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# Morphological and chromatographic characterization of molecularly imprinted monolithic columns.

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***Abstract:*** The chromatographic stationary phases based on molecularly imprinted monoliths were prepared by free radical polymerization and subsequently characterized. The mixture of commonly used comonomers of ethylene dimethacrylate/methacrylic acid was polymerized in presence of various porogenic mixtures. Tosyl-L-phenylalanine was selected as a model template. Polymerization steps were carried out in the glass columns (i.d. of 3.3 mm) enabling UV initiation. Difficulties encountered with polymerization *in situ*, e.g. volume contraction and adhesion of polymer onto the glass inner wall, were successfully solved. The morphology of monoliths was investigated by electron microscopy, mercury porosimetry and surface area measurements. The influence of polymerization conditions on monolith morphology and subsequent chromatographic properties was evaluated. Polymers prepared by UV polymerization showed higher total porosity and the most frequent pore radius compared to the same polymers prepared thermally. The prepared monoliths by UV irradiation were also significantly more permeable for mobile phase during the chromatography tests. Morphology of monoliths prepared in dodecanol/toluene porogenic mixture markedly depended on the temperature of polymerization; the most frequent pore radius decreased with increasing temperature. From chromatographic point of view, monolithic columns were tested by separations of standard hydrophobic solutes. Height equivalent to a theoretical plate reached the values of approx. 25 µm, Walters indices of hydrophobicity laid slightly above 3. Prepared monoliths were compared to the conventional columns, filled with the particles prepared from the polymer of the same composition and preparation. In many cases the monolithic columns showed better chromatographic parameters.

*Key words:* Molecularly imprinted polymers, Monolithic columns, Morphology.

## Introduction

Due to their ability to separate target molecules with high selectivity, stationary phases based on molecularly imprinted polymers (MIPs) have recently received great attention, especially in enantioselective separations of biologically active molecules [1].

The preparation of MIPs is based on the procedure, where the template molecule to be determined is added to a reaction mixture consisting of a monomer, a cross-linker, solvents (also called porogenes) and an initiator. The monomer carries certain functional groups capable of interacting with the template. During polymerization the binding sites are constructed according to the shape [2] and chemical properties [3,4] of the target molecules. After removal of the template, these specific binding sites exhibit high selectivity and affinity to the molecules with similar structure as the template [5-7].

The conventional approach for preparation of molecularly imprinted chromatographic columns involves the preparation of polymer particles and their subsequent packing into the columns [8]. In general, conventional methods are tedious, time-consuming and suffer from problems connected with particle irregularities [9]. Although some complications have been partly solved by using the uniform polymer beads prepared by precipitation polymerization [10, 11], the most promising strategies to simplify the molecular imprinting technique is polymerization *in situ* [12-14]. Polymer rods (so called “monoliths”) were prepared directly inside the separation columns and subsequently employed as chromatographic media. A general methodology of monolithic materials, principles of thermodynamics and effects of reaction kinetics during polymerizations are in detail reviewed in a comprehensive book by F. Svec [15].

Monolithic stationary phases have usually been prepared in capillaries of small diameters for nano/micro-HPLC [16]. Relatively a few studies were focused on the preparation of the classically sized imprinted monoliths [17-20]. Moreover, these monoliths were synthesized exclusively inside the steel columns since this material is convenient with regard to a favourable adhesion of the monolith to the column wall and to an uncomplicated connection with the HPLC apparatus. The limitation of steel material resides in using the thermally initiated polymerizations only, as steel is not transparent for UV irradiation.

In our study we investigated methacrylic monolithic phases prepared in the classically sized columns made from the glass. This material enables the preparation of monoliths by both thermal and photo initiation. The second one appears to be more promising as the preparation of the monoliths is faster and particularly the reaction can proceed at the broad temperature range. It is important with respect to the kinetics of the imprinting procedure, as the change of temperature affects significantly the interaction between template-monomer and template-porogene [21-24]. Various temperatures affect chain polymerization kinetics as well, resulting in the different growth of the polymer agglomerates and subsequently in different polymer morphology (surface areas, distribution of pore sizes etc.) [15, 25]. Regardless of the facts mentioned above, the selection of a suitable solvent also is of a crucial importance [26]. Cosolvent pairs of dodecanol/cyclohexane or dodecanol/toluene belong to commonly used porogenes.

Although MIP proved to be superior for separations of various chiral drugs [27], the role of nonspecific interaction during separations was often underestimated. Our paper also deals with these effects and estimates a hydrophobicity index of stationary phases using Walters indices [28].

## Results and discussion

The derivatization of the column inner wall was necessary for sufficient adhesion of the polymer. Two ways of glass modification were tested: glycidylisation by GMA (washing by NaOH, filling by GMA for 60 hours, 20 $^{O}$C) and silanisation by γ-MAPS (washing by NaOH and HCl, filling by γ-MAPS for 60 hours, 20 $^{O}$C).

The monomer part of the polymerizing mixture (38 wt. %) consisted of EDMA and MA (9.2/1 w/w). On the basis of previous work the porogenic component (57.6 wt.%) consisted of dodecanol and cyclohexanol (1/3 w/w) [17, 20] or dodecanol and toluene (4/1 w/w) [9]. Together with the template tosyl-L-phenylalanine (4 wt.%) and initiator (0,4 wt.%) Darocur 1173 (photo initiation) or ABIN (thermal initiation) the mixture was stirred for 10 min, degassed in ultrasonic bath for 10 min and bubbled by nitrogen for 10 min. Afterwards, the mixture was placed into the 3.3 x 150 and 3.3 x 30 mm i.d. columns. For elimination of free spaces arising during volume contraction of the polymer, columns were connected with silicon tubes and the system was pressurized by clamps. Polymerization was initiated thermally (heating in the water bath at 65 $^{O}$C for 12 hours), or by irradiation (water bath with various temperatures in the UV lamp for 30 minutes).

