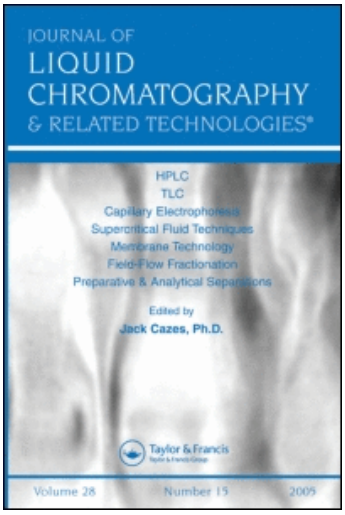

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Abstract: A new approach of immobilized particle separation media for capillary HPLC and CEC has been developed. A mixture of porogenic solvents and methacrylate based monomers is pumped through a single tapered packing column, following a polymerization step by microwave irradiation. Furthermore, the chromatographic behaviors of the prepared column were thoroughly evaluated by changing the flow rate and the percentage of mobile phase by the developed capillary HPLC system. With the optimal experimental conditions, baseline separation of the model analytes including thiourea, benzene, methylbenzene, ethylbenzene, was obtained with a column efficiency of theoretical plate height less than 100 μm for the last eluted analyte of ethylbenzene in capillary HPLC and CEC. The developed capillary column was found to be stable and could easily be operated continuously up to a pressure of 30 MPa without column damage and the capillary can be cut to any desired length.

Keywords: Capillary electrochromatography, Capillary liquid chromatography, Immobilization, Microwave irradiation, Octadecylsilane, Slurry packing

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INTRODUCTION

The miniaturization of liquid chromatography (LC) through the use of packed, open tubular, and monolithic capillary columns has opened up a number of possibilities for the rapid analysis of nanoliter and subnanoliter samples.[1–4] However, its application has been restricted by difficulties associated with column preparation and complication of the design of homemade and commercial capillary HPLC and CE systems. Capillary HPLC, as an intermediate technique between conventional liquid chromatography and microchip separations, has recently attracted significant research interest because of both reduced analysis times and reduced sample consumption. For the commercial capillary HPLC instrument, it generally includes several main parts, such as nanopump, nanoinjector, capillary column, nanoflow cell, and so on. If the conventional analytical HPLC is expanded to micro HPLC, three main problems should be solved effectively. One is how to prepare a capillary column of high efficiency for different applications, the second is how to realize the nanoliter injection, the third is that dead volume should be decreased, possibly by using suitable linkers of a small inner diameter, on-column detector or expansive nanoflow cell.

Packed, fused silica capillaries with an inner diameter of 50 μm to 530 μm are typically used as columns and a common design for their application includes packed, monolithic and open tubular columns.[5,6] The latter can display excellent efficiencies but require in situ synthesis of the stationary phase bonded to the walls of the capillary, which have a limited sample capacity. Because of the availability of numerous packing materials for conventional analytical HPLC columns, the packing columns and entrapped microsphere columns are applied more popularly due to their larger sample capacity.[7–9] The entrapped microsphere was usually realized by flushing the reactant as a matrix and followed with a polymerization reaction by thermoinitiation, photoinitation, or solgel technologies. G. S. Chirica and V. T. Remcho made a fritless capillary column using a methacrylate based monomer and ternary solvent system to entrap C_{18} particles for micro HPLC and CEC. After the packing column was forced into an organic polymer matrix, it should be placed in an oven at 60°C for 48 h.[10] Xu and coworkers also applied sol-gel technology to produce submicrometer sized organic inorganic hybrid silica packing particles for CEC.[10] The hybrid silica particles were prepared using TEOS and vinyltriethoxysilane as precursors and were encapsulated with a layer of polymerization product between styrene and divinylbenzene (DVB).[11] Bakry, R. et al. demonstrated the immobilization of continuous silica beds including C_{18} reversed phase, anion exchange, and chiral stationary phases. It was achieved by *in situ* polymerization of styrene and divinylbenzene in the presence of

decanol as a porogen and AIBN as thermal initiator. It should be noted, that the capillaries should also be placed in a water bath at 70°C for 24 h.[12] Furthermore, similar monolithic capillaries by thermal initiation were carried out at about 50 ~ 70°C for 12 ~ 72 hours or 30 min.[13–18] To our knowledge, R.X. Xie and R. Oleschuk introduced the fastest method for entrapping octadecylsilane microsphere columns for CEC recently.[20] The polymerization reactant with the butyl acrylate monomer was photo-induced and the polymerization under UV illumination could be carried out in several minutes.

