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Bonded-phase photopolymerized sol-gel monoliths for reversed phase capillary electrochromatography

A porous chemically modified photopolymerized sol-gel (PSG) monolith enhanced the capillary electrochromatographic separations of two test mixtures, one containing eight alkyl phenyl ketones and the other containing thiourea and three polyaromatic hydrocarbons. Derivatization of the PSG surface with silane coupling reagents resulted in bonded phases of pentafluorophenylpropyldimethyl, pentafluorophenyl, 3,3,3-trifluoropropyl, *n*-octadimethyl, perfluorohexyl, and aminopropyl. The fabrication of the bonded-phase PSG column is easy to do with the silanization reaction proceeding at room temperature for not longer than 60 minutes. The hydrophobicity of the PSG was altered without degrading its chromatographic performance. The bonded-phase PSG monoliths have higher stability at pH values below 4 as compared to the parent (underivatized) PSG. Separations of different mixtures containing nucleosides, positively charged peptides, and taxol derivatives illustrate the potential of bonded-phase PSG columns for the analyses of biologically and pharmaceutically important compounds. We report column efficiencies of up to 180 000 plates/meter and retention factors as large as 28.6 for decanophenone.

Key Words: Bonded phase; Monolith; Photopolymerized sol-gel; Capillary electrochromatography

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1 Introduction

In capillary electrochromatography (CEC), monoliths [1] such as continuous polymer beds [2, 3] polymer rods [4], silica rods [5], and continuous column supports [6] are being used to effect separations with advantage compared to packed columns. Monoliths obviate the need for retaining frits, the porosity is tunable, and fabrication is easy. Monoliths have been developed for the separation by CEC of neutral aromatics [1, 10–14], oligosaccharides, and peptides [11, 14]. The use of acrylates in free-radical polymerization is common in the preparation of porous organic polymer monoliths [15, 16]. Alternatively, we reported that photopolymerized sol-gel (PSG) can be used for the preparation of porous organic-inorganic hybrid monoliths [10]. This paper describes an extension of this previous work in which the PSG is chemically modified to improve chromatographic performance.

Several variables, such as the separation mode and pore size can be optimized to improve the CEC separation of complex mixtures. The bonded phase is also an important variable that can be manipulated to optimize a separation. The nature of the stationary phase has a major influence on the separation and retention factors, hence on the resolution of a separation. For example, the enantiomeric CEC separation of dansyl-dl-amino acids was made possible through the use of polyacrylamide with covalently bound β-cyclodextrin on the polymer surface [17].

The PSG monoliths bearing hydroxyl groups are good candidates for bonded-phase chemistry. The hydroxyl groups can be easily derivatized with organochlorosilane or organoalkoxysilane coupling reagents in the classical silanization approach commonly used in the modification of silica-based particles [18]. The effects of silanization reaction times and the nature of the bonded phase are determined by carrying out separations of two mixtures, one containing eight neutral alkyl phenyl ketones and the other three polycyclic aromatic hydrocarbons (PAHs). The usefulness of the bonded-phase PSG columns for the analyses of biological and pharmaceutical compounds is illustrated in the separation of a mixture of six positively charged peptides and a mixture of taxol and two of its precursors. The separation of a mixture of four nucleosides was achieved using an aminopropyl-bonded phase PSG with reversed electroosmotic flow.

2 Experimental

2.1 Apparatus

All of the electrochromatograms were obtained using a Beckman P/ACE 2000 capillary electrophoresis (CE)

