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High-efficiency liquid chromatography–mass spectrometry separations with 50 mm, 250 mm, and 1 m long polymer-based monolithic capillary columns for the characterization of complex proteolytic digests

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ABSTRACT

In this study, high-efficiency LC–MS/MS separations of complex proteolytic digests are demonstrated using 50 mm, 250 mm, and 1 m long poly(styrene-*co*-divinylbenzene) monolithic capillary columns. The chromatographic performance of the 50 and 250 mm monoliths was compared at the same gradient steepness for gradient durations between 5 and 150 min. The maximum peak capacity of 400 obtained with a 50 mm column, increased to 485 when using the 250 mm long column and scaling the gradient duration according column length. With a 5-fold increase in column length only a 20% increase in peak capacity was observed, which could be explained by the larger macropore size of the 250 mm long monolith. When taking into account the total analysis time, including the dwell time, gradient time and column equilibration time, the 50 mm long monolith yielded better peptide separations than the 250 mm long monolithic column for gradient times below 80 min (n_c = 370). For more demanding separation the 250 mm long monolith provided the highest peak production rate and consequently higher sequence coverage. For the analysis of a proteolytic digest of *Escherichia coli* proteins a monolithic capillary column of 1 m in length was used, yielding a peak capacity of 1038 when applying a 600 min gradient.

1. Introduction

Shotgun proteomics, in which proteins are digested and the resulting peptides are separated by high-performance liquid chromatography (HPLC) and detected by tandem mass-spectrometry detection (MS/MS), has become the standard approach in proteomics research [1–4]. The number of peptides present in a single sample of proteolytic protein digests can be enormous. For example, hundreds of thousands of peptides can be present in a sample after digestion of a cell lysate. In addition, the concentration range can encompass many orders of magnitude [5,6]. When analyzing complex peptide mixtures, the identification and quantification of (low abundant) peptides is often hindered by ion-suppression effects. To improve the LC–MS/MS analysis of complex proteomic samples much effort is directed to the development of novel column technology [7–9].

The use of columns packed with porous silica particles in gradient LC is a widely accepted approach for peptide mapping. To increase the separation efficiency, longer columns and columns of the same length packed with smaller particles have been proposed [10,11]. However, the preparation of stable column packings and robust frits are well-know problems [12,13]. Although excellent separation performance has been demonstrated with sub-2-micron packed columns [14–16] the technical difficulties associated with the preparation of packed capillary and nano-LC columns have spurred the development of new approaches, such as monolithic column technology. Monolithic separation materials have already been developed since the late 1960s and they have now become a viable alternative for packed column technology. Due to their continuous structure that can be attached to the capillary walls, frits are no longer necessary, hence increasing the robustness of the columns.

Monolithic materials for chromatography can be roughly divided into two main categories – silica-based and polymer-based monolithic stationary phases. Silica monolithic phases are typically prepared using sol–gel technology [17,18]. After hydrothermal treatment of the silica backbone a mesoporous structure with micrometer-sized interconnected skeletons is obtained with a

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distribution of flow-through pores. Different polymer-based monolithic phases, for example based on acrylamide, methacrylate and styrene monomers, have been developed for HPLC [19–22]. Often a single-step copolymerization is employed using a reaction mixture comprising monomers, crosslinkers, porogens, and the initiator. Due to the absence of mesopores (and thus the absence of mass transfer based on diffusion), polymer-based monoliths may provide high-efficiencies for gradient separations of large molecules, such as peptides and proteins. The efficiency and porosity can be influenced by changing the composition of the polymerization mixture or the reaction conditions [23].

Poly(styrene-*co*-divinylbenzene) monolithic stationary phases were introduced for protein separations in molded columns with a typical inner diameter of 4.6 mm by Svec and Fréchet [24,25]. Huber and coworkers refined the preparation of poly(styrene-*co*-divinylbenzene) monoliths in capillary column formats and demonstrated high-efficiency LC–MS separations of tryptic peptides, oligonucleotides, and proteins [26,27]. 20 μm I.D. monolithic columns (100 mm in length) were developed by Karger et al. and employed for the analysis of protein digests with high mass sensitivity [28]. In gradient LC coupled on-line to an ion-trap mass spectrometer about 10 amol of peptides could be detected in both the MS and MS/MS modes. For in-line enzymatic digestion of proteins, porous polymer monolithic microreactors with immobilized trypsin and endopoteinase LysC have been developed [29,30]. In the present study, the LC performance of capillary poly(styrene-*co*-divinylbenzene) monolithic columns of 50 mm, 250 mm, and 1 m length was evaluated for reversed-phase gradient-elution LC–MS/MS separations of complex peptide samples. The effects of flow rate, gradient time, and column length on peak width and peak capacity are discussed. The potential of long monolithic columns in one-dimensional LC coupled to tandem-mass-spectrometry detection is demonstrated by the analysis of tryptic-digest proteomic samples of varying complexity. Furthermore, the retention-time stability of the chromatographic method was investigated.

