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Facile preparation of octadecyl monoliths with incorporated carbon nanotubes and neutral monoliths with coated carbon nanotubes stationary phases for HPLC of small and large molecules by hydrophobic and π–π interactions

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ABSTRACT

Two approaches for incorporating carbon nanotubes into monolithic columns for HPLC are described in this report. They pertain to the investigation of carbon nanotubes either (i) as entities to modulate solute retention on monolithic columns bearing well defined retentive ligands or (ii) as entities that constitute the stationary phase responsible for solute retention and separation. Approach (i) involved the incorporation of carbon nanotubes into octadecyl monolithic columns while approach (ii) concerns the preparation and evaluation of an ideal monolithic support and coating it with carbon nanotubes to yield a real "carbon nanotube stationary phase" for the HPLC separation of a wide range of solutes. First, an octadecyl monolithic column based on the *in situ* polymerization of octadecyl acrylate and trimethylolpropane trimethacrylate was optimized for use in HPLC separations of small and large solutes (e.g., proteins). To further modulate the retention and separation of proteins, small amounts of carbon nanotubes were incorporated into the octadecyl monolith column. In approach (ii), an inert, relatively polar monolith based on the *in situ* polymerization of glyceryl monomethacrylate (GMM) and ethylene glycol dimethacrylate (EDMA) proved to be the most suitable support for the preparation of "carbon nanotube stationary phase". This carbon nanotube "coated" monolith proved useful in the HPLC separation of a wide range of small solutes including enantiomers. In approach (ii), a more homogeneous incorporation of carbon nanotubes into the diol monolithic columns (i.e., GMM/EDMA) was achieved when hydroxyl functionalized carbon nanotubes were incorporated into the GMM/EDMA monolithic support. In addition, high power sonication for a short time enhanced further the homogeneity of the monolith incorporated with nanotubes. In all cases, nonpolar and π interactions were responsible for solute retention on the monolith incorporated carbon nanotubes.

1. Introduction

Monolithic columns are continuously attracting strong interest due to their unique characteristics such as high permeability [1,2] and excellent mass transfer properties that arise from their flow throughpores [3,4] in contrast with columns packed with microparticles that are characterized by slow diffusional mass transfer in the porous particles and large void space between the packed particles [5–8] which translate into a relatively increased band broadening. Polymer-based monolithic columns, which are the subject of this investigation, have evolved significantly in the last decade and have proven useful in the separation of a wide range of mixtures [9–14]. Although polymer-based monolithic columns can be readily prepared and confined in columns in all sizes, these media suffer from low surface area and in turn limited retention toward small solutes. However, their low surface areas make monolithic columns ideal for the efficient separations of biopolymers (e.g., proteins) using gradient elution in HPLC [6,15] or simply isocratic elution in capillary electrochromatography (CEC) [16,17].

Some of the characteristics of polymer-based monoliths, namely their high permeability and throughpores can be exploited to design stationary phases of different selectivities than the simple nonpolar monolithic columns with alkyl ligands by incorporating into their structure nano-entities to the extent that these entities would not obstruct the porous structures of the original

Abbreviations: AIBN, 2,2′-azobis(isobutyronitrile); BMA, butyl methacrylate; Dns, DL-dansyl; EDMA, ethylene glycol dimethacrylate; GMM, glyceryl monomethacrylate; MMA, methyl methacrylate; MWCNTs, multiwalled carbon nanotubes; ODA, octadecyl acrylate; TFA, trifluoroacetic acid; TRIM, trimethylolpropane trimethacrylate

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monoliths or disrupt the realization of a mechanically stable monolithic structure. Under these conditions, the incorporated nano entities may not increase significantly the surface area of the monolith but rather they would provide other interactions in the aim of achieving different selectivity that would compensate for the limited retention generally observed with small molecules. In another thought, the ideal flow characteristics of monoliths should make them suitable support for nano entities that can afford distinct selectivity toward a wide range of solutes with the aim of realizing "nano entities-based stationary phases".

A few nano entities, including carbon nanotubes and nanoparticles, fullerenes and nanodiamonds as well as metal oxide and gold nanoparticles have been reported in nanomaterials-based separation media for gas chromatography, HPLC, CEC, CE and microchip electrophoresis and the field has been reviewed recently in 2012 and 2013 by Speltini et al. [18], Nesterenko et al. [19] and by Pauwels and Van Schepdael [20]. As far as HPLC with organic-based polymer monolithic columns having incorporated carbon nanoparticles is concerned, only a few attempts have been made in this area (see Svec's recent review article [21]). After a brief study reported in 2005 by Li et al. [22] which involved nano-LC with carbon nanotubes entrapped into a monolithic capillary column made of poly(chloromethylstyrene-co-ethylene dimethacrylate), Chambers et al. [23] reported in 2011 the incorporation of carbon nanotubes in porous polymer monolithic capillary columns consisting of poly (glycidylmethacrylate-co-ethylene dimethacrylate) monoliths for the chromatographic separation of some alkylbenzenes. More recently, Arrura et al. [24,25] introduced monolithic cryopolymers with neutral and charged embedded nanoparticles for capillary liquid chromatography of proteins under a hydrophobic interaction chromatography mode as well as under ion exchange conditions. Although nonpolar polymer based monoliths allows the rapid separation of proteins by linear gradient elution HPLC, the nonpolar monolithic phases may benefit from fine tuning their selectivity by incorporating adequate amount of nano entities into their structures such as carbon nanotubes.

Thus, it is the aim of this investigation to optimize nonpolar octadecyl monolith for HPLC separations by adjusting the fabrication conditions of these monoliths and by further incorporating into their structure an adequate amount of carbon nanotubes. Also, reported here are some initial studies on the fabrication of a blank monolith "void" of strong interactions with the solutes of interest in the aim of functioning as an ideal support for realizing columns coated with "carbon nanotube stationary phases" for the HPLC of small solutes including some enantiomers.

2. Materials and methods

2.1. Apparatus

The HPLC setup consisted of a quaternary solvent delivery system Model Q-Grad pump from Lab Alliance (State College, PA, USA), a multiple solvent delivery system Model CM4000, and a Model SpectroMonitor 3100 UV–vis variable wavelength detector from Milton Roy, LDC division (Riviera Beach, FL, USA) and a Rheodyne injector Model 7010 (Cotati, CA, USA) equipped with a 20 μL loop. A constant pressure air-driven pump Model Shandon from Southern Products Limited (Cheshire, UK) was used for column packing. A Branson1510 ultrasonic cleaner from Branson Ultrasonic Corp. (Danbury, CT, USA). A water bath equipped with a Fisher Scientific Isotemp 2100 immersion circulator and a high power sonicator Model 50 Sonic Dismembrator were from Thermo Fischer Scientific (Waltham, MA, USA).

2.2. Reagents and materials

Multiwalled carbon nanotubes were purchased from Sun Innovation Inc. (Fremont, CA, USA). Alkylbenzenes, phenoxy acid herbicides, cyanobenzene derivatives, benzonitrile, aniline derivatives, trifluoroacetic acid (TFA), 2,2′-azobis(isobutyronitrile) (AIBN), octadecyl acrylate (ODA), butyl methacrylate (BMA), trimethylolpropane trimethacrylate (TRIM), ethylene glycol dimethacrylate (EDMA), ethylene glycol, methyl methacrylate (MMA), chlorophenols, 1-dodecanol, cyclohexanol, DL-dansyl (Dns) amino acids, were purchased from Sigma Aldrich (Milwaukee, WI, USA). Glyceryl monomethacrylate (GMM) was from Monomer–Polymer and Dajac Labs (Trevose, PA, USA). Egg white lysozyme, bovine serum albumin, ribonuclease A, ovalbumin, horse heart cytochrome C, bovine erythrocytes carbonic anhydrase, bovine milk β–lactoglobulin A and B and bovine milk α-lactalbumin were purchased from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile and isopropyl alcohol were purchased from Pharmco Aaper (Brookfield, CT, USA). Stainless steel tubing of 4.6 mm id was obtained from Alltech Associates (Deerfield, IL, USA).

