Chromatographia (2011) 74:1–8
DOI 10.1007/s10337-011-2047-x

ORIGINAL

Preparation and Evaluation of Long Chain Alkyl Methacrylate Monoliths for Capillary Chromatography

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Received: 14 January 2011 / Revised: 8 April 2011 / Accepted: 18 April 2011 / Published online: 15 May 2011

Abstract This work describes the fabrication of long chain alkyl methacrylate monolithic materials for use as stationary phases in capillary liquid chromatography. After capillary inner wall surface activation with 3-(trimethoxysilyl)propyl methacrylate, monoliths were formed by copolymerization of either lauryl or stearyl methacrylate (LMA or SMA) with ethylene dimethacrylate (EDMA) as crosslinker, in the presence of azobisisobutyronitrile (AIBN) as initiator and a mixture of porogenic solvents including water, 1-propanol and 1,4-butanediol. The composition of the polymerization mixture was changed in terms of monomer, crosslinker and porogen ratio composition, in order to compare the influence of these parameters. The monoliths were prepared in 320 μm i.d. and 200 mm length capillaries. The column morphology was characterized by optical microscopy and scanning electron microscopy (SEM). Total porosity and permeability of each column were calculated using uracil as unretained material by measuring the pressure drop across the columns as a function of linear velocity. The microglobule average size for each column was also determined using Hagen–Poiseuille equation and compared with the SEM images. As expected, a decrease of the porogen to monomer ratio corresponded to smaller microglobules and a lower total porosity. The columns were then chromatographically evaluated; good results were obtained when these capillaries were used to separate mixtures of phenols, aromatics and drug compounds.

Keywords Capillary liquid chromatography · Monolithic column · Lauryl methacrylate · Stearyl methacrylate · Ethylene dimethacrylate

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Introduction

In the past few years, capillary high-performance liquid chromatography (HPLC) has been one of the most important developments in separation and analysis technology. Capillary HPLC offer several advantages over conventional normal scale HPLC. The advantages include increased chromatographic resolution, higher efficiency, lower sample and solvent consumption, the ability to analyze and isolate rare compounds of interest, greater mass sensitivity and ease of on-line connection to mass spectrometer [1–3].

Traditionally, capillary HPLC uses fused silica capillaries prepared with a variety of different stationary phases; these phases seem to be very promising in separating a wide variety of analytes in different application fields, including pharmaceutical, clinical, environmental, agrochemical and biological samples [4–9]. However, the successful development of these techniques is closely related to the technical challenges associated with the column manufacturing.

Monolithic columns have rapidly become highly popular separation media in chromatography. Hjertén et al. [10] first introduced the use of monoliths with capillary liquid chromatography in 1989, and since that time, monolithic

columns have been extensively studied for use in capillary HPLC [11–15]. Several different monolithic supports were described in the literature. In brief, two general categories of monolithic columns have been developed for chromatography: porous organic polymer-based monolithic columns produced by a simple molding process [11] and silica-based monolithic columns made by using the sol–gel approach [12]. Due to the simpler preparation process and easier adaptability of column selectivity, the organic polymeric approach exhibits more potential advantages compared with silica-based monoliths.

Monolithic columns consist of one single piece of highly porous material with bimodal pore size distribution, μm-sized through pores (macropores) and nm-sized mesopores. The monolith structure does not contain interparticular voids. As a result, all the mobile phase must flow through the stationary phase. This unique structure exhibits many improvements including high porosity and surface area, fast mass transfer kinetics between the mobile and stationary phases and a high binding capacity. On the other hand, the lower resistance to hydraulic flow reduces the back pressure drop across the column, while the absence of end frits, a low consumption of chemicals and better exchange of the mobile phase (during gradient formation) are other advantages. In addition, the in situ preparation process of monolithic stationary phases from liquid precursors is relatively easy [16–18].

Although they were evaluated as polymeric chromatographic packing as early as 1978 by Švec et al. [19], methacrylate-based polymers were originally introduced for HPLC analysis in the early 1990s by Švec and Fréchet [11, 20]. Methacrylates are now one of the most widely popular monoliths used as chromatographic separation media [21, 22]. There are several advantages associated with using methacrylate-based polymers as monolithic stationary phases, including high stability in a wide range of mobile phase pH (2–12), fast and simple preparation and easy functionalization. The methacrylate monolithic columns have also various selectivities towards monomers with wide ranging polarities [23, 24].

