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Fritless Capillary Columns for HPLC and CEC Prepared by Immobilizing the Stationary Phase in an Organic Polymer Matrix

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A new design of immobilized particle separation media for capillary liquid chromatography and electrochromatography has been developed. A mixture of porogenic solvents and methacrylate-based monomers is pumped through a packed column to provide, following a polymerization step, an organic matrix capable of holding the sorbent particles in place, thus rendering the end frits unnecessary. The new columns demonstrate excellent chromatographic performance in both CEC (reduced plate height [*h*] =1.1−1.5) and micro LC modes (*h* = 2.2−2.5), while minimizing secondary interactions encountered when silica-based entrapment matrixes are employed. In addition to delivering mechanically robust columns, the methacrylate matrix provides a mechanism for fine tuning of the electroosmotic flow velocity when 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) is incorporated into the polymerization mixture.

Capillary electrochromatography (CEC) draws its strengths from well-established predecessors: high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). The use of stationary phases commonly employed in HPLC provides a wide choice of selectivity, while the replacement of pressure-driven flow with electroosmotic flow provides a more ideal profile and affords the use of smaller diameter particles, thus providing a significant improvement in chromatographic performance.[1]

A better understanding of the limitations and virtues of the technique will smooth the path for specific CEC applications, which may promote the technique to more routine use.[2] Separations using submicrometer-size particles,[3] molecular imprint polymers (MIP),[4] perfusive media,[5] etc. have been demonstrated. There are, however, technical problems that must be surmounted, and column preparation and maintenance is recognized as a major limitation.[6]

Common designs of capillary columns for micro LC and CEC include packed and open tubular columns. The latter can display excellent efficiencies but require in situ synthesis of stationary phase bonded to the walls of the capillary, have a limited sample capacity, and often require long analysis times. Packed columns seem to be the format of choice for most applications since they use currently available HPLC sorbents, offer good efficiencies (reduced plate height [*h*] = 2 or less for CEC), and impart significantly larger sample capacity. Unfortunately, their design for the most part requires the presence of frits to hold the particles in place. Current frit fabrication procedures introduce side effects such as nonspecific (secondary) interactions, increased backpressure during micro LC analyses, gas-bubble formation during CEC analyses, column-to-column reproducibility problems, increased fragility of capillaries, and poor column performance due to disruption of the structure of the separation medium.

As a result, numerous studies have been directed toward the development of fritless columns, in both continuous rod[7−9] and immobilized packed bed format,[10−15] or the design of new types

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of frits[16] which eliminate the difficulties encountered using the widely employed water−glass-based frits.

Monolithic columns, both silica-[7] and organic-polymer-based,[8,9] have been prepared in situ by pumping a monomeric solution into a capillary column blank. Upon further treatment, these solutions polymerize to render a continuous porous polymer. The desired stationary-phase composition can be achieved through initial incorporation of functional monomers or by further derivatization of the polymeric rod.

Elaborate design of monolithic poly (butyl methacrylate-*co*-ethylene dimethacrylate) columns by Peters et al.[9] demonstrates that it is possible to tailor the pore size and, to a certain degree, the surface chemistry of the rods to obtain remarkable chromatographic performance. However, at least for the moment, their applications are limited to reversed-phase and size-exclusion chromatography.

In 1997 the first two designs of immobilized packed beds for capillary chromatography were introduced: the entrapment of packed sorbent particles within a water−glass matrix[10] and cementing in situ silica-based reversed-phase particles through a thermal treatment to form a monolithic structure.[11] The irreversible agglomeration of particles obtained later by Asiaie et al., following a sintering and subsequent octadecylation procedure, provided columns appropriate for both CEC and micro LC separations.[12] Another approach is to encapsulate sorbent particles in a porous sol−gel matrix.[13] This design yielded monolithic capillaries by simply loading a suspension of particles and sol−gel precursors into the capillary tubing at low pressure. Following the path of earlier results with water−glass entrapment of a tightly packed bed,[10] we used sol−gel chemistry to "glue" sorbent particles together.[14] The procedure provided stable columns that preserve the high performance characteristics typical of the classic packed columns in both CEC and micro LC operation. Similar results were obtained when supercritical carbon dioxide was used to dry the sol−gel matrix in a thorough study conducted by Tang et al.[15]

Not surprisingly, however, the surface of the silica-based entrapment matrixes renders separation media prone to nonspecific adsorptive interactions.

