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THE STRUCTURE OF A POLY(STYRENE-*CO*-DIVINYLBENZENE) MONOLITHIC CHROMATOGRAPHIC SUPPORT AND ITS EFFECT ON THE CHROMATOGRAPHIC PROPERTIES

STRUKTURA POLI(STIREN-*KO*-DIVINILBENZEN) MONOLITNEGA KROMATOGRAFSKEGA NOSILCA IN NJEN VPLIV NA KROMATOGRAFSKE LASTNOSTI

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The rapid development of new chromatographic supports has been stimulated by the need for fast and efficient separations of various types of solutes.

In this paper we report on the preparation of a new, commercially available monolithic chromatographic support that is based on styrene-divinylbenzene chemistry and is applicable in reversed-phase chromatography (CIM® RP-SDVB disk monolithic column).

The mechanism of the formation of the porous, monolithic structure and the major factors affecting this porous structure are discussed. The influence of the pore size on the specific surface area and on the specific volume of the pores is presented.

The precisely defined structure and the short length of the monolithic column result in a low pressure drop, even when using very high mobile phase velocities.

The applicability of a reversed-phase monolithic chromatographic column of only 3 mm in length is clearly demonstrated in the very fast baseline separation of our four test proteins.

Key words: poly(styrene-*co*-divinylbenzene), monolith, structure, porous support, chromatography, reversed-phase

Cilj razvoja novih kromatografskih nosilcev je hitrejša in učinkovitejša separacija različnih tipov vzorcev.

Pripravili smo nov, tržno dostopen monolitni kromatografski nosilec na osnovi kopolimera stirena in divinilbenzena za uporabo v kromatografiji na reverzni fazi (CIM® RP-SDVB-disk monolitna kolona). Prikazan je mehanizem tvorbe polimernega, poroznega monolita, študij parametrov, ki vplivajo na tvorbo porozne strukture, ter vpliv velikosti por na specifično površino in specifični volumen por.

Zaradi definirane porozne strukture monolita in kratke dolžine monolitne kolone je povratni tlak izredno nizek in ostane tak tudi pri visokih pretokih mobilne faze.

Učinkovitost trimilimetrske monolitne kolone za uporabo na reverzni fazi smo prikazali z zelo hitro separacijo štirih preskusnih proteinov.

Ključne besede: poli(stiren-*ko*-divinilbenzen), monolit, struktura, porozni nosilec, kromatografija, reverzna faza

1 INTRODUCTION

The attention of most researchers who are dealing with the preparation of HPLC chromatographic supports is mainly focused on improving the chemical stability, enhancing the mass transfer of the solute in the pores, and increasing the efficiency of the chromatographic support. Despite many improvements over the last decades, conventional porous particles still show some drawbacks [1,2]. A slow diffusional mass transfer and a large void volume between the packed particles diminish the speed of the separation and cause a reduction in the resolution. This is especially true for larger molecules separated at a high mobile phase velocity [3]. Additionally, the isolation of labile, unstable samples needs to be performed quickly and under mild conditions. Improvements of the properties of conventional particles and the design of new stationary phase configurations have been introduced to overcome the limitations mentioned above.

Decreasing the size of the porous particles and therefore decreasing the diffusion distances enhanced the column's efficiency [4]. However, the infinite lowering of the particle size is restricted by the high pressure drop [5].

Non-porous particles are the ultimate solution to the problem of diffusion into the pores of the particle supports [6,7]. In any case, the absence of intraparticle pores diminishes the binding capacity [8,9].

Since the progress with porous and non-porous particles has become a bottleneck, chromatographers are demanding new types of supports. Improvements have led to the introduction of micropellicular [3,10,11], superficially porous [4], superporous [12], perfusion [13] and 'gel in a shell' [14] gigaporous supports.

The configuration of the micropellicular supports and the superficially porous supports is a spherical layer supported by a fluid-impervious, rigid micro sphere. These supports show a rapid mass transfer between the mobile and stationary phase because of the short diffusion distance in the thin retentive layer of the particles.

Superporous beads contain two sets of pores, diffusion pores and so-called superpores or flow pores, in which the mobile phase can transport solutes to the interior of each individual bead. These superpores, whose diameter is 1/3 to 1/10 of the particle diameter, allow an improved mass transfer.

