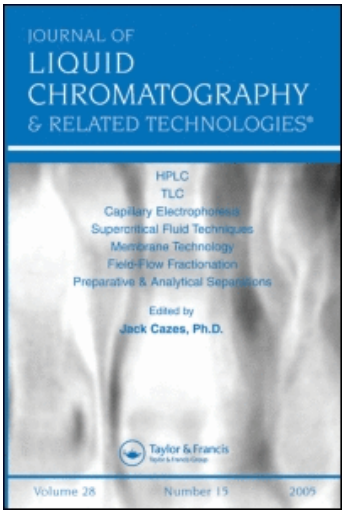

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APPLICATION OF VERY SHORT MONOLITHIC COLUMNS FOR SEPARATION OF LOW AND HIGH MOLECULAR MASS SUBSTANCES

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ABSTRACT

Convective Interaction Media® (CIM) disk monolithic columns are specific among the chromatographic columns because of their monolithic structure and extremely short column length. In this work, HETP values and *Z* factors for different groups of molecules—proteins, DNA, oligonucleotides, peptides, and organic acids on strong anion exchange CIM disk monolithic columns were determined. Results are discussed in terms of the molecule structures and applied to develop different approaches for successful separation of abovementioned group of molecules on these types of columns.

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Key Words: Monoliths; Short columns; Chromatographic columns; Stationary phases; HETP; *Z* factors

INTRODUCTION

In recent years, a demand for fast and efficient separation of different substances has increased dramatically. With the development of combinatorial chemistry, thousands of chemical substances are synthesized daily and the impurities are removed from the target substances for their characterization. In addition, requirements for fast separations of proteins and polynucleotides like DNA and RNA in the fields of genomics and proteomics, increased too. In both cases, separation or purification steps become a bottleneck of the entire process. Therefore, it is not surprising that the development of the abovementioned areas was, and still is, accompanied with the development of different separation techniques, among which High Performance Liquid Chromatography (HPLC) plays a very important role. Since one of the key components of any HPLC system is an HPLC column, many different stationary phases were introduced in the last decade. The main target was to increase the chemical stability, as well as, to enable fast and efficient separations. To achieve the first goal, stationary phases based on polymers, zirconia, or a combination of silica and polymer, just to mention some of them, were introduced.[1] To accomplish the second goal, besides optimizing the chemical composition, an optimization of the matrix structure is a key feature.[2]

Conventional stationary phases for HPLC consist of a few micron-sized particles. The pores within the particles are important to enlarge surface area and, consequently, to increase the binding capacity of the matrix. However, the pores are closed from one side and the molecules to be separated can reach the active sites on the surface only by diffusion. Since this process is rather slow, especially for large molecules, this becomes a limiting step when fast analyses are required. Having this fact in mind, different matrices were introduced. Preparation of so-called perfusion particles[3] was one of the first steps toward the elimination of diffusion bottleneck. Perfusion particles contain, besides small, diffusive pores, also large, perfusion pores, through which the liquid can flow. A transport inside these pores is governed by convection that significantly improves the characteristics of such a media. However, because of the particle nature, most of the liquid still flows through the voids between the particles, diminishing in this way the beneficial effect of convection.[4]

Another attempt to improve the stationary phase hydrodynamics are so-called monoliths introduced around 1990.[5–7] Monoliths are not in the form of particles anymore, but they consist of a single piece of porous material. The pores are open and form a highly interconnected network of channels. As a

consequence, all the mobile phase is forced to flow through the bed and convection becomes a dominant transport mechanism. Especially, methacrylate based monoliths, which exhibit excellent mechanical and chemical stability[8] were extensively studied. It was shown that this type of supports exhibits flow unaffected separation efficiency and dynamic binding capacity.[8–11] In addition, extremely fast separations were performed on short monolithic columns at room temperature.[12] As such, they were successfully applied in different areas such as for separation and purification of proteins,[12–29] DNA,[30] smaller molecules like organic acids,[10,31] hydroxybenzoates,[32] oligonucleotides, and peptides,[9,29,32] as well as sensors incorporated in a FIA system.[19,33–35] Methacrylate based monoliths are commercialized under the trademark Convective Interaction Media® (CIM).

In this work, the separation of different types of molecules on extremely short CIM polymeric monolithic ion-exchange columns has been studied. Height Equivalent of Theoretical Plate (HETP) values and *Z*-factors were used as a guideline for the development and further optimization of the separation method.