For preparation of the conventional columns packed with the polymer particles, the porous block of polymer (dodecanol/cyclohexanol as porogene, initiation by UV irradiation at 25 $^{O}$C) was crashed into small pieces. The soluble compounds were removed from the pores by extraction with toluene and dried under the vacuum at 60 $^{O}$C over night in order to obtain breakable particles. The polymer was subsequently ground at a mortar, fractionalized and placed into the glass columns. In this manner stationary phases with fraction of particles 7-12 µm and 25-35 µm were prepared.

In the next step, columns were connected to the chromatography apparatus and the template was washed out with acetonitrile, until a stable signal of the detector was obtained. Acetonitrile was used for testing of monolith permeabilities as well.

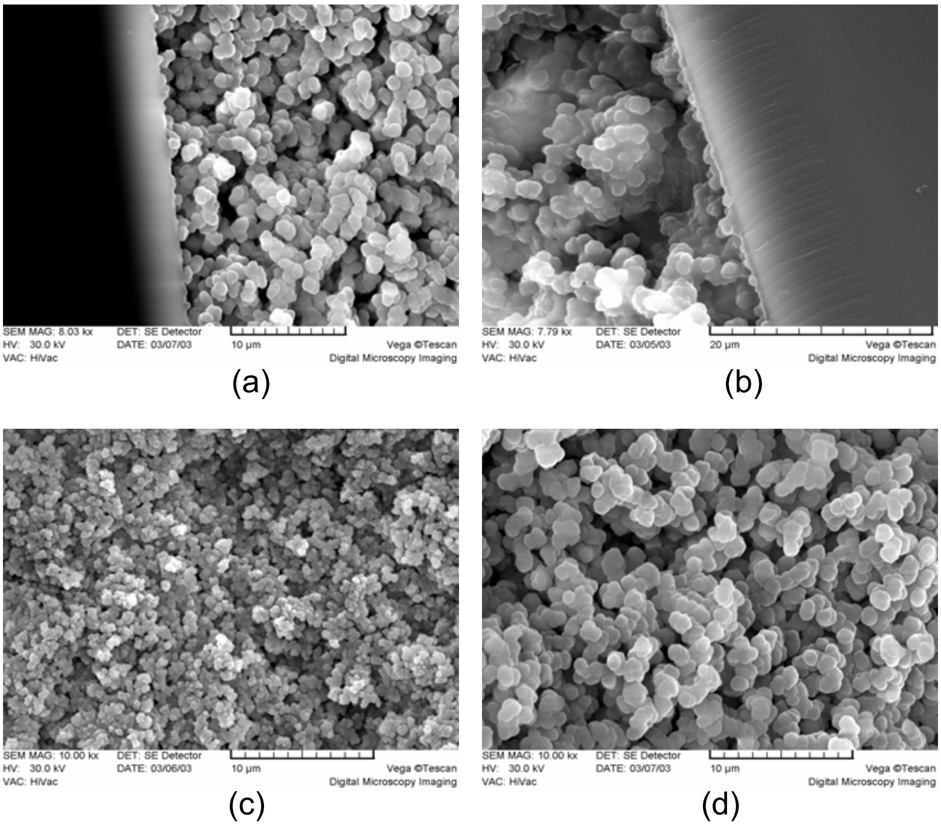
For characterization of separation performance of monoliths, acetonitrile/water (65/35 % (w/w)) mixture was chosen as eluent for separation of solutes differing in polarities (mixture of uracil, cinnamic acid and tosyl-L-phenylalanine or uracil, tosyl-L-phenylalanine, benzene, naphtalene and anthracene) and acetonitrile/acetic acid (99.5/0.5 w/w) for separation of L and D isomers of tosylphenylalanine. For testing of the columns by Walters test [28] for reversed stationary phases was acetonitrile/water (65/35 % (v/v)) used as eluent and index of hydrophobicity was calculated as $k_{\text{anthracene}}/k_{\text{benzene}}$.

Samples for characterization of polymer morphology were prepared in the same way as chromatographic columns with the exception that the glass inner wall was not modified. The soluble compounds were rinsed with the acetonitrile, the bottom column fitting was removed and the monolithic rod was pushed out by the methanol under pressure. The rods were cut into the appropriate length and dried under the vacuum at 60 $^{O}$C overnight. The polymer surfaces for scanning electron microscopy measurements were prepared by braking of the polymer rod in the liquid nitrogen to preclude secondary changes of the polymer structure. The porosity was investigated by mercury intrusion porosimetry and specific surface area was calculated from nitrogen adsorption/desorption isotherms. The mean pore radius was calculated as 2000*pore volume/specific surface area and the mean globule radius as 3000/specific surface area.

### *In situ polymerization inside the glass columns*

*In situ* polymerization performed directly inside the glass columns of conventional size (i.d. 3.3 mm) brought some initial problems which had to be solved. The common glass columns, without any modification of inner wall, were shown to be unsuitable due to the low adhesion of polymer to the glass. Prepared monoliths were unstable, the polymer rod was not fixed inside the column and it was washed out during the chromatographic process.

For this reason two types of chemical modification of glass inner wall were examined: glycidylisation with glycidyl methacrylate (GMA) and silanisation with (trimethoxysilyl)propyl-methacrylate (γ-MAPS). In the first case the epoxy group of bifunctional molecule of glycidyl methacrylate is covalently bonded to the silanol groups of the glass surface. During *in situ* polymerization the second methacrylate part of the molecule copolymerizes with the monomers of polymerization mixture. Similar covalent linkage arises in the case of γ-MAPS. Molecule of γ-MAPS is bonded by siloxan link to the silanol groups of the glass and the methacrylate part is incorporated to the monolith.



(a) (b)

(c) (d)

**Fig. 1.** SEM images of prepared monoliths: a) adhesion of the polymer to the glass inner wall modified by glycidylisation, b) adhesion of the polymer to the glass inner wall modified by silanisation, c) morphology of the polymer initiated thermally, d) morphology of the polymer initiated by UV irradiation.

