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Polymethacrylate monolithic and hybrid particle-monolithic columns for reversed-phase and hydrophilic interaction capillary liquid chromatography☆

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ABSTRACT

We prepared hybrid particle-monolithic polymethacrylate columns for micro-HPLC by *in situ* polymerization in fused silica capillaries pre-packed with 3–5 μm C_{18} and aminopropyl silica bonded particles, using polymerization mixtures based on laurylmethacrylate–ethylene dimethacrylate (co)polymers for the reversed-phase (RP) mode and [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl) zwitterionic (co)polymers for the hydrophilic interaction (HILIC) mode. The hybrid particle-monolithic columns showed reduced porosity and hold-up volumes, approximately 2–2.5 times lower in comparison to the pure monolithic columns prepared in the whole volume of empty capillaries. The elution volumes of sample compounds are also generally lower in comparison to packed or pure monolithic columns. The efficiency and permeability of the hybrid columns are intermediate in between the properties of the reference pure monolithic and particle-packed columns. The chemistries of the embedded solid particles and of the interparticle monolithic moiety in the hybrid capillary columns contribute to the retention to various degrees, affecting the selectivity of separation. Some hybrid columns provided improved separations of proteins in comparison to the reference particle-packed columns in the reversed-phase mode. Zwitterionic hybrid particle-monolithic columns show dual mode retention HILIC/RP behaviour depending on the composition of the mobile phase and allow separations of polar compounds such as phenolic acids in the HILIC mode at lower concentrations of acetonitrile and, often in shorter analysis time in comparison to particle-packed and full-volume monolithic columns.

1. Introduction

Introduction of monolithic chromatographic stationary phases marked a major advance in column technology [1]. Monolithic (continuous bed) separation media consisting of one piece of rod with flow-through pores can be divided into two general categories: (1) silica-based monolithic columns and (2) rigid organic polymer-based monolithic columns. The key advantages of monolithic columns in the capillary format include the absence of frits necessary to retain the packed bed and a moderate column back pressure at high mobile phase flow-rates [2].

Organic polymer (most often polystyrene or polymethacrylate) monolithic capillary columns can be prepared by *in situ* polymerization of suitable organic monomers in fused silica capillaries with inner diameters of 0.1–0.4 mm. The polymerization reaction mixtures usually consist of a combination of monomers and cross-linkers, an initiator and a porogen solvent mixture [3,4]. Methacrylate monolithic columns for capillary HPLC and electrochromatography were synthesized using various substituted methacrylates and ethylene dimethacrylate as the cross-linking reagent [1,5–9]. Parameters such as temperature, composition of the porogen solvent mixture, and concentration of the cross-linking agent allow tuning the average pore size in the monolithic columns within a broad range, from tens to thousands of nanometres [10–13]. For more details on preparation, testing and applications of polymethacrylate monolithic columns for capillary liquid chromatography see, e.g., recent review [14].

Silica beads can be used as sacrificed material for templating of porosity [15]. Templated monolithic stationary phases are prepared by *in situ* polymerization of styrene/divinylbenzene or methacrylate/ethylene dimetacrylate monomers in the presence of 10 μm silica beds pre-packed in a capillary. When the polymerization is finished, the silica particles are dissolved and removed by flushing the column with alkali hydroxide solution [16]. The porosity of the monolith and other properties of the finished separation media are affected by the surface characteristics of the templating silica beads and by the nature and composition of the polymerization mixture. Recently, silica nanoparticle-templated (poly) methacry-

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late ion-exchange monoliths were prepared, where templating was reported to result in 33-fold increase in ion-exchange capacity [17].

Earlier, we prepared butylmethacrylate–ethylene dimethacrylate monoliths in the presence of sorbent particles, but in contrast to using silica as templating sacrificed material, we kept the original particles in the "hybrid" capillary columns [18]. Hybrid capillary columns can also be prepared by encapsulation of silica or other porous particles in a poly(styrene-divinylbenzene) matrix [19].

The monolithic phase formed by polymerization fills essentially the space in between the particles in the column and "glues" the entrapped sorbent particles together and to the fused silica capillary walls, so that the column end frits are not necessary. The size of the pores inside the particles is generally too small to accommodate the monolithic structure with large through pores, even though partial penetration of the organic polymer into the inner particle pores at their edge cannot be ruled out. Hybrid columns are resistant against shrinking of the polymer monolithic phase and against building of voids in between the monolithic bed and the capillary walls, occasionally observed with full-volume monolithic columns prepared in capillaries with relatively larger inner diameters, 0.3–0.5 mm. Monolithic filling in the interparticle volume increases the ratio of the stationary to the mobile phase in the column, which may enhance the retention and improve the separation of weakly retained compounds.

"Controlled surface porosity" (CSP) sorbents with a solid core sphere and a porous adsorbent outer layer became popular in HPLC in early 1970s, as they enabled preparing reproducible HPLC columns by dry-packing into empty column tubes. We found the CSP-based hybrid capillary columns with bare silica gel or C_{18}-bonded silica porous layers on a solid core sphere (37–50 μm) suitable for fast separations of proteins [18]. However hybrid columns prepared using relatively large CSP particles showed limited efficiency.

In the present work, we extended the preparation of hybrid interparticle monolithic separation media to fused-silica capillaries packed with small-particle chemically bonded C_{18} and NH_2 materials (3 and 5 μm). We fabricated columns with lauryl methacrylate monoliths, providing stronger reversed-phase retention in comparison to butyl methacrylate materials. Further, we prepared hybrid columns with zwitterionic polymethacrylate monoliths, suitable for hydrophilic interaction (HILIC) liquid chromatography of polar compounds. We investigated chromatographic properties of the new hybrid columns and application possibilities for separations of alkylbenzenes, proteins and phenolic acids.

Reversed-phase separation of large-molecular proteins differs in some aspects from the chromatography of low-molecular compounds. Isocratic separations are usually difficult to accomplish, because the retention of large molecules is very sensitive to even very small changes in the composition of aqueous–organic mobile phases and proteins with different molecular weights require mobile phases with different compositions to accomplish the elution [20]. Hence, gradient elution is usually necessary for successful and reproducible reversed-phase separations of proteins.

Generally, organic-polymer monolithic columns show better separation efficiency for biopolymers than for porous particulate or monolithic silica-based columns, because of a very low proportion of mesopores with respect to large flow-through pores, in which the transfer of the molecules from the mobile to the stationary phase is attributed to convection rather than to diffusion (generally slow for large molecules) [21]. Therefore it is interesting to investigate simultaneous effects of the particulate chemically bonded and monolithic stationary phases in a single column on the separation of proteins.

Hydrophilic interaction chromatography is essentially aqueous normal-phase chromatography on polar columns with occluded diffuse water layer, combining adsorption and partition distribution mechanism between the stationary and the mobile phase (usually buffered acetonitrile with low concentration of aqueous component). Columns with zwitterionic sulfobetaine stationary phase chemically bonded on silica gel support (available under the trade name ZIC-HILIC) provided very successful HILIC separations of polar samples [22]. Recently, we prepared HILIC monolithic sulfobetaine columns [23]. In the present work we investigate the properties of hybrid sulfobetaine monolithic HILIC columns prepared in fused silica capillaries packed with non-polar (C_{18}) and polar (NH_2) particles.

Table 1
Composition of polymerization mixtures used for the preparation of capillary monolithic and hybrid columns, % (w/w).

Column	% LMA	% EDMA	% BUT	% PROP	% H_2O
MON/LMA-EDMA	10.50	15.01	35.00	39.94	–
Column	% MEDSA	% EDMA	% BUT	% PROP	% H_2O
MON/MEDSA-EDMA	19.98	15.01	24.97	23.15	14.91

2. Experimental

2.1. Materials

Ethylene dimethacrylate (EDMA), lauryl methacrylate (LMA), [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide (MEDSA), 3-(trimethoxysilyl)propyl methacrylate, 1-propanol, and uracil were purchased from Sigma–Aldrich (Milwaukee, WI, USA). 1,4-Butanediol, hydrochloric acid, toluene, acetic acid, phenol and radical polymerization initiator AIBN were obtained from Fluka (Buchs, Switzerland). Gallic, protocatechuic, p-hydroxybenzoic (PHBA), 4-hydroxyphenylacetic (4-HPAC), vanillic, ferulic and sinapic acids were obtained from Fluka (Buchs, Switzerland). Caffeic acid was purchased from Sigma–Aldrich (St. Louis, MI, USA). Polyimide-coated 320 μm i.d. fused silica capillaries were purchased from J & W (Folsom, CA, USA).

