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Super-high-speed liquid chromatography of proteins and peptides on non-porous Micra NPS-RP packings

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Abstract

The new generation of non-porous silica RP packings commercially available from Micra Scientific was tested for separations of peptides and proteins by means of the gradient HPLC. Extremely high-speed separations were achieved using conventional chromatographic equipment: six proteins could be completely separated within six seconds. Tryptic digest peptides could be resolved in more then 40 components within 2–3 min. The effect of the experimental parameters such as temperature, flow rate etc. was investigated.

Keywords: Stationary phases, LC; Proteins; Peptides

1. Introduction

Since the very first high-performance liquid chromatographic separation by Huber in 1964 [1] performed with particles of a mean size of ca. 40 μm, the diameter of HPLC packings was steadily reduced to 5 or even 3 μm. The main benefit of the reduction in particle size was an increase in the column efficiency and a decrease in the analysis time.

As is well known, the efficiency of a chromatographic column, expressed as the number of the theoretical plates N, relates to the length of the column L and to the height of the theoretical plate H: $N = \frac{L}{H}$ [1]. Under an optimal linear flow rate u_{Opt} for a well packed chromatographic column the height of the theoretical plate H is ca. $2d_p$ [1]. Therefore, the number of the theoretical plates is inversely proportional to the particle size [1]:

$$N \approx L/2d_p \tag{1}$$

The number of theoretical plates rises quite significantly for smaller particles because the speed of changes $\mathrm{d}N/\mathrm{d}(d_p)$ is inversely proportional to the second power of the particle size: $\mathrm{d}N/\mathrm{d}(d_p) = L/2(d_p)^2$.

The analysis time under isocratical elution is given by:

$$t_R = L/u\,(1 + k') \tag{2}$$

where k' is the retention factor of the analyte. The optimal flow rate depends on the analyte used and on the particle size of the packing [2]:

$$U_{\mathrm{opt}} = 3D_m/d_p \tag{3}$$

where D_m is the analyte diffusivity in the mobile phase. Combining Eqs. (2) and (3), it can be easily seen that the analysis time under optimal conditions is directly related to the particle size of the adsorbent used:

$$t_R = d_p L(1 + k')/3D_m \tag{4}$$

Eqs. (1) and (4) clearly demonstrate the advantage of

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finely dispersed adsorbents and suggest the application of even smaller and smaller particles. Neither Eq. (1) nor Eq. (2) impose any restrictions on the size of packings which can be used in HPLC. The restrictions are presented by the parameter which is not probably so important from the theoretical point of view, but extremely important for practical work. This parameter is the column pressure drop.

The inlet pressure, which has to be applied to achieve a particular flow rate on the column, can be calculated according to Darcy's law:

$$\Delta P = \frac{uL\eta\psi}{d_P^2} \tag{5}$$

where η is the viscosity of the mobile phase and ψ is the parameter given by:

$$\psi = \frac{180(1+\omega)(1-\varepsilon^2)}{\varepsilon^2} \tag{6}$$

where ε is the interstitial porosity, commonly 0.4 for randomly packed columns, and ω is the ratio of the intraparticulate and interstitial void volumes. Upon comparing Eqs. (1), (4) and (5), one can see, that the pressure drop on the column at a constant column length L is inversely proportional to the square of the particle size, while the efficiency of the column and the analysis time depend on the first order of the particle size. Therefore, a quick increase in the pressure drop can be expected with a decrease of the particle size. Modern chromatographic pumps can commonly deliver a liquid at a pressure of up to 500 bar. Using small particle adsorbents one has to reduce the column length so does not to exceed the allowed pressure range. The column length itself as not provide information about the resolving ability of the column. It is more reliable to use the number of the theoretical plates which is required to resolve the given mixture of analytes as a fixed parameter in equations above. The column length L can be

