Chromatographia (2015) 78:481–486
DOI 10.1007/s10337-015-2861-7

ORIGINAL

Sporopollenin Microparticle-Based Monolithic Capillary Columns for Liquid Chromatography

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Received: 30 October 2014 / Revised: 7 January 2015 / Accepted: 2 February 2015 / Published online: 17 February 2015

Abstract In this study, sporopollenin microparticles were used as the stationary phase in chromatography for the first time. Sporopollenin microparticles were incorporated into a mixture containing hexyl methacrylate and ethylene dimethacrylate monoliths to enhance the liquid chromatographic separation of different molecules. Five columns with increasing amounts of sporopollenin, from 0 to 3.0 mg mL^{-1}, were prepared by thermal polymerization in 0.32 mm i.d. × 150 mm fused silica tubing. The morphology of the columns was thoroughly characterized by optical and scanning electron microscopy. The chromatographic properties were investigated and the columns were successfully applied to assess the separation efficiency of different mixtures. The results showed that incorporation of sporopollenin microparticles reduced the retention and enhanced the column efficiency by a factor of up to 5. This effect also corresponded to an improved resolution and a full separation of the solutes.

Keywords Capillary liquid chromatography · Sporopollenin microparticles · Organic monolithic polymer · Hexyl methacrylate · Ethylene dimethacrylate

Introduction

Monolithic columns represent a relatively new and innovative type of column for rapid chromatographic analysis [1]. In contrast to traditional columns and stationary phases, monoliths are formed from a block of continuous materials made of highly porous rods with two types of pore structure (macropores and mesopores), giving them favorable properties for chromatography [2–4].

Pollen and spores are the male reproductive particles of flowering and non-flowering plants, respectively [5, 6]. Pollen and spores possess a protective double-walled outer coating, the inner layer (called intine) of which consists mainly of pure cellulose, while the outer layer (called exine) is formed from a unique polymer known as sporopollenin [7]. The inner cellulose layer can be removed to isolate the exine by treatment with acids and bases [8, 9].

The name sporopollenin is used to describe the resistance of the inert exine material of pollens and spores [10]. The exact chemical structure of sporopollenin has not been fully defined, because of its extreme resistance to most chemicals [11]. Most current researchers agree that sporopollenin is a complex of biopolymers consisting of unbranched aliphatics with a variable amount of aromatics and containing mainly phenylpropanoids, phenolics, carotenoids, carotenoid esters, and long chain fatty acids [12].

Sporopollenin is extremely stable, insoluble, and does not swell in organic and aqueous solvents. One of the most important physical characteristics of spores and pollen grains is their consistency of size, within one species [13]. Depending upon their origin, spores and pollen grains can vary widely in size from approximately 1.2 µm as in *Bacillus subtilis* to 250 µm as in *Pumpkin cucurbita* [14, 15]. Sporopollenin obtained from *Lycopodium clavatum*, which is used in this work, is a natural polymer that has a constant particle size of about 25 µm [14, 15].

All of these properties in addition to its commercial availability should make sporopollenin an ideal natural material for a wide range of applications, including as a

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separation medium for chromatography. To the best of our knowledge, the application of sporopollenin microparticles as a separation material has not been reported in the literature.

Experimental

Chemicals and Columns

Formic acid, acetone, benzene, and naphthalene were of analytical grade and purchased from BDH (Lutterworth, UK). Acetophenone, butyrophenone, and anthracene were obtained from Aldrich (Steinheim, Germany). *L. clavatum* spores with 25 μm particle size mesh were supplied by Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were obtained from BDH (Lutterworth, UK).

HPLC-grade acetonitrile was obtained from BDH (Lutterworth, UK). Purified water used throughout all experiments was prepared on a Milli-Q system (Advantage with Elix, Millipore S.A.S. 67120 Molsheim, France), then filtered with 0.2 μm nylon membrane filters (Whatman, Maidstone, UK).

Fused silica tubing (0.32 mm i.d.) was purchased from Restek (Bellefonte, USA). The following chemicals used for the preparation of the monolithic materials were obtained from Aldrich (Steinheim, Germany): 3-(trimethoxysilyl)propyl methacrylate 98 %, ethylene dimethacrylate 98 % used as crosslinker, hexyl methacrylate 98 % as monomer, and 2,2′-azobisisobutyronitrile (AIBN) as thermal initiator. Toluene, hydrochloric acid, sodium hydroxide, 1-propanol, and 1,4-butandiol were acquired from BDH (Lutterworth, UK). All chemicals were used without further purification.