The particulate column packing materials, Corasil II (silica gel, Co/S) and Corasil II C_{18} (Co/C18), both with particle size 37–50 μm, were obtained from Waters (Milford, MA, USA), Luna C_{18}, 3 μm, from Phenomenex, Torrance, CA, USA, Zorbax Eclipse XDB, 5 μm, from Agilent, Palo Alto, CA, USA and Biospher NH_2, 5 μm from LABIO, Prague, Czech Republic, a commercial sulfobetaine ZIC-HILIC column, 5 μm, 150 mm × 4.6 mm i.d., was purchased from SeQuant, Umea, Sweden.

Acetonitrile for HPLC (LiChrosolv grade) was obtained from Merck (Darmstadt, Germany), ammonium acetate (TraceSelect, 99,995%) was obtained from Fluka (Buchs, Switzerland) and formic acid (98%), from Riedel-de Haën (Seelze, Germany). Distilled water was purified in a Demiwa 5ROI station (Watek, Ledeč nad Sázavou, Czech Republic).

2.2. Instrumentation

A modular micro-liquid chromatograph was assembled from the following components: (i) two LC10ADvp pumps (Shimadzu, Kyoto, Japan) with a high-pressure gradient controller; (ii) a microvalve injector with a 60 nL inner sample loop (Valco, Houston, USA) equipped with a pneumatic actuator and an electronic time switch for injection of lower sample volumes; (iii) a restrictor capillary inserted as a mobile phase flow splitter closely before the injector; (iv) a variable wavelength LCD 2083 UV detector adapted for capillary electrophoresis, with a silica capillary flow-through cell, 50 μm i.d. (ECOM, Prague, Czech Republic); (v) a personal computer with a chromatographic CSW Data Station for Windows, version 1.5 (Data Apex, Prague, Czech Republic). The capillary columns were connected to the detector using a zero-volume PTFE capillary connection.

Table 2
Characteristics of the capillary monolithic and hybrid columns.

Column	L [mm]	i.d. [mm]	V_M [μL] (RP)	ε_T (RP)	K_F (10^{10}) [cm^2]	[N/m] 70% ACN
MON/LMA-EDMA	193	0.32	7.3	0.835	7.47	5,900
Luna/LMA-EDMA	137	0.32	4.86	0.441	0.512	33,000
Eclipse/LMA-EDMA	144	0.32	4.22	0.364	0.773	24,000
Biospher-NH_2/LMA-EDMA	132	0.5	20.64	0.795	6.75	6,800
Luna	157	0.32	7.81	0.618	0.806	162,000
Eclipse	141	0.32	5.87	0.518	1.15	247,000
Biospher-NH_2	170	0.5	23.45	0.703	9.72	85,000
COR-SIL/LMA-EDMA	190	0.32	7.27	0.476	3.94	4,100
COR-C18/LMA-EDMA	190	0.32	6.76	0.443	3.14	1,400
Column	**L [mm]**	**i.d. [mm]**	**V_M [μL] (HILIC)**	**ε_T (HILIC)**	**K_F (10^{10}) [cm^2]**	**[N/m] 95% ACN**
MON-HILIC/MEDSA-EDMA	191	0.32	11.67	0.759	4.85	3,900
Biospher-NH_2/MEDSA-EDMA	132	0.32	5.91	0.532	4.61	1,900
Biospher-NH_2	170	0.32	8.41	0.615	9.72	9,800
Zorbax C18/MEDSA-EDMA	153	0.32	1.92	0.156	0.26	1,400

L: column length, V_M: hold-up volume, ε_T: total porosity measured with toluene (TO) in 98% acetonitrile (HILIC mode) and with acetone (AC) in 50% acetonitrile (RP mode), K_F: permeability, N: number of theoretical plates, efficiency measured for sinapic acid in 95% acetonitrile (HILIC mode) and for acetone (AC) in 70% acetonitrile.

2.3. In situ preparation of capillary columns

2.3.1. Preparation of full-volume capillary monolithic columns

Full-volume monolithic stationary phases were prepared by *in situ* polymerization in fused silica capillaries, 0.32 mm i.d. Before polymerization, the inner wall surface of a polyimide-coated fused silica capillary was modified to improve the adhesion of the monolith bed to the capillary walls. As recommended by Lee et al. [24], the capillaries were rinsed with acetone and water, activated by flushing with 0.2 mol/L sodium hydroxide for 30 min at 0.25 μL/min, washed with water, 0.2 mol/L HCl for 30 min (0.25 μL/min), and finally with ethanol. 20% solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol (pH adjusted to 5 with acetic acid) was then pumped through the capillary at 0.25 μL/min using a syringe pump (KD Scientific, New Hope, PA, USA) for 1 h. The modified capillary was washed with ethanol, dried with a stream of nitrogen, and left at room temperature for 24 h.

The monolithic media for reversed-phase LC were prepared from polymerization mixtures containing LMA and EDMA as the monomer and cross-linker and a binary porogen solvent mixture,

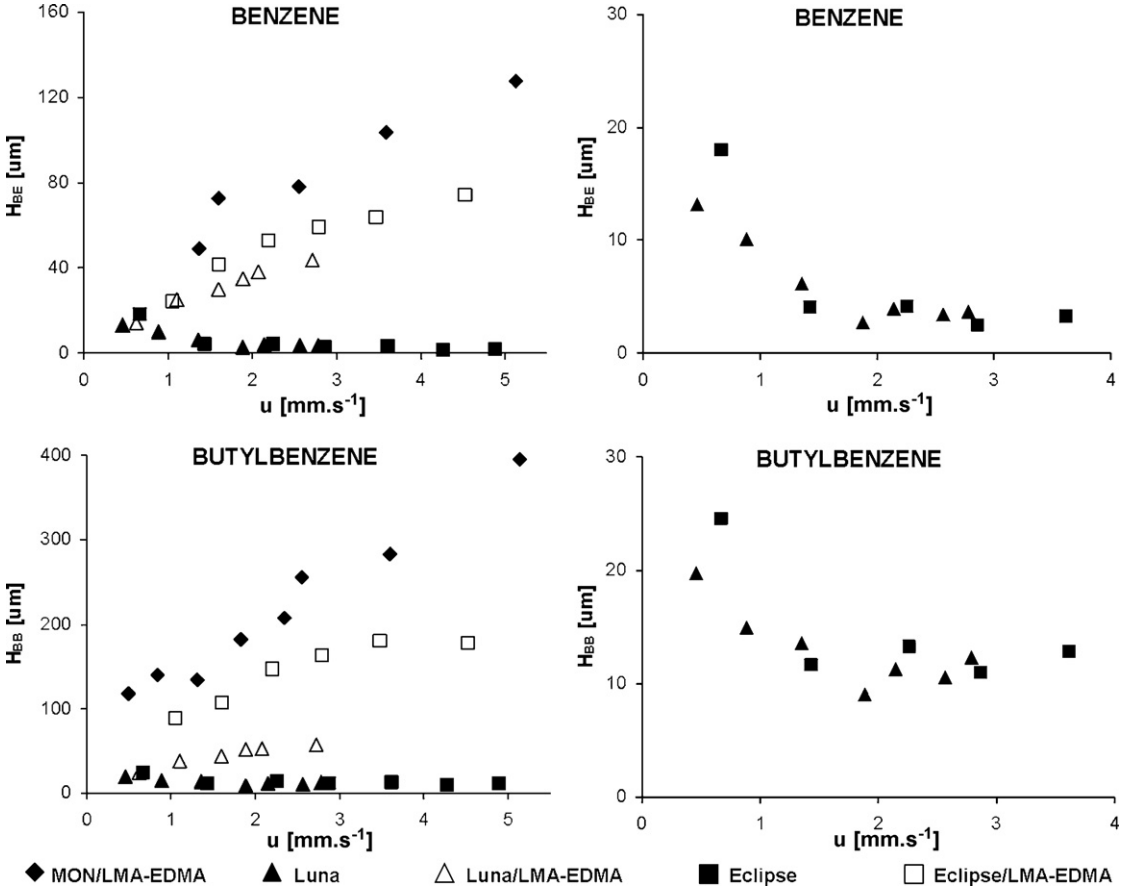

Fig. 1. Van Deemter H-u plots for benzene and butylbenzene on monolithic capillary column MON/LMA-EDMA, hybrid capillary columns Luna/LMA-EDMA, Eclipse/LMA-EDMA and packed capillary columns Luna and Zorbax, isocratic, 70% acetonitrile in water, UV-detection (λ = 254 nm).

consisting of 1-propanol (PROP) and 1,4-butanediol (BUT). The polymerization mixtures for the preparation of monolithic HILIC sulfobetaine columns contained MEDSA and EDMA as the monomer and cross-linker, respectively, and a ternary porogen solvent mixture, consisting of 1-propanol, 1,4-butanediol and water. Using an aqueous–organic porogen solvent mixture was dictated by limited solubility of MEDSA in purely organic solvents [23]. The exact composition of the polymerization mixtures for RP and HILIC monolithic columns is given in Table 1. 2,2′-Azo-bis-isobutyronitrile (AIBN) was used as the initiator of the polymerization reaction and was added to all polymerization mixtures at a constant concentration (1%, w/w, relative to the sum of monomers).