2. Experimental procedures

2.1. Chemicals and materials

Ammonium bicarbonate (min. 99%), dithiothreitol (min. 99%), iodoacetic acid (approx. 99%), guanidine-HCl, sodium chloride (analytical reagent grade), cytochrome *c* (bovine heart), apotransferrin (bovine, ≥98%), and iodoacetic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Lysozyme (hen egg white), alcohol dehydrogenase (yeast), serum albumin (bovine, assay > 96%), β-galactosidase (*E. coli*), sodium phosphate monobasic dihydrate (analytical reagent grade) were obtained from Fluka (Buchs, Switzerland). *Escherichia coli* (*E. coli*, *strain K12*) protein sample, (lyophilized) was obtained from Bio-Rad Laboratories (Veenendaal, The Netherlands). Acetonitrile and trifluoroacetic acid (TFA, ≥99.95%) were purchased from Biosolve (Valkenswaard, The Netherlands). Trypsin (sequencing grade modified) was obtained from Promega (Madison, WI, USA). Water was purified using a MilliQ Gradient A10 system (Millipore, Molsheim, France). Fused-silica capillary tubing was obtained from Polymicro Technologies (Phoenix, AZ, USA). Polyetheretherketone (PEEK) capillary tubing, tubing sleeves, micro-tight unions, and micro-tight fittings were purchased from Upchurch Scientific (Oak Harbor, WA, USA). Experiments were performed using poly(styrene-*co*-divinylbenzene) monolithic capillary columns with the following dimensions; 50 mm × 0.2 mm; 250 mm × 0.2 mm (Dionex, Amsterdam, The Netherlands). A 1 m long monolith was created by coupling four 250 mm monolithic columns with PEEK micro-tight fittings.

2.2. Digestion procedure

2.7 mg *E. coli* was dissolved in 1000 μL Guanidine (7 M). After adding 10 μL reducing agent (1 M DTT), the solution was mixed and maintained at 60 °C for 60 min. After adding 20 μL alkylating reagent (1 M iodoacetic acid), the solution was mixed and stored in the dark for 30 min. 40 μL 1 M DDT was added to consume any unreacted iodoacetic acid. After adding 100 μL trypsin solution (80 μg in 100 mM ammonium bicarbonate) the solution was gently mixed and digestion was carried out for 20 h at 37 °C.

The tryptic digest of a mixture of six proteins (transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme and cytochrome *c*) was obtained from Dionex Benelux, Amsterdam, The Netherlands.

2.3. LC–MS/MS instrumentation and configuration

Experiments were performed using an UltiMate 3000 Proteomics MDLC system (Dionex, Germering, Germany) consisting of a dual-ternary gradient pump with membrane degasser, a thermostatted flow-manager module equipped with a 1:100 flow splitter, a well-plate autosampler, and a variable-wavelength detector equipped with a 3-nL z-shaped flow cell. The system was coupled on-line to an ion-trap mass spectrometer (HCTultra, Bruker Daltonics, Bremen, Germany) for peptide identification.

The separations were performed using the "direct injection" setup applying a 1 μL full-loop injection, with the column placed in the oven, and connected to the injector with 50 μm I.D. connection tubing and to the UV detector using 20 μm I.D. connection tubing. The column temperature was maintained at 60 °C. To increase robustness of the electrospray interface, the nano interface using spray needles was replaced by a modified capillary interface, where the metal needle in the electrospray source was replaced by a 20 μm I.D. × 90 μm O.D. fused-silica capillary. The capillary was connected to the flow-cell outlet by a grounded stainless-steel micro-tight union (Fritz Gyger Swiss, Gwatt-Thun, Switzerland). The mass spectrometer was operated in the positive ionization mode in the scan range from 300 to 1600 m/z where two precursor ions were selected for MS/MS fragmentation applying a scan range of 100–2800 m/z. The scan speed in MS and MS/MS mode was 26 000 m/z pro sec. Other MS settings were: dry gas at 4 L min^{-1}, nebulizer gas pressure of 20 psi, dry temperature of 300 °C, and a target mass set to 800 m/z.