EXPERIMENTAL

Materials

High purity water and chemicals of analytical grade quality were used throughout the experimental work. *Tris*(*Tris*(hydroxymethyl)methyl amine) was purchased from Aldrich (Steinheim, Germany), K_2HPO_4 and KH_2PO_4 were from Kemika (Zagreb, Croatia), and sodium chloride from Fluka (Buchs, Switzerland). The actual compositions of the applied mobile phases are given in Table 1.

Citric and pyruvic acid (sodium salt) were supplied by Sigma (St. Louis, MO), α-ketoglutaric acid (di-sodium salt) and malic acid was purchased at Fluka (Buchs, Switzerland). Synthetic peptide, a kind gift from the Institute of Applied Microbiology (IAM), Vienna, Austria, with the following amino acid sequence was used: EYIKWEEFK (Pep-9). The oligonucleotides containing a different number of nucleic bases, synthesized and purified at the National Institute of Chemistry, Ljubljana, Slovenia, were applied. They had various lengths of 2–12, 14, 15, and 16 mers. The sequence of the 16th mer was the following: GCC GAG GTC CAT GTC $T^{3'}$. The sequence of the other oligonucleotides is simply obtained by deleting the proper number of nucleic bases from the left side of the 16th-mer sequence.

Bovine serum albumin (BSA) was purchased from Fluka, the clotting factor IX (FIX) was obtained from Octapharma Prod.m.b.H. (Vienna, Austria). DNA with the size of 5.7 kb pairs was extracted from *E. coli*.

Table 1. Height Equivalent of Theoretical Plate Values and *Z* Factors of Different Substances Determined on CIM Disk Monolithic Columns

Molecule	MW	HETP Value	*Z* Factor	Mobile Phase Range (M)
BSA	67,000	112	6.6	0.22–0.32
FIX	66,000	268	16.3	0.5–0.6
DNA	3,500,000	90	19.1	0.8–0.88
Oligo 9 mer	2,791	19	5.9	0.3–0.45
Oligo 4 mer	1,110	18	1.7	0.06–0.21
Peptide 9	1,192	31	1.3	0.02–0.1
Citric acid	193	59	3.0	0.15–0.3
Malic acid	134	49	1.9	0.1–0.3

Samples were dissolved in appropriate mobile phase composition. Mobile phase range defines the conditions where the experiments were performed.

Bovine serum albumin was dissolved in a working buffer at a final concentration of 4 mg/mL and filtered through a 0.45 μm filter (Macherey Nagel, Düren, Germany) prior to use. Factor 1X was first desalted and equilibrated against the working buffer using PD-10 columns containing Sephadex G-25 (Amersham Pharmacia Biotech, Uppsala, Sweden) and then adjusted to the final concentration of 4 mg/mL in the case of IgG and 100 IU/mL in the case of FIX.

Instrumentation

A gradient HPLC system built with two Pumps 64, an injection valve with a 20 μL SS sample loop, a variable wavelength monitor with a 10-mm optical path set to 210, 215, 260, or 280 nm (depending on samples) with a 10 μL volume flow-cell, the response time set to 0.1 s and HPLC hardware/software (data acquisition and control station), all from Knauer (Berlin, Germany) was used. The instrument delivers precise flow rates up to 10 mL/min. Actual flow rates were additionally controlled for each experimental setting using a validated digital flow meter (K-3773, Phase Separations, UK). To minimize the extra-column effects, the Knauer mixing chamber with its relatively large dead volume was replaced by the PEEK Mixing Tee with an extra low-dead volume (VICI Jour Research, Onsala, Sweden). The CIM disk monolithic column was connected directly to the detector by means of a zero dead volume connector, while the connections between the manual injector and the monolithic column was made of 0.25 mm I.D. PEEK capillary tube (the final length did not exceed 10 cm).

Columns

CIM disk monolithic columns bearing strong anion quaternary amine (QA) groups were used throughout the experimental work (BIA Separations, Ljubljana, Slovenia). CIM disk monolithic columns consist of a disk-shaped poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous polymer matrix that is seated in a non-porous self-sealing fitting ring (stationary phase). The bed volume of one disk is 0.34 mL with diameter of 12 mm and thickness of 3 mm, 62% of which resides in the highly interconnected, flow through pores. The disk is connected to an HPLC system through a specially constructed CIM housing (BIA Separations, Ljubljana, Slovenia). By placing a different number of disk shaped units in the same housing[8] the length of a disk monolithic column can easily be varied.

Procedures

The sample of each substance was dissolved in the appropriate mobile phase used for the particular experiment and injected to the monolithic column under isocratic conditions. The mobile phase composition (ionic strength) was then changed in order to vary the retention time of the particular substance (see Table 1). These experiments were repeated in 5–6 different mobile phase compositions covering, in this way, a relatively wide range of k' values. Additionally, experiments under non-retained conditions were carried out in order to determine the delay time (t_s) for components with different molecular masses under the actual experimental conditions.