The polymerization mixture was filled into a fused-silica capillary with modified internal walls. Both ends of the filled capillary were sealed with rubber stoppers and the capillary was placed in a circulated-air thermostat. The polymerization reaction was performed at 60 °C for 20 h. Then, both ends of the capillary were cut, the monolithic column was washed with acetonitrile and finally with the mobile phase.

2.3.2. Preparation of hybrid interparticle monolithic capillary columns

For the preparation of hybrid columns with small particles (3–5 μm), fused silica capillaries were pre-packed using supercritical CO_2 technique as follows [25]: One end of a fused silica capillary (i.d. 320 μm, 30 cm in length) was closed by porous ceramic frit, prepared from mixture of water glass (Kittfort Prague, Czech Republic.), water and formamide (6:3:1, v/v, respectively) and dried at 80 °C for 24 h. The length of the frit was approximately 10 mm. The frits used for the fabrication of the hybrid columns only served to hold the packing particles during the polymerization process and eventually were cut-off from the finished hybrid columns.

Using a Valco ZU1T union, the column end with frit was connected to a fused-silica capillary restrictor, 10 μm i.d., 150 mm in length. The other end was inserted into a stainless-steel filling reservoir, containing ca. 20–30 mg of sorbent. The reservoir was connected to a HPLC piston pump HPP 5001 (Laboratory Instruments, Prague, Czech Republic) filled with liquid CO_2 (SIAD, purity 4.8) through a needle valve.

Table 3
The methylene selectivity, α, and the benzene (end group) selectivity, β, in the homologous alkylbenzene series (Eq. (1)) calculated for full-volume monolithic (LMA-EDMA), particle (Luna, Eclipse, Biospher-NH_2) and hybrid interparticle-monolithic columns for alkylbenzenes at 70% acetonitrile.

	α	β	R^2
MON/LMA-EDMA	1.24	0.27	0.991
Luna	1.50	2.13	0.999
Luna/LMA-EDMA	1.51	3.08	0.999
Eclipse	1.54	1.40	0.999
Eclipse/LMA-EDMA	1.53	1.97	0.999
Biospher-NH_2/LMA-EDMA	1.43	0.20	0.999

R^2: correlation coefficient.

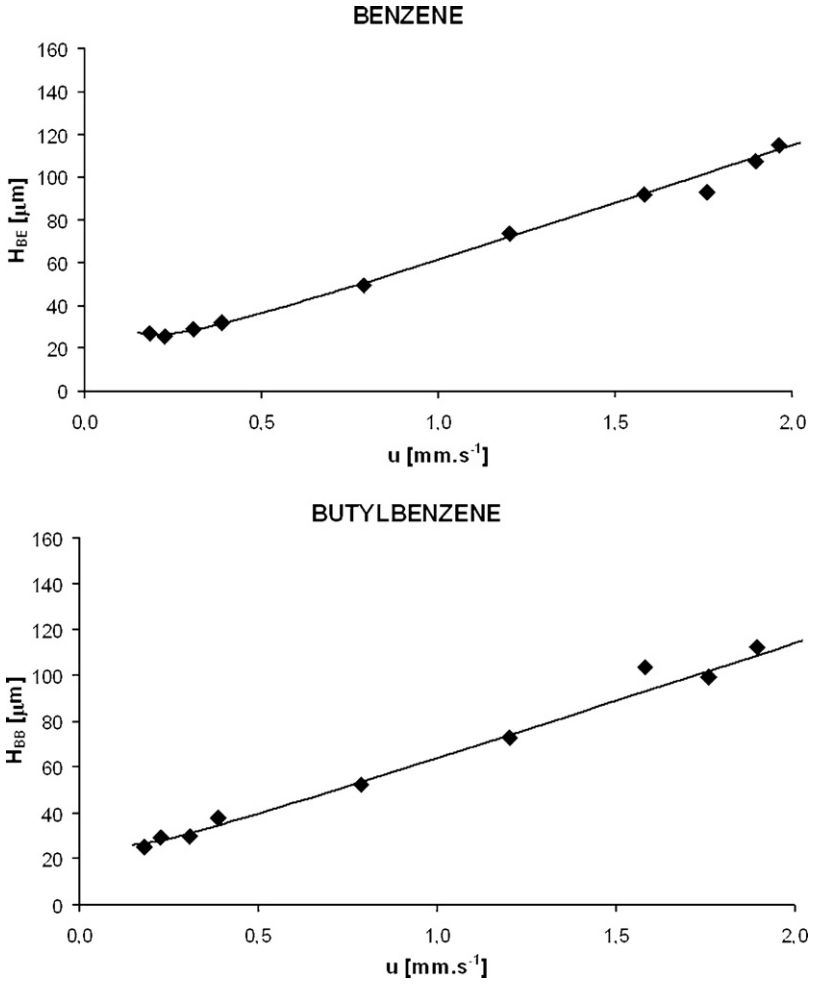

Fig. 2. Van Deemter H-u plots for benzene and butylbenzene on hybrid capillary column Biospher-NH_2/LMA-EDMA, 5 μm (132 mm × 0.5 mm i.d.), isocratic, 70% acetonitrile in water, UV-detection (λ = 254 nm).

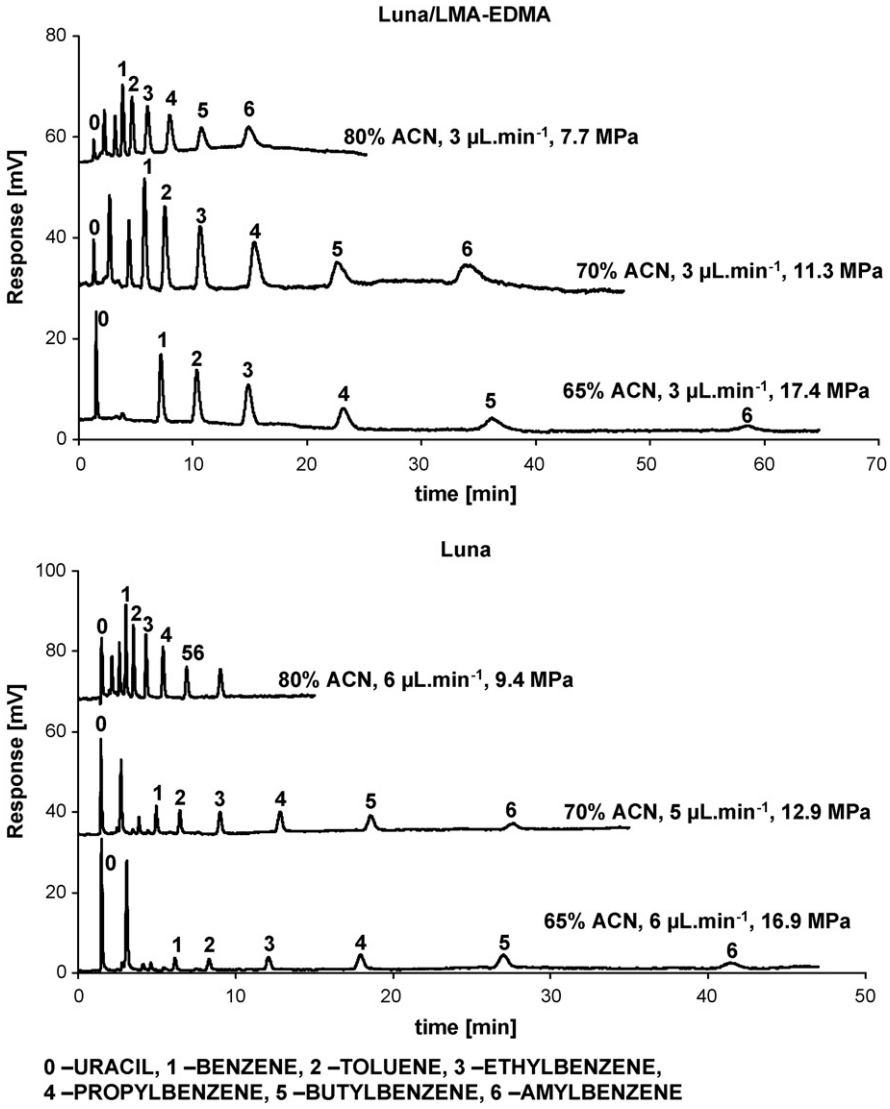

Fig. 3. Separation of alkylbenzenes. Conditions: UV-detection (λ = 254 nm), laboratory temperature; isocratic: ACN in water; Capillary hybrid column Luna/LMA-EDMA, 3 μm (133 mm × 0.32 mm i.d.). Capillary packed column Luna, 3 μm (157 mm × 0.32 mm i.d.).

