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Comparison of styrene–divinylbenzene-based monoliths and Vydac nano-liquid chromatography columns for protein analysis

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Abstract

This report details the fabrication of polystyrene-based nano-LC monolithic columns for the separation of protein molecules. The report focuses on the practical advantages of monolithic columns when compared to conventional packed columns. Capillary columns were made to 100 and 50 μm i.d. and used to analyse a mixture of proteins, these separations were then compared with a conventional protein phase under the same conditions. A second functionalised monolithic polystyrene-based column was also manufactured and compared for the analysis of proteins, under the same analytical conditions, to the standard non-functionalised bare polystyrene monolith. Nano-LC polystyrene monolithic columns were found to be advantageous to conventional phases for the analysis of protein molecules, with a one-step fabrication process, faster analysis times, lower limits of detection hence higher sensitivity.

Keywords: Monolithic columns; Stationary phases, LC; Poly(styrene–divinylbenzene); Proteins

1. Introduction

Monolithic columns have been an area of extensive research within the past 5 years. These columns have many advantages, they are easy to manufacture, the monolith being formed in situ, often via a one-step reaction process, and its properties such as porosity, surface area and functionality can be tailored. Many applications have been investigated including solid-phase extraction, sample pre-concentration, and separation analysis in fields such as pharmaceutical, environmental, and more extensively, biomolecules [1–4].

In the last 3 years, the group of Premstaller et al. [5,6] have made big advances for the chromatographic separation of biomolecules such as proteins and nucleic acids. The group synthesised a styrene-based monolith that enabled the highly efficient separation of biomolecules by reversed-phase micro-high-performance liquid chromatography (μ-RP-HPLC). Their key discovery was the use of a mixture of tetrahydrofuran and decanol as porogens for the fabrication of a micropellicular poly(styrene–divinylbenzene) (PS–DVB) backbone.

The poly(styrene–divinylbenzene) polymer is prepared by free radical cross-linking copolymerisation of styrene and divinylbenzene monomers in the presence of a diluent, which can be a solvent, a non-solvent or a linear polymer, and is the pore forming agent as shown in Fig. 1. In a solvating diluent, macroporous polymers are only produced when the divinylbenzene concentration is high, and the monomer concentration is diluted. However, when non-solvating diluents are used, the macroporosity appears at lower concentrations of divinylbenzene and with less diluted monomers.

Recently, a 200 μm i.d. column manufactured from PS–DVB has been made available in the market for the analysis of peptides and proteins.

Following this work, we have studied three new aspects of the styrene monolith. Our first study focused on the successful fabrication of columns with internal diameters of 100 and 50 μm i.d. This is done due to the increase in the sensitivity of the analysis achieved as the column internal diameter decreases.

In 1995, Ryan proposed a model to adapt normal flow in chromatography to micro-flow chromatography [7]. The theory states that when going from a column of diameter X to a smaller diameter Y the gain in sensitivity would be equivalent to the partition of the squares of the internal diameters $[X^2/Y^2]$. Thus, in practical terms, when adapting a method from a 2.1 to a 0.5 mm i.d. column there would

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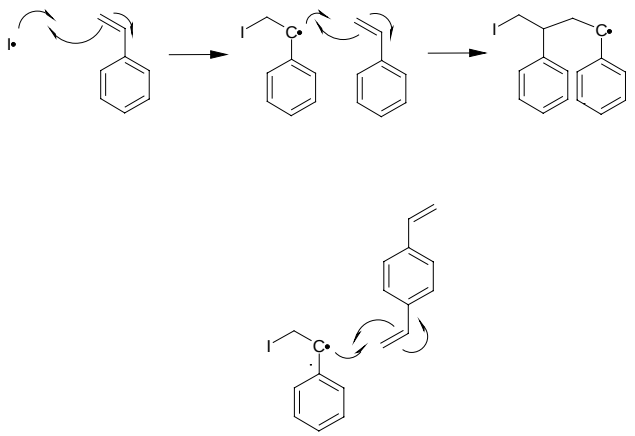

Fig. 1. Radical step-wise polymerization of styrene. Cross-polymerization of styrene with divinylbenzene.

be a sensitivity gain of $2.1^2/0.5^2 = 17$, i.e., 17 times more counts would be observed. From a 2.1 to a 0.3 mm i.d. column the sensitivity increase could be as big as 49 times.

