Monolithic and small particle column materials for application in proteomics

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Monolithic and small particle column materials for application in proteomics

Monolithische en kleine deeltjes kolommaterialen voor toepassing in proteomics

(met een samenvatting in het Nederlands)

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Chapter 1

General introduction

1 Introduction

Systems biology is an interdisciplinary study field that focuses on the interactions in biological systems. Genomics, metabolomics and proteomics are some examples of widely used studies to collect data to elucidate the different biological processes in organisms. System biology integrates these 'omics' to give an overview of the state of an organism. In *genomics* the sum of all individual genes are studied at the DNA, RNA or protein level allowing the prediction of a (higher) disease risk. A milestone in this field was reached in the 2000s when the Human Genome Project was finished. As a result lifestyle advice or medication preventing the expected disease can be administered after a genomic study of a patient. In *metabolomics* all metabolites, intermediates and end products in cellular processes, are analyzed. In contrast to genomics this approach gives up-to-date information on the physiological state of the organism. In *proteomics* the total proteome, *i.e.* the full complement of proteins including modifications in the quantitative and qualitative way, is studied. In contrast to the genome the proteome differs from cell to cell and from time to time and gives a much better understanding of the actual state of an organism. Proteomics is also suitable for the identification of biomarkers, proteins whose concentration indicates the presence of a certain disease [1-7]. In general, the main research topics in proteomics are [8]:

- improvement of the dynamic range, so low abundance proteins are analyzed among abundant components
- improvement of the sensitivity
- high throughput measurements
- analysis of posttranslational modifications
- protein interactions
- protein quantification

A typical proteomic workflow consists of sampling, prefractionation, fractionation, separation and identification of analytes. For better identification and quantification each step in this workflow should be optimized. So efficient methods for lysing the cell, for fractionation and purification of cellular components and for digestion of

the proteins in combination with a fast and sensitive method for separation, detection and identification of the obtained peptides are required [9].

A part of proteomic research focuses on the improvement of separation techniques to better resolve complex proteomes. This study of proteins can be divided into two classes: the top-down and bottom-up approach. In the first strategy the intact protein is studied. The main benefit of this approach is that the intact protein preserves the biochemical characteristics and modifications. In the bottom-up approach proteins are (usually tryptic) digested. The obtained, often highly complex, peptide mixtures are usually chromatographic or electrophoretic separated to simplify the identification and/or quantification with mass spectrometry (MS) [8].

2 Mass spectrometry

MS is the preferred identification method in proteomics. In recent years large progress is made like [9]:

- developing of hybrid instruments (a combination of coupled mass analyzers)
- increasing the sequencing speed so all eluting peaks can be targeted
- increasing the resolution and mass accuracy
- more efficient fragmentation techniques
- upgrading of the ion inlet and transfer optics

Popular soft ionization techniques for proteomics research are Matrix-Assisted Laser Desorption/Ionization (MALDI) and Electrospray Ionization (ESI), the latter technique most often combined with HPLC. As mass analyzers quadrupole time-of-flight MS and quadrupole ion-trap MS are frequently used. The obtained MS-MS data are converted to (for example) the MASCOT generic format and searched in online databases (like SwissProt) to identify peptide sequences and the original protein [8].

3 Top-down approach

The oldest (since 1975) and standard technique to study proteins in the top-down approach is two-dimensional electrophoresis (2-DE) [10]. The proteins are separated based on their isoelectric point (pI) by isoelectric focusing (IEF) in the first and on their molecular weight (MW) through varying the pore size of the gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension [10]. In 1982 the technique is further refined for the first dimension by using commercial available immobilized pH gradients (IPG) strips instead of carrier ampholytes [11]. This orthogonal method separates thousands of proteins in a single run and with high resolution. Small differences in pI or MW *e.g.* due to phosphorylation or glycosulation or metabolism, can be separated [10]. Typically, the proteins are made visible as spots by staining (for example Coomassie blue) and can be transferred to the MS for further analysis. Also enzyme cleavage with *e.g*. trypsin or pepsine on the spot is possible. The major drawbacks are the limited applicability to hydrophobic proteins, extreme MW or pI [12], the low sensitivity (in the μM range) so only high abundant proteins are made visible [13, 14], the presence of carrier ampholytes makes the direct connection with the MS complicated and the difficulty to automate [14]. The sample throughput of the system is rather low [8].

4 Bottom-up approach

'Shotgun' proteomics is the most common bottom-up approach. High resolution (multidimensional) separation techniques in combination with MS-MS is the preferred analysis technique [8].

In recent years much attention has been paid to the improvement, *e.g.* separation performance and throughput, of chromatographic columns focusing on one dimension (1-D), 2-D and even multidimensional HPLC systems. Due to the increased signal-to-noise ratio, reduced sample volume, reduced amount of stationary phase and the splitless combination with MS the internal diameter column is down-sized from narrow-bore (a few mm) to capillary (50 – 100 μm) or even smaller dimensions [15].

Columns with silica-based *reversed-phase* (RP) particles with a diameter (d_p) of 3 to 5 μm are the most widely used material for the separation of peptides. The separation is based on the hydrophobic interaction between the peptide and the packing material/eluent composition. An important trend is to decrease this particle diameter to 1.7-1.8 μm (to obtain *ultra small particle* columns) so the eddy diffusion (A-term) and resistance to mass transfer (C-term) in the Van Deemter curve is improved. Due to the extreme high backpressure (up to 1000 bar) special UPLC (Ultra Pressure/Performance Liquid Chromatography) equipment is needed.

4.1 Bottom-up approach – one-dimensional separation systems

Affinity columns can be used for selective purification, separation and enrichment of a specific peptide of group of peptides from a complex mixture of peptides (*e.g.* from a cell lysate). The column consists of a support to which a ligand is attached. For example an antigen to capture an antibody or the covalent binding of specific amino acid side chains (Cys, His, Trp) from the peptide with transition metal ions bound to the column (IMAC, Immobilized Metal Ion Affinity Chromatography) [16]. Hydrophilic Interaction Chromatography (*HILIC*) is a variant of normal-phase LC using a polar column and an organic mobile phase (like acetonitrile with a small amount of water). Polar compounds have a stronger interaction with the stationary phase and are eluted last, while the less polar compounds elute first [16]. *Monolithic* columns consist of a single, porous rod that can be polymeric- or silica-based. Due to a good permeability and mass transfer these columns have a high chromatographic performance and short analysis times than conventional particle-packed columns.

Reversed phase silica-based monolithic columns, commercialized by Merck (Darmstadt, Germany) under the name Chromolith, have a chromatographic efficiency corresponding to a 3-5 μm particulate column due to the mesopores where the analytes diffuse in and out. They have macro- or through-pores which account for a high porosity corresponding to a column packed with 10-15 μm particles [17]. Due to this high porosity peptides can be analyzed using long columns and high flow-rates.

The most popular monolithic polymer columns are poly(styrene-divinylbenzene), *PS/DVB*, and *methacrylate* columns. Due to the intrinsic hydrophobic properties of the PS/DVB column this stationary phase can directly be used for RP separations and is comparable with a C4 or C8 RP column. This column type can be divided into continuous bed columns (commercialized by LC Packings/Dionex, Amsterdam, The Netherlands) and homemade porous layer open-tubular columns, a wall coated open tubular column. The latter type allows longer lengths, a lower backpressure and a much higher chromatographic performance [18].

Due to the absence of commercially available methacrylate columns the desired stationary phase is tailor-made for each application. All kind of groups can be attached to the monolithic material during the preparation process. For example they are a support for on-line enzyme reactors and for affinity chromatography [18].

The newest type of columns combines the low backpressure (as in monolithic materials) and the high resolution of ultra small particle columns. These columns, for example Kinetex (Phenomenex, Torrance, CA, USA) and Ascentis (Sigma-Aldrich/Supelco, St. Louis, MO, USA), available in the capillary mode, are based on *silica core-shell* technology. They combine a solid core (d_p=1.7 μm for Ascentis) and a 0.5 μm (Ascentis) porous shell with a short diffusion path to minimize peak broadening [18].

4.2 Bottom-up approach - multidimensional separation systems

Due to the increased complexity of peptide mixture two (and even multi) dimensional separation systems have been developed. The selectivity of two separation mechanisms must differ substantially in order to maximize the chromatographic performance. Fractions can be transferred between the two (or more) separation systems in the off-line or on-line mode. Off-line methods are simple in design and operation. Via a fraction collector samples are collected from the first separation system and are directly re-injected or after manipulation (concentration/drying, buffer exchange, chemical reaction, etc.) into the second. These manipulations allow the combination of a wide range of separation mechanisms and make it possible to isolate specific classes of peptides. A

disadvantage is the potential sample loss. In the on-line method the transfer is fully automated through the use of switching valves. A disadvantage is that the compatibility requirements are very stringent. For example, the solvent in which the peptides elute from the first separation system must be a weak eluent in the second system. Solvents should be miscible and not cause salt precipitation.

In proteomics the combination of strong-cation exchange (SCX) column with RP-LC is the most used multidimensional liquid separation. The SCX column is performed under low pH conditions so the peptides are positively charged and bind to the SCX stationary phase. They are subsequently eluted in fractions by increasing salt concentration or by increasing pH [19]. In 2001 Yates *et al.* [20] described their multidimensional protein identification technology (MudPIT) system. SCX and RP materials are sequentially packed together in a single capillary with a pulled tip at the end. The tip maintains the stationary phase and serves as the ESI needle for the MS. After loading a sample into this capillary, a step gradient of buffer with increasing ionic strength is applied to elute the peptides from the SCX part to the RP part. A linear acetonitrile gradient is applied for the elution of peptides from latter part into the MS. This method removes all sampling-handling steps and the transfer between the two columns is fully automated. An alternative for the MudPIT approach is an on-line column switching method. Fractions eluting from the SCX column are trapped on top of a small RP trapping column and washed to remove the salts. Then the trapping column is placed in the flow path of the RP-LC pump and peptides are flushed onto the analytical RP-LC column. A disadvantage compared to the MudPIT method is the possibility of sample loss onto the enrichment column.

Other two-dimensional combinations are HILIC combined with RP-LC, size exclusion chromatography (SEC) combined with RP-LC and affinity chromatograph with RP-LC [19].

An example of a three-dimensional approach is the separation of a serum protein tryptic digest using off-gel isoelectric focusing (OGE-IEF) in the first dimension. In OGE-IEF analyte separation takes place in a two-phase system with an upper liquid phase that is divided in compartments and a lower phase that is an IPG gel strip. The sample is diluted and loaded into all wells and charged according to their

pI and the pH imposed by the gel. An electric field is applied between two electrodes located on the beginning and end of the gel. There is no open fluidic connection between the wells so the analytes are forced to migrate through the gel where the actual separation takes place. Once the analytes reach the well in which $pH_{well}=pH_{strip}=pI_{analyte}$ the compounds will lose their charge and can be recovered from that particular compartment for further processing [21, 22]. For example in the first dimension 20 fractions are collected by OGE-IEF. Each fraction can be further fractioned into for example 7 fractions by SCX that are each analyzed by RP-LC-MS-MS (making a total of 140 fractions) [23].