2.4. Database search and sequence analysis

Proteins were identified using ProteinScape (Version 2.1, Bruker) with Mascot (Version 2.2, Matrix Science, London, UK) as search engine and the SwissProt database (SwissProt_56.1.fasta). The following settings were applied: 0.8 Da MS tolerance, 0.6 Da MS/MS tolerance, 2+ and 3+ peptide charge, trypsin as the enzyme, carboxymethyl as fixed modification (for C), oxidation as variable modification for M, and allowing up to one missed cleavage. The taxonomy was set as "All Organisms" to analyze the digest originating of six proteins, and "*Escherichia coli*" to analyze the *E. coli* digest. The ProteinExtractor algorithm of ProteinScape was used for compiling the protein lists using the following parameters:

- Peptide ID: ion score > 20, minimum length 7 amino acids, threshold $p < 0.05$.
- Protein ID: protein score > 60. Minimum 2 peptides, and at least one of them has an ion score > 40.

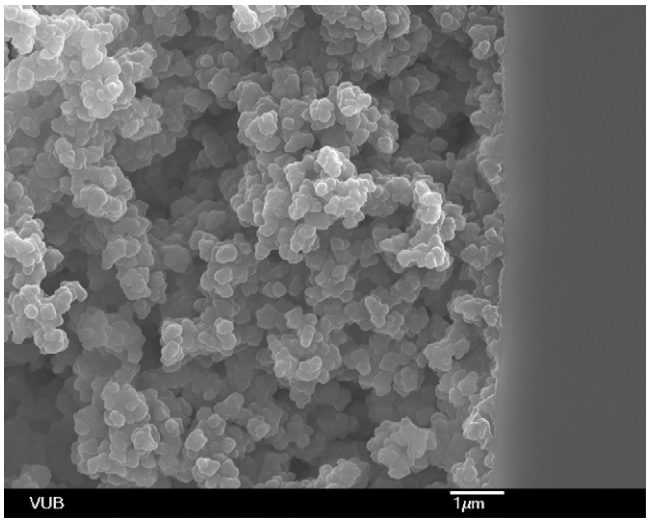

Fig. 1. Scanning electron micrograph of the cross-section of a poly(styrene-*co*-divinylbenzene) monolithic capillary column.

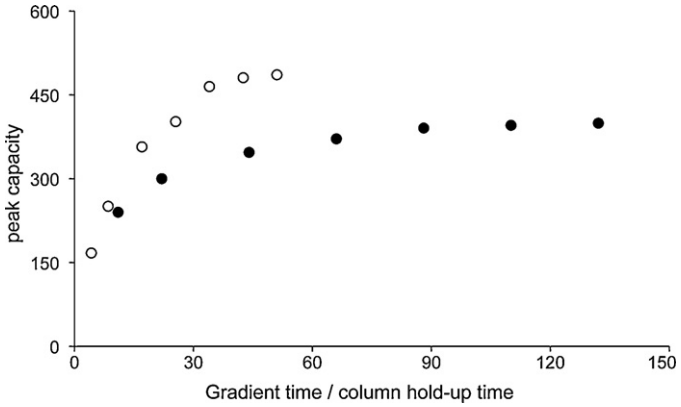

Fig. 2. Effect of gradient time on peak capacity recorded on a 50 (closed symbols) and 250 monolithic column (open symbols). Sample: six-protein-mix digest (transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme and cytochrome *c*), 1 μL injection (concentration 0.5 pmol/μL); flow rate: 2 μL/min; aqueous acetonitrile gradient from 1% to 35% with 0.05% TFA ion-pairing agent; column temperature: 60 °C.

3. Results and discussion

Poly(styrene-*co*-divinylbezene) monoliths were prepared *in situ* in 200 μm I.D. capillary columns from liquid precursors through a thermally-initiated free-radical copolymerization reaction [31]. Fig. 1 shows a scanning electron micrograph of the monolithic chromatographic bed (250 mm long monolith) close to the fused-silica wall. The monolith is covalently bonded to the fused-silica capillary surface via a 3-(trimethoxysilyl)propyl methacrylate spacer. The morphology of the polymer monolith features macropores (flow-through pores) and polymer microglobules. Conceptually, this material is very well suited to perform large-molecule separations since mass transfer is driven by convection, rather than by diffusion as in the case of porous particles. In addition, the size of the macropores and microglobules can be tuned independently to optimize the morphology to yield high-efficiency separations [32]. Since the columns are prepared from liquid precursors, virtually any column size or channel format is easily accessible.