Two types of gigaporous particles have been introduced so far. One is a rigid, gigaporous support, often termed a perfusive support, involving convective mass transfer in gigapores and diffusive mass transfer in smaller pores of each particle. Although only a small part of the mobile phase flows through the gigapores, the hydrodynamic properties are much improved, which considerably increases the mass transfer and consequently contributes to the high speed of the separation [15]. The other type, termed a 'gel-in-a-shell' support, is also a gigaporous, rigid particle support in which gigapores are filled with a retentive hydrogel, responsible for the high binding capacity of the solute [16].

Membrane technology was introduced as a technological progress in both membrane filtration (ultrafiltration) and fixed-bed liquid chromatography. Several extensive papers on membrane technology were published [17, 18, 19]. The separation processes in membranes are usually one or more orders of magnitude faster than in packed particles due to the predominantly convective mass transport of the solute in the flow-through pores [20]. Additionally, the membranes are very thin and, consequently, the pressure drop observed is very low.

One of the newest generations of chromatographic supports is the monolithic support. Monoliths are continuous stationary phases that are cast as a homogenous column in a single piece and prepared in various dimensions with an agglomeration-type or fibrous microstructure [21].

The silica-based monolithic beds were introduced as solid rods of silica monolith, prepared by a sol-gel process, which is based on the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers [22].

Another type of monolithic beds was introduced as a swollen polyacrylamide gel compressed in the shape of columns [23, 24]. The polymerization of monomers to such a bed is performed directly in the chromatographic column. The polymeric chains, in the presence of salt, form aggregates by hydrophobic interactions and create voids between the bundles of aggregates. The voids or to be more precise, the channels between the bundles are large enough to permit a high hydrodynamic flow.

Tennikova and co-workers [25] introduced the third type of monolithic support, based on a rigid, macroporous methacrylate polymer, which was synthesised by radical bulk polymerization of glycidyl methacrylate and ethylene glycol dimethacrylate in the presence of porogenic solvents. They developed 1-mm-thick membranes, which are particularly appropriate for separating large molecules like proteins [25].

Reversed-phase monolithic columns are prepared either by direct polymerization of hydrophobic monomers, which results in a polymeric, porous monolith of an appropriate chemistry [26], or by suitable further derivatization of the prepared monolith.

An ideal chromatographic support should fulfil the following requirements: efficient separation, high-flow-unaffected resolution and capacity, low pressure drop, stability under any applied conditions, as well as ease of handling. We wanted to meet these requirements by preparing an efficient, reversed-phase monolithic chromatographic support based on styrene-divinyl-benzene chemistry.

The preparation of a rigid, macroporous, polymeric monolith based on styrene-divinylbenzene chemistry was first introduced as early as 1993 [26]. Further investigations and optimisations resulted in the CIM® RP-SDVB disk monolithic column. This product has filled the void created by the lack of commercially available, styrene-divinylbenzene-based, monolithic chromatographic supports.

In this work, the preparation of a poly(styrene-*co*-divinylbenzene) monolith by free radical polymerization will be presented. The factors that affect the porous structure and consequently the characteristics of the prepared monolithic column will be discussed. The important properties of the monolithic column will be studied and a potential application will be demonstrated.

2 EXPERIMENTAL

2.1 The synthesis of a poly(styrene-co-divinylbenzene) monolith.

Materials for the synthesis. Styrene (>99%), divinylbenzene (~80%) and 1-dodecanol (~97%) were all purchased from Fluka (Buchs, Switzerland), while 2,2-azobisizobutyronitrile (>98%) was obtained from Merck (Darmstadt, Germany).

The initiator, 2,2-azobisizobutyronitrile (1wt % with respect to the monomers) was dissolved in a mixture of monomers (50:50 v/v), consisting of styrene and divinylbenzene. The porogenic solvent, 1-dodecanol, was admixed slowly to the monomers. The polymerization mixture was purged with nitrogen for 15 minutes, in order to remove the oxygen and then poured into a closed mould. The bulk polymerization was performed for 24 hours at a temperature in the range of 55-80 °C. After the polymerization was completed,

ethanol was pumped through the resulting monolith to wash out the porogenic solvent and other soluble compounds present in the monolith. The commercial product with an optimum structure, the CIM® RP-SDVB disk, has a diameter of 12 mm, a length of 3 mm and is surrounded by a non-porous, self-sealing fitting ring (BIA Separations d.o.o., Ljubljana, Slovenia).

2.2 Characterisation of the porous properties.

The morphology of the prepared monolith was investigated using a scanning electron microscope (JEOL Ltd., Tokyo, Japan). The pore size distributions, the specific surface area and the specific volume of the macroporous polymeric monolith were determined with a mercury porosimeter (ThermoQuest Italia S. p.A., Rodano, Italia).

