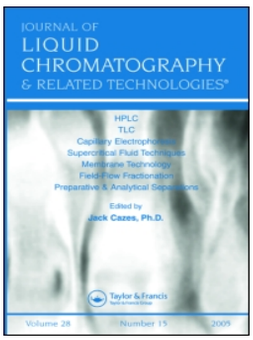

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Effect of sporopollenin microparticle incorporation into the hexyl methacrylate-based monolithic columns for capillary liquid chromatography

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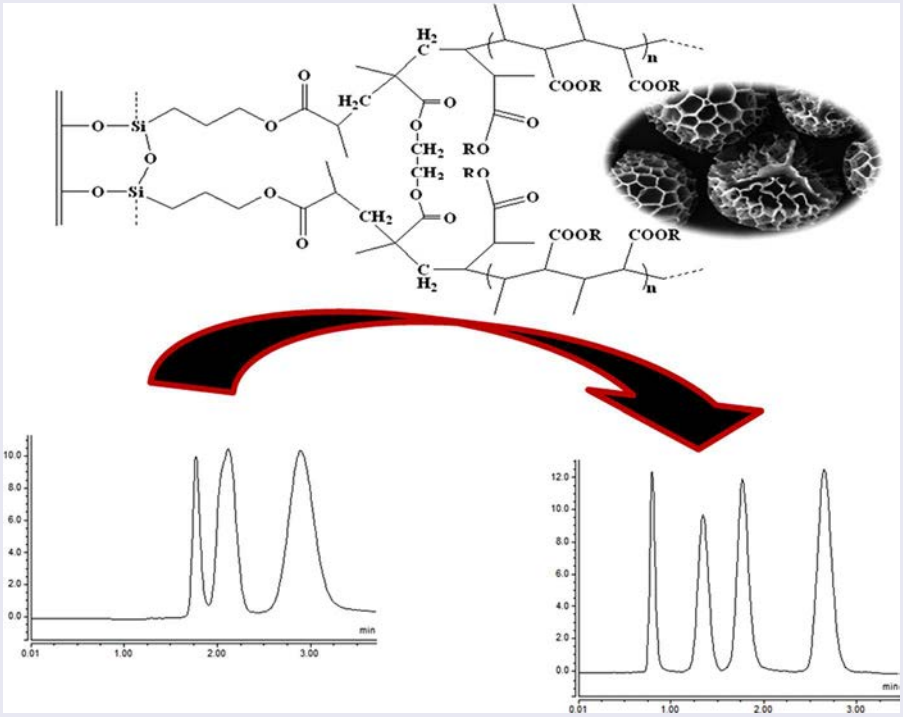
ABSTRACT

Sporopollenin microparticles have been prepared form *Lycopodium clavatum* spores, defatted and incorporated into a porous methacrylate polymer monolith to enhance liquid chromatographic performance of different sets of small neutral molecules. A stable suspension between sporopollenin microparticles and porogenic solvents composed of 1-propanol and 1,4-butandiol has proved before preparation, and seven compositions with increasing sporopollenin microparticles were prepared inside fused silica tubing. After optimizing of the preparation conditions, the structure of the stationary phase was characterized by scanning electron microscopy, surface area analysis, thermodynamic study, short- and long-term precision, and hydrodynamic properties including mechanical stability, porosity, and permeability. The columns were successfully applied to improve the separation efficiency of different mixtures using capillary liquid chromatography. Addition of very small amount of sporopollenin microparticles to the methacrylate mixture enhanced the column efficiency from 3 to 5 times for ketonic and phenolic compounds and reduced the retention with the corresponding better resolution and peak shapes for all studied compounds.

KEYWORDS

Capillary liquid chromatography; *Lycopodium clavatum* spores; polymethacrylate; porous polymer monolith; sporopollenin microparticle

GRAPHICAL ABSTRACT



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Introduction

Polymer monolithic columns are relatively new and innovative high-permeable columns for rapid chromatographic analysis. The concept of monolithic columns have been identified and rapidly become very attractive in the last few years.[1,2] In contrast to traditional particulate packed stationary phases, monoliths are highly porous rod structures made from single- and continuous piece materials, giving them favorable properties for chromatographic applications especially for separation of large molecules such as peptides and proteins.[3–9] However, bare monoliths experience from the lack of small mesopores, and hence sufficient interaction sites that are necessary to deal with the small molecules.

Today, many scientific and technological fields are becoming more complicated and facing new challenges, making the need to analyze more complex substances and to enhance the resolution and sensitivity of the analytical methods, in addition to reduce the analytical costs and time. Such needs may increase as more and more materials and products are developed. Recent development in separation science concerns with the introduction, improvement, and development of new and versatile stationary-phase materials. Great efforts were made especially in chromatographic techniques,[10–12] and several substances were reported and contributed to many significant developments in this area.

Spores and pollen grains are fine powdery substances that represent the male reproductive units of both nonflowering and flowering plants, respectively.[13–15] All spores and pollen grains possess a protective double-walled outer structure made up of a bilayer. The outer layer is called exine, and is formed from a unique polymer known as sporopollenin, while the inner layer, which is called intine consists mainly of pure cellulose and a few other polysaccharides.[14,16] To isolate the exine layer alone, the cellulose and polysaccharides could be removed by treatment with various acids and bases. Several methods have been developed for this purpose, some involve harsh treatments with strong acids and bases at relatively high temperatures, and others use gentle procedures,[17–23] each of these methods have their advantages and limitations.

