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Highly Efficient Separations on 5 μm Internal Diameter Open Tubular Capillaries Coated with Thick Polyacrylate Stationary Phases

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Key Words

Column liquid chromatography
Open tubular columns
Cross-linked polyacrylate stationary phases
Photopolymerization
PTH-amino acids

Summary

Fused silica capillaries with an internal diameter of 5 μm were coated with polyacrylate films containing either alkyl or ether functionalities. Polymer networks were created by *in situ* photopolymerization of acrylic monomers. Capillaries with phase ratios up to 1.3, having excellent separation efficiencies, could be prepared. The performance of these capillaries was demonstrated by the separation of various solute mixtures. Nineteen phenylthiohydantoin (PTH) amino acid derivatives could be separated in less than 10 minutes when using UV detection. Laser Induced Fluorescence detection was used for the separation of polycyclic aromatic hydrocarbons, dansyl (Dns) derivatized amino acids and *o*-phthalaldehyde (OPA) derivatized aliphatic amines which could be separated with approximately 310,000 plates in less than 3.5 minutes corresponding to around 1500 plates per second.

Introduction

In previous papers we have reported a method by means of which it is possible to immobilize thick (0.5–1.9 μm) polymer layers in approximately 10 μm I.D. fused silica capillaries by using *in situ* photopolymerization of acrylates [1, 2]. Of the various stationary phases considered that prepared from a mixture of silicone- and ethoxyethylacrylate (SiA-EEA) showed the best performance in terms of separation efficiency and coating success. With this stationary phase capillaries up to 5 m long could be prepared, showing more than

one million plates for anthracene ($k' = 0.32$). According to Tijssen et al. [3] the separation speed of open tubular columns should be utilised in two different ways. Rapid separations can be performed best in relatively short capillaries (< 2 m) with a small internal diameter (< 5 μm), whereas with longer (5–10 m) columns with an internal diameter of about 10 μm very large plate numbers can be generated with acceptable inlet pressures. When keeping the phase ratio constant, the efficiency in Open Tubular Liquid Chromatography (OT-LC) is, in theory, inversely proportional to the square of the capillary diameter. Thus, in order to fully exploit the potentials of OT-LC, reduction of the capillary internal diameter to approximately 5 μm is desirable.

In this paper we report on the performance of 5 μm I.D. fused silica capillaries coated with a thick polyacrylate stationary phase. Several classes of compounds were chosen to demonstrate the efficiency of the coated columns and to study the retention behaviour of the polyacrylate stationary phases.

Experimental

Apparatus

The OT-LC set-up used for the chromatographic measurements consisted of two HPLC pumps (Spectroflow 400, Kratos, Rotterdam, The Netherlands) which were controlled by a solvent programmer (model 450, Kratos). A splitting device was used to reduce the flow from ml/min to $\mu\text{l/min}$; a second splitting device was used to attenuate the injection volume. The pressure was monitored with a digital pressure sensor (type 4041A200, Kistler, Winterthur, Switzerland) combined with a current amplifier (type 4061, Kistler) and a recorder (model BD111, Kipp & Zonen, Delft, The Netherlands). Two on-column detection systems were employed, a UV absorbance detector (Kratos 757) using a home built on-column detection cell with adjustable slit [4] (the detector rise time was set to 0.1 sec) and Laser Induced Fluorescence detection (LIF)

Table I. Experimental coating conditions of the prepared fused silica columns.

Capillary no.	length (cm)	monomer conc. (% v/v) ¹	light int. (mW)	irradiation time (sec)	d _c ² (μm)	d _f ³ (μm)	V _s /V _m
1	112	10 % EEA/10 % SiA	0.14	385	4.16	0.70	0.79
2	87	10 % EEA/10 % SiA	0.14	385	4.16	0.70	0.79
3	94	10 % EEA/10 % SiA	0.14	385	4.18	0.69	0.78
4	140	10 % EEA/10 % SiA	0.14	360	3.58	0.91	1.27
5	120	10 % EEA/10 % SiA	0.14	360	3.56	0.92	1.30
6	96	15 % EHA/15 % SiA	0.14	385	3.98	0.77	0.92

¹ DMPA concentration: 3.0 mg/ml for all solutions. Solvent: acetone-pentane (1 : 1)

² d_c: diameter of the column after coating

³ d_f: thickness of the acrylate film

For abbreviations see Experimental section

with a helium-cadmium laser, $\lambda_{\text{ex}} = 325$ nm (model 356XM, Omnicrome, Chino, Calif., USA) as the light source; the emission wavelength was set at 420 nm. The fluorescence yield was measured with a photomultiplier tube (type 6225 S, EMI, Hayes, UK).