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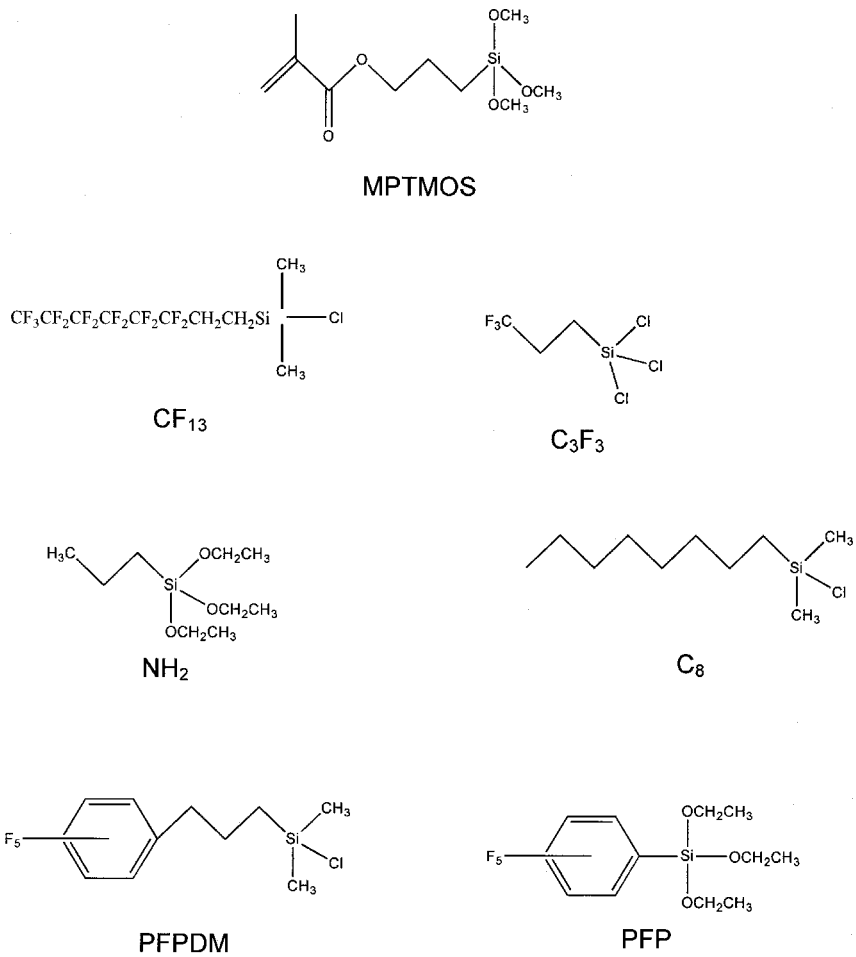

Figure 1. Structures of the silane precursor and silane coupling reagents.

instrument equipped with a UV absorbance detector (Beckman Coutler, Inc. Fullerton, CA). A Spectrolinker XL-1500 equipped with six 365-nm low-pressure mercury bulbs (Spectronics Corp., Westbury, NY) was used in the photopolymerization reactions. A Philips Model 505 scanning electron microscope was used to analyze the physical structure of the PSG monoliths in the capillary columns. For that purpose each column was coated with a 10-nm thickness of gold/palladium using a Denton Vacuum, LLC Desk II Cold Sputter/Etch unit and carbon evaporation accessory (Moorestown, NJ).

2.2 Materials and chemicals

Fused-silica capillaries of 75-μm ID × 365-μm OD were purchased from Polymicro Technologies (Phoenix, AZ). The PSG precursor, methacryloxypropyltrimethoxysilane (MPTMOS) and the silane coupling reagents, pentafluorophenyldimethylchlorosilane (PFPDM), pentafluorophenyltriethoxysilane (PFP), 3,3,3-trifluoropropyltrichlorosilane (C_3F_3), *n*-octadimethylchlorosilane (C_8), (tridecafluoro-1,1,2,2-tetrahydrooctyl)dimethylchlorosilane (CF_{13}), and *n*-propylaminotriethoxysilane (NH_2), were purchased from Sigma-Aldrich (Milwaukee, WI) or Gelest (Tullytown, PA) and used as received. **Figure 1** shows the structures of these reagents. The photoinitiator, Irgacure 1800, was donated by Ciba (Tarrytown, NY). All solvents were of spectroscopic grade from Sigma-Aldrich. Thiourea, alkyl phenyl ketones, naphthalene, phenanthrene, pyrene, nucleosides, taxol, taxol derivatives, and the peptides were purchased from Sigma-Aldrich and used as received.