First, the reservoir was positioned vertically in the holder with the restrictor directed "up". When the valve was slowly opened, the system was let at the working pressure 15 MPa for approximately 1 min. After balancing of pressure, the filling reservoir and column with the restrictor were directed "down" and immersed into an ultrasonic bath (50 °C). In a few minutes, the suspension of sorbent particles penetrated into the capillary column. The total filling time was about 30 min, and the next 30 min were used to consolidate the column packing by ultrasonic vibration under the flow of CO_2. After this time the valve was closed and the column was allowed to depressurize slowly and spontaneously at 25 °C overnight outside of the sonic bath. Finally, the column was shortened to the required length and a ceramic frit was made at the inlet end of the packed capillary columns used as the reference. For the preparation of hybrid interparticle monolithic columns, the second frit was omitted.

Hybrid columns were prepared in silica capillaries packed either with fully porous or with CSP solid core particles by *in situ* polymerization using the same polymerization mixture as for the preparation of the full-volume monolithic columns (Table 1). Hybrid columns with Corasil Silica and Corasil C_{18} particles were prepared in a simple way, by pushing a slurry of particles in the polymerization mixture (mixed in the ratio 1:1) into a fused capillary by pressurized helium from a pressure vessel, before the *in situ* polymerization. Hybrid particle-monolithic columns with small-diameter particles were prepared by pushing the polymerization mixture from an attached small container, applying the pressure of helium, into a pre-packed fused-silica capillary. Both ends of the filled capillary were sealed with rubber stoppers and the capillary was placed in a circulated-air thermostat. Then, the polymerization reaction was performed at 60 °C for 20 h, as with the full-volume monolithic columns. Finally, the end-frit was cut-off.

2.4. Microcolumn LC operation conditions

Sample solutions were prepared in the mobile phase at concentrations yielding adequate detector response. Volumes of 60 nL or lower were injected onto the columns.

For isocratic separations of alkylbenzenes, pre-mixed mobile phases containing various volume fractions of acetonitrile in aqueous–organic mobile phases were used. For reversed-phase HPLC of proteins, gradients of acetonitrile in water, both with addition of 0.15% (vol.) trifluoracetic acid, were generated in the Shimadzu high-pressure binary gradient system. For the analyses of phenolic acids, mixtures of acetonitrile and acetate buffer (0.01 M

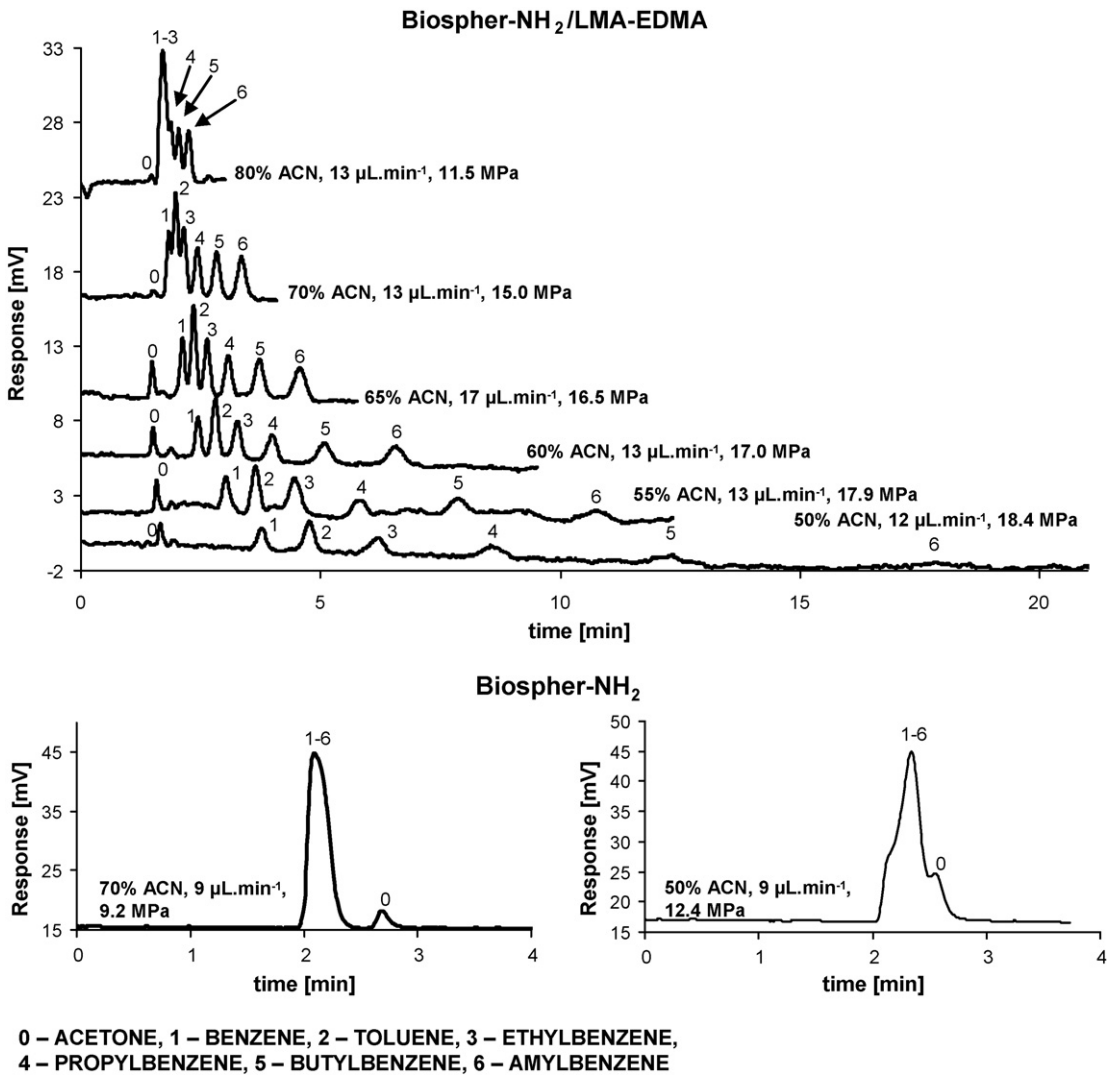

Fig. 4. Separation of alkylbenzenes. Conditions: UV-detection (λ = 254 nm), laboratory temperature; isocratic: ACN in water; Capillary hybrid column Biospher-NH_2/LMA-EDMA, 5 μm (132 mm × 0.5 mm i.d.). Capillary packed column Biospher-NH_2, 5 μm (170 mm × 0.5 mm i.d.).

CH_3COONH_4 in water adjusted with formic acid to pH 3) were used as the mobile phase.

Before use, all mobile phases were filtered through a Millipore 0.45 μm filter and degassed by ultrasonication (UC 405 BJ1, TESLA, Prague, Czech Republic). The actual flow-rate of the mobile phase, in the range between 1 and 25 μL/min, was measured using a stopwatch and a calibrated 100 μL microburette. The column hold-up volumes, V_M, were determined from the elution volumes of non-retained marker compounds, uracil in the RP LC and toluene in the HILIC modes. All chromatographic separations were carried out at laboratory temperature. The retention times and the peak widths at the half peak height were evaluated using the CSW Data evaluation software (Data Apex, Prague, Czech Republic). All experiments were repeated in triplicate and the arithmetic means of the retention volumes, V_R, were used for calculations of the retention factors, $k = (V_R - V_M)/V_M$, and other column characteristics.

3. Results and discussion

3.1. Porosity, flow properties and efficiency of hybrid columns

We varied the composition of the polymerization mixtures containing laurylmethacrylate–ethylene dimethacrylate monomer and cross-linker to optimize the efficiency, selectivity and flow resistance (permeability) of the monolithic bed in similar way as described earlier for poly(butylmethacrylate) columns [20]. The composition of polymerization mixture in non-aqueous binary porogen solvent that provided the best monolithic column for reversed-phase chromatography, MON/LMA-EDMA (Table 1) was used to prepare all hybrid reversed-phase columns.