2.3. Preparation of monolithic columns

In all cases, and for the preparation of ODM based monoliths, polymerization mixtures consisting of 5.5 g each were prepared by weighing monomers and porogens as follows. 7-wt% ODA and 14.5-wt% TRIM were added to 78.5-wt% ternary porogen of cyclohexanol, ethylene glycol and water. While the % water was maintained constant at 3.2-wt%, cyclohexanol composition was decreased in the range of 54.2–50-wt% and that of ethylene glycol was increased in the range of 20.9–24.48-wt% so that the total porogen amounted in total to 78.5-wt%. All polymerization solutions for making the monoliths were vortexed for 1 min, sonicated at 40 °C for 15 min, purged with nitrogen for 5 min and introduced into stainless steel columns with dimensions of 25 cm × 4.6 mm I.D. that function as a mold for the monolith. Both column ends were plugged tightly and heated at 60 °C in a water bath for 15 h. The monolithic column was washed with acetonitrile for 30 min followed by isopropyl alcohol. The monolith was transferred from 25 cm mold to a shorter column of 10 cm × 4.6 mm I.D. by connecting the two columns with ¼ -union and passing isopropyl alcohol using a constant pressure pump starting at 6000 psi until the monolith was completely transferred. The transfer of monoliths from column-to-column has been performed in our laboratory for many HPLC column applications without noticeable adverse effect on column performance; for typical recent references see [26,27]. This is because by producing the initial monolithic mold in a longer column than the final column length, any shrinkage due to the formation of the monolith will be at the inlet of the mold. In the transfer process, the shorter column is connected to the bottom of the mold *via* a union. Under this condition, the final monolithic column is filled totally with the monolith without any void in its structure. Different amounts and types of MWCNTs were added to the ODM monolith as discussed later.

For the monoliths with retention characteristics due to the incorporated carbon nanotubes, polymerization mixtures of 6 g each were prepared by weighing monomers and porogens as described below. All the mixtures were first vortexed for 1 min, sonicated at 40 °C for 15 min, purged with nitrogen for 5 min and then introduced into a stainless steel column of dimensions 25 cm × 4.6 mm I.D. that functions as a mold for the monolith. Both column ends were plugged tightly with column end fittings and thereafter heated at 50–60 °C in a water bath for 15–20 h. The monolithic columns thus obtained were washed as stated in the preceding section. The monolith was transferred from 25 cm mold to a shorter column of 10 cm × 4.6 mm I.D. as explained above.

As discussed below, a series of monolithic compositions at various amounts and types of MWCNTs were tested. In order to homogenize the nanotubes and temporarily minimize their aggregation, the MWCNTs were dissolved in 1-dodecanol and subjected to high power sonication for 1 min, 15 min or 30 min while keeping the vial in ice to prevent evaporation and then add the monomers and the rest of the materials to prepare the given monolith.

3. Results and discussion

3.1. Optimizing an octadecyl monolithic column for use in HPLC

3.1.1. Chromatographic evaluation and optimization

The major criteria that must be met for using a column in HPLC include, among other things, good mechanical stability and permeability at elevated flow velocity and most importantly ensuring good separation efficiency. On this basis, an octadecyl monolith (ODM), which was originally developed and optimized by Karenga and El Rassi [28] for CEC separations of small molecules and proteins using capillary tubes of 100 μm I.D., was prepared at a larger scale in stainless steel tubing of 4.6 mm I.D. and tested in HPLC. First, the ODM column for HPLC was fabricated form a polymerization mixture having the same composition as for the CEC ODM column, which consisted of 7-wt% ODA and 14.5-wt% TRIM in a ternary porogen of cyclohexanol, ethylene glycol and water at 54.3-wt %, 21-wt% and 3.2-wt%, respectively, in the presence of 1-wt% AIBN with respect to monomers and heated at 60 °C for 15 h. The ODM column thus obtained exhibited low permeability and in turn high backpressure. Furthermore, this ODM column did not show good separation efficiency for both proteins and alkylbenzenes. This may be due to the high mass transfer resistance in small pores as it was manifested by the relatively high backpressure observed with the column. In CEC, the electroosmotic flow permits the mobile phase to flow in narrow channels, a fact that allows the solute to be transported by the flow throughout the column with much less mass transfer resistance than encountered in pressure driven flow in HPLC whereby the mobile phase is rather stagnant in narrow channels and the solutes face enormous amount of mass transfer resistance (controlled by diffusion) that can cause excessive band broadening. Therefore, it is not surprising to observe that the ODM monolith that proved useful for CEC was not suitable for HPLC.

In order to generate an HPLC column of higher permeability (i.e., monolith with increased throughpores content), the porogen composition was changed to 53.58-wt% cyclohexanol and 21.8-wt % ethylene glycol while keeping the % of water the same at 3.2-wt% and the ODA and TRIM monomers composition the same at 7-wt% and 14.5-wt%, respectively. The column thus obtained (designated ODM-1) did not show much improvement over the ODM column in terms of protein or alkylbenzene separations. However, and under isocratic elution with a mobile phase at 65% v/v ACN in water, a noticeable increase in solute retention factor (nearly 40% on the average) for seven alkylbenzenes (i.e., C1–C7) was observed on the ODM-1 column when compared to the ODM column. Under gradient elution, the retention times of four standard proteins, namely ribonuclease A, cytochrome C, bovine serum albumin and ovalbumin did not show much change when going from ODM to ODM-1 column. Following these findings, further decrease in the cyclohexanol concentration to 52.4-wt% and increase in the concentration of ethylene glycol to 22.9-wt% were undertaken. The ODM-2 column thus prepared showed narrower peaks for proteins (Fig. 1A) than ODM and ODM-1, but was not the optimum column for the separation of alkylbenzenes. When compared to the ODM-1 column, the ODM-2 column yielded moderately higher retention factor for alkylbenzenes (nearly 10% increase on the average) but did not show efficient peaks under the same isocratic elution conditions. A fourth column designated ODM-3 was prepared by further decreasing the cyclohexanol content to 51.5-wt% and increasing the ethylene glycol content to 23.8-wt% while keeping the content of the other constituents (i.e., water, ODA and TRIM) in the polymerization mixture the same. The ODM-3 column showed decreased performance for proteins (Fig. 1B vs. 1A) but was ideal for alkylbenzenes in terms of retention and peak shape (Fig. 1C). The retention factors for the first seven homologs of the alkylbenzenes series (i.e., C1–C7) increased on the average by 35% when going from ODM-2 column to ODM-3 column under otherwise the same isocratic elution conditions. The retention times of the four standard proteins were almost the same on the ODM-3 column than the ODM-2 column (compare Fig. 1A and B). This may indicate that the porosity of ODM-3 is ideal for alkylbenzenes but less favorable for larger size protein solutes. The columns obtained by further decreasing the cyclohexanol and increasing the ethylene glycol contents of the polymerization mixture showed less performance for the separations of both proteins and alkylbenzenes.

In short, shaper peaks were observed for proteins using the ODM-2 column while the ODM-3 column showed enhanced separation for alkylbenzenes. The above chromatographic results indicate that small changes in the porogen contents (~2.8-wt% in cyclohexanol and ethylene glycol) may have affected significantly the morphology of the ODM monolith in terms of total porosity as was manifested in a quite large increase in the retention of alkylbenzenes under isocratic elution. In other words, it could be envisioned on the basis of the chromatographic results that the total porosity was increased, which in turn increased the phase ratio too. This effect is not apparent in the case of proteins that were separated by gradient elution, which is well known to obliterate major differences in retention. Adsorbents with higher retention (i.e., larger phase ratio) provide efficient columns [29]. The fact that ODM-3 offered the best performance toward alkylbenzenes may suggest that the ODM-3 column has an increased number of mesopores that are easily accessible by small molecules such as alkylbenzenes.