To our knowledge, this paper reports the first effort to immobilize reversed-phase sorbents within the walls of fused silica capillary tubes using an organic-based entrapment matrix. Our goal was to develop an immobilization procedure that would have a minimal effect on the chromatographic performance of the starting separation media, while at the same time yielding a stable fritless packed column.

EXPERIMENTAL SECTION

Chemicals and Materials. Butyl methacrylate (BMA), methyl methacrylate (MMA), ethyl methacrylate (EMA), ethylene dimethacrylate (EDMA), 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), and 2,2′-azobisisobutyronitrile (AIBN) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received. All analyte compounds and tris(hydroxymethyl)-aminomethane (TRIS) used for buffer preparation were obtained from Sigma Chemical Co. (St. Louis, MO).

The solvents employed in the CEC and micro LC runs were HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA) or Mallinckrodt (St. Louis, MO).

Fused silica tubing of 75-μm and 250-μm i.d. with 375-μm o.d. was purchased from Polymicro Technologies (Phoenix, AZ). Capillaries cut to a total length of 35 cm were filled with packing material as described herein. The reversed-phase sorbent, Nucleosil (Macherey-Nagel, Düren, Germany) C18 5-μm diameter was purchased from MetaChem Technologies (Torrance, CA).

Temporary frits were prepared by tapping one end of the capillaries in a paste prepared from silica-based particles (1000 Å pore size, 10-μm diameter, purchased from MetaChem) and potassium silicate solution (Kasil no. 1; PQ Corp., Valley Forge, PA).

Instrumentation. All CEC runs were performed on a Hewlett-Packard HP^{3D} CE (Waldbronn, Germany) instrument. The instrument is modified such that pressures up to 12 bar can be applied to the inlet and/or outlet vials. This feature was used only to obtain micro LC runs, since during operation the entrapped columns presented no gas-bubble generation problems. The cassette temperature was held at 25 °C. Electrokinetic injection (3 kV for 3 s) was used for sampling the analytes. Data acquisition and processing were done using HP ChemStation software (Hewlett-Packard).

The entrapment setup consists of a glass vial of 4-mm diameter placed in a typical-solvent-rinsing kit described elsewhere.[17] Detection windows were obtained using an arc fusion splicer (Fujikura FSM 05S, Alcoa Fujikura, Duncan, SC). For better coupling, a Hewlett-Packard capillary column cutter with rotating diamond blade was used to obtain clean, straight cuts of fused silica tubing.

Preparation of Entrapped Columns. In view of our goal, i.e., designing an immobilization procedure that would have minimal negative impact on the chromatographic performance of a typical packed column, the capillaries were prepared using a standard-slurry-packing method. A very porous frit was prepared at the inlet, and the slurry made of packing dispersed in 50% acetonitrile, 50% water was forced into the capillary using a syringe pump. The pressure was gradually increased from 200 to 6000 psi. During packing, the chromatographic bed was sonicated for several minutes. When the desired length of packed bed was achieved, the pump was stopped and the column was slowly depressurized to ensure minimal movement of the particles.

The entrapment mixture used in this study to immobilize the particles was prepared using various amounts of methyl-, ethyl-, or butyl- ("R") methacrylate (MA), along with EDMA and the free radical initiator, AIBN. These were added to a ternary solvent system consisting of water, 1-propanol, and 1,4-butanediol. This system offers several advantages: it allows for handling the monomeric mixture at room temperature for several hours without initiating the polymerization reaction; it dissolves both the hydrophilic AMPS and hydrophobic RMA and EDMA monomers to form a homogeneous mixture; and, it is readily miscible in the mobile phases employed in micro LC or CEC separations, allowing for quick column washing and equilibration as indicated by the efforts of Peters et al. in designing monolithic columns.[9]