In a general sense, polymerization can be realized by the use of electromagnetic irradiation as the energy source for the polymerization of monomers, oligomers, and polymers. Although ultraviolet light and thermal initiation are mainly used for this purpose, it can also be induced by ionizing radiation (e.g., electron beam, γ and x-ray), infrared, microwave, or even ultrasound.[5,21] Initiation by conventional heating presents the disadvantage of a long reaction time due to the slow convection of heat, whilst photopolymerization necessitated use of capillaries with UV transparent outer coatings and special UV cross linkers tend to be more expensive than conventional polyimide coated capillaries. Compared with conventional means, microwave heating has the advantages of being volumetric, direct, selective, and instantaneously controllable. The interaction between materials and microwaves is direct and occurs as soon as the electromagnetic field is established. All the molecules of material are subject to the electromagnetic field, although the field strength decreases as it gets deeper into the material.

Here, a novel method to immobilize reversed phase particles within the walls of fused silica capillary tubes was developed using microwave irradiation. It provided a viable alternative to the traditional methods, with the competitive advantages of simplicity, high efficiency, and low expense.[22,23] The chromatographic performance of the prepared column was evaluated with the help of the developed HPLC, which was reinstalled with a simple splitting system and on-capillary column detector.

EXPERIMENTAL

Instrumentation

Micro HPLC experiments were carried out by using a HP1100 Series HPLC system (Agilent Technologies, Inc., Walbronn, Germany) equipped with a quaternary pump and an injector with a 20 μL quantitative tube (Rheodyne 7725i). A CL3030 UV on-capillary column detector was used with a changeable ultraviolet visible wavelength in the range of 190–700 nm (Beijing Cailu Scientific Instrument Ltd., China). A precision

flow rate up to 0.001 mL/min could be provided in all experiments. A T union was used to split delivering appropriate amounts of the mobile phase. The separation column inlet was connected with one outlet of the T union, using a PEEK sleeve (0.5 mm. i.d., 1.6 mm o.d.) and a screwed joint. Another capillary (4 m × 100 μm i.d) for splitting the injection sample was linked to another outlet of the T union. Chromatograms were recorded using the computer software N2000 chromatography data system supplied by Zhida Information Engineering Ltd., Zhejiang University, China. All CEC experiments were performed on an Agilent ^{3D}CE system (Agilent Technologies, Inc., Walbronn, Germany) equipped with a diode array detector and the capability to apply up to 1.2 MPa pressure to one or both ends of the capillary. A pneumatic pump (RPL-ZD10, Dalian Replete Scientific Instrument Co., Ltd., Dalian, China) and an ultrasonic bath (KQ-500E, Kunshan Ultrasonic Instrument Co., Ltd., Kunshan, China) were used to drive solvent and slurry into the capillary during column preparation. The irradiation step was carried out in a home microwave oven (Midy Co., Ltd., Guangdong, China) with a largest output power of 700 W and a frequency of 2450 Hz. An FEI QUANTA 200 scanning electron microscope (Philips-FEI Corporation, Netherlands) was used to study the morphology of the monolith. A capillary with the monolith was sectioned into 10 mm segments without sputtering with gold prior to SEM analysis.

Materials and Chemicals

Fused silica capillaries (100 μm inside diameter, 375 μm outside diameter) were purchased from Yongnian Ruipu Optic Fiber Plant (Yongnian, Hebei Province, China). Butyl methacrylate (BMA) and ethylene dimethacrylate (EDMA) were obtained from New Jersey, USA. BMA and EDMA were extracted with 5–10% aqueous sodium hydroxide and water before use. 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), azobisisobutyronitrile (AIBN), and 1,4-butanediol, 1-propanol, acetonitrile (ACN), Tris(hydroxymethyl)aminomethane, thiourea, benzene, toluene, and ethylbenzene were purchased from Beijing Bailingwei Chemical Reagent Company and Tianjing Chemical Reagent Company, China.