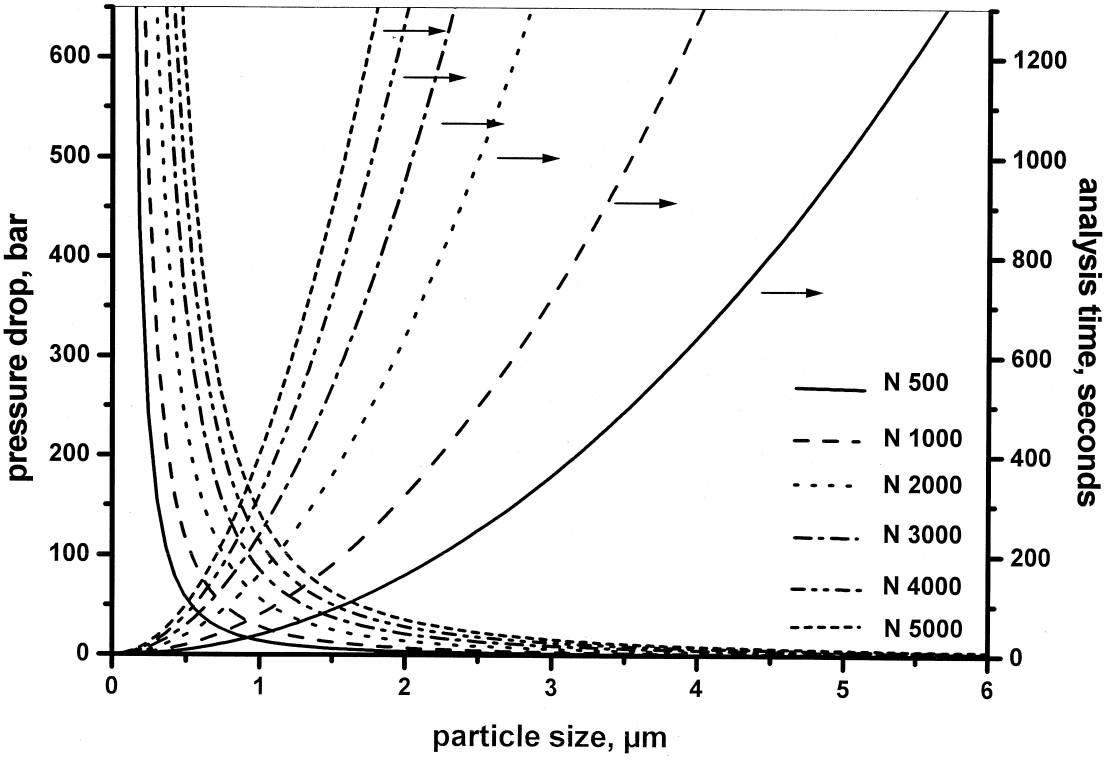

Fig. 1. Dependence of analysis time and pressure drop on the column on particle size at a given plate number for non-porous packings. $D_m = 5\times10^{-7}$ cm^2 s^{-1}, $\eta = 1$ cP, $\varepsilon = 0.4$, $\psi = 945$, $k' = 3$ and the required N is shown in the figure.

eliminated between the Eqs. (1), (4) and (5) and the analysis time and the pressure drop on the column depending on the number of theoretical plates required for the analysis can be given under optimal conditions by:

$$t_R = 2Nd_p^2(1 + k')/3D_m \quad (7)$$

$$\Delta P = 6D_m N\eta\psi/d_p^2 \quad (8)$$

These equations are similar to those obtained earlier by Cheng and Horváth [2] but they consider the number of theoretical plates rather then the column length as a fixed parameter. Fig. 1 displays the corresponding dependencies. It is seen that particles of diameter 2 μm and lower only can be used to perform an analysis within one minute or quicker with sufficient efficiency. For example, for a separation which requires 2000 theoretical plates and should be performed within 200 s, the largest particle size is about 1 μm. The corresponding column length is only 0.4 cm and the pressure drop on the column is ca. 70 bar. Such small particles have not yet been utilised in the HPLC of proteins and peptides.

Adsorbents of particle size smaller then 2 μm were introduced in chromatographic praxis more then 10 years ago by Unger et al. [21], by Horváth et al. [22] and by Danielson and Kirkland [14]. These groups demonstrated the potential ability of small size packings in the acceleration of the chromatographic analysis. C. Horváth et al. described in a series of publications [3–6] the usage of silica and polymeric non-porous beads of 2 μm in diameter in the high speed analysis of proteins. The column length was about 3 cm and the flow rate was about 5 ml/mm. A high back-pressure was observed under these conditions. To reduce the back-pressure, the separations had to be performed under increased temperature. Almost complete separation of four proteins could be achieved, at 120°C and with a flow rate of 5 ml/mm within 12 s! [6]. This is the fastest separation of proteins ever reported in literature. The pressure drop on the column was 240 bar in spite of the high temperature applied.

Unger and co-workers investigated modified non-porous silicas in reversed-phase [7–9] and ion-exchange [10] chromatography of proteins on columns of 3–5 cm in length. Up to six proteins could be separated within ca. 1 min. A further decrease of the analysis times was prevented by a high pressure drop on the column (500 bar) achieved at a flow rate of 2.5 ml/mm. Similar results were also reported by other research groups [11–14].

It appears that the progressively increased pressure drop on the column packed with fine particles is the most restrictive parameter in high speed HPLC. MacNair et al. [15] tried to overcome this disadvantage of small sized packings by creating a chromatographic system that was operated at a pressure of 5000 or even 7000 bar. Eighty-thousand theoretical plates were achieved with the system within ca. 15 min for the column of 25 cm length and packed with 1 μm non-porous RP packing. Whether this technique will be established as a routine one or not, is still to be seen.