Sporopollenin is a major component of the inert exine-layer material of spores and pollen grains,[14] the name used to describe one of the most known resistant biological polymers. Great efforts have been made to characterize the physical and chemical nature of sporopollenin. However, the extreme resistance of sporopollenin to chemical treatments prevented its exact chemical structure knowledge.[24,25] From the earliest theory at 1930s and till present, several theories have been established to describe sporopollenin structure. In summary, sporopollenin was initially related to polyterpenoids and then more precisely to polymer of carotenoids and carotenoid esters.[14,26,27] After that, it was believed to have lipidic- and lignin-like moieties.[28]

Some researchers suggested the possibility for the presence of two different kinds of sporopollenins; one mainly composed of phenolic constituents and the other mainly of aliphatic units. Several works also took in consideration the hypothesis of aromatic and aliphatic moieties to be linked in a common mixed monomer.[29,30] After solid-state NMR studies, the aliphatic lipidic polymer nature of sporopollenin was put forward again.[31] However, most of current research works agree that sporopollenin is a complex family of chemical-material-related polymers. Analyses have revealed a mixture of biopolymers including unbranched aliphatic compounds with variable amounts of aromatics; consisting mainly of long chain fatty acids, phenylpropanoids, phenolics, carotenoids, and carotenoid esters.[32,33]

Several studies have demonstrated the chemical and physical properties of sporopollenin microparticles; most of these investigations reveal that sporopollenin has a constant chemical structure regardless of source, and only consisting of three elements; carbon, hydrogen, and oxygen, in the absence of nitrogen and metals.[34,35] The empirical formula is of the order 3, 5, and 1 for carbon, hydrogen, and oxygen, respectively.[36,37] Many researchers described sporopollenin as one of the most known organic substances resistant to chemical and physical attack.[38,39]

Sporopollenin is extremely stable material, nonsoluble, and it does not swell in aqueous and organic solvents and even in most chemical reagents such as hot alkali and strong acid. One of the most important physical properties of pollen grains and spores is their similarity of particle size, within the same original species, as well as their high consistency and monodispersity.[40–42] Depending on their original sources, pollen grains and spores can vary in particle size starting from 1.2 μm as in the *Bacillus* or grass *subtilis* to 250 μm as in *Pumpkin Cuburbita*.[43–45] Other natural sources would provide a wide range of constant particle diameter such as *Abies* (125 μm), *Lycopodium clavatum* (25 and 40 μm), *Epicoccum* (20 μm), *Ganomerma* (5–6.5 μm), *Aspergillus niger* (4 μm), and *Penicillium* (3–5 μm).[43–45]

The sporopollenin exine layer contains a variety of functional groups allowing specific modifications; it is also perforated with nanopores that make its membrane permeable. Thus, sporopollenin can be easily functionalized using simple chemistry and cheap reagents.[46] All mentioned properties, in addition to their natural and commercial availability, would make sporopollenin microparticles good candidate and an ideal material for a wide range of applications, in our opinion as a separation media in chromatographic applications. Many researchers advised to optimize the use of sporopollenin particles for various purposes such as solid-phase synthesis, medicine and drug delivery, catalyst support, ion and ligand exchange, microencapsulation, food industry, and in cosmetics.[47–58]

Based on their amazing properties, this study aims to enhance the liquid chromatographic performance of methacrylate monolith stationary phases by incorporation with sporopollenin microparticles. This work demonstrates a reasonable procedure to prepare sporopollenin microparticles form *L. clavatum* spores, and maintain a uniform composite between both materials inside the capillary tubings. Investigation and physical properties including thermodynamic parameters of the prepared columns were completely described and discussed. The performance of the incorporated columns was evaluated and compared with the unmodified monoliths for separation of different model compounds. More

experiments and extensive efforts are soon necessary to develop functionalized sporopollenin particles, which could be used for more general and selective applications.

Experimental

Chemicals and columns

Benzene, toluene, ethylbenzene, naphthalene, 4-aminophenol, formic acid, hydrochloric acid, sodium hydroxide, 1,4-butandiol, and 1-propanol were of analytical grade and obtained from BDH (Lutterworth, UK). Phenol, m-cresol, m-nitrophenol, anthracene, propylbenzene, butylbenzene, pentylbenzene, hexylbenzene, acetophenone, and butyrophenone were purchased from Aldrich (Steinheim, Germany). *L. clavatum* spores were supplied by Fluka A.G. (Buchs, Switzerland).

High-performance liquid chromatography-(HPLC)-grade solvents, methanol, acetone, and acetonitrile were obtained from Merck KGaA (Darmstadt, Germany). Ultrapure water was produced by a Milli-Q water system (Advantage with Elix, Millipore S.A.S. 67120 Molsheim, France), then filtered through 0.20-µm nylon membrane filter Whatman (Maidstone, UK).

Fused silica capillary tubing of 320 µm of i.d. and 370 ± 40 µm of o.d. with outer coating polyimide was provided from Restek (Bellefonte, USA). 3-(trimethoxysilyl) propyl methacrylate, ethylene dimethacrylate, hexyl methacrylate, and azobisisobutyronitrile (AIBN) were obtained from Aldrich (Steinheim, Germany). All chemicals were used without further purification.

Preparation of sporopollenin

Sporopollenin particles have been prepared from *L. clavatum* as reported by Hamad et al.[59] with some modification, 10 g of spores was dried and refluxed with 75 mL of acetone for 4 hr to remove the hydrophobic layer. The defatted spores were then filtered off and treated with 75-mL (6 wt%) potassium hydroxide under reflux for 12 hr, while the solution was filtered and replaced after 6 hr. Then the mixture was filtered and washed with hot water and hot absolute ethanol. The solid residue was suspended in 75-mL (85 wt%) ortho-phosphoric acid and stirred under reflux for 5 days. Then, it was filtered again and washed with water, acetone, 2.0 mol/L hydrochloric acid, 2.0 mol/L sodium hydroxide, water, acetone, and absolute ethanol, and finally dried at 65°C under vacuum until constant weight. The prepared particles were used in the next experiments.