Preparation of Sporopollenin

Dry spores were refluxed with acetone for 4 h to remove the hydrophobic layer. Then the defatted spores were filtered and treated with KOH under reflux for 12 h, then filtered and washed with hot water and absolute ethanol. The solid residue was suspended in H_3PO_4 and stirred under reflux for 7 days. Then, it was filtered again and washed with water, acetone, 2 M HCl, 2 M NaOH, and absolute ethanol and dried at 60 °C under vacuum until constant weight. The resulting particles were used in the next experiments.

Preparation of Capillary Monolithic Columns

The inner surface of the fused-silica capillaries (150 × 0.32 mm i.d.) were activated with a 20 % 3-(trimethoxysilyl)propyl methacrylate in toluene solution. The monomer mixture was prepared from 12 % hexyl methacrylate, 12 % ethylene dimethacrylate, and 1 % AIBN as initiator (all v %). The porogenic mixture comprised 75 % of the total solution volume and was prepared as a 50:50 (v/v) mixture of 1-propanol and 1,4-butandiol. Sporopollenin particles were suspended in the porogenic solvents and mixed with the monomer mixture into a homogenous solution. While maintaining a uniform reaction mixture, we immediately filled the capillary columns with the reactant solution and the polymerization was performed at 70 °C for 15 h.

After the polymerization, the resulting columns were washed with acetonitrile and water to remove any unreacted materials and porogenic solvents. Five columns (C_1–C_5) were prepared with different sporopollenin particle contents ranging from 0 to 3.0 mg mL^{-1}; these corresponded to 0.0, 0.05, 0.1, 0.2, and 0.3 % content of sporopollenin particles, respectively. The column without sporopollenin particles was used as a control.

Characterization of Prepared Columns

The porosity and permeability of the synthesized monoliths were characterized as described in previous studies [3, 16]. The flow method was used to evaluate the total column porosity and, in this study, uracil was used as an unretained marker. On the basis of Darcy's equation, the permeability was calculated using acetonitrile as the mobile phase at 10 μL min^{-1}.

After chromatographic experiments were performed, the monolith rods in the tubes were washed and cut into small pieces then dried. The dried columns and monolith materials were subjected to optical microscopy and scanning electron microscopy (SEM). The optical microscope images were obtained using a Micromaster Fisher Scientific optical microscope (G2009-A 702-042, Shanghai, China) typically with 100-fold magnification. For optical microscopy imaging, a piece of the monolith was extruded from the capillary column and examined. The pore properties and microscopic morphology of the prepared columns were examined by a Jeol (JSM-7600F) field emission scanning electron microscope (Tokyo, Japan) at 5 kV.

HPLC Conditions

All chromatographic analyses were performed on a Shimadzu HPLC system (Kyoto, Japan) including a pump (LC-20AD), Valco automatic injector (EHMA) fixed at 4 nL, and a UV detector (SPD-M20A). The HPLC system was equipped with a micro flow cell corresponding to a 3-mm path length and 210-nL volume. The detector was set at different wavelengths according to the type of analyzed compounds. Acetonitrile/water solutions with or

without acid additives at different ratios were used as the mobile phase. All solutions were filtered through 0.2 μm nylon membrane filters (Whatman, England) prior to use. All experiments were carried out at room temperature.

Results and Discussion

Preparation and Optimization of Monolithic Columns

Five monolithic columns were prepared by incorporation of increasing amounts of sporopollenin particles from 0.05 to 0.3 mg mL^{-1} as mentioned in the "Preparation of Capillary Monolithic Columns" section.

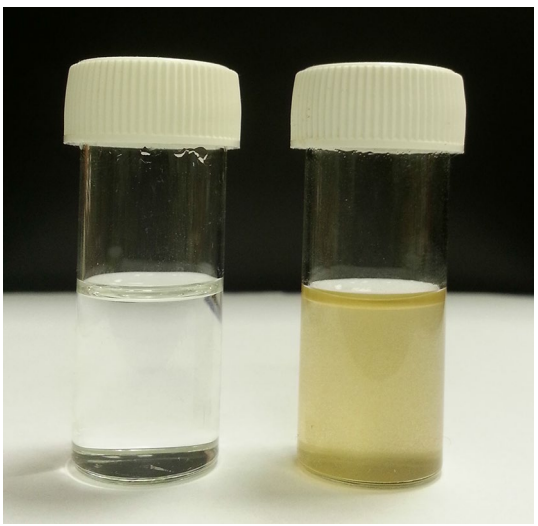

Fig. 1 Porogenic mixture without sporopollenin particles (*left*) and with 1.0 mg mL^{-1} sporopollenin particles (*right*)