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Table 1. Performance Characteristics of Immobilized Bed Columns Packed with 5-μm Nucleosil C18 Particles and Compositions of the Corresponding Entrapment Matrixes

entrapment composition[a]	solvent (%v)	EDMA (%v)	alkyl methacrylate (RMA %v)	AMPS (wt %)	s_0[b]	s_F[b]	s_N[b]	h_0[c]	h_F[c]	h_N[c]
D2	91.5	8.5			0.81 ± 0.06	0.72 ± 0.08	0.69 ± 0.06	1.2	2.3	2.3
DM1	91.1	4.4	4.5 (MMA)		0.92 ± 0.05	0.78 ± 0.12	0.76 ± 0.09	1.6	2.3	3.3
DM2	91.1	4.4	4.5 (MMA)	0.07	0.89 ± 0.02	0.67 ± 0.07	0.69 ± 0.07	1.4	2.6	3.4
DM3	91.0	4.5	4.5 (MMA)		0.70 ± 0.08	0.63 ± 0.10	0.61 ± 0.08	2.0	2.7	3.1
DM4	91	4.0	5.0 (MMA)	0.07	0.86 ± 0.03	0.70 ± 0.02	0.68 ± 0.02	2.2	3.2	3.8
DE1	91.7	3.6	4.7 (EMA)		0.85 ± 0.05	0.84 ± 0.08	0.78 ± 0.09	3.6	3.4	3.6
DB1	90.9	4.4	4.7 (BMA)		0.93 ± 0.01	0.92 ± 0.04	0.92 ± 0.01	2.4	2.7	2.7
DB7	89.0	4.4	6.6 (BMA)		0.92 ± 0.03	0.94 ± 0.04	0.88 ± 0.03	1.4	1.7	1.5
DB10	89.1	4.4	6.5 (BMA)	0.10	0.94 ± 0.04	0.91 ± 0.04	0.84 ± 0.03	1.7	1.9	2.3

[a] The nomenclature used for each composition contains one or two letters designating the initials of the methacrylate monomers used (D for ethylene dimethacrylate, M for methyl methacrylate, E for ethyl methacrylate, and B for butyl methacrylate) and the current number of the trial. [b] s_O, s_F, and s_N represent the average symmetry factors and corresponding standard deviations for thiourea, fluorene, and naphthalene, respectively; these values were calculated by averaging data (assessed using the Chemstation software) acquired from running 6−20 chromatograms for each entrapment composition at 10−30 kV. [c] h_O, h_F, and h_N represent the average reduced plate heights, calculated for 6−20 runs for thiourea, fluorene, and naphthalene, respectively.

The goal of this procedure was to introduce a minimal amount of polymer that would ensure particle immobilization during routine CEC column operation. Therefore, the proportion of solvents to monomers was varied such that, after polymerization, the bed could withstand pressures of about 300 psi. The polymerization mixture used for entrapment was obtained by dissolving RMA (0−6 wt %), EDMA (5−30 wt %), AIBN (2 wt % with respect to monomers), and in some cases, AMPS (0.03−0.12 wt %) in the ternary solvent system of water (7−10 wt %), 1-propanol (0−50 wt %), and 1,4-butanediol (30−80 wt %). The homogeneous mixture was purged with nitrogen for 10 min to remove dissolved oxygen, after which, part of the mixture was transferred into the 4-mm diameter glass vial and placed into the solvent rinsing kit. The remaining liquid was poured into a 20-mL vial, which was capped and set in the refrigerator (∼10 °C) for later use.

A dried packed capillary was mounted in the solvent rinsing kit with the fritless end in the entrapment solution. On application of 50 psi of gas pressure, about 10 column volumes of the liquid was forced through the capillary. For a 25-cm-long capillary packed with 5-μm particles, the flushing step requires about 2 min. Next, the column was sealed at both ends and placed in an oven at 60 °C for 48 h. After the matrix polymerized, the frit was removed and the column was cut to the desired length and coupled to an open tubular capillary segment on which a detection window was made.