The ODM-2 column was further evaluated with a few other standard proteins to explore its potentials in resolving complex protein mixtures using linear ACN gradient. As shown in Fig. 1D, the column was able to successfully separate 7 proteins including ribonuclease A, cytochrome C, lysozyme, transferrin, BSA, β-lactoglobulin A and ovalbumin with the retention times of 6.8, 7.72, 8.14, 8.54, 8.82, 9.27 and 10.5 min, respectively. The elution order of the proteins shown in Fig. 1D reflects the expected RPC behavior on the ODM-2 column. Ribonuclease A and cytochrome C are hydrophilic proteins with relatively low molecular weights of 12, 200 and 13,500, respectively, and therefore, they are expected to elute faster than other proteins. Other proteins having higher molecular weights such as BSA, β-lactoglobulin A, ovalbumin are hydrophobic proteins and consequently exhibit more hydrophobic interactions with the ODM-2 monolithic stationary phase leading to more retention for these three proteins. Even though it has a higher molecular weight than ovalbumin and β-lactoglobulin A, BSA eluted faster than these two proteins. Similarly, despite the fact that ribonuclease A has a higher molecular weight than cytochrome C it eluted faster. This observation for RPC retention indicates that the native protein hydrophobicity or its size are not the only factors determining the retention of the solutes, since proteins can undergo denaturation in a hydro-organic mobile phase. The acid (e.g., TFA) and organic solvent in the mobile phase partially denature the proteins, thereby exposing the more hydrophobic interiors of the protein molecules to the nonpolar stationary phase [30–32].

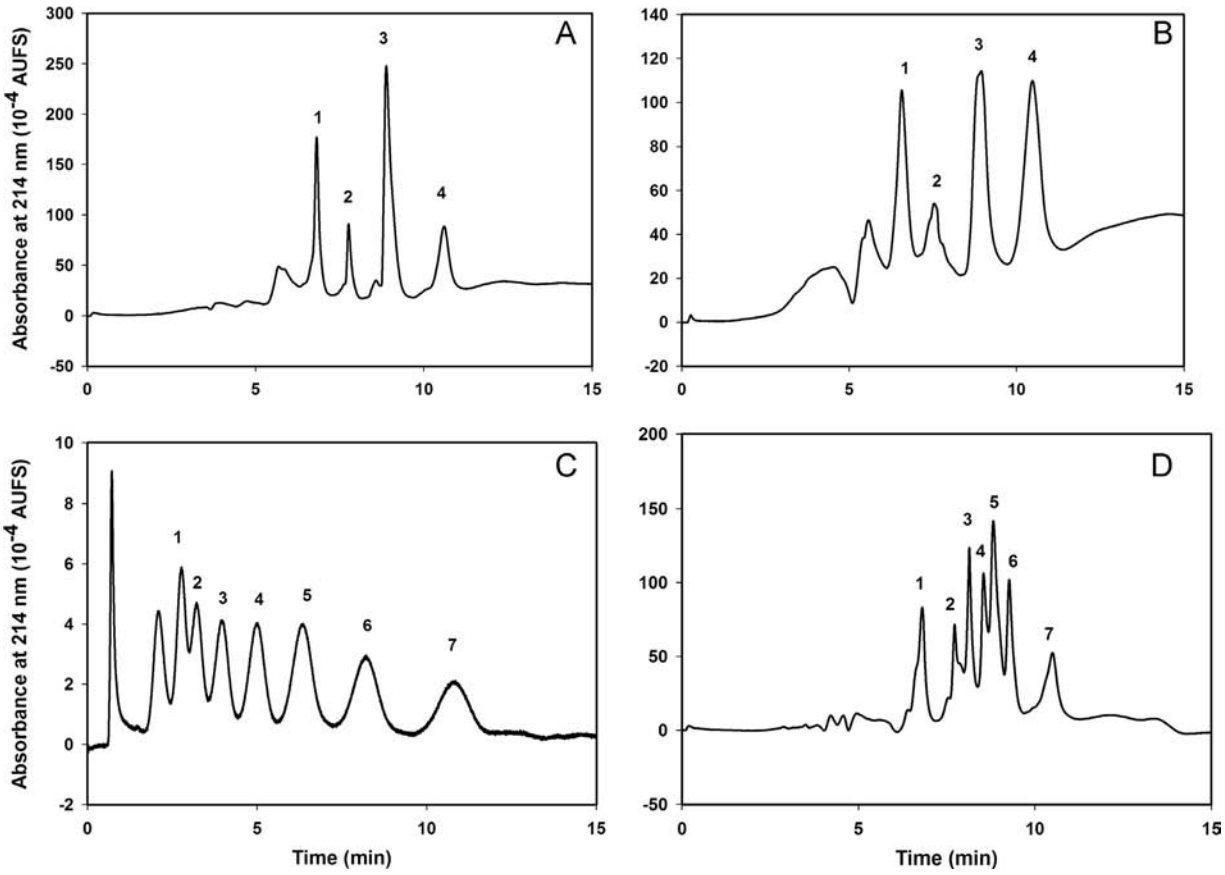

Fig. 1. Chromatograms of standard proteins in (A), (B) and (D) and alkyl benzenes in (C) obtained on ODM-2 in (A) and (D) and ODM-3 in (B) and (C). Column dimensions, 10 cm × 4.6 mm id; flow rate, 1 mL/min. Proteins were chromatographed using a linear ACN gradient while alkyl benzenes were chromatographed using isocratic elution. Linear ACN gradient was carried out by increasing the % mobile phase B in mobile phase A from 0 to 75% v/v in 12 min. Mobile phase A consisted of H_2O:ACN at 95:5 (v/v) containing 0.1% TFA and the mobile phase B consisted of ACN:H_2O at 95:5 (v/v) containing 0.1% TFA. Isocratic elution in (C) was carried out with a mobile phase consisting of ACN:H_2O at 65:35 (v/v) containing 0.1% TFA. Solutes in (A) and (B): (1), ribonuclease A; (2), cytochrome C; (3), bovine serum albumin; (4), ovalbumin. Solutes in (C): (1), toluene; (2), ethylbenzene; (3), propylbenzene; (4), butylbenzene; (5), amylbenzene; (6), hexylbenzene; (7), heptylbenzene. Solutes in D: (1), ribonuclease A; (2), cytochrome C; (3), lysozyme; (4), transferrin; (5), bovine serum albumin; (6), β-lactoglobulin A; (6), ovalbumin.

3.1.2. ODM-2 monolithic column with incorporated carbon nanotubes

The ODM-2 column, which showed the best performance for the separation of proteins was further optimized by incorporating MWCNTs. The goal of adding MWCNTs to the monolith was to modulate the retentive property of the monolith since the MWCNTs, which are carbon allotropes with cylindrical structures, are hydrophobic in nature [33], and also would establish π–π interactions with aromatic and π-bond rich solutes including proteins with aromatic amino acid residues (e.g., tryptophan, phenylalanine and tyrosine). On this basis, it was expected to enhance retention and in turn resolution of some proteins with the addition of MWCNTs to the ODM-2 column.

First, a series of columns was prepared by adding different amounts of MWCNTs of part #SN2302 to the ODM-2 monolith. The SN2302 MWCNTs have the following physical properties: an outer diameter (OD) of 10–20 nm, inner diameter (ID) 5–10 nm with a length of 0.5–2 μm. In a first attempt, 150 mg of MWCNTs with part #SN2302 was added to the polymerization mix of ODM-2 monolith. This mix exhibited thick appearance and the resulting monolith showed very high backpressure. It did not yield any separation for proteins or alkylbenzenes. This may indicate that the nanotubes at such large amount of 150 mg have blocked the pore structure of the monolith, thereby decreasing its permeability and leading to poor chromatographic retention and selectivity. This finding called for decreasing the amount of added nanotubes, and as a result the column performance improved in terms of pressure and also showed improved separation of proteins as the amount of nanotubes was decreased. Finally, an enhanced performance was achieved at 8 mg MWCNTs with part #SN2302 as shown in Fig. 2A.

