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Non-particulate (continuous bed or monolithic) acrylate-based capillary columns for reversed-phase liquid chromatography and electrochromatography

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Abstract

Three approaches are described to synthesize acrylic non-particulate beds (also called continuous beds or monoliths) in aqueous polymerization media for reversed-phase capillary liquid chromatography/electrochromatography. In the first, hexyl acrylate comonomer was dissolved together with water soluble polar comonomers using a non-ionic detergent. In the second, a new alkyl ammonium salt comonomer, (3-allylamino-2-hydroxypropyl)dodecyldimethylammonium chloride was used, which is water soluble and has detergent properties itself. The alkyl group of this comonomer provides hydrophobicity while the ionic groups generate electroosmosis in the non-particulate bed. In the third approach, the alkyl comonomer was used as a detergent to dissolve another hydrophobic comonomer in an aqueous polymerization medium. All three approaches were evaluated with respect to hydrophobicity, efficiency and electroosmotic properties of the beds. Hydrophobicity expressed as methylene group selectivity for the three types of the beds in 50% methanol mobile phase was 1.86, 1.16 and 1.78, electroosmotic mobility -5.14×10^{-5}, 6.89×10^{-5} and 6.37×10^{-5} $cm^2\ V^{-1}\ s^{-1}$ and efficiency for the retained compound (methylparabene) 67,000, 93,000 and 110,000 plates m^{-1} correspondingly. The columns were tested using pressure driven capillary chromatography and capillary electrochromatography. The influence of polymerization temperature on hydrodynamic permeability, separation impedance and inverse size exclusion porosimetry characteristics were used to evaluate the separation columns. The increase of the polymerization temperature resulted higher permeability of the bed, separation impedance and lower polymeric skeleton porosity. Further characterisation was provided by examining the separation efficiency observed for a series of benzoic acid esters and alkyl parabens.

Keywords: Reversed-phase capillary electrochromatography; CEC; Capillary liquid chromatography; Inverse size exclusion chromatography; Continuous beds; Monoliths; Hydrophobicity; Porosimetry

1. Introduction

The flexibility of the non-particulate bed technique lies in the almost limitless selection of potential comonomers containing the functionalities of interest, which can be incorporated in the resultant stationary phase and utilized for generation of the required selectivity. Due to the fact that the vast majority of chromatographic applications are performed in reversed-phase (RP) conditions [1], much experimental effort to date has been directed towards designing RP continuous beds.

The non-particulate bed technique first proposed in 1989 [2] has demonstrated its excellent compatibility with capillary or microchip format separations [3–5]. Non-particulate beds (also called continuous beds or monoliths, and for nomenclature details see Ref. [5]) are essentially polymerized in situ by filling the chromatographic column with an aqueous solution of monovinyl and bivinyl comonomers and initiator. During the polymerization the continuous bed precursors

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undergo phase separation. The spongy morphology and size of the resultant flow-through channels may be regulated by adjusting the ionic strength of the polymerization medium. Other approaches to perform the phase separation resulting in non-particulate beds of similar morphologies were proposed, namely the use of pore-forming solvents (porogens) to polymerize monoliths in organic media [6–8] or sol–gel technologies to form inorganic beds [9,10].

Due to a limited solubility of the nonpolar monomers in aqueous polymerization media, it is difficult to incorporate the hydrophobic ligands necessary for RP chromatography into the stationary phase, when the continuous bed technique is used (see Ref. [5]). To overcome this problem several solutions have been proposed to date by different research groups.

One solution reported the use of hydrophobic ligands being attached to the continuous bed containing epoxy functionalities [11]. Different surface chemistries can be generated using the continuous bed approach, however the additional step(s) of surface functionalization makes the synthesis of the bed more complicated and therefore less attractive and also increases the risk of irreproducibility. A one-step continuous bed preparation for RP capillary electrochromatography (CEC) was described employing an aqueous micellar polymerization medium in order to dissolve the nonpolar comonomers with polar counterparts [12]. Triton X-100 was used as a neutral surfactant above its critical micellar concentration (CMC) in order to solubilize the nonpolar comonomer but the continuous beds were of moderate hydrophobicity. To increase the hydrophobic ligand density even further another method for RP continuous beds for CEC and capillary liquid chromatography (cLC) was employed [13]. First a continuous bed matrix was created in the Bind Silane (methacryloxypropyl trimethoxysilane) pretreated capillary using 2-hydroxyethyl methacrylate (HEMA) and piperazine diacrylamide (PDA) as comonomers. In the second step additional in situ polymerization of allyl glycidyl ether, HEMA and PDA was carried out in the presence of dextrane or dextrane sulfate as hydroxyl-rich or hydroxyl-rich and electroosmosis-creating substances in order to immobilize dextrane polymer. The next step was covalent attachment of octadecyl ligands via in situ reaction with the 1,2-epoxyoctadecane in the organic medium.