Columns preparation

Before the preparation, the fused-silica capillary (0.32 mm i.d.) was rinsed with acetone, 0.20 mol/L NaOH, water, 0.20 mol/L HCl, ethanol, and toluene. The capillary was then flushed with a 20% 3-(trimethoxysilyl)propyl methacrylate in toluene solution for 1 hr and made to stand with the same solution for 4 hr, then rinsed with toluene and dried with a stream of nitrogen.

The monomeric mixture comprised of 12% hexyl methacrylate, 12% ethylene dimethacrylate, and 1% AIBN initiator. The porogenic mixture was 75% of the total solution and comprised (v%) of 50% 1-propanol and 50% 1,4-butandiol. Five common solvents were tested to allow a stable suspension of the sporopollenin particles in the monomeric mixture namely, toluene, 1-propanol, 1-dodecanol, cyclohexanol, and 1,4-butandiol. The best stable mixture was obtained with a binary 1,4-butandiol and 1-propanol mixture. Seven columns with different sporopollenin particle contents have been prepared as listed in Table 1. Using the same procedure, the control monolithic column was prepared without sporopollenin particle incorporation.

Sporopollenin microparticles were added in the monomeric mixture including the porogenic solvents. While maintaining a uniform mixture, the treated capillaries were immediately filled with the mixture and both ends were sealed. The polymerization was performed in a first step at 70°C for 2 hr in water bath with sonication, and then the polymerization was completed to 15 hr in the same water bath but without sonication. After the polymerization, both seals were removed, and about 1 cm were cut from both capillary ends. The resulted columns were finally washed with acetonitrile and water to remove any porogenic solvent or unreacted material.

Characterization of the prepared columns

Hydrodynamic parameters of the synthesized monoliths including porosity and permeability were evaluated as described elsewhere.[12,60] Flow method was used to determine the total porosity ε_T of the prepared columns, as unretained material, uracil was used in this study. Based on Darcy's equation that links the solvent viscosity, pressure drop and ε_T to permeability K^0, column permeability was determined at 10 µL/min flow rate using acetonitrile as mobile phase.

After the separation experiments, the monolith rods in the columns were washed with acetonitrile and water, cut into small pieces and dried. The dried pieces and the composite stationary phase were subjected to scanning electron microscope (SEM). A Jeol (JSM-7600 F) Field Emission Scanning Electron Microscope (Tokyo, Japan) was used at typically 5 kV to examine the pore properties and microscopic morphology of the prepared columns.

Adsorption–desorption isotherm of liquid nitrogen was used for calculation and comparison of the surface areas of the stationary-phase materials using a Gemini VII 2390 surface area analyzer (Micromeritics, Norcross, Georgia, USA) at −196°C. For this purpose, stationary-phase samples

Table 1. Sporopollenin microparticle contents of the prepared capillary columns. Porosities ε_T (using uracil as unretained material) and permeabilities K^0 (using acetonitrile as mobile phase at a flow rate of 10 µL/min).

Column	Sporopollenin (mg/mL)	Sporopollenin (%)	ε_T	K^0 (m^2
C_1	0.00	0.00	0.91	1.32×10^{-13}
C_2	0.50	0.05	0.86	9.96×10^{-14}
C_3	0.75	0.08	0.84	9.76×10^{-14}
C_4	1.00	0.10	0.83	9.63×10^{-14}
C_5	1.50	0.15	0.85	1.03×10^{-13}
C_6	2.00	0.20	0.87	1.09×10^{-13}
C_7	3.00	0.30	0.89	1.17×10^{-13}

corresponding to control column (C_1) and 0.1% sporopollenin-added column (C_4) were grinded and degassed at 300°C before the measurements.

HPLC conditions

All separations and evaluations were performed on a Dionex Ultimate 3000 HPLC system (Sunnyvale, CA, USA) equipped with a 45-nL detector cell volume. The detector was set at different nanometers according to the analyzed compound type. In all cases, fixed volume was injected at 30 nL. Water and acetonitrile solvents with/without formic acid additives were used as the mobile phase. Before use, all solutions were filtered through 0.20-µm nylon membrane filter (Whatman, England). The column temperature has been changed in some cases to improve the separation efficiency.

Calculation of the thermodynamic parameters

Standard partial molar enthalpy change of transfer (ΔH, kJ/mol), standard partial molar entropy change of transfer (ΔS, J/mol/K), and Gibbs free energy change of transfer (ΔG, kJ/mol) were measured to evaluate the thermodynamics for the transfer of the solutes from the mobile-phase solvents to the sporopollenin-incorporated monolith stationary phase.[61,62] Liquid chromatography experiments were performed over five different temperatures in the range of 30–70°C. Then, the van't Hoff equations (1) and (2) were used to calculate ΔH, ΔS, and ΔG:

$$\ln k' = -\Delta H/(\mathrm{RT}) + \Delta S/R + \ln \Phi, \quad (1)$$

$$\Delta G = \Delta H - T\,\Delta S, \quad (2)$$

where, k' is the retention factor, R is the gas constant, T is absolute temperature, and Φ is the phase ratio.