3.1. Optimizing peak capacity in gradient LC

A good performance criterion for gradient separations of complex mixtures is the peak capacity (n_c), which is defined as the maximum number of peaks that can be separated with a unit resolution and elute within the applied gradient window. In the simplest form the peak capacity is defined as [33]:

$$n_c \approx \frac{t_G}{W} + 1 = \frac{t_G \cdot \sqrt{L}}{4 \cdot t_0(1 + k_e) \cdot \sqrt{H}} + 1 \quad (1)$$

where t_G is the gradient time and W the average 4σ peak width, L the column length, t_0 the column hold-up time, k_e the retention factor of the analyte at the moment of elution (which – to a first approximation – may be assumed equal for all analytes), and H the plate height. Eq. (1) shows that maximizing the separation performance involves optimization of column technology, such as the morphology (reflected in the plate-height) and the column length and tuning LC conditions, including gradient time and composition window (affecting k_e), and flow rate and column temperature (affecting k_e as well as H).

The peak capacity was experimentally calculated from MS data by averaging the 4σ peak width for at least five peptides and applying Eq. (1). The peptides selected for this calculation eluted evenly spread (start, middle, and end) over the gradient window. A column temperature of 60 °C was maintained during all experiments, leading to faster mass transfer and, consequently, narrower peaks than when working at room temperature. The decrease in retention observed could be counteracted by shifting the gradient window. When operating a 250 mm long (×0.2 mm I.D.) monolithic column at 2 μL/min the peak capacity was significantly higher than when applying a flow rate of 0.5 or 1 μL/min. At a flow rate of 2 μL/min the column yielded a maximum column pressure of 31 MPa, which is close to the pressure limit of the system.

3.2. Effect of column length and morphology

When comparing gradient-elution results obtained on columns of different dimensions several effects play a role. If the composition window remains constant, the effective steepness of the gradient is the ratio of the gradient duration and the column hold-up time (*i.e.* t_G/t_0). From this perspective, gradient-elution experiments can be performed five times faster on the 50 mm long column to achieve similar results. However, the other effect is the plate count of the column. If the column quality (*i.e.* H) is the same for both columns we would expect that the peak capacity increases with $\sqrt{5}$ when increasing the column length with a factor 5 (see Eq. (1)).

Fig. 2 shows the effect of gradient time normalized for the column hold-up time on peak capacity measured on a 50 and 250 mm long monolithic capillary column. This representation ensures that gradient steepness is comparable. t_0 was estimated from the injection pulse. With increasing gradient slope a strong increase in peak capacity was observed for short gradients, but this effect levels off at longer gradient duration. This is caused by the linear increase in peak width (measured at half height) with increasing gradient time. The maximum peak capacity obtained with a 50 mm column was 400 and increased to 485 when using the 250 mm long column. The observation that the peak capacity does not increase with a factor $\sqrt{5}$ is explained by a difference in morphology. To operate the 250 mm long monolithic column at a flow rate of 2 μL/min yielding a maximum pressure drop of 31 MPa without reaching the pressure limit of the HPLC instrumentation, the polymerization mixture was optimized (changing the ratio of porogen). This adjustment results in an increase of the macropore size [34] of the 250 mm long monolith compared to that of the 50 mm long monolith (consequently H is not the same for both columns) and with a 5-fold increase in column length only a 2-fold increase in column pressure was observed.