Both modifications of glass inner wall guaranteed sufficient adhesion of imprinted polymer to the glass inner wall (Fig. 1). When the pressure on the end of the column was applied, the bottom column fitting was not necessary and the polymer rod stayed inside. No difference between glycidylisated and silanisated glass was observed. SEM images showed that even in the case of rough removal of the polymer from the glass the disruption arose in the matter of polymer and not in the junction of the polymer and the glass.

In the next step, the optimisation of the preparation of monolithic columns was necessary. The oxygen inhibiting radicals were removed out from the polymerization mixture in ultrasound bath with a stream of nitrogen. Subsequently it was necessary to keep the polymerization mixture out of contact with the air. The method of filling of the columns and ending of the tubes to keep the polymerization mixture inside was carried out. When the proper facility in preparation of the columns was achieved, the polymers were reliable and highly reproducible (Fig. 1).

The microscope images demonstrated high level of homogeneity of prepared monolithic rods and similar particle size in various parts of the column. No difference between the structures in the periphery and in the middle of the rod caused by insufficient transparency of water bath, glass wall or polymer mixture for UV irradiation was observed. Also no artefacts which could arise due to the volume contraction during the polymerization were found.

### *Morphology of prepared monolithic columns*

The structure of monolithic stationary phases plays very important role in their chromatographic behaviour. Total porosity, character of the pores and specific surface area are major factors affecting separation process. Appropriate volume of the stationary phase provides sufficient capacity of active sites but on the other hand free volume in the column is necessary for satisfactory permeability for mobile phase. The character of porosity is crucial - large throughout pores of the monolith are analogous to the volume between particles in the packed columns and enable flow of the mobile phase. The smaller mesopores are filled with the stagnant mobile phase and provide access of the solute molecules to the active sites. Morphology of prepared monoliths in the dry state was investigated by mercury porosimetry and surface area measurements and the results were compared to the properties of the monoliths swollen by mobile phase during the chromatographic process.

Mercury porosimetry revealed notable differences in the pore volume of prepared monoliths. The contrast between polymers prepared thermally and by UV irradiation was the most significant. UV irradiation created monoliths with total pore volume approx. 1.7 – 2.0 $cm^3/g$, whereas thermally initiated ones had pore volume about 1.2 $cm^3/g$, using dodecanol/toluene porogene mixture. Even more distinct difference was observed for monoliths prepared with dodecanol/cyclohexanol. Thermally initiated polymers exhibited only 1.0 $cm^3$ pore volume per gram, compared to 1.3 – 1.6 $cm^3/g$ for monoliths initiated by UV irradiation. Expressed in ratio of free volume to polymer matter, the porosity was about 50% for thermally initiated polymers compared to 80% for UV initiated ones (see Tab. 1). Total pore volumes together with the distribution of pore sizes had important effect on the permeability for mobile phase during chromatographic process.

Having investigated the pore sizes of prepared monoliths, two characters of the porosities were found (Fig. 2). Using dodecanol/cyclohexanol (1/3 w/w) as porogene mixture, the distribution curves showed pores with the sizes up to approx. 80 nm. The most frequent pore radius was found between 20 and 50 nm and no dependence of pore radius on the reaction temperature was observed (Tab. 1). In the contrast with these results, a sharp peak appeared on the pore size distribution curve measured on monoliths prepared from dodecanol/toluene porogene (4/1 w/w). This effect is in accordance with ref. [29]. Porogene mixture with lower polarity, containing toluene and higher percentage of dodecanol, is more potent precipitant in polymerization mixture and causes earlier onset of the polymer separation. The new phase preferentially swells with the molecules of monomers (or template) because they are better solvents for the polymer than the porogene solvent. The polymerization proceeds in these swollen nuclei rather than in the solution. Those nuclei are absorbed by the pre-globules formed by coalescence of many nuclei and further increase in their size. The globules that are formed in this system are larger and consequently the pores between them are larger as well.

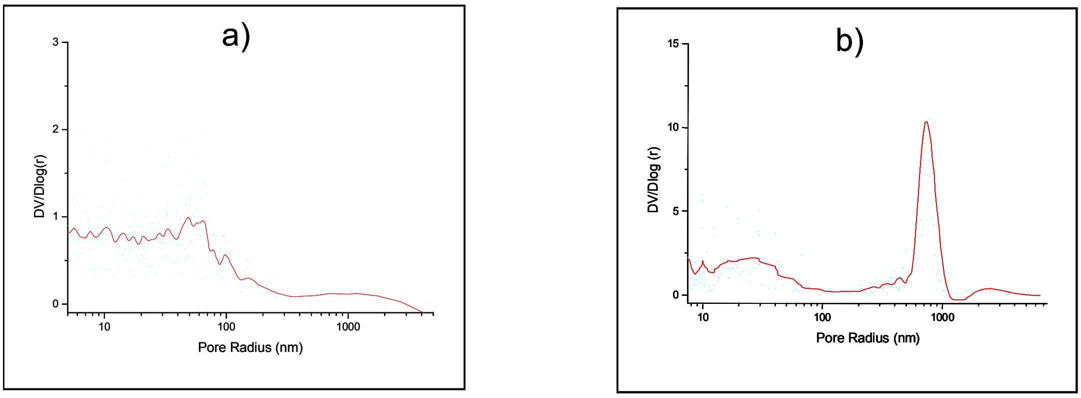


**Fig. 2.** Pore size distribution curves of the monolith initiated by UV irradiation at 25 °C , prepared with a) dodecanol/cyclohexanol 1/3 w/w and b) dodecanol/toluene 4/1 w/w as porogene mixtures.