We compared the flow properties and efficiencies of the hybrid columns prepared in fused silica capillaries pre-packed with the Corasil Silica and Corasil C_{18} CSP particles [26] or with fully porous 3 μm or 5 μm octadecyl silica or aminopropyl silica particles, of the pure monolithic laurylmethacrylate–ethylene dimethacrylate column (MON/LMA-EDMA), and columns packed with fully porous 5 μm (Zorbax Eclipse) and 3 μm (Luna) C_{18} particles (Table 2). The monolithic MON/LMA-EDMA column has higher porosity (ε_T = 0.84), and better permeability than the columns packed with fine particles, which show the total porosity $\varepsilon_T \approx 0.5$–0.6. The hybrid columns generally show chromatographic properties in between the parent particulate and the pure monolithic columns. The hybrid columns with CSP particles have $\varepsilon_T \approx 0.44$–0.48; the lowest porosity was measured for the hybrid columns with fully porous small particles, $\varepsilon_T \approx 0.36$–0.44. This decrease in porosity indicates that the monolithic phase fills essentially only the inter-particle volume in the hybrid columns, as significantly larger drop in the total porosity is expected should the organic polymer fill

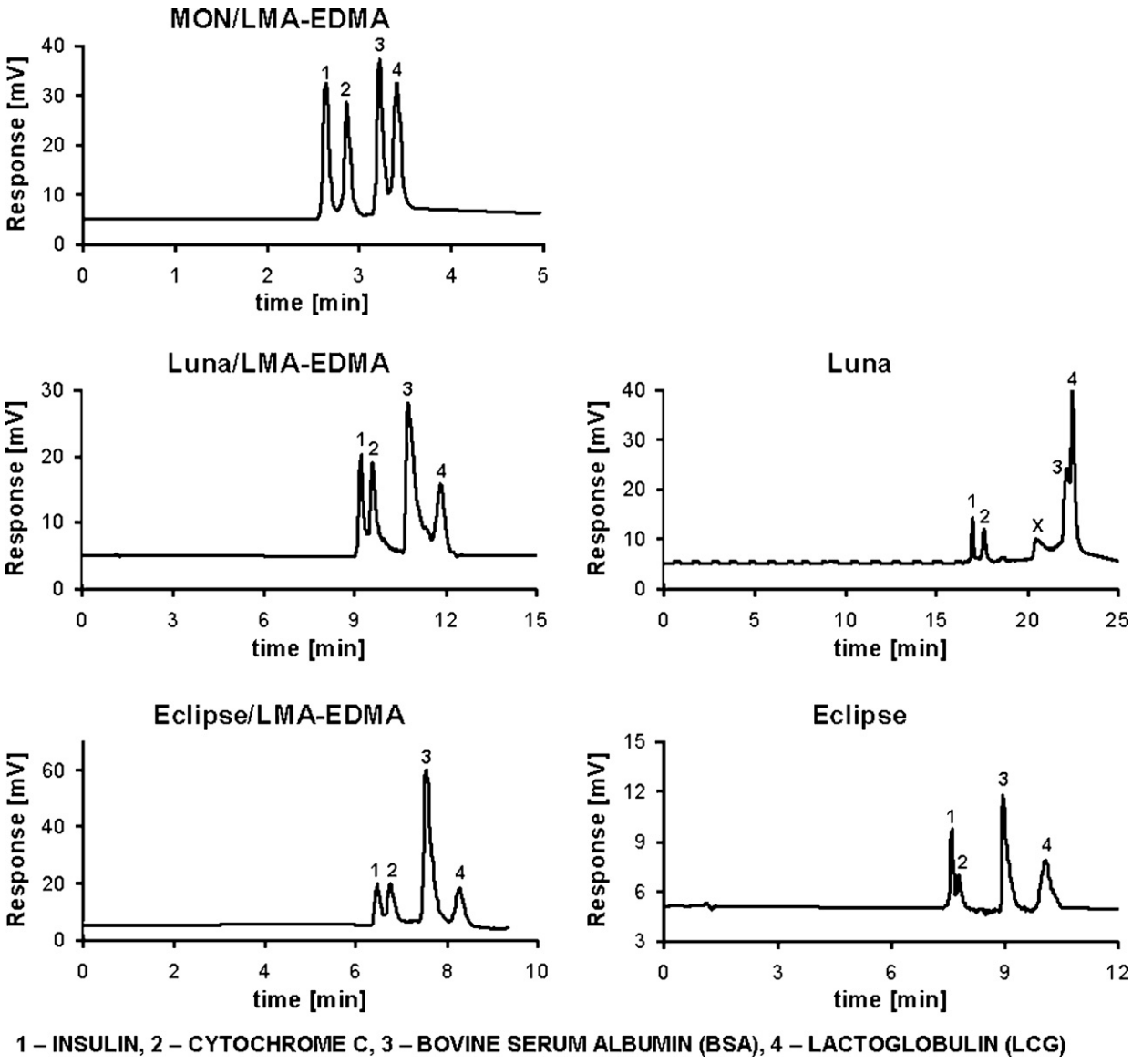

Fig. 5. Separation of proteins. Conditions: UV-detection (λ = 220 nm), laboratory temperature; gradient: acetonitrile (B) in water + 0.1% TFA (A); MON/LMA-EDMA: 20–80% B in 4 min, 17 μL min^{-1}, Δp = 21 MPa, G = 6.45; Luna/LMA-EDMA: 20–80% B in 15 min, 3 μL.min^{-1}, Δp = 16 MPa, G = 6.45; Luna: 15–50% B in 20 min, 3 μL min^{-1}, Δp = 15 MPa, G = 4.49; Eclipse/LMA-EDMA: 20–80% B in 10 min, 5 μL min^{-1}, Δp = 15 MPa, G = 5.08; Eclipse: 20–80% B in 15 min, 5 μL min^{-1}, Δp = 12 MPa, G = 3.2. G = gradient slope in % acetonitrile per column hold-up volume (V_M) of effluent (V_G/V_M), V_G = the volume of the mobile phase from the start to the end of gradient.

in a significant part of the inner particle pores. The permeability, K_F, of hybrid columns with CSP particles is approximately half the K_F of the pure monolithic column MON/LMA-EDMA. The columns packed with small-diameter particles show significantly lower permeabilities than the purely monolithic column, surprisingly not much higher than the K_F of the hybrid columns prepared on their basis (Table 2).

Table 2 gives the numbers of theoretical plates, N, calculated from the experimental retention times and peak widths of acetone and Fig. 1 illustrates the efficiency of the full-volume monolithic, particle packed and hybrid interparticle monolithic in terms of van Deemter plots of the height equivalent to theoretical plate, H, of benzene and butylbenzene *versus* the linear flow velocity of the mobile phase, u, in 70% acetonitrile. The hybrid columns show intermediate efficiency, lower than the corresponding "mother" packed micro-particulate columns (Luna 3 μm, Eclipse 5 μm), but higher (lower H) than the pure monolithic column MON/LMA-EDMA. This agrees with generally observed worse performance of organic polymer monolithic columns for small molecules than for large biopolymers [22]. The hybrid monolithic interparticle lauryl methacrylate–ethylene dimethacrylalyte column prepared with 5 μm aminopropyl silica particles shows similar efficiency as the small C_{18} particle-based hybrid columns, with maximum efficiency (minimum H) of approximately 4 times the particle diameter (h = 4) at the linear flow velocity of 0.2 mm s^{-1}, determined by fitting the well known van Deemter equation, $H = A + B/u + Cu$, to the experimental data (Fig. 2).

3.2. Non-polar selectivity of hybrid columns for reversed-phase LC—separation of alkylbenzenes

The chromatograms in Fig. 3 show the separation of benzylalcohol, benzaldehyde, benzene (1) and five homologous *n*-alkylbenzenes (2–6), on a hybrid interparticle laurylmetacrylate–ethylene dimethacrylalyte small-particle column and on the "mother" Luna C_{18} 3 μm column with various concentrations of acetonitrile in water as the mobile phase. The hybrid column shows comparable selectivity and retention as the particle-packed