From these experiments, the k', Z, and HETP values were calculated according to the following equations:

$$k' = \frac{t_r - t_s}{t_s} \tag{1}$$

where k' is the capacity factor for isocratic elution, t_r is the retention time, and t_s is the value of t_r for the solute when it is unretained by the stationary phase.

Retention in ion-exchange chromatography involves a competition between solute and mobile phase ions for ion-exchange sites on the stationary phase and can be described with the following expression for k' vs. mobile phase salt concentration:[36]

$$\log k' = \log K - Z \log c \tag{2}$$

The parameter K is the equilibrium distribution constant for ion-exchange retention, while Z represents the ratio between the charge on solute ion and the charge on mobile phase ion.

Height equivalent of theoretical plate (HETP) value was calculated from equation:

$$\sigma_t = \sqrt{\frac{\text{HETP}}{L}} t_r \tag{3}$$

where σ_t is peak width (standard deviation) and L is the column length. The experimentally determined peak widths were plotted against the retention times and the obtained linear relationship was fitted with a linear function. From the slope of the line, the HETP value was calculated for each component.

RESULTS AND DISCUSSION

An HETP value is commonly used as a measure for column efficiency. Under the same operating conditions, the more efficient column is characterised by a lower value of HETP. In HETP, value effects of column packing efficiency, axial dispersion, pore diffusion, and adsorption rate are cumulative.[37] Although, there are developed methods for determining the HETP value under gradient conditions,[38] as well as under non-binding conditions,[39] determination in isocratic mode under binding conditions is routinely used for the column evaluation.

Height Equivalent of Theoretical Plate is normally applied for Gaussian shaped peaks, while short columns normally yield a non-Gaussian distribution.[37] This is especially true for non-binding conditions, mainly due to asymmetry caused by the HPLC system itself (Fig. 1). Connecting CIM columns to the system, symmetry significantly increases despite the extremely short column length of only 3 mm (Fig. 1), thus, HETP values seems to be indicative. For large molecules like proteins and DNA, high peak symmetry could not be obtained (asymmetry above 1.7) and HETP values are not so accurate.[37] For those molecules, HETP values were also calculated by using and exponentially modified Gaussian equation[40] suggested for peaks with high asymmetry. The HETP values determined in this way were in all cases higher (data not shown), but the overall order of molecules obtained with the HETP values calculated from the assumption of the Gaussian shape has remained the same. The question is, however, whether these values are more correct, since the peak asymmetry is caused by asymmetry of the input signal into the column rather then by the column itself. Because of this fact, and because the order of molecules was the same independently of the applied equation, the former values were used for further examination.

In Table 1, the HETP values of different substances obtained on a CIM QA disk monolithic column are presented. Five different groups of substances