However, although several solutions have been proposed by different research groups [16, 18, 25–28], most reported methacrylate-based monoliths were based on short alkyl chain monomers due to the limited solubility of long alkyl chain methacrylates [29–31]. In this study, long chain methacrylate monolithic columns were prepared by in situ copolymerization of either LMA or SMA with EDMA in the presence of a suitable porogen using 200 mm length and 320 μm i.d. fused silica tubing. Characterization and physical properties of the prepared monolithic columns were thoroughly investigated. The columns were then evaluated and applied to the separation of different mixtures, including phenols, aromatics and drug samples.

Experimental

Chemicals and Columns

Formic acid, phosphoric acid, benzene, naphthalene, phenanthrene, 4-aminophenol, 2-naphthol, *m*-cresol, *m*-nitrophenol and other chemicals used in the experiments were of analytical grade and were purchased from BDH (Poole, UK). Phenol and 4-chlorophenol were provided by Merck Schuchardt (Darmstadt, Germany).

Drug standard aspirin and vitamin C were obtained from BDH while the aspirin-C tablets were purchased at the local pharmacies (Riyadh, Saudi Arabia). These tablets were powdered and stored in dark vacuum packages. Aspirin-C tablets contained aspirin and vitamin C compounds and were used as a real sample.

Standard solutions were prepared as follows: all phenol compounds were dissolved in purified water at 0.2% level. Aromatic compounds were dissolved in HPLC grade hexane to provide 8 μg mL^{-1} solutions.

Drug tablets and standard solutions were prepared in purified water at a concentration of 0.3% (*w/v*) for aspirin and vitamin C; the resulting solutions were filtered through 0.45 μm membrane filters and injected without further pretreatment to the HPLC system.

HPLC grade acetonitrile and hexane were purchased from BDH. The purified water used throughout all experiments was prepared on a Milli-Q system (Advantage with Elix, Millipore S.A.S., Molsheim, France), then filtered with 0.2 μm nylon membrane filter (Whatman, Maidstone, UK). The mobile phase was always filtered using a vacuum glass filtration system through the same nylon membrane filters and degassed ultrasonically for 30 min prior to use.

Fused silica tubing (0.32 mm i.d.) was purchased from Restek (USA). The chemicals used for monolithic materials preparation in this work were purchased from Aldrich (Steinheim, Germany) as follows: 3-(trimethoxysilyl)propyl methacrylate (TMSM) 98%, ethylene dimethacrylate 98% used as crosslinker, stearyl and lauryl methacrylate 98% as monomers and AIBN as initiator. Toluene, hydrochloric acid, sodium hydroxide, 1-propanol and 1,4-butandiol were acquired from BDH. All chemicals were used without further purification.

Preparation of Capillary Monolithic Columns

In order to clean and activate the inner surface of capillary, the fused-silica capillary (0.32 mm i.d.) was rinsed first with 1.0 M NaOH solution for 30 min and let for 15 min with the same solution, then rinsed with water and dried in air for 10 min, twice for each step. The tubing was then flushed with 1.0 M HCl for 30 min and dried with air for 5 min. After that, the capillary was rinsed with toluene for

Table 1 Composition of polymerization mixtures used for the preparation of capillary monolithic columns, % (*w/w*)

Column	Monomer (%)	EDMA (%)	Porogen[c] (%)	AIBN[d] (%)
L_1^a	20	15	64	1
L_2^a	25	15	59	1
S_1^b	20	15	64	1
S_2^b	25	15	59	1

[a] Lauryl methacrylate

[b] Stearyl methacrylate

[c] 60% 1-propanol, 30% 1,4-butandiol and 10% water

[d] Azobisisobutyronitrile

15 min then flushed with 20% 3-(trimethoxysilyl)propyl methacrylate solution in toluene for 30 min and let with the same solution for 2 h, then rinsed with toluene for 10 min and dried with air for 5 min. The activated capillary was then cut into four pieces (200 mm length each) with a razor blade. The monomer and porogen mixtures were prepared (*wt%*) as described in Table 1.