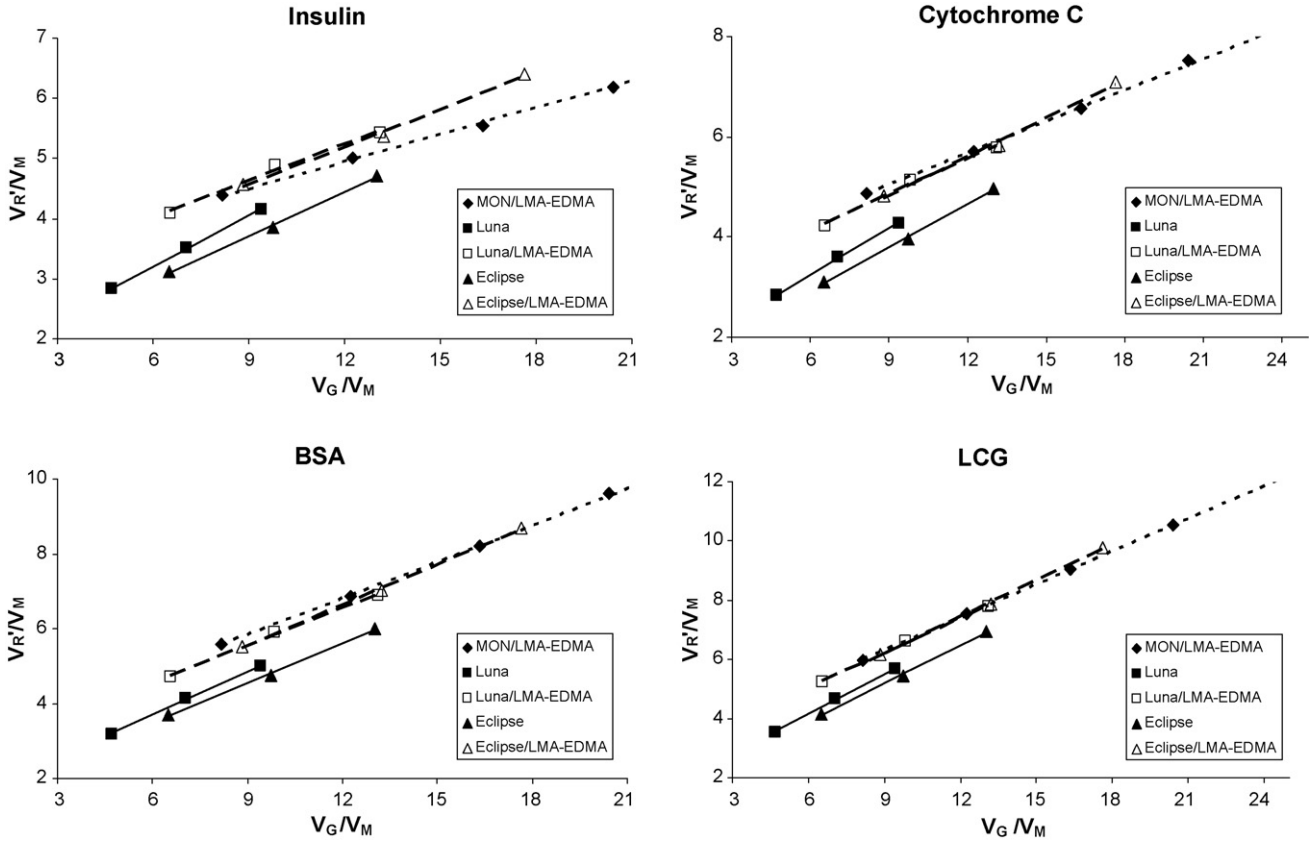

Fig. 6. Normalized elution volumes, V_R/V_M, of proteins on the monolithic MON/LMA-EDMA, hybrid interparticle C_{18}/LMA-EDMA and particle C_{18} columns in gradient elution (20–80% acetonitrile) at varying gradient volumes, V_G/V_M.

column. The ratios of the retention times on the two columns in various mobile phases are inversely proportional to the mobile phase flow-rates on the two columns. Similar behaviour was observed with the hybrid lauryl-metacrylate–ethylene dimethacrylalyte column in comparison to the "mother" column packed with Zorbax Eclipse C18, 5 μm, particles (not shown).

In Table 3, the methylene selectivity, α, and the benzene (end group) retention, β, in the homologous alkylbenzene series (Eq. (1)) are compared for a full-volume monolithic column (MON/LMA-EDMA), the C_{18} columns packed with 5 μm (Zorbax Eclipse XDB) and 3 μm (Luna) particles and the hybride-interparticle columns polymerized in the pre-packed small-particle columns [27].

$$\log k = \log \beta + n_c \log \alpha \quad (1)$$

The methylene (lipophilic) selectivity, α, was determined as the increase in the retention, $\log k$, per one carbon atom in alkylbenzenes containing n_c carbon atoms in the alkyl chain (Eq. (1)). α is approximately the same for the particle-packed and the hybrid columns (1.50–1.53), but is significantly higher than with the pure monolithic laurylmethacrylate–ethylene dimethacrylate column, MON/LMA-EDMA (1.24). On the contrary, the aromatic (benzene) end-group retention, β, has the lowest value for the full-volume methacrylate column (0.27), but is higher on the hybrid columns (2.0 and 3.1 for the Luna and the Eclipse columns, respectively) than on the "mother" small-particle columns (1.4 and 2.1). Hence, the methylene selectivity of the hybrid LMA-C_{18} columns can be primarily attributed to the "mother" C_{18} particles, whereas the interparticle monolithic phase enhances the aromatic (benzene) retention. Obviously, the hybrid LUNA/LMA-EDMA column does not offer any practical advantage for the separation of alkylbenzenes with respect to the particulate LUNA column, which shows higher porosity, permeability and efficiency, except for the fritless design.

On the other hand, the bonded particle aminopropyl silica Biospher NH_2 column does not retain alkylbenzenes which all elute earlier than acetone, but the hybrid NH_2/LMA-EDMA column prepared using the bonded aminopropyl silica particles shows significant methylene selectivity ($\alpha = 1.43$), even slightly higher than the LMA-EDMA monolithic column. In contrast to LUNA/LMA-EDMA hybrid column, the reversed-phase retention and selectivity for alkylbenzenes on the hybrid aminopropyl-silica based column is controlled primarily by the properties of the interparticle monolithic phase. Relatively high permeability, porosity and low retention allows isocratic separation of alkylbenzenes on the hybrid NH_2/LMA-EDMA column in less than 5 min in 65% acetonitrile, which is considerably faster than the separation on the hybrid C_{18}/LMA-EDMA, or on a particulate Luna C_{18} column (Fig. 4).

3.3. Comparison of the reversed-phase separation of proteins on hybrid, particle-packed and pure monolithic columns

We compared the chromatographic behaviour of four proteins with different molecular weights on particle-packed, monolithic and hybrid columns under reversed-phase gradient elution conditions, corresponding to the gradient steepness in between 3.2 and 6.45% acetonitrile per one column hold-up volume unit of the effluent. The lauryl-metacrylate–ethylene dimethacrylalyte monolithic column, MON/LMA-EDMA, provides complete separation of insuline, cytochrom C, bovine serum albumine (BSA) and lactoglobulin (LGB) in considerably shorter time (3.5 min) with larger differences in the relative retention and, consequently, better resolution in comparison to the C_{18} particle-packed Luna 3 μm and Eclipse 5 μm C_{18} column s, even though less steep gradients (which generally enhance the resolution under gradient conditions) were used with the particle-

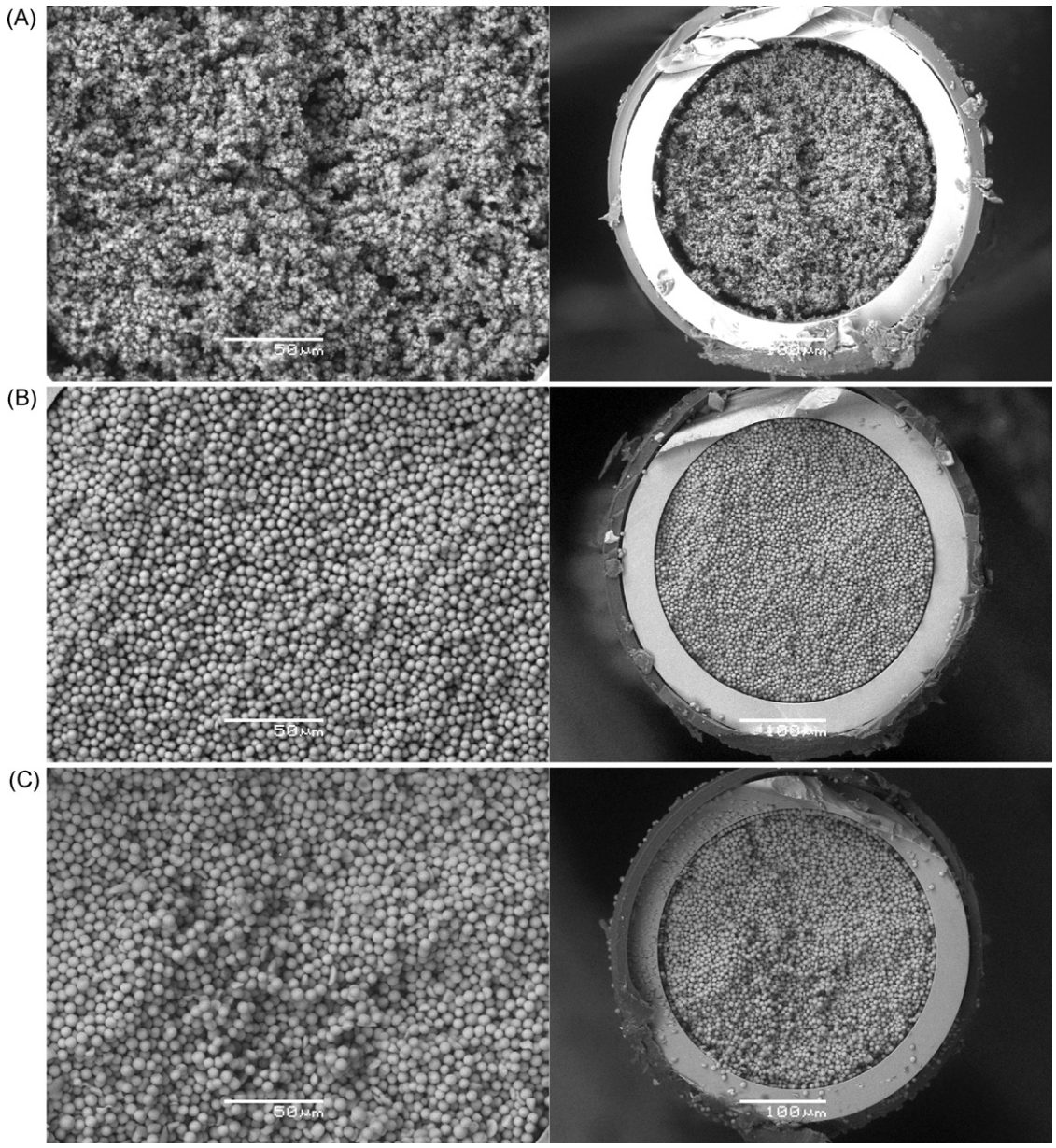

Fig. 7. Microphotographs of the cross-section of HILIC capillary columns (0.32 mm i.d.). (A) Monolithic capillary column; HILIC (MON/MEDSA-EDMA); (B) Hybrid capillary column Zorbax C18/MEDSA-EDMA; (C) Hybrid capillary column Biospher-NH_2/MEDSA-EDMA.