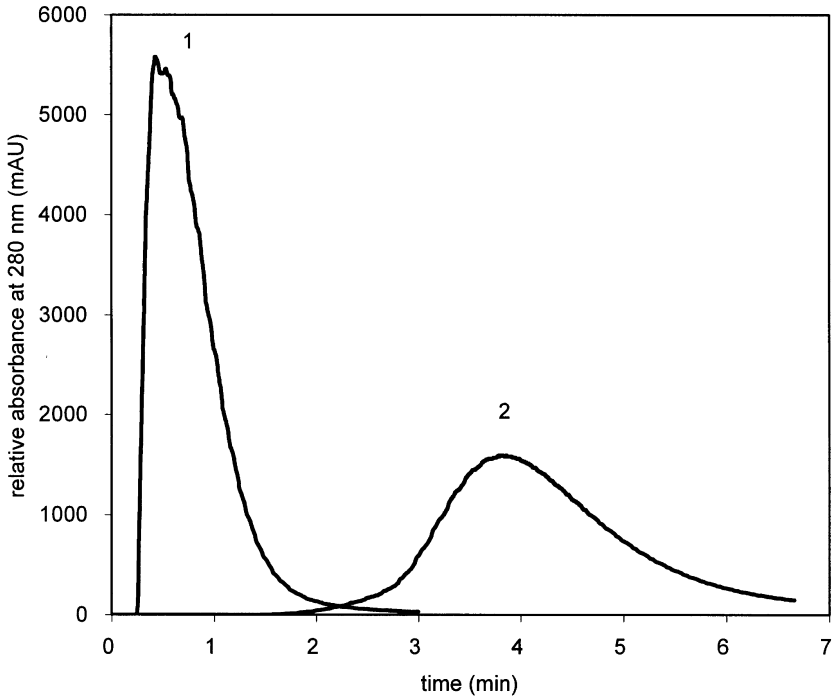

Figure 1. Comparison of the peak shape for HPLC system without and with CIM monolithic column. Hundred micro liter of myoglobin with the concentration of 3 mg/mL dissolved in 20 mM *Tris*-HCl buffer containing 2 M NaCl, pH 7.4 was injected into HPLC system when no CIM monolithic column was coupled to the system (peak 1), and with the column under non-retained conditions (peak 2). Flow rate: 0.1 mL/min; Detection: UV at 280 nm.

were examined: organic acids, peptides, oligonucleotides, polynucleotides, and proteins. It can be observed that HETP values within the groups are of the same range, but they vary significantly between the groups. For molecules consisting of the same units, for example peptides and proteins built up from aminoacids, large difference in molecular mass result in differences in HETP, which can be attributed to higher molecule mobility. The same trend is observed for molecules composed of nucleic bases, namely comparing oligonucleotides and DNA. It is interesting to notice, however, that in general, HETP values do not correlate with the molecular mass of the substances. This is somehow contradictory to the results of the reverse phase mode, where there is a direct linear correlation in-between.[41] For example,

inside the group of proteins, BSA and FIX differ significantly in HETP value, although they have almost identical molecular mass. An interesting case between the groups is comparison of proteins and DNA. Although DNA has the largest molecular mass, its HETP value is lower than of all the tested proteins. The same can be observed by comparing the groups of organic acids and oligonucleotides. Actually, oligonucleotides exhibit the lowest HETP value of all the tested substances. A possible reason for such a result might be in the structure of oligo and polynucleotides.

In contrast to proteins and organic acids, which can be described as spheres, polynucleotides are long but very thin molecules. The asymmetry ratio in the case of DNA is around 15 in contrast to proteins, which are much more spherical with the asymmetry ratio between 1 and 7.[42] Because of that and because of the flexibility of the molecular chain, we can speculate, that average distance between the molecule and the active surface is lower in the case of DNA. Actually we can imagine that molecules of oligonucleotides and DNA are like flexible ticks, which rotate during their passage through the pores. Therefore, more interactions with stationary phase are realized, in this case, on the same column length compared to spherical molecules. It seems that besides mobility related to molecular mass, also the molecule shape plays an important role.

The values of HETP vary from 18 μm for oligonucleotide containing four nucleic bases up to 268 μm for clotting FIX. As already discussed, the correct HETP values for proteins and DNA cannot be determined because of the high peak asymmetry. Additionally, there is a very narrow interval of the mobile phase composition within which the proteins are isocratically eluted. Nevertheless, the HETP range between 100 to 300 μm indicates that no successful isocratic separation for proteins can be expected on short columns.

In contrast to proteins, small molecules like organic acids are routinely separated in isocratic mode.[43] Conventional HPLC columns filled with porous particles used for this purpose are several centimeters long, while the length of CIM disk monolithic column is of only 3 mm. As demonstrated in Fig. 2, successful separation can be accomplished in less than 2 min. Furthermore, it was demonstrated that even shorter monolithic columns of only 0.3 mm are enough for separation of oligonucleotides.[32] Because of that, one might speculate that organic acids, having the HETP value around 50 μm, could also be separated successfully on 3 mm CIM monolithic columns. However, all the attempts to separate them were unsuccessful. It seems that, besides low HETP value, also large enough difference in Z factor, calculated from Eq. 2, should be present. In Table 1, there are Z factors for the same group of molecules. Z factor represents a measure for the tightness of binding or, more precisely, it defines a ratio of charges between molecule to be separated and the mobile phase. If the difference between the molecules is larger, also the retention difference is bigger. It can be

