ISSN 0036-0244, Russian Journal of Physical Chemistry A, 2015, Vol. 89, No. 9, pp. 1688–1692. © Pleiades Publishing, Ltd., 2015.
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PHYSICAL CHEMISTRY OF SEPARATION PROCESSES: CHROMATOGRAPHY

Monolithic Capillary Columns Based on Pentaerythritol Tetraacrylate for Peptide Analysis

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Received November 26, 2014

Abstract—Monolythic medium–polar capillary columns based on pentaerythritol tetraacrylate were optimized for separation of peptides. The synthesis temperature and time, the fraction of monomer in the initial polymerization mixture, and the nature of alcohol contained in the complex porogen were chosen as optimization parameters. The highest efficiency was attained for columns obtained with 33 and 34% monomer at a polymerization time of 75 min and a temperature of 75°C. The columns with the optimum structure were effective in separation of a model mixture of five peptides. The sensitivity of the method was 200 ng of peptide per column.

Keywords: HPLC, monolithic sorbents, peptides, capillary chromatography.
DOI: 10.1134/S0036024415090198

INTRODUCTION

Peptide analysis is performed by various methods, mainly electrophoresis (thin layer and capillary) and reversed-phase HPLC, which exceed many other techniques (ion-exchange, exclusion chromatography, isoelectric deposition, etc.) in resolution, separation efficiency, and analysis rate. New requirements to substances separation and determination methods have appeared due to the development of analytical chemistry and life sciences. Today, it is often required to analyze the ultrasmall samples when the researcher has to analyze the content of only one cell [1, 2]. The techniques used for these analyses should be extremely sensitive and highly selective, while the analytical equipment should have minimum extracolumn communications. All these requirements are satisfied by capillary liquid chromatography. Using a monolithic capillary column allows optimization of the sorbent structure for separation of the definite compounds and also easy regulation of the surface chemistry and hence the separation selectivity; it also leads to highly effective separations at relatively low pressures [3].

The authors of [4] optimized the sample preparation and separation of peptide mixture for analysis of small amounts of the sample. They effectively performed quantitative analysis of peptide mixtures of 250 and 500 cells, the limit of quantitative determination was 30 ng. However, the separation was performed on a narrow short column, and all peptides were eluted as a single unseparated peak.

The monolithic sorbents which are most often used for separation of biopolymers are sorbents based on the styrene–divinylbenzene copolymer PSDVB and on polar organic polymer matrices. The possibility of using of commercially available monolithic columns (50 mm × 200 μm, PepSwift, Dionex) based on PSDVB for separation of peptides and proteins by ultra high pressure chromatography (UHPLC) was shown in [5]. The analysis time was only 60 min for analysis of hydrolysate of mixture of six proteins at the flow rate of the mobile phase of 2 μL/min with gradient elution: A—0.05% aqueous formic acid; B—ACN : H_2O (80 : 20) + 0.04% aqueous formic acid. The relative standard deviation of the retention time was less than 0.09%.

Monolithic columns (200 × 1.02 mm) were synthesized from the copolymer of hexyl methacrylate and ethylene glycol dimethacrylate as reported in [6]; they were used for fast and effective separation of ribonuclease A, cytochrome C, transferrin, and ovalbumin. The 20 cm column had efficiency of 3000 TP at flow rate of the mobile phase of 50 μL/min and pressure of less than 1 MPa. In addition, the obtained columns were stable even at pressure of 15 MPa and flow rate of the mobile phase exceeding the above mentioned rate by a factor of 15 to 20. For example, the authors separated the above mentioned proteins at a flow rate of the mobile phase of 1000 μL/min using gradient elution.

Table 1. Synthesis conditions and characteristics of several monolithic capillary columns

Column no.	Porogen	L, cm	Polymerization conditions			$B_0 \times 10^{-14}$, m^2	ε, %
			t, min	T, °C	Δ,%		
PE5	nonanol	51	60	75	33	0.53	75.0
PE7	decanol	50	45	75	33	0.23	60.3
PE8	decanol	50	90	75	33	0.61	74.5
PE9	decanol	50	30	75	33	0.55	81.7
PE10	decanol	50	75	75	33	0.48	85.3
PE11	decanol	50	60	75	33	0.55	77.1
PE12	decanol	44	75	70	33	1.66	94.9
PE13	decanol	50	75	73	33	0.45	92.1
PE14	decanol	50	75	78	33	–	–
PE15	decanol	50	75	67	33	1.87	88.9
PE16	decanol	50	75	75	34	0.47	75.3
PE17	decanol	50	75	75	32	0.88	78.8
PE18	decanol	50	75	75	31	1.34	90.8
PE19	decanol	50	75	75	35	0.25	81.5

L is column length, t is time, T is temperature, Δ is monomer fraction in the polymerization mixture, B_0 is permeability, and ε is porosity.