A further one-step continuous bed synthesis for RP CEC was described by Palm and Novotny [14]. The comonomers were dissolved and polymerization was carried out in the polar organic solvent, formamide or *N*-methylformamide, which are compatible with the water-based radical polymerization and capable of dissolving the organic/inorganic salts present. The beds demonstrated efficiency up to almost 400,000 plates m^{-1} for alkyl phenones in RP mode. The beds suffered from hydrodynamic impermeability, therefore, electroosmotic conditioning had to be utilized. This could be a remarkable drawback if the capillary is dried during chromatographic use since such continuous beds could not be reconditioned by means of pressure nor by electroosmosis.

A similar one-step approach and comprehensive evaluation of the acrylamide-based monolithic columns was published by Hoegger and Freitag [15]. They used PDA, *N*,*N*-dimethylacrylamide, MA, butyl acrylate and hexyl acrylate dissolved either in aqueous buffer or its mixture with formamide to form the RP continuous beds. Porosimetric evaulation of the beds revealed that the size of flow-through channels could be reproducibly adjusted by selection of ionic strength of the monomer solution.

In our previous study to synthesize a one step RP continuous bed for capillary format chromatographic separations, we used *N*,*N*-dimethylformamide or 50% (v/v) 2-propanol in *N*,*N*-dimethylformamide, which are compatible with free-radical polymerization [16]. The beds were synthesized with stearyl methacrylate, methacrylamide, vinylsulfonic acid as comonomers and PDA as crosslinker and were applied for gradient capillary LC using mixtures of proteins, peptides and extract of sage as a source containing a mixture of naturally occurring antioxidants [17].

The task of this study was to further develop our one-step synthesis strategy for RP continuous beds to be used in either capillary format LC or capillary electrochromatography. A secondary aim to achieve this was to test the applicability of an alkyl ammonium salt which is not limited by poor solubility in an aqueous polymerization mixture. Finally, success would be measured by determining the influence of polymerization temperature on the morphology and efficiency of the resultant continuous beds using a series of benzoic acid esters and alkyl parabens.

2. Experimental

2.1. Chemicals

Ammonim persulfate (APS) was of analytical grade from Lachema (Neratovice, Czech Republic). *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) was from Reanal (Budapest, Hungary). PDA, MA, *N*-(hydroxymethyl)acrylamide (HMAM), butyl methacrylate and diallyldimethylammoniumchloride (DDAC) were bought from Fluka (Buchs, Switzerland). Hexyl acrylate and stearyl methacrylate were from Aldrich (Milwaukee, WI, USA). Methanol (MeOH) of HPLC grade, glacial acetic acid (HOAc), triethylamine (TEA), methacryloxypropyl trimethoxysilane (Bind Silane A 174), Triton X-100, ammonium sulfate, benzene (for LC, 99.9%), tetrahydrofuran (for LC, 99.9%), dodecyl methacrylate and methyl- and ethyl-parabens were purchased from E. Merck (Darmstadt, Germany). 3-Chlor-2-hydroxypropyldimethyldodecylammoniumchloride (Q342) (40% aqueous solution) was kindly supplied by Degussa Hüls Corporation (Frankfurt, Germany). (3-Allylamino-2-hydroxypropyl)dodecyldimethylammonium chloride monomer (alkyl ammonium salt comonomer) was synthesized from allylamine and cationizing alkyl chlorohydrine

in alkaline conditions. 2.5 ml Q342 was diluted with 3.2 ml 4% NaOH and 0.167 ml of allylamine was added to the stirred solution. The reaction mixture was stirred for 24 h at 20 °C. Modified Q342 was neutralized with phosphoric acid solution. The required amount of this solution was used in polymerization mixture. Final concentration of modified Q342 was 18%.

Fused silica capillary (50 μm i.d. × 375 μm o.d.) was obtained from Polymicro Technologies (Phoenix, USA). Polystyrene standards of M_r 484, 1560, 3470, 10,300, 18,000, 34,000, 65,000, 125,000, 226,000, 564,000, 1,070,000, 2,530,000, 5,423,000 and 11,000,000 were bought from Polymer Standards Service (Mainz, Germany). Methyl, ethyl, propyl, butyl and isopentyl benzoic acid esters were kindly donated by Prof. U. Pyell from Marburg University (Germany).