2.3 Monolith Preparation

2.3.1 Parent PSG

The parent PSG columns were prepared with a precursor stock solution of 575 μL MPTMOS and 100 μL of 0.12 M HCl that was stirred at room temperature for 15 minutes. An 80/20 toluene precursor solution (*v/v*) was prepared by mixing 60 μL of the precursor stock solution with 30 mg of the photoinitiatior dissolved in 240 μL of toluene. The

resulting solution was stirred at room temperature in the dark for 5 minutes. A PSG capillary column with a 5-, 10-, or 15-cm stripe of polyimide removed from its exterior is prepared as described in a previous report [10].The total length of the capillary was 25.6 cm (18.8 cm from inlet to the detector window). Burning off the polyimide with fuming sulfuric acid created the detector window, which was located at the outlet of the PSG monolith.

2.3.2 Bonded phases

The bonded phase was prepared on the parent PSG column that was rinsed with toluene prior to the silanization reaction. The rinsed column was then treated with the silane coupling reagent by continuous flow of a neat solution of the reagent with a syringe in a hand-held vise. The reagent was allowed to react with the PSG surface for 15, 30, 60, 70, and 90 minutes at room temperature. Any unreacted silane coupling reagent was removed from the bonded-phase PSG column by flushing with toluene using a syringe. The following bonded-phase PSG monoliths were prepared by this procedure: PSG-PFP, PSG-PFPDM, PSG-C_8, PSG-C_3F_3, PSG-CF_{13}, and PSG-NH_2.

2.4 PSG column conditioning

A PSG capillary column was carefully installed into a P/ACE cartridge. Although the column is without a complete coating of polyimide, it still maintains good mechanical strength. Care is taken, however, in handling these capillaries to prevent breakage during installation into the capillary cartridge. Once in the cartridge, the capillary is first conditioned with the separation solution using a syringe. In the CE instrument the column is rinsed further for about 2 minutes at 20 psi followed by electrokinetic conditioning at 5 kV for 10 minutes. The columns were thermostated at 20°C.

2.5 Sample and separation solutions

The alkyl phenyl ketone sample solution was prepared with 1.42 μM acetophenone, 25 μM propiophenone, 15 μM butyrophenone, 20 μM valerophenone, 5.5 μM hexanophenone, 16.5 μM heptanophenone, 15.3 μM octanophenone, and 3.5 μM decanophenone in a solution of 50 mM ammonium acetate (pH 6.5)/water/acetonitrile (1/3/6). Using a lower volume of acetonitrile resulted in lowering the solubility of the sample. The PAH sample solution was prepared with 25 mM thiourea, 5.05 mM naphthalene, 4.90 mM phenanthrene, and 4.95 mM pyrene in the separation solution. The nucleoside sample mixture consisted of inosine, uridine, guanosine, and cytidine in concentrations of 1 mg/mL each in water. Short-chain peptides, angiotensin I, bradykinin, angiotensin II, gly-gly-gly, val-tyr-val, and methionine enkephaline, were used as cationic analytes. The concentration of each of the peptides is 16 μg/mL. Taxol, baccatin III, and acetylbaccatin were each prepared as 1 mg/mL solutions in acetonitrile. The separation solution used in the experiments were prepared with various ratios of 50 mM ammonium acetate (pH 4.3 or 6.5), phosphate (pH 8), water, and acetonitrile and were sonicated for about 2 minutes prior to use.

3 Results and discussion

The surface of the parent PSG monolith was modified with a variety of silane coupling reagents having mono- or trifunctionality. These reagents covalently bind to the free hydroxyl groups on the PSG surface, resulting in bonded phases that alter the hydrophobicity of the PSG monolith. The reaction between a silane coupling reagent and the surface hydroxyl group is believed to follow the same complex mechanism involved in other silanization reactions [19].

Derivatization of the PSG surface serves two purposes. First, it creates a bonded phase on the PSG surface that can enhance its resolution. Secondly, the PSG surface is somewhat deactivated by derivatization thereby decreasing the surface activity caused by the existing negative charge (from the ionization of the free hydroxyl groups). The bonded-phase PSG supports electroosmotic flow (EOF) in the column.