2.3 High-performance reversed-phase liquid chromatography of test proteins.

Chromatographic system. A gradient HPLC system (Knauer, Berlin, Germany) built of two K-500 pumps, an injection valve with a 20-µl sample loop, a UV-VIS K-2500 detector set to a response time of 0.1 s, with a 10-mm optical path, operated at 280 nm, and with a 10-µl volume flow-cell, connected by means of 0.25-mm I.D. PEEK capillary tubes and HPLC hardware/software (data acquisition and control station) were used in all separations. The Knauer mixing chamber with its relatively large dead volume was replaced by the PEEK mixing tee with an extra low dead volume (VICI Jour Research, Uppsala, Sweden).

Chromatographic column. The CIM® RP-SDVB disk monolithic column, consisting of CIM® RP-SDVB disk and an appropriate housing (BIA Separations d.o.o., Ljubljana, Slovenia), was used in the chromatographic experiments.

Mobile phase. High-purity water and high-purity chemicals were used throughout the experimental work. HPLC-grade acetonitrile was purchased from Rathburn (Walkerburn, Scotland), while trifluoroacetic acid was purchased from Fluka (Buchs, Switzerland).

Samples. Ribonuclease A, Cytochrome C and Chicken Egg Albumin were purchased from Sigma (St. Louis, MO, USA), while Bovine Serum Albumin was purchased from Fluka (Buchs, Switzerland).

3 RESULTS AND DISCUSSION

The chromatographic properties significantly depend on the structural properties of the monolith. A large number of smaller pores are needed to provide a large surface area, which results in a high binding capacity of the solute. On the other hand, larger pores enable liquid to flow through the support at a low pressure drop. Therefore, an ideal monolithic chromatographic support should have a bimodal pore size distribution, which is

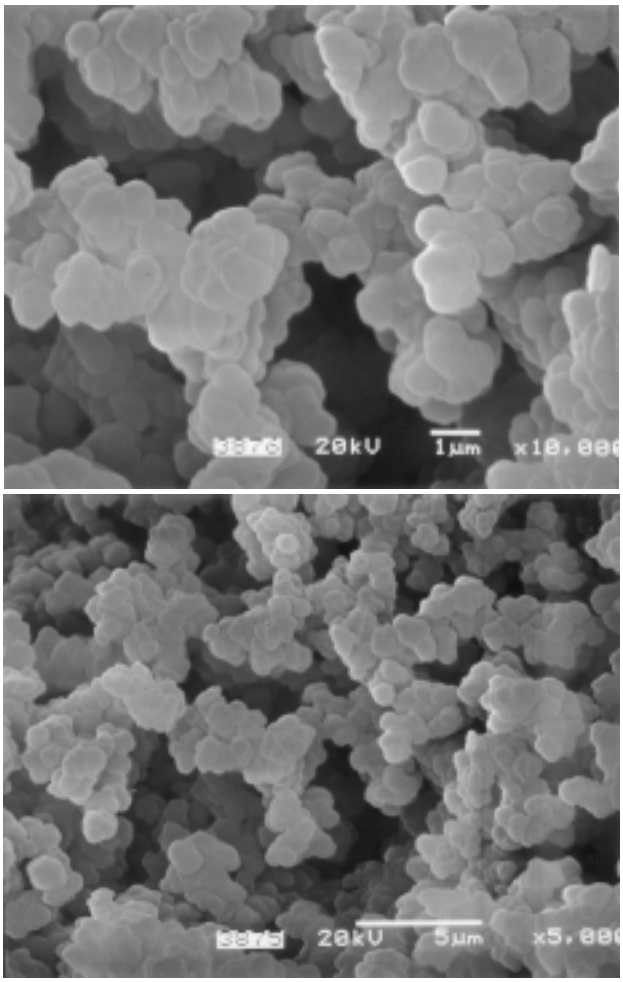

Figure 1: SEM micrographs of poly(styrene-*co*-divinylbenzene) monolith
Slika 1: SEM posnetka poli(stiren-*ko*-divinilbenzen) monolita

demonstrated in the poly(styrene-*co*-divinylbenzene) monolith (the preparation of the monolith is described in the Experimental Section). As can be seen (**Figure 1**), the pores in the polymeric monolith are actually irregular voids between clusters as well as within each cluster. For a better understanding of the structure of the polymeric monolith, a closer examination of the pore formation is needed [27].