packed columns. On the hybrid particle-monolithic columns, Luna/LMA-EDMA and Eclipse/LMA-EDMA, the relative retention of proteins is similar as on the pure monolithic MON/LMA-EDMA column, with lower retention volumes, but higher retention times (due to a lower flow-rate dictated by lower column permeabilities). The resolution is better and the retention times are shorter on the hybrid columns than on the packed C_{18} 5 μm Eclipse (insulin/cytochrome) or 3 μm Luna (BSA/LCG) columns (Fig. 5). Poor resolution of BSA and LCG on the Luna column probably causes the differences in their relative intensities with respect to the other columns tested; the small peak detected between the peaks 2 and 3 probably belongs to an impurity. On the other hand, the separation of proteins is better on the monolithic MON/LMA-EDMA column than on the hybrid columns. This behaviour suggests that the selectivity and efficiency of separation of proteins on the hybrid columns is more significantly contributed by the interparticle monolithic stationary phase than by the C_{18} particles, unlike the separations of alkylbenzenes.

In Fig. 6, the net gradient elution volumes normalized in the column hold-up volume units, V'_R/V_M, are plotted *versus* the normalized dimensionless gradient volume (again in the column hold-up units), V_G/V_M. The plots show regular linear increase of retention with increasing molecular weights of proteins on all columns. The plots for the hybrid columns are similar to each other and much closer to the plots for the monolithic lauryl-methacrylate column (MON/LMA-EDMA). On the particle-packed columns, the gradient volume has less effect on the increase in the retention of proteins in terms of V'_R/V_M. In this respect, too, the properties of the hybrid stationary phases depend more on the monolithic interparticle phase than on the properties of the "mother" particle-packed columns.

The retention of proteins in gradient elution RP-HPLC can be characterized by concentrations of acetonitrile at the time of elution, φ_e, which increase with the molar masses of proteins and are higher for particulate and hybrid columns than for the pure monolithic column. However, the differences between the elution

Table 4
Elution concentration of acetonitrile in gradient elution of proteins (20–80% acetonitrile).

Column	Elution concentration [% ACN]			
	Insulin	Cytochrome C	BSA	LCG
MON/LMA-EDMA	26.3	30.0	36.4	38.8
Luna	36.9	37.9	42.3	46.4
Luna/LMA-EDMA	34.5	36.0	40.7	44.2
Eclipse	35.6	36.3	41.1	45.4
Eclipse/LMA-EDMA	35.2	37.2	42.4	46.0
COR-SIL/LMA-EDMA	31.2	33.4	39.7	42.8
COR-C18/LMA-EDMA	24.0	26.6	32.9	35.2

concentrations of individual proteins, characterizing the reversed-phase separation selectivities for proteins, are higher on the hybrid than on the particle-packed columns (Table 4).

3.4. Hybrid columns for HILIC separations of polar compounds

Hybrid zwitterionic columns were prepared in fused silica capillary columns pre-packed with 5-μm Zorbax Eclipse C_{18} and Biospher Amine as the support particulate materials by co-polymerization of the sulfobetaine methacrylate, MEDSA, with ethylene dimethacrylate in the same way as the reference pure monolithic sulfobetaine zwitterionic phase (MON/MEDSA-EDMA) in the empty capillary [23] (Table 1). Fig. 7 compares the microphotographs of the hybrid interparticle columns and of the monolithic zwitterionic MON/MEDSA-EDMA column. Closer inspection of the pictures of the hybrid columns can reveal small monolithic globules in the space between the 5-μm adsorbent spheres. However, the type of the particles has surprising effect on the column permeability, which is much better with the aminopropyl silica than with the C_{18}-based hybrid column (Table 2).

On all the monolithic and hybrid columns we observed dual retention mode, based on the HILIC mechanism in mobile phases rich in acetonitrile and on the reversed-phase mechanism in water-rich mobile phases, with U-shape dependence of the retention on mobile phase composition. Fig. 8 shows two examples of the U-shape retention plots for gallic and caffeic acids. The points represent the experimental data and the lines show the best-fit dependence of the $\log k$ *versus* the volume fraction of buffer, φ(buffer), derived earlier, Eq. (2) [23], which were determined by non-linear regression over the full range of the mobile phase composition:

$$\log k = a_1 + m_1 \cdot \varphi(\text{buffer}) - m_2 \cdot \log \varphi(\text{buffer}) \quad (2)$$

The parameter m_1 characterizes the effect of increasing concentration of water in the mobile phase on the contribution of reversed-phase mechanism to the retention. The parameter m_2 is a measure of decreasing HILIC contribution to the retention in highly organic mobile phases; a_1 is an empirical constant without specific physical meaning. Interesting features of hybrid sulfobetaine monolithic stationary phases are a broader HILIC mobile phase range and higher retention in comparison to the zwitterionic pure monolithic column, MON/MEDSA-EDMA.

We applied the hybrid sulfobetaine columns to the HILIC separation of phenolic acids. The pure monolithic and hybrid columns offer a broader reversed-phase retention range for phenolic acids

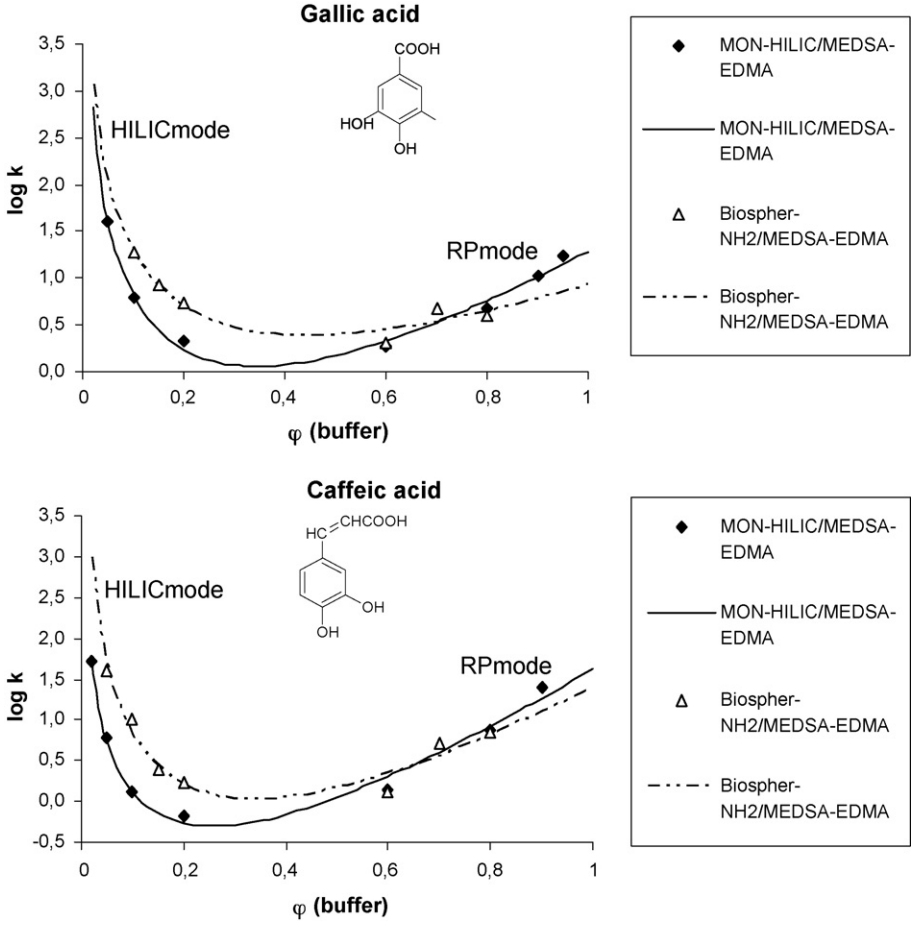

Fig. 8. U-shape plots of the retention factors of phenolic acids, k, versus the volume fraction of buffer, φ (buffer).

than the ZIC-HILIC particulate column, whereas the aminopropyl silica column does not show significant retention of phenolic acids in the RP-mode. The retention in the HILIC mode in acetonitrile-rich mobile phases increases with increasing number of phenolic OH-groups, i.e., with increasing sample polarities on all the columns tested (Fig. 9), even though the individual columns show important differences in the concentration of buffer required for the separation, peak symmetry and efficiency, and minor differences in the relative retention (separation selectivity). The retention in the HILIC mode increases in the following order: conventional particle-packed ZIC-HILIC column, pure monolithic MON/MEDSA-EDMA capillary column, hybrid C_{18}/MEDSA-EDMA and hybrid NH_2/MEDSA-EDMA columns. Higher retention in comparison to the MON/MEDSA-EDMA column results in better HILIC separation of phenolic acids, especially of the less retained ones, on the hybrid Zorbax Eclipse C_{18}/MEDSA-EDMA capillary column (Fig. 9).