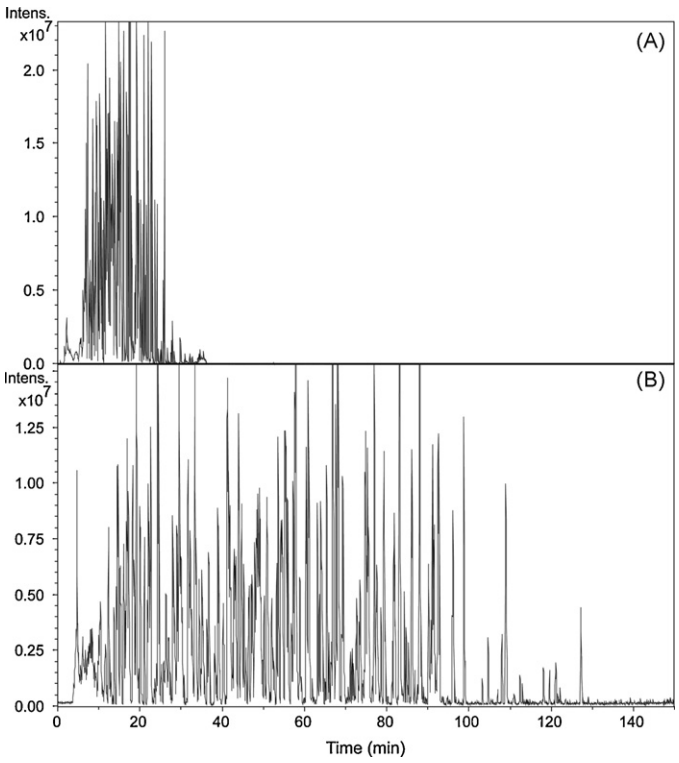

Fig. 3. Base-peak chromatogram of the LC–MS separations of a tryptic digest of a mixture of six proteins on a 50 mm column applying a gradient time of 30 min (A) and 250 mm long monolithic column with a 150 min gradient (B). The gradient is scaled according to column length. Further conditions as described in Fig. 2.

Table 1
Comparison of the sequence coverage and ions scores of a six-protein-mix tryptic digest measured on the 50 and 250 mm monolithic columns when scaling the gradient steepness according column length.

Column length (mm)	50		250	
Gradient time (min)	30		150	
Protein	Seq. cov. (%)	Ion score	Seq. cov. (%)	Ion score
Transferrin	74	3289	89	4126
Bovine serum albumin	82	3272	89	3607
β-Galactosidase	64	2756	81	3739
Alcohol dehydrogenase	66	1212	70	1255
Lysozyme	76	756	90	766
Cytochrome *c*	70	618	74	729

Fig. 3 shows base-peak chromatograms of separations of a tryptic digest of a mixture of six proteins (transferrin, bovine serum albumin, β-galactosidase, alcohol dehydrogenase, lysozyme and cytochrome *c*) on a 50 and 250 mm long monolithic column, respectively, scaling the gradient according to the column length. The number of peptides resolved on the longer column (same gradient steepness as 50 mm monolith) is significantly higher, which resulted in improved peptide identification, as demonstrated in Table 1.

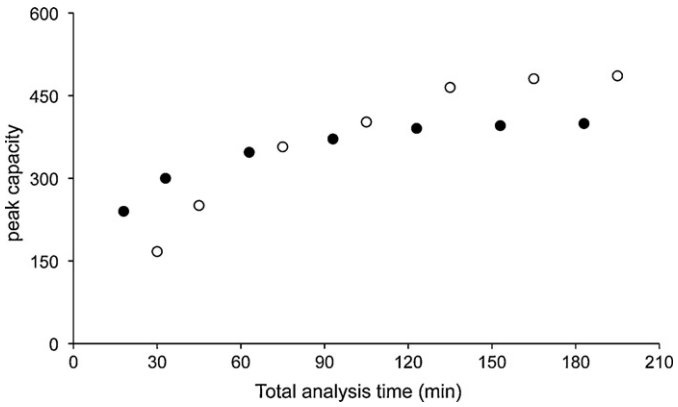

Fig. 4. Peak capacity *versus* total analysis time obtained on a 50 (closed symbols) and 250 mm monolith (open symbols). Conditions as described in Fig. 2. $t_{0,50\,mm\,column}$ = 0.6 min, t_{delay} = 1.4 min, $t_{eq,50\,mm\,column}$ = 2 min, $t_{0,250\,mm\,column}$ = 2 min and $t_{eq,250\,mm\,column}$ = 10 min.

