture obtained on axial and radial monolithic columns. Conditions: sample: myoglobin (first peak), conalbumin (second peak), trypsin inhibitor (third peak); mobile phase: buffer A: 20 m*M* Tris–HCl buffer, pH 7.4; buffer B: 20 m*M* Tris–HCl buffer + 0.7 mol/l NaCl, pH 7.4; columns: CIM disk monolithic columns with the monolith volumes A—0.34 ml, B—0.68 ml, C—1.02 ml, D—1.36 ml and CIM tube monolithic columns with the monolith volumes E—8 ml, F—80 ml and G—800 ml. Description of the gradients and other conditions are given in Table 1. Spectrophotometric detection was done at 280 nm.

The first set of experiments was performed with the mixture of standard proteins using the method described in detail in Table 1. Different amounts of samples were injected to obtain the chromatograms shown in Fig. 2. The resolutions between the peaks are presented in Table 2. We can see that the chromatograms look very similar confirming the similar resolutions reported in Table 2. A slight splitting of the second peak is due to different conalbumin conformations present in the sample, which do not significantly influence the resolution.

Another set of experiments was performed using a real sample of *P. chrysosporium* fermentation broth. This fungus excretes different proteins, among which LiP and MnP enzymes are especially interesting. These peroxidases are involved in lignin degradation [25]. Each peroxidase appears in the medium as a family of different isoforms having almost identical catalytic properties but slightly different molecular mass and isoelectric points [26]. All isoforms contain one haem group, which enables their spectroscopic detection at 409 nm. Besides these proteins, the medium contains a significant amount of other proteins, some of which are retained under the applied conditions [20] and might consequently influence LiP or MnP enzyme separation.

Separation of the cultivated growth medium containing LiP isoforms was performed using a salt gradient. Details of the method applied on each column are shown in Table 1, while the chromatograms and corresponding resolutions are presented in Fig. 3 and Table 2, respectively. We can conclude that the proposed method transfer gives similar separation of target proteins out of the complex sample preserving the resolution.

Finally, a sample containing MnP isoforms was tested. In contrast to the salt gradient, separation of MnP isoforms was performed applying a pH gradient [21]. A pH gradient was prepared simply by using sodium acetate buffers of two different pH values. Although this type of buffer does not form an entirely linear pH gradient, it was selected because it is usually used for the separation of MnP isoforms [27]. The gradient time was calculated according to Eq. (6) and the real pH gradient produced by the HPLC system was measured. Measured pH gradients are shown in Fig. 4 together with the linear fit through the region where MnP isoforms are eluted. The linear fit of obtained pH gradient slope was compared to the gradient slope calculated by Eq. (6) as shown in Table 3. We can conclude that the values match quite well and that further estimation of the resolution is meaningful. Results of the separation are presented in Fig. 4 and in Table 3. Again, very similar separations were obtained on both columns.

4. Conclusions

The applied transfer approach gives similar resolution with the tested protein samples on all CIM monolithic columns despite their short bed length. Furthermore, resolution was similar for axial and radial columns, which can be attributed to flow-independent chromatographic characteristics of the monoliths. Since salt and pH gradients were tested,

Table 2
Resolution, R_s, calculated according to Eq. (7) for the separation of standard protein mixture and LiP isoforms performed on axial and radial CIM monolithic columns of different volumes

Sample	Peak No.	Monolith volume (ml)						
		0.34	0.68	1.02	1.36	8	80	800
		R_s						
Standard proteins	1–2	2.87	2.96	2.94	2.83	2.98	3.0	2.98
	2–3	3.19	3.23	3.14	3.11	3.14	3.1	3.23
LiP	1–2	3.44	3.52	3.43	–	3.38	3.28	–
	2–3	1.39	1.28	1.24	–	1.27	1.39	–
	3–4	3.10	3.19	3.13	–	3.10	3.13	–

For conditions see Table 1.