5 Describing the chromatographic performance

In isocratic HPLC the chromatographic performance is described by the resolution (R_s) and the plate number (N) or plate height (H). See Eqn (1.1) - (1.3) where ($t_{r,B}–t_{r,A}$) is the retention time difference between two adjacent analytes and W_b is the mean peak width of these two analytes [24]. For gradient elution, where the composition of the eluent changes in time, the quality of the separation is expressed by other chromatographic parameters, like peak capacity (PC) mentioned in Eqn (1.4), sample peak capacity (PC**) mentioned in Eqn (1.5) and the throughput or productivity (P) mentioned in Eqn (1.6). The PC describes the number of peaks fitting in a chromatogram from start to the end (gradient time t_g). The PC** describes the number of peaks between the first $t_{r,1}$ and last $t_{r,n}$ eluting peptide. P illustrates the number of compounds per minute leaving the column [25-29].

$$R_s = (t_{r,B}–t_{r,A})/W_b \qquad \text{Eqn (1.1)}$$

$$N = L/H \qquad \text{Eqn (1.2)}$$

$$H = L/N \qquad \text{Eqn (1.3)}$$

$$PC = 1+(t_g/W_b) \qquad \text{Eqn (1.4)}$$

$$PC^{**} = (t_{r,n} - t_{r,1})/W_b \quad \text{Eqn (1.5)}$$

$$P = PC/t_g = PC^{**}/(t_{r,n} - t_{r,1}) \quad \text{Eqn (1.6)}$$

6 Scope and outline of the thesis

In this thesis, by using the bottom-up 'shotgun' approach, we focus on the influence of the separation on the identification of protein digests. Different column materials, like (ultra small) particle columns and polymer/silica based monolithic columns, are studied and compared. Focus is laid on the chromatographic performance, expressed as (sample) peak capacity and productivity, and MS protein identification scores. Chromatographic variables like column length, gradient time/steepness and flow-rate are optimized in time and in performance using the above mentioned parameters. The chromatographic performance in combination with the MS identification scores lead to overall conclusions on the quality of the separation and identification of proteins.

Chapter 2 gives an overview, covering the scientific literature from 2004 to the beginning of 2011, of capillary monolithic silica and organic (polystyrene and methacrylate) columns for LC in proteomics. Attention is paid to recent developments in column technology, materials, applicability and future trends.

Chapter 3 clearly shows the main benefit of silica monolithic columns: a low backpressure allowing a high flow-rate and still a sufficient separation and identification power. A method for the fast separation of a simple digest is described using a silica RP-18 monolithic column by optimizing the eluent composition and increasing the flow-rate. *Intraday* repeatabilities and *interday* reproducibility for this type of column is studied.

Chapter 4 describes the adaptation of a simple univariate optimization method to gain a high separation power for flow-rate and eluent composition using a capillary silica monolithic column. Some critical comments on this optimization method are stated and the influence of the PC** on the protein identification score is discussed.

Chapter 5 illustrates a simple, fast and cost-effective method to modify a conventional commercial UPLC system to a capillary one for the analysis of a

digest. The performance of this system is compared with a commercial capillary UPLC system.

Chapter 6 compares the performance of different lengths silica monolithic columns and an ultra small particle column. A scaling factor is introduced to correctly compare these columns with various lengths and internal diameters.

In *Chapter 7* the performance of a short polystyrene and silica monolithic column of the same length are compared.

Chapter 8 discusses a substitute of 2-DE, the off-gel electrophoresis – isoelectric focusing system (OGE-IEF) including benefits, shortcomings, applications and some preliminary experiments.

The thesis ends with overall conclusions and recommendations for future research (*Chapter 9*).

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Chapter 2

Silica-based and organic monolithic capillary columns for LC: recent trends in proteomics

Rozenbrand , J., Van Bennekom, W. P., *J. Sep. Sci.* 2011, *34*, 1934-1944

Abstract

The use of monolithic Liquid Chromatography (LC) columns for proteomics, covering the scientific literature from 2004 to the beginning of 2011, is reviewed. Attention is paid to recent developments in column technology and materials, focusing on silica-based and organic (polystyrene and methacrylate) monolithic capillary columns for proteomics. The applicability of these columns is illustrated by examples of the analysis of (complex) protein digests and proteins conveniently summarized in tables. Furthermore, characteristics of column materials are compared and future trends and prospects are presented.

1 Introduction

For the identification of proteins in proteomic research often SDS-PAGE is used as a first step to separate the proteins of a sample. A spot, possibly containing several proteins, is selected and processed for identification. The protein(s) can directly be identified using high resolution MS (*e.g.* MALDI-TOF) or indirectly, *i.e.* via the peptides formed after enzymatic digestion. However, most frequently, the proteins are digested and again through the peptides, but now separated using LC (Liquid Chromatography) and identified with ESI-MS (Electrospray Ionization MS), protein identification is accomplished. For complex protein samples two-dimensional LC, *e.g.* strong-cation exchanger with a reversed-phase column in series, can be used [1, 2, 3].

Column types in use in proteomics are particulate columns (either with the standard 3-5 µm or nowadays with the more advanced 1.7/1.8-µm particles) and monolithic columns [4], introduced in the late 1980s. In these columns a capillary is filled with a single piece of porous material usually prepared from silica or organic polymers like polystyrene or methacrylate. Main benefits of these monolithic materials are the absence of retaining frits, high permeability and low resistance to mass transfer leading to a small C-term in the Van Deemter equation and as a consequence to an almost horizontal slope of the Van Deemter curve at higher flow-rates.

Protein digests are almost exclusively separated with gradient elution. The peak capacity PC expresses the performance of the gradient system and is defined by the gradient time (t_g) and (the average baseline) peak width (W_b) as PC = $1+(t_g/W_b) \approx t_g/W_b$ when $t_g >> W_b$, which normally is the case in gradient elution of peptides [23]. When the abundance of the different peptides is neither too high nor too low, the PC is very well approximated by the ratio of t_g and W_b (Eqn 2.1) [5-9]

$$PC = t_g/W_b \qquad \text{Eqn (2.1)}$$

The PC is also dependent on column length, temperature and flow-rate. It has been demonstrated that the PC increases linearly with the square root of the column length [10]. Working at higher temperatures decreases solvent viscosity and increases diffusion coefficients leading to higher plate numbers and a higher PC [11, 12]. Various strategies for optimization of the PC have been published including an elegant, simple and efficient univariate method proposed by Wang *et al.* [11].

A complication with the PC as performance parameter is the dependence on the gradient time. A long gradient time, according to Eqn (2.1) always lead to a high PC. For example Miyamoto *et al.* [13] compared a 28-cm (t_g=30 min) and 300-cm (t_g=300 min) monolithic silica capillary column. The PC for the 300-cm column was the highest but the number of identified peptides was the same. Therefore the performance of the system is better described by the sample peak capacity PC**, defined as the ratio of the retention time difference (Δt_r) of the first and last eluting peptide (Eqn (2.2)) or by the production rate P defined as the quotient of PC and t_g or of PC** and Δt_r. P is nothing more than the reciprocal baseline peak width W_b (Eqn (2.3)).

$$PC^{**} = \Delta t_r/W_b \qquad \text{Eqn (2.2)}$$

$$P = PC/t_g = PC^{**}/\Delta t_r = 1/W_b \qquad \text{Eqn (2.3)}$$

Usually the gradient systems are characterized with PC, PC** and P, although some authors (like Rieux *et al*. [14] and Rozenbrand *et al*. [15]) prefer the term resolution (R_s). Besides these chromatographic parameters, however, in proteomics also the number of identified peptides and proteins (identification score) or the sequence coverage of a protein (obtained by MS-MS and computer algorithms like Mascot) are used as an indirect optimization parameter of the chromatographic system. It is assumed that higher identification scores and sequence coverages are found when the peptides are better LC-resolved.

This review is dealing with recent trends in the separation of peptides and proteins in capillary monolithic LC columns. Advantages of capillary columns are the better signal-to-noise ratio and the less sample amount needed [4, 16]. Developments and applications using monolithic *e.g.* silica, methacrylate and poly(styrene-divinylbenzene) (PS/DVB) materials in proteomic research, will be discussed. The use of monolithic supports for enzyme immobilization will be discussed only briefly for the methacrylate columns, because Svec [17] and Krenkova and Svec [18] published excellent reviews on this subject. Recently, Tao *et al*. [3] reviewed micro/nano-scale LC for proteome research. Their main focus was multidimensional HPLC and briefly silica and PS/DVB monolithic columns have been discussed. Nischang *et al*. [19] published a review on the preparation of polymer monoliths. Their focus was on the morphological aspects; only slightly applications for LC and proteomics have been reported.

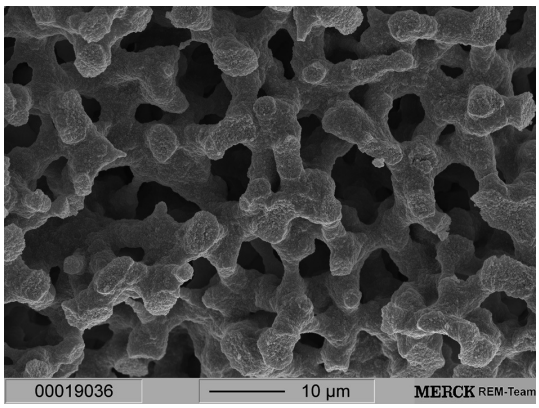
2 Monoliths

In general monolithic columns are prepared by polymerization of monomers (in the presence of an initiator) in a so-called porogenic solvent in which the monomers and not the polymers are soluble. Monolithic functionality can be tailored through the use of various kinds of monomer precursors, chemistry and surface reactions [2]. The recent developments and applications for proteomics using silica-based and organic-based monolithic columns are discussed. Tables 2.1, 2.2 and 2.3 show various applications of monolithic columns in proteomics.