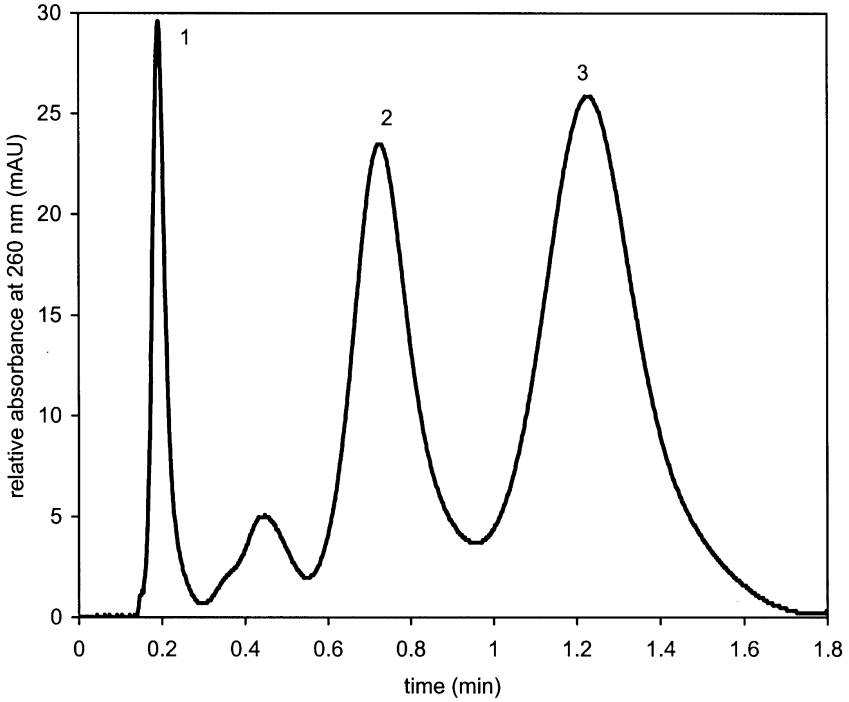

Figure 2. Isocratic separation of oligonucleotides CIM disk monolithic column of 3 mm length. Conditions: Mobile phase: 0.46 M NaCl in 20 mM *Tris*-HCl buffer, pH 8.5; Flow rate: 9 mL/min; Stationary phase: CIM disk monolithic column comprising of a single disk; Sample: 50 μg/mL of oligo 8-mer (peak 1), 150 μg/mL of oligo 10-mer (peak 2) and 450 μg/mL of oligo 12-mer (peak 3) in buffer A; Injection volume: 20 μL; Detection: UV at 260 nm.

seen, that the difference between two oligonucleotides is bigger than the difference between organic acids and, consequently, a lower number of interaction steps can provide separation.

Obviously, to achieve separation of e.g., organic acids, longer monolithic columns should be used. In this case, a unique possibility to adjust CIM disk column length can be applied.[10] Convective Interaction Media disk monolithic columns consist of a housing in which so-called CIM disk is placed. In this way, a 3 mm monolithic column is formed. However, because of the housing construction, from one up to four CIM disks can be placed together. Consequently, the column length can vary from 3 to 12 mm (Fig. 3). Since the 12 mm CIM monolithic column is formed from four CIM disks of 3 mm length,

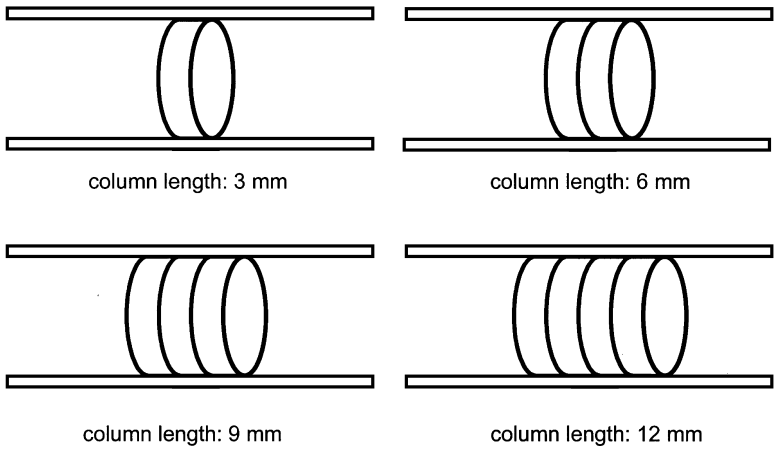

Figure 3. Construction on the CIM disk monolithic column of adjustible column length. Different number of CIM disks can be inserted into a single CIM housing forming a column of 3, 6, 9 or 12 mm length.

we examined if the efficiency of the column is different from the efficiency of the CIM column consisting of a single CIM disk. This was verified by determining the HETP for monolithic columns of length 3, 6, 9, and 12 mm. The results presented in Fig. 4, demonstrate that the efficiency remains almost constant over the change of the column length. Therefore, such a column can be indeed considered as a column comprising of a single monolith with the length up to 12 mm.

As demonstrated in Fig. 5, with the 12 mm CIM disk monolithic column, a good isocratic separation of organic acids can be achieved in less than 4 min. This column length is, therefore, enough to separate molecules with HETP values around 50 μm, but similar Z factors.