The second study comprised of a comparison of the sensitivity achieved by a 100 μm i.d. monolith column versus a conventional stationary phase packed column such as Vydac. Both columns being the same internal diameter allowed for the direct comparison of the lower limits of detection in this way assessing the performance of the new stationary phase.

Following this work, a third study was performed on a new form of the polystyrene monolith, by derivatising using *N*,*N*-dimethylbutylamine. The purpose being to alter the surface functionality of the monolith, and as such a quaternary ammonium group and a butyl chain were introduced onto the chromatographic surface.

A monolithic column that is manufactured solely from styrene and divinylbenzene will consist of many benzene rings on the surface of the column. These benzene rings all carry delocalised electrons, forming an electron cloud over the surface, which may act as a form of cation exchange functionality.

By introducing chloromethylstyrene in place of styrene into the polymer mixture, it is possible to derivatise the surface after the polymer has been formed, therefore, introducing further chromatographic functionality to the column. This column was then directly compared to the original non-functionalised PS–DVB monolith for the separation of proteins.

2. Experimental

2.1. Chemicals

For analysis: Deionised water came from an ELGA water purifier, acetonitrile to HPLC standard was from Rathburn. Trifluoroacetic acid (TFA) was purchased from Fluka.

For monoliths: Sodium hydroxide, acetic acid 99% purity, monomer 3-(trimethoxysilyl)propylmethacrylate, tetrahydrofuran, styrene 99% purity, divinylbenzene 80% purity, chloromethylstyrene, *N*,*N*-dimethylbutylamine, decanol and azobisisobutyronytrile were all purchased from Sigma.

2.2. Instrumentation

The instrumentation used was an LC Packings (Dionex, Sunnyvale, CA, USA) capillary-HPLC with an Ultimate detector and a 49 nl flow cell. The fused silica capillaries of 100 and 50 μm i.d. were purchased from SGE, Australia.

2.3. Procedure

The columns were produced following modifications to the method published in [5]. Parameters such as the silanisation process were adapted from Coufal et al. and the temperature and time of polymerisation were changed [8].

2.3.1. Silanisation process

The required length of capillary was filled with a solution of 1 M NaOH using a syringe and placed in an oven for 24 h at 50 °C. After this the column was cleaned for 30 min by flushing with water using a HPLC pump. The column was then dried for 30 min under N_2 gas. Forty microliter of the monomer 3-(trimethoxysilyl)propylmethacrylate mixed with 10 ml of 6 M acetic acid was inserted in the dried column. Septa were then placed at both ends and the column was placed in an oven at 60 °C for 20 h. After this the capillary was washed with water and acetone and dried under N_2 gas for 1 h.

2.3.2. Polymer mixture

2.3.2.1. Standard PS–DVB monolith. For the standard PS–DVB monolith, the polymer mixture consisted of 2 μl tetrahydrofuran, 5 μl styrene, 5 μl divinylbenzene, 13 μl decanol and 10 mg/ml of initiator (0.25 mg azobisisobutyronytrile).

A length of the silanised capillary was then filled using a syringe. Septa were then placed at both ends of the column to seal the capillary and left to polymerise for 20 h at 60 °C.

For the analysis of small molecules, the total cross-linker and monomer concentration in the mixture remained to the same proportion, however, the percentage between monomer and cross-linker ranged from 50 to 85% cross-linker.

2.3.2.2. Derivatised PS–DVB monolith. For the functionalised monolith, the polymer mixture consisted of 2 μl tetrahydrofuran, 5 μl chloromethylstyrene, 5 μl divinylbenzene, 13 μl decanol and 10 mg/ml of initiator (0.25 mg azobisisobutyronytrile).