RESULTS AND DISCUSSION

Preparation of the Single Tapered Column and Slurry Packing

Prior to frit fabrication, the capillaries with a length of 25 cm and an inner diameter of 100 μm were rinsed with 1 M NaOH for 30 min and

then with 0.1 M HCI for 30 min. After subsequent flushing with H_2O for about 30 min, they were dried by passage of nitrogen gas. The procedure for the single tapered capillary column was developed by our lab, which was described as follows: One end of a polyimide coated fused silica capillary with 100 μm i.d. was slowly heated to the melting stage using a butane/oxygen torch (ShanDong Province Construct High Pressure Vessel Co., Ltd., China) and then quickly withdrawn. A tip of $10 \sim 20$ μm i.d. was obtained by removing the end of the tip using a ceramic cutter or fine emery cloth. This could be monitored and confirmed by observation under a microscope (Figure 1, step 1). A "stone arch" structure can be formed in the next slurry packing due to the single narrow capillary head.[24] A fine spray under pressure should be observed for successful frits, indicating minimum flow resistance and good porosity to allow maximum packing material flow. If no such spray was

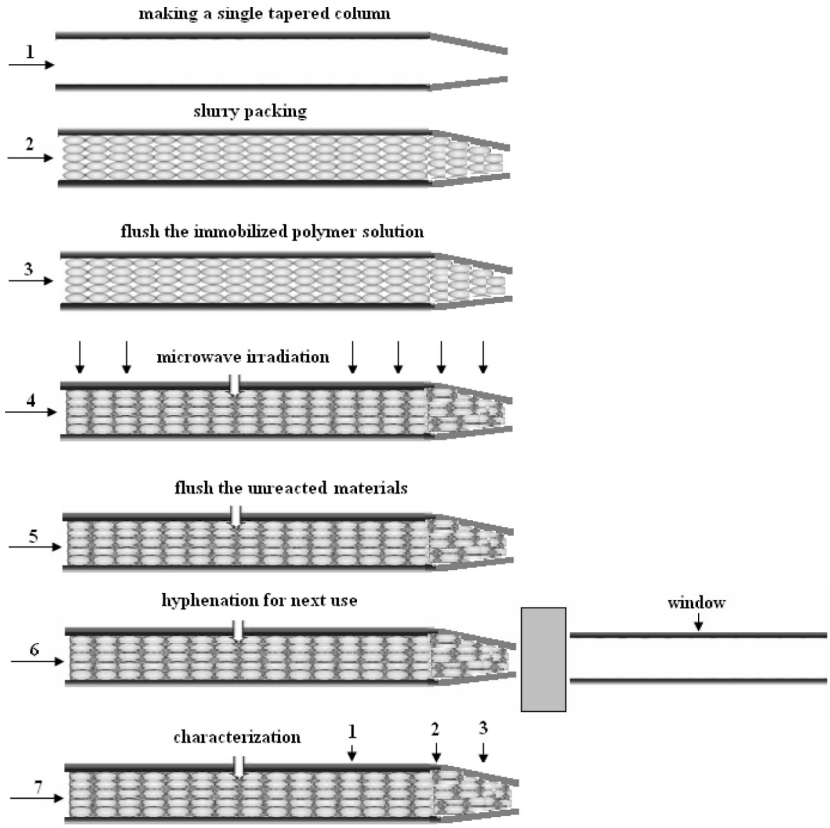

Figure 1. Schematic column preparation procedure.

observed, the frit was removed by cutting and the procedure repeated so as to obtain a satisfied tappered tip. Then the prepared column was slurry packed with 4 μm ODS silica gel in a 90 mm × 1 mm i.d. stainless steel column as slurry reservoir. The other capillary end was connected to the slurry reservoir by means of a screwed joint and a piece of 1/16″ PEEK tubing as sleeve. The slurries (20% stationary phase in toluene/cyclohexanol, 1/1, *v*/*v*) were sonicated for 10 min and filled into the slurry reservoir. After rapidly closing the reservoir, the capillary was immersed into the ultrasonic bath while the capillary with the end fitting was kept outside in a vertical position. The packing procedures could be carried out by the flush of slurry liquid, followed by methanol within 1 h.