Materials

Fused silica capillaries with an inner diameter of approximately 5 μm were obtained from Philips Laboratories (Eindhoven, The Netherlands) and Polymicro Technologies (Phoenix, Ariz., USA). The external coating of both type of capillary possessed enough UV-transparency for in situ photopolymerization. The capillaries obtained from Polymicro are protected with an 'UV transmitting buffer coating', which is at least 95 % transparent from 190 nm to beyond 800 nm.

To initiate the polymerization reaction, α, α -dimethoxy- α -phenylacetophenone (DMPA) was used (Irgacure 651, Ciba, Basel, Switzerland).

HPLC-grade methanol and acetonitrile were obtained from Janssen (Beerse, Belgium); ethoxyethylacrylate (EEA), ethylhexylacrylate (EHA) and 3-(methacryloxy)propyltrimethoxysilane (γ -MPS) were purchased from Fluka (Buchs, Switzerland). Silicone acrylate (SiA) (Tegomer V-Si2150) was obtained from Goldschmidt (Essen, Germany). Phenylthiohydantoin (PTH) and dansyl(Dns) derivatized amino acids were purchased from Sigma (ST. Louis, Mo., USA) and *o*-phthalaldehyde (OPA) from Aldrich (Brussels, Belgium).

Coating procedure

The photopolymerization of acrylates to immobilize a polymer film in fused silica capillaries has been described in more detail in a previous publication [1]. Briefly the procedure consists of the following successive steps:

- Etching of the fused silica surface to increase the number of silanol groups.
- Silylation of the surface with a silyl reagent containing an acrylate group (γ -MPS). The acrylate group on the surface is needed to anchor the polymer layer.

- Filling the capillary with a mixture of acrylate monomers in acetone-pentane (1 : 1) containing photoinitiator (DMPA). The capillary is then irradiated with UV light to initiate the polymerization.
- Evaporation of the solvent.
- Curing at elevated temperature.

Capillaries of different length were treated according to this procedure as described. By using the residence time of an unretained solute in the bare and the coated capillary and the inlet pressure it is possible to calculate the internal diameter of the capillary by applying the Poiseuille equation. The polymer film thickness was determined by subtracting the radius of the coated capillary from the initial capillary radius. Coating conditions and capillary dimensions are laid out in Table I.

Derivatization of Primary Amines with *o*-Phthalaldehyde

The fluorescent reagent was prepared according to Jones et al. [5]. 50 mg *o*-phthalaldehyde was dissolved in 1.25 ml methanol and added to 11.2 ml borate buffer, pH = 9.0. Alkylamines were dissolved in acetonitrile and subsequently mixed with the OPA reagent.

Chromatography

Pure methanol, acetonitrile and aqueous mixtures of these solvents, were used as the mobile phase in separations of nonionic compounds. For the separation of amino acids either a 0.1 M sodium acetate buffer or 0.45 % acetic acid (pH = 3.0) was added to the water-organic modifier mixture. In general, solutes were dissolved in the mobile phase used. Salicylate was used as the unretained, fluorescent solute.

For both detection techniques used it was necessary to remove the outside coating of the capillary for increased sensitivity. For the capillaries obtained from Philips, this was done by immersing the detection side of the capillary in methanol and stripping off the protection layer mechanically. The protective coating of the capillaries purchased from Polymicro could easily be burned off with a match.

Figure 1

Experimental and theoretical plate heights of 1,2-benzanthracene on a SiA-EEA coated Capillary 1 (●) and a SiA-EHA coated Capillary 6 (○) using methanol as mobile phase. Solid lines represents theoretical Golay curves. Detection: LIF.

Distribution coefficients, K_i , were calculated from the capacity factor and phase ratio according to $K_i = k'_i \cdot V_m/V_s$.

Experimental plate heights for the test solutes were calculated according to $H = L \cdot \sigma^2/t_r^2$, where L is the column length and σ is half the peakwidth at 0.607 of the peak height. Column efficiency was evaluated by fitting the experimental plate heights to the extended Golay equation.