RESULTS AND DISCUSSION

Column Preparation. The incidence of voids in the chromatographic bed of a packed column is not infrequent when CEC and micro LC capillary columns are prepared. Voids can alter peak shape and significantly decrease efficiency. Possible reasons for occurrence of these defects include poor packing technique (nonhomogeneous slurries, problems with the apparatus, etc.) or frequent column pressurization−depressurization cycling between CEC runs. Particle immobilization or "entrapment" provides a stable bed that increases column lifetime and allows length customization. Moreover, in the case of the presently described columns, there is no need for column pressurization during CEC runs.

Two techniques have been described for immobilized-bed column fabrication: in situ treatment of a prepacked bed[10−12,14−15] and particle loading of the capillary.[13] The latter approach, though a one-step procedure, requires that there be no particle segregation in the loading slurry. This limits the range of ideal immobilization matrixes to those that, in the monomeric stage, have densities that allow the formation of a stable suspension with the sorbent material. In addition, the degree of matrix shrinkage during polymerization and relatively low packing density of the sorbent result in disruptions of the chromatographic bed: cracks, voids, or nonuniform distribution of stationary phase throughout the length of the column. A two-step process, such as the in situ treatment of a consolidated packed bed, offers increased control over the homogeneity of the packed bed and ensures a more uniform dispersal of the immobilization matrix, though it is a more laborious process. Increased efficiency and improved peak shape can be achieved[10,14−15] in exchange for the extra effort. This method also allows for more flexibility in selection of immobilization matrixes. As such, organic- or silica-based polymers can be employed without concern that alternative compositions of the entrapment solution might cause it to fall out of the preferred or optimal density domain.

Designing the Optimum Polymeric Mixture. In what follows, we present the chromatographic performance of columns packed with Nucleosil C18 particles subsequently immobilized in matrixes in which the nature of the monomer or the solvent-to-monomer ratio has been varied. Table 1 summarizes the main features of selected entrapment compositions employed in packed-bed immobilization. The nomenclature used for each composition contains one or two letters designating the initials of the methacrylate monomers used (D for ethylene dimethacrylate, M for methyl methacrylate, E for ethyl methacrylate, and B for butyl methacrylate) and a figure designating the current number of the trial. Two to three columns were entrapped with each immobilizing composition. Retention times varied between columns by less than 5% relative standard deviation.

The mixtures based on ethylene dimethacrylate (Table 1 lists the composition which displayed the best characteristics, namely D2) offered an extremely rigid support for the particles, which were confined within the walls of silica at pressures up to 3000 psi. On the other hand, the retention factors were much smaller than those measured for a nonentrapped column, indicating that,

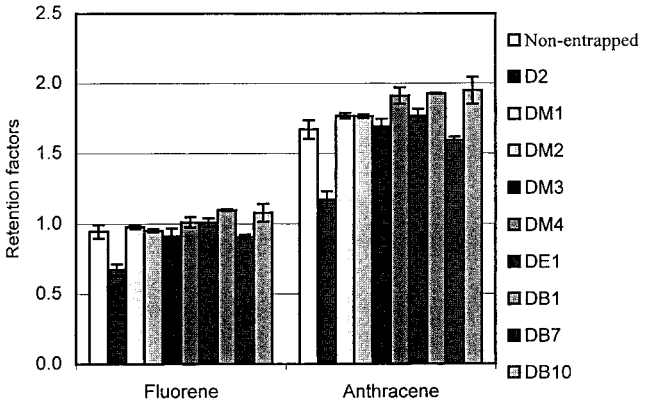
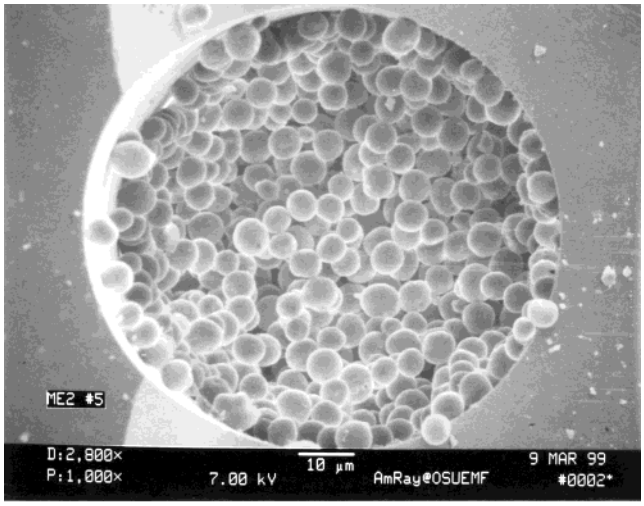