In order to maintain a uniform polymer matrix and good solubility of the monomeric mixture, the selected porogenic solvent was a binary solution composed of 1-propanol and 1,4-butandiol (50:50, v/v). By using this binary mixture, the sporopollenin particles were well dispersed and the suspension was homogenous and stable for about 2 h after mixing, as can be seen in Fig. 1. This situation was then confirmed inside the capillary columns by optical microscopy.

The composition range of sporopollenin particles was set on the basis of back pressures determined for each column using acetonitrile as the eluent at different flow rates, as depicted in Fig. 2. These experiments showed that with a sporopollenin content above 3.0 mg mL^{-1}, the corresponding monolith content was not sufficient to ensure good stability of the prepared material; however, it was stable in the range 0.05–0.3 %.

Sporopollenin and Columns Characterization

Figure 2 evaluates the mechanical stability and permeability of the monolithic materials. An excellent linear dependence of inlet pressure versus the flow rate was indicated by a regression factor *R* better than 0.999 for all measured curves. All prepared columns exhibit back pressure less than 20 psi at 1 μL min^{-1} up to less than 650 psi at 30 μL min^{-1} acetonitrile flow rate. In comparison with carbon nanotube columns, incorporation of sporopollenin particles resulted in smaller increases in column back pressures [16, 17].

For determination of the total porosity of all prepared monolithic columns, uracil was injected as a non-retained marker with acetonitrile/water (50:50, v/v) as the mobile phase. It was found that the total porosity of the prepared

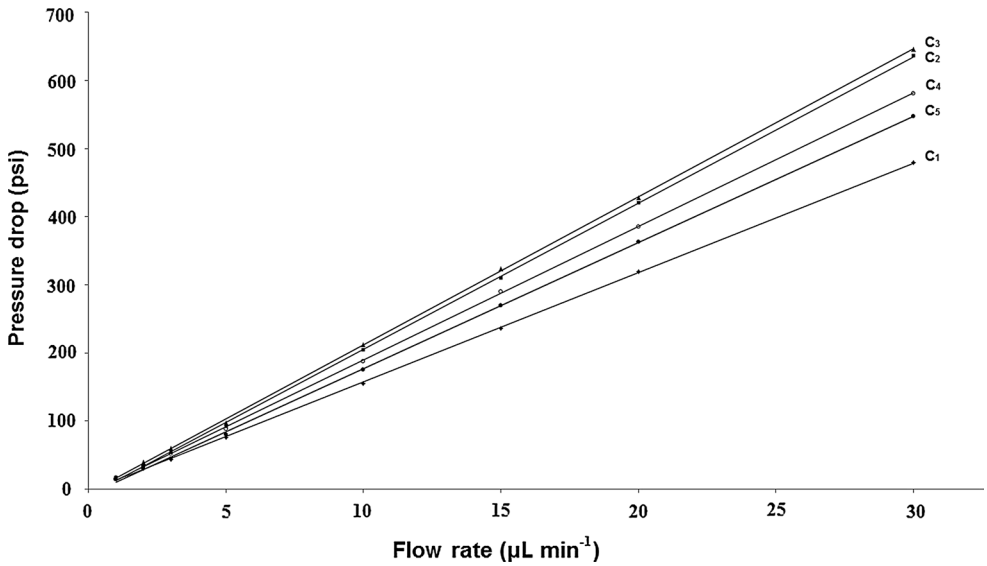

Fig. 2 Plots of pressure drop (psi) versus flow velocity (μL min^{-1}) for each column using acetonitrile as mobile phase, temp. 24 °C