The dependence of the value of the most frequent pore radius on the polymerization temperature for dodecanol/toluene porogene is depicted in Fig. 3. The values are in the range between 600 and 850 nm, whereas the most frequent radius decreases with increasing temperature of polymerization. This can be explained by the fact that higher temperature produces higher number of free radicals and subsequently higher number of nuclei and globules are formed. Since the amount of monomers in the system is the same, the higher number of globules translates into their smaller size and the pore radius decreases as well [29].

**Tab. 1.** Results of mercury porosimetry and specific surface area measurements.

| Porogene | dodecanol/cyclohexanol | | | | | dodecanol/toluene | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Initialization | Thermal | UV irradiation | | | | Thermal | UV irradiation | | | |
| Reaction temperature (°C) | 65 | 5 | 15 | 25 | 35 | 65 | 5 | 15 | 25 | 35 |
| Pore volume (cm$^3$/g) | 1.01 | 1.49 | 1.43 | 1.33 | 1.66 | 1.20 | 1.83 | 1.69 | 1.98 | 1.78 |
| Total porosity (%) | 52.7 | 80.7 | 83.7 | 79 | 82.2 | 59.8 | 84.4 | 81.0 | 86.8 | 87.0 |
| Specific surface area n$^2$/g) | 204.3 | 442.8 | 433.2 | 471.7 | 528.5 | 9.0 | 12.9 | 8.9 | 13.3 | 10.3 |
| Most frequent pore radius | 4.3 | 32.5 | 32.6 | 22.5 | 48.1 | 81.9 | 826.1 | 808.1 | 640.1 | 628.9 |
| Mean pore radius (nm) | 2.14 | 6,7 | 6,6 | 5.6 | 6.3 | 262.0 | 283.1 | 380.4 | 297.5 | 343.8 |
| Mean globule radius (nm) | 14.8 | 6.8 | 6.9 | 6.4 | 5.7 | 333.3 | 232.2 | 337.8 | 225.5 | 290.2 |

The texture of monolithic stationary phases can also be characterized by the specific surface area. Measurements based on the nitrogen adsorption/desorption provided the values corresponding to the porosity characteristics. Generally, polymers prepared from dodecanol/cyclohexanol porogene mixture have higher specific surface area. In case of thermal initiation it was about 200 m$^2$/g, for polymer initiated by UV irradiation it was even 500 m$^2$/g. The same pore volume created by the pores

with higher radius in case of dodecanol/toluene polymers was followed by lower specific surface area. Therefore the measured surface was around 10 $m^2$/g.
With approximation that the matter of monolith is created by the globules of the same size and the spherical shape it is possible to calculate mean (average) pore radius and mean globule radius (see Tab. 1). These values generally correspond to the characteristics mentioned above, some differences from other measurements are caused just by approximation.

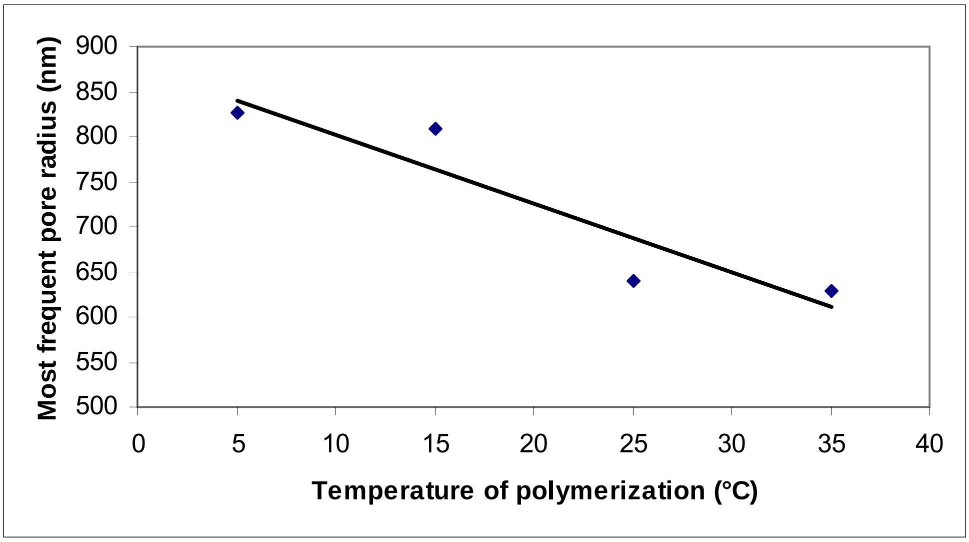


**Fig. 3.** Dependence of the most frequent pore radius on the temperature of polymerization for UV initiated monolith prepared with dodecanol/toluene as porogene mixture.

### *Chromatography*

Thermal initiation of polymerization brought monoliths with significantly different texture compared to the photo initiated ones. Morphological measurements found much lower values of the most frequent and average pore radius practically on the border of micropores. Together with notably lower pore volume it caused a very low permeability for mobile phase as can be seen in Fig. 4. Maximum permissible flow rate of acetonitrile (ACN) mobile phase through the 3.3 x 30 mm thermally initiated monolith prepared with dodecanol/toluene was 0.6 ml/min with backpressure about 17 MPa. In the case of monolith prepared with dodecanol/cyclohexanol as porogene the maximum flow rate was only 0.5 ml/min with backpressure of about 18 MPa. The permeability of the 150 mm long columns was very low and such columns could not be tested under the common chromatographic conditions.

Monoliths prepared by UV initiation were significantly more permeable. The dependence of permeability on the composition of porogene mixture was obvious (see Fig. 4). If the dodecanol/cyclohexanol and dodecanol/toluene monoliths were prepared under the same conditions, more permeable columns were prepared from dodecanol/toluene porogene. It corresponds with the pore size distribution curves (Fig. 2).

The influence of the polymerization temperature on dodecanol/toluene polymer was also pronounced. The permeability significantly increased with decreasing temperature of the polymerization. This effect corresponds to the relationship between pore size distribution curve and the temperature of polymerization (Tab. 1). The value of the most frequent pore radius increased with decreasing temperature. The larger pores enable higher flow rates through the monolith under the same pressure.