Equation (3) was used to calculate the retention factor k', where t is the retention time and t_0 is the column void time. The void time was determined by injecting a small plug of uracil that is unretained material in this case,

$$k' = (t - t_0)/t_0. \quad (3)$$

Equation (4) was used to calculate the phase ratio Φ (that is the volume of stationary phase divided by the volume of mobile phase), where V_s is the volume of the stationary phase in the column, which is equal to the void volume V_0 subtracted from the geometrical column volume V_{Col} as in Eq. (5), on the other hand, V_0 is the void volume of the column (the volume of mobile phase within the column), which was evaluated according to Eq. (6), where F is the flow rate of the mobile phase.

$$\Phi = V_s/V_0. \quad (4)$$

$$V_s = V_{Col} - V_0. \quad (5)$$

$$V_0 = t_0 \times F. \quad (6)$$

Results and discussion

Preparation and optimization of the columns

Seven capillary columns have been prepared by incorporation of increasing sporopollenin microparticles contents as summarized in Table 1. The composition percentages of the monomeric mixture other than sporopollenin microparticles were fixed for the seven columns as mentioned in the experimental section. The time of the polymerization was set at 15 hr corresponded to the maximum monomeric mixture conversion[5] within two stages; for the first 2 hr in water bath with sonication at 70°C to maintain a uniform suspension inside each column.

To select the most suitable incorporation mixture, five solvents were tested. The best medium should allow a good adjustment between sporopollenin particle suspension (to ensure uniform matrix) and a good solubility of monomeric mixture. The most stable mixture was maintained using binary porogenic solvents composed of 1-propanol and 1,4-butandiol (50/50%, v/v). Under these conditions, the sporopollenin microparticles were well dispersed inside the capillaries, and the mixture was homogenous with no precipitation for about 2 hr after mixing, as it can be seen in Figure 1a, which confirmed with different sporopollenin particle contents.

The content of sporopollenin microparticles was set based on the determination of backpressures for each column using acetonitrile as eluent at different flow rates, as depicted in Figure 2a for C_1, C_4, and C_7 columns. Various experiments showed that the columns prepared with sporopollenin microparticle content above 0.3% were not stable, because of the corresponding monolith content was not sufficient to maintain a good stability of the prepared phase, while the prepared compositions inside the columns were stable in the range 0–0.3%.

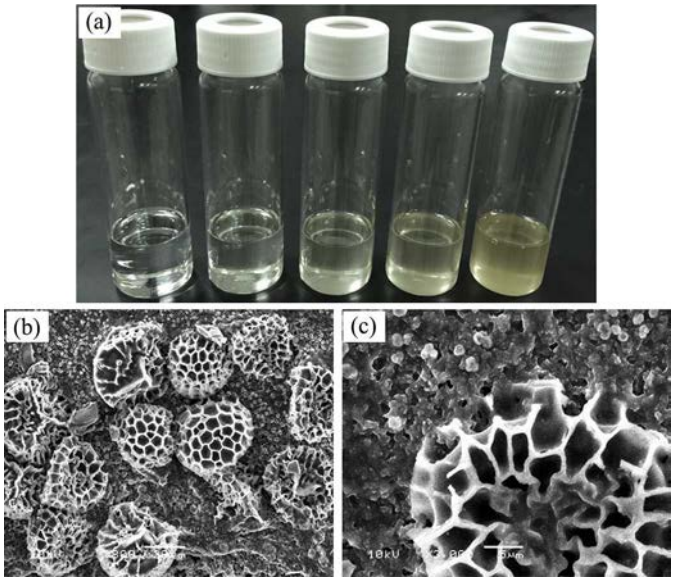

Figure 1. (a) Porogenic mixture (from left to right) without sporopollenin particles, with incorporated 0.1, 0.25, 0.5, and 1.0 mg/mL sporopollenin particles, respectively, (b) SEM image of *Lycopodium clavatum* sporopollenin produced after treatment and defatting, and (c) SEM micrograph of one sporopollenin particle incorporated with the bulk polymer monolith inside C_4 column. *Note*: SEM, scanning electron microscope.

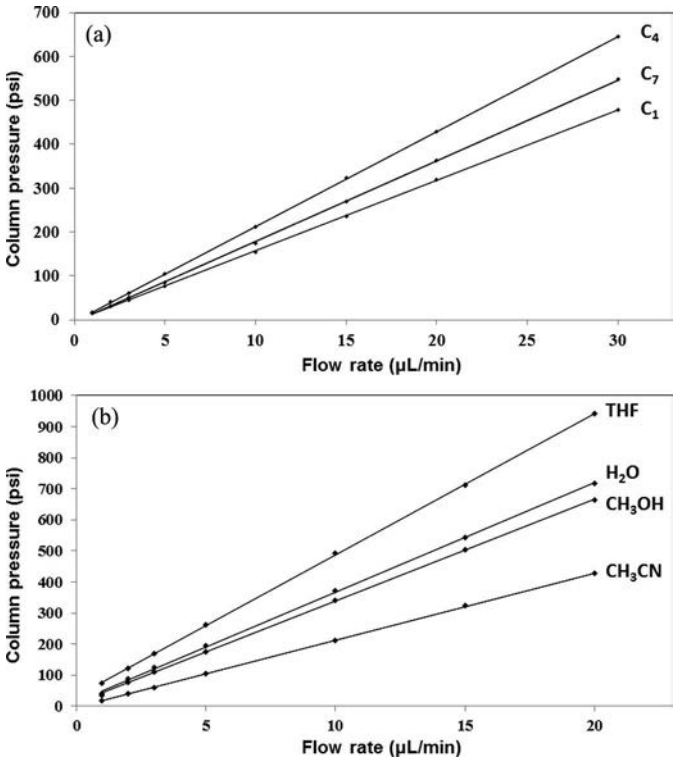

Figure 2. (a) Pressure drop (psi) versus flow rate (µL/min) of acetonitrile as eluent plots for three of the prepared columns C_1, C_4, and C_7, temperature, 24°C and (b) mechanical stability of C_4 column using acetonitrile, methanol, water, and THF as eluents, temperature, 24°C.