Immobilized Microphere Column Preparation

After slurry packing, the capillary was forced with the polymerization mixture, respectively (Figure 1, steps 2 and 3). Here, the polymerization mixtures of BMA, EDMA, and AMPS were used following a similar recipe developed by several groups.[13–18,22,23] The procedure to prepare the reactant solution was described as follows: The entrapped mixture was made up of the monomer mixture (A) including 270 μL butylmethacrylate (BMA) as a functional monomer, 150 μL ethylene glycol dimethacrylate (EDMA) as a crosslinking agent, 0.004 g AIBN (1% monomer, *wt*%) as an initiator and porogenic mixture (B) including 217 μL 1,4-butanediol, 400 μL 1-propanol, and 6 0 μL water. The weight ratio (A/B, *w*/*w*) is kept 40 to 60. The reactant solution should be mixed ultrasonically into a homogenous solution, respectively, followed by purging with nitrogen for 10 min before a small part of the reactant solution was introduced into each capillary, respectively.

After several column volumes of the polymerization mixture had passed through the capillary, the packing pump was turned off and the outlet of the capillary was sealed with a septum. Then, the microsphere packed capillary was exposed to a home microwave oven for microwave irradiation about several minutes, which can facilitate microsphere entrapment (Figure 1, step 4). Irradiation for the prepared column was carried out in the home microwave oven with 80% output of a largest power and irradiation about 4 min. After that, methanol or acetonitrile was flushed through the capillary column back and forth with an HPLC pump, to remove unreacted monomeric materials and non-entrapped microspheres, and the column was cut to the desired length and coupled to an empty capillary segment on which a detection window was made for the next use and characterization (Figure 1, steps 5, 6, and 7). To prevent the prepared column from drying, it was stored by carefully immersing both ends of the capillary into vials containing acetonitrile solvent.

Prior to micro HPLC and CEC experiments, the prepared capillary should be coupled with another empty capillary by a commercial connector with a void volumn of 4 nL (P772, Upchurch Company, USA). A window about 1 cm length was created by scraping the polyimide coating of the empty capillary with 150 mm × 100 i.d. (from the window to the capillary outlet = 8.5 cm). A capillary with a length of 4 m, 100 μm i.d.; 375 μm o.d. was used to split the injection sample and mobile phases before the eluates flowed through the separation column. The hyphenated capillaries were flushed with mobile phase for 30 min before experiments. A preconditioning step was performed by applying a stepwise increase in voltage up to 30 kV over the column, until a stable current was observed in the mode of CEC.

Column Characterization

To avoid the use of frits in a packed CEC column, and to prevent particles flowing out of the column, a column with a single tapered tip was prepared for the next packing. Strict control of the morphology of the immobilized stationary phase is important to obtain a generic porous material that provides good separation efficiency and a low resistance to flow, since it enables easy flushing of the column with liquids that are used in the subsequent separation in the modes of capillary LPLC and CEC. The key variables that allow controlling the pore size are the percentage of crosslinking monomer and the composition of porogenic solvent. Entrapped microsphere columns have the advantage of immobilizing the chromatographic material, preventing the formation voids within the column, and eliminating the need for either an inlet or outlet frit. Coverage of the packing material during microsphere immobilization will affect column performance and has been reported by other researchers using alternative immobilization schemes.[10,12,22] The three typical cross sections of the prepared column (see Fig. 1, step 7) were thoroughly characterized in Figure 2. The sample preparation process for SEM resulted in the beads being scattered on the capillary surface and the inner diameter in the tapered section is narrower than that in the untapered section (100 μm). The organic polymer matrix was observed between the beads that both ''glued'' them together, whilst still leaving the majority of the surface to participate in a regular chromatographic participation. Although the capillary internal walls were not silanized and not covalently bound with the methacrylate ester polymerization mixture, the entrapped stationary phases could endure a high pressure over 30 MPa due to the formed polymer in the packing column and the effect of a single outlet taper. The former could prevent the formation