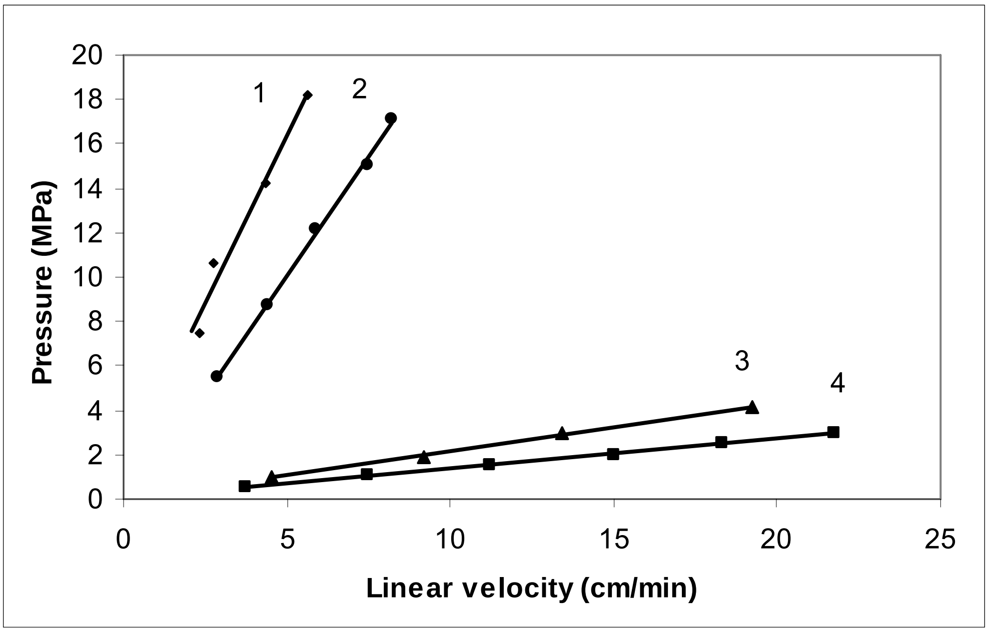


**Fig. 4.** Comparison of linear dependencies of the back pressure on the linear velocity of eluent at prepared monolithic columns. Thermally prepared (65 $^{O}$C) 3.3 x 30 mm monolith with porogene solvent dodecanol/cyclohexanol 1/3 (w/w) (1) and dodecanol/toluene 4/1 (w/w) (2), by UV irradiation prepared 3.3 x 150 mm monolith at 25 $^{0}$C with porogene solvent dodecanol/cyclohexanol 1/3 (w/w) (3) and dodecanol/ toluene 4/1 (w/w) (4).

Porogene mixture dodecanol/cyclohexanol provided monoliths with the permeability practically similar to the UV initiated dodecanol/toluene monoliths prepared at the highest temperature; however the permeability was the same during the whole range of polymerization temperatures. This fact also corresponds to the morphological observations – the shapes of the pore size distribution curves were not influenced by the preparation temperature.

The conventional columns packed with particles were prepared for comparison with the monolithic columns. Polymer based on the same composition of polymerization mixture as the monoliths was initiated by UV irradiation, dried and subsequently ground and sieved. The particles of two fractions were placed into the 3.3 x 150 mm columns. The first column was packed with 25-35 µm particles and the second one with 7-12 µm particles.

The various pressures applied on the packed columns to reach efficient flow rates were compared. The pressure drop of column packed with 25-35 µm particles was close to the most permeable monolithic column prepared with dodecanol/toluene porogene initiated with UV irradiation at 5 $^{0}$C (Fig. 5). The flow rate of 1 ml/min was reached at the pressure slightly more than 1 MPa. Although on the both columns the baseline separation of the test mixture was not achieved, chromatogram obtained on monolith had a notably better course. Low pressure drop can predetermine this type of stationary phases for example for pre-concentration purpose or for solid phase extraction.

In case of the column packed with the 7-12 µm particles the pressure drop was higher than for the monoliths initiated by UV irradiation. In comparison of the packed and monolithic columns with conformable separation properties it was necessary to use notably higher pressures on the packed columns.

The differences in pressure drop are evidently caused by the different character of pores, because the pore volumes calculated from retention times of void marker and flow rates were found to be very close. For the column packed with smaller particles the porosity was 78.7% and for the conformable monolith initiated with UV irradiation and prepared with dodecanol/cyclohexanol it was 77.6%. The column packed with 25-35 µm particles showed 81.2% of pore volume whenever its pressure drop was significantly lower. Comparison of these values with the results of mercury porosimetry measured on dry polymers shows low swelling of EDMA/MA polymer in ACN/water mobile phase.