Based on these results, sporopollenin microparticle content was set in the range of 0.5 and 3.0 mg/mL (0.05–0.3%). As a control column, C_1 has been prepared with the same monomeric composition but without incorporated particles.

Sporopollenin and column characterization

Column stability and permeability have been evaluated using various eluents; the influence of acetonitrile, methanol, water, and tetrahydrofuran (THF) flow rate on the column backpressure was investigated. Figure 2a and b shows this effect through C_1, C_4, and C_7 columns. A linear relationship between column backpressure and each eluent flow rate indicated by a regression factor of about 0.999 for all measured curves proves column stability.

It can be observed that increasing the sporopollenin particle content induced first a higher backpressure from C_1 to C_4. On the other hand, although C_6 and C_7 columns have a higher sporopollenin particle content of 0.2 and 0.3%, respectively, they exhibit a lower backpressure in all the flow rate range. This could be explained by the relatively large size of the incorporated sporopollenin particles that induces a higher interparticle void volume. The same effect is observed in Table 1, where increasing the sporopollenin particle content corresponds to a decrease in porosity from C_1 to C_4, then an increase for C_6 and C_7.

All columns exhibit backpressure less than 20 psi and up to about 650 psi at 1.0 and 30 µL/min acetonitrile flow rates, respectively. In comparison with previously reported works, incorporation of sporopollenin microparticles into polymer monoliths showed significantly lower backpressures than monolithic columns incorporated with carbon nanotubes.[12,63]

The permeability values of the prepared columns were also determined using acetonitrile eluent at 10 µL/min flow rate. The pressure drops were measured, and the permeability values were calculated for the seven columns, all measured values are summarized in Table 1. The determined values show that the larger average pore sizes exhibited higher permeabilities. As shown in Figure 2a and b, the good linear relationship between all column backpressure and eluent flow rate proves that the prepared composites were mechanically stable.

The total porosity values of the prepared columns were calculated using uracil as unretained marker and composition of water/acetonitrile (50/50, v/v) mobile phase. As shown in Table 1, the total porosity values of the prepared columns ranged from 83% for column C_4 corresponding to 0.1% sporopollenin particle content to 91% for column C_1 that had no sporopollenin particles. This trend confirms the significant influence of an even small content of sporopollenin microparticles on the column porosities.

On the other hand, the rigidity and homogeneity of the prepared composite are very important to obtain high-efficiency columns. Therefore, the morphology and surface property of the composites were investigated using optical microscopy, SEM, and specific surface area. SEM images of sporopollenin microparticles are shown in Figure 1b and c, both figures represent sporopollenin particles after treatment, in which the hollow and the scars are apparent, while the inner layers present in the untreated spores were completely removed. The figures show also that the produced sporopollenin microparticles remained intact and the original morphology of the untreated spores did not alter after the purification method. However, some spores had become distorted or slightly burst, this could be probably explained by the high vacuum encountered in the SEM itself. Figure 1c, corresponding to the bulk composite sample prepared inside column C_4, confirms that the sporopollenin structure remained intact and does not damaged after the polymerization with the monomeric mixture. Moreover, it can be clearly seen that the average diameter of the sporopollenin microparticles is about 36 µm.

The specific surface area of the stationary phase corresponding to columns C_1 and C_4 was determined using liquid nitrogen physisorption based on Brunauer, Emmett and Teller (BET) method. The specific surface area was significantly increased 51.01% by incorporation of only 0.1% sporopollenin; the obtained values were 4.950 and 10.104 m^2/g for C_1 and C_4, respectively. These results confirmed that the incorporation of sporopollenin microparticles into the monomeric mixture induced a significant increase in its specific surface area.

Separation and column efficiency

To monitor any change in the chromatographic properties of monolithic columns after incorporation of the defatted sporopollenin microparticles, we selected hexyl methacrylate that is relatively nonpolar monomer. Several parameters were investigated, and model analytes were selected regarding to their

Table 2. Separation parameters and column efficiency evaluation expressed in terms of t_R, N, and R_s.

	C_1 column			C_4 column			
	t_R (min)	N	R_s	t_R (min)	N	R_s	N_{C4}/N_{C1}
Phenols							
4-Aminophenol	1.77	9,200	—	0.87	7,700	—	0.84
Phenol	2.13	—	—	1.40	4,900	2.86	—
m-Cresol	2.20	—	—	1.81	3,400	1.49	—
m-Nitrophenol	7.53	1,100	2.47	2.69	5,300	3.33	4.82
Ketones							
Acetone	1.28	3,600	—	0.89	13,400	—	3.72
Acetophenone	1.42	1,600	0.65	1.07	7,500	1.61	4.69
Butyrophenone	1.88	1,600	0.71	1.41	4,900	1.92	3.06
Aromatics							
Benzene	1.29	4,600	—	1.05	13,100	—	2.85
Naphthalene	1.63	2,400	0.82	1.23	6,200	1.52	2.58
Anthracene	2.04	1,400	0.98	1.68	3,000	1.74	2.14
Alkylbenzenes							
At 40°C							
Toluene	2.23	7,600	0.99	2.01	8,500	1.28	1.12
Ethylbenzene	2.82	6,000	1.18	2.55	6,900	1.44	1.15
Propylbenzene	3.48	4,300	1.26	3.19	5,200	1.45	1.21
At 50°C							
Toluene	2.04	8,000	1.10	1.86	11,200	1.41	1.40
Ethylbenzene	2.45	7,700	1.24	2.24	9,100	1.52	1.18
Propylbenzene	3.07	5,600	1.42	2.84	6,800	1.63	1.21
At 60°C							
Toluene	1.87	12,100	1.13	1.72	14,000	1.44	1.16
Ethylbenzene	2.21	9,700	1.26	2.04	11,400	1.55	1.18
Propylbenzene	2.72	7,000	1.54	2.53	8,700	1.76	1.24
At 70°C							
Toluene	1.74	14,400	1.27	1.61	17,000	1.50	1.18
Ethylbenzene	2.02	11,900	1.45	1.87	14,100	1.68	1.18
Propylbenzene	2.44	8,800	1.68	2.28	11,100	1.89	1.26

polarity; mixtures of phenolic, ketonic, aromatic, and alkylbenzene compounds were tested to check and evaluate the effect of sporopollenin incorporation. The sample injection volume used was 30 nL in all cases, while the obtained results are presented in Table 2.