A length of the silanised capillary was then filled using a syringe. Septa were placed over the ends of the column to seal the capillary and left to polymerise for 22 h at 70 °C.

Following this the column was flushed with acetonitrile, and then filled using a syringe with *N*,*N*-dimethylbutylamine, and heated at 70 °C for 30 min.

3. Results and discussion

3.1. Standard PS–DVB monolith

The monolith morphology consists of globules, which are cross-linked to form a continuous mesoporous network as shown in Fig. 2. This stationary phase structure is composed of benzene rings linked by sp^3 carbons. This conformation gives the backbone a hydrophobic character ideal for reversed-phase chromatography.

The fabricated monoliths exhibited good mechanical strength and high porosity. The best columns in terms of efficiency were those of internal diameters of 100 and 50 μm i.d. and length of 33 cm. These columns could withstand pump pressures up to 600 bars and a maximum flow rate of 120 μl/min was observed.

In a previous study reported by Premstaller et al. [5], short columns of 6 cm length and 200 μm i.d. were assessed for effectiveness against these monoliths. The selectivity of the monolith versus a packed column was studied. The study consisted of a comparison between a PS–DVB monolith and octadecylated PS–DVB particle filled conventional column. The columns that were compared were of different internal diameter (6 cm × 200 μm versus 5.3 cm × 400 μm) and a different method of analysis was used for each column. The outcome concerned the number of proteins resolved in each case, both the monolith and the conventional column capable of separating 16 proteins in less than 15 min.

As our concern is with smaller diameter columns and in order to directly compare with monolithic and conventional phases, the internal diameters of the columns and the methods used were the same. When adapting a method from a normal bore column to a nanoscale column the flow rate must be reduced to find the optimal efficiency. On the other hand, the sensitivity gained by changing from a diameter of 4.6 mm i.d. to a diameter of 100 μm i.d. column can be as high as 20,000-fold [9,10].

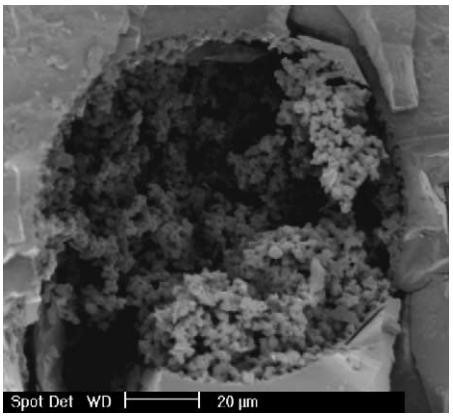

Fig. 2. SEM of PS–DVB monolith showing the cauliflower structure.