Results and Discussion

Polymerization

The experimental coating conditions and determined layer thicknesses of our columns are given in Table I. Two types of fused silica capillaries with different outside protective coatings were used: columns 1–3 and 6 were made out of Philips capillaries and columns 4 and 5 from Polymicro Technologies capillaries. From Table I it can be seen that with the Polymicro capillaries significant thicker layers were obtained than with the Philips capillaries. The Polymicro capillaries have a five times thinner outside protective coating, which results in a larger photon flux during the irradiation and, consequently, a larger extent of polymerization. In a previous article [1] we reported that there was an indication that the polymerization reaction proceeds in the dark. In order to investigate this effect in more detail with the present stationary phase, a 2 m long capillary was filled with a SiA-EEA solution and irradiated. Then the solvent was evaporated in the dark until 1 m was emptied. This empty part, capillary 2, was cut off and cured at elevated temperature. The remaining part, capillary 3, was reconnected to the vacuum pump to evaporate the solvent. The found

phase ratios, (V_s/V_m) , of both columns are given in Table I. As can be seen, the phase ratio of both capillaries is very similar. This indicates that the polymer conversion over the length of the capillary is constant but it also shows that polymerization in the dark does not proceed during the evaporation of the solvent.

Kinetic Performance

In previous papers [1, 2] we have emphasized the importance of a large diffusivity of the immobilized layer in thick coated capillaries. This can be seen in the third term of the extended Golay equation which describes the efficiency in OT-LC:

$$H = \frac{2D_m}{u} + \frac{(1 + 6k' + 11k'^2) d_c^2 u}{96D_m(1 + k')^2} + \frac{2k' d_f^2 u}{3D_s(1 + k')^2} \quad (1)$$

where:

- H = theoretical plate height
- u = the linear velocity of the mobile phase
- d_c = internal diameter of the capillary
- d_f = film thickness of the stationary phase
- D_m = diffusion coefficient in the mobile phase
- D_s = diffusion coefficient in the stationary phase
- k' = capacity factor

The first term in this equation describes axial diffusion of the solute which is negligible at moderate mobile phase velocities. The second and third term represent mass transfer in the mobile- and stationary phase respectively.

The performance of the immobilized stationary phases was determined by measuring plate heights at different mobile phase velocities. The experimental plate heights were fitted with the extended Golay equation, using D_m values calculated by means of the Wilke-Chang equation [6]. From the fit, the diffusion coefficient of the solutes, D_s , in the layer can be estimated. Figure 1 shows the theoretical and experimental plate height of 1,2-benzanthracene on a SiA-EHA coated and on a SiA-EEA coated capillary. Although the capacity factors for 1,2-benzanthracene are somewhat different on Capillaries 6 and 1, the difference in plate heights can be attributed almost fully to a difference in diffusivity in the stationary phases occurring in Eq. (1). Diffusion coefficients for 1,2-benzanthracene of 0.58 and $1.50 \cdot 10^{-10} \text{ m}^2 \text{ s}^{-1}$ are found on the SiA-EHA and SiA-EEA stationary phase respectively. These values are quite similar to diffusion coefficients previously estimated on $8 \mu\text{m}$ I.D. columns, coated with identical stationary phases, namely 0.62 [1] and $1.15 \cdot 10^{-10} \text{ m}^2 \text{ s}^{-1}$ [2]. In Table II estimated diffusion coefficients for polycyclic aromatic hydrocarbons (PAHs) on various columns are listed. Note that there is a correlation between the diffusion coefficients in both stationary phases. On the SiA-EEA phase the diffusion coefficients for the PAHs are found to be 2–3 times higher compared to those on the SiA-EHA phase. Elsewhere,

Table II. Diffusion coefficients ($\times 10^{-10} \text{ m}^2 \text{ s}^{-1}$) of PAHs in SiA-EHA and SiA-EEA stationary phases using methanol as mobile phase.

	Capillary no.		
	6	1	4
9-anthracenemethanol	0.26	0.85	1.10
9-anthracenecarbonitrile	0.55	1.30	1.40
anthracene	0.80	1.80	1.80
fluoranthene	0.60	1.45	1.45
9-phenylanthracene	0.37	1.00	1.20
1,2-benzanthracene	0.58	1.50	1.50

Table III. Distribution coefficients (K_i) and selectivity factors ($\alpha_{j,i} = K_j/K_i$) of PAHs on 5 μm I.D. polyacrylate coated columns using methanol as mobile phase.