The monomer mixture and the porogen solvents were mixed into a homogenous solution then sonicated and purged with helium gas for 5 min. Each capillary column was then filled with the corresponding reactant solution and both ends were plugged with a piece of rubber. The polymerization was performed in a water bath at 60 °C for 20 h. After the polymerization, the seals were removed; the resulting columns were connected to an HPLC pump and thoroughly washed with acetonitrile to remove the unreacted materials and porogenic solvents.

Porosity and Bed Permeability

The total column porosity (ε_T) is an important parameter for column evaluation. In the literature, various methods are available to measure ε_T, such as the flow method [32], the conductivity method [33] and the gravimetric method [34]. In this study, the flow method was used to evaluate ε_T. This method is based on the retention volume determination of an unretained marker and the geometrical volume of the empty column (since it can be considered as a long cylindrical tube), after correction for extra-column volume contributions, depending on the end frits and connection tubes used.

The calculation of ε_T in this work was based on the following equation:

$$\varepsilon_T = \frac{4Ft_0}{\pi d^2 L} \quad (1)$$

where F is the volumetric flow rate, t_0 is the retention time of an unretained marker (uracil was used in this work), d is the column inner diameter and L is the column length.

The permeability (K^0) of a porous medium is a measure of its capacity to transmit a fluid driven by an imposed pressure drop across the column. Darcy's law links the solvent viscosity η and column porosity ε_T to K^0 [35], which is calculated as follows:

$$K^0 = \frac{u\eta L\varepsilon_T}{\Delta P} \quad (2)$$

In this equation, u is the linear velocity of the mobile phase, η is the dynamic viscosity of the eluent, L is the column length and ΔP is the pressure drop.The Hagen–Poiseuille equation is a physical law that gives the pressure drop in a fluid flowing through a long cylindrical pipe. The average velocity of mobile phase over the channel section (v) is obtained from integration of the momentum transport equation that derived on the basis of Navier–Stokes equation [36]:

$$v = \frac{\Delta P R^2}{8\eta L} \quad (3)$$

where R is the average diameter of monolith channels (macropores).

Characterization of the Monolithic Columns

After chromatographic experiments were finished, the monolith rods in the tubes were washed, cut into small pieces and then dried. The dried columns and monolith materials were subjected to optical microscopy and SEM.

The optical microscope images were obtained using a Micromaster Fisher Scientific optical microscope (G2009-A 702-042, China) with typically 100-fold magnification. The pore properties and microscopic morphology of the polymers were examined by a Jeol (JSM-7600F) field emission scanning electron microscope (Japan) at 3 kV after the column samples were sputtered with platinum.

Chromatographic Conditions

All analyses were performed using a Shimadzu HPLC system including a pump (LC-6A, Kyoto, Japan), a Rheodyne 7125 manual injector, a UV detector (SPD-6A) and a C-R6A integrator. The detector was equipped with a 2-cm path length and 1 µL volume homemade cell and set at different wavelengths according to the analyzed compounds. 500 nL of each sample and standard solutions were injected in the monolithic column using the manual injector of the HPLC instrument. As mobile phase, acetonitrile/water solutions with or without acid additives at different ratios were used. All solutions were filtered through 0.2 µm nylon membrane filter (Whatman) prior to use. All experiments were carried out at room temperature, while each sample or standard was injected five times.

Results and Discussion

Preparation of the Monolithic Columns

The general scheme for preparation of the four capillary monolithic columns is illustrated in Fig. 1, which includes four steps: (a) empty capillary wall modification, (b) filling the tubing with the monomer mixture, (c) polymerization of functional monomer and crosslinker at the surface of column and (d) connecting the column to the HPLC system and washing it with acetonitrile.