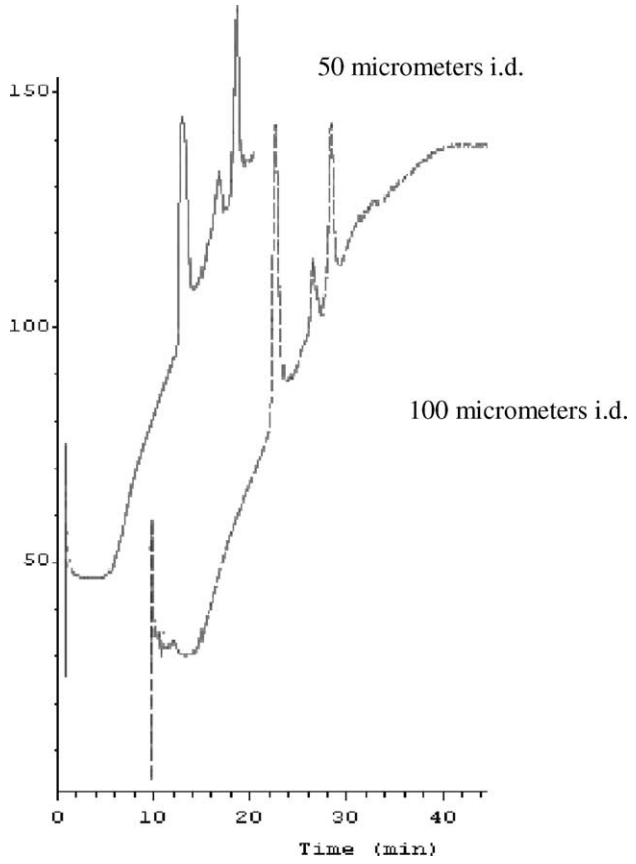

Fig. 3. Separation of three proteins in a monolithic capillary column. Columns: monolithic PS–DVB; length 33 cm, 0.10 mm i.d. and length 30 cm, 0.05 mm i.d. Mobile phase: (A) 10% acetonitrile, 0.10% TFA in water; (B) 50% acetonitrile, 0.10% TFA in water. Linear gradient, 0–100% (B) in 30 min; flow rate: 6 μl/min; temperature: 30 °C; detection: UV, 214 nm; 0.2 μL injection. First peak ribonuclease A (M_r: 13,700), second peak cytochrome *c* (M_r: 12,400) and third peak lysozyme (M_r: 14,300).

The monolith column of internal diameter 100 μm and another monolith of smaller internal diameter 50 μm were used to perform a separation of the three proteins, Fig. 3 shows the chromatograms achieved.

The separations were achieved at room temperature, in under 30 min and were highly reproducible with a R.S.D. of <1%. We can observe how the chromatography is very similar for both monolithic columns of different internal diameters. It was observed that the smaller diameter column was less efficient (LYS, the last eluted peak was 35% less efficient than the 100 μm column). As expected the elution times were also different as both analysis were run at the same flow rate and the internal diameters were not the same. The first eluted protein was at 14.7 min in the smaller diameter and at 16.0 min for the bigger diameter. A possible reason for the discrepancy in efficiency between the columns could be that this was not the optimised flow rate for the monoliths but the flow rate used for comparison with a Vydac conventionally packed column.

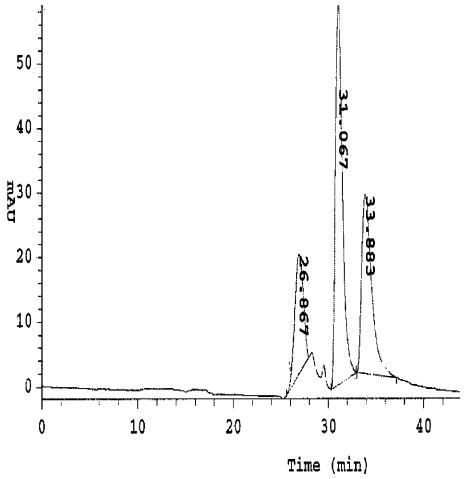

Fig. 4. Shows the chromatogram of a mixture of three small neutral molecules. Peaks in the order of elution: thiourea, dimethyl pthalate, and anisole. Column is PS–DVB monolith 80% crosslinker, 999 mm × 0.1 mm i.d. Eluent 60% acetonitrile–water (60:40); detection 210 nm; temperature 30 °C; flow 100 μl/min; injection 10 nl.

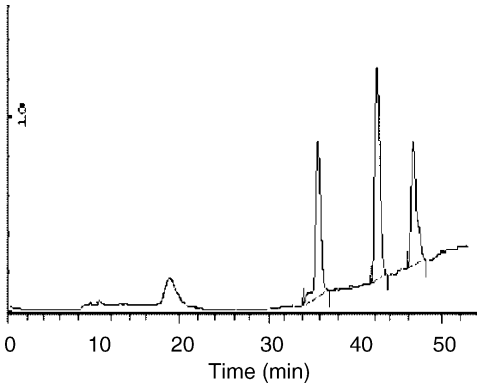

Fig. 5. Separation of three proteins with a conventional phase column. Column: Vydac, length 15 cm, 0.1 mm i.d. Mobile phase: (A) 10% acetonitrile, 0.10% TFA in water; (B) 50% acetonitrile, 0.10% TFA in water; Linear gradient: 0–100% (B) in 30 min; flow rate: 1.2 μl/min; temperature: 30 °C; detection: UV, 214 nm; 0.2 μl injection. First peak ribonuclease A (M_r: 13,700), second peak cytochrome *c* (M_r: 12,400) and third peak lysozyme (M_r: 14,300).