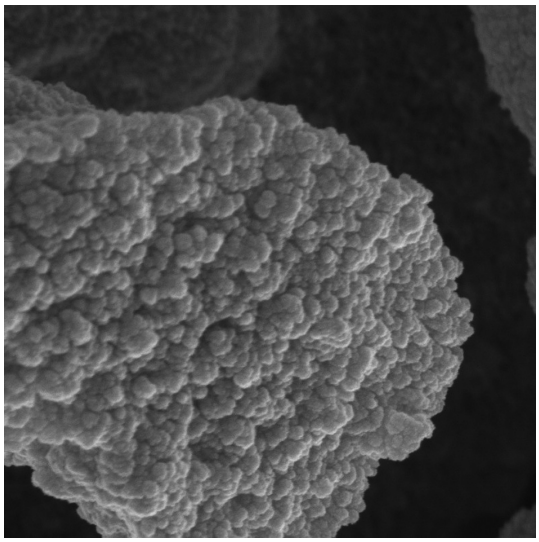
2.1 Silica-based monoliths

The group of Tanaka [20] described the first LC application using silica-based monolithic rods. The rods are prepared, by hydrolytic polymerization of silanes, like tetra(m)ethoxysilane, catalyzed by aqueous acetic acid in the presence of polyethylene glycol. The sol formed is converted to monolithic silica having network structures attached to the tube wall in a fused-silica capillary. Then ammonia is introduced allowing the formation of mesopores (where the analytes diffuse in and out), so creating a large specific surface area and a chromatographic efficiency corresponding to a 3-5 µm particulate column. Chemical modification of the polar material with ODS (octadecyl silane) to create a reversed-phase (RP) column is often performed. The columns have large macro- or through-pores which account for a high porosity corresponding to a column packed with 10-15 µm particles. Fig. 2.1 shows scanning electron microscope (SEM) pictures of the mesopores and through-pores [21].

(a)



(b)



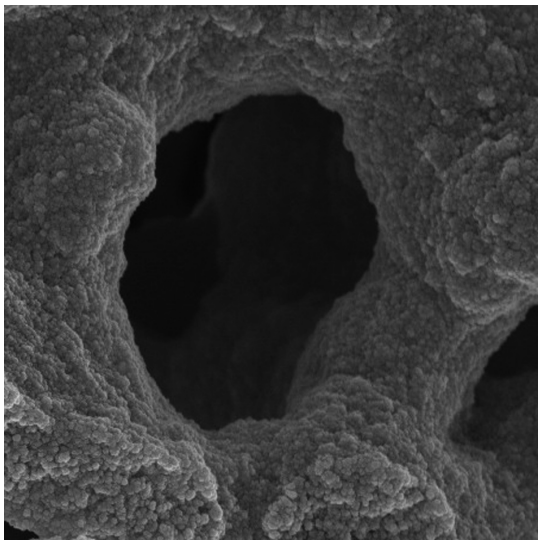
(c) 

Fig. 2.1. SEM picture of (a) the typical porous structure of a monolithic silica column, (b) mesoporous structure and (c) the through-pore structure [21].

Due to the high porosity (or low back-pressure) of this type of stationary phase protein digests can be analyzed using long columns and high flow-rates. Like all silica columns working at high pH values is not possible.

In Table 2.1 some selected LC-applications of silica-based monoliths for digests have been summarized. Examples of the fast analysis in a short time of tryptic digests are performed by Rozenbrand *et al*. [15] and Rieux *et al.* [14] using commercially available Chromolith (Merck, Germany) RP columns. The analysis time is reduced by increasing flow-rate and the gradient steepness to obtain an acceptable separation within a short time. Rozenbrand *et al*. [15], used a 15-cm column, a flow-rate of 2.8 µL/min and a gradient slope of 16%/min to analyze and identify a Myoglobin digest within 4 min. The back-pressure remained under 300 bar. Under these conditions the identification score was sufficient (Mascot score is 115) and a mean PC** of about 9 was obtained. Rieux *et al*. [14] used a longer column (56 cm), a flow-rate of 1.0 µL/min (maximum 1.95 µL/min) and a gradient slope of 9%/min to analyze a more complex Cytochrome C digest within 3 min. The resolution (R_s) was used for optimization. Rieux *et al*. [14] were able to determine a tryptic serum digest from a cervical cancer patient in 30 min using a flow-rate of 1.5 µL/min and gradient slope of 3%/min. The base peak chromatogram is shown in Fig. 2.2. The highest flow-rate for a Chromolith column

Table 2.1. Selected LC applications using silica-based monolithic columns

Sample	*Precolumn*	*Analytical column*	*Packing*	*Analysis time (min)*	*Flow-rate (µL/min)*	*MS detection mode*	*Remarks*	*Reference*
Myoglobin digest	0.5 cm x 300 µm i.d. C18 trap (d_p=5µm)	C18 15 cm x 100 µm i.d.	Chromolith	4	2.8	ESI-Iontrap MS-MS		[15]
Human serum digest	0.5 cm x 300 µm i.d. C18 trap (d_p=5µm)	C18 56 cm x 50 µm i.d.	Chromolith	30	1.5	ESI-Iontrap MS-MS		[14]
BSA digest and Myoglobin digest	no	C18 15 cm x 100 µm i.d ; 64 cm x 100 µm i.d.	Chromolith	26-110	1.0	ESI-Iontrap MS-MS		[23]
BSA digest and Casein digest	no	C18 15 cm x 100 µm i.d ; 75 cm x 100 µm i.d.	Chromolith	3-225	0.5	ESI-Iontrap MS-MS		[24]
BSA digest	0.5 cm x 300 µm i.d. C18 trap (d_p=5µm)	C18 15 cm x 100 µm i.d; 75 cm x 200 µm i.d.	Chromolith	3-75	0.5; 2.0	ESI-Iontrap MS-MS	UV detection	[25]
BSA digest	no	C18 28.4 cm x 100 µm i.d; 300 cm x 100 µm i.d.	homemade	30; 300	1.2; 0.7	ESI-Iontrap MS-MS		[13]
Escherichia coli digest	no	C18 350 cm x 100 µm i.d.	homemade	2470	0.5	ESI-Iontrap MS-MS		[26]
Shewanella oneidensis digest	4 cm x 50 µm i.d C18 trap (on-line; d_p=5µm)	C18 25 cm x 10 µm i.d.	homemade	240	0.01	ESI-Iontrap MS-MS	integrated monolithic ESI emitter	[28]
Shewanella oneidensis digest	4 cm x 50 µm i.d. monolithic trap (on-line)	C18 70 cm x 20 µm i.d.	homemade	200-750	0.04	ESI-Iontrap MS-MS		[29]
Shewanella oneidensis digest	4 cm x 50 µm i.d. C18 trap (off-line; d_p=5µm)	C18 25 cm x 10 µm i.d.	homemade	180	0.01	ESI-Iontrap MS-MS	integrated monolithic ESI emitter	[27]

(15 cmx100 µm i.d.) is published by Guryca *et al*. [22]: 5.0 µL/min with a back-pressure of (not mentioned) probably 300 bar.

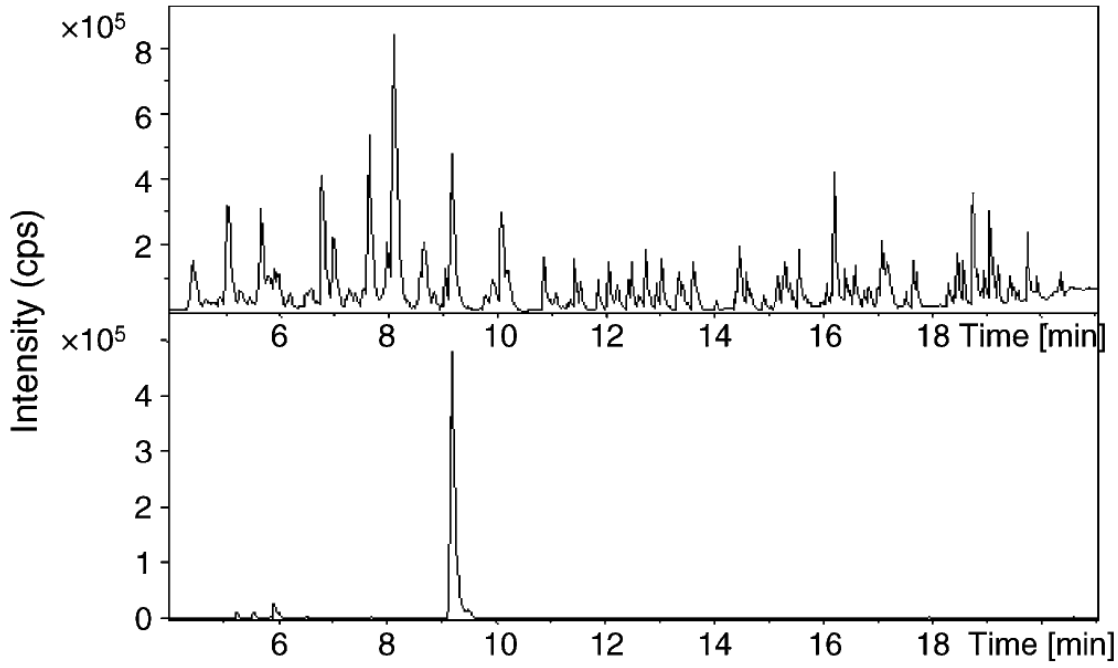


Fig. 2.2. Base peak chromatogram (top) and extracted ion chromatogram (peptide fragment at m/z=694.4; bottom) of a tryptic serum digest from a cervical cancer patient using a 56-cmx50 µm i.d. silica monolithic column and a flow-rate of 1.5 µL/min [14].

The influence of column length (Chromolith) on peak capacity and MS identification scores has been studied [23, 24]. The back-pressure of these columns is rather low, *e.g.* for the 75-cm column (200 µm i.d.) less than 200 bar using a flow-rate of 2.0 µL/min [25]. Rozenbrand *et al*. [23] used 15- and 64-cm columns. The PC** for a BSA digest, using the same gradient slope for both columns, increased from 66 to 100 and the BSA sequence coverage from 34% to 48%, respectively. Van de Meent *et al.* [24] compared columns of 15 and 75 cm (100 µm i.d.). When comparing gradient times of 3, 9 and 45 min (15-cm column) with 15, 45 and 225 min (75-cm column), the same gradient slope, an increase of the PC from 11, 27, 87 to 43, 90, 238, respectively is observed which is almost in accordance with Wang *et al*. [10], who claimed that the ratio of the PC should be close to the square root of the column length ratio. The sequence coverage for BSA changed from 11%, 21%, 27% to 26%, 32%, 26%, respectively. Comparison

of the same columns but with different internal diameters (100 vs 200 µm) shows the same trend: for simple digests long gradient times increase the PC but the MS identification score flattens out [25]. For complicated digests increasing the gradient time is more beneficial for protein and peptide identification. For example, Iwasaki *et al.* [26] identified 22 196 peptides (2602 proteins) of *E. coli* in 41 h using a 350 cmx100 µm i.d. home made analytical column (Fig. 2.3).