The prepared capillary columns were tested for separation mixture of four phenolic compounds using different experimental conditions. Figure 3a shows the separation of aminophenol, phenol, cresol, and nitrophenol using C_1 (left) and C_4 (right) columns using a binary mobile-phase mixture; acetonitrile/water (30:70, v/v) with 0.1% formic acid at 15 µL/min flow rate and a detection wavelength of 260 nm at 70°C column temperature. While incomplete separation was obtained for phenol and cresol using unmodified column, the C_4 column (corresponding to 0.1% sporopollenin content) allowed a full separation of all phenolic compounds in a run time shorter by about 1.0 min. Addition of 0.1% sporopollenin to the monomeric solution improved all peak shapes and enhanced the column efficiency five times calculated for the retained nitrophenol peak.

Figure 3b illustrates the separation of three ketonic compounds (acetone, acetophenone, and butyrophenone) at a flow rate of 15 µL/min and 280-nm detection wavelength using mixture of (45:55, v/v) acetonitrile/water mobile phase and 0.1% formic acid additive. Unmodified monolithic column affords only 3,600 plates/m for acetone, a column efficiency of 13,400 plates/m achieved at a content of only 0.1% sporopollenin determined at a flow rate of 15 µL/min. Again, incorporation of sporopollenin microparticles into a monolith

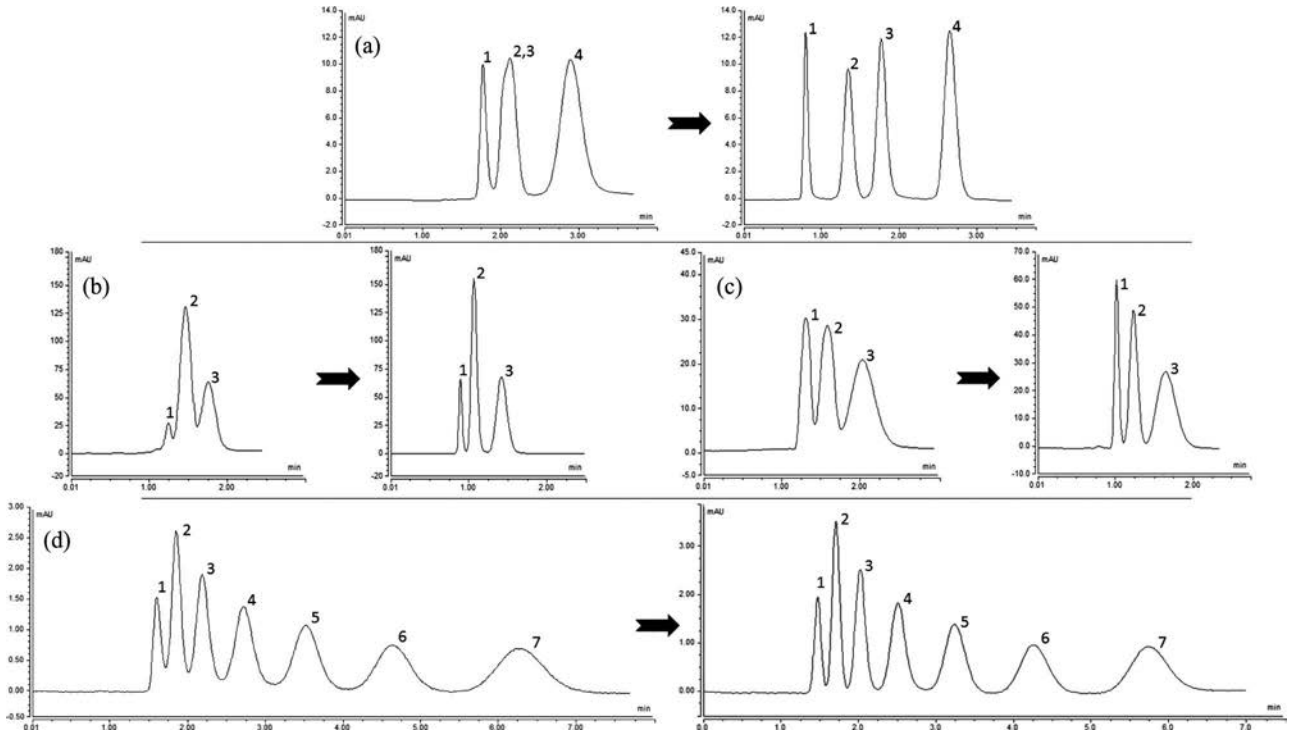

Figure 3. Separation using control column C_1 (left) and one of the 0.1% sporopollenin incorporated column (right) of: (a) phenolic compounds using a binary mobile-phase composition; acetonitrile/water (30:70, v/v) with 0.1% formic acid at 15 µL/min, where: (1) 4-aminophenol, (2) phenol, (3) m-cresol, and (4) m-nitrophenol, (b) ketones using acetonitrile/water (45:55, v/v) with 0.1% formic acid mobile phase at 15 µL/min, where: (1) acetone, (2) acetophenone, and (3) butyrophenone, (c) aromatic hydrocarbons using acetonitrile/water (60:40, v/v) mobile phase at 15 µL/min, where: (1) benzene, (2) naphthalene, and (3) anthracene, and (d) alkylbenzenes using acetonitrile/water (50:50, v/v) mobile phase at 13 µL/min, where: (1) benzene, (2) toluene, (3) ethylbenzene, (4) propylbenzene, (5) butylbenzene, (6) pentylbenzene, and (7) hexylbenzene.

results in an increase in both resolution and efficiency for the three studied ketones.