Figure 1. The effect of entrapment composition on the retention factors (*k*) of electrochromatographic separation of two PAHs. The mobile phase employed was 80% acetonitrile, 20% TRIS 25 mM (pH = 8.0), and flow velocities varied from 0.4 to 1.2 mm/s. Each histogram represents the average *k* and standard deviation for 6−20 runs performed on columns entrapped with corresponding matrixes (for matrix composition see Table 1).

to a certain degree, the accessibility of the stationary phase was hindered by the entrapment procedure (Figure 1). Such results are similar to those observed with earlier immobilization procedures,[10,14] all of which were silica-based. Possible causes in these instances included coverage of the stationary phase or occlusion of a fraction of the pores in the packing material with the entrapment polymer, hydrolysis of bonded phase during the bed-flushing step when alkaline entrapment solutions are employed, and loss of bonded phase during the matrix-polymerization step, often a high-temperature process (120−360 °C). Some of these earlier methods necessitate a subsequent postderivatization step to restore the carbon content of the stationary phase,[12] while others recover part of the active surface by flushing the column with a weakly alkaline solution[10] to dissolve the excess entrapment matrix. There is a limited range of options, as far as minimization of undesirable surface coverage is concerned. However, replacement of the silica-based immobilization matrix with an acrylate matrix reduces the stationary-phase alteration that occurs during the immobilization procedure, since the alkaline solutions are replaced with a mixture that is inert, insofar as bonded-phase hydrolysis in concerned. In addition, the organic-based matrix requires much lower temperatures for polymerization (60 °C).

The next step in improving the entrapment composition was to produce monomeric mixtures in which ethylene dimethacrylate was partially replaced by methyl methacrylate (compositions DM1−DM4 in Table 1) or ethyl methacrylate (composition DE1 in Table 1). As a result, column permeability was greatly improved (Figure 2); the retention factors grew close to the values characteristic of a nonentrapped column, and the separation efficiency increased. However, an unexpected tailing of polyaromatic hydrocarbons (PAH) was observed. This problem could not be solved by varying the volume of solvent relative to monomers, but rather was alleviated by replacing the methyl or ethyl methacrylate monomers with butyl methacrylate. The use of such monomers evidently increased the effective carbon loading of the separative media. This could partially account for the observed proximity of retention factors determined for entrapped columns versus those for nonentrapped columns. In fact, Peters et al.[9] demonstrated that butyl methacrylate based monolithic columns



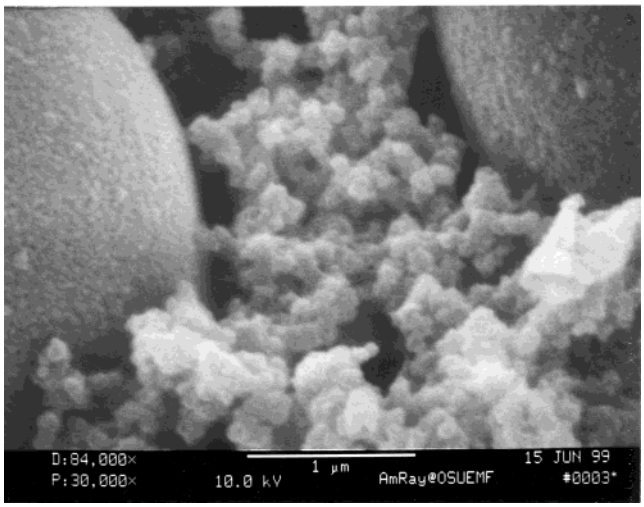


Figure 2. Scanning electron micrographs of a capillary column (75-mm i.d.) packed with ODS Nucleosil particles of 5-μm diameter and entrapped with composition DM2 (see Table 1). A small amount of organic polymer deposited in the entrapment procedure is responsible for the immobilization of the sorbent particles. These images reveal that the entrapment is a mild technique that leaves the original packed bed largely unmodified.