	SiA-EHA		SiA-EEA	
	K	$\alpha_{j,i}$	K	$\alpha_{j,i}$
9-anthracenemethanol	0.32		0.41	
9-anthracenecarbonitrile	0.95	2.95	0.72	1.75
anthracene	1.13	1.19	0.79	1.10
fluoranthene	1.44	1.27	1.00	1.27
9-phenylanthracene	1.71	1.19	1.08	1.08
1,2-benzanthracene	1.82	1.07	1.24	1.15

Figure 2

Separation of PAHs using LIF detection. (A) 100 % methanol on Capillary 6 (SiA-EHA), (B) 100 % methanol on Capillary 1 (SiA-EEA), (C) 100 % acetonitrile on Capillary 1. Solutes: 1 = salicylate; 2 = 9-anthracenemethanol; 3 = 9-anthracenecarbonitrile; 4 = anthracene; 5 = fluoranthene; 6 = 9-phenylanthracene; 7 = 1,2-benzanthracene.

with polysiloxane phases, e.g. PMSC₁₈ and PS 255 [7], the diffusion coefficients of 9-phenylanthracene were found to be about $10^{-12} \text{ m}^2 \text{ s}^{-1}$ which is smaller by a factor of 100 than in the SiA-EEA and SiA-EHA phases. The high performance of the SiA-EEA and SiA-EHA coated 5 μm I.D. columns is illustrated in Figures 2 (A, B and C) showing fast separations of anthracene derivatives. Chromatograms A and B were obtained with 100 % methanol as mobile phase. The difference in selectivity of the SiA-EHA (A) and the SiA-EEA (B) stationary phase towards these compounds is clear to see. Chromatogram C shows a separation on a SiA-EEA coated capillary using 100 % acetonitrile as mobile phase. The plate numbers of the first four retained solutes in this chromatogram range from 215,000 to 245,000 which corresponds to a separation speed of around $1200 \text{ plates s}^{-1}$. On this stationary

phase 9-phenylanthracene and 1,2-benzanthracene coelute when pure acetonitrile is used as the mobile phase.

Retention and Selectivity

Distribution coefficients and selectivity factors of various PAHs are listed in Table III. The variation in distribution coefficients obtained on three separately coated 5 μm I.D. SiA-EEA capillaries is within 2 %, which illustrates the good reproducibility of the acrylate layers. The selectivity factors of PAHs on polyacrylate stationary phases in the 5 μm I.D. capillaries do not differ from those as previously found for 10 μm I.D. coated capillaries [1, 2]. This result differs from the findings of Göhlin et al. who reported significant differences in selectivity between 5 and 10 μm I.D. capillaries, coated with PS 264 and PS 255 stationary phases [7].

Figure 3
Experimental plate heights for 9-anthracenemethanol on column 1 using methanol as mobile phase and 'on-column' UV (■) and LIF (□) detection.

Figure 4
Separation of nineteen PTH-amino acids on Capillary 2 using UV detection (206 nm). Mobile phase: acetonitrile-100 mM NaAc (pH = 4.6) 14 : 86, v/v for the first nine solutes and 50 : 50, v/v for the other solutes. Elution order of PTH-amino acids: 1 = Arg; 2 = His; 3 = Cys; 4 = Asn; 5 = Gln; 6 = Asp; 7 = Ser; 8 = Thr; 9 = Glu; 10 = Gly; 11 = Ala; 12 = Tyr; 13 = Met; 14 = Pro; 15 = Val; 16 = Phe; 17 = Ile; 18 = Leu; 19 = Trp.

UV Detection

In a previous publication [2] we demonstrated the applicability of UV detection in 8 μ m I.D. capillaries. Compared to LIF detection, UV detection gave an extra peakbroadening which caused a loss in resolution of about 5 %. To assess the applicability of on-column UV detection for 5 μ m I.D. capillaries, plate heights of

anthracenemethanol were measured with UV and LIF detection and are given in Figure 3. As can be seen, with UV detection a significant loss in resolution occurs. The extra contribution is comparable to that previously found for the 8 μ m I.D. columns [2]. At moderate velocities the difference in plate height is about 75 % which results in a loss in resolution of around 25 %. To demonstrate that nevertheless, on-column UV detection is still useable for 5 μ m I.D. columns, a separation of nineteen PTH-amino acids is shown in Figure 4. The separation of PTH-amino acids is generally performed on 4.6 mm I.D. C₁₈ columns, often using gradient elution, because of the large polarity difference of the PTH-amino acids [8].