3.1 Porous bonded-phase PSG monoliths

Figure 2.a and **Figure 2.b** present scanning electron microscope (SEM) micrographs of a parent PSG monolith and a C_8-bonded-phase PSG monolith, respectively. Both the parent and C_8-bonded phase PSG monoliths have nearly identical structures. The cross-sectional images reveal porous structures consisting of interconnecting 1-μm diameter spheres. The bonded-phase PSG monolith, however, contains some areas of higher monolith density. SEM analyses of the other bonded-phase PSG monoliths (not shown) indicate that the porous monolith structure is similar in all the bonded-phase PSG monoliths.

3.2 Effect of the bonded phase on column performance

A comparison of the five bonded-phase PSG columns (**Figure 3**), namely PSG-C_3F_3 (Figure 3.b), PSG-CF_{13} (Figure 3.c), PSG-PFP (Figure 3.d), PSG-C_8 (Figure 3.e), and PSG-PFPDM (Figure 3.f) to the parent PSG column (Figure 3.a) reveals enhanced resolution of the test analytes, particularly for the more hydrophobic ones, with the

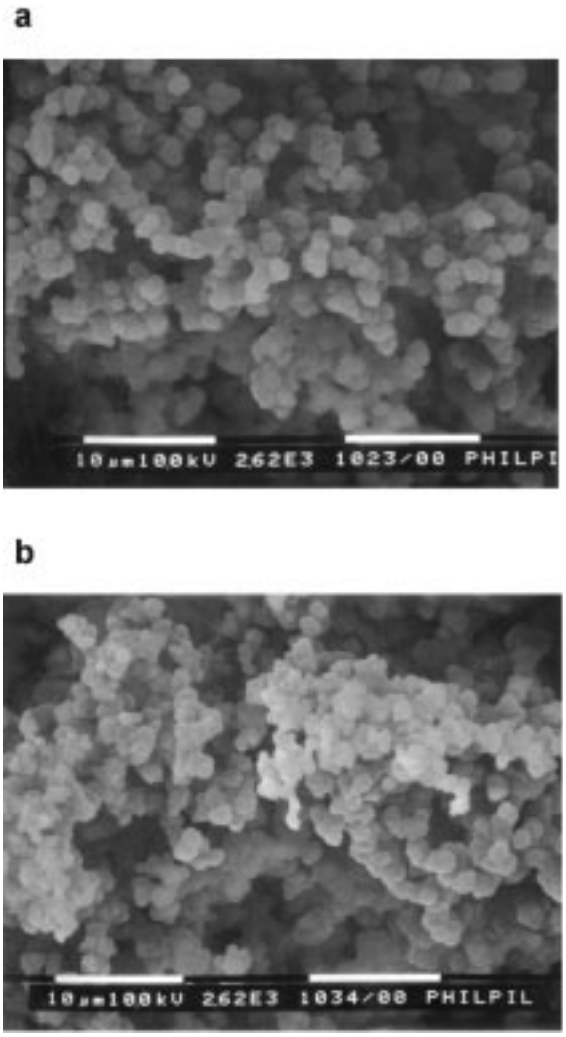

Figure 2. Scanning electron microscope micrographs of the cross-section of a (a) parent PSG monolith and (b) C_8 bonded phase PSG monolith in 75-µm ID capillaries.

highest enhancement occurring for PSG-PFPDM. Resolution was determined from the expression

$$R_S = \frac{\sqrt{N}}{4}\,\frac{(\alpha - 1)}{\alpha}\,\frac{k}{(k+1)},$$

where N is the efficiency (theoretical plate number), α is the separation factor, and k the retention factor for a particular analyte. The separation factor α is given by k_2/k_1. The retention factor $k = (t_R - t_0)/t_0$ was determined in the usual way, where t_R is the analyte retention time and t_o is the retention time of an unretained marker (for which we used thiourea). Resolutions for acetophenone (peak 1) and hexanophenone (peak 5) are 2.02, 2.15, 2.64, 3.13, and 3.71 for PSG-C_3F_3, PSG-CF_{13}, PSG-PFP, PSG-C_8, and PSG-PFPDM, respectively. This performance is an increase of up to 50% from the parent PSG (R_S = 1.86). In all cases, the alkyl phenyl ketones elute in order of increasing hydrophobicity: acetophenone (peak 1), propiophenone (peak 2), butyrophenone (peak 3), valerophenone (peak 4), hexanophenone (peak 5), heptanophenone (peak 6), octanophenone (peak 7), and decanophenone (peak 8). All of the alkyl phenyl ketones are eluted from the bonded-phase PSG capillary columns within 30 minutes at relatively high field strengths (> 1 000 V/cm). The lowest resolution of the alkyl phenyl ketones is observed for the parent PSG column (Figure 3.a), whereas the highest resolution of the alkyl phenyl ketones is achieved on PSG-PFPDM with octanophenone eluting after 13 minutes (Figure 3.f) and decanophenone (not shown) eluting after 25 minutes.