When comparing the column performance of different column lengths at the same gradient slope it should be noted that the total analysis time ($t_0 + t_{delay} + t_{gradient} + t_{eq}$) may dramatically differ. Fig. 4 shows a comparison between the LC performance of a 50 and 250 mm monolith taking into account the total analysis time. For separation with analysis times below 80 min the 50 mm long monolith provides superior separations. For more demanding separations (peak capacities > 370) the use of 250 mm long monoliths are recommended. Within a total analysis time of 120 min the 250 mm monolith provides a maximum peak capacity of approximately 450. To achieve similar performance with a column packed with 3 μm particles, a column length of 500 mm is required [35]. For relative simple peptides mixtures, such as a tryptic digest originating from six proteins, the LC–MS/MS analysis with 50 mm or 250 mm long monolithic columns and applying a 30–150 min gradient often suffice to identify all proteins with enough confidence (Table 1). For more complex mixtures the requirements of the HPLC separation are much more stringent. Fig. 5 shows the base-peak chromatogram of a separation of *E. coli* peptides on a 1 m long monolithic column applying a 600 min gradient. The peak capacity exceeded one thousand, resulting in a very-high-resolution separation, extending the dynamic range of the method since less peptides are influenced by ion-suppression effects. It should be noted that the 1 m long monolith was operated at a volumetric flow rate of 0.5 μL/min due to back pressure limitation of the LC system, which is a factor 4 below its optimum flow velocity. It is likely that when using the 1 m monolith at the optimum flow rate a 20% increase in peak capacity ($n_{c,\,max} \sim 1250$) can be obtained, as was observed for shorter columns. The increase in separation performance clearly leads to a higher number of peptides and proteins identified, as shown in Table 2.

3.3. Run-to-run repeatability

Having excellent retention-time repeatability is essential to be able to map small differences in complex peptide samples that are alike. Also, when correlating retention time models to MS/MS

Table 2
MASCOT identification scores for the separation of an *E. coli* tryptic digest measured on a 250 mm and 1 m long monolith. The false positive rate is 3.0 ± 0.1% (proteins above homology or I.D. threshold).

Column length (mm)	Flow rate (μL/min)	Gradient time (min)	No. of peptides identified	No. of proteins identified
250	2	120	859	134
250	2	360	1399	216
1000	0.5	360	1781	234
1000	0.5	600	2053	283