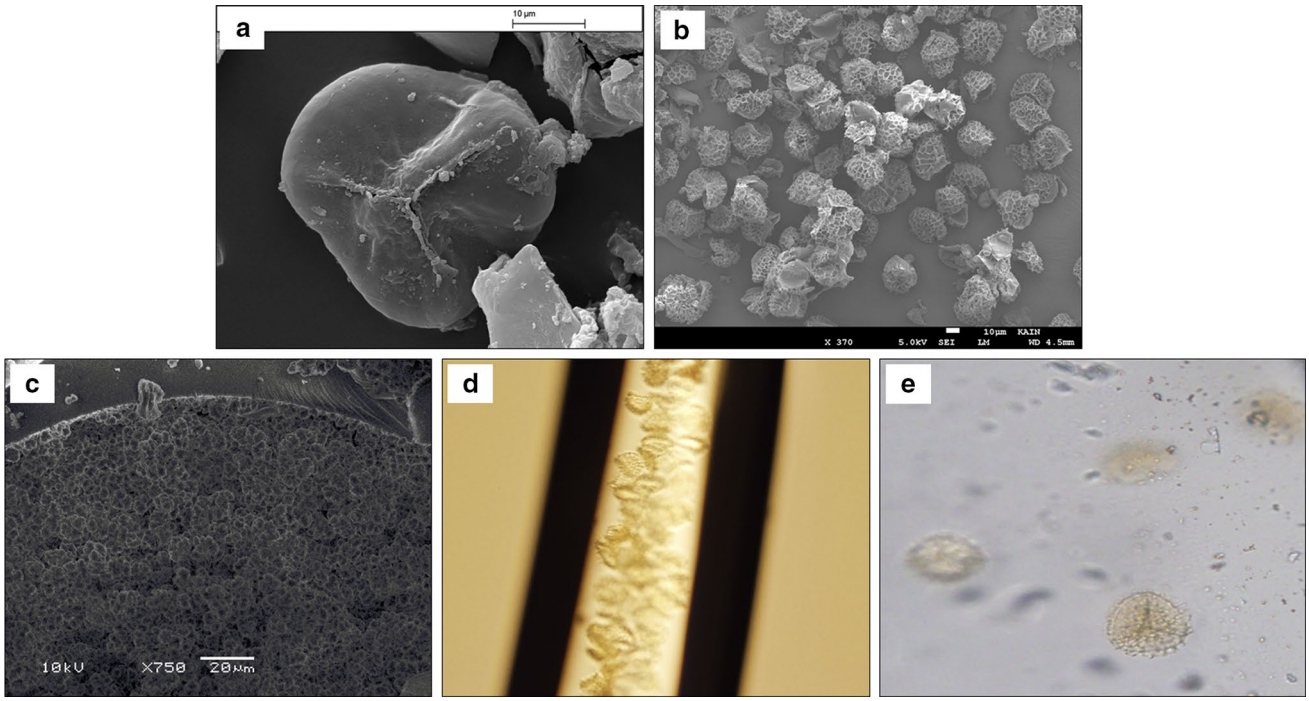

Fig. 3 SEM images of **a** *L. clavatum* spores before treatment, **b** sporopollenin produced after treatment, and **c** C_1 embedded monolith; optical micrographs of **d** the polymeric monolithic layer extruded from the fused silica tubing of C_3 column, **e** the bulk polymer sample prepared for column C_3

columns ranged from 83 % for C_3 column corresponding to a 0.1 % content of sporopollenin particles to 91 % for C_1 (the control column). The total porosity values for C_2, C_4, and C_5 were 86, 87, and 89 %, respectively. This trend confirmed the major influence that the sporopollenin particles exerted on the column porosity, in spite of their relatively low content.

It is important to investigate and control the column morphology that governs the homogeneity and rigidity of the polymer bed. Therefore, the morphology and surface properties of the monoliths were evaluated by optical and scanning electron microscopy.

Figure 3a shows a typical SEM image of untreated spores from *L. clavatum*. A representative SEM image of sporopollenin microparticles after treatment is shown in Fig. 3b, wherein each sporopollenin particle is hollow and the trilite scars are also visible. SEM micrographs also showed the complete removal of the intine present in the untreated spores. Most of the produced sporopollenin particles remained intact as the untreated spores. However, some had slightly burst or had become distorted, but this could be due to the high vacuum encountered in the electron microscope. These results indicate that the purification method used did not alter the structural framework of the sporopollenin.