In the case of even larger molecules, like proteins or polynucleotides, HETP values are too high to achieve any isocratic separation even on the 12 mm CIM monolithic column. Although, the number of theoretical plates for macromolecules for such column is comparable to the number of plates for oligonucletides for 3 mm CIM monolithic columns, which were successfully separated isocratically, in practice such separation cannot be realized. The reason is in an extremely narrow mobile phase concentration window within which the protein is retained, but not irreversibly bound. Since such a window is different for different proteins it is unlikely that a single mobile phase for isocratic separation can be found.[44] However, large biomolecules differ significantly in the Z-factor (see Table 1). The reason is in high molecular size

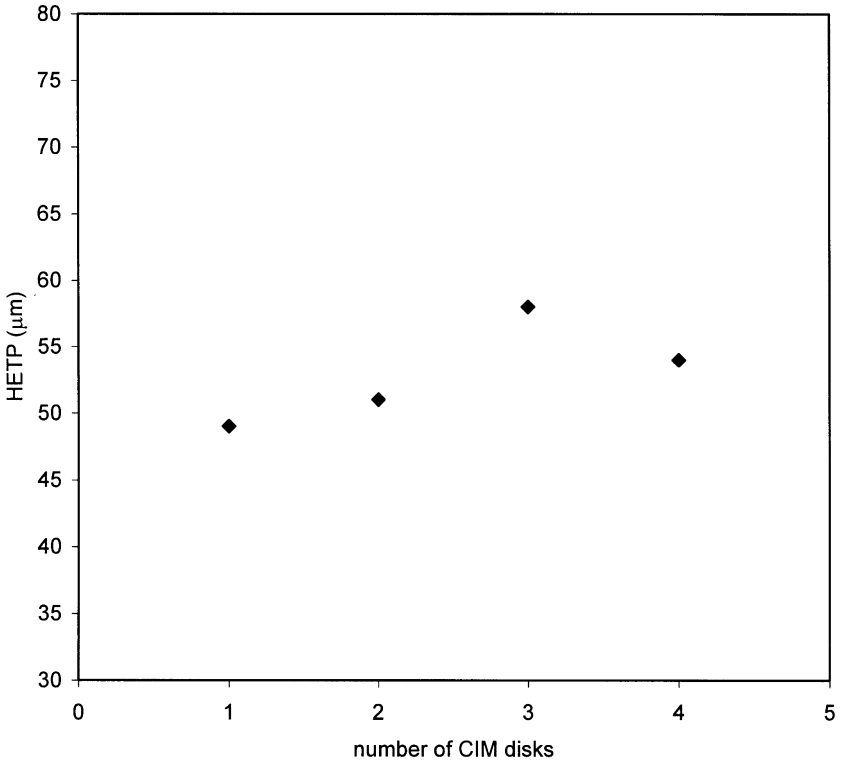

Figure 4. Effect of the CIM disk monolithic column length on the HETP value. Convective Interaction Media disk monolithic column of different length was constructed placing different number of CIM disks in single CIM housing. Citric acid was injected in the mobile phase and HETP value was calculated according to Eq. 3. Conditions: Mobile phase: 130 mM NaCl in 20 mM phosphate buffer, pH 8.0; Stationary phase: CIM disk monolithic column comprising of one up to four disks; Flow rate: 5 mL/min; 0.75 g/L citric acid; Injection volume: 20 μL; Detection: UV at 210 nm.

and, consequently, higher heterogeneity of the charged groups on a protein surface. Due to these differences, the binding tightness is significantly different and separation can, therefore, be achieved via selective elution applying gradient separation. It was shown that in gradient separation mode, column length does not play an important role.[45] Although many successful separations of proteins using CIM disk columns were already demonstrated, no separation of combination of proteins and DNA was demonstrated so far. From Table 1, can be seen that the highest Z factor belongs to DNA, which can be explained with an extremely high number of charged phosphate