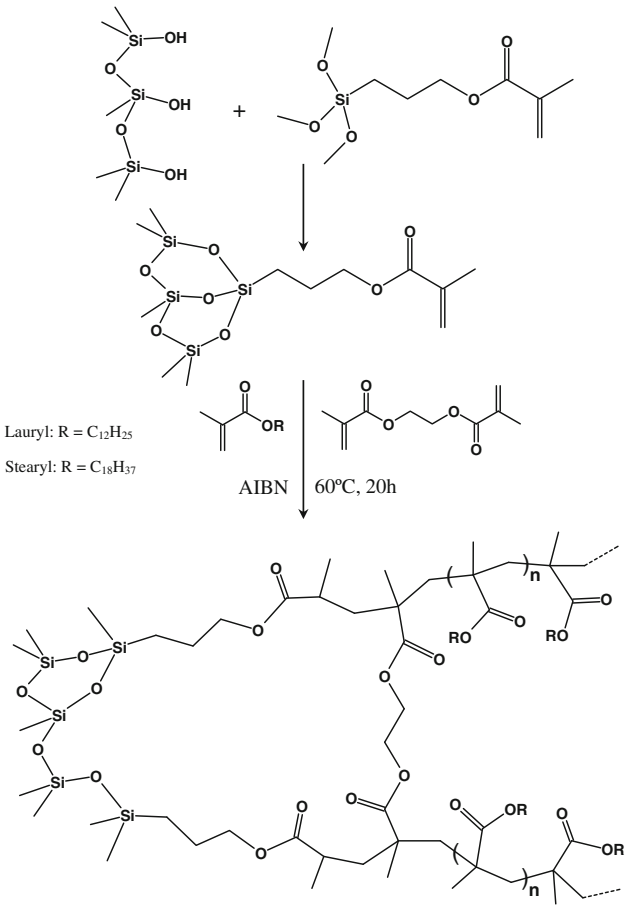

Fig. 1 Schematic representation of preparation procedure of monolithic columns

Varying the polymerization mixture composition generates monolithic columns with different properties (e.g., physical and chemical properties) from which different chromatographic performances are expected. The monomer and porogen composition used for columns preparation are shown in Table 1.

Characterization of the Monolithic Columns

In contrast to particle columns preparation, where common particle size classification is required after polymerization is completed, monoliths are prepared with a bulk polymerization and their structure is defined already by monomer composition and polymerization temperature without further processing [37, 38].

Determination of the Column Porosity and Bed Permeability

Total porosity is an important parameter for column evaluation. In summary, the mobile phase linear velocity was calculated using uracil as inert dead volume tracer while the volumetric flow rate was accurately measured for each column. Next, with the known empty tube dimensions, the total porosities ε_T were calculated using Eq. 1. As shown in Table 2, the total porosities of the examined monoliths L_1, L_2, S_1 and S_2 were 76%, 72%, 75% and 69%, respectively. These results confirm that, as mentioned by previous works, increasing the porogen ratio in the polymerization mixture corresponds to a decrease of the monomer content and induces a higher porosity of the monolith [18, 39]. Porosity values were calculated after extra volume (5.9 μL) corrections.

Acetonitrile and water were used for the measurement of the pressure drop across the columns at different flow rates, in order to evaluate the mechanical stability and permeability of our monolithic materials. Figure 2 shows the effect of the flow rate through each column on backpressure using either water or acetonitrile as mobile phase. An excellent linear dependence of the four columns inlet pressure on the flow rate is indicated by a regression factor R better than 0.999 for all measured curves.

Table 2 Pore characteristics of monolithic columns (columns L_1, L_2, S_1 and S_2)

Column	ε_T	K^0 using acetonitrile (m^2)	K^0 using water (m^2)	R (μm) using acetonitrile	R (μm) using water
L_1	0.76	5.55×10^{-14}	6.50×10^{-14}	0.77	0.83
L_2	0.72	1.77×10^{-14}	1.98×10^{-14}	0.44	0.47
S_1	0.75	5.96×10^{-14}	6.67×10^{-14}	0.80	0.84
S_2	0.69	1.17×10^{-14}	1.20×10^{-14}	0.36	0.37

ε_T total porosity, K^0 permeability, R average diameter of monolith channels (macropores) using acetonitrile and water as eluents