Figure 3c shows a SEM image of the embedded monolith for C_1; the monolith bed has a uniform structure and porosity and is well attached to the inner surface of the capillaries. On the other hand, the optical micrograph in Fig. 3d shows that the polymeric monolithic layer extruded from the fused silica tubing is a long strip of material in which the sporopollenin particles are clearly visible and their distribution in the polymeric strip is uniform. Another optical micrograph of the bulk polymer sample is shown in Fig. 3e. Moreover, these micrographs confirm that the sporopollenin structure remained intact and was not damaged after the polymerization process. It can be clearly seen from these figures that the average diameter of the sporopollenin particles is about 25 µm, as stated before.

Separation and Efficiency of Columns

The efficiencies of the columns fabricated in this study were also investigated. Model compounds were selected with regard to polarity; ketonic and aromatic compounds including acetone, acetophenone, butyrophenone, benzene, naphthalene, and anthracene were tested as model solutes to check and evaluate the efficiency of the prepared columns.

The prepared columns were tested for their ability to separate three ketones using different experimental conditions. As an example, Fig. 4a, b shows the separation of the three components on C_1 (a) and C_3 (b) columns at a flow rate of 20 µL min^{-1} and a detection wavelength of 260 nm

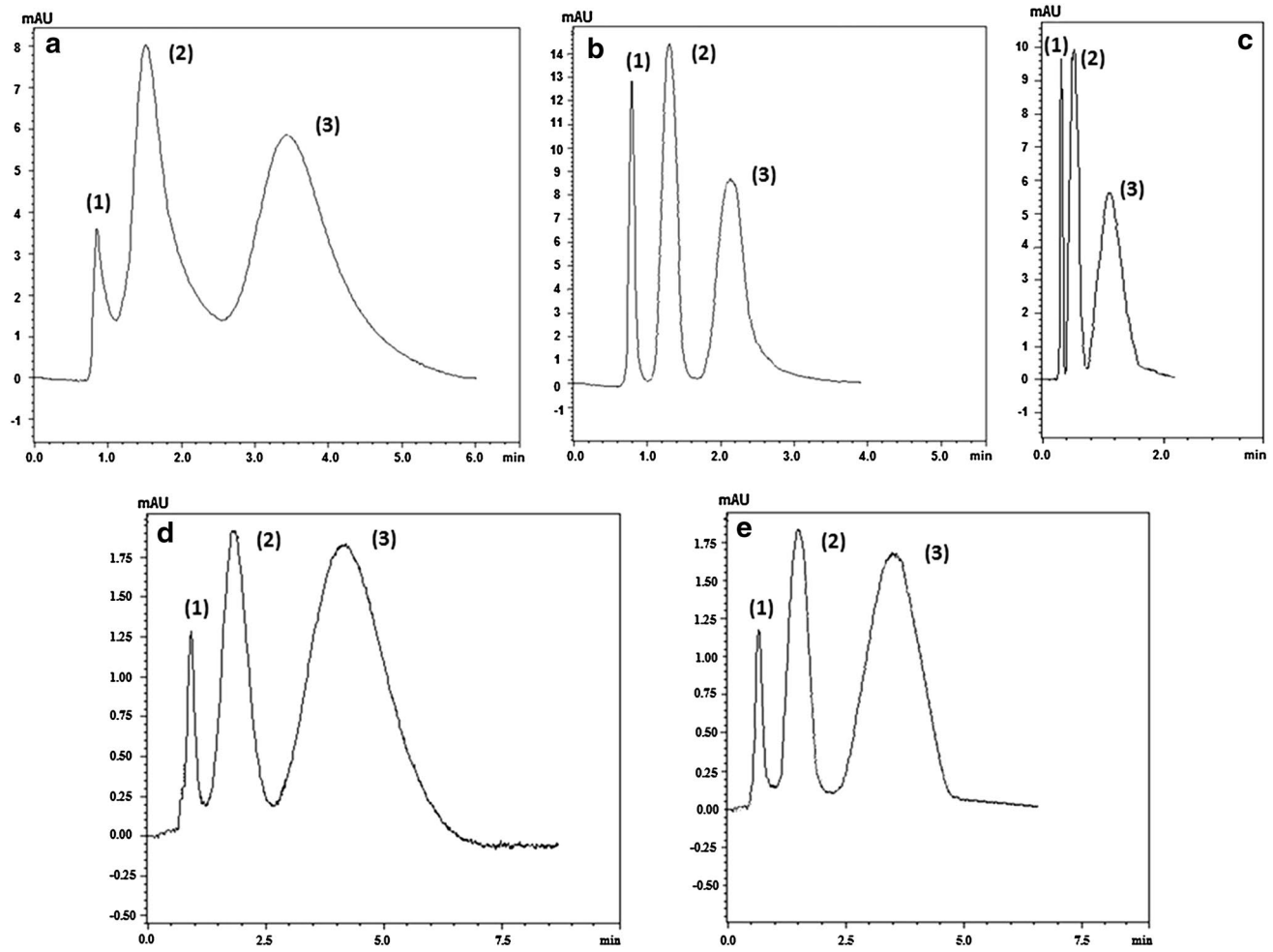

Fig. 4 Chromatograms obtained using columns C_1 (**a**, **d**) and C_3 (**b**, **c**, and **e**) for the separation of **a** and **b** ketones (20 μg mL^{-1} of each component) with a binary acetonitrile/water (50:50, v/v) with 0.1 % formic acid as the mobile phase at 20 μL min^{-1}, **c** fast separation of ketones at 50 μL min^{-1} mobile phase flow rate (*1* acetone, *2* acetophenone, and *3* butyrophenone), **d** and **e** aromatics using acetonitrile/water (50:50, v/v) as the mobile phase at 40 μL min^{-1} (*1* benzene (10 μg mL^{-1}, *2* naphthalene 40 μg mL^{-1}, and *3* anthracene 40 μg mL^{-1})

using a binary acetonitrile/water (50:50, v/v) mixture with 0.1 % formic acid as the mobile phase. While incomplete separation was obtained on the control column C_1, the C_3 column allowed the full separation of the three solutes in a run time shorter by approximately 2 min.

On the other hand, incorporating sporopollenin microparticles into the monolithic material obviously showed a marked enhancement of the column efficiency by increasing the plate number by a factor between 3 and 5, at flow rates between 5 and 50 μL min^{-1}. The fastest separation for the three compounds was achieved in approximately 1.5 min using the C_3 column at a flow rate of 50 μL min^{-1} as shown in Fig. 4c. The best enhancement was obtained for acetone injected on the C_1 column at 10 μL min^{-1} which corresponded to a column efficiency of 1,072 plates m^{-1}, compared with 5,347 plates m^{-1} for the C_3 column using the same chromatographic conditions.