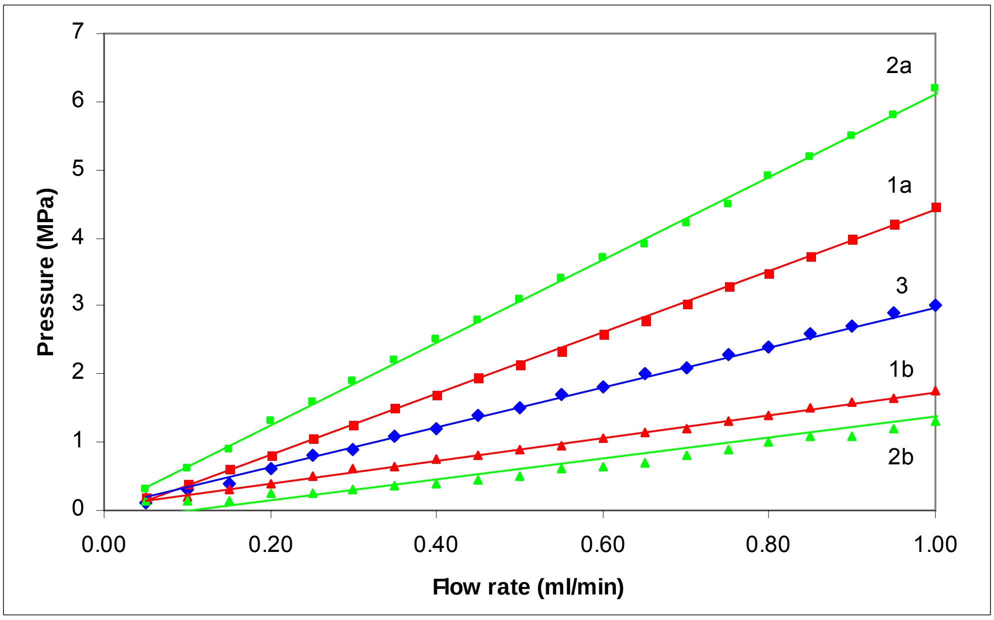


**Fig. 5.** Back pressure of the monolithic columns (3.3 x 150 mm) as a function of flow rate. The curves 1 belong to the monolithic columns prepared with dodecanol/toluene 4/1 (w/w) as porogene solvent, polymerized at 35 $^0$C (1a) and 5 $^0$C (1b); the curves 2 belong to the conventional columns filled with the polymer particles at the fraction of 7-12 µm (2a) and 25-35 µm (2b). The curve 3 corresponds to the commercial column packed with 7 µm spherical silica C18 particles.

### *Separation and retention characteristics*

After the physical characterization the prepared columns were tested for the chromatographic purposes. Due to the chemical character of monolithic columns the reversed-phase separation mode realized by acetonitrile/water was applied.

Generally very low permeability was observed for the thermally initiated monoliths, the 150 mm long columns were not permeable enough to characterize their separation properties. Chromatographic apparatus enabled testing of 30 mm long monoliths, but the columns of this length were too short to separate the simple mixture of test compounds (uracil, tosyl-L-phenylalanine and cinnamic acid). Also the retention profile of L- and D- form of imprinted compound was identical, they eluted practically with the void marker.

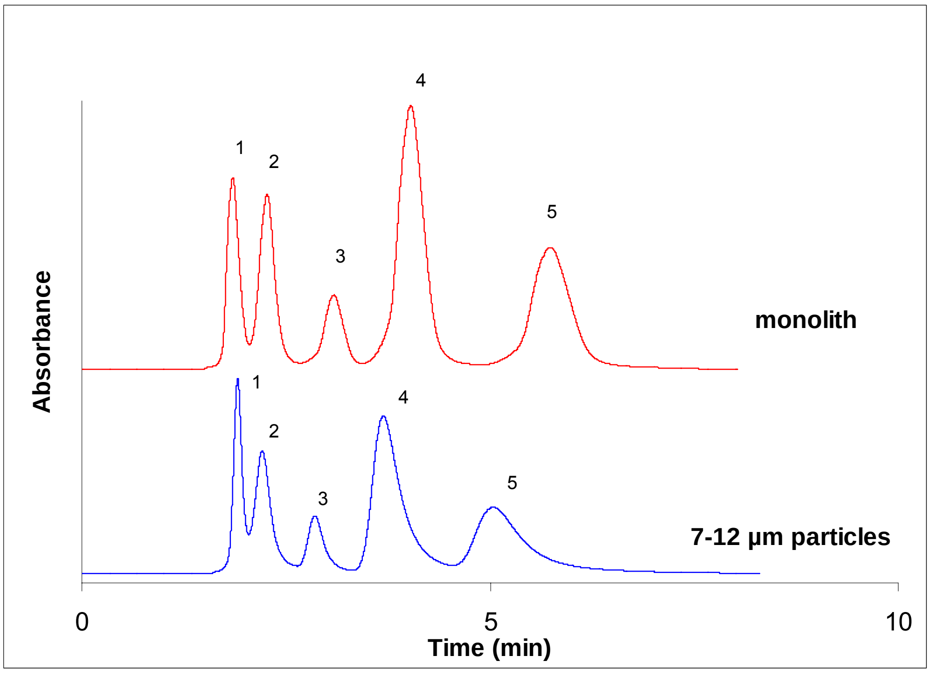


**Fig. 6.** Comparison of the chromatograms obtained on the 150 mm long monolithic column and the same column packed with 7-12 µm particles of the same polymer. Polymerization was initiated by UV irradiation, mixture of dodecanol/cyclohexanol was used as porogene solvent. Separation of uracil (1), tosyl-L-phenylalanine (2), benzene (3), naphthalene (4) and anthracene (5) at flow rate 0,55 ml/min, mobile phase ACN/water 65/35 v/v, UV detection at 254 nm.

The 150 mm long monoliths initiated with UV irradiation exhibited much better chromatographic properties. Dodecanol/cyclohexanol porogene mixture provided monoliths enabling practically base-line separation of the test mixture of uracil, tosyl-L-phenylalanine, benzene, naphtalene and anthracene (see Fig. 6, Tab. 2). Using ACN/water 65/35 (v/v) mobile phase the mean number of theoretical plates reached the values more than 5000 and high equivalent to a theoretical plate was less than 30 µm. No dependence of separation behaviour on the temperature of *in situ* polymerization was observed, which corresponds to the morphological characterization.

Monoliths initiated by UV irradiation and prepared from dodecanol/toluene showed worse peak broadening. Although the test compounds did not elute with the void marker, the broadness of peaks precluded base-line separation. The retention profile even got worse with lower temperature of polymerization. This significant difference in the monoliths behaviour was apparently caused by the different structure. Together with markedly lower specific surface area it negatively influenced the separation properties.

The most appropriate separation parameters exhibited the monolithic column prepared with dodecanol/cyclohexanol porogene mixture and initiated by UV irradiation. This monolithic column was compared to the column packed with 7-12 μm particles prepared by grinding of the polymer with the same composition and the same way of preparation. The columns showed some differences in chromatographic properties. For lower flow rates and early eluting compounds the efficiency of packed columns was higher. However, for higher flow rates and especially for later eluting compounds the number of theoretical plates was significantly higher for monoliths than for the packed columns.