A more conventional way used nowadays is the usage of shorter columns, which are compatible with commercially available chromatographic equipment. Recently, the firm Micra Scientific (Northbrook, IL, USA) presented a new generation of monodisperse non-porous RP silicas [16]. The column of 33 mm in length and packed with non-porous monodisperse silica Micra NPS-RP of a mean diameter of 1.5 μm generates ca. 10 000 theoretical plates within ca. 45 s on the separation of aromatic hydrocarbons under isocratical conditions and a pressure of ca. 200 bar [17].

The aim of this work was to elucidate the potential of the new NPS material in high-speed bio-separations using commercially available chromatographic equipment. Both a synthetic mixture of proteins and tryptic digest peptides were investigated and the results obtained are presented below.

2. Experimental

2.1. Materials

33×4.6 mm and 15×4.6 mm columns packed with Micra NPS-RP material were a gift from Micra Scientific.

Non-porous silica coated with polystyrene was prepared from native monodisperse non-porous silica supplied by Micra Scientific. The coating procedure

was essentially the same as described by us earlier for the modification of porous silica beads [18]. The modified material was packed in stainless steel columns by a conventional slurry technique under a pressure of 700 bar using acetone as the slurry liquid.

2.2. Equipment

The high pressure gradient chromatographic system used throughout the study was built up from two analytical HPLC pumps Model 2250, a variable-wavelength UV detector Model 1010 operated at 210 nm and equipped with a micro-cell of 0.8 μl cell volume and 3 mm optical path length, a dynamic high pressure mixer, a central processor unit Model 1152, a column thermostat Model 4110 (all from Bischoff Analysentechnik und Geräte GmbH, Leonberg, Germany) and an injector valve Model 7413 equipped with a 0.5 μl loop (Rheodyne, USA).

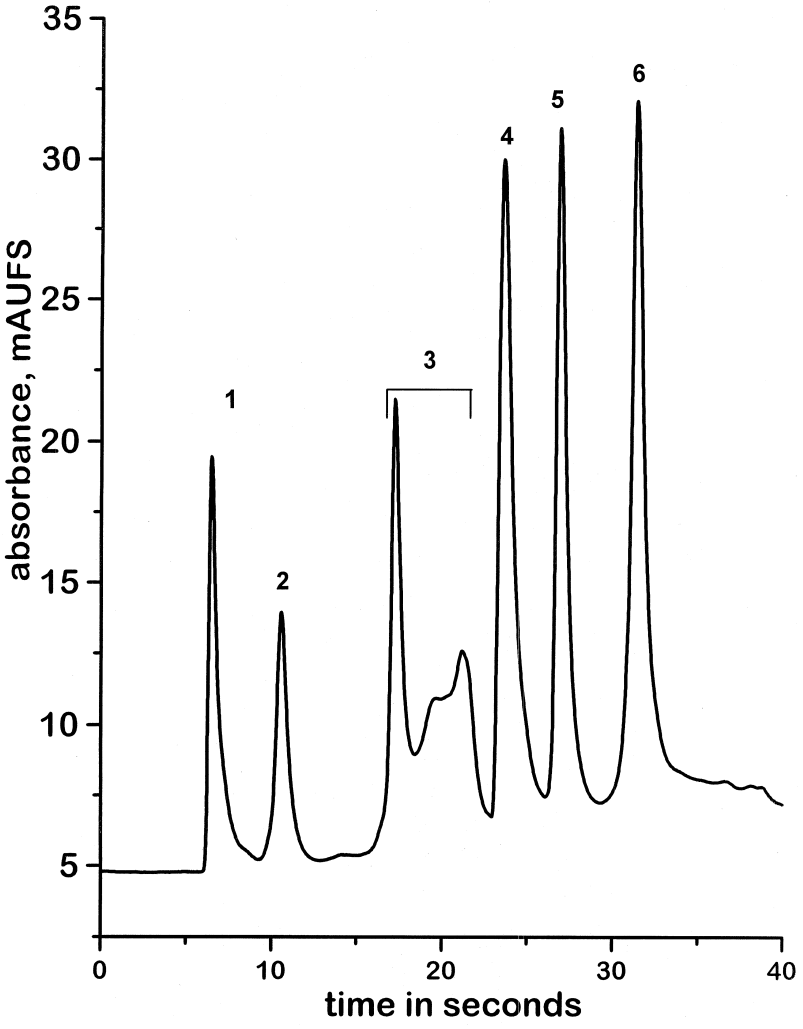

Fig. 2. Separation of a mixture of six proteins on Micra NPS RP column. Conditions: column 33×4.6 mm, flow rate 1.9 ml/min, gradient from 31 to 100% of acetonitrile in water with 0.1% TFA in 30 s, pressure 450 bar, sample size 0.5 μl, analytes: 1=ribonuclease A, 2=cytochrome *c*, 3=lysozyme, 4=bovine serum albumin, 5=chymotrypsinogen, 6=albumin egg (ovalbumin).