2.2. Equipment

All chromatographic experiments were performed using a capillary liquid chromatography setup suitable for capillary electrochromatography and capillary pressure driven chromatography and consisted of HPLC units as follows: a Model Linear UVIS 200 detector (Reno, NV, USA) modified by installing ball lenses for on-capillary detection, a Model PALM 897 high voltage source designed by Per-Axel Lidström at Uppsala University (Sweden) or a Model LC 250 Binary Pump (Perkin-Elmer, USA) and a model SpectraSystem AS3000 Autosampler (Thermo Separation Products, USA). To split the mobile phase stainless-steel T-unit with flow splitting capillary (100 μm i.d.) was installed. For data registration and calculations BioFocus (BioRad, USA) or Chromstar software (Brucker, Germany) was used. For calculation of the porosimetric data the inverse size exclusion cLC was performed and Krušinskas and Maruška© computing software based on theoretical model described by Gorbunov et al. [18] was employed. For the calculations two sets of size exclusion distribution coefficients K_{SEC} were measured in automated manner using benzene as low molecular weight compound and polystyrene standards in THF. The R.S.D.% for elution volumes using this equipment was bellow 1.1% [19].

2.3. Preparation of capillary columns

In order to prepare hydrophobic acrylate-based stationary phases the procedure essentially described in Ref. [20] was employed. The respective comonomers, crosslinker and additives (salt, surfactant) were dissolved in aqueous buffer (see Table 1) and degassed using vacuum for 5 min. Then 20 μl portions of 10% aqueous solutions of APS and TEMED were added to the monomer solution. The fused silica capillaries with Bind Silane modified inner surfaces [21] were filled with the monomer solution by means of vacuum. The capillary ends were sealed with silicone grease and left to polymerize for 24 h while maintaining temperature at 25 °C by means of a water bath unless indicated otherwise.

3. Results and discussions

In this study we report three different approaches to synthesize acrylic continuous beds for RP CEC/cLC, the details of which are outlined in Table 1.

In the first approach (continuous bed named *RPM1*) a procedure similar to that proposed by Hjertén et al. [12] was utilised. Neutral surfactant Triton X-100 was used to dissolve the hydrophobic comonomer in an aqueous polymerization medium. Hexyl acrylate was employed as nonpolar comonomer instead of the stearyl or butyl methacrylate used in the earlier work [12]. PDA was used as crosslinker and dimethyldiallylammonium chloride as ionic comonomer for synthesis of the *RPM1* continuous bed. Sufficient hydrophobicity for separation of benzoic acid esters or parabens in RP mode was obtained for hexyl ligand-continuous beds, therefore no surfactant additive in the mobile phase was required to increase the hydrophobic interaction in contrast to the beds synthesized using octadecyl or butyl ligands. This is due to sufficient solubility in the aqueous micellar medium and hydrophobicity of hexyl acrylate comonomer. The resultant beds demonstrated moderate efficiency of up to 70,000 plates m^{-1} for the retained compounds (see Table 2).

In the second approach a water-soluble alkyl ammonium salt comonomer obtained by the reaction of allyl amine and an appropriate chlorohydrine in alkaline conditions was utilized. The monomer utilised and shown in Fig. 1 contained both an alkyl chain required for reversed-phase separation and a charged group for the generation of electroosmosis (two cationic groups: secondary amino and quaternary ammonium). Stationary phase *RPM2* prepared using these comonomers resulted in efficiency values twice that of *RPM1*, however hydrophobicity of the phase was low (Table 2). Efficiency, hydrophobicity and electroosmotic mobility was cal-

Table 1
Composition of polymerization mixtures used in synthesising the three types of acrylic continuous beds for RP CEC/cLC

Name	$(NH_4)_2SO_4$ (mg)	HMAM (μl)	PDA (mg)	HA (μl)	DDAC (μl)	Triton X (μl)	Alkyl ammonium salt comonomer[a] (μl)	Buffer[b] (ml)
RPM1	10	59	92	27.5	10	19	–	0.5
RPM2	–	37.5	75	–	–	–	500	–
RPMmix	5	37.5	75	27.5	10	–	300	0.2

[a] 18% solution neutralized with phosphoric acid, pH 7.
[b] 50 mM phosphate buffer, pH 7.