The free-radical initiator decomposes at a particular temperature and the initiating radicals start the polymerization in the solution of monomers and porogenic solvent. The polymeric chains precipitate after they become insoluble in the solution. The precipitated nuclei are swollen with the monomers and therefore, the polymerization continues in the solution of monomers and porogenic solvent as well as within the swollen nuclei. The nuclei enlarge and cross-link the neighbouring nuclei thus forming clusters. The size of the clusters increases until they are large enough to cross-link with the neighbouring clusters and form the final, highly interconnected network. The fraction of

voids within the porous polymeric network at the end of the polymerization is close to the volume fraction of the porogenic solvent in the initial reaction mixture. The reason for this lies in the capturing of inert porogenic solvent molecules within the forming polymeric network during the polymerization.

To fulfil the chromatographic requirements for the porous structure of the prepared styrene-divinylbenzene monolithic support, we optimised the experimental parameters such as the styrene-to-divinylbenzene ratio, the monomers-to-porogenic solvent ratio, the choice of porogenic solvent and the polymerization temperature.

A decrease in the styrene-to-divinylbenzene ratio affects the porous structure of the resulting polymeric monolith [27]. A higher content of divinylbenzene results in an earlier phase separation due to the increased formation of cross-linked copolymer at an earlier stage of polymerization. The formed porous polymeric monolith consists of smaller nuclei and, as a result, it has smaller pores. Generally, the percentage of smaller pores increases with a larger amount of divinylbenzene.

The choice of porogenic solvent is another tool for controlling the size of the polymeric monolith pores. Pores with larger sizes are obtained by using a poorer porogenic solvent (1-dodecanol is known to be a poor solvent for poly(styrene-*co*-divinylbenzene)) [27]. These pores form because of an earlier onset of the phase separation. More precisely, the formed polymeric chains are locally swollen with the monomers, which are better solvents than the porogenic solvent. The polymerization preferably proceeds on these polymeric chains rather than in the surrounding solution. This results in the formation of larger clusters and, consequently, larger voids between these clusters.

The porosity of the polymeric monolith is controlled by the ratio of monomers to porogenic solvent. To obtain a large number of pores this ratio should be low enough, leading to a high permeability and a lower pressure drop. High porosity can, therefore, be achieved by increasing the amount of porogenic solvent, however, the mechanical stability is decreased when this approach is used [27].

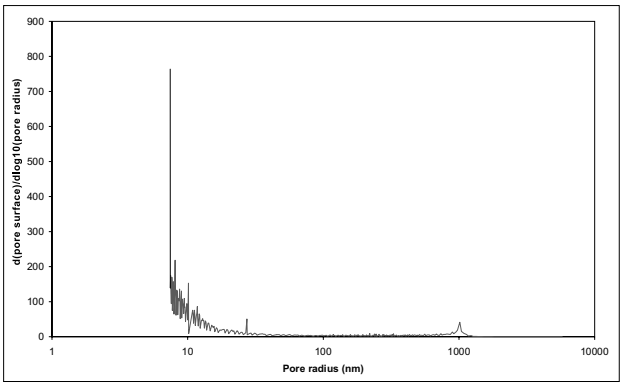

Figure 3: The effect of the size of the pores on the specific surface area of the pores of the poly(styrene-*co*-divinylbenzene) monolith
Slika 3: Vpliv velikosti por na specifično površino por poli(stiren-*ko*-divinilbenzen) monolita

Figure 2 shows that the change in the polymerization temperature considerably shifts the maximum of the radius of the pore size distribution to lower values, in our case, by two-to three-folds.

As we can see from the figure, the higher polymerization temperature results in smaller pores. This can be explained as follows: at a higher polymerization temperature the rate of initiator decomposition is faster, which leads to a higher number of radicals and consequently to a higher number of nuclei. Since the amount of monomers in the polymerization mixture is constant, a higher number of nuclei results in their size being smaller. The temperature control of the pore size is therefore straightforward, allowing us to obtain the desired pore size distribution.

Taking into account all the above-mentioned variables, we prepared an optimised poly(styrene-*co*-divinylbenzene) monolith with an appropriate porous structure and mechanical properties for use as a chromatographic support commercialised under the trade mark CIM. The microstructure is shown in **Figure 1**.