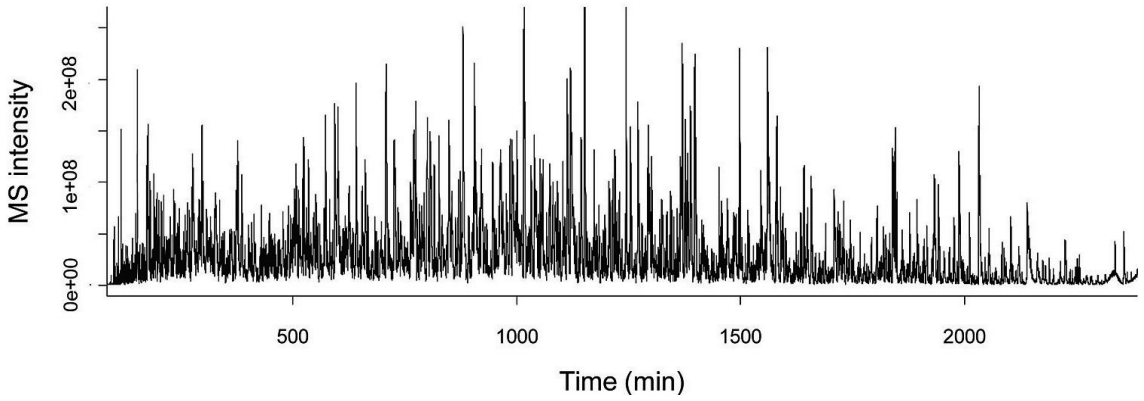


Fig. 2.3. Base peak chromatogram of a digested E. coli cell lysate using a 350-cmx100 µm i.d. silica monolithic column and a flow-rate of 0.5 µL/min [26].

Luo *et al*. [27-29] use longer columns (25–70 cm) and smaller internal diameters (10–20 µm) in combination with low flow-rates (10–40 nL/min) and shallow gradients or long gradient times (*e.g.* 3–4 h). Next to the increased separation efficiency, due to this low flow-rate smaller droplets are formed, which evaporate more rapidly and efficiently produce more ions and increase MS sensitivity. At the same time undesired matrix or ionization suppression effects are reduced [27]. The group used loading columns, sometimes directly coupled with the analytical column to minimize post-column dead volume, and (integrated) ESI emitters leading to the identification of 5164 peptides (1332 proteins) in 3 h [27]; 1184 peptides (428 proteins) in 3.5 h [29]; 5510 peptides (1443 proteins) in 4 h [28] and 2367 peptides (855 proteins) in 10 h [29] of an *S. oneidensis* digest. A PC of 420 can be reached (Fig. 2.4) [29]. Xie *et al*. [30] identified 5501 peptides (1323 proteins) in about 7 h of a *S. cerevisiae* digest using a 60-cm column (75 µm i.d.). The integrated ESI emitter leads to a 20% increased separation efficiency.

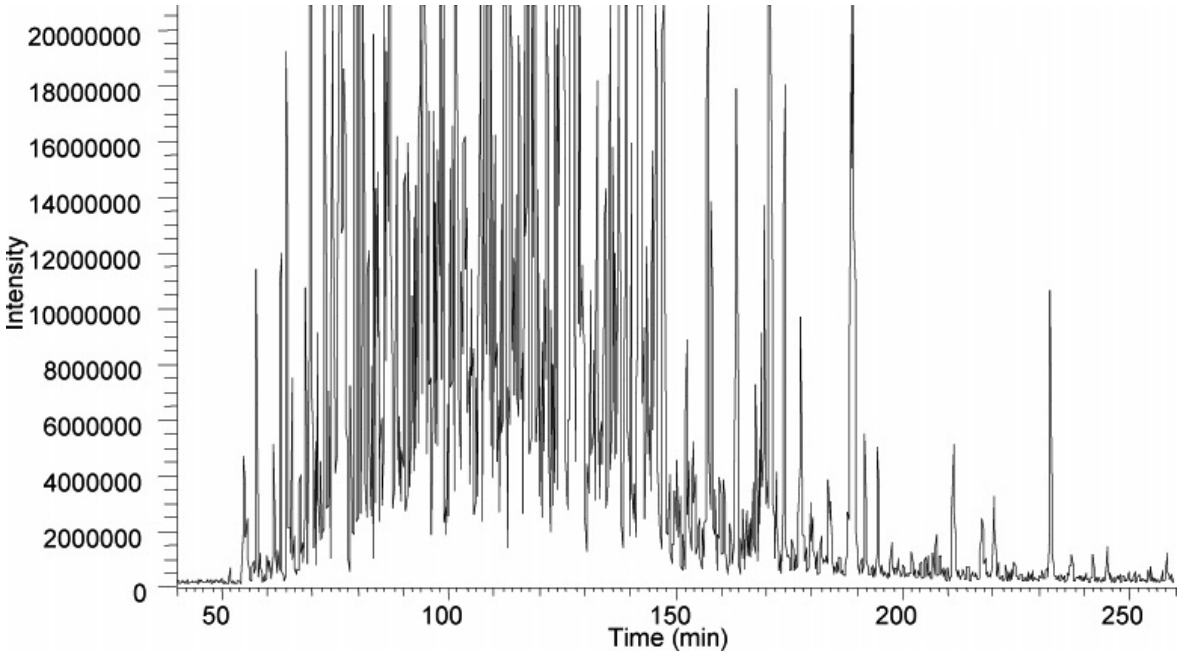


Fig. 2.4. Base peak chromatogram of a S. oneidensis digest using a 70 cmx20 µm i.d. silica monolithic column and a flow-rate of 0.04 µL/min [29].

Monolithic trapping columns [29] and particle-fixed monolithic trapping columns for proteins [1] or peptides [31] have been studied. Short trapping columns increase sensitivity, the amount of sample that can be loaded and minimize loading time [1]. Guan *et al.* [1] prepared such a column (0.5 cmx320 µm i.d.) by entrapping 5-µm C8 particles in the monolithic phase. The monolithic material serves as a 'glue' to create a bridge between particles and capillary wall thereby eliminating the need for retaining frits, which are often a source of band broadening (Fig. 2.5). Gu *et al.* [31] prepared in the same way peptide trapping columns (0.5 cmx320 µm i.d. and 530 µm i.d.) by using 5-µm C18 particles.

It can be concluded that for high-throughput analysis (with a maximum of several hundred analyses per day) and the identification of (complex) protein digests silica monoliths are very suitable. These LC columns are usually coupled with ESI and ion-trap MS-MS. Due to large through-pores the back-pressure is rather low so longer columns and higher flow-rates can be used. Columns (Chromolith, Merck) with fixed lengths and diameters are commercially available. Probably this is the reason that hardly homemade columns have been reported. However, the few ones published show benefits of smaller internal diameters, longer lengths and the possibility of integrated ESI emitters to reduce dead volume. Coupling of medium

length Chromolith columns in series, despite the increment of the void volume due to extra connection tubing or frits, is another possibility of creating a longer column. Monolithic and particulate precolumns and monolithic materials mixed with particles are available for trapping of analytes.

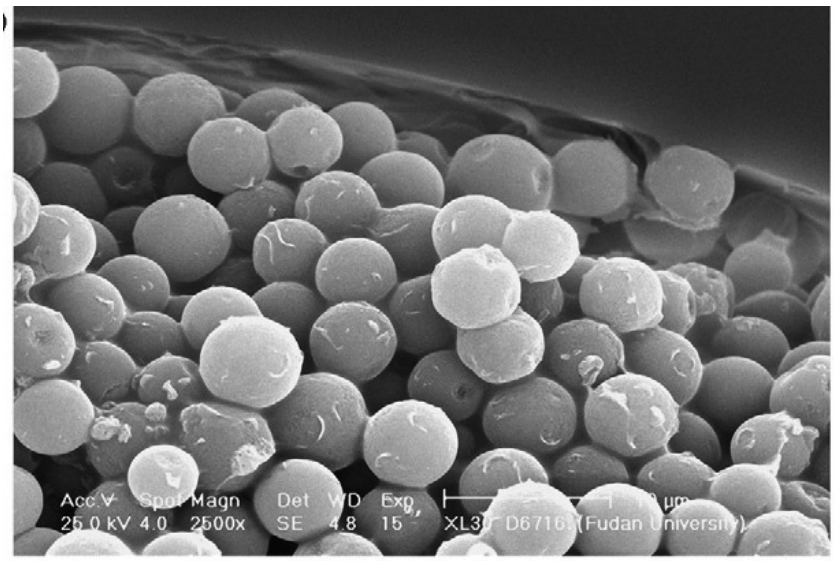


Fig. 2.5. SEM picture of a silica monolithic trapping column packed with 5-µm C8 particles [1].

Due to the need for a broader range of applications also organic monolithic polymer columns have been developed. In the next paragraphs we will focus on the most popular ones, PS/DVB and methacrylate.

2.2 Organic polymer monoliths

Organic monoliths are prepared by filling a capillary column with monomers, cross-linker and initiator in a porogenic solvent. Chain polymerization starts using heat or UV light. Porosity, surface area and pore size are controlled by adjusting the initial monomer solution and polymerization conditions. For columns with special functionalities different precursors and chemistries can be used [2]. For a detailed review on the preparation of monolithic polymer columns we refer to Nischang *et al*. [19].

2.2.1 Methacrylate-based monoliths

For the preparation of methacrylate-based monoliths glycidyl methacrylate (GMA) is frequently used as a monomer. The reactive epoxide group allows modification to produce various functionalities. For example, AX (anion-exchange) columns were prepared using poly(GMA-co-EDMA) by reaction with diethylamine; SCX (strong cation-exchange) columns by grafting AMPS onto hydrolyzed poly(GMA-co-EDMA) and a RP column by alkylation of poly(GMA-co-EDMA) [2]. Fig. 2.6 shows a SEM picture of a methacrylate column. Urban and Jandera [32] published an excellent review on the preparation and LC applications of methacrylate columns.

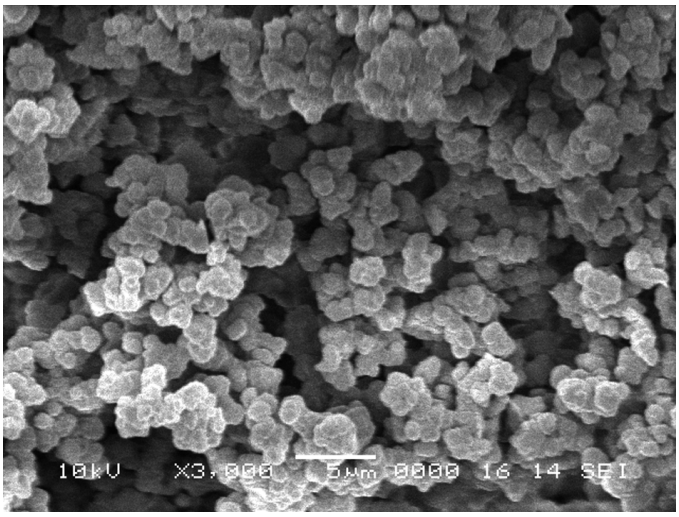


Fig. 2.6. SEM picture of a methacrylate column [32].