Table 2
Electrochromatographic characteristics of the acrylic RP continuous bed stationary phases[a]

Continuous bed	%TCC[b]	Efficiency (acetone) (plates m^{-1})	Efficiency (methylparaben) (plates m^{-1})	Hydrophobicity α_{CH_2}	Electroosmotic mobility ($\times 10^{-5}$ $cm^2/V\,s$)
RPM1	27.4	40,000	67,000	1.86	5.14
RPM2	29.9	100,000	93,000	1.16	6.89
RPMmix	27.3	96,000	110,000	1.78	6.37

[a] In 50 % aqueous MeOH with 0.35% TEMED in acetic acid, pH 3.7 as mobile phase.
[b] %TCC is total concentration of comonomers plus crosslinker; g/100 mL.

culated as average values from two chromatographic runs. Run-to-run reproducibility ($n = 5$) gave %R.S.D. values for the plate number 4.4, hydrophobicity 0.76 and electroosmotic mobility 0.91. Optimization of the organic modifier concentration in the mobile phase showed, that only 30% MeOH was required using *RPM2* stationary phase to separate electrochromatographically a mixture of four parabens compared with the required 65% MeOH, when *RPM1* was evaluated. Reversed-phase electrochromatographic separations of neutral compounds obtained using *RPM2* type stationary phase are exemplified in Fig. 2. Sample in load was performed using so-called diffusion–extraction based injection, which was performed immersing the capillary inlet into the sample solution for 15 s [21]. The tailing observed in Fig. 2a can be due the sample overload, however it is not reflected in the efficiency since the plate counts were measured at half height of the peaks.

In the third approach the alkyl ammonium salt comonomer was used as a surfactant above its CMC to solubilize the additional highly nonpolar comonomers (for instance hexyl acrylate) which allowed full incorporation into the continuous bed. This approach resulted in beds of increased hydrophobicity and higher observed peak efficiency values than those obtained using the common neutral surfactant to solubilize the nonpolar comonomer (Table 2).

As mentioned above, a general problem with CEC continuous beds for reversed-phase separations often is allow ligand density, which results in low electroosmotic mobility or hydrophobicity. This, however, was not the case when the hexyl acrylate was combined with the alkyl ammonium salt comonomer. Hexyl acrylate is not soluble in a water solution, however when using the alkyl ammonium salt comonomer additive, which is a surfactant itself, no problems were observed with solubility in the aqueous solution, hydrophobicity or electroosmotic mobility.

Upon changing hydrophobic monomers (from butyl- to stearyl methacrylate) or their molar ratio in the polymerization mixture it was found that hydrophobicity of the continuous beds could be regulated (expressed as methylene group selectivity (α_{CH_2}) [16]) (Fig. 3). At a fixed comonomer molar ratio in the polymerization mixture, the hydrophobicity increased with the length of the alkyl chain in the nonpolar comonomer (curves I and III). Some decrease in the hydrophobicity was observed using longer chain alkyl comonomers, when a total concentration (TCC%) of the

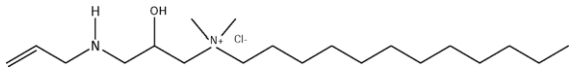
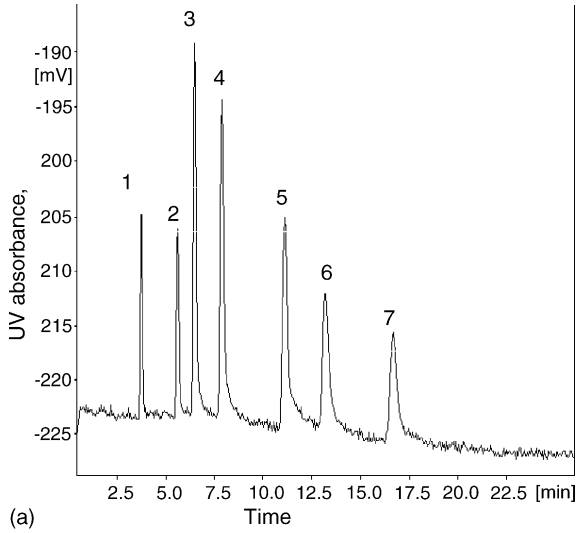


Fig. 1. Structure of the water soluble alkylammonium salt monomer used for the preparation of *RPM2* and *RPMmix*.