3. Result and discussion

3.1. Separation of proteins

Separation of proteins has been investigated in depth during the last two decade [18,19]. Acetonitrile gradient with a small additive of trifluoracetic acid was found to be the best tool to perform a separation on RP packings. Performance of separations does not depend on the column length on elution under gradient conditions [20]. It makes this technique particularly suitable for application with small particle size adsorbents.

A standard Micra NPS-RP column of 33×4.6 mm was tested first. The extra column contributions were reduced to a minimum by the use of very short connections between the column and the injector and the detector. The sampling rate of the detector was

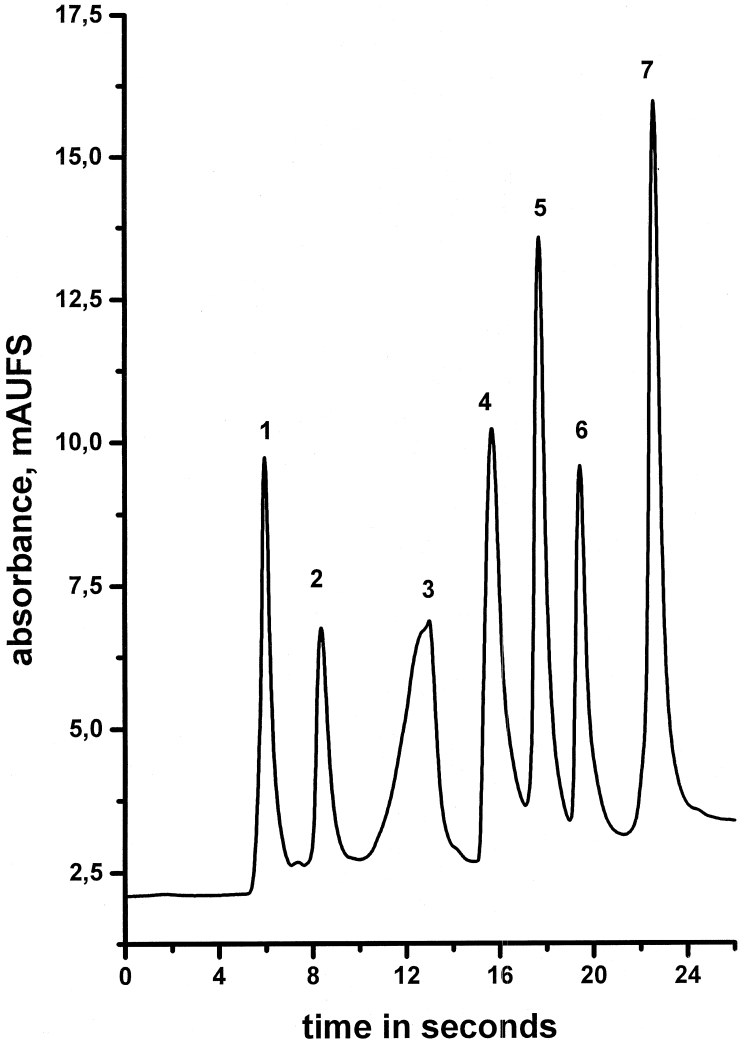

Fig. 3. Separation of a mixture of seven proteins on a column packed with Micra NPS silica coated with polystyrene. Conditions: column 33×4.6 mm, flow rate 2.5 ml/min, pressure 440 bar, gradient from 30 to 100% of acetonitrile in water with 0.1% TFA in 30 s, sample size 0.5 μl, analytes: 1=ribonuclease A, 2=cytochrome *c*, 3=lysozyme, 4=conalbumin, 5=chimotrypsinogen, 6=catalase, 7=albumin egg (ovalbumin).

chosen to be 20 ms to obtain a sufficient number of data points in high-speed separations. Under these conditions the fastest separation of the test protein mixture could be realized within ca. 30 s (Fig. 2). The first eluted component appeared in six seconds close to the dead volume of the column. It was not possible to decrease this time further due to the maximum pressure of ca. 500 bar achieved at flow rate of 2 ml/mm.

Slightly better results than above were obtained with a column packed with Micra NPS silica modified with polystyrene (Fig. 3). The column had a higher permeability and allowed a higher flow rate of the eluent. The mixture of seven proteins could be separated at a flow rate of 2.5 ml/mm within 24 s. It is worth noting the broad peak shape of lysozyme observed. We suppose that this is a result of some slow association processes or any other interconversions of the analyte molecule. This point is supported by the dependence of the peak shape on the eluent