**Tab. 2.** Chromatographic characteristics of 150 mm monolithic column dodecanol/ cyclohexanol as porogene mixture, initiation by UV irradiation and column filled with the 7-12 μm particles prepared from the polymer of the same composition. Mobile phase was ACN/water 65/35 (v/v) at flow rate 0.55 ml/min.

| | compound | $t_R$ (min) | $k$ | $\alpha$ | $N$ | $R_{i/j}$ | $b/a_{10\%}$ |
|---|---|---|---|---|---|---|---|
| monolithic column | uracil | 1.81 | 0.00 | | 4377 | | 1.49 |
| | L-TPA | 2.27 | 0.25 | | 4762 | 0.94 | 0.89 |
| | benzene | 3.09 | 0.71 | 2.84 | 6127 | 1.69 | 1.02 |
| | naphtalene | 4.03 | 1.23 | 1.73 | 5193 | 1.38 | 1.06 |
| | anthracene | 5.74 | 2.17 | 1.76 | 5286 | 1.80 | 1.08 |
| column filled with 7-12 μm particles | uracil | 1.86 | 0.00 | | 9365 | | 1.24 |
| | L-TPA | 2.21 | 0.19 | | 3730 | 0.67 | 1.70 |
| | benzene | 2.86 | 0.54 | 2.84 | 5249 | 1.10 | 1.30 |
| | naphtalene | 3.69 | 0.98 | 1.81 | 3676 | 0.90 | 2.22 |
| | anthracene | 5.04 | 1.71 | 1.74 | 2608 | 0.87 | 1.88 |

$t_R$- retention time, $k$ – retention factor, $\alpha$ – separation factor, $N$ – numer of theoretical plates, $R_{i/j}$ – resolution, $b/a_{10\%}$ - asymmetry factor, L-TPA – tosyl-L-phenylalanine

The values of resolution and retention factor were notably higher and the asymmetry factors lower compared to the packed column. This effect predicts about lower contribution of eddy dispersion to the chromatographic process in case of monolithic columns. The van Deemter plots showing dependence of heigh equivalent to a theoretical plate on the linear velocity of mobile phase are depicted on Fig. 7.

One of the important parameters of stationary phase is hydrophobicity/hydrophilicity which can be characterized by Walters test [28]. The Walters index of hydrophobicity (HI) is defined as the ratio of retention factors of benzene to anthracene in a mobile phase consisting of ACN/water 65/35 (v/v). The found indices of all tested monoliths were almost identical and reach the value 3.15 ± 0.10; the packed column reached 3.25. The HI values indicate slightly lower hydrophobicity compared to the methacrylate stationary phases commonly used for reversed phase chromatography [30, 31].

Two monolithic columns prepared from dodecanol/toluene and dodecanol/ cyclohexanol porogene mixtures were tested with chiral separation of L and D form of imprinted compound. The complete separation of the racemic mixture of tosylphenylalanine was not achieved on any column; however, the difference in retention times and the shapes of the peaks was observed.

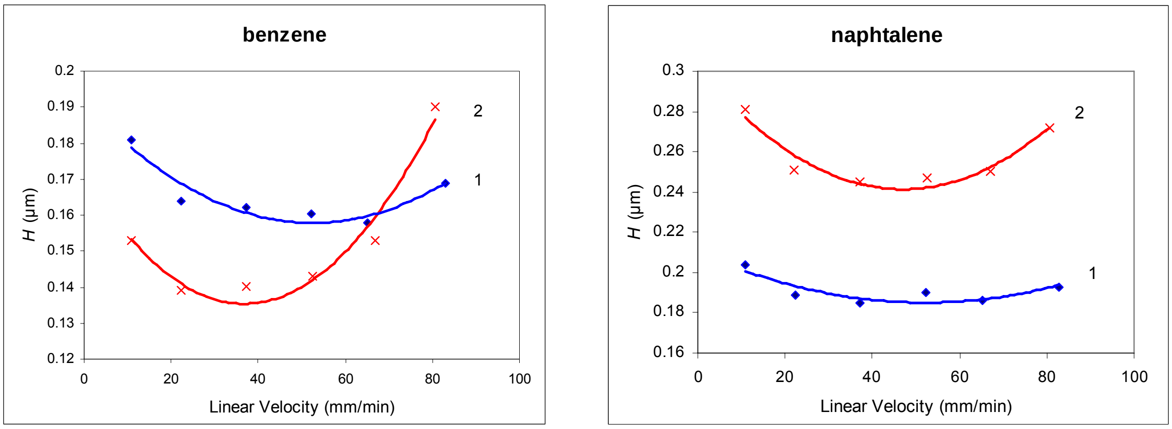


**Fig. 7.** Van Deemter plots of plate heights (*H*) vs. mobile phase linear velocity obtained with benzene and naphthalene as solutes. The plots marked 1 belongs to the monolithic column and the plots 2 to the column packed with 7-12 µm particles. Polymerization was initiated by UV irradiation, mixture of dodecanol/cyclohexanol was used as porogene solvent.

Even though dodecanol/toluene porogene solvent built up the monoliths with worse separation properties for test mixture of compounds with various polarities, the difference between retention of L and D form of tosylphenylalanine was more significant. When the enantiomers were injected separately, the imprinted L form was apparently more retained (see Tab.3). This difference obtained on monolith with less appropriate texture should be explained by stronger imprinting effect caused by lower polarity of porogene component in the polymerization mixture. Porogene mixture dodecanol/toluene 4/1 is less polar compared to dodecanol/cyclohexanol 1/3 and worse solvent for imprinted molecules of tosyl-L-phenylalanine. During polymerization the molecules of template are consequently more solvated with monomer instead of porogene and the imprinting effect is strengthened.