The aim of the present study was to optimize the structure of medium-polar monolithic sorbents based on pentaerythritol tetraacrylate for peptide separation by RP HPLC.

EXPERIMENTAL

Monolithic capillary columns were synthesized by the procedure of [7]. The inner surface of a quartz capillary ($d_{in.}$ = 100 μm) was treated with 1% hydrofluoric acid; then it was silanized with 3-(trimethoxysilyl)propyl methacrylate for covalent binding of the monolith with the capillary surface. The silanized capillary was filled under vacuum with a polymerization mixture consisting of an initiator (2,2'-azodiisobutyronitrile, 1 wt % of the amount of the monomer), monomer (pentaerythritol tetraacrylate), and porogen (a mixture of methyl ethyl ketone and one of higher alcohols). The capillary ends were sealed and the capillary was placed in a water thermostat for some time. The polymerization conditions and the structural parameters (permeability and porosity) of the prepared monolithic columns are listed in Table 1. The chromatographic experiments were performed on a Shimadzu liquid chromatograph (Japan) equipped with an LC-10AD isocratic high-pressure pump and an SPD-10A UV detector with a variable wavelength combined with a capillary cell.

RESULTS AND DISCUSSION

The effects of polymerization conditions on the properties of monolithic capillary columns in the RP HPLC mode were studied using the van Deemter curves. According to [8, 9], the polymerization temperature and time, the relative amount and nature of the monomer and porogen in the polymerization mixture are the main parameters that affect the efficiency of monolithic columns. Figure 1 shows the changes in the shape of the van Deemter curves for monolithic capillary columns obtained at different polymerization times; according to Fig. 1, this mainly affects the slope of the right-hand branch of the van Deemter curve and, to a much lesser extent, the minimum value of HETP. The gradual increase in the column synthesis time from 30 to 75 min led to a decrease in the coefficient C of the van Deemter equation (the slope of the right-hand part of the curve), which pointed out to the increased rate of mass transfer between the mobile and stationary phases. The most flat curve was observed at polymerization time of 75 min (H_{min} = 16 μm, C = 0.18 min). Further increase of the synthesis time up to 90 min leads to a drastic increase in the coefficient C

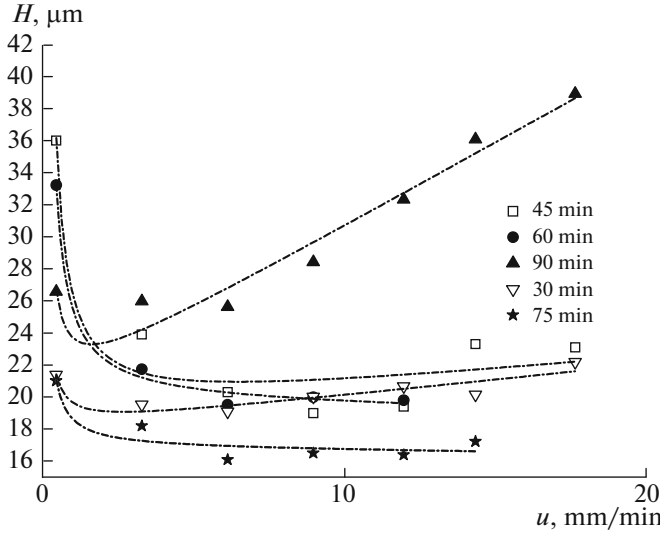

Fig. 1. Dependence of the shape of the van Deemter curves on the polymerization time. Polymerization conditions: relative monomer content 33%, temperature 75°C, porogen—1-decanol. Experimental conditions: composition of the mobile phase H_2O : MeCN (75 : 25%) + 0.1% TFAA (trifluoroacetic acid), detection wavelength 220 nm, sorbate is uracyl.