The efficiency of the prepared columns was measured for each injected compound at different flow rates ranged between 5 and 50 µL/min. Incorporating sporopollenin microparticles into the monolithic polymer obviously exhibited a notable increasing of the column efficiency for both phenolic and ketonic compounds by enhancement of the plate numbers by factors between 3 and 5. This note could be observed by comparing the number of theoretical plates between C_1 and C_4 columns, as it can be seen in Table 2.

Comparison of the (left) and (right) chromatograms in Figure 3a and b obviously shows a marked influence on their resolution. The results summarized in Table 2 indicate that the resolution increases by factors between 1.3 and 2.7 for both groups of analytes. On the other side, the fastest runs were achieved in approximately 1.45 min for phenolic and in less than 1.0 min for ketonic compounds using the C_4 column at 50 µL/min flow rate. Asymmetric parameter was in the range between 0.94 and 1.12 for all studied peaks.

The prepared columns were finally checked for separation of three aromatic and seven alkylbenzene compounds. As an example, Figure 3c and d shows the separation of the two groups on C_1 (left) and C_4 (right) columns using different experimental conditions and at various temperatures as in Figure 4a. The results indicated that the peak shapes have been improved, and the resolution increased about 1.8 times for the three aromatics and more than 1.2 times for the first four eluted alkylbenzenes (benzene, toluene, ethylbenzene, and propylbenzene).

As noted with the phenolic and ketonic analytes, the retention time of both aromatic and alkylbenzene compounds has

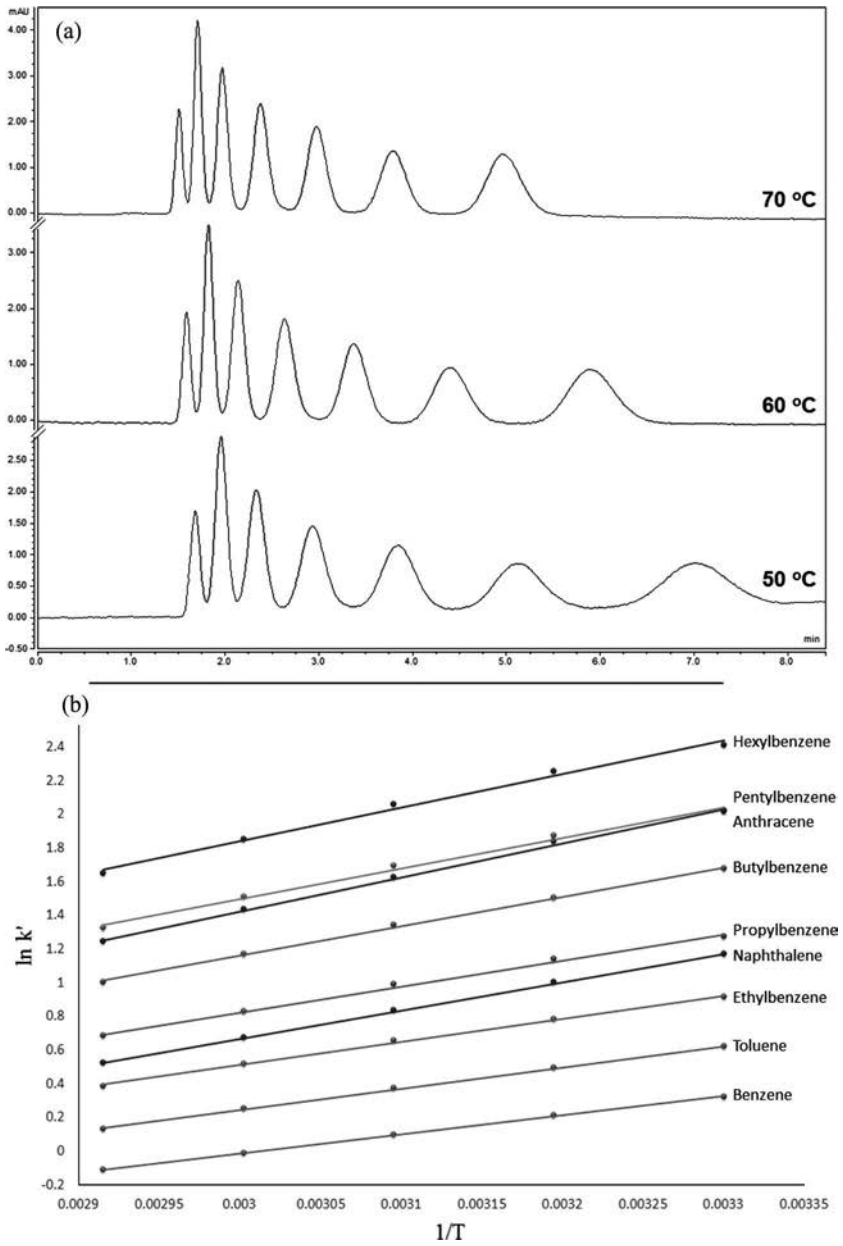

Figure 4. (a) Separation of alkylbenzenes using a binary mobile-phase composition; acetonitrile/water (50:50, v/v) at a flow rate of 13 µL/min and different column temperatures and (b) van't Hoff plots for alkylbenzenes as well as benzene, naphthalene, and anthracene on C_4 column.

been reduced by about 1 min using C_4, the column efficiency and resolution are not greatly affected by the incorporation of sporopollenin microparticles to the polymer monolith. This could be explained by the defatted sporopollenin microparticles' hydrophilic interaction character, which do not well retain the hydrocarbon compounds, while the decrease in retention time should be due to the lower content of hexyl methacrylate and ethylene dimethacrylate monomers.