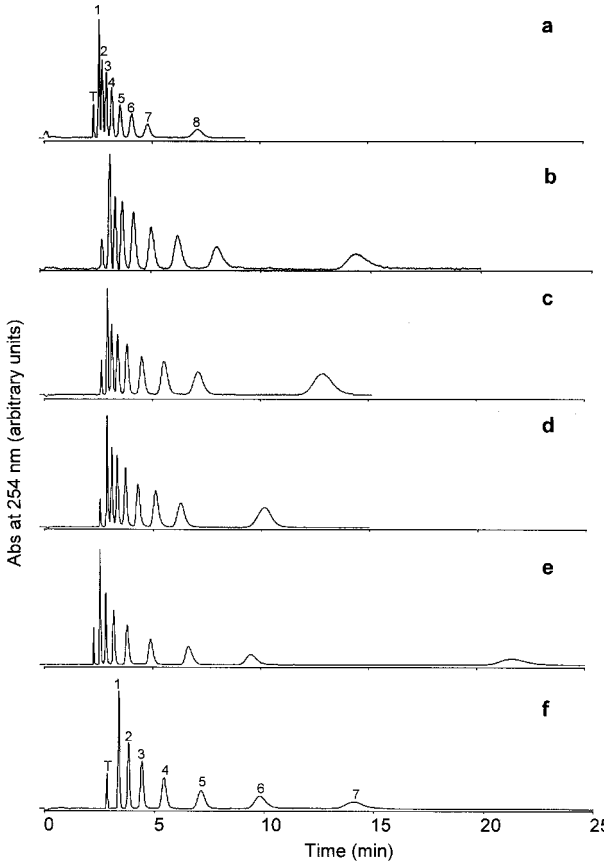

Figure 3. Electrochromatograms of the separation of thiourea and alkyl phenyl ketones on PSG capillary columns: (a) parent, (b) C_3F_3, (c) CF_{13}, (d) PFP, (e) C_8, and (f) PFPDM. The peaks are thiourea (peak T), acetophenone (peak 1), propriophenone (peak 2), butyrophenone (peak 3), valerophenone (peak 4), hexanophenone (peak 5), heptanophenone (peak 6), octanophenone (peak 7), and decanophenone (peak 8). Injection is at 1 kV for 5 seconds; the field strength is 769 V/cm; the separation solution is 1/5/4 (*v/v/v*) 50 mM ammonium acetate (pH 6.5)/water/acetonitrile. The sample solution is 1/3/6 (*v/v/v*) 50 mM ammonium acetate (pH 6.5)/water/acetonitrile.

It has been reported that fluorinated bonded phases interact with aromatic compounds based on electrostatic interactions between the π electrons of the aromatic rings and

the lone pair electrons (in the 2p orbital) on the fluorine atom of the bonded phase [20]. This behavior appears not to be the case for the fluorinated bonded phases, PFP and PFPDM. Instead, separation of the alkyl phenyl ketones is caused by the increasing hydrophobicity of the compounds as the number of carbons of the alkyl functional group increases. The elution order follows this increase in the hydrophobicity for these compounds.

PSG-C_8 shows good resolution for the alkyl phenyl ketones and shorter analysis times for all eight alkyl phenyl ketones with decanophenone eluting within 20 minutes (Figure 3.e). PSG-C_8 combines high resolution with fast elution times for the alkyl phenyl ketones and has an EOF of 1.76×10^{-4} cm^2/V · s, which is 15% higher than the EOF values for the other bonded-phase PSG monoliths, but the same as that of the parent PSG.