In another set of experiments, MWCNTs of part #SN 6957838 were incorporated into the ODM-2 monolith. The SN 6957838 MWCNTs have an OD of 20–30 nm, an ID of 5–10 nm and a length of 1–2 μm. ODM-2 monoliths were prepared by adding 25 mg, 12.5 mg or 8 mg of SN6957838 MWCNTs. It was observed that the optimum separation was achieved with the monolith containing 12.5 mg of MWCNTs, see Fig. 2B. The optimum incorporated amount of MWCNTs seems to depend on the physical characteristics of the carbon nanotubes, which in this case seems to depend on the carbon nanotubes outer diameter. The larger outer diameter nanotubes (i.e., SN6957838 MWCNTs) provided sharper peaks for proteins but necessitated the addition of a larger amount of nanotubes amounting to 12.5 mg as opposed to 8 mg of the MWCNTs with part #SN2302. This may be due to the fact that the specific surface area of the large OD nanotube is smaller than that of the narrower OD nanotubes ($>120\ m^2/g$ for SN6957838 vs. $>200\ m^2/g$ for #SN2302).

The ODM-2 monolith with incorporated 12.5 mg SN6957838 MWCNTs was produced in duplicate ($n=2$). The column-to-column reproducibility in terms of % RSD of retention times obtained in gradient elution for seven standard proteins (see Fig. 2B) including ribonuclease A, cytochrome C, lysozyme, transferrin, bovine serum albumin, β-lactoglobulin A and ovalbumin were 5.0%, 4.2%, 3.7%, 3.3%, 2.8%, 3.2% and 2.7%, respectively.

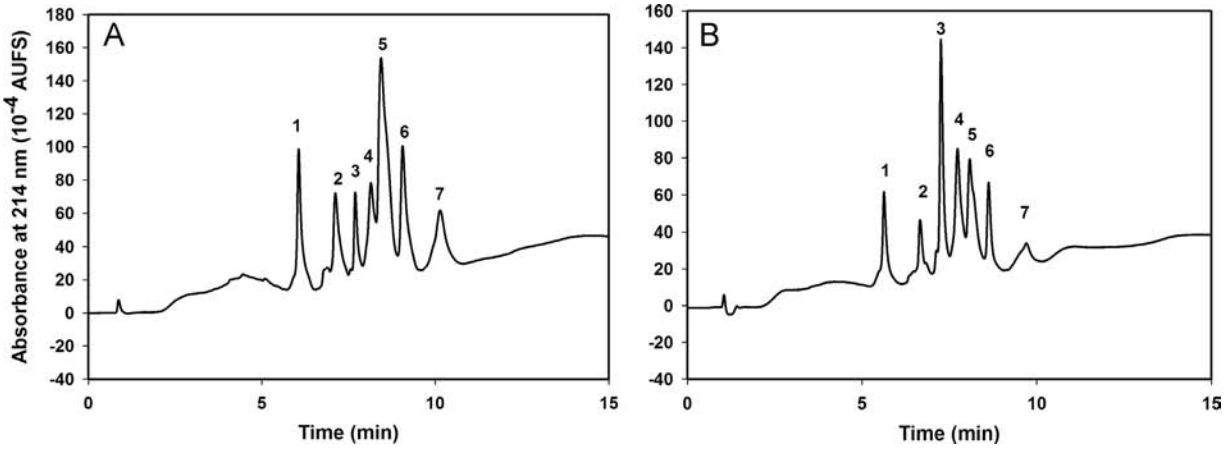

Fig. 2. Chromatograms of proteins obtained on ODM-2 incorporated with 8 mg MWCNTs #SN2302 and 12.5 mg MWCNTs #SN6957838 in (A) and (B), respectively. Column dimensions, gradient elution condition and flow rate as in Fig. 1; solutes as in Fig. 1D.

In summary, The ODA/TRIM (i.e., ODM) column was successfully scaled up/optimized for use in RPC separations by HPLC. A series of monolithic columns was developed and the performance of each column was evaluated with alkylbenzenes and standard proteins. The ODM-2 column showed optimum performance towards proteins while the ODM-3 column yielded better separation for alkylbenzenes. The addition of MWCNTs to the ODM-2 monolith generally resulted in enhanced separation for proteins. The column, which was prepared by adding 12.5 mg of SN6957838 MWCNTs to the ODM-2 monolith was quite reproducible and showed an optimum performance for the separation of a mixture of seven standard proteins (see Fig. 2B). The enhanced separation of proteins on the ODM-2 monolith incorporating MWCNTs is believed to be due to the concurrence of both hydrophobic and π–π interactions.

3.2. *Monolithic columns with retention principally due to incorporating MWCNTs*

3.2.1. *Finding the suitable monolithic "support"*

This part of the investigation is aimed at developing a monolith that functions as an "ideal" support for incorporating nanotubes to serve as "nanotube" stationary phases for HPLC separations. Thus, the support monolith should meet some criteria, which include, among other things, minimum contribution from the monolith backbone to the overall solute retention, acceptable permeability at high flow velocity, and useful retention and selectivity due in large part to the incorporated nanotubes for a wide range of solutes. In order to find the monolithic support best suited to incorporate MWCNTs, a series of monolithic columns of different monomer compositions incorporating different nanotubes was tested with alkylbenzenes and standard proteins. Also, a blank monolith (i.e., without incorporated nanotubes) was prepared each time and tested with the same set of probe solutes to examine the contribution of the monolith backbone to solute retention. A brief trial and error among various monomers and crosslinkers was undertaken. This involved the *in situ* polymerization of BMA (30-wt%)/EDMA (20-wt%) in the presence of the ternary porogen composed of 1-propanol (30-wt%), 1,4-butanediol (15-wt%) and water (5-wt%) at 60 °C for 15 h or the *in situ* polymerization of MMA (12-wt%)/TRIM (28-wt%) in the presence of 1-propanol (36-wt%), water (6-wt%) and 1,4-butanediol (18-wt %) at 60 °C for 15 h. While the BMA/EDMA did not meet the criteria as a blank monolith, since it showed some retention toward alkylbenzenes and proteins, the MMA/TRIM monolith yielded as expected much less residual retention toward the test solutes. MMA has a smaller alkyl chain than BMA, and therefore, it should generate a less hydrophobic monolith. Although the MMA/TRIM with its permeability and weak hydrophobicity was a suitable blank monolith, it did not allow the incorporation of MWCNTs as a homogeneous dispersion in the monolith, a fact that led to a monolith with low chromatographic performance.

All of the above monoliths were unsatisfactory because they showed either residual hydrophobicity as a blank monolith (e.g., BMA/EDMA monolith) or exhibited low chromatographic performance in terms of separation efficiency upon incorporating nanotubes (e.g., MMA/TRIM monolith). Finally, the suitable blank monolith was achieved by the *in situ* polymerization of the monomers GMM (18-wt%)/EDMA (12-wt%), in the presence of the binary porogen composed of cyclohexanol (35-wt%) and dodecanol (35-wt5) at 55 °C for 15 h. As expected, the blank monolith thus obtained (referred to as MN0) yielded no retention towards alkylbenzenes as shown in Fig. 3A.

3.2.2. *Incorporating MWCNTs into the MN0 monolith.*

The blank MN0 possesses diol groups on its surface, which confer to this monolith some hydrophilic character and as a result the MN0 exhibited no significant retention toward alkylbenzenes as shown in Fig. 3A. Using the nanotubes of batch #SN2302 and #SN6957838, which dispersed nicely in the ODM monoliths were not the ideal ones for incorporating them in the polar MN0. An OH functionalized MWCNT of batch #SN32547 was chosen instead. This OH functionalized MWCNT dispersed more uniformly in the polymerization mixture used for the *in situ* polymerization of the MN0 in the presence of nanotubes may be due to the mutual interaction of the OH groups of the monomer GMM and the SN32547 nanotubes.