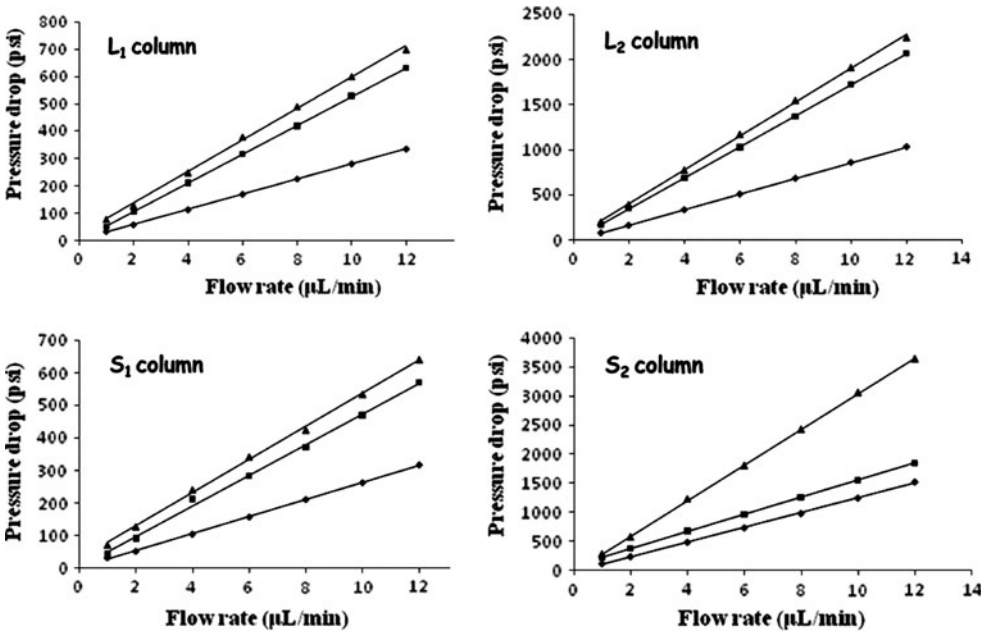

Fig. 2 Graph illustrating plots of pressure drop versus flow velocity for each column using water and acetonitrile as mobile phase, (*filled diamonds* acetonitrile, *filled squares* acetonitrile:water (50/50), *filled triangles* water), temperature: 24 °C

When the plot of the pressure drop versus the velocity of the fluid shows a linear relationship, this indicates that permeability and mechanical stability of the monolith are both excellent, and so Darcy's law for laminar flow through porous media is applicable for the calculation of the permeability [40]. Numerical values for the permeability of the monolithic capillary columns were determined; acetonitrile and water were passed through the column at a volumetric flow rate of 4 μL min^{-1} (corresponding to a 8.3×10^{-4} ms^{-1} linear flow velocity) and a temperature of 24 °C (room temperature). The measured pressure drops were of 1.72×10^6, 5.36×10^6, 1.66×10^6 and 8.45×10^6 Pa for water and 7.79×10^5, 2.32×10^6, 7.17×10^5 and 3.37×10^6 Pa for acetonitrile for the four columns L_1, L_2, S_1 and S_2, respectively. The permeabilities K^0 of the four columns are summarized in Table 2. The permeability calculated with water is higher than with acetonitrile in all cases. This indicates that some swelling of the stationary phase occurs with acetonitrile, inducing a restriction of the accessible pore volume.

The Hagen–Poiseuille's law (Eq. 3) was also used to estimate the average diameter of the monolith channels, and the determined values are presented in Table 2. The values calculated for the average through pore diameter R are about 10% higher for water than acetonitrile, confirming the presence of a solvent layer for the latter due to the polymer swelling. These results are in good agreement with the solubility parameters given for both solvents and polymethacrylate polymers according to Hildebrand equation [41]. The good linear response between backpressure and flow rate for both solvents as shown in Fig. 2 clearly demonstrates that the monoliths were mechanically stable. This linear behavior is in accordance with both Darcy's and Hagen–Poiseuille laws, which state that the pressure drop across a column is directly proportional to the viscosity of the eluent. As expected, the values indicated in Table 2 confirm that increasing the porogen ratio induced larger macropores while the total porosity and permeability increased.

Morphology of the Monolithic Columns

Morphology of the monolith is one of the key factors affecting the separation capability of the polymeric monolithic column. To obtain high efficiency, homogeneity and rigidity of the polymer bed are needed [42]. Therefore, it is important to investigate and control the morphology governing parameters during the synthesis of the monoliths. Following the investigations above, the prepared monoliths were first checked by optical microscopy then characterized with SEM.