Comparison of the chromatograms in Fig. 4a and b shows the noticeable influence of incorporating sporopollenin microparticles on the separation of the ketones. Moreover, the peak shape of the three ketones was improved and the resolution increased by a factor between 1.7 and 4.2.

The prepared columns were also used for the separation of three aromatic compounds (benzene, naphthalene, and anthracene). The three aromatics were fully separated using columns C_1 (Fig. 4d) and C_3 (Fig. 4e) with a resolution of more than 1.2 over all the studied flow rate range.

Although the retention time of the aromatic compounds decreased by about 1 min using C_3, other results show that the incorporation of sporopollenin microparticles does not greatly affect the column efficiency and resolution. This could be explained by the hydrophilic character of the defatted sporopollenin microparticles which do not interact

greatly with the aromatic hydrocarbons, while the decrease in retention should be due to the lower content of polymethacrylate material.

In comparison with the other used incorporated monolithic materials (carbon nanotubes and metal organic frameworks), only sporopollenin particles had the advantage of decreasing the retention times for the separated solutes [16–20].

Conclusion

In this work, we introduce sporopollinin microparticles as a stationary phase in chromatography for the first time. The effect of sporopollinin incorporation into the polymerization mixture was investigated in the range 0.05–0.3 %. In comparison with neat polymer columns, their incorporation produced monolithic columns exhibiting an improvement of the column performance of about fivefold for retained acetone. In addition, they showed a notable decrease in run time for both ketonic and aromatic compounds.

This work will undoubtedly contribute to open up new avenues of research in the field of separation science by incorporation of sporopollenin microparticles into either monolithic or packed stationary phases. Other trends could be the use of modified particle surfaces by functionalization of the sporopollenin for specific separations or the use of different types of sporopollenin from various sources and species.

Acknowledgments The authors acknowledge the financial support of this work by King Saud University through National Plan for Science and Technology grant # NPST 14-ADV931-02.

Conflict of interest The authors of this publication has research support by King Saud University through National Plan for Science and Technology grant # NPST 14-ADV931-02. The terms of this arrangement have been reviewed and approved by King Saud University at Riyadh, Kingdom of Saudi Arabia in accordance with its policy on objectivity in research.

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