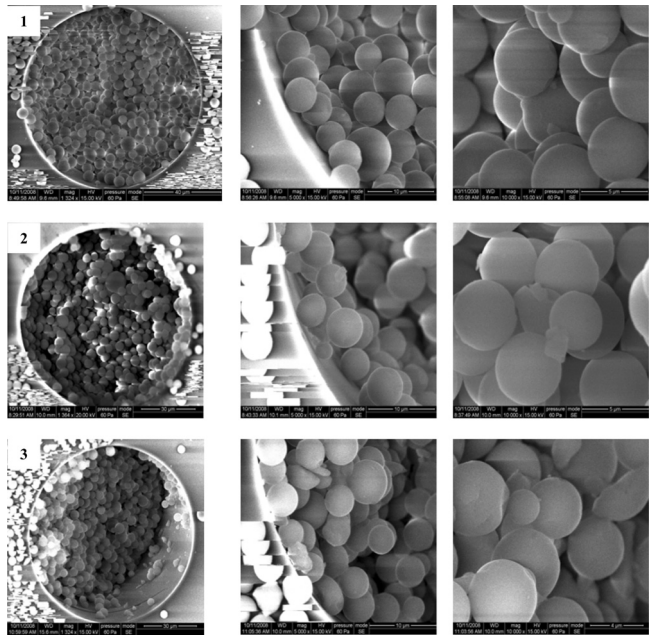

Figure 2. SEM of the different cross sections using the packing column immobilized within organic based polymer matrix. 1: Cross Section of the immobilized stationary phases, 2: Cross Section near to the taper, 3: Cross Section of the taper.

voids between the packing beads, whilst the latter could cause a stone arch, so as to avoid the loss of beads in the chromatographic runs.

Evaluation of Chromatographic Performance

A reversed phase mechanism was observed using the prepared column throughout all separation experiments. Solution partitioning between the mobile and stationary phases was the mechanism responsible for their retention of the model compounds. In general, sample injection was carried out by a nanoinjector in commercial micro HPLC instrument. In our experiment, eluent flow through the capillary separation column was controlled by a custom built adjustable flow splitter, based upon a T piece connector with another void capillary. The backpressure enforced on the separation column can be adjusted by the change of another capillary inner diameter and length. The splitting capillary is linked to the waste container under the atomsphere. Splitting ratio was calculated by weighing the eluates from the capillary outlets, which were collected in two sealed vials. Generally, the volume eluted from the T union was

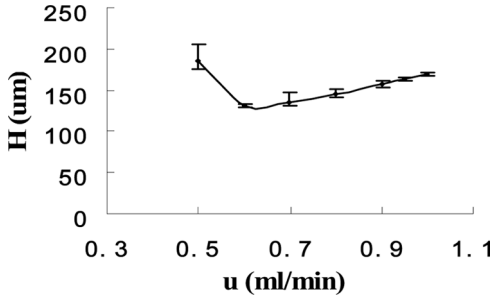

Figure 3. The relationship between the flow rate impelled by the high pressure pump and the theoretical plate hight (H) for last eluted analyte of ethylbenzene. Capillary: Total capilary Length = 34 cm, effective length = 26.5 cm (form capillary inlet to the window), the length of immobilized stationary phases = 19 cm, mobile phase: ($ACN/H_2O = 65/35$, *v*/*v*).

greater than that eluted from the separation column, due to the presence of back pressure from the separation column.

Figure 3 showed the relationship between the flow rate impelled by the high pressure pump and the theoretical plate height (*H*) of the last eluted analyte of ethylbenzene. With the flow rate changed from 0.500 mL/min to 1.000 mL/min with a stepwise of 0.100 mL/min, the back pressure of the prepared column increased from 9.4 MPa to 19.0 MPa. The curves were plotted using 7 points, with each point repeated at least three times, respectively. Baseline separation of all model compounds could be achieved in all experiments with the constant percentage of mobile phases (ACN/H2O = 65/35, *v*/*v*). The relevant theoretical plates were changed in the range of 5414 N/m to 7577 N/m for the last eluted component of ethylbenzene.

Figure 4 showed that the *H* values were changed with the percentage of mobile phase (ACN/H_2O, *v*/*v*) with a constant flow rate of 0.8 mL/min. The curves had similar trends over the full range of the percentage of mobile phase. The curves were plotted using 7 points, with each point repeated at least three times, respectively. With the change of mobile phase pencentage in the range of 80/20 to 50/50 with a stepwise of 5% ACN, the back pressure of the prepared column increased from 11.3 MPa to 18.7 Mpa. The relevant theoretical plates were changed in the range of 5032 N/m to 9216 N/m for the last eluted component of ethylbenzene, whilst the resolutions for each peak pair were apparently improved with the increase of the polar component (H_2O) in the mobile phase. Furthermore, the dead time by the prepared column was almost kept unchanged throughout all experiments, which was obtained taking thiourea, an unretained component, as the marker. The relative standard