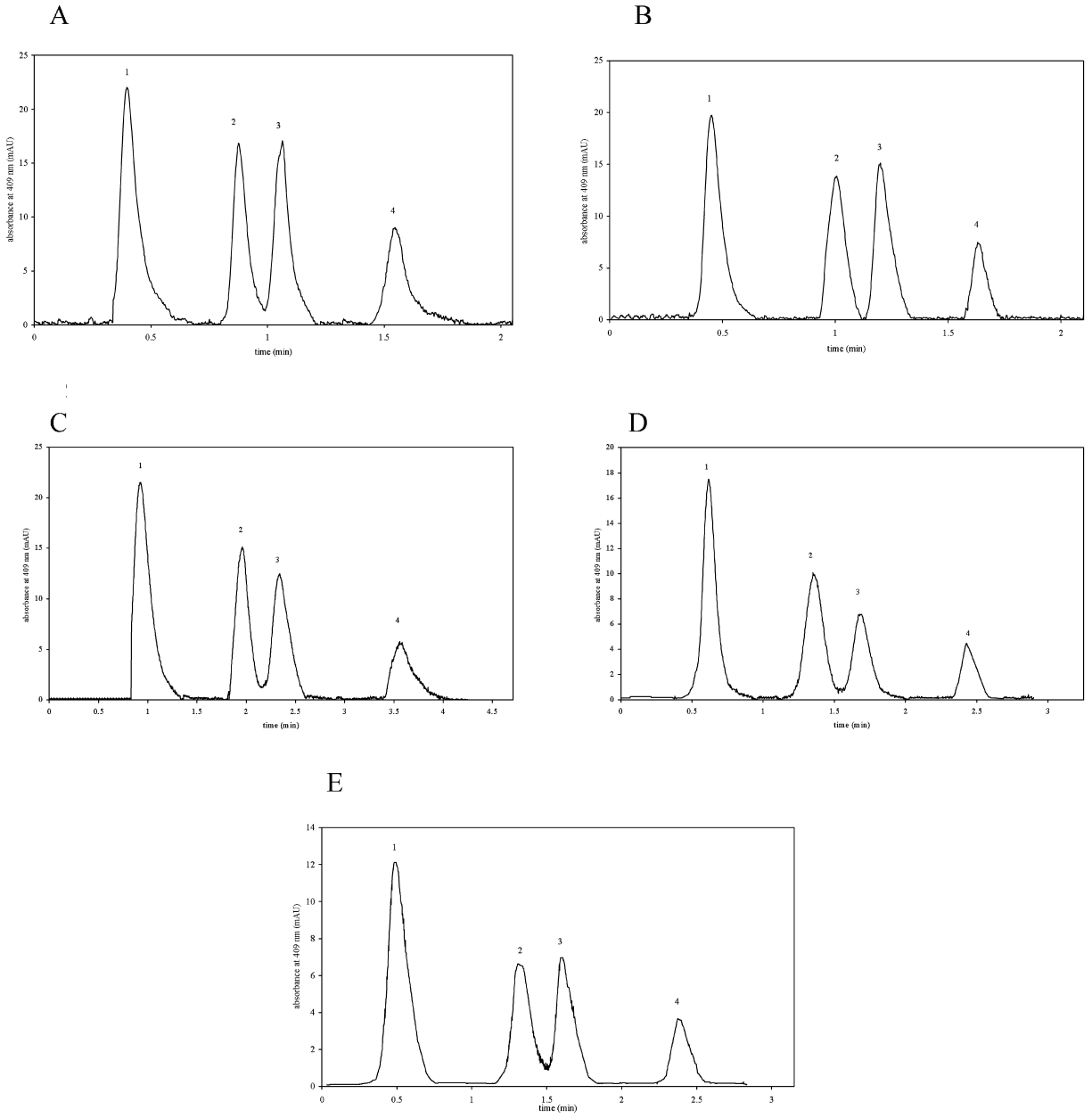

Fig. 3. Chromatograms of LiP isoforms obtained on axial and radial monolithic columns. Conditions: sample: lignin peroxidase H2 (first peak), lignin peroxidase H6 (second peak), lignin peroxidase H8 (third peak), lignin peroxidase H10 (fourth peak); mobile phase: buffer A: 10 m*M* sodium acetate buffer, pH 6; buffer B: 1 mol/l sodium acetate buffer, pH 6; columns: CIM disk monolithic columns with the monolith volumes A—0.34 ml, B—0.68 ml, C—1.02 ml and CIM tube monolithic columns with the monolith volumes D—8 ml and E—80 ml. Description of the gradients and other conditions are given in Table 1. Spectrophotometric detection was done at 409 nm.