We observed no bubbles or drying out of the bonded-phase PSG columns; even at high field strengths employed in the separation of the alkyl phenyl ketones. The bonded-phase PSG monoliths are more stable to highly acidic conditions (pH 2–4) than the parent PSG monolith. A plot of the current versus field strength for each of the bonded-phase PSG monoliths is linear (data not shown). This linearity suggests the absence of Joule heating, which can lead to peak broadening. In each of the separations, the efficiency of the alkyl phenyl ketones decreases with increasing *k* values. Diffusional effects that cause the analytes to elute slowly off the bonded-phase PSG monolith may explain the low efficiencies. Higher resolution can be attained with a bonded-phase PSG monolith having a length of 15 cm, but at the expense of longer elution times. A linear relationship exists between monolith length and *k* (correlation coefficients, r^2, greater than 0.990 for all the PAHs). The separation factors for monoliths of lengths 15, 10, and 5 cm are 2.55, 2.52, and 2.40, respectively.

3.3 Effect of silanization reaction time on separation factor

Table 1 presents the effect of the silanization reaction time on the separation factor of PSG-C_8 for acetophenone and hexanophenone. The separation factors increase as a function of the reaction time up to 60 minutes with the separation factors for 0 and 15 minutes differing only slightly. Separation factors greater than 1 indicate successful separation of the analytes. With longer reaction times of 70 and 90 minutes, the separation factors decrease. The same trend in resolution of acetophenone and hexanophenone is observed for increasing reaction times. The resolution of the two analytes increases linearly with reaction time ($r^2 = 0.974$) in the range of 0 to 60 minutes. Column-to-column reproducibility ($n = 3$ or 4) for bonded-phase PSG columns prepared with 15, 30, and 60 minute reaction times was better than 3% RSD. The resolution decreases for reaction times of 70 and 90 minutes.

Table 1. Effect of silanization reaction times on the separation of acetophenone (1) and hexanophenone (5) on a 5-cm PSG-C_8 monolith.

Reaction time (min)	k_1	k_5	$\alpha_{1/5}$	R_S(1/5)
0	0.15	0.74	4.91	2.11
15	0.16	1.37	4.73	2.33
30	0.14	1.17	8.36	2.46
60	0.18	1.77	9.85	3.31
70	0.18	1.67	9.20	2.83
90	0.15	1.41	9.47	2.69

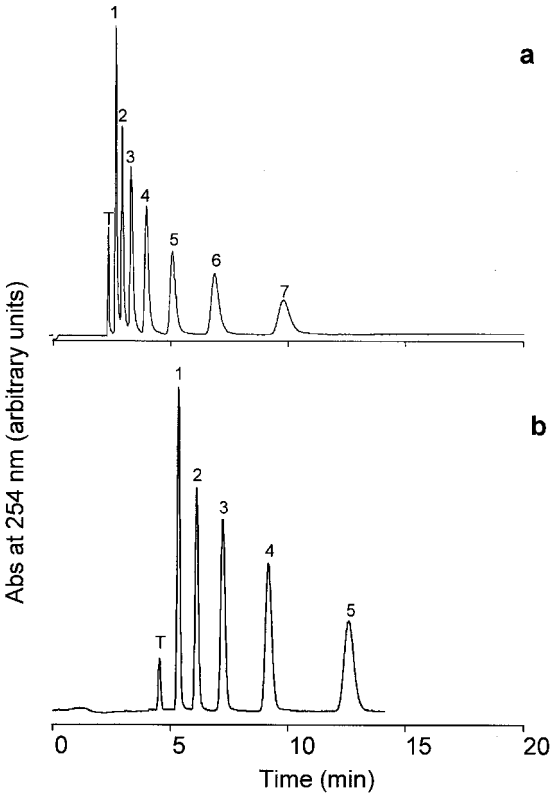

Figure 4. Electrochromatograms of the separation of five to seven alkyl phenyl ketones on a 5-cm PSG-C_8 monolith for (a) 30 min and (b) 90 min. The separation conditions and peak identities are the same as in Figure 3.