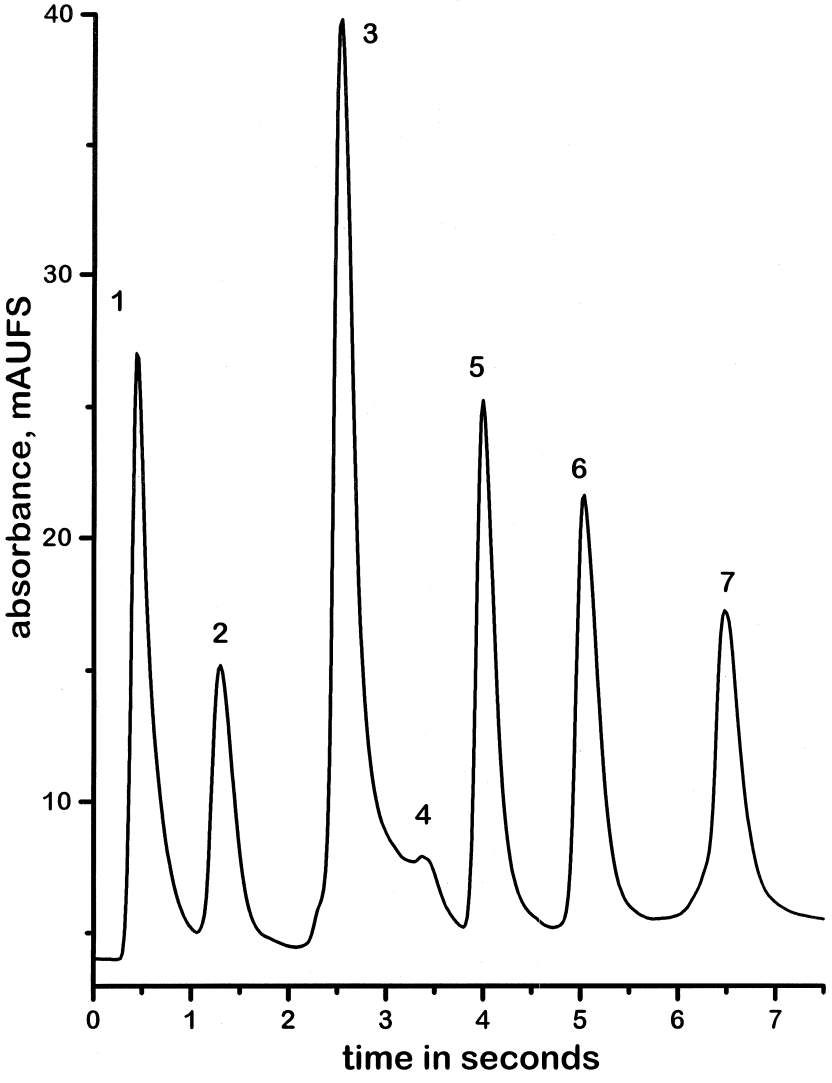

Fig. 4. Separation of a mixture of six proteins on a short Micra NPS-RP column. Conditions: column 15×4.6 mm, flow rate 4 ml/min, pressure 405 bar, gradient from 30 to 100% of acetonitrile in water with 0.1% TFA in 5 s, analytes: 1=ribonuclease A, 2=cytochrome *c*, 3=lysozime, 4=unknown, 5=bovine serum albumin, 6=catalase, 7=albumin egg (ovalbumin).

flow rate. Up to three badly resolved bands for lysozyme could be observed under particular gradient conditions (Fig. 2).

Further decrease the analysis time by an increase of the eluent flow rate was not possible because of the high back-pressure on the column. Therefore, we decided to reduce the column length since it is not a critical parameter under gradient elution. Using a column of 15 mm only the flow rate of ca. 5 ml/mm could be achieved at a pressure of 450 bar. The elution time of the dead volume is now 0.5 s and extremely high-speed separations can be performed. Fig. 4 demonstrates separation of a standard mixture of six proteins, which was realized within 6 s. All proteins are base line separated. Furthermore, the peak capacity of the column was not yet completely exhausted and small admixtures were observed resolved from the main proteins (Fig. 4).