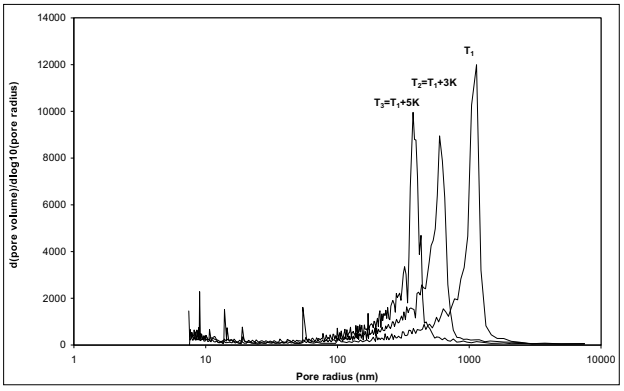

Figure 2: The influence of the polymerization temperature on the pore size distribution of the poly(styrene-*co*-divinylbenzene) monolith
Slika 2: Vpliv temperature polimerizacije na porazdelitev velikosti por poli(stiren-*ko*-divinilbenzen) monolita

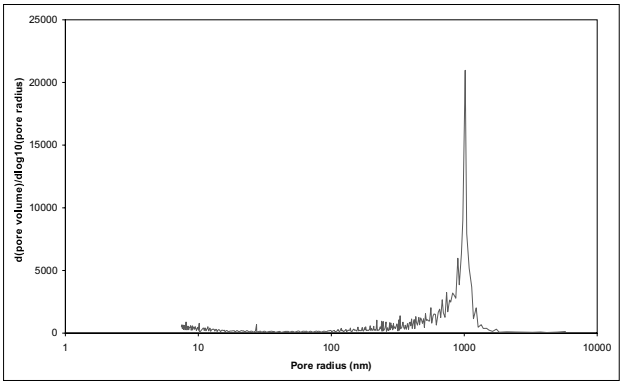

Figure 4: The effect of the size of the pores on the specific volume of the pores of the poly(styrene-*co*-divinylbenzene) monolith
Slika 4: Vpliv velikosti por na specifični volumen por poli(stiren-*ko*-divinilbenzen) monolita

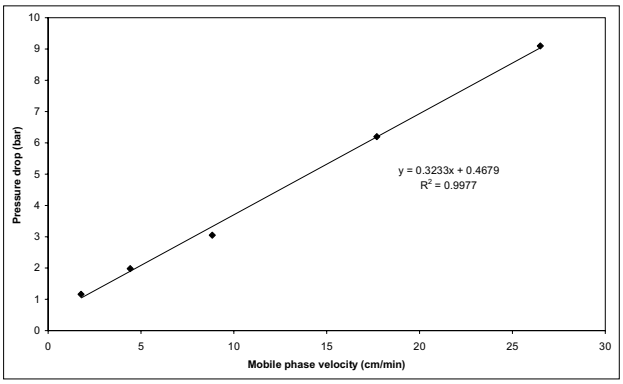

Figure 5: Pressure drop vs. mobile phase velocity for a poly(styrene-*co*-divinylbenzene) monolith
Conditions: Stationary phase: CIM® RP-SDVB disk monolithic column (BIA Separations d.o.o., Ljubljana, Slovenia); Mobile phase: 20% acetonitrile + 0,15% trifluoroacetic acid; Flow monitoring: digital flowmeter (K-3773, Phase Separations, UK).
Slika 5: Odvisnost povratnega tlaka od pretoka mobilne faze za poli(stiren-*ko*-divinilbenzen) monolit
Pogoji: Stacionarna faza: CIM® RP-SDVB-disk monolitna kolona (BIA Separations, d.o.o., Ljubljana, Slovenija); Mobilna faza: 20 % acetonitril + 0,15 % trifluoroocetna kislina; Kontrola pretoka: digitalni merilnik pretoka (K-3773, Phase Separations, VB).