In Table 2.2 some selected methacrylate LC-applications are presented. Due to the absence of commercially available columns, the desired stationary phase is tailor-made for each application. For example, (1) Moravcova *et al.* [33] prepared very short (3-cm) and very wide (320 µm i.d.) lauryl methacrylate columns for desalination, preconcentration and separation of peptides for a BSA digest and an in-gel digest of *Hordeum vulgare*. Separated fractions were collected on MALDI target spots and analyzed using TOF. The sequence coverage for BSA increased from 17% (no column) to 63%; for *Hordeum vulgare* the 14 kDa spot revealed 16 proteins (with column) instead of 3 (without separation). (2) Daley *et al*. [34] made 10-cm methacrylate columns containing fluorous (F) compounds. Due to highly specific F-F interactions F-tagged analytes can selectively be separated.

Table 2.2. Selected LC applications using organic-based/methacrylate monolithic columns

Sample	*Precolumn*	*Analytical column*	*Packing*	*Analysis time (min)*	*Flow-rate (µL/min)*	*MS detection mode*	*Remarks*	*Reference*
BSA digest; in-gel digest *Hordeum vulgare*	included in analytical column	RP (C12) 3 cm x 320 µm i.d.	homemade	40	1	MALDI-TOF/TOF MS		[33]
BSA digest, yeast protein digest	included in analytical column	RP (C12) 20 cm x 100 µm i.d.	homemade	40-120	0.25-0.3	ESI-Iontrap MS-MS	integrated ESI emitter	[37]
Myoglobin, Lysozyme, Ribonuclease A	no	250 µm i.d.	homemade	40	2.5	no	UV detection	[35]
BSA; *Saccharomyces cerevisiae*	home made 10 cm x 100 µm i.d. methacrylate trypsin reactor	RP (C18) 12 cm x 75 µm i.d. (particulate, d_p=5 µm)	homemade	60-100	0.12	ESI-Iontrap MS-MS		[39]
Cytochrome C, Myoglobin, Lactalbumin, BSA	home made 5 cm x 100 µm i.d.methacrylate trypsin or protease V8 or trypsin/protease V8 reactor	no	homemade		0.2-1.0	MALDI-MS; ESI-MS		[38]
BSA	home made 10 cm x 180 µm i.d. methacrylate C4-trypsin reactor		homemade		0.5	MALDI-TOF-MS		[40]

(3) Hemstrom *et al*. [35] varied porogen and monomer concentrations to optimize the separation of the intact proteins Myoglobin, Ribonuclease A and Lysozyme for Hydrophobic Interaction Chromatography (HIC). (4) An unusual but highly interesting application of this methacrylate material is to prevent clogging of the emitter tip for ESI and to decrease void volumes. Therefore an integrated monolithic frit (1 cm, C12) was placed into a 12-cm particulate (d_p=1.5 μm) capillary column. From this frit an integrated ESI tip was pulled [36]. The group also prepared an integrated emitter in a C12-methacrylate trapping/separation column [37].

Due to the fast mass-transfer properties and low back-pressure methacrylate materials are often used as a support for on-line enzyme reactors [38]. Because of the lack of autodigestion the concentration of enzyme inside the reactor can be rather high, leading to short digestion times [39]. Feng *et al.* [39] prepared a pH-robust on-line capillary methacrylate trypsin enzyme reactor coupled to a C18 particle separation column. An incubation time of 15 min showed for BSA a sequence coverage of about 77%; for *Saccharomyces cerevisia*, a yeast protein extract, 1578 peptides (541 proteins) were identified within an incubation time of 1 min. Lin and Skinner [38] prepared three enzyme reactors containing trypsin, protease and trypsin/protease (5 cmx100 μm i.d.) using various flow-rates (0.2-1 μL/min) and reaction times (0.24-1.4 min), linked with ESI-MS and MALDI-MS. They also showed an example of a top-down analysis: leading separated proteins of the first dimension to the enzyme reactor in the second dimension. Zhang *et al.* [40] combined an enzyme reactor and trapping column by introducing C4 groups on the monolithic surface. These columns showed improved digestion efficiency. With the addition of these groups 30% more peptide sequences of a BSA digest were analyzed compared to the same columns without C4.

Intact proteins can also be attached to methacrylate columns for affinity chromatography. Bedair and El Rassi [41] immobilized mannan for the separation of mannose-binding proteins. After isolation of these proteins from, for example, rabbit serum they are further separated by SDS-PAGE. An isolated band is digested and analyzed using MALDI-MS. Okanda and El Rassi [42] used immobilized lectines in methacrylate material for the isolation of glycoproteins.

Methacrylate columns are, as far as we know, not commercially available. The columns reported are therefore exclusively homemade for a wide variation of purposes. All kind of groups, like alkyl groups, proteins, enzymes and, for example, fluorous compounds can be attached to the monolithic material during the preparation process. For proteomics, intact proteins and peptides from digests, can be analyzed with this material in a wide pH range. Like silica monoliths, the material can also be mixed with particles to increase trapping properties. Integrated ESI emitters have been developed.

2.2.2 Poly(styrene-divinylbenzene)-based monoliths

This monolithic material is prepared from a mixture of styrene monomer, p-divinylbenzene cross-linker, initiator 2,2'-azobisisobutyronitrile (AIBN) in various porogenic solvents. Polymerization starts on heating [2]. Fig. 2.7 shows a SEM picture of the PS/DVB column [43]. Due to the intrinsic hydrophobic properties of the poly(styrene-divinbylbenzene) this stationary phase can directly be used for reversed-phase separations [44] and is comparable with a C4 or C8 RP column [45]. In Table 2.3 some LC-applications using PS/DVB monoliths have been included. Columns (5 and 25 cm) with internal diameters of 100 or 200 µm are available from LC Packings/Dionex (Amsterdam, The Netherlands) and often used for protein and peptide analysis. We report on optimizations and applications using common monolithic column material (continuous-bed), either homemade or from LC Packings. In the last part applications of a new type column, *i.e.* porous-layer open-tubular, will be discussed.

Continuous-bed columns

Walcher *et al.* [46] studied the optimum conditions (temperature and mobile phase additives) for the analysis of peptides and proteins for homemade 6-cm (200 µm i.d.) columns. They found the smallest peak widths (and therefore the highest PC) at a temperature of 70^{o}C. The ion-pairing reagent TFA (trifluoracetic acid) turns out to be the best compromise between chromatographic and mass spectrometric performance.

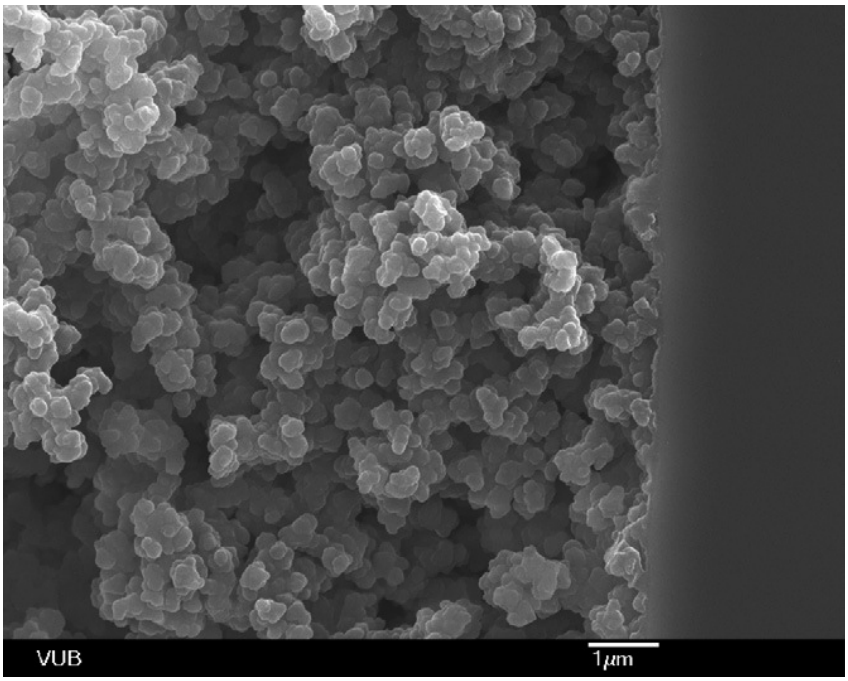


Fig. 2.7. SEM picture of the cross section of a continuous bed PS/DVB monolithic capillary column [43].

Swart from LC Packings studied the separation of five intact model proteins using columns of 5, 10 and 25 cm (200 µm i.d.). Their optimum temperature was 60°C: at that condition the smallest W_b's were obtained without observing any degradation. The maximum PC obtained was respectively 330, 440 and 760 for the studied columns [47]. Connecting 4 columns of 25 cm in series, for the analysis of an *E. coli* digest, yielded a PC of 1038. 2053 Peptides were identified (283 proteins) using a gradient time of 600 min and a flow-rate of 0.5 µL/min (Fig. 2.8) [43]. About the same PC (1000) was obtained for the analysis of ten peptides using the same column length and a gradient time of 1-5 h [48]. Coupling of a 5-cm column with field-flow electrophoresis [49] shows in the first dimension separation of intact histones (proteins found in cell nuclei which package and order the DNA into structural units) according to their iso-electric point (pI). Selected fractions were loaded on a precolumn and separated with the analytical column. This 5-cm column in combination with a monolithic precolumn (5 mmx200 µm i.d.) was also off-line coupled with MALDI-TOF/TOF-MS for the fast and efficient protein identification of digests [45]. Without a precolumn an in-gel tryptic digest of an SDS-PAGE separation of a yeast lysate was injected in the 5-cm column and

Table 2.3. Selected LC applications using organic-based/polystyrene-divinylbenzene monolithic columns