3.2. Separation of tryptic digest peptides

Tryptic digest of proteins is a widely used tool for

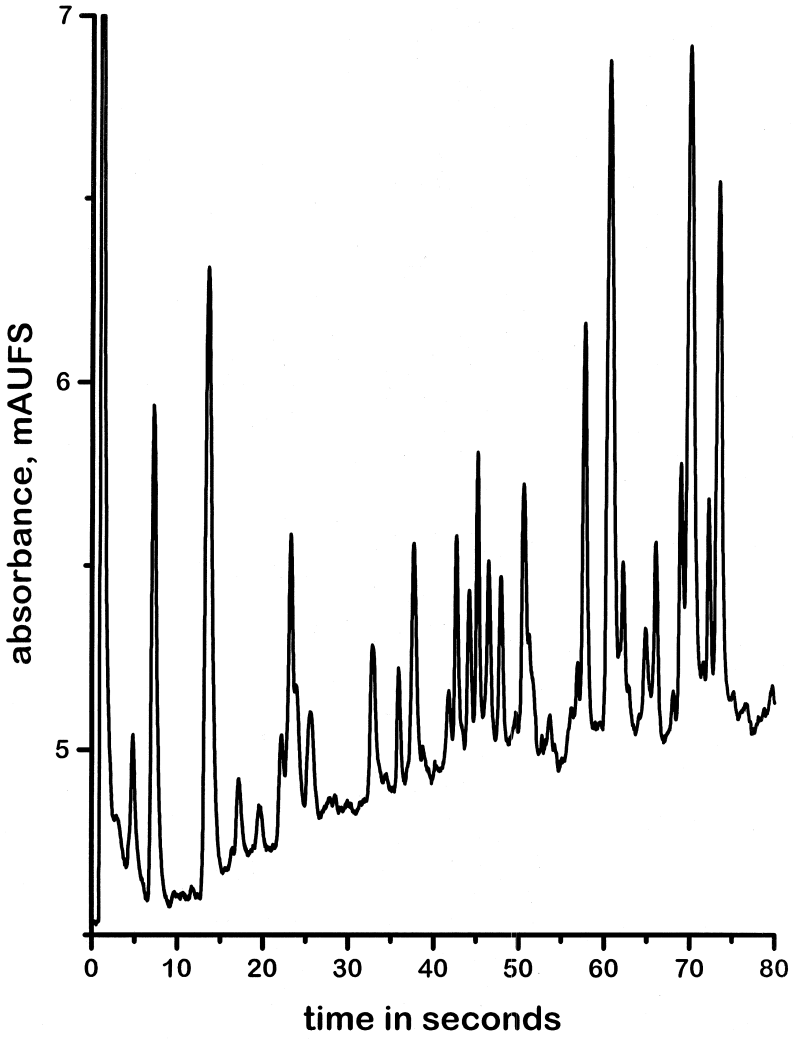

Fig. 5. Separation of tryptic digest of hemoglobin A on a short Micra NPS-RP column. Conditions: column 15×4.6 mm, room temperature, flow rate 4 ml/min, pressure 400 bar, gradient of acetonitrile in water with 0.1% TFA according to program (% acetonitrile, time in seconds): 1% 0; 20% 20; 30% 90; 40% 100; 40% 120; 60% 130.

the recovery of protein structure. A complex mixture of peptides after digestion can be analysed by means of any liquid separation techniques, such as capillary electrophoresis, high-performance liquid chromatography or planar chromatography. Two-dimensional planar chromatography and two dimensional electrophoresis are particularly popular as a tool for the separation of tryptic digest proteins due to their high resolution ability; which is superior to other chromatographic techniques. At the same time, they are