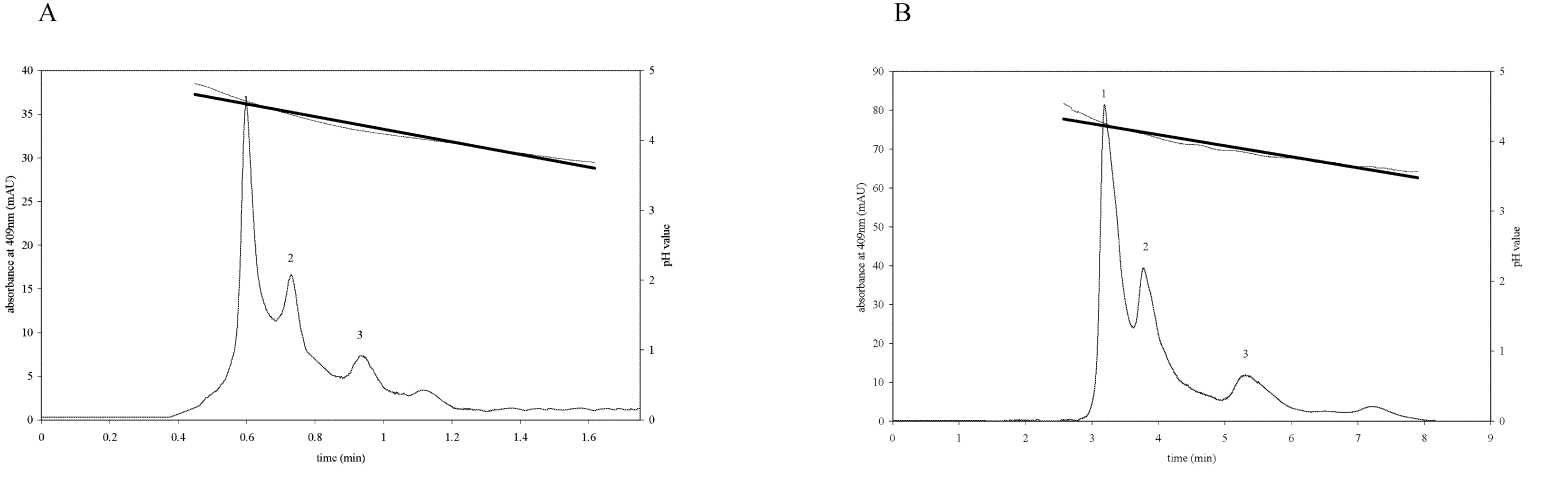

Fig. 4. Chromatograms of MnP isoforms obtained on axial and radial monolithic columns. Conditions: sample: manganese peroxidase MnP1 (first peak), manganese peroxidase MnP2 (second peak), manganese peroxidase MnP3 (third peak); mobile phase: buffer A: 10 m*M* sodium acetate buffer, pH 5; buffer B: 10 m*M* sodium acetate buffer, pH 3.5; columns: CIM disk monolithic column with the monolith volume A—0.34 ml and CIM tube monolithic column with the monolith volume B—8 ml. Description of the gradients and other conditions are given in Table 3. Spectrophotometric detection was done at 409 nm.

Table 3
Gradient chromatographic method and resolution R_s, calculated according to Eq. (7) for the MnP isoforms separation performed on axial and radial CIM monolithic column of different volumes

Column type	Monolith volume, V (ml)	Column length, L (cm)	Flow-rate, F (ml/min)	Gradient time, t_g (s)	Sample volume (μl)	Fit pH gradient slope	R^2	Theoretical pH gradient slope	Peak No.	R_s
Axial	0.34	0.3	4	84	200	−0.906	0.96	−0.908	1–2	1.03
									2–3	1.33
Radial	8	0.65	9	405	5000	−0.158	0.94	−0.179	1–2	0.94
									2–3	1.41

The data are given also for the slopes of on-line measured pH gradient and fit slopes of pH gradient.

this approach seems to give a general and simple guideline for the transfer of the gradient chromatographic methods between ion-exchange CIM monolithic columns.

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