Collapse [18] of the bonded phase may explain the decrease in the resolution for reactions times of 70 and 90 minutes. As the silanization reaction time is increased, more of the *n*-octyldimethylsilane is allowed to bond to the hydroxyl groups of the monolith, thereby increasing the number of *n*-octyldimethyl groups on the monolith surface. As more of the bonded phase is bound on the PSG surface, the closer the ligands approach one another. The

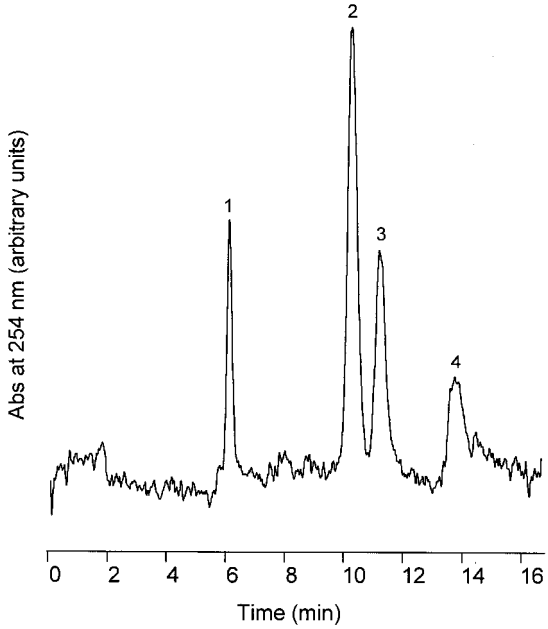

Figure 5. Electrochromatograms of the separation of four nucleosides, inosine (peak 1), uridine (peak 2), guanosine (peak 3), and cytidine (peak 4) on a 10-cm PSG-NH_2 monolith. The separation solution is 0.5/3.5/6 (*v/v/v*) 50 mM phosphate (pH 8)/water/acetonitrile. The injection plug length is 0.1 mm and the field strength is −577 V/cm.

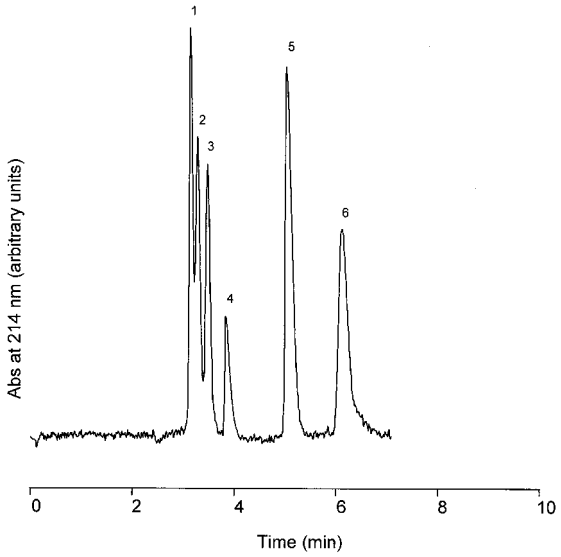

Figure 6. Electrochromatograms of the separation of five cationic peptides, angiotensin I (peak 1), bradykinin (peak 2), angiotensin II (peak 3), gly-gly-gly (peak 4), val-tyr-val (peak 5), and methionine enkephalin (peak 6) on a 15-cm PSG-PFP monolith. The separation solution is 1/4/5 (*v/v/v*) 50 mM ammonium acetate (pH 4.3)/water/acetonitrile. Injection is for 30 s at 0.5 psi and the field strength is 385 V/cm.

fact that the k_1 and k_5 values increase by 17% and 58%, respectively, from 0 to 60-minute reaction time indicates that the monolith surface is becoming increasingly hydrophobic.

Figure 4 illustrates the effect of 0-minute reaction time (Figure 4.a) and 60-minute reaction time (Figure 4.b) for a PSG-C_8 monolith on the separation of an alkyl phenyl ketone mixture. The *k* values for decanophenone, having a 10-carbon chain, are extremely high. For a 30-minute reaction time *k* is 23.0, whereas for a 60-minute reaction time *k* is 28.6, an increase of 24%. No loading measurements were performed to assess the surface coverage of the bonded phase on the PSG monolith, but it can be inferred from the chromatographic data that there should be a higher coverage of the bonded phase on the PSG monolith particularly for the reaction times 15–60 minutes, hence an increased number of chromatographic sites for the separation of the test compounds.