Although this monolith is regarded as a liquid separation media for biomolecules, it has also been successfully adapted for the separation of small compounds by gas chromatography [11]. We attempted the separation of small molecules by altering the cross-linker concentration to achieve smaller pores, and then by hyphenating two and three columns to form one very long column. Such long columns can only be composed of the monolith phase due to lower backpressures being experienced. Small internal diameters such as 100 μm generally develop high backpressures since it is difficult to pump the mobile phase through them when fully packed. The instrument used allowed the pump to work as high as 400 bar. This is a big problem when using conventional columns and it was observed that packed columns at this diameter could only be of a maximum of 15 cm length.

When the 1 m column was made, by linking three monoliths composed by 80% cross-linker monomer, the separation of three small neutral compounds could be achieved. Fig. 4 shows an example chromatogram. The best chromatogram achieved an efficiency of 55,000 plates/m for the second peak. This is encouraging because it highlights the possibility of using long monolith columns for analysis. A possible application would be linking a HPLC instrument post-separation to a MS instrument. Achieving in this way no extra band broadening of the solutes, and increasing separation and sensitivity in difficult separation mixtures.

3.2. Comparison of PS–DVB monolith with conventional protein phase

In order to gain a direct comparison of the monolithic phase with a conventional phase used for protein separations, a column of 3 μm Vydac phase was manufactured. Vydac is a large pore (300 Å) stationary phase, which is alkylated with C_8 groups and is the stationary phase of choice for proteomic studies. The Vydac column of 100 μm i.d. was fabricated following the guidelines published in a previous study [9], and the separation conditions were repeated for the protein separation as previously used.

Fig. 5 shows the separation obtained with the 3 μm particle size Vydac stationary phase column and Table 1 shows mean values of 10 consecutive runs of retention times, heights and widths of the same fresh sample of three proteins when analysed with two columns of the same diameter and different stationary phase.

The most important and expected finding from the experiment was that the monolith actually performed a much quicker analysis. The Vydac column allowed only very low flow rates due to the backpressures achieved being at the

Table 1
Mean values of retention times (t_R) and peak widths at half-height and height of three proteins separated by RP-HPLC on a monolithic and on a Vydac packed column

Protein (abbreviation)	PS–DVB monolithic column				Vydac column			
	t_R (min)	Height (mAU)	Width (min)	R.S.D. (%)	t_R (min)	Height (mAU)	Width (min)	R.S.D. (%)
Ribonuclease A (RIB)	16.0	26	0.7	<1	35.3	6.6	0.6	<1
Cytochrome *c* (CYT)	20.4	6	0.6	<1	42.8	11.8	0.7	<1
Lysozyme (LYS)	22.6	15	0.6	<1	47.3	4.6	0.6	<1

limit of the instrument (400 bar). The flow rate permitted with the monolith was more than four times the maximum flow rate allowed by the Vydac column, without compromising the analysis.

The second most important finding was the difference in the outcome when the columns were loaded with samples of the same concentration. There are two main factors to consider for this phenomenon, the interaction between the proteins and the stationary phase and the gradient elution used in the analysis.

In chromatography, it is common that proteins attach themselves in an irreversible manner to the stationary phase, and this is a problem because it reduces the sensitivity of an analysis. In this case, it was observed that the analysis was more sensitive when the monolithic column was used. The proteins interact less with the PS–DVB phase and as a consequence they can elute at an earlier stage of the gradient.