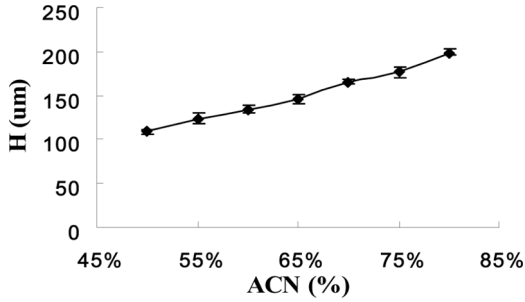

Figure 4. The relationship between percentage of mobile phases (ACN/H_2O) and the theoretical plate height (H) for last eluted analyte of ethylbenzene. Flow rate = 0.8 mL/min, other experimental conditions are the same as Figure 3.

deviation of retention time for thiourea was calculated equal to 2.08% (n = 33), which indicated no apparent change of flow rate in the separation capillary tube during all experiments. The typical chromatograms were shown in Figure 5. Moreover, it should be noted that the splitting

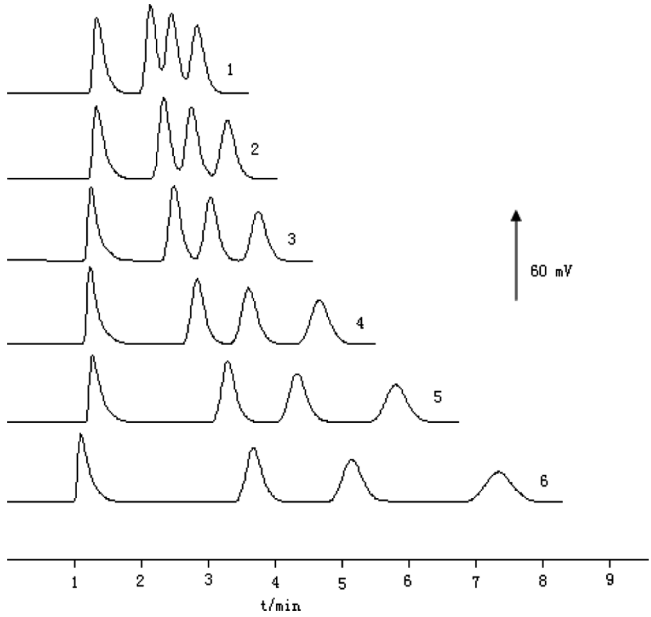

Figure 5. The typical chromatograms using different percentages of mobile phases. 1–6 stand for 80:20, 75:25 ,70:30, 65:35, 60:40, 55:45 of ACN/H_2O (v/v). Other experimental conditions are the same as figure 4. Peak order: 1. thiourea, 2. benzene, 3. toluene, 4. ethylbenzene.

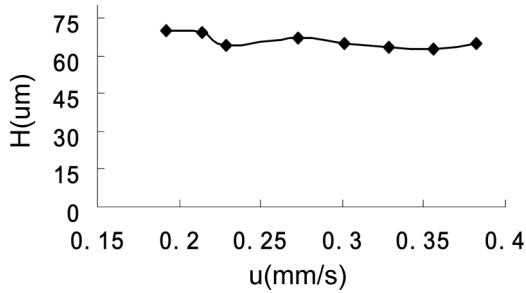

Figure 6. Van Deemter plot of ethylbenzene by CEC. Experimental condition: buffer: ACN/ 2 mM Tris (70/30, *v*/*v*); injection: 10 kV × 10 s; operation voltage:16–30 kV with a stepwise of 2 kV, both ends = 1.2 MPa, 14°C, DAD detector (wavelength 200 nm). Other experimental conditions are the same as Figure 3.

ratio increased apparently with the increase of flow rate rather than with the change of mobile phase percentage. It was probably attributed that the former led to apparent changes of column back pressures, whilst only small changes of column back pressures in the latter.