**Tab. 3.** Retention characteristics of L and D form of tosylphenylalanine.

| Solute | $t_R$ (min) | $W_{1/2}$ (min) | $b/a_{10\%}$ | $k$ | $\alpha$ |
|---|---|---|---|---|---|
| D-tosyl phenylalanine | 7.97 | 0.81 | 0.92 | 1.53 | |
| L-tosyl phenylalanine | 8.59 | 1.32 | 1.14 | 1.73 | 1.13 |

3.3 x 150mm UV initiated monolith prepared with dodecanol/toluene as porogene mixture. Mobile phase was acetonitrile/acetic acid (99.5/0.5 v/v), UV detection at 254 nm, $t_R$- retention time, $W_{1/2}$ – width of peak at 1/2 of heigh, $b/a_{10\%}$ - asymmetry factor, $k$ – retention factor, $\alpha$ – separation factor

### *Repeatability and reproducibility of prepared monolithic columns*

The repeatability and reproducibility of the preparation of EDMA/MA molecularly imprinted monolithic columns were studied. Four monolithic columns with the best chromatography properties (dodecanol/cyclohexanol porogene mixture, initiation by UV irradiation) were prepared from one polymerization mixture and four monoliths from another mixture but with the same composition. The repeatability and reproducibility presented in Tab 4. were expressed as relative standard deviations of the same chromatographic parameters measured with the same set of analytes as those used for testing of separation properties (see Tab. 2). The run-to-run repeatability was evaluated with one column from 10 parallel measurements. The column-to-column repeatability was calculated within two independent sets of four columns. The run-to-run repeatability was lower than 6% for all tested parameters and the column-to-column repeatability was below 17% within the first column set and below 16% within the second set. The reproducibility of eight monolithic columns prepared from two different polymerization mixtures of the same composition was better than 5% regarding the retention times and retention factors, around 10% in the asymmetry factors, 15% in resolution and 17% considering the number of theoretical plates.

**Tab. 4.** Repeatability and reproducibility of molecularly imprinted EDMA/MA monolithic columns expressed as relative standard deviations (R.S.D., %) of measured parameters.

| Parameter | Repeatability | | | Reproducibility |
|---|---|---|---|---|
| | Run-to-run | Column-to-column | | Mixture-to-mixture |
| | | 1st set (n=4) | 2nd set (n=4) | 2 sets (n=8) |
| $t_R$ (min) | 3.3 | 4.5 | 3.6 | 4.1 |
| $k$ | 2.9 | 3.8 | 3.4 | 3.7 |
| $N$ | 4.8 | 17.0 | 15.4 | 16.5 |
| $R_{i/j}$ | 2.2 | 16.6 | 14.1 | 14.9 |
| $b/a_{10\%}$ | 6.1 | 14.4 | 8.9 | 10.1 |

$t_R$- retention time, $k$ – retention factor, $N$ – numer of theoretical plates, $R_{i/j}$ – resolution, $b/a_{10\%}$ - asymmetry factor

## Conclusions

The primary problems with preparation of EDMA/MA molecularly imprinted monoliths inside the glass columns were successfully solved. After the modification of the column inner wall and optimization of filling process, the monolithic columns were successfully prepared and tested in the chromatographic process. The results showed that it is possible to obtain monoliths with the same chemistry, even with the same composition of the polymerization mixture, but with considerably different morphologies.

The type of initiation of polymerization significantly influenced the structure of final monolith and its subsequent behaviour in chromatographic process. Thermally initiated 3.3 x 150 mm monolithic columns showed high specific surface area but they were not permeable enough for testing in chromatographic separations. The polymers initiated with UV irradiation were much more permeable. For these polymers the significant influence of the used porogene was observed. Using dodecanol/toluene as porogene solvent the pore radius and consequent permeability for mobile phase was controllable by the temperature of polymerization. Although these columns were not able to separate the mixture of test compounds, the difference in retention of D form and imprinted L form of tosyl-phenylalanine was observed. Monoliths initiated with UV irradiation and prepared with dodecanol/cyclohexahol porogene showed morphology which was not influenced by the temperature of polymerization. On these columns the base-line separation of the test mixture was achieved. Height equivalent to a theoretical plate reached the values about 30 µm. Walters test revealed slightly lower hydrophobicity compared to the commonly used methacrylate stationary phases. In terms of retention characteristics, this column was compared to the column packed with 7-12 µm particles made from the polymer of the same composition and the same way of preparation. In most aspects the monolithic column exhibited better chromatographic properties than the column packed with the particles. Prepared monolithic columns exhibited a good repeatability and reproducibility of preparation with R.S.D. values below 15% in majority of the investigated chromatographic parameters.

## Experimental part

HPLC system PYE UNICAM (UK) was consisted of LC-XPD pump and PU 4020 UV detector (254nm). Glass columns, i.d. 3.3 x 150 mm and 3.3 x 30 mm, were provided by Tessek (Czech Rep.). UV initiation unit obtained 6 mercury lamps RVK 125W from Tesla (Czech Rep.). Morphology was investigated by scanning electron microscope TS 5130 VEGA, TESCAN (Czech Rep.) and mercury porosimeter Pascal 440, Thermo Finnigan (Italy). Specific surface areas were measured by Nitrogen BET Quantasorb, Quantachrome (USA).

Methacrylic acid (MA), ethylene dimethacrylate (EDMA), cyclohexanol, dodecanol, α,α´-azo-bis(isobutyronitrile) (AIBN) and acetonitrile (ACN) were supplied by Fluka. Glycidyl methacrylate (GMA) and 3-(trimethoxysilyl)propyl-methacrylate (γ-MAPS) were purchased from Merck and 2-hydroxy-2,2-dimethylacetophenone (Darocur 1173) from Aldrich. Toluene and acetic acid were provided by Lachema. Tosyl-phenylalanine (L, D form) was synthesized from p-toluenesulfonyl chloride and phenylalanine (Fluka).

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