Nanotubes are inert, hydrophobic in nature and may undergo strong van der Walls and π–π interactions with the solutes. Due to these characteristics, the MWCNTs are expected to achieve the desired retention for solutes under RPC mobile phase conditions that consist of hydro-organic mobile phases. First, 12.5 mg of MWCNTs batch #SN32547, which consists of OH-MWCNTs and has the following specifications: 10–20 nm OD, 5–10 nm ID, and 10–30 μm length were incorporated into the MN0 to yield MN1. The monolithic column thus prepared with OH-MWCNT showed retention and partial separation, see Fig. 3B. To obtain a column with optimized retention characteristics, the amount of OH-MWCNTs added to the polymerization mixture was varied. By decreasing the amount of nanotubes from 12.5 mg to 6 mg to yield MN2, the separation of the alkylbenzenes was improved as shown

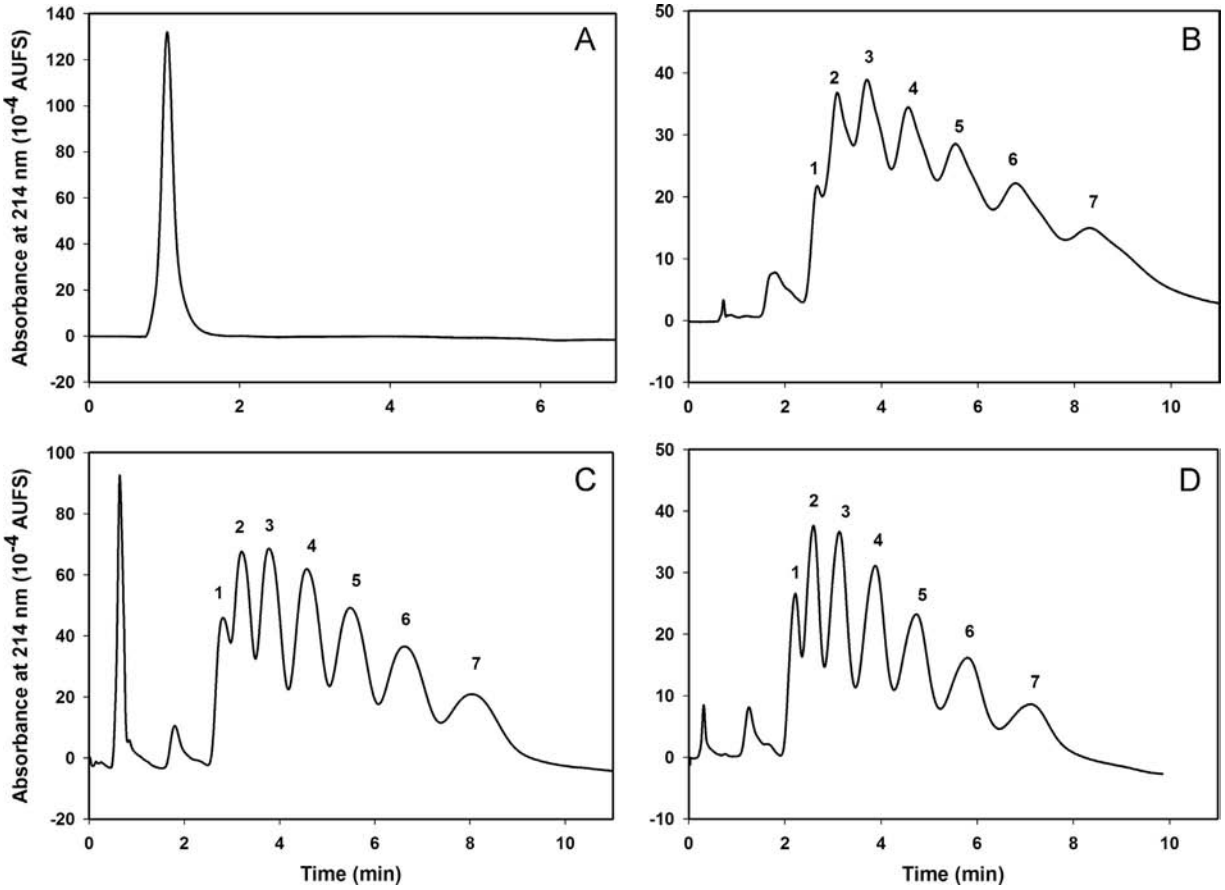

Fig. 3. Chromatograms of a mixture of 7 alkylbenzenes (same as in Fig. 1) obtained on MN0, MN1, MN2 and MN3 monoliths incorporated with zero, 12.5 mg, 6 mg and 3 mg OH-MWCNTs SN32547, respectively. Mobile phase, ACN:H_2O at 35:65 (v/v) containing 0.1% TFA; column dimensions, 10 cm × 4.6 mm I.D.; flow rate, 1 mL/min. The solutes which are not resolved in (A) are numbered in (B)–(D) as in Fig. 1D.

in Fig. 3C. The separation was further improved (see Fig. 3D) by decreasing the amount of nanotubes to 3 mg to yield MN3. Out of these monoliths, MN3 with 3 mg of OH-MWCNTs showed the best retention and separation for alkylbenzenes, see Fig. 3D. Decreasing the amount of nanotubes may have resulted in decreasing their destabilizing effect on the morphology of the monolith thus yielding improved separation. The column made with 1 mg of OH-MWCNTs, poorly separated alkylbenzenes, a fact that further confirms that MWCNTs are the main contributors to the separation of alkylbenzenes. The results in terms of k' values for alkylbenzenes obtained on the various monoliths as a function of amount of nanotubes added to the monoliths are plotted in Fig. 4. As can be seen in this figure, by increasing the amount of nanotubes incorporated into the monolith from 1 to 12.5 mg, and using isocratic elution at 1 mL/min with a mobile phase at 35% v/v ACN in water, the k' values obtained for the alkylbenzenes under investigation increased by about 20%. The increase in the k' values is not commensurate with the increase in the amount of nanotubes incorporated in the monolith, a fact that indicate that the nanotubes at high amount are rather not accessible to solute interactions. The increase in the k' values of the alkylbenzenes under RPC elution conditions is believed to arise primarily from the hydrophobic character and π–π interactions of the column, which increased upon increasing the amount of OH-MWCNTs in the polymerization mixture.

In order to achieve a better dispersion of the incorporated nanotubes, the OH-MWCNTs were subjected to a high power sonication at varying sonication times before adding the nanotubes to the polymerization mixture. 1 min and to a larger extent 15 min high power sonication times resulted in sharper peaks (see

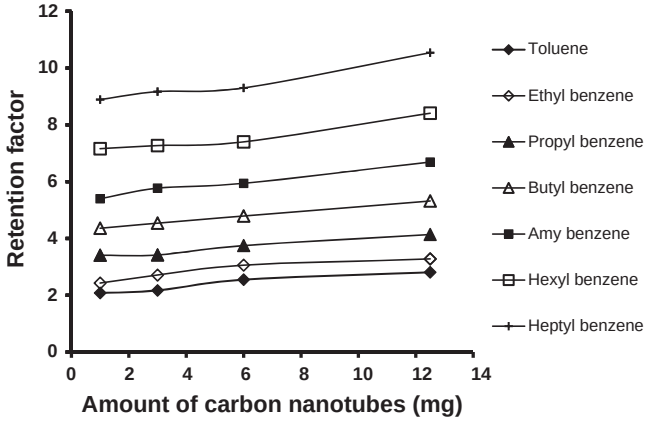

Fig. 4. Retention factors of alkylbenzenes obtained on the GMM/EDMA monolithic columns incorporating various amounts of OH-MWCNTs. Other conditions are as in Fig. 3.