A reliable measure of the sensitivity concerns the lower limits of detection (LODs) achieved by a chromatography set-up and it depends on the instrumentation used and the column used. In the case of the monolith, the LOD was 7.3 fmol for ribonuclease A. The LOD for the Vydac column was higher and was in the order of 41.3 fmol for ribonuclease A.

Another important factor concerns the loadability of the columns. Both stationary phases showed good performance at 8 ng per column and this was double the amount loaded onto those published in a previous study. This difference is possible because the 100 μm columns were highly porous. There was not a loadability difference between the monolith and the Vydac column. An explanation for this is that the monolith phase structure is a porous continuous rod with pores in the macroscale and mesoscale range. Its surface area is accessible for both small and large molecules, as the structure is believed not to have micropores. Therefore, the loading capacity of monolithic columns varies only slightly as a function of the molecular mass. On the other hand, the Vydac column does have pores in the microscale range and it can load in the order of 10 times more small molecules. This important difference did not affect the study since our focus at this stage was large biomolecules [6].

3.3. Comparison of PS–DVB monolith with derivatised PS–DVB monolith

The monolithic column was manufactured by substituting styrene in the original recipe, with chloromethylstyrene. After the monolith was formed, it was then derivatised to achieve a surface with both quaternary ammonium groups and butyl chains. Again the monolith was made to achieve a column of the same internal diameter as previously used, to allow a direct comparison between the two types of monolith.

The morphology of the final polymeric bed was examined by scanning electron microscopy (SEM). Fig. 2 shows an SEM of the original PS–DVB monolith, while Fig. 6 shows the SEM of the derivatised monolith.

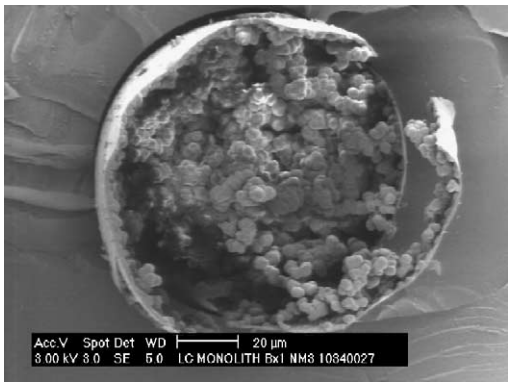

Fig. 6. SEM of derivatised PS–DVB monolith.

Both monolith morphologies are quite different, the original monolith consisting of small polymer globules, which are extensively cross-linked to form a cauliflower-like structure, with a microporous and mesoporous structure and a higher surface area. While the derivatised monolith displays a pronounced mesoporous network, with the whole structure consisting of larger cross-linked polymer clusters of nearly spherical shape.

Both monoliths possess an interconnected porous network, creating continuous porous channels through the monolithic bed. This allows for high throughput of mobile phase while experiencing low backpressures. These fabricated monoliths exhibited good mechanical strength and displayed high porosity.

When the porosity of the derivatised PS–DVB monolith was studied by inverse size-exclusion chromatography (ISEC), the porosity was comparable to most protein phases. It displayed an average pore size distribution of 270 Å, which is ideal for the separation of proteins.

In order to gain a direct comparison of protein separations with each monolithic phase, the separation conditions were repeated as before. No retention was achieved when running this gradient, so the starting concentration of the gradient was altered, to remove all acetonitrile and hence gain the maximum retention, which should be total retention on the column. When the gradient was run, then the proteins should release at a set concentration of acetonitrile.

Fig. 7 shows the chromatogram obtained for the three proteins with the derivatised PS–DVB column.

It is clear that the chromatography is very different for this column from the standard PS–DVB column. Even at 0% acetonitrile there is still no retention on the column, as there was with the original monolith. This is unexpected, as the presence of butyl groups would be expected increase the retention, although the introduction of an anion exchange group to the benzene rings, may well have altered the electron density on the surface of the monolith.