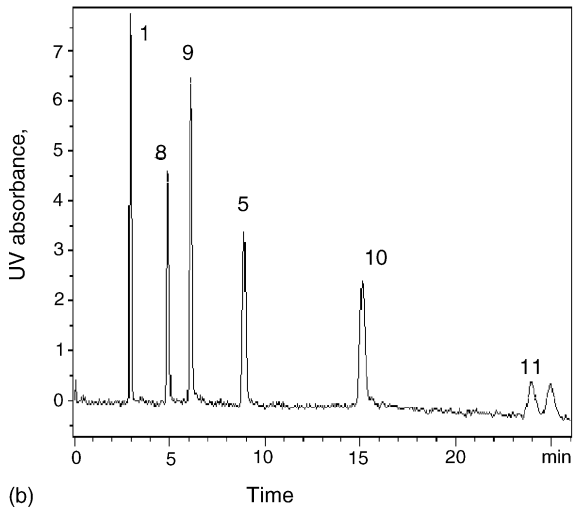


Fig. 2. Reversed-phase CEC separation of neutral compounds: (a) 1, acetone; 2, acetophenone, 3, propiophenone; 4, butirophenone; 5, propyl benzoate; 6, methyl paraben; and 7, ethyl paraben in their elution order. (b) 1, Acetone; 8, methyl; 9, ethyl; 5, propyl; 10, butyl; and 11, isopentylbenzoate using continuous bed of *RPM2* composition, but without HMAM in the comonomer mixture. Voltage: (a) 5 kV and (b) 7 kV. MeOH (30%) in 0.35% TEMED and acetic acid buffer pH 3.7. Column effective length 10.4 cm. Efficiency (b) for acetone (unretained marker) 190,000 plates m^{-1} and 150,000 plates m^{-1} for retained compounds.

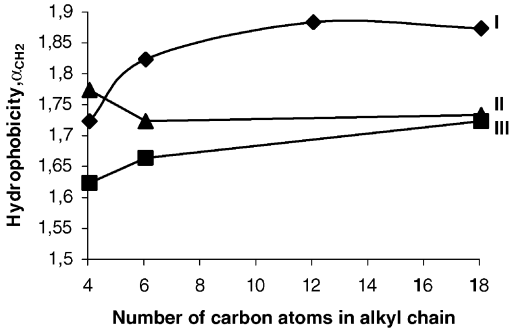


Fig. 3. Hydrophobicity of RP stationary phases expressed as methylene group selectivity: (I) *RPM1* (constant molar ratio of comonomers); (II) *RPMmix* (constant volume of comonomers); and (III) *RPMmix* (constant molar ratio of comonomers). Mobile phase: 50% of MeOH.

comonomers was fixed (curve II). This could be due to lower percentage and density of the longer chain alkyl comonomers in the bed. Due to the flexibility of the synthesis concerning the comonomers and their concentrations a variety of RP stationary phases were obtained, with hydrophobicity (α_{CH_2}) ranging from 1.6 to 1.9 when a 50% MeOH in aqueous media is employed as mobile phase. Such hydrophobicity is comparable with conventional silica based RP C18 stationary phases [16].

In order to optimize the synthetic conditions, the polymerizations were carried out at different temperatures. The effect of polymerization temperature on continuous bed permeability is shown in Fig. 4. As shown, the morphology of the continuous beds is highly dependent on temperature. The linear velocity plotted against polymerization temperature has a slightly parabolic dependence. The highest linear velocity was obtained for *RPM2*, the capillary column prepared at 50 °C. Low permeability indicates very narrow flow-through channels, which may substantially prolong the chromatographic analysis (using pressure driven mode) and conditioning of the bed.

The stationary phase morphology was evaluated using scanning electron microscopy (SEM) (Fig. 5). The images indicate that the largest skeletons and flow-through channels were formed at the elevated temperature (50 °C). The influence of temperature can be characterised similarly to the salt additive, which was found to increase hydrophobic interaction between the polymeric chains which in turn results in broader channels in the bed. In a previous study [22] the optical microscopic observations investigating the influence of ionic strength on the continuous bed morphology were reported. It was shown that the phase separation process is based on the polymer-dissalting phenomenon, which is favoured by the increase of the salt concentration. This results in a higher roughness of the continuous bed surface observed by means of optical microscopy, indicating larger channels and higher bed hydrodynamic permeability. An advantageous use of the ionic strength for the regulation of the phase separa-

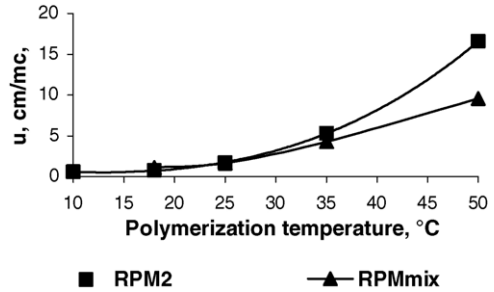


Fig. 4. Dependance of mobile phase (water) linear velocity on polymerization temperature at pressure 187 bar for *RPM2* and 177 bar for *RPMmix*.