Fig. 5A and B). The column made using OH-MWCNTs at 30 min sonication exhibited decrease in the performance (see Fig. 5C). High power sonication is known to improve the dispersion of nanotubes in solutions when performed for short duration [34,35]. However, prolonged high power sonication may shorten the length of the nanotubes and may cause defects and changes in the properties of nanotubes [36,37]. Therefore, for the rest of the investigation, 15 min sonicated nanotubes were used, and the MN3 column whose incorporated nanotubes were sonicated for 15 min was designated as MN3-15. This MN3-15 column was

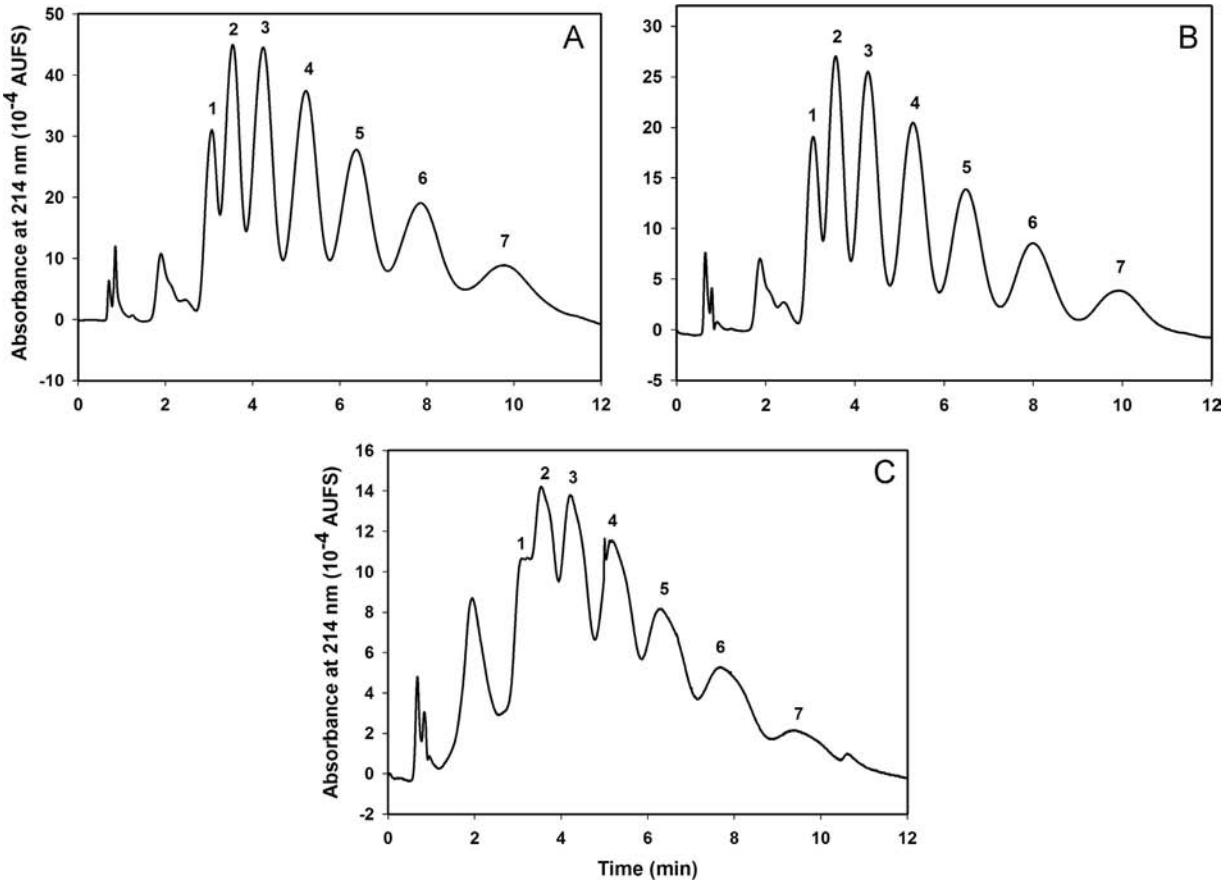

Fig. 5. Chromatograms of alkylbenzenes obtained on the MN3 monolithic column using 1 min, 15 min, and 30 min high power sonication in (A), (B) and (C), respectively. Mobile phase, ACN:H_2O at 35:65 (v/v) containing 0.1% TFA. Solutes and conditions are as in Fig. 3.

prepared in triplicate ($n=3$) and tested with alkylbenzenes for its reproducibility. The % RSD of the retention factors obtained in isocratic elution under the conditions of Fig. 5B were 4.4%, 3.6%, 4.3%, 4.2%, 3.2%, 3% and 2.8% for toluene, ethylbenzene, propylbenzene, butylbenzene, amylbenzene, hexylbenzene and heptylbenzene, respectively.

3.2.3. Chromatographic evaluation of the optimized MN3-15 column
3.2.3.1. Evaluating hydrophobic and π–π interactions of the MN3-15 using some solute probes. To analyze the hydrophobic and π–π interactions exhibited by the MN3-15 stationary phase, aromatic compounds having different substituents as well as electron donating/withdrawing groups on the aromatic ring(s) were chromatographed on the column. Electron-donating substituents on the benzene ring make the ring more π donating than benzene. These groups are called benzene ring activating groups [38,39]. They increase the electron density on the benzene ring. Typical examples of strongly activating groups are $–NH_2$, –NHR, $–NR_2$, OH and O^- while representative moderately activating groups are $–NHCOCH_3$, –NHCOR, $–OCH_3$ and –OR and weakly activating substituents are $–CH_3$, $–C_2H_5$, –R, and $–C_6H_5$. Electron-withdrawing groups decrease the electron density on the benzene ring by making the ring able to accept more π electrons. These are called deactivating groups and are classified into (i) weakly deactivating groups such as –F, –Cl, –Br and –I; (ii) moderately deactivating substituents such as –CN, $–SO_3H$, –COOH, –COOR, –CHO and –COR; and (iii) strongly deactivating groups such as $–NO_2$, $–NR_3$, $–CF_3$, and CCl_3 [38].

In the first set of measurements, 4 toluene derivatives, namely *p*-toluidine (has both strongly, i.e., $–NH_2$, and weakly, i.e., $–CH_3$, benzene ring activating groups), *p*-tolualdehyde (has a strongly activating group, i.e., $–NH_2$, and a weakly deactivating group, i.e., –CHO), *p*-tolunitrile (has a moderately deactivating substituent, i.e., –CN) and toluene were used as model solutes. When the mixture of these 4 solutes was injected onto the column, the observed elution order was *p*-toluidine, *p*-tolualdehyde, *p*-tolunitrile and toluene with k' values of 0.44, 1.68, 1.91 and 3.9, respectively, using a mobile phase consisting of water at 20% ACN v/v containing 0.1% TFA at 1 mL/min. Regarding hydrophobicity, toluene is more hydrophobic than the other 3 solutes, and eluted last. *p*-Toluidine, *p*-tolualdehyde and *p*-tolunitrile eluted in the order of increasing deactivation of the benzene ring by their respective substituents which indicate π–π interactions in addition to nonpolar interactions with the nanotubes on the surface of the monolith, while toluene being the most hydrophobic and carrying the weakly activating methyl group was the last eluting compound, a fact that indicates mainly nonpolar interactions of toluene with the surface carbon nanotubes.

In another set of experiments, a mixture of *m*-substituted toluene compounds including *m*-toluidine, *m*-tolualdehyde, *m*-tolunitrile, toluene and *m*-nitrotoluene was separated on the column and the k' values for these compounds were 0.31, 2.42, 2.8, 3.9, 4.52, respectively, using a mobile phase consisting of water at 20% ACN v/v containing 0.1% TFA at 1 mL/min. *m*-Nitrotoluene is more retained on the column than toluene. This may indicate that the retention mechanism is due to hydrophobic interactions and π–π interactions. The strong electron-withdrawing group on *m*-nitrotoluene decreases the π electron density on the aromatic ring, making it a soft Lewis acid that can accept π-electrons from OH-MWCNTs, which is having high π-electrons density resulting in π-donor-π-acceptor complexes. The π–π interactions make the *m*-nitrotoluene more retained on the column than other solutes [40,41].