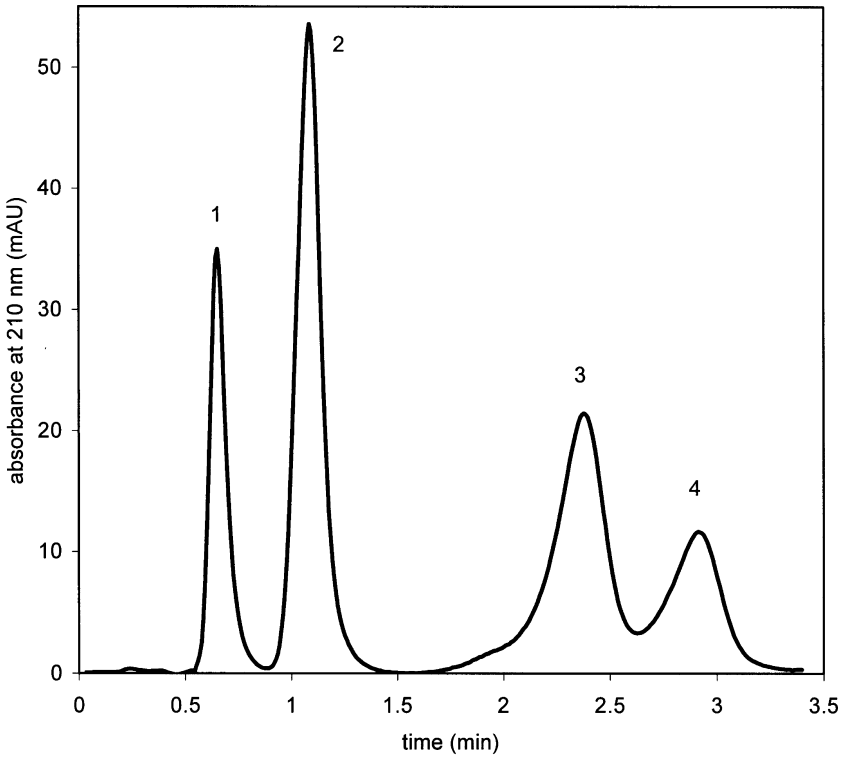

Figure 5. Isocratic separation of organic acids on CIM QA disk monolithic column with the length of 12 mm. Conditions: Mobile phase: 130 mM NaCl in 20 mM phosphate buffer, pH 8.0; Stationary phase: CIM disk monolithic column comprising of four disks; Flow rate: 5 mL/min; Sample: 0.06 g/L pyruvic acid (peak 1), 1.0 g/L malic acid (peak 2), 0.125 g/L α-ketoglutaric acid (peak 3) and 0.75 g/L citric acid (peak 4); Injection volume: 20 μL; Detection: UV at 210 nm.

groups. DNA is followed by clotting FIX, which is, as can be seen from *Z* value, highly charged protein. To DNA and FIX, also BSA as the third most charged protein of the tested ones was added. Gradient separation of the mixture is presented in Fig. 6. Although molecular masses of biomolecules were extremely high, baseline separation was achieved in less than a minute without any blocking of the column.

To exploit full potential of short columns, a careful method optimization including isocratic and gradient separation should be performed.[37] To demonstrate this, a complex gradient combined with the isocratic elution was developed for the separation of oligonucleotides. A detailed study of