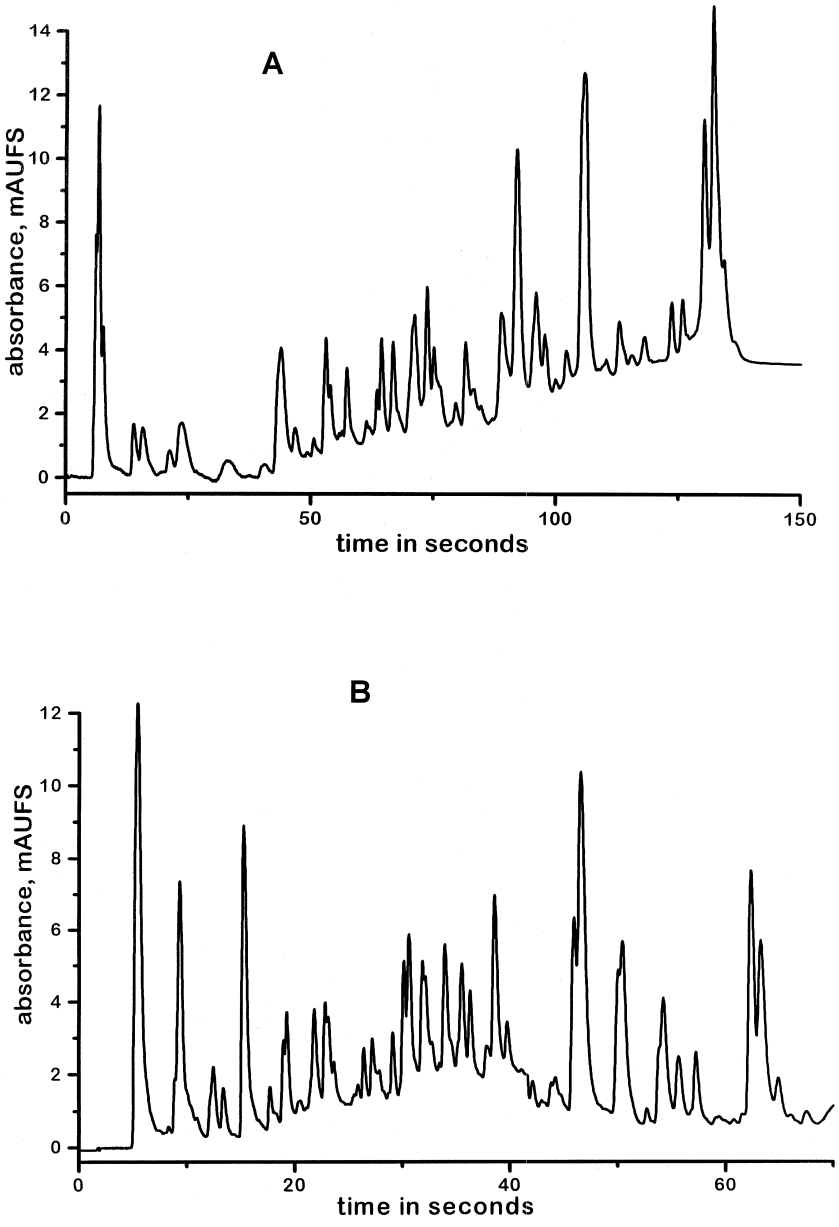

Fig. 6. Separation of tryptic digest of hemoglobin A on a long Micra NPS-RP column at a room temperature (A) and at 70°C (B). Conditions: column 33×4.6 mm, flow rate 2.5 mm, pressure 495 bar (room temperature) and 250 bar (70°C), gradient of acetonitrile in water with 0.1% TFA according to the same program as in Fig. 5

extremely time consuming methods. Separation of a single sample lasts many hours. Therefore, development of a separation technique of high speed and at the same time of high resolving power remains still a challenge in the analysis of tryptic digest peptides.

Accounting for the good results achieved in the high-speed separation of proteins on the short column we performed a separation of tryptic peptides on a small column of 15 mm length. The separation is seen in Fig. 5. More than 30 components could be separated within 75 s. Nevertheless, accounting for the complex composition of the mixture analysed, not all components were completely resolved. Therefore, we checked a longer column of 33 mm.

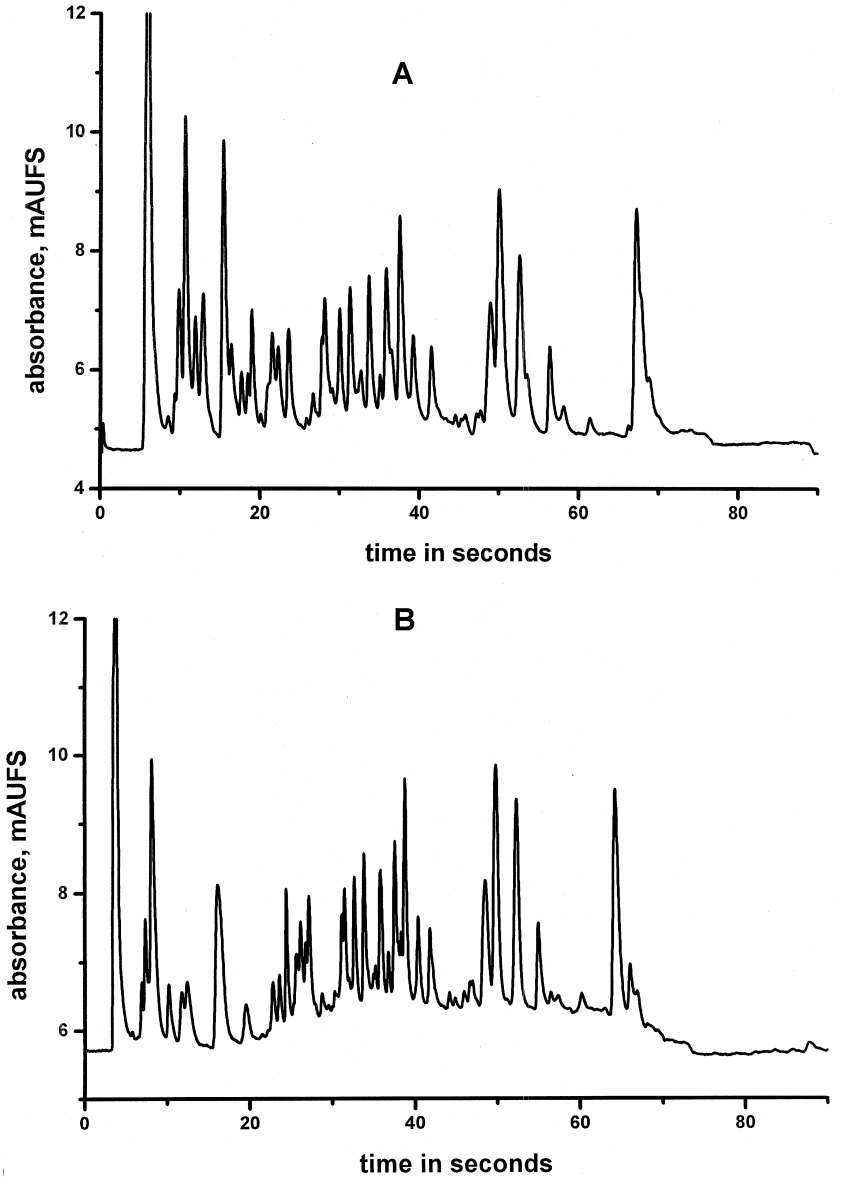

Fig. 7. Separation of tryptic digest of hemoglobin A on a long Micra NPS-RP column at two different flow rates 2.5 ml/min (A) and 4 ml/min (B). Other conditions as in Fig. 6.