have polarities approximating those for ODS sorbents. The alkyl methacrylate monomers, however, represent less than 6% of the entire entrapment solution. The fact that *k* values for analytes on entrapped columns approach those specific to a conventional octadecylated stationary phase (for identical eluent composition) suggests a minimal contribution of the matrix to the separation process. Columns in which the packed bed was immobilized using composition DB1 produced micro LC separations with reduced plate heights of 2.2−2.5. Except for the entrapment composition, all other parameters unchanged, the shorter retention time of the flow marker recorded in micro LC separations indicates an improvement in column permeability. All micro LC runs were performed in the HP^{3D} CE instrument by applying 6−12 bar of N_2 headpressure on the inlet vial, which resulted in a surprisingly stable and reproducible flow. In an effort to further improve performance, the relative amounts of porogenic solvent and monomer were further varied. Consequently, mixture DB7 provided a monolithic column that produced very efficient separations over a rather broad range of flow velocities as illustrated in Figure

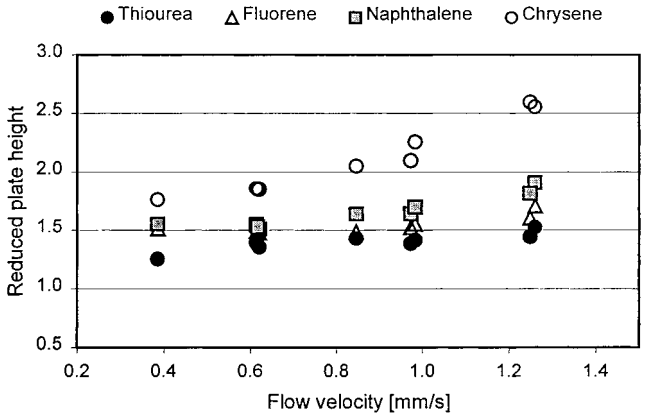


Figure 3. Variation of reduced plate height with electroosmotic flow velocity for CEC separation of PAHs using a column packed with 5-μm ODS Nucleosil particles subsequently immobilized with matrix DB 7 (see Table 1 for detailed description of DB7 entrapment matrix). Mobile phase: 80% acetonitrile, 20% TRIS 25 mM (pH 8.0).

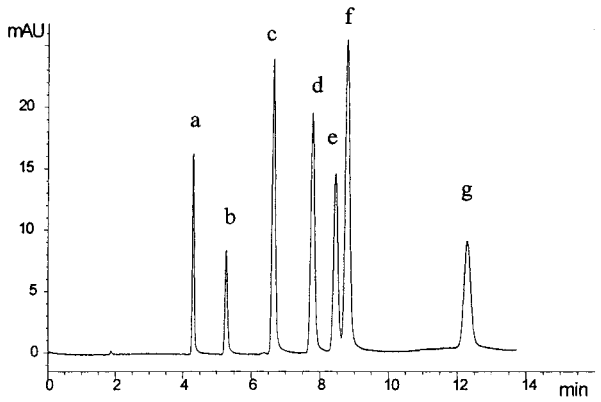


Figure 4. High-efficiency CEC separation achieved on a column packed with 5-μm ODS Nucleosil particles subsequently entrapped with DB7 (see Table 1 for detailed description of the immobilizing composition). Solutes and corresponding reduced plate height: (a) thiourea ($h = 1.0$), (b) butylparaben ($h = 1.1$), (c) fluorene ($h = 1.2$), (d) naphthalene ($h = 1.3$), (e) phenanthrene ($h = 1.4$), (f) anthracene ($h = 1.5$), and (g) chrysene ($h = 1.7$). $L_{eff} = 16$ cm, $L_{tot} = 26$ cm. UV detection at 254 nm. Mobile phase: 80% acetonitrile, 20% TRIS 25 mM (pH 8.0).