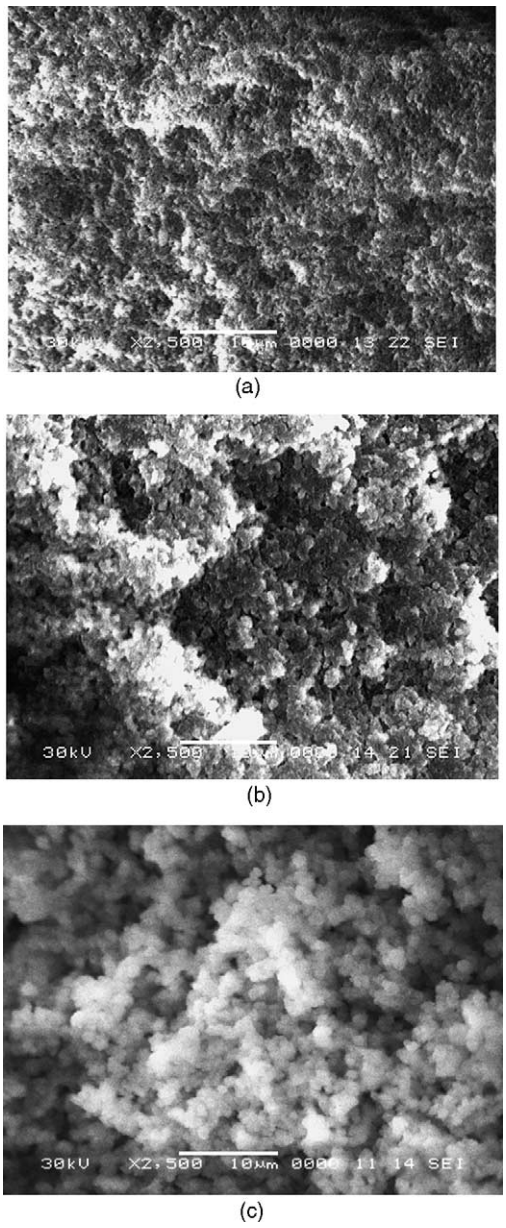


Fig. 5. SEM images of *RPMmix* continuous beds synthesized at different temperatures: (a) 10 °C; (b) 35 °C; and (c) 50 °C. Magnification ×2500.

tion process during the polymerization and optimization of the continuous bed morphology was indicated by Hjertén's group in their early studies. This fact was confirmed later in the detailed study [23], where the same phenomenon was observed increasing the temperature. Hydrophobic interaction of amphiphilic or hydrophobic monomers in aqueous media is higher at higher temperature.

Hydrophobicity, expressed as α_{CH_2}, ranged from 1.38 to 1.56 for *RPM2* (at 30% MeOH in aqueous media) and from 1.7 to 1.77 for *RPMmix* (at 50% MeOH in aqueous media using pressure driven capillary chromatography). Methylene group selectivity was dependent on polymerization temperature with the maximum observed at 25 °C for both continuous beds.

In Fig. 6a plate height dependence on the mobile phase velocity (*H*/*u* curves) are plotted for the *RPMmix* continuous beds obtained at different polymerization temperatures. Plate heights were measured for dimethylformamide as unretained marker using pressure driven chromatography. The same *H*/*u* curve character was characteristic also for the retained compounds. All beds formed demonstrated very similar *H*/*u* dependencies except the bed formed at 50 °C. Due to the high hydrodynamic resistance it was difficult to measure plate heights at higher linear velocities for the beds formed at lower temperatures, namely RPMmix10 °C, RPMmix18 °C and RPMmix25 °C. Almost the same optimum linear velocity (at a minimum value of the plate height) was characteristic for all the beds, since the monomer composition, their total concentration and other polymerization conditions (except the temperature) were the same. The bed *RPMmix prepared at* 50 °C, however, demonstrated high efficiency, which was nearly constant in the broad range of linear velocities. This is an advantage and characteristic feature of the non-particulate beds, since the fraction of the stationary phase in the non-particulate beds is much smaller comparing to the particulate packings. The turbulence originating in the broad channels of the continuous beds at high mobile phase velocities enhances the mass transfer without a sharp drop in efficiency. If the continuous bed is of low hydrodynamic resistance a much longer bed may be used to generate higher plate counts per short period at high linear velocities of the mobile phase. This parameter, separation impedance, integrates both hydrodynamic and kinetic properties of the stationary phase. Separation impedance (*E*) [24] was calculated for capillary columns, synthesized at different temperatures (Fig. 6b). *E* is directly dependent on back pressure and reciprocally proportional to the square of efficiency:

$$E = \frac{t_R \Delta p}{N^2 \eta (1 + k)}$$

where t_R is the retention time, Δp the back pressure, *N* the efficiency expressed as plate number, η the viscosity of mobile phase and *k* the retention factor. The *E* values, calculated for the beds synthesized at 35 and 50 °C were very different (Fig. 6b) from that of other beds. This is due to the fact, that hydrodynamic resistance was very dependent on the polymerization temperature (see Fig. 4), although efficiency was less influenced by temperature (Fig. 6a). Separation impedance for the capillary column synthesized at 50 °C temperature increases slowly at the flow rates up to 30 mm/s. From the results shown in Figs. 4 and 6a and b we can see, that having the same composition of RP continuous bed while adjusting polymerization temperature the hydrodynamic resistance of capillary columns can be regulated. High back pressure is less critical in CEC, however, while in cLC the low back pressure of capillary columns is desirable.

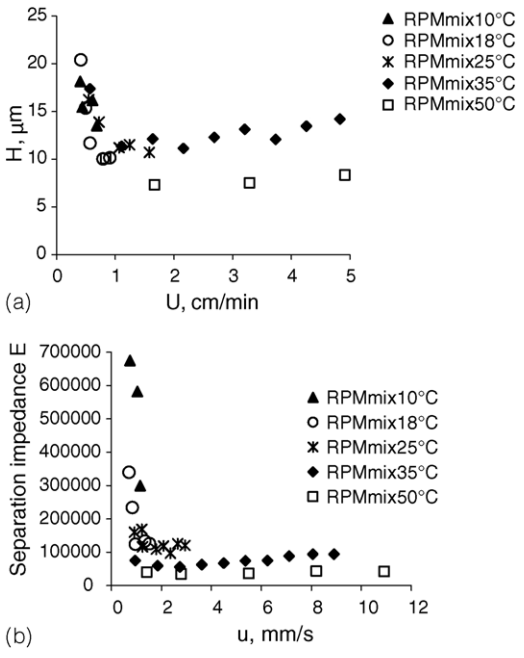


Fig. 6. Dependance of efficiency (a) and separation impedance *E* (b) on polymerization temperature for *RPMmix* composition. *H*/*u* curve performed for unretained DMF, *E* calculated for butyl benzoic acid ester.

Porosimetry data were also collected using inverse size exclusion chromatography (ISEC) with polystyrene standards and tetrahydrofuran as a mobile phase. To fulfill size exclusion chromatography conditions any interaction of the solutes with the packing material should be excluded. Due to hydrophobic properties of the reversed phase beds, the nonpolar mobile phase and nonpolar polymeric standards were selected for evaluation of the pore sensitive characteristics of the continuous beds. Due to high crosslinker concentration in the polymeric skeleton (crosslinker concentration in the comonomer mixture for *RPMmix* beds was 41%), probability of swelling or shrinkage of the beds in different polarity media is low. On the other hand, this was a comparative study and all the beds were evaluated using the same ISEC conditions, which reveal differences of their porous structure and the influence of the synthesis parameter, which was varied. All ISEC calculations are based on the calibration of the packing material investigated with the polymeric standards of different M_r. Size exclusion chromatography calibration data for three continuous beds *RPMmix* formed at different tem-