We first separated the first nine polar amino acids isocratically and then applied a step gradient to elute the less polar amino acids. The polar amino acids could be separated with a mixture of acetonitrile/100 mM NaAc, pH = 4.6 (14 : 86 v/v) whereas the non-polar amino acids could be separated with mobile phases containing 50 to 60 % acetonitrile. The resolution between phenylalanine and isoleucine could be improved by increasing the content of the organic modifier after the step gradient, however this at the same time decreased the resolution between proline and valine. The relative standard deviation of the retention times of the PTH-amino acids was below 2 % during a day. To restore the column after the step gradient, flushing of the column with only one column-volume of the starting mobile phase was sufficient.

LIF Detection of OPA-derivatized Amines

OPA can be used to derivatize primary amines into strongly fluorescent compounds. The OPA derivatized compounds can be excited with the 325 nm line of a He-Cd laser. Amino acids [5], catecholamines [9] and alkylamines [10, 11] have been successfully derivatized this enabling their sensitive detection in liquid chromatography.

On Capillary 2 several OPA derivatized alkylamines could be baseline separated in 5 minutes with up to 400,000 plates and 1500 plates s⁻¹ using acetonitrile-water (80 : 20) as mobile phase. Figure 5 A and B show the separation of several OPA derivatized alkylamines on Capillary 2 using mixtures of acetonitrile-water (80 : 20 and 50 : 50 respectively). The chromatogram in Figure 5B shows that peak shapes are still well preserved up to capacity factors of 30.

The effect of the alkyl chain in these derivatives on the retention is shown in Figure 6. As can be seen, the logarithms of the capacity factors increase linearly with increasing alkyl chain length. The log *k'* increment per methylene group in the aliphatic chain is 0.05 and 0.10 using 90 : 10 and 80 : 20 (v/v) mixtures of methanol-water as mobile phase respectively. P. Jandera et al. [11] found increments of 0.10 and 0.135 with similar mobile phases and solutes in HPLC. This indicates that our SiA-EEA coated OT-LC columns are less selective

Figure 5
Separation of OPA derivatized alkylamines on Capillary 2 using acetonitrile-water 80:20, v/v (Figure 5A) and 50:50, v/v (Figure 5B) as mobile phase. Elution order: 1 = salicylate; 2 = n-propylamine; 3 = n-butylamine; 4 = n-pentylamine; 5 = n-hexylamine; 6 = n-octylamine; 7 = n-decylamine.

Figure 6
Relation between $\log k'$ and chain length of aliphatic amines for various methanol-water mixtures; n = number of carbon atoms in the alkyl chain.

Figure 7
Separation of five Dns-amino acids on Capillary 4. Mobile phase: acetonitrile-0.45% acetic acid (40:60, v/v). Peaks: 1 = Arg; 2 = Asn; 3 = Ala; 4 = Pro; 5 = Ile.

than the C₁₈ column used in their study. However, due to a stronger dependence of retention on percentage water in the mobile phase of OT-LC compared to HPLC for these compounds, selectivity of the SiA-EEA stationary phase seems to improve compared to the C₁₈ HPLC column with more aqueous mobile phases.

Figure 7 shows a separation of five dansyl derivatized amino acids with an acetonitrile-acetic acid mixture (40 : 60 v/v). Using a 70 : 30 v/v acetonitrile-acetic acid mixture, the Dns-amino acids could be separated within 2 minutes (chromatogram is not shown). Arginine did not show any retention with the mobile phases employed.

Conclusions

5 µm I.D. coated capillaries for OT-LC with good properties in terms of kinetic performance and mass loadability can be prepared without any difficulty. The polyacrylate stationary phases possess reversed phase characteristics and have been shown to be suitable for the separation of a variety of compounds. We are now investigating a stationary phase that contains reactive groups which should make it practicable and easy to manipulate its selectivity.

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