Some optical microscope and SEM images of these monolithic columns are shown in Fig. 3. SEM pictures demonstrate that the procedure for synthesis renders permeable monoliths with a uniform structure and porosity, completely bonded to the inner capillary walls. As shown in the figures, the formed monoliths were well attached to the inner surface of the capillaries. Cross section of intact and homogeneous column bed can also be seen. The microglobules which appear in the figures have an approximate diameter in the range 1–2 μm. On the other hand, the values of the macropore diameters estimated from SEM images (Fig. 3) are in good agreement with the calculated values given in Table 2.

Optical microscopy (at magnification of 100 times) examinations revealed that the continuous beds were

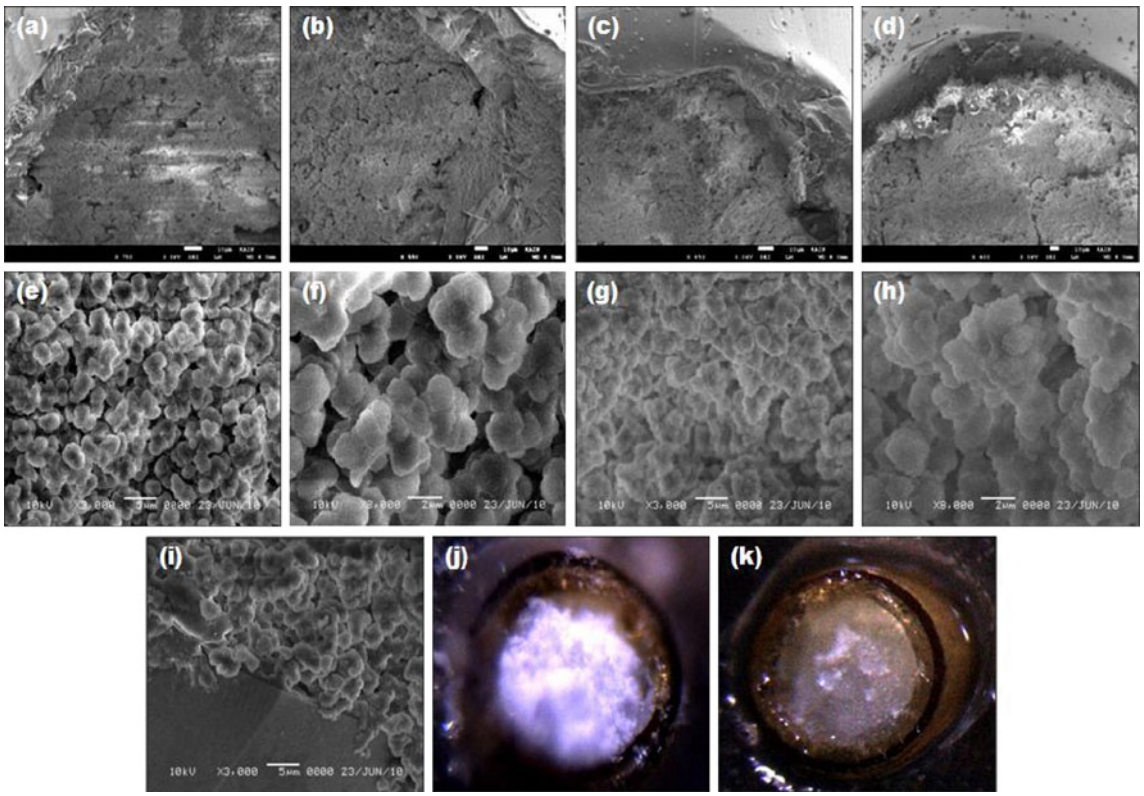

Fig. 3 **a**–**d**, **i** Cross-section SEM images of L_1, L_2, S_1, S_2 and L_1 columns, respectively. **e**, **f** bulk region SEM images of L_1 column. **g**, **h** bulk region SEM images of S_1 column. **j**, **k** optical micrographs (with magnification x100) of L_1 and S_1 columns, respectively