Comparing the long-term stability of the monolithic columns, they were both mechanically robust after 100

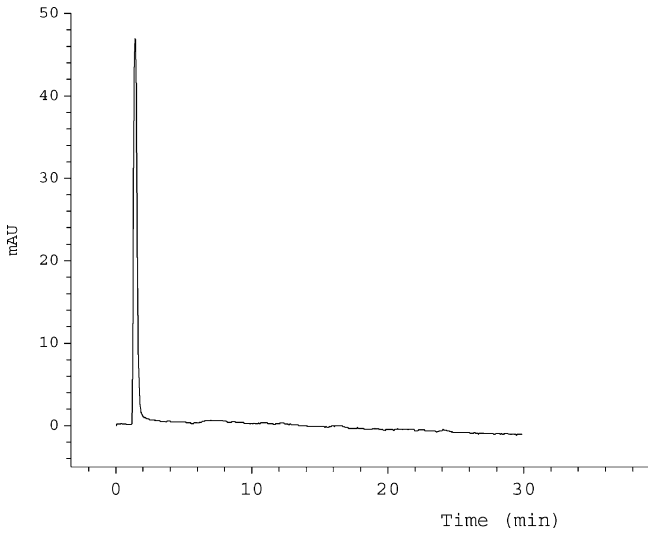

Fig. 7. Analysis of three proteins on a monolithic capillary column. Column: Derivatised monolithic PS–DVB, 35 cm × 0.10 mm i.d. Mobile phase: (A) 10% acetonitrile, 0.10% TFA in water; (B) 50% acetonitrile, 0.10% TFA in water. Linear gradient: 0–100% (B) in 30 min; flow rate: 6 μl/min; temperature: 30 °C. Detection: UV, 214 nm; ribonuclease A (M_r: 13,700), cytochrome *c* (M_r: 12,400), and lysozyme (M_r: 14,300).

repeat injections. Over this period of repeat injections, the R.S.D. remained <1%, and the efficiencies were ±5% of the initial injections.

4. Conclusions

Nano-LC columns for separation of proteins can be easily fabricated in a one step polymerisation process. When comparing monolith PS–DVB columns in the nano-LC range with their column analogue of a conventional phase we can deduce three important advantages when analysing proteins: monoliths perform a much quicker analysis because of higher throughput; they can achieve lower limits of detection than the Vydac stationary phase, therefore, they are more sensitive; and they have the same loading ability as the conventional phases when analysing proteins.

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References

[1] E.F. Hilder, F. Svec, J.M.J. Frechet, Electrophoresis 23 (2002) 3934.
[2] C. Legido-Quigley, N. Marlin, V. Melin, A. Manz, N.W. Smith, Electrophoresis 24 (2003) 917.
[3] H. Oberacher, C.G. Huber, Trends Anal. Chem. 21 (2002) 166.
[4] P.J. Oefner, C. Huber, J. Chromatogr. B 782 (2002) 27.
[5] A. Premstaller, H. Oberacher, C.G. Huber, Anal. Chem. 72 (2000) 4386.
[6] A. Premstaller, H. Oberacher, W. Walcher, A.M. Timperio, L. Zolla, J.P. Chervet, N. Cavusoglu, A. van Dorsselaer, C.G. Huber, Anal. Chem. 73 (2001) 2390.
[7] T.W. Ryan, J. Liq, Chromatogr. 18 (1995) 51.
[8] P. Coufal, M. Cihak, J. Suchankova, E. Tesarova, Z. Bosakova, K. Stulik, J. Chromatogr. A 946 (2002) 99.
[9] C. Legido-Quigley, N.W. Smith, D. Mallet, J. Chromatogr. A 976 (2002) 11.
[10] A. Braithwaite, F.J. Smith, Chromatographic Methods, Chapman & Hall, London, 1996.
[11] F. Svec, T.B. Tennikova, Z. Deyl (Eds.), Monolithic Materials—Preparation, Properties and Applications, Elsevier, Amsterdam, 2003 (J. Chromatogr. Libr. 67 (2003)).