3. The best run produced reduced plate heights of 1.1 for acetone and 1.2−1.8 for retained test probes (Figure 4).

Aside from the separation of PAHs (used here as representative standards for reversed-phase column evaluation), the performance of these columns was evaluated using caffeine and several analgesics. Baseline separation of acetaminophen, caffeine, and acetone was achieved with 80% acetonitrile, 20% TRIS 25 mM pH 8.0 (Figure 5). Acetaminophen, caffeine, aspirin, flurbiprofen, naproxen, and ibuprofen were separated after switching to an acidified mobile phase: 70% acetonitrile, 30% sodium acetate 10 mM (pH 3.0). The peaks are sharp and have symmetry factors of 0.89 ± 0.05 (Figure 6).

Another demonstration of the favorable characteristics exhibited by these organic-polymer-entrapped columns is provided by the micro LC analysis of a UNG (Uracil DNA Glycosylase) tryptic digest mixture. A complete description of the protocol used to obtain the digest and the instrumental setup employed to obtain the gradient separation is given elsewhere.[18] The chromatogram shown in Figure 7 was obtained at a flow rate of 0.4 μL/min

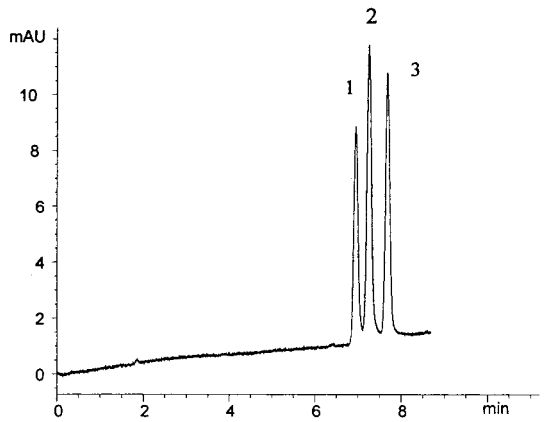


Figure 5. CEC separation of (1) acetaminophen, (2) caffeine, and (3) acetone on a 75-μm i.d. column packed with 5-μm ODS Nucleosil particles and subsequently entrapped with composition DB10 (see Table 1 for detailed description of matrix composition). Mobile phase: 80% acetonitrile, 20% TRIS 25 mM (pH 8.0). UV detection at 254 nm; $L_{eff} = 17$ cm; $L_{tot} = 26$ cm. Note that the electrophoretic mobilities of acetaminophen and caffeine are such that they elute ahead of an EOF flow-velocity marker, acetone.

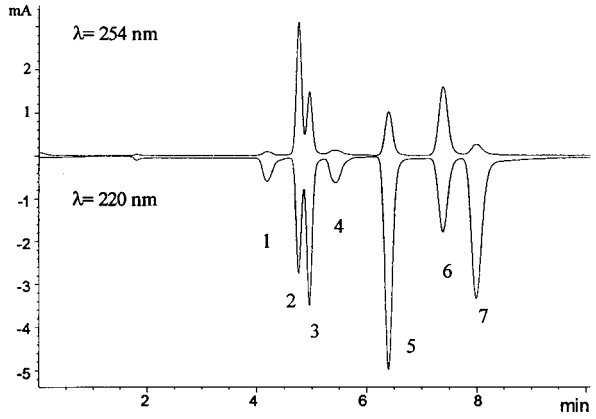


Figure 6. Nonsteroidal antiinflammatory drugs, acetaminophen, and caffeine separated by electrochromatography on a 75-μm i.d. column packed with 5 μm ODS Nucleosil particles immobilized within a matrix with composition DB10 (see Table 1 for detailed description of entrapment mixture). Mobile phase: 70% acetonitrile/30% acetate, 10 mM (pH 3.0). UV detection at 254 and 220 nm (upper trace and, respectively, lower trace); applied voltage, 20 kV; $L_{eff} = 17$ cm, $L_{tot} = 26$ cm. The sample contains (1) an unknown impurity, (2) acetaminophen, (3) caffeine, (4) aspirin, (5) naproxen, (6) flurbiprofen, and (7) ibuprofen.