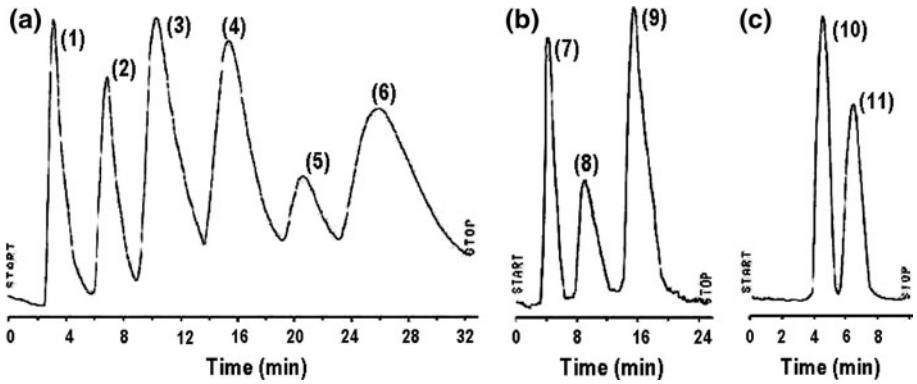

Fig. 4 Chromatograms of **a** phenol separation: *1* 4-aminophenol, *2* phenol, *3* *m*-cresol, *4* *m*-nitrophenol, *5* 4-chlorophenol and *6* 2-naphthol. **b** aromatics' separation: *7* benzene, *8* naphthalene and *9* phenanthrene. **c** drug compound separation: *10* vitamin C and *11* aspirin

homogeneous and also close to the wall. A homogeneous, opalescent continuous bed is observed if the synthesis is made properly.

Separation of Phenolic Compounds

The column efficiency is the key factor for column evaluation; different monolith materials have been already used for separation and evaluation of low-, medium- and high-molecular-weight analytes [43, 44]. The prepared capillary columns were tested for separation of a phenol mixture using a binary acetonitrile/water (40:60, *v/v*) with 1% formic acid mobile phase. As an example, Fig. 4a shows separation of the six components on column S_1 at 4 μL min^{-1} flow rate in 30 min and 254 nm detection wavelength. The plate height H was then calculated for each phenol constituent at different flow rates. For the six phenolic compounds, the height equivalent to a theoretical plate remains between 0.2 and 0.45 mm on the investigated flow rate range which was 2–7 μL min^{-1}. This fact indicates that the column performance is slightly affected by increasing the mobile phase velocity in this range, as it has been previously established for capillary columns in both liquid chromatography and gas chromatography [45–48].

As their plate number remains almost constant while increasing the flow rate, capillary columns allow faster analysis without reduction of their separation ability.

Separation of Aromatic Compounds

The prepared capillary columns were used to separate a mixture of aromatic hydrocarbons using different experimental conditions. As an example, Fig. 4b shows the chromatogram obtained with benzene, naphthalene and phenanthrene injected into column L_2 using an acetonitrile/water mobile phase (50:50, *v/v*). The 3 hydrocarbons are completely separated in 20 min at 3 μL min^{-1} flow rate and 260 nm detection wavelength. By varying mobile phase flow rate, the column efficiency was calculated for each compound; the best performance was obtained for phenanthrene at 1.5 μL min^{-1} which corresponded to a column efficiency of 8,300 plates m^{-1}. The plate number H fluctuates very slightly when flow rate is increased, meaning that the separation ability of the column is not seriously affected by using a higher mobile phase velocity. These results show that capillary monolithic columns could be an interesting and economic alternative to conventional columns for separation and determination of priority pollutants such as polycyclic aromatic hydrocarbons.

Separation of Drug Compounds

Column S_1 was tested for separation of aspirin and vitamin C which are widely used active principles and often associated in pharmaceutical preparations. The two compounds were completely separated in less than 8 min at a flow rate of 3 μL min^{-1} and 230 nm detection wavelength using 0.1% phosphoric acid as mobile phase (Fig. 4c). Application of micro-scale liquid chromatography for routine quality control of food and drugs is promising as the use of capillary columns offers several advantages over conventional packed columns.

Conclusion

Lauryl and stearyl methacrylate monolithic columns were prepared by in situ free radical polymerization in fused silica tubing using different compositions. The procedure proved to be rapid, simple and efficient; it needed only small quantities of solvents and reagents. The prepared monoliths were characterized by optical microscopy and SEM. Their porosity and permeability were also determined and compared with the morphology parameters obtained from the micrographs. The capillary columns were successfully applied for separation of different kinds of compounds such as phenols, aromatic hydrocarbons and drugs; the performance of each column was also evaluated.

Acknowledgments The Authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the Research Group No RGP-VPP-043.

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