Separation achieved on the long column at room temperature was not better then on the short one (Figs. 5 and 6). Peaks observed were rather broad and complete separation required ca. 2 min. The resolution was significantly improved by increasing the temperature. Up to 40 components could be separated at 70°C within 70 s with much better peak symmetry (Fig. 6). The flow resistance of the column was decreased with the temperature increase and higher flow rates up to 4 ml/mm could be applied. Surprisingly no improvement in the separation could be detected with a high flow rate. Separations performed at 50°C with the flow rates 2.5 and 4 ml/mm and a gradient time of 120 s looked very similar (Fig. 7A and B). It appears that the gradient shape and the temperature are the most critical parameters in the separations. When a short analysis time is not the aim and a shallow gradient can be applied, separations can be performed even at a room temperature. Fig. 8 displays a separation of an old tryptic digest sample of hemoglobin (digestion time ca. 7 days) on a Micra NPS-RP column at a room temperature and a gradient time of 3 min. More than 60 components were resolved within ca. 3 min demonstrating the high resolving potential of the non-porous packing.

4. Conclusions

The results obtained in this work clearly show the great potential of non-porous adsorbents in the acceleration of the chromatographic analysis. The lack of the corresponding chromatographic equipment such as fast and sensitive detectors, high speed integrators, high pressure pumps etc. prevented the invention of these packings in the routine chromatographic analysis in the past. Nowadays, these problems have been overcome. Very high-speed separations of proteins and peptides can be realised using commercially available chromatographic equipment and commercially available chromatographic columns packed with non-porous micron size particles. Application of these adsorbents in micro-column and

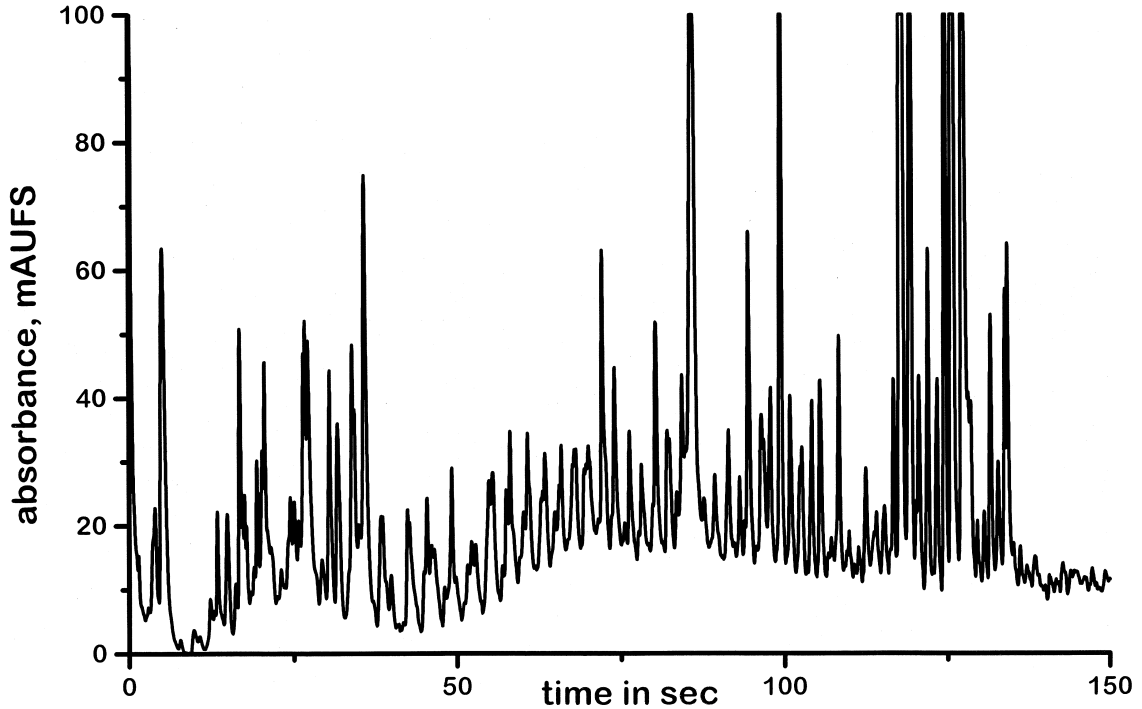

Fig. 8. Separation of an old sample of tryptic digest of hemoglobin A on a Micra NPS-RP column. Conditions as in Fig. 6, besides the temperature 30°C and the flow rate 1 ml/min.

capillary HPLC promises an even further decrease of the analysis times.

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