A comparative separation by electrochromatography was carried out using the prepared column. The Van Deemter plot for the last migrated analyte of ethylbenzene was shown in Figure 6. The small change of H throughout all experiments was mainly attributed to the dead volume of the coupling connector, and a typical electrogram was obtained using the selected experimental conditions in Figure 7. Although the

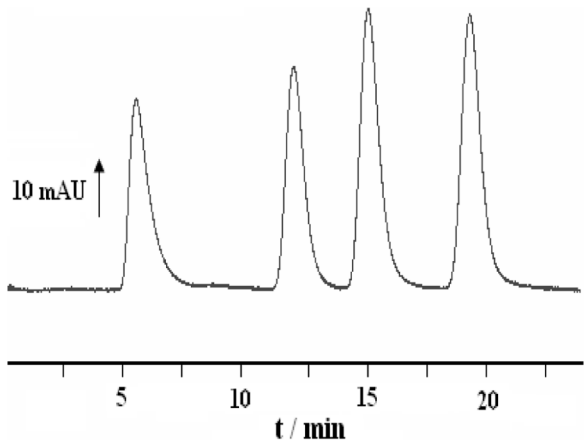

Figure 7. Electrochromatograms of the separation of the analytes. Experimental condition: operation voltage:20 kV. Other experimental conditions are the same as Figure 6.

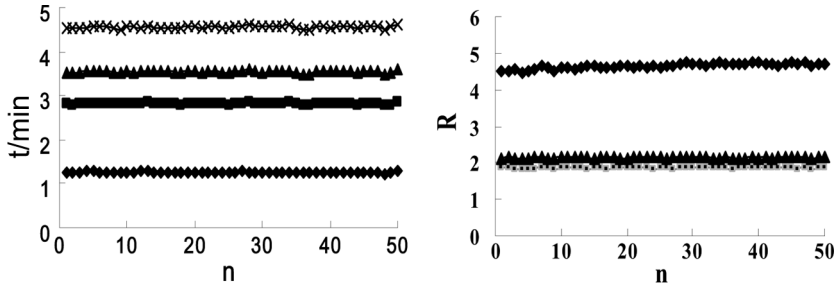

Figure 8. Reproducibility of retention time for four model analytes and resolution for three peak pairs (n = 50). Experimental Conditions:ACN:H_20 = 65:35, flow rate: 0.8 mL/min, splitting ratio: 747. Symbol: Thiourea(□), benzene(■), toluene(▲), ethylbenzene(×); R_{12} (□), R_{23} (▲), R_{34} (■).

chromatogram and electrogram shown here did not have very high separation efficiencies, the structure of the capillary column presented in this paper determined that it could minimize the dead volume and thus, the column should produce higher column efficiency if it was better packed and avoided the usage of the hyperlinked capillary. The effect of outlet taper and immobilized polymers was sufficiently stable enough to safely hold back the packing bed, and no bleeding of the particles was observed in at least 300 repeated operations. Since the aim of this work was to demonstrate that a simple method to immobilize the stationary phases by microwave irradiation can withstand high pressure and retain the packing material inside the fused silica capillary, the above experimental results are satisfactory. The prepared column was selected for the investigation of reproducibilities in micro HPLC (Figure 8). The relative standard deviations of the migration time and resolution for each analyte (n = 50) were calculated between 0.64% ~ 0.82% and 0.88% ~ 1.58%. The results indicated good run to run reproducibilities could be easily obtained and the immobilized stationary phases were stable enough for long runs.

CONCLUSIONS

Capillary HPLC with inner column diameter as low as 50 μm has established itself as a complementary technique to conventional LC columns routinely used in HPLC. It possesses some advantages such as high performance, rapid sensitive analysis with low cost operation, and environmental compatibility. An easy and fast method to retain the C_{18} particles in place using polymethacrylate base has been initially

attempted by microwave irradiation with the polymerization time about 4 min. The resulting stationary phase is well suited for the separation of a variety of neutral compounds in both capillary HPLC and CEC. Our intention in this technical note is simply to demonstrate that our approach is valid to prepare the fritless capillary column quickly. It exhibited good potential instead of the traditional thermal and UV light initiations. The main advantage of microwave irradiation as an energy source is its short polymerization time, simplicity, and no need to use brisk and expensive UV transparent capillaries. Furthermore, some application such as the immobilization for HPLC-MS and solid phase microextraction are going on in our lab. To better understand microwave heating, how electromagnetic fields are established inside the capillary and materials, and how they interact with the material at the molecular level, further studies should be necessarily carried out in the next research.

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