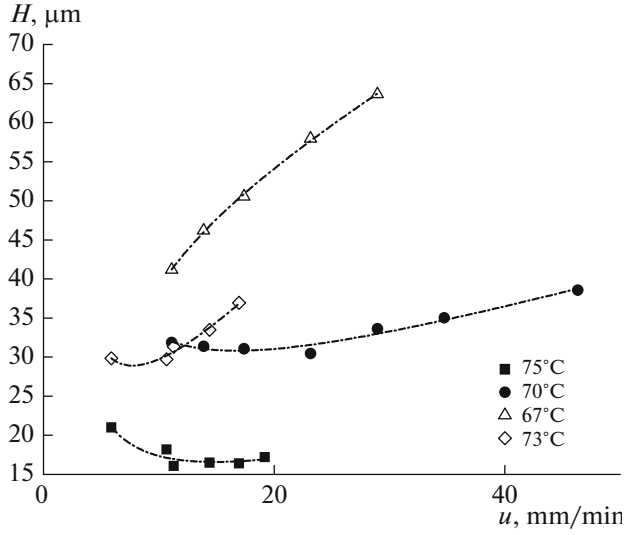

Fig. 2. Dependence of the shape of the van Deemter curves on the polymerization temperature. Polymerization conditions: relative monomer content 33%, time 75 min, porogen—1-decanol. Experimental conditions: composition of the mobile phase H_2O : MeCN (75 : 25%) + 0.1% TFAA, detection wavelength 220 nm, sorbate is uracyl.

to 1.05 min, i.e., to a retardation of mass exchange between the mobile and stationary phases and a 1.5-fold decrease in the column efficiency at the optimum rate of the mobile phase to $H = 25$ μm.

Figure 2 presents the van Deemter curves for columns synthesized at different temperatures. An increase of the synthesis temperature from 67 to 75°C led to decrease of the slope of the right-hand side of the curve (acceleration of mass transfer between the mobile and stationary phases) and to decrease of HETP from 40 to 16 μm. Further increase of the synthesis temperature to 78°C led to the formation of a very dense structure of the monolith characterized with low permeability. Therefore, the van Deemter curve could not be plotted for the column synthesized at 78°C. Also note that the sequential increase of the polymerization temperature led to a shift of the van Deemter curves upward along the ordinate axis; this may indicate to increase of the coefficient A of the van Deemter equation, which characterizes the contribution of eddy diffusion to the peak broadening and depends on the diameter of the sorbent particles in the case of the column packed with the particulated sorbent. At the same time, the structure of the monolithic sorbent is formed by domains, but not by individual particles; the eddy diffusion in this case characterizes the size of these domains and their distribution. Therefore, the increase of the coefficient A in the van Deemter equation at increasing synthesis temperature of the monolithic sorbent is caused by an increase of the size of the monolith structure-forming domains.

Figure 3 presents the van Deemter curves for monolithic columns obtained at different monomer contents in the polymerization mixture. The minimum HETP occurred to be rather sensitive to the monomer content in the polymerization mixture. The minimum HETP ($H \approx 16$ μm) was obtained at 33%

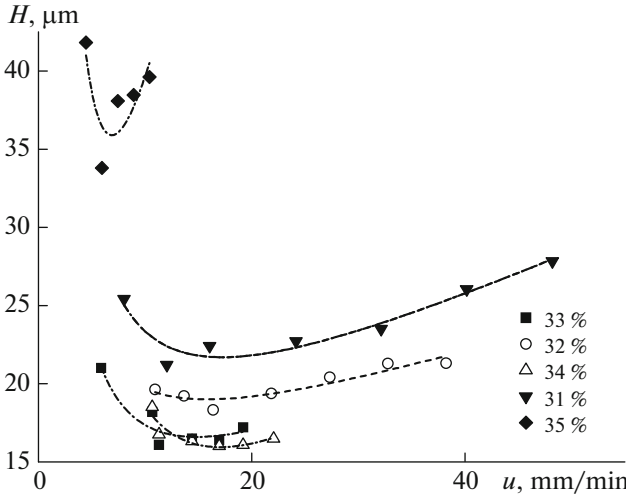

Fig. 3. Dependence of the shape of the van Deemter curves on the eluent flow rate at various relative monomer contents in the initial polymerization mixture. Polymerization conditions: temperature 75°C, time 60 min, porogen—1-decanol. Experimental conditions: composition of the mobile phase H_2O : MeCN (75 : 25%) + 0.1% TFAA, detection wavelength 220 nm, sorbate uracyl.