Sample	*Precolumn*	*Analytical column*	*Packing*	*Analysis time (min)*	*Flow-rate (µL/min)*	*MS detection mode*	*Remarks*	*Reference*
BSA digest and Casein digest; in-gel digest *M. acetivorans*	4 cm x 50 µm i.d.PS/DVB (off-line)	420 cm x 10 µm i.d. PLOT	homemade	60-210	0.02	ESI-Iontrap MS-MS		[56]
in-gel digest cervical cancer cell line	4 cm x 50 µm i.d. PS/DVB (on-line)	320 cm x 10 µm i.d. PLOT	homemade	100	0.02	ESI-Iontrap MS-MS		[55]
in-gel digest cervical cancer cell line	100 µm i.d. SCX (d_p=5 µm) - 4 cm x 50 µm i.d. PS/DVB (on-line)	320 cm x 10 µm i.d. PLOT	homemade	100	0.02	ESI-Iontrap MS-MS		[55]
in-gel digest cervical cancer cell line	2 cm x 75 µm i.d. C18 (d_p=5 µm) - 2 cm x 75 µm i.d. SCX (Polysulfoethyl) (d_p=5 µm) - 4 cm x 50 µm PS/DVB (on-line)	320 cm x 10 µm i.d. PLOT	homemade	100	0.02	ESI-Iontrap MS-MS		[54]
glycans	no	250 cm x 10 µm i.d. amine bonded PLOT	homemade	60	0.02	ESI-Iontrap MS-MS	hydropilic interaction	[57]
Ribonuclease, Lysozyme, Trypsinogen, Myoglobin, Carbonic Anhydrase, *E. coli*	no	5; 10; 25 cm x 200 µm i.d.	LC Packings	25-210	2.0		UV	[47]
Escherichia coli digest	no	100 cm x 200 µm i.d.	LC Packings	600	0.5	ESI-Iontrap MS-MS		[43]
intact histones	0.5 cm x 200 µm i.d. PS/DVB	5 cm x 200 µm i.d.	LC Packings	25	2.0	FT-ICR MS	1st dimension: FFE	[49]
digest	0.5 cm x 200 µm i.d. PS/DVB	5 cm x 200 µm i.d.	LC Packings		3.0	MALDI-TOF/TOF MS		[45]
yeast lysate in-gel digest	no	5 cm x 100 µm i.d.	LC Packings	10	1.0	MALDI-TOF/TOF MS		[50]
in-gel digest *S. fredii*	no	5 cm x 100 µm i.d.	LC Packings	20	1.1	ESI-MS-MS (on-line)		[51]
in-gel digest *S. fredii*	no	5 cm x 200 µm i.d.	LC Packings	20	3.0	MALDI-MS-MS (off-line)		[51]
Myoglobin (digest) in human serum	25 cm x 4000 µm i.d.SAX (off-line)	6 cm x 200 µm i.d.	homemade	15	2.0	ESI-Iontrap MS		[52]
β-Casein, Epidermal Growth Factor Receptor digest	no	10 cm x 20; 50 µm i.d	homemade	40	0.02-0.1	ESI-MS		[63]
Antenna proteins	no	6 cm x 100 µm i.d. and 200 µm i.d.	homemade	16	0.5-2.5	ESI-MS		[53]

off-line continuous deposited at a MALDI plate and analyzed (TOF-TOF). In all, 386 peptides corresponding with 128 proteins were identified [50].

Rodrigues *et al*. [51] showed that MALDI-TOF and ESI-MS are complementary techniques. After separation of the intact proteins of *S. fredii* by SDS gel electrophoresis the different bands are digested and analyzed by LC-ESI-MS-MS and LC-MALDI-TOF (off-line). Both techniques use 5-cm capillary columns and identified the same proteins but via different peptides.

The group of Huber [52, 53] used a 6-cm homemade LC Packings type column. They quantified Myoglobin in human serum by strong-anion exchange (SAX) for sample cleanup in the first dimension, digested off-line the Myoglobin fraction and analyzed this digest by LC in the second dimension [52]. They also applied liquid extraction and ultracentrifugation followed by RP-HPLC-ESI-MS for the analysis of intact antenna proteins; 66 to 76% of the proteins were identified within an analysis time of 16 min [53].

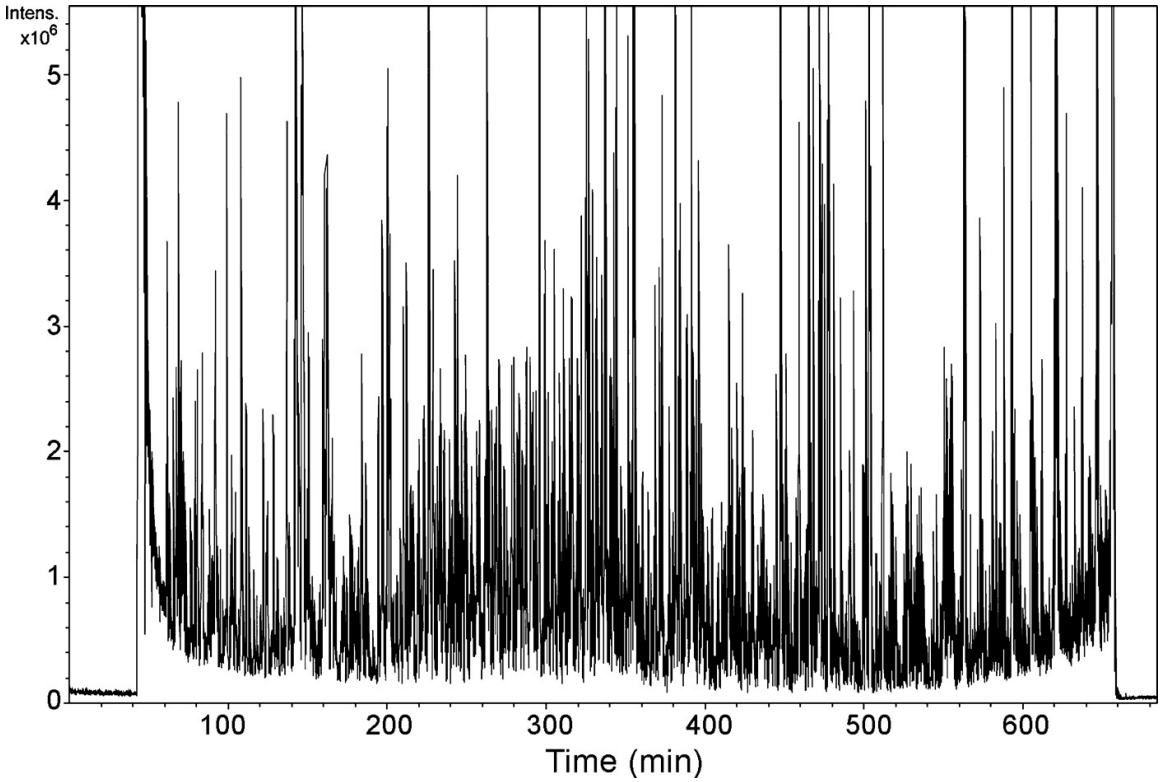


Fig. 2.8. Base peak chromatogram of an E. coli digest using a 100 cmx200 µm i.d. PS/DVB monolithic column and a flow-rate of 0.5 µL/min [43].

Porous-layer open-tubular columns

In analogy with the excellent performance of wall-coated open-tubular columns (WCOT) in Gas Chromatography (GC) Luo *et al.* [54-57] prepared reproducibly porous-layer open-tubular (PLOT) columns by a one step in-situ copolymerization of styrene-divinylbenzene (Fig. 2.9). The authors do not use the word 'monolithic' for these columns, but the columns walls have all the characteristics of monoliths. These extreme low back-pressure columns of several meters are connected with a precolumn and ESI-MS. In their first application [56], a sample was manually loaded off-line onto a monolithic PS/DVB capillary precolumn and eluted to the 4.2-m long analytical column (10 µm i.d). Again, they used a low flow-rate (20 nL/min) to increase the ionization efficiency for the MS. An in-gel digest of *M. acetivorans* revealed, with an analysis time of 3.5 h, 689 peptides (238 proteins) using 4 ng of sample and 1793 peptides (512 proteins) using 50 ng of sample. A PC of about 400 was obtained.

Luo *et al.* [55] also prepared a 3.2-m PLOT column connected with an on-line SPE column and an on-line SCX (strong cation-exchange)-SPE column. In the first system 638 peptides (343 proteins) and in the second one 1071 peptides (536 proteins) of a cervical cancer cell line were identified. They also connected a triphasic trapping column (RP/SCX/SPE) to decrease the void volume to the analytical PLOT column. From the in-gel digest of the cervical cancer cell line 4497 peptides (1209 proteins) were identified [54]. A 2.5-m hydrophilic amine-bonded PS/DVB column is developed by the same group for the analysis of glycans [57].

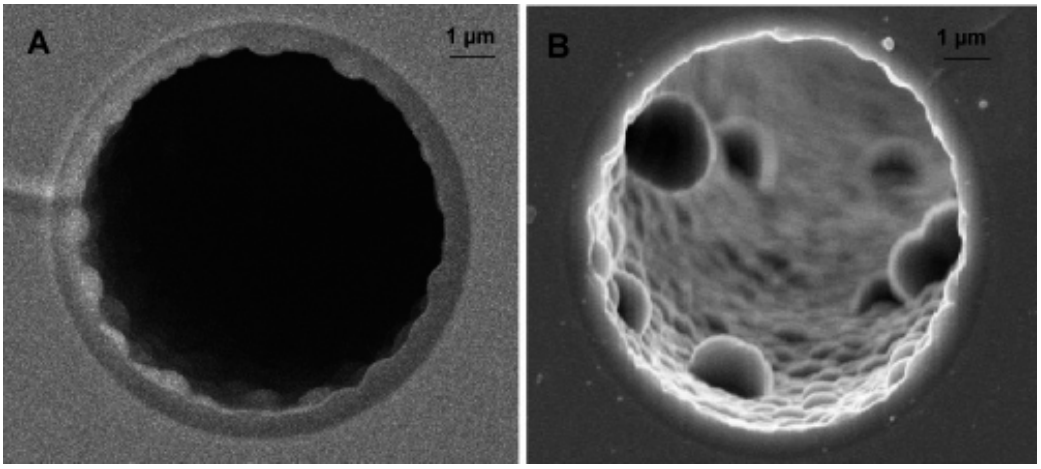


Fig. 2.9. SEM picture of the cross section of a PS/DVB porous-layer open-tubular (PLOT) monolithic capillary column, (a) middle section, (b) end section [56].

It turns out that due to their intrinsic hydrophobic character many applications using monolithic PS/DVB deal with the analysis of peptides *and* intact proteins in the RP mode. These columns are commercial available from LC Packings/Dionex and in most applications their 5-cm columns are used. Longer columns are either home made (*e.g.* 6-cm) or can be bought (25-cm) or tailor-made at the Dionex company. A new type of column, porous-layer open-tubular PLOT, is developed by Luo *et al*. [54-57]. Due to the extreme high porosity very long columns, up to several meters, with small column diameters (down to 10 µm i.d.) are developed. In combination with low flow-rates and long analysis times this results in very high MS protein identification scores.

3 Comparison of column materials

In several studies the performance of column material is compared. Toll *et al.* [58] studied the performance of a homemade capillary PS/DVB 6-cm column (100-200 µm i.d.) and compared the column with a 7.5-cm C18 silica particle column (d_p=3 µm; 75 µm i.d.) for a digest of ten model proteins. The monolithic column showed the highest identification score and the largest number of identified peptides. In contrast, Guryca *et al.* [22] calculated for a Cytochrome C digest a PC** of only 65 for the PS/DVB column (inferior peak shapes) and about 140 for the particulate (d_p=3 µm) column and silica monolith. Also the number of identified proteins for the PS/DVB column is lower compared to the silica and Chromolith column (197 vs 270) for an *A. thaliana* digest. However, it should be noticed that their PS/DVB column is 10 cm shorter than the other two columns. Van de Meent *et al*. [59] found a much higher peak capacity (170) for their PS/DVB column when working at higher temperatures.