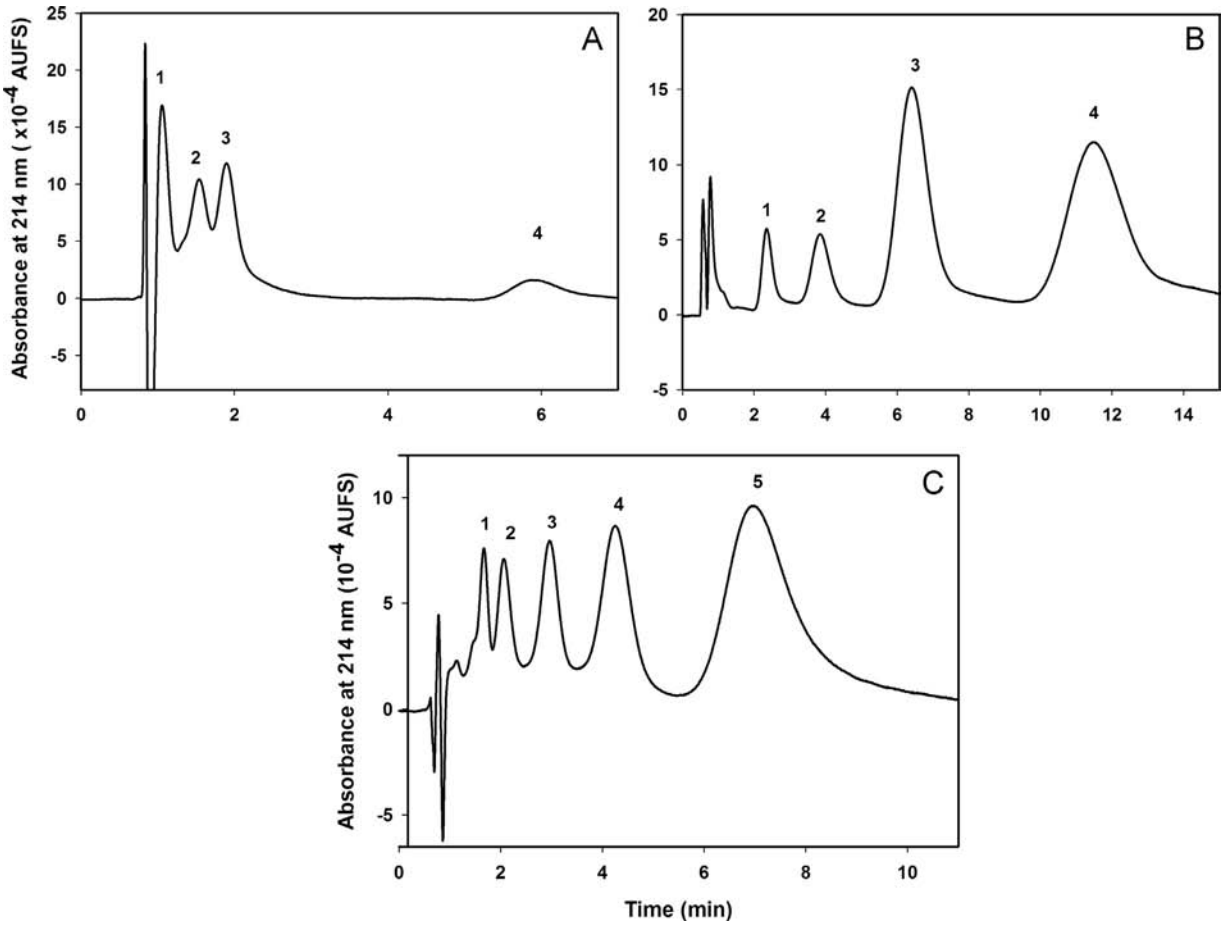

Fig. 6. Chromatograms of anilines in (A), phenoxy acid herbicides in (B) and phenols in (C) obtained on MN3-15 column. Column dimension and flow rate are as in Fig. 3. Mobile phase in (A): ACN:H_2O at 20:80 (v/v) containing 0.1% TFA; mobile phase in (B): ACN:H_2O at 40:60 (v/v) containing 50 mM sodium dihydrogen phosphate, pH 2.12; mobile phase in (C): ACN:H_2O at 40:60 (v/v) containing 0.1% TFA. Solutes in (A): (1), aniline; (2), 4-ethylaniline; (3), 4-bromoaniline; (4), 2,4-dichloroaniline. Solutes in (B): (1), 2-phenoxypropionic acid; (2), 2-(2-chlorophenoxy)propionic acid; (3), 2-(4-chloro-2-methylphenoxy)propionic acid; (4), 2-(2,4,5-trichloropenoxy)-propionic acid. Solutes in (C): (1), phenol; (2), 2-chlorophenol; (3), 4-nitrophenol; (4), 2,4,5-trinitrophenol; (5), pentachlorophenol.

3.2.3.2. Evaluation with typical weak bases, weak acids and slightly polar compounds.. The retention properties of the MN3-15 column were further studied using a mixture of some anilines, see Fig. 6A. The order of elution was aniline (pK_a=4.63), *p*-ethylaniline (pK_a=5.1), *p*-bromoaniline (pK_a=3.86) and 2,4-dichloroaniline (pK_a=2.05). This retention order shows the typical RPC behavior whereby the weakest halogenated aniline bases are more retained than *p*-ethylaniline and aniline, which are slightly stronger bases. This once again demonstrates that hydrophobicity plays major role in solute retention. The selectivity factor α (where $\alpha = k'_2/k'_1$) was quite high amounting to 2.9, 1.5 and 4.7 for the solute pairs *p*-ethylaniline/aniline, *p*-bromoaniline/*p*-ethylaniline and 2,4-dichloroaniline/*p*-bromoaniline, respectively.

Besides their environmental importance [42], phenoxy acid herbicides such as 2-phenoxypropionic acid, 2-(2-chlorophenoxy) propionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid and 2-(2,4,5-trichlorophenoxy)propionic acid constitute a group of weak aromatic acid compounds whose chromatographic retention would shed some light on the retention characteristics of the MN3-15 column, see Fig. 6B. Since the pK_a of the phenoxy acid herbicides is generally ~3.5, these compounds are very slightly ionized to about the same degree in the hydro-organic mobile phase consisting of sodium dihydrogen phosphate, pH 2.12. As can be seen in Fig. 6B, the column was able to separate 2-phenoxypropionic acid, 2-(2-chlorophenoxy)propionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid and 2-(2,4,5-trichlorophenoxy)propionic acid with the k' values of 2.01, 3.93, 7.19, 11.74, respectively. Although the peaks are relatively broad (especially for the last eluting peak), better than baseline resolution is achieved among the adjacent peaks (R_s in the range 1.9–2.1), mainly due to the relatively high selectivity factors α for 3 solute pairs. In fact, the values of α for the solute pairs 2-(2-chlorophenoxy)propionic acid/2-phenoxypropionic acid, 2-(4-chloro-2-methylphenoxy)propionic acid/2-(2-chlorophenoxy) propionic acid and 2-(2,4,5-trichlorophenoxy)propionic acid/2-(4-chloro-2-methylphenoxy)propionic acid were 1.9, 1.8 and 1.6, respectively. As expected, the retention of these phenoxy acid herbicides paralleled their hydrophobicity and electron density on the benzene ring. In fact, that 2-(2,4,5-trichlorophenoxy)propionic acid with three chlorine atoms attached to the benzene ring eluted last preceded by 2-(4-chloro-2-methylphenoxy)propionic acid with methyl and chlorine substituted benzene ring, while the monochloro substituted 2-(2-chlorophenoxy)propionic acid eluted just after the early eluting 2-phenoxy propionic acid. This may indicate that chlorine substitution increase the hydrophobicity of the phenoxy acid herbicide and can also induce π–π interactions with the stationary phase and increase solute retention.

Furthermore, some phenols were analyzed on the MN3-15 column, see Fig. 6C. The observed elution order was phenol, 2-chlorophenol, 4-nitrophenol, 2,4,5-trichlorophenol and pentachlorophenol with the k' values of 1.14, 1.64, 2.76, 4.46, 7.78, respectively. The selectivity factors of the solute pairs 2-chlorophenol/phenol, 4-nitrophenol/2-chlorophenol, 2,4,5-trichlorophenol/4-nitrophenol and pentachlorophenol/2,4,5-trichlorophenol

were 1.4, 1.7, 1.6 and 1.7, respectively. These relatively high selectivity factors (see also the preceding section) demonstrate the utility of carbon nanotube stationary phases under investigation. Phenol eluted faster than the substituted phenols such as 2,4,5-trichlorophenol and pentachlorophenol, which yielded higher retention due to their hydrophobic and π–π interactions. It is observed that the degree of halogenation and the size of the solute control the retention. The more halogenated phenols were retained for longer time on the stationary phase showing the typical RPC behavior.