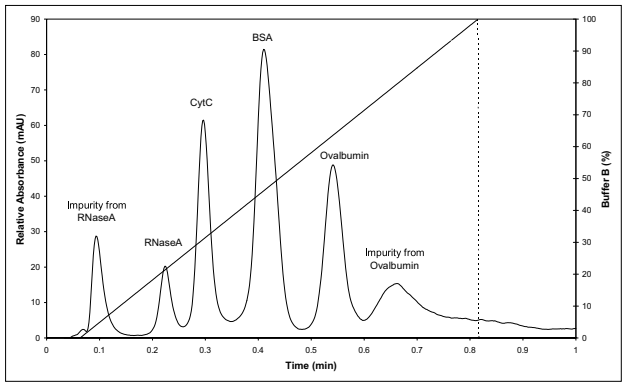

Figure 6: A 1 minute separation of test proteins
Conditions: Stationary phase: CIM® RP-SDVB disk monolithic column (BIA Separations d.o.o., Ljubljana, Slovenia); Buffer A: 20% acetonitrile + 0,15% trifluoroacetic acid; Buffer B: 70% acetonitrile + 0,15% trifluoroacetic acid; Flow rate: 10 ml/min; Gradient: as shown in the Figure; Detection: UV at 280 nm; Temperature: room temperature; Injection volume: 20 µl; Flow monitoring: digital flowmeter (K-3773, Phase Separations, UK); Sample of proteins: 1) Impurity from Ribonuclease A (Impurity from RnaseA), 2) 1,5 mg/ml Ribonuclease A (RNAseA), 3) 0,5 mg/ml Cytochrome C (CytC), 4) 2,5 mg/ml Bovine Serum Albumin (BSA), 5) 3,0 mg/ml Chicken Egg Albumin (Ovalbumin) and 6) Impurity from Ovalbumin. Sample of proteins was dissolved in distilled water.
Slika 6: 1-minutna separacija preskusnih proteinov
Pogoji: CIM® RP-SDVB-disk monolitna kolona (BIA Separations, d.o.o., Ljubljana, Slovenija); Pufer A: 20 % acetonitril + 0,15 % trifluoroocetna kislina; Pufer B: 70 % acetonitril + 0,15 % trifluoroocetna kislina; Pretok: 10 ml/min; Gradient: kot je prikazano na grafu; Detekcija: UV pri 280 nm; Temperatura: sobna temperatura; Injekcijski volumen: 20 µl; Kontrola pretoka: digitalni merilnik pretoka (K-3773, Phase Separations, VB); Vzorec proteinov: 1) nečistoče iz ribonukleaze A (Impurity from RnaseA), 2) 1,5 mg/ml ribonukleaze A (RNAseA), 3) 0,5 mg/ml citokroma C (CytC), 4) 2,5 mg/ml albumina iz govejega seruma (BSA), 5) 3,0 mg/ml albumina iz kokošjega jajca (Ovalbumin) in 6) nečistoče iz albumina iz kokošjega jajca (Impurity from Ovalbumin). Vzorec proteinov je bil raztopljen v destilirani vodi.

Macropores between the clusters enable improved convective mass transfer of the solute, which considerably increases the speed of the separations. Although the macropores are very important for high-speed separations, the most important factor for a high binding capacity is a large surface area, which mainly comes from mesopores with a radius smaller than 25 nm (**Figure 3**). As shown in **Figure 3**, macropores, with radii larger than 25 nm, contribute insignificantly to the surface area. In comparison, the volume of the pores is the largest in the macropore region (**Figure 4**), which leads to the low flow resistance of the support. It is then possible to apply a high mobile phase velocity in excess of 20 ml/min (17.7 cm/min), since the prepared monolith is mechanically stable and the observed pressure drop remains very low (**Figure 5**). Furthermore, the pressure drop is a linear function of the mobile phase velocity (**Figure 5**), which is often not the case for particle supports where the pressure drop could increase exponentially with a higher mobile phase velocity. The compression of the bed of particles and, consequently, the occurrence of the non-homogeneity of the support as well as an increase in the pressure drop are the effects that influence the flow through the column [28]. The linear dependency of the pressure drop vs. the mobile phase velocity of the prepared polymeric monolith is maintained even at high velocities, which confirms that the monolith is not compressed but remains rigid.

Since the length of the support significantly affects the pressure drop, the column length should be carefully adjusted. It was shown [29] that the column length has no influence on the quality of the gradient separation using reversed-phase monolithic columns, which indicates the potential for short monolithic supports (3 mm in length) in fast, efficient separations. In **Figure 6**, a 1 minute separation of four proteins using a monolithic column based on styrene-divinylbenzene chemistry is presented. Due to the low pressure drop (3 bar per disk at 10 ml/min water solution of 20% acetonitrile + 0,15% trifluoroacetic acid), high mobile phase velocities can be applied, which decreases the analysis time with no loss of resolution.

4 CONCLUSIONS

An efficient poly(styrene-*co*-divinylbenzene) monolithic chromatographic support applicable in reversed-phase chromatography was prepared. The CIM® RP-SDVB disk monolithic column with a bimodal pore size distribution was shown to have excellent chromatographic characteristics. The efficiency of the separation, the high speed of the separation, and the low pressure drop are the consequences of a proper monolithic support design and a short column length. The advantages displayed by this monolithic column

make it the tool of choice for the separation of biopolymers.

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