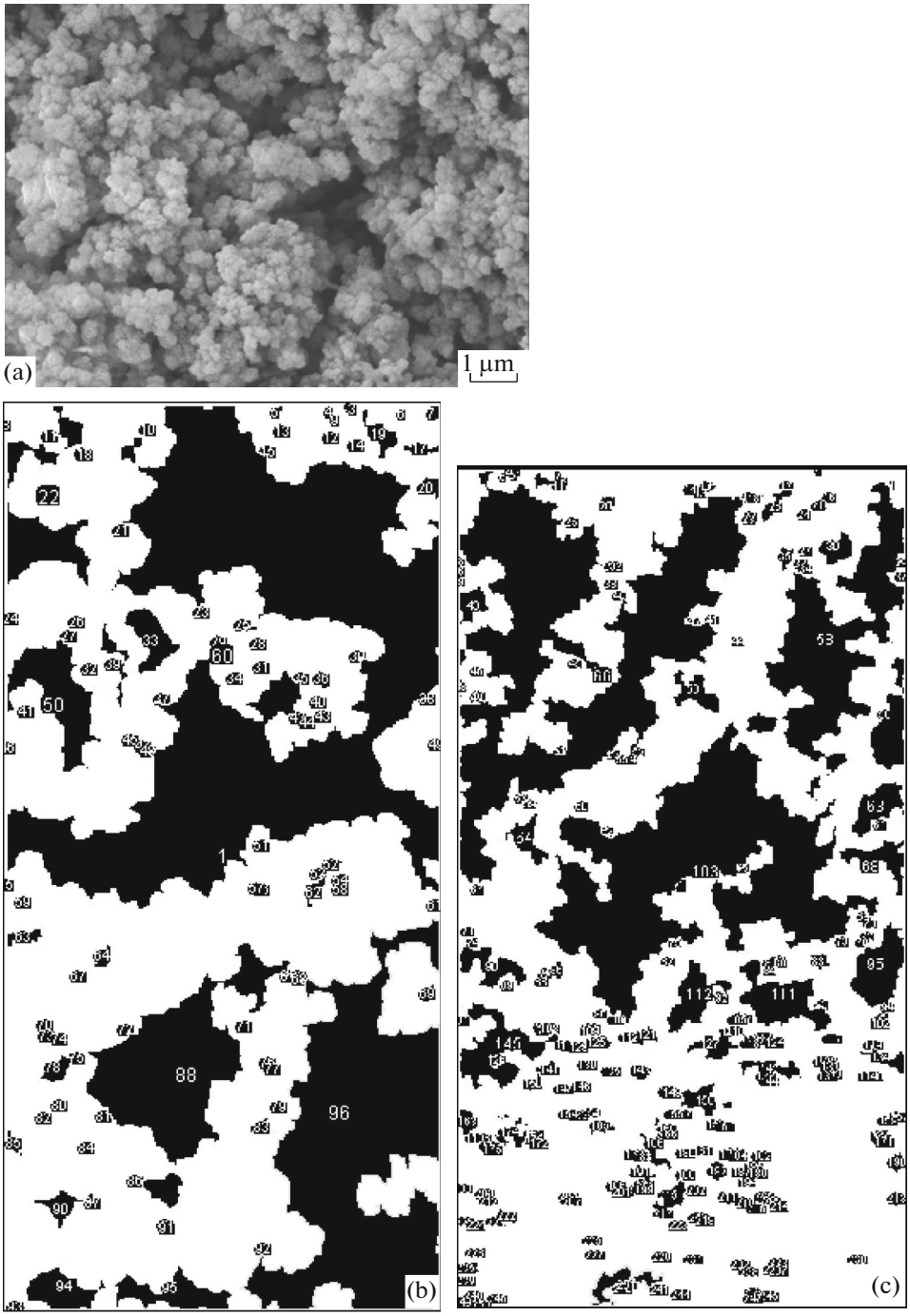

Fig. 4. SEM microphotograph of the cross-section of the monolithic capillary column: (a) the original image for the PE10 column (75°C, 75 min, 33%), (b) projection of the cross-section for the PE10 column, and (c) projection of the cross-section for the PE18 column.

monomer in the polymerization mixture, while already at 35% monomer, the HETP was of about 34 μm. In addition, for the column with 35% monomer in the polymerization mixture, the shape of the van Deemter curved drastically changed. This change may be caused by the fact that the conditions for the preparation of monoliths suitable for chromatographic separation lie in a very narrow range of values, and even small deviations cause a dramatic deterioration of the chromatographic characteristics of the sorbent. This is just observed for the column obtained using 35% monomer in the starting polymerization mixture.