3.2.4. Enantioseparations

Despite recent studies on the enantiorecognition capability of carbon nanotubes [43–45], their potentials in enantioseparations are still yet to be demonstrated and exploited in a wide range of chiral compounds. The chirality is believed to arise from the spiral alignment (either left- or right handed) of the hexagonal rings along the nanotube axis [46,47]. Thus far, only limited attempts have been reported in recent years involving capillary electrophoretic [48,49] and flow injection analysis systems [50].

Similar to the achiral separations described in the above sections, the MN3-15 with 3 mg incorporated OH-MWCNTs that were power sonicated for 15 min yielded the best performance for chiral compounds too. It has been demonstrated that the sonication of carbon nanotubes may impart enhanced chirality in these nanostructures [51]. This is shown in Fig. 7 that shows that the MN3-15 column was able to successfully separate four enantiomeric compounds including 2-(2,4-dichlorophenoxy)-propionic acid, Dns-methionine, Dns-phenylalanine and the pharmaceutically important compound bupivacaine, with the selectivity factor α of 2.2, 1.4, 1.6 and 3.9, respectively. The two enantiomers of 2-(2,4-dichlorophenoxy)propionic acid were separated with an R_s ~1.3, see Fig. 7A. Since the pK_a value of phenoxy acid herbicides is around 3.5, these compounds are partially ionized at the pH of the mobile phase, a condition that favored enantioseparation. Also, Dns-phenylalanine and Dns-methionine were separated with an R_s of ~1.3 and ~1.1, respectively, see Fig. 7B and C. The enantiomeric separation (R_s~0.9) of the relatively strong base bupivacaine (pK_a=8.1) was achieved at acidic pH with a mobile phase consisting of ACN:H_2O at 55:45 (v/v) containing 0.1% TFA. The observed resolution values in the range of 0.9–1.3 for 4 enantiomeric solutes were readily achieved due to the relatively high selectivity factors in the range of 1.4–3.9 (see above). Despite the relatively pronounced peak broadening in the case of bupivacaine, an acceptable R_s of ~0.9 was obtained primarily due to the relatively high α value of 3.9.

4. Conclusions

The composition of the porogenic solvent for an optimal monolithic column (e.g., ODM column) for CEC was not suitable to produce a useful ODM column for HPLC. In the latter case, the monolith must possess an increased content in throughpores to yield a column with acceptable permeability for pressure driven flow. Under this condition, solute diffusional mass transfer resistance in and out of stagnant mobile phase would diminish thus leading to improved separation efficiency. The amounts of throughpores in a given monolith depend largely on the composition of the porogen. In CEC, the solute as well as the

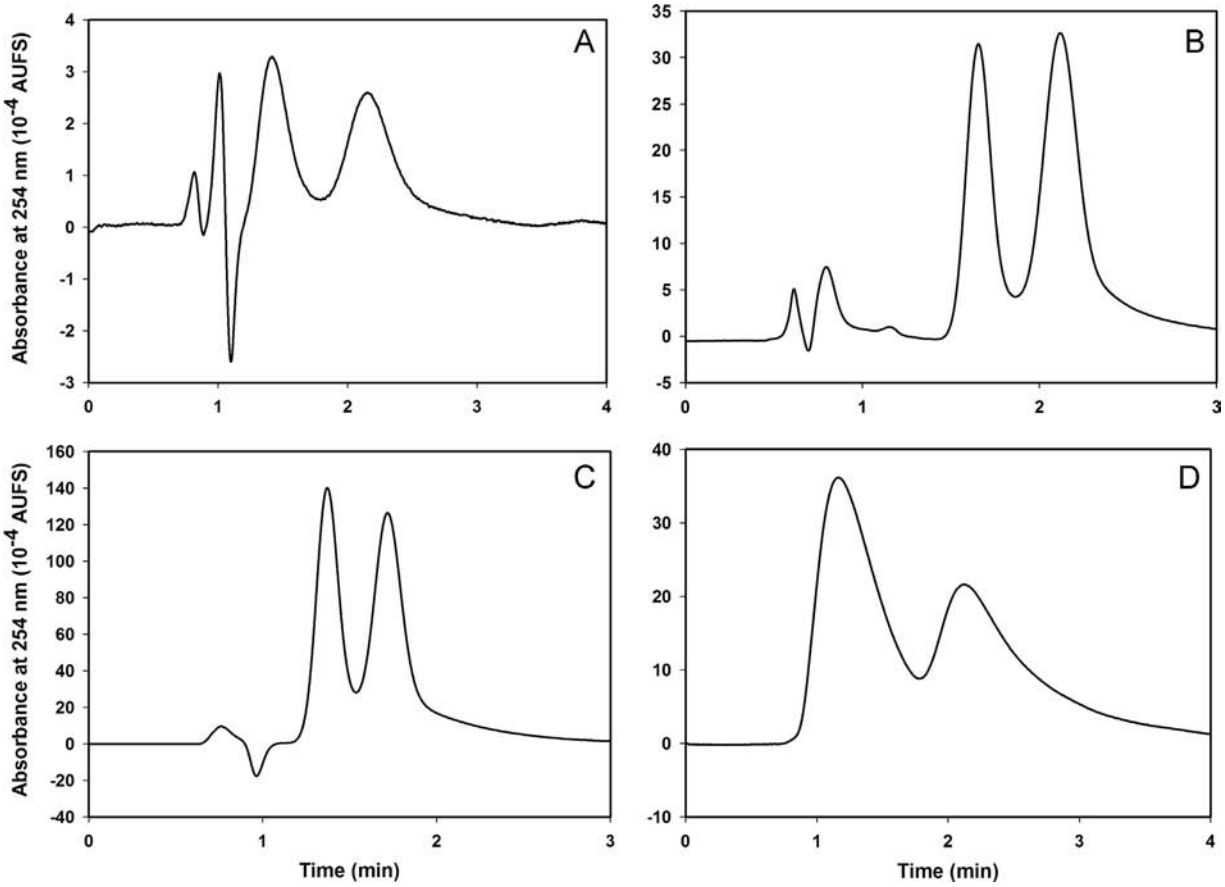

Fig. 7. Separation of the enantiomers of 2,4-dichlorophenoxypropionic acid, Dns-phenylalanine, Dns-methionine and bupivacaine in (A), (B), (C) and (D), respectively, obtained on MN3-15 monolithic column. Column dimensions and flow rate are as in Fig. 3. Mobile phase in (A), ACN:H_2O at 40:60 (v/v) containing 50 mM sodium acetate, pH 4.1; mobile phase in (B) and (C): ACN:H_2O at 35:65 (v/v) containing 25 mM sodium acetate, pH 4.1; mobile phase in (D): ACN:H_2O at 55:45 (v/v) containing 0.1% TFA.

mobile phase can be readily transported throughout the monolith by virtue of the electroosmotic flow regardless of the width of the channels. When added in small amounts to the ODM column, carbon nanotubes enhanced the separation of proteins in linear gradient elution at increasing acetonitrile concentration in the mobile phase. It is believed that the incorporated carbon nanotubes modulated the retention of proteins by additional π-interactions with the carbon nanotubes and consequently improved their separation profile.

GMM/EDMA monolith proved to be a very useful neutral, relatively hydrophilic monolith to serve as an ideal monolithic support for incorporating carbon nanotubes with the aim of yielding a true "carbon nanotube stationary phase" for the separation of a wide of solute including chiral compounds. In this case, the homogeneity of the monolith with incorporated carbon nanotubes was insured by selecting hydroxyl functionalized MWCNTs that associated with the hydroxyl functions of the GMM/EDMA monolith (i.e., like dissolves like). Also, the homogeneity of the monolith with incorporated nanotubes was enhanced by high power sonication of the nanotubes for a short duration before mixing them with the polymerization solution. The retention of solutes and the selectivity of their separation were governed by hydrophobic and π-interactions with the carbon nanotube stationary phase.

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