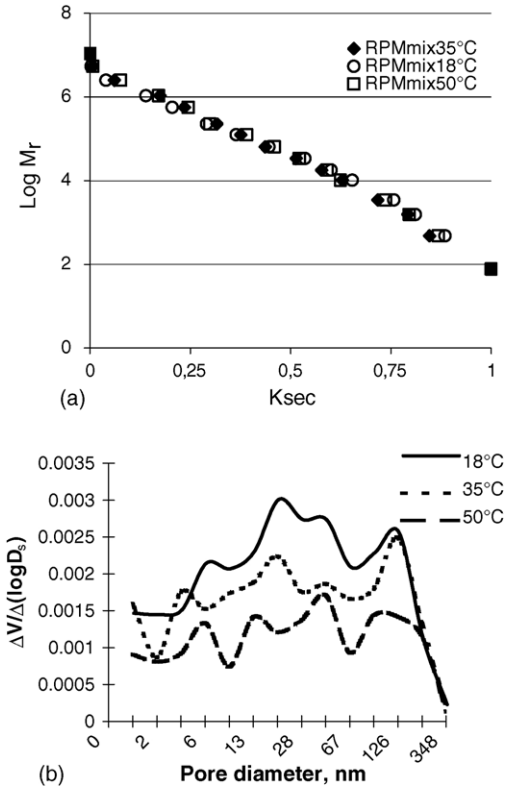


Fig. 7. Inverse size exclusions porosimetric evaluation of the beds polymerized at different temperature: (a) size exclusion chromatography calibration graphs; (b) pore size distribution curves, presented as derivatives of the cumulative curves of the calibration graphs. Columns: L_{ef} = 9.8 cm, 50 μm i.d.; samples: 0.5 mg/ml benzene and polystyrene standards.

peratures are presented in Fig. 7a. The main characteristics calculated from the calibration graphs using ISEC porosimetry program were average pore diameter (D_s) and pore size polydispersity (U). This parameter reflects a width of the pore size distribution function. It is equal to unity (U = 1), when porous matrix has unimodal pore size, i.e. all pores are of the same diameter. The surface-per-pore volume was calculated from D_s, assuming that pores are of cylindrical shape. Detailed explanation of the ISEC porosimetry model is given in Ref. [18]. Polymeric skeleton porosity was calculated from the difference of elution volumes of benzene (M_r = 78) and polystyrene standard (M_r = 11,000,000).

As shown in Fig. 7b the beds have extremely high pore size distribution, which ranges from micropores (pore diameter less than 2 nm) up to a remarkable fraction of mesopores (diameters greater than 50 nm). According to the pore size distribution curves presented in Fig. 7b, the polymeric skeleton formed at 50 °C has the lowest porosity (smallest area under the curve). The lower synthesis temperature increased the skeleton porosity, which is manifested in the upward shift of the curves (see also Table 3). Due to the same total concentration of the monomers the phase ratio for all three beds *RPMmix* in the capillary columns was constant $V_{stationary}/V_{mobile}$ = 0.38 (v/v). Since the beds are formed in aqueous medium, the phase separation process is affected by hydrophobic interaction between the polymerizing hydrophobic oligomers. The hydrophobic interaction is increased at elevated temperatures, which manifests in formation of more compact and less porous skeletons and broader perfusive channels in the bed. Flow through channel fraction was increasing from 35.4% (v/v) at 18 °C to 40.9% (v/v) at 35 °C and 46.9% (v/v) at 50 °C. This is in accordance with the permeability data presented in Fig. 4.

The inverse size exclusion chromatographic porosimetry calculations data are presented in Table 3. The average pore size for all three continuous beds is very similar. It ranges between 25.5 and 28.9 nm. The pore size polydispersity parameter was extremely high U = 4.51–6.29. It is quite common, that this function for the non-particulate beds is very broad, although for the particulate matrices U rarely exceeds 1.5. Due to the similar pore size polydispersity and mean pore sizes (D_s), the surface to pore volume ratio was also similar for all the beds evaluated. It was in the range 69.3–78.4 m^2/ml, however due to greater porosity of the beds synthesized at lower temperature their surface areas per bed volume are considerably higher: for RPMmix18 °C 38.1 m^2/ml, RPMmix35 °C 34.1 m^2/ml and RPMmix50 °C 23.8 m^2/ml.

4. Conclusions

Several approaches for one-step continuous bed synthesis for RP were described and utilized to obtain highly nonpolar and efficient stationary phases for cLC/CEC. The alkyl ammonium salt comonomer including both ionic groups and hydrophobic moieties was shown to be suitable for simultaneous generation of electroosmotic flow and hydrophobic interaction. The properties of the alkyl ammonium salt comonomer, namely solubility in aqueous polymerization medium, allowed it to be utilized as a surfactants to solubilize other lipophilic comonomers. Polymerization temperature was shown to remarkably affect both the morphology and hydrodynamic properties of the continuous beds where an increase in temperature resulted in larger flow-through channels resulting in favourable continuous bed permeability.

Table 3
Characteristics of porous structure

Capillary column	Polymeric skeleton porosity (%, v/v)	Mean pore sizes D_s (nm)	Pore size polydispersity (U)	Surface/pore volume (m^2/ml)
RPMmix18 °C	57.0	26.6	4.51	75.1
RPMmix35 °C	53.0	25.5	6.29	78.4
RPMmix50 °C	47.6	28.9	4.97	69.3

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