The retention on the hybrid NH_2/MEDSA-EDMA column in the HILIC mode is strongly affected by the properties of the aminopropyl silica support, which may be used alone as the material for HILIC separations. Generally, the separation of phenolic acids is better on the particle-packed "parent" Biospher-NH_2 column than on the less efficient hybrid NH_2/MEDSA-EDMA column, which however shows higher separation selectivity for some compounds, such as caffeic, 4-hydroxyphenylacetic and protocatechuic acids, which are poorly resolved on the particulate Biospher NH_2 column. Further, the peaks of strongly retained polyphenolic acids such as gallic acid are more symmetrical on the hybrid Biospher-NH_2/MEDSA-EDMA column. The improved peak shape can be probably attributed to suppressed ion-exchange interactions of the basic amino groups by electrostatic or hydrogen-bonding interactions with the acidic -SO_3^- groups in the monolithic zwitterionic interparticle phase. The Zorbax C18/MEDSA-EDMA hybrid column shows the best HILIC separation of phenolic acids of all the capillary columns tested, better than both the monolithic MON-HILIC/MEDSA-EDMA and the particle-packed Biospher aminopropyl silica gel column, and comparable to the commercial ZIC-HILIC conventional analytical column. This hybrid column allows separations of phenolic acids with lower concentration of

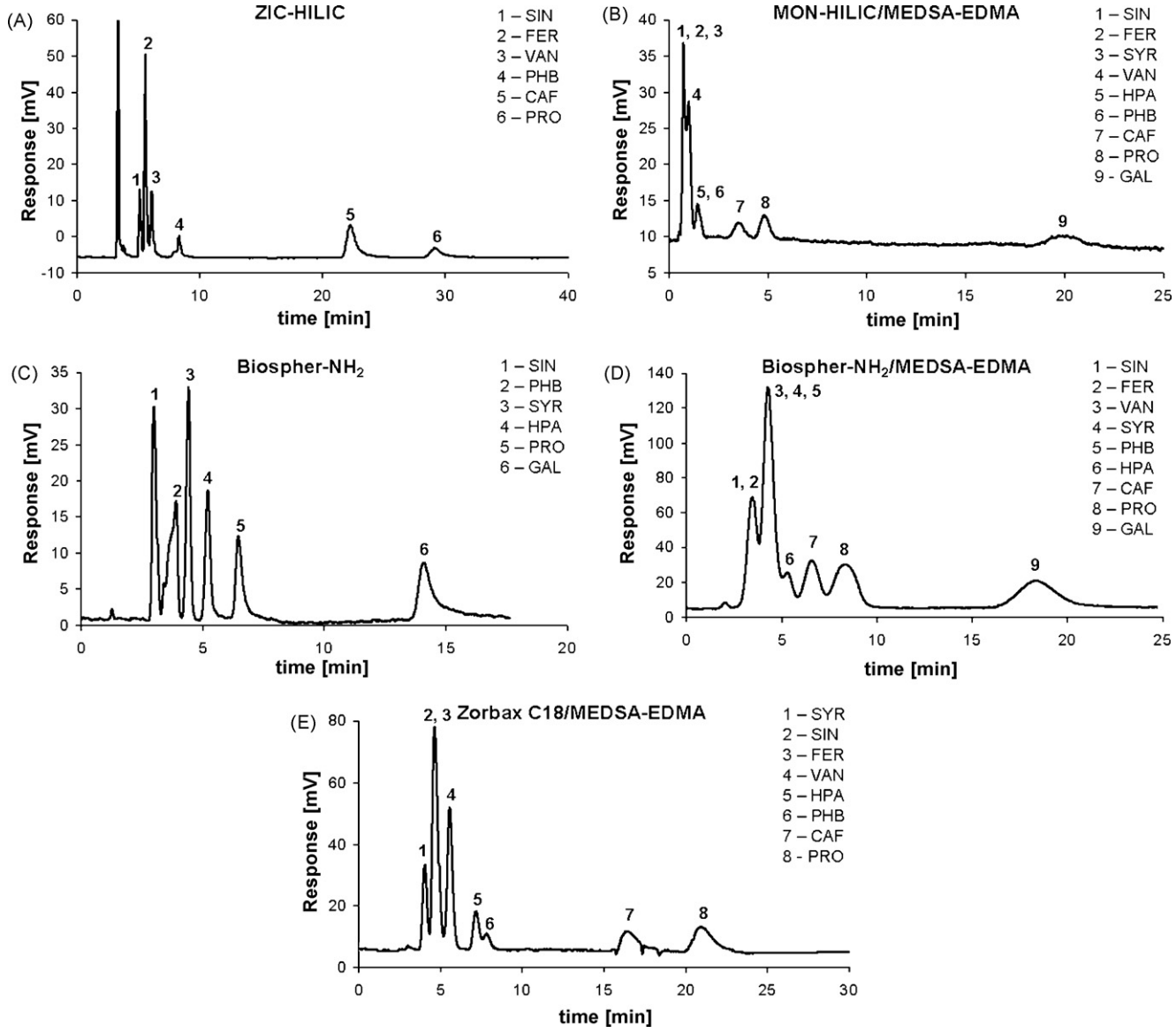

Fig. 9. Isocratic HILIC separation of phenolic acids. Conditions: UV-detection (λ = 220 nm), laboratory temperature; Mobile phase: aqueous 10 mM ammonium acetate buffer (pH ~3) (B) in acetonitrile (A); ZIC-HILIC: 3.5% B, 0.5 mL min^{-1}, Δp = 16 MPa; MON-HILIC/MEDSA-EDMA: 5% B, 18.5 μL min^{-1}, Δp = 6 MPa; Biospher-NH_2: 10% B, 7.1 μL min^{-1}, Δp = 10 MPa; Biospher-NH_2/MEDSA-EDMA: 15% B, 4.7 μL min^{-1}, Δp = 8.6 MPa; Zorbax Eclipse C18/MEDSA-EDMA: 5% B, 0.15 μL min^{-1}, Δp = 18 MPa.

acetonitrile in the mobile phase and at lower flow-rates in comparison to pure monolithic or particle-packed ZIC-HILIC columns.

4. Conclusions

An important advantage of the hybrid monolithic-particle columns with respect to the particle-packed capillary columns is that they do not need column end frits, which are tricky to fabricate and may be source of extra-column band broadening. The monolithic moiety "glues" the entrapped sorbent particles together and to the fused silica capillary walls. The results of the present work show that the permeability and efficiency of the hybrid interparticle monolithic columns prepared with small particles (5 and 3 μm), even though lower than with the particle-packed columns, are generally sufficient for applications in capillary HPLC, so that the fabrication of the hybrid columns is not limited to large particle materials. The efficiency of the hybrid columns is generally in between the efficiency of particle-packed and monolithic columns.

In addition to the fritless design, the hybrid columns combine some retention and selectivity properties of the particle and monolithic components, which may be useful for specific separation problems, as demonstrated by several examples in this work. The separation efficiency of the hybrid capillary columns is generally in between the packed and monolithic columns. The properties of the hybrid columns are still not at optimum, however, some application examples of the present work seem encouraging and show that the efficiency and permeability are good enough to allow separation times and resolution comparable or even better than with packed capillary columns. For example, the Zorbax C18/MEDSA-EDMA hybrid column shows HILIC separation of phenolic acids better than the particle-packed aminopropyl silica gel column (Fig. 9). Further, separation of proteins on the hybrid Eclipse/LMA-EDMA hybrid column is better than on the "parent" Eclipse C18 column. Hence we believe that this type of column is worth further future research aimed to improve their dynamic flow properties and to prepare new separation media suitable for other types of analytes.

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