3.4 Application to the analysis of charged and uncharged analytes

A 10-cm long PSG monolith was derivatized with *n*-propylaminotriethoxysilane. The resulting monolith, PSG-NH_2, has an amine-bonded phase (pI 9–10) that is positively charged under our experimental conditions. The positive charge allows for reversal of the EOF. Although chromatographic materials with an amine group functionality has found some application in normal phase and ion-exchange chromatography, PSG-NH_2 under our conditions behaves as a reversed-phase material. The alkyl phenyl ketones elute in the same order as compared to the other bonded-phase PSG monoliths described above. **Figure 5** shows the separation of a mixture of four nucleosides on a PSG-NH_2 column with reversed EOF. The separation of this mixture was not possible in capillary zone electrophoresis in the same separation solution.

Figure 6 demonstrates the utility of a PSG-PFPDM column for the separation of four positively charged peptides, which is achieved in less than 15 minutes. Note that the cationic peptides are eluted from the column despite the anionic nature of the monolith. The bonded phase essentially creates a layer on the monolith to shield the negative charge of the ionized hydroxyl groups from the peptides. Thus, charge interactions have been diminished between the cationic peptides and the anionic monolith.

A 15-cm long PSG-C_3F_3 monolith was used for the separation of a mixture of taxol, baccatin III, and acetylbaccatin. **Figure 7** illustrates the complete separation of this mixture, which is achieved within 8 minutes. This separation demonstrates the potential of a bonded-phase PSG column for the analysis of pharmaceutical drugs.

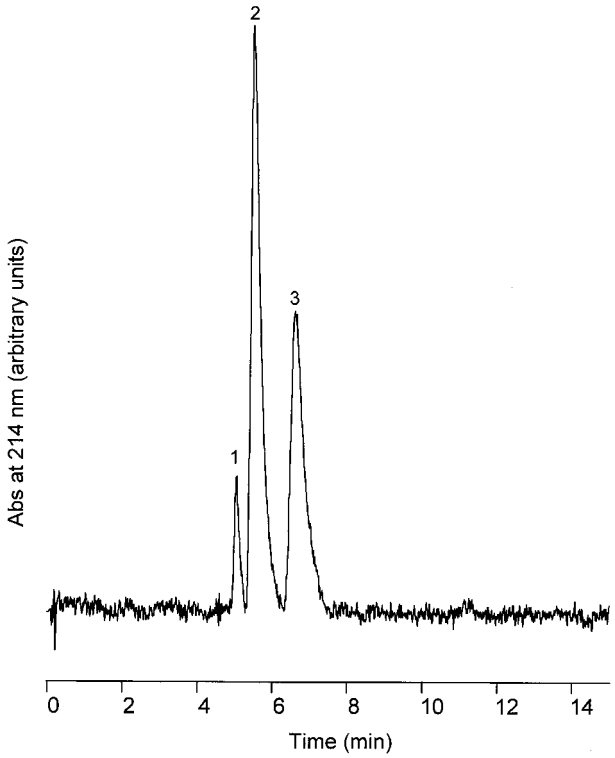

Figure 7. Electrochromatogram of the separation of baccatin III (peak 1), taxol (peak 2), and acetylbaccatin (peak 3) on a 15-cm PSG-C_3F_c monolith. The separation solution is 1/4/5 (*v/v/v*) 50 mM ammonium acetate (pH 6.5)/water/acetonitrile. Injection is for 5 s at 0.5 psi and the field strength is 385 V/cm.

4 Conclusions

Separations of neutral test compounds were improved by optimizing the PSG monolith through the use of bonded phases. Bonded-phase PSG monoliths are prepared by means of a simple synthetic procedure. These bonded-phase PSG monoliths are useful for the separation of biologically and pharmaceutically important compounds such as peptides, nucleosides, taxol, and its precursors.

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