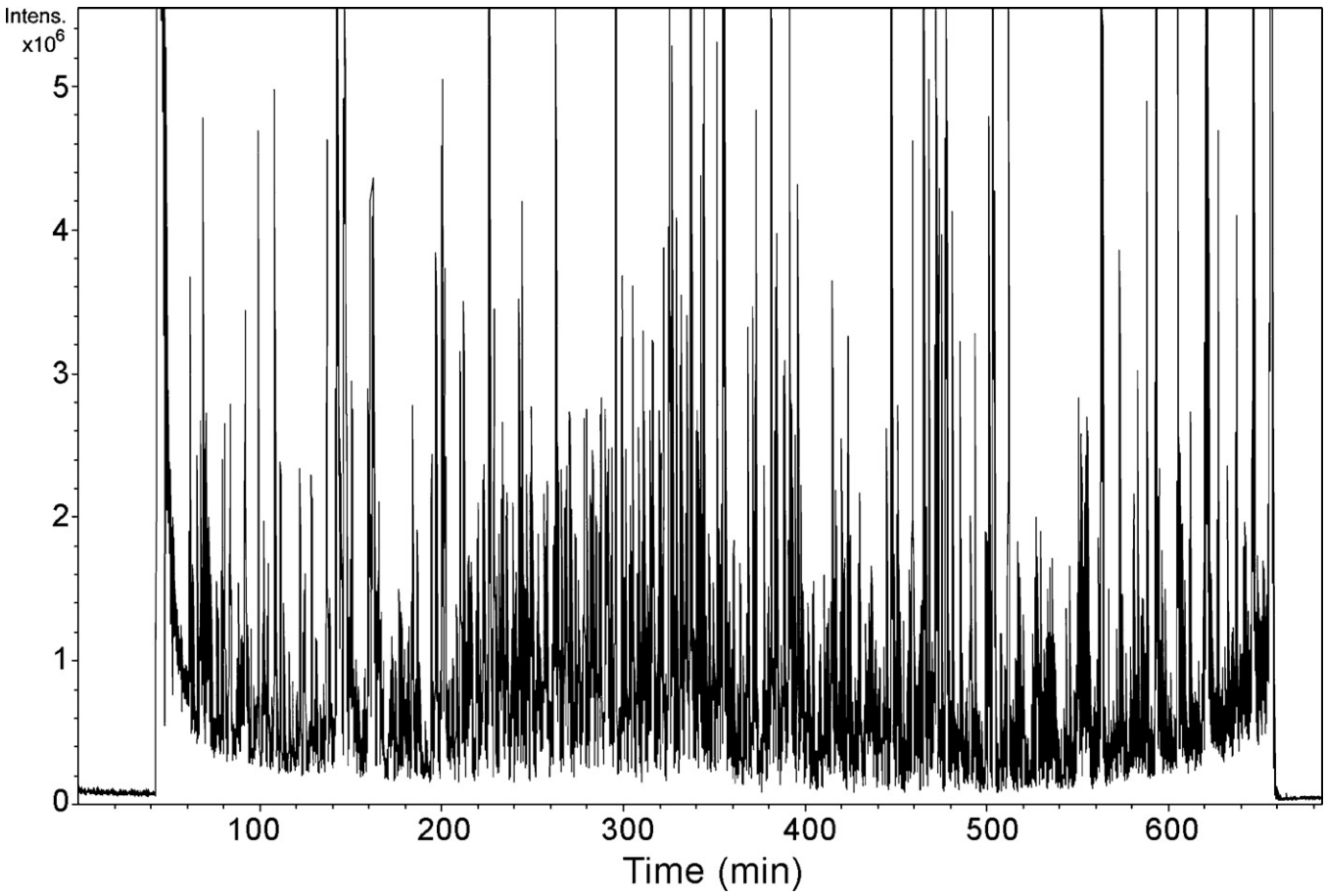

Fig. 5. High-efficiency separation of an *E. coli* digest on a 1 m monolithic column operating at a flow rate 0.5 μL/min applying a 600 min gradient. 1 μL injection (concentration 2 μg/μL). Base-peak chromatogram.

data to improve confidence in peptide identification, it is important that the applied method is robust and always provides excellent retention-time stability. Retention-time repeatability was recorded on a single 250 mm long monolithic column for 20 consecutive injections applying a 120 min gradient. Fig. 6 shows an overlay of five consecutive injections (base-peak chromatograms of a six-protein-mix digest sample). To demonstrate the retention-time repeatability, two model peptides were selected that eluted at the start and at the end of the gradient. The standard deviation of a peptide (KFWGK) eluting at an average retention time of 12.760 min was 0.070 min. For a peptide (GLVLIAFSQYLQQCPFDEHVK) eluting at 109.788 min the standard deviation was only 0.107 min. The RSD in peak capacity was only 2.1%.

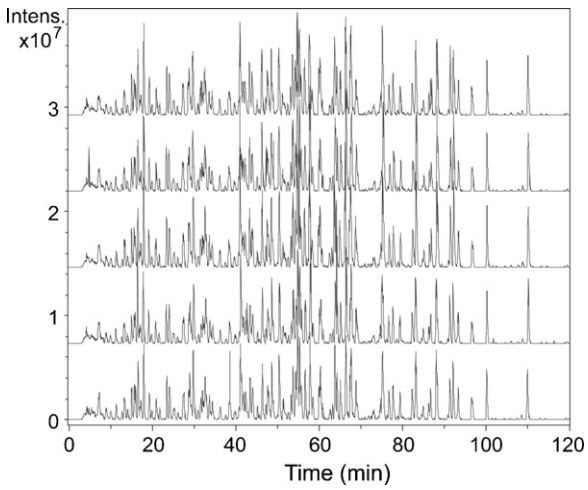

Fig. 6. Overlay of five base-peak chromatograms of the separation of a six-protein-mix digest on a 250 mm long monolithic column applying a 120 min gradient. Conditions as described in Fig. 2.

4. Conclusions

This study shows the effect of column length and gradient duration on the separation performance and sequence coverage of protein enzymatic digest with LC–MS/MS using poly(styrene-*co*-divinylbenzene) monolithic columns. Maximizing peak capacity involves optimizing LC conditions (including flow rate, gradient time, and column temperature) and column technology, *i.e.* column length and morphology.

For high-throughput LC–MS/MS screening of relatively uncomplicated proteomics samples that require peak capacities below 365 the 50 mm long monolith provides the best peak-capacity-per-unit-time (best performance within the shortest possible time frame). For more demanding separations the 250 mm monolith is recommended. The maximum peak capacity that can be achieved with 250 mm long monolithic columns is 485 within a total analysis time of 210 min. Peak capacity over 1000 can be reached using 1 m long monolithic column and applying gradient duration > 10 h. Especially the robustness of these monolithic materials, that are covalently bonded to the capillary wall, and their excellent retention-time stability (standard deviation below 0.1 min for 120 min gradients) makes this column technology attractive in comparison with packed capillary columns.

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