In comparison with carbon nanotubes (single- and multi-walled) and metal organic frameworks, which have been recently incorporated into the monolithic materials and studied for separation of various analytes,[12,63–66] only sporopollenin-polymer monolith composite showed advantage in terms of decreasing retentions of the separated solutes.

Thermodynamics of separation on prepared columns

To investigate the thermodynamics of column separation, a series of hydrocarbon compounds including benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, pentylbenzene, hexylbenzene, naphthalene, and anthracene were separated using C_4 column (corresponds to 0.1% sporopollenin incorporated methacrylate monolithic column) in the temperature range between 30 and 70°C.

As shown in Figure 4a, the retention time of all analyte separation decreased as the temperature increased, which indicates an exothermic separation process. van't Hoff plots shown in Figure 4b of the examined compounds show a linear relation between ln k' and $1/T$ over all studied temperatures, indicating that no change in separation mechanism. On the other hand, the negative values of ΔG indicating a spontaneous separation process, in which all solutes distributed from mobile phase to stationary phase. More negative ΔG values provides an evidence for stronger retained analytes; this could be explained as the alkyl chain becomes longer and the multiplicity of aromatic rings gets bulkier. The estimated values of ΔH, ΔS, and ΔG are listed in Table 3.

Column repeatability and reproducibility

Qualitative and quantitative investigations have been evaluated by the repeatability (short-term precision) and reproducibility (long-term precision) of column preparation. Repeatability and reproducibility were assessed through the relative standard deviation (RSD) of some selected parameters for the four phenolic compounds, C_4 column was again selected as model column. The chromatographic conditions were acetonitrile/water (30:70, v/v) with 0.1% formic acid additive as mobile phase at a flow rate of 15 µL/min.

The repeatability values evaluated by injecting the phenolic compounds on the same column, t_R, H, and R_s were measured and expressed in terms of RSD, run-to-run and day-to-day results were less than 2.2 and 3.8% ($n = 5$), respectively. The reproducibility results on the other hand were expressed in terms of RSD values for ε_T and K^0; column-to-column preparation from the same batch of polymerization mixture, and batch-to-batch preparation of C_4 column were 5.2 and 13.6% ($n = 3$), respectively. The complete results are listed in Table 4.

Table 3. Values of ΔH, ΔS, ΔG, and R^2 (linear correlation coefficient) of the van't Hoff plot for separation of alkylbenzenes as well as benzene, naphthalene, and anthracene using acetonitrile/water (50:50, v/v) as mobile phase, on C_4 column at a flow rate of 13 µL/min.

Analyte	ΔH (kJ/mol)	ΔS (J/mol/K)	ΔG (kJ/mol)	R^2
Toluene	−10.53	26.27	−19.00	0.9985
Ethylbenzene	−11.43	28.59	−20.65	0.9977
Propylbenzene	−12.86	31.33	−22.95	0.9971
Butylbenzene	−14.54	34.30	−25.59	0.9986
Pentylbenzene	−15.15	37.15	−27.12	0.9943
Hexylbenzene	−16.57	40.15	−29.51	0.9935
Benzene	−9.45	24.01	−17.18	0.9993
Naphthalene	−13.95	30.14	−23.66	0.9998
Anthracene	−16.83	36.72	−28.66	0.9978

Table 4. Repeatability and reproducibility of C_4 column preparation expressed in terms of RSD on t_R, H, R_s, ε_T, and K^0 for the phenolic analytes.

Repeatability (%RSD)		
Parameter	Run-to-run ($n = 5$)	Day-to-day ($n = 5$)
4-Aminophenol (a)		
t_R	0.1	0.5
H	0.4	0.8
Phenol (b)		
t_R	0.3	0.6
H	0.4	0.8
$R_{s\ (a–b)}$	2.2	2.7
m-Cresol (c)		
t_R	0.3	0.3
H	1.0	3.7
$R_{s\ (b–c)}$	1.0	2.1
m-Nitrophenol (d)		
t_R	0.4	0.5
H	1.2	3.8
$R_{s\ (c–d)}$	1.2	2.2
Reproducibility (%RSD)		
Parameter	Column-to-column ($n = 3$)	Batch-to-batch ($n = 3$)
ε_T	5.2	13.6
K^0	3.4	12.6

RSD, relative standard deviation.

Conclusions

This study demonstrates high potentials for using sporopollenin microparticles as stationary phase in capillary liquid chromatography. In comparison with the unmodified monolithic column, we conclude that the incorporation of very small amount of sporopollenin microparticles exhibiting an improvement of both columns' efficiency and resolution relative to the column made without sporopollenin. The performance of the modified monolithic columns enhanced from 3 to 5 times for ketonic and phenolic compounds. In summary, the presence of sporopollenin microparticles showed a noticeable decreasing in the run time for all studied compounds and an enhanced efficiency for separation of polar solutes.

Until this time, the chemistry of sporopollenin is not well known. However, it is amazing and intrinsic chemical and physical characteristics such as unusual stability, large surface area, constant particle size within the same species and a variety of functional groups that allowing specific modifications make it a strong candidate to enter the world of chromatography. This work should not stop at this point, various trends could contribute to open up new aspects in the field of liquid and even gas chromatography; starting from using

sporopollenin from different sources and species and not ending by functionalization of the sporopollenin surface for specific separations.

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