employing a gradient from 100% H_2O (0.04% TFA) to 100% acetonitrile (0.04% TFA) in 45 min. In this particular micro LC run (Figure 7), micropreparative fractions of the digest mixture were collected and later identified using MALDI-TOF mass spectrometry.[18]

Amending the Electroosmotic Flow. A benefit of using organic polymers in the fabrication of chromatographic media is the ease of incorporating monomers with various functionalities. In particular, ionizable groups can be quantitatively added to the immobilization matrix by introducing 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) or similar reagents. The thorough study conducted by Peters et al.[9] on monolithic columns reports on the complex effect of AMPS on flow velocity and pore size of the polymeric material.

(18) Doneanu, C.; Barofsky, D., manuscript in preparation.

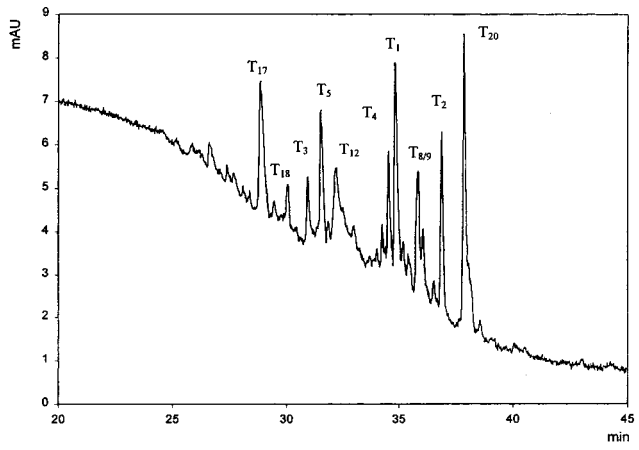


Figure 7. Micro LC separation of tryptic digest fragments (noted T_1-T_{20} starting from the N terminal) of UNG (Uracil DNA glycosylase) achieved on a column entrapped with mixture DM2. The setup (described in detail elsewhere[18]) was used to separate, collect, and identify (using a MALDI-TOF instrument) each of the recorded fractions. Conditions: eluent A (100% H_2O, 0.04% TFA), eluent B (100% acetonitrile, 0.04% TFA); gradient from 0 to 100% B in 45 min; applied pressure, 1000 psi; UV detection at 200 nm; injected volume, 1 μL.

Table 1 contains two representative entrapment mixtures that include this monomer: DM4 (0.07 wt % AMPS with respect to monomer content) and DB10 (0.10 wt % AMPS with respect to monomer content). Two columns packed with ODS Nucleosil particles were immobilized with each of these entrapment solutions. The results were referenced to mixture DB7 and DM2, respectively, which contain essentially the same amount of all constitutive materials, with the exception of AMPS. To preserve the ratio of solvents to monomers in DB7 and DM2, AMPS was replaced with the corresponding amounts of ethylene dimethacrylate. The expected increase in electroosmotic flow velocity, which amounts to 10−15%, was observed for the methyl (DM 4) and butyl methacrylate (DB10) based matrixes.

CONCLUSION

Organic-polymer-entrapped capillaries offer an attractive variety of possibilities for fritless columns. The major advantages of this novel separation medium include mechanical robustness, on-the-fly length customization, relative ease of preparation (by simply flushing a packed capillary with monomeric mixture and subsequent heat curing of the column), and minimal alteration of original properties of the sorbent. These capillaries can be used without the need of column pressurization, since no gas-bubble formation was observed. These salient features make organic-polymer-entrapped columns a viable option for micro LC, CEC, or pressurized CEC.

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