Ro *et al.* [60] compared homemade PS/DVB columns with methacrylate columns of the same length (10 cmx100 µm i.d.) using ESI-MS and a BSA tryptic digest. The sample loading of the digest for the PS/DVB was the highest (irregular and rough surface) and separation efficiency (due to a superior surface morphology and smaller pore diameter) the best.

Rozenbrand *et al.* compared a 15-cm Chromolith column with 5-µm (16-cm) [15] and 1.8-µm (15-cm) [23] particle columns using a Myoglobin digest. At moderate

flow-rates the resolution for a Myoglobin digest for the Chromolith and 5-µm particle column are about the same. For the ultra small particle column the PC** was much higher. For more complex samples the ultra small particle column also shows the highest PC** and sequence coverage compared to a 64-cm Chromolith column.

The decision to use 1.7/1.8-µm particle columns or silica, PS/DVB or methacrylate monolithic material is mainly dependent on the problem to be solved, the equipment available in the laboratory (including LC) and column preparation tools and skills.

For high-throughput or fast analysis of protein digests we suggest to use commercial Chromolith columns at high flow-rates or commercial available small 1.7/1.8-µm particle columns at low flow-rates. For the latter special LC equipment is needed available by, for example, Agilent and Waters. For the one dimension analysis of complex protein digests we suggest either to use long silica monoliths or long PLOT PS/DVB columns to obtain the lowest possible back-pressure or relatively short 1.7/1.8 µm-particle columns. For the separation of intact proteins homemade methacrylate or commercial available PS/DVB are the best monolithic columns. For special applications tailor-made methacrylate columns are the best choice. All kind of groups can be attached to this monolithic material.

4 Concluding remarks and future perspectives

This review deals with recent development and applications of the most popular monolithic column materials. For better throughput (with a maximum of several hundred analyses per day)/low back-pressure analysis of peptides usually (commercially available) silica monolithic columns are applied. Homemade columns, sometimes mixed with particles for trapping of the analytes and long columns for the separation of complex digest sample are developed. Methacrylate and PS/DVB columns are used for separation of peptides and proteins in a wide pH range. The first are tailor-made and all kind of groups can be attached to this material making it suitable for all kind of applications. The PS/DVB column type is commercially available (LC Packings/Dionex). A new more porous PS/DVB column with an open core has been developed allowing longer lengths.

Moreover, another new type of column combining low back-pressure (as in monolithic materials) and the high resolution of ultra small particle columns has been developed. These columns, Kinetex (Phenomenex, Torrance, CA, USA) and Ascentis (Sigma-Aldrich/Supelco, St. Louis, MO, USA), available in the capillary mode, are based on silica core-shell technology. They combine a solid core (d_p=1.7 μm for Ascentis) and a 0.5 μm (Ascentis) porous shell with a short diffusion path to minimize peak broadening. Gritti and Guiochon [61] and Ruta *et al*. [62] studied the performance of these columns for the analysis of peptides.

Monolithic materials, especially organic columns, (will) play an important role in the further development of microfluidic devices in proteomics. Due to the easy preparation (single step, photochemically initiated polymerization) into a specific area, no need for retaining frits and the possibility, especially for methacrylate columns, to attach all kind of groups to the material, makes these columns very suitable for miniaturized analytical on-chip systems. Methacrylate systems containing in one column on-line digestion, trapping and separating of the peptides and an integrated ESI emitters can be developed. Pressure-driven LC systems might be replaced by electrokinetic CEC (Capillary Electrochromatography) and MS remains the main identification technique.

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Chapter 3

Fast LC separation of a Myoglobin digest: a case study using monolithic and particulate RP-18 silica capillary columns

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Abstract

A method was developed for the fast separation of a Myoglobin digest using a monolithic RP-18 silica capillary column of 100 µm i.d. The results were compared with those obtained with a particulate RP-18 silica capillary column of 100 µm i.d. at a flow-rate between 0.6 and 1.2 µL/min. The digest was analyzed at the monolithic column at a flow-rate up to 2.8 µL/min. This high flow-rate could not be applied to the particulate column due to the high back-pressure. When the starting composition of the gradient was changed from 0 to 20% and a gradient steepness of 16%/min was used, the analysis time was less than 4 min. A positive Mascot identification score of 115 was achieved for the MS–MS data. When a lower gradient steepness was employed, the chromatographic resolution and the peak capacity did not increase for most compounds. The *intraday* repeatability for the retention time of the monolithic column was better than 1.5% at 2.8 µL/min and even less than 0.5% using a flow-rate of 0.6 or 1.0 µL/min. For the particulate column, it was between 0.5 and 1.4% for a flow-rate of 0.6 µL/min, probably due to the high column back-pressure. The *interday* reproducibility for the retention time of the monolithic column was less than 0.9% using a flow-rate of 1.0 µL/min.

1 Introduction

One area of proteomics research focuses on the improvement and optimization of analytical methodologies to allow higher throughput analyses in shorter times. The conventional method used to analyze proteomic samples is 2-D gel electrophoresis, which can be used for the separation of thousands of proteins in a single run. The proteins are separated based on their pI by isoelectric focusing in the first dimension and based on their molecular weight by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. However, this method is time consuming and difficult to automate.

An alternative method is multidimensional LC, which has high resolving power, is highly reproducible and can be coupled on-line with electrospray mass spectroscopy (MS). The limited amount of sample, low abundance of proteins of interest and the increased sensitivity has accelerated the development of capillary separation systems like nanoLC. The protein is first digested, and then the

peptides are separated and introduced into the mass spectrometer for analysis and identification using a database search like Mascot. The MS–MS results can be used to identify the original protein.

For better performance, more sophisticated separation power demands better column materials, which can be obtained by using more uniform packing materials and by decreasing the particle size of the silica packing in order to increase efficiency and speed [1–3]. The Ultra Performance LC (UPLC) system contains columns with particle sizes of 1.7 µm [4] and Shen *et al.* [5] even use particle sizes of 0.8 µm. A major disadvantage of these smaller particles is the higher column back-pressure associated with them, and special equipment is needed to deal with this. Another approach is to use monolithic materials with large macro through-pores and smaller mesopores, as developed by Nakanishi and Soga [6]. This approach is based on the hydrolysis and polycondensation of alkoxysilanes in the presence of water-soluble polymers. Tanaka *et al.* [7] demonstrated that this method allows the preparation of chromatographic columns with internal diameters in the mm, µm and nm ranges. The mesopores possess a specific surface area of 200–300 m^2/g and an efficiency corresponding to a 3- to 5-µm particulate column [8] can easily be obtained. The macropores account for a very low pressure drop (*i.e.,* corresponding to a column packed with particles of 10 to 15 µm).

Monolithic columns are very suitable for high-throughput analyses. Several applications using a 50 to 150 mm×4.6 mm i.d. Chromolith columns have been reported. Wu *et al.* [9] discussed some practical aspects of the fast analysis of some test compounds and gave some examples of fast (compared to 5-µm particulate columns) separations of pharmaceutical process samples. For ketamine and its metabolites, a sixfold reduction in run time was achieved while the R_s remained unaffected [10]. Borges *et al.* [11] separated bupropion from its metabolites within 23 s using a flow-rate of 5 mL/min, and Bugey and Staub [12] separated benzodiazepines in 4 min. Bacitracin, a cyclic polypeptideantibiotic, was analyzed within 6 min using a flow-rate of 6 mL/min [13]. Gerber *et al.* [14] noticed some drawbacks using high flow-rates. Loss of efficiency for larger molecules and the maximum data acquisition rate for the diode array detector were the limiting factors for very fast separations. Xiong *et al.* [3] separated a Cytochrome C tryptic

digest using a 45-min gradient at a flow of 1 mL/min. At a flow of 10 mL/min, the analysis time was decreased by a factor of 10 while the back-pressure did not exceed 150 bar. The R_s of a representative peptide peak pair decreased from 4.4 to 3.4. Barosso *et al.* [15] provided chromatograms of a tryptic digest of Cytochrome C obtained with a flow-rate of 2 and 4.5 µL/min with a Chromolith CapRod column of 150×0.1 mm i.d. There was no significant effect on the separation, but the analysis time decreased from 50 to 30 min. In the middle part of the chromatogram there are no peaks, so it still took a relatively long time to analyze the sample. Also, a protein mixture was separated by polyacrylamide gel electrophoresis and digested with trypsin. The peptides were separated by the Chromolith CapRod column and successfully identified by the LC–MS–MS system. Rieux *et al.* [16] have measured a Cytochrome C digest within 18 min using a capillary monolithic silica column and a flow-rate of 1 µL/min. For isocratic elution they have found a minimum HETP of 5–10 µm using the same digest for this column. Davis and Lee [17] have also analyzed a Cytochrome C digest using a 5-µm C18 particulate column placed in the microspray needle of the MS. At a flow rate of 1.3 to 1.5 µL/min and an analysis time of 6 min, the protein was identified by a database search of the MS–MS data.

The present paper describes the analysis of a Myoglobin digest with a homemade particulate Aqua C18 silica capillary column and a commercially available monolithic RP-18 silica capillary column (Chromolith CapRod). We have compared both columns despite the different types of stationary phase. Our goal is to decrease the analysis time of the digest by increasing the flow-rate and to optimize the gradient and still obtain a good Mascot identification score [18]. The chromatographic resolutions (R_s) and the sample peak capacities (PC**) of the peak pairs are shown and discussed. Finally, the *intraday* repeatibilities for the retention times of both columns and the *interday* reproducibility for the retention time of the Chromolith column have been measured.

2 Experimental

2.1 Materials

The monolithic column, a Chromolith CapRod column, RP-18 endcapped, 150×0.1 mm i.d., was purchased from Merck (Darmstadt, Germany). The particulate column, Aqua C18 (column material received from Phenomenex, Torrance, CA, USA), 5 µm, 160×0.1 mm i.d., was constructed in our department according to Meiring *et al*. [19].

The acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands) and the trifluoroacetic acid (TFA, biochemical grade) was from Acros (Geel, Belgium).