The structure of the monolithic sorbent was visualized by scanning electron microscopy. Figure 4a shows

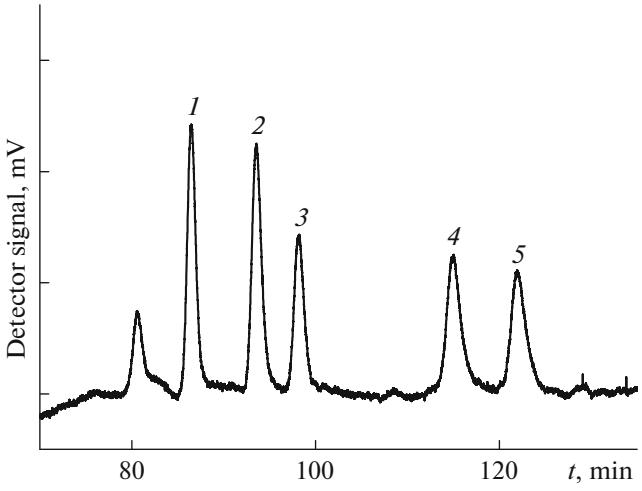

Fig. 5. Chromatogram of the model mixture of peptides. Determination conditions: PE10 column (33% PE4A + 1% AIBN, decanol/MEK 7/3, 75°C, 75 min); mobile phase: H_2O : MeCN (72.5 : 27.5%) + 0.1% TFAA; mobile phase flow rate 0.040 mL/min; detection wavelength: λ = 220 nm. Sorbates: (*1*) Gly-Tyr, (*2*) Val-Tyr-Val, (*3*) Met-enkephalin, (*4*) Leu-enkephalin, and (*5*) angiotensin II.

that the size of domain particles that form the monolith structure is much smaller than the diameter of the transport channels in the structure. The morphology of the monolith was evaluated by the procedure for analyzing the graphical images described in [10]. Figures 4b and 4c present the projections of the cross-sections of the monolithic capillary columns used for analysis. The calculated data are given in Table 2 for two columns with high and low permeability. According to Table 2, the domain structures of the columns are characterized by low circularity coefficient and can be described as fractal-like. The Feret diameter is much smaller for the more permeable column, which is rather unexpected. This suggests that the flow-through pore diameters are independent of the monolith skeleton parameters.

Table 2. Morphological characteristics of the porous structure of the monolith

Column	Shape coefficients			d_{Feret}, μm	
	K_{circ}	AR	K_{conv}	min	max
PE10	0.163	2.235	0.679	2.4	5.2
PE7	0.205	2.666	0.535	0.7	1.6

K_{circ} is the circularity coefficient, *AR* is the characteristic ratio of equivalent ellipse, K_{conv} is the convexity coefficient, and d_{Feret} is the Feret diameter.

A model mixture of five peptides with molecular masses from 200 to 1000 used for efficiency evaluation was separated for all columns at the optimum flow rate of the mobile phase, i.e., at the highest efficiency. Figure 5 shows an example of such separation for one of the most effective columns. For the optimum column, a calibration curve was constructed, and the sensitivity of the method was determined (200 ng per column).

CONCLUSIONS

Monolithic capillary columns were prepared from the medium polar monomer pentaerythritol tetraacrylate. Their structure was optimized for peptide separation in the RP HPLC mode. Columns with the best chromatographic properties were obtained at 33% monomer in the initial polymerization mixture (polymerization in a capillary for 75 min at 75°C). A morphology analysis showed that preparation of the optimum structure of the monolith requires strict control of polymerization conditions and mixture composition. In separation of peptides, the detection limit was 0.10 mg/L.

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Translated by L. Smolina