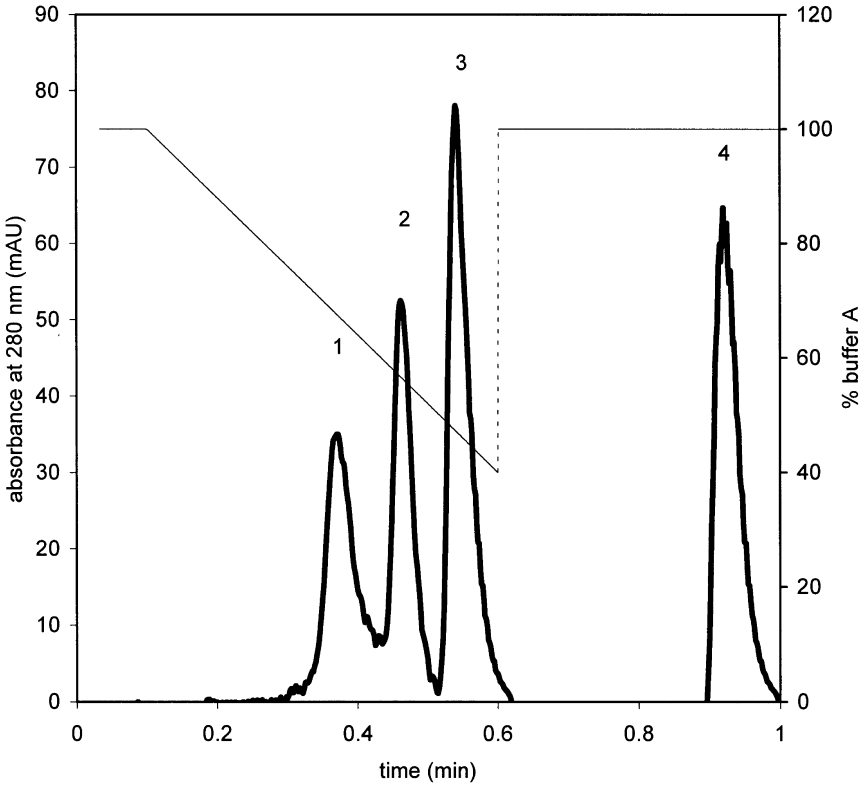

Figure 6. Gradient separation of proteins and DNA on CIM QA monolithic column with the length of 3 mm. Conditions: mobile phase: buffer A: 20 mM Tris, pH = 8.0; buffer B: 2 M NaCl in 20 mM Tris, pH = 8.0; Stationary phase: CIM disk monolithic column comprising of a single disk; Flow rate: 4 mL/min; detection: UV at 280 nm; Sample: BSA (peak 1), FIX (peak 2), DNA (peak 3), solvent peak (peak 4); Injection volume: 20 μL.

oligonucleotide behaviour on short CIM monolithic columns revealed, that while HETP is comparable, there is a linear correlation between oligonucleotide length and Z factor according to equation.[9]

$$Z \text{ factor} = \text{oligonucleotide length} * 0.995 - 2.26 \quad (4)$$

Such significant difference in Z factor enables selective elution applying shallow gradient, while low HETP enables isocratic separation also on a CIM monolithic column of 3 mm length. In Fig. 7, a separation of 14 oligomers is presented. Different gradient slopes were applied to achieve optimal separation. In addition, for separation of 6 mer from 7 mer an isocratic elution was required.

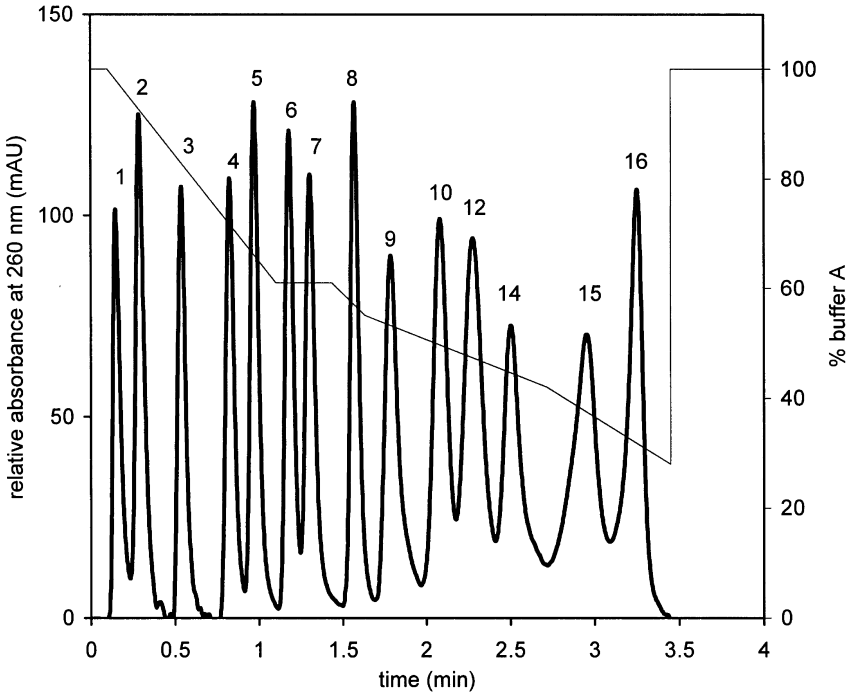

Figure 7. Separation of oligomers using optimised gradient conditions. Conditions: Mobile phase: buffer A: 20 mM *Tris*-HCl buffer, pH 8.5; buffer B: 1 M NaCl in 20 mM *Tris*-HCl buffer, pH 8.5; Flow rate: 4 mL/min; Stationary phase: CIM disk monolithic column comprising of a single disk; Sample: oligonucleotides of different lengths (see Material and Methods. Number near the peak represents the oligonucleotide length); Gradient: as shown in Figure; Injection volume: 20 μL; Detection: UV at 260 nm.

Despite the complex gradient, the entire analysis was completed within 4 min, demonstrating high peak capacity of short monolithic columns.

CONCLUSIONS

CIM disk monolithic columns are versatile units and can be used efficiently for various separations despite their extremely short length. The same column type can be used for the separation of molecules with a broad range of molecular masses. Determination of HETP value and *Z*-factor gives us a hint, which separation mode or combination would be advantageous for analysis of particular substances. It is important to emphasize that molecules like organic acids, as well

as proteins and DNA, can be separated using a single column. User adjustable column length with constant column efficiency is advantageous for particular method optimization.

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