2.2 Methods

To prevent contamination of the digest with enzyme or with fragments of the enzyme caused by autodigestion, the trypsin (Sigma Aldrich, Zwijndrecht, The Netherlands) was immobilized on carbonyldiimidazol (CDI, Sigma Aldrich, St. Louis, MO, USA) Sepharose CL6B (Sigma Aldrich). Pre-activated Sepharose (20 mL) was washed with a series of solutions (~40 mL) of acetone (Merck), 70% acetone/water (MQ), 50% acetone/water, 30 mL acetone/water, water, and finally with cold 100 mM phosphate buffer, pH 7.0. Excess liquid was removed from the gel by applying vacuum on a sintered glass filter (no. 3). The gel was not completely dried. Trypsin (80 mg, ~14 kU/mg, Sigma Aldrich) was weighed in a beaker and dissolved in 20 mL ice-cold 100 mM phosphate buffer, pH 7.0, containing 1 mM benzamidine. The wet gel (~20 mL) was added to the trypsin solution and stirred overnight in a cold room (4°C). The non-immobilized trypsin was removed from the gel by vacuum suction on the glass filter, and the gel was washed three times with 100 mM phosphate buffer, pH 7.0. The gel was redissolved in an equal volume of 100 mM phosphate buffer pH 7.0 with azide and stored at 4°C until use. The Myoglobin (2 mg/mL) was dissolved in borate buffer pH 8.5 and digested by the immobilized trypsin for 67 h at 37°C. Demineralized and filtered (cellulose acetate filter, 0.2 µm from Sartorius, Göttingen, Germany) water with 0.1% trifluoroacetic acid (TFA; solvent A) and 90% (m/m) acetonitrile with 0.1% TFA (solvent B) were used for the mobile phases.

To increase the flow-rate, the elution volume was set to 12 µl, according to the gradient scheme below. To get a valid comparison for starting percentages of 0 or 20% B at 2.8 µL/min, the gradient steepness was kept the same for both (*e.g.*, 16%/min).

Gradient for 0.6 µL/min was: 0 min 0%B, 1 min 0%B, 21 min 70%B, 25 min 0%B; gradient for 1.0 µL/min was 0 min 0%B, 1 min 0%B, 13 min 70%B, 17 min 0%B; gradient for 1.2 µL/min was: 0 min 0%B, 1 min 0%B, 11 min 70%B, 15 min 0%B; gradient for 2.8 µL/min was: 0 min 0%B, 1 min 0%B, 5.3 min 70%B, 9.3 min 0%B or 0 min 20%B, 1 min 20%B, 4.07 min 70%B, 8.07 min 0%B.

To increase the steepness when using a flow-rate of 2.8 µL/min, the following gradient schemes were used. For 4%/min the gradient was: 0 min 20% B, 1 min 20%B, 13.5 min 70%B, 17.5 min 0%B; for 8%/min the gradient was: 0 min 20%B, 1 min 20%B, 7.25 min 70%B, 11.25 min 0%B; for 12%/min the gradient was: 0 min 20%B, 1 min 20%B, 5.17 min 70%B, 9.17 min 0%B; for 16%/min the gradient was: 0 min 20%B, 1 min 20%B, 4.07 min 70%B, 8.07 min 0%B; for 20%/min the gradient was: 0 min 20%B, 1 min 20%B, 3.5 min 70%B, 7.5 min 0%B.

All chromatographic separations were performed on an Agilent 1100 Series nanoLC with an Agilent LC/MSD ionTrap XCT (Agilent, Palo Alto, CA, USA). The voltage of the MSD was –3 kV, the flow of the drying gas was 6 L/min, and the temperature was kept at 150°C; the peptide scan mode was used for MS–MS. Fifty nanoliters of the Myoglobin digest (100 ng) was loaded on a 5-µm Zorbax 300 SB-C18 trapping column (5×0.3 mm i.d.) using a high precision pump (Gynkotek, Germering, Germany, model 300) with a flow of 0.01 mL/min water. After 1 min the valve is switched and the digest is desorbed by the eluent to the analytical column.

The chromatographic resolution (R_s), sample peak capacity (PC**) and asymmetry factor (A_s) have been calculated according to Eqn (3.1), (3.2) and (3.3). W_b is the peak width, t_r is the retention time, Δt_r is the time between the first and last eluting peak in each chromatogram, $W_{0.05}$ is the peak width at one-twentieth of the peak height and d is the distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

$$R_s = 2(t_{r,B} - t_{r,A})/(W_{b,B}+W_{b,A}) \qquad \text{Eqn (3.1)}$$

$PC^{**} = \Delta t_r / W_b$ Eqn (3.2)

$A_s = W_{0.05}/2d$ Eqn (3.3)

3 Results and discussion

3.1 Influence of the flow-rate

The Myoglobin digest was separated at flow-rates of 0.6 to 1.2 µL/min. The retention behaviors of the digest obtained using the particulate and monolithic column was compared for a flow-rate of 1.2 µL/min and the chromatograms are shown in Fig. 3.1. Peaks in the different base peak chromatograms with the same mass spectrum have the same identity and peak number. The chromatogram for the monolithic column showed 13 (partially resolved) peaks and the particulate column showed 12 (partially resolved) peaks. The total analysis time was 11–12 min and the gradient time was 6 min for both columns. The back-pressure for the monolithic column was 59 bar, as expected due to the high permeability, and the back-pressure for the particulate column was ~185 bar. Due to this high column back-pressure, the particulate column did not allow a higher flow-rate, in contrast to the monolithic column.

Table 3.1 shows the R_s of the peaks in Fig. 3.1. When the R_s was insufficient or one of the compounds was not observed, the R_s was not determined (n.d.). The columns contained different kinds of stationary phase, which explained the slightly different retention behaviors. Peaks 1–2 were not separated on the particulate column, in contrast to the monolithic column (R_s=1.0). Peaks 3–4 nearly coeluted on the monolithic column (R_s=0.6) and were better separated on the particulate column (R_s=1.1). Peaks 9-10 and 11-12 were much better separated with the monolithic column. Due to this extra peak, the R_s of the peak pair 10-11 was not determined.

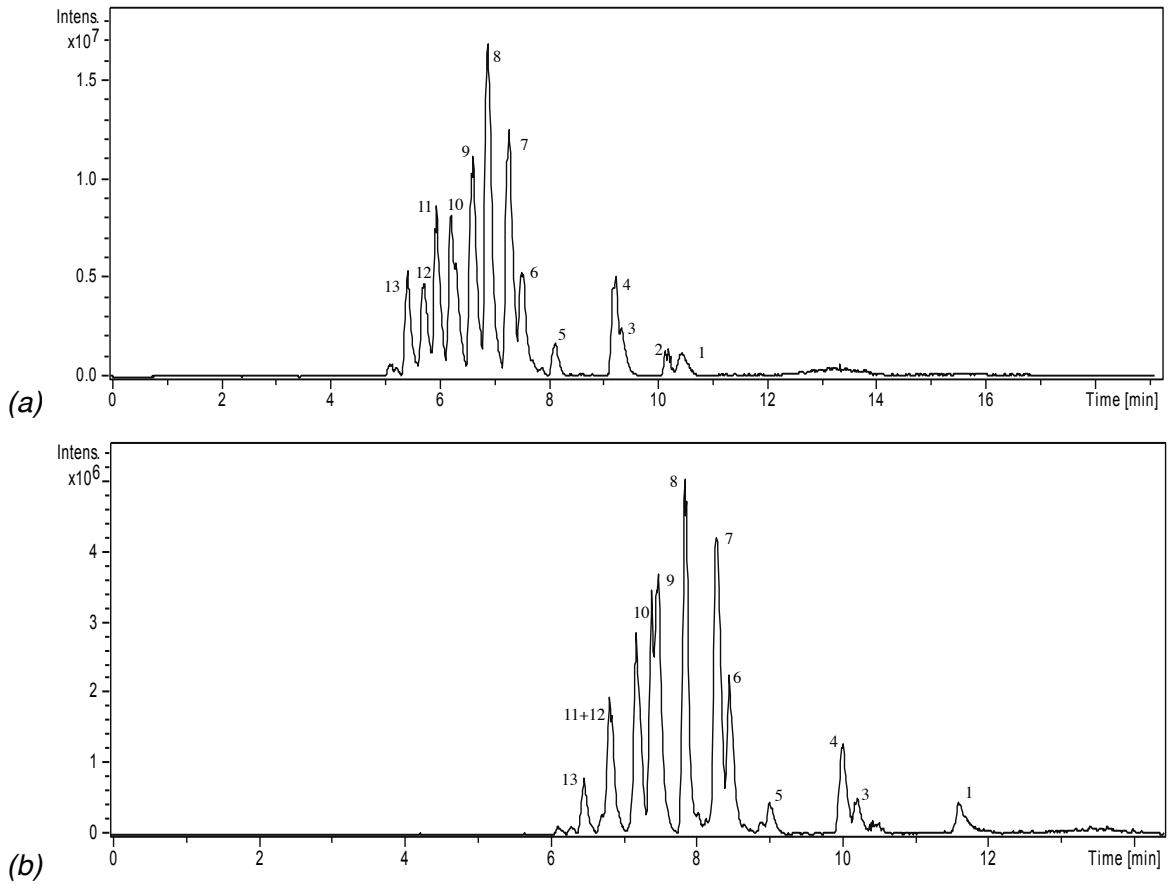


Fig. 3.1. Base peak chromatogram of a Myoglobin digest obtained using a flow-rate of 1.2 µL/min and (a) a capillary Chromolith CapRod RP-18 column and (b) a capillary Aqua particulate C18 column. For gradient scheme and experimental conditions, see text.

Table 3.1. R_s values of the peaks from the monolithic and particulate columns obtained with a low flow-rate of 1.2 µL/min and with B_0=0%

Peak pair	*Monolithic column*	*Particulate column*
13 - 12	1.3	1.8
12 - 11	1.2	n.d.
11 - 10	1.1	n.d.
10 - 9	1.7	0.7
9 - 8	1.1	2.0
8 - 7	1.5	2.0
7 - 6	1.0	1.0
6 - 5	2.9	3.2
5 - 4	4.3	5.1
4 - 3	0.6	1.1
3 - 2	4.9	n.d.
2 - 1	1.0	n.d.

n.d.: not determined

Due to the low back-pressure for the monolithic column, the flow-rate could be increased to 2.8 μL/min (Fig. 3.2[a]). Even with this high flow-rate, the back-pressure was still far below 300 bar. Compared to the chromatogram for 1.2 μL/min (Fig. 3.1[a]) the number of peaks decreased from 13 to 11. As can be seen in Table 3.2, compared to the values for the same column when using 1.2 μL/min, the R_s values decreases, as expected for this higher flow-rate. However, all of the peaks, except for the pairs 1-2 and 3-4, were still resolved. In Fig. 3.1[a], the peak pair 3-4 elute at almost the same time, and in Fig. 3.2[a] they cannot be separated. An advantage of this higher flow-rate was that the analysis time for the digest was less than 5 min, in contrast to the 11 min of analysis time required for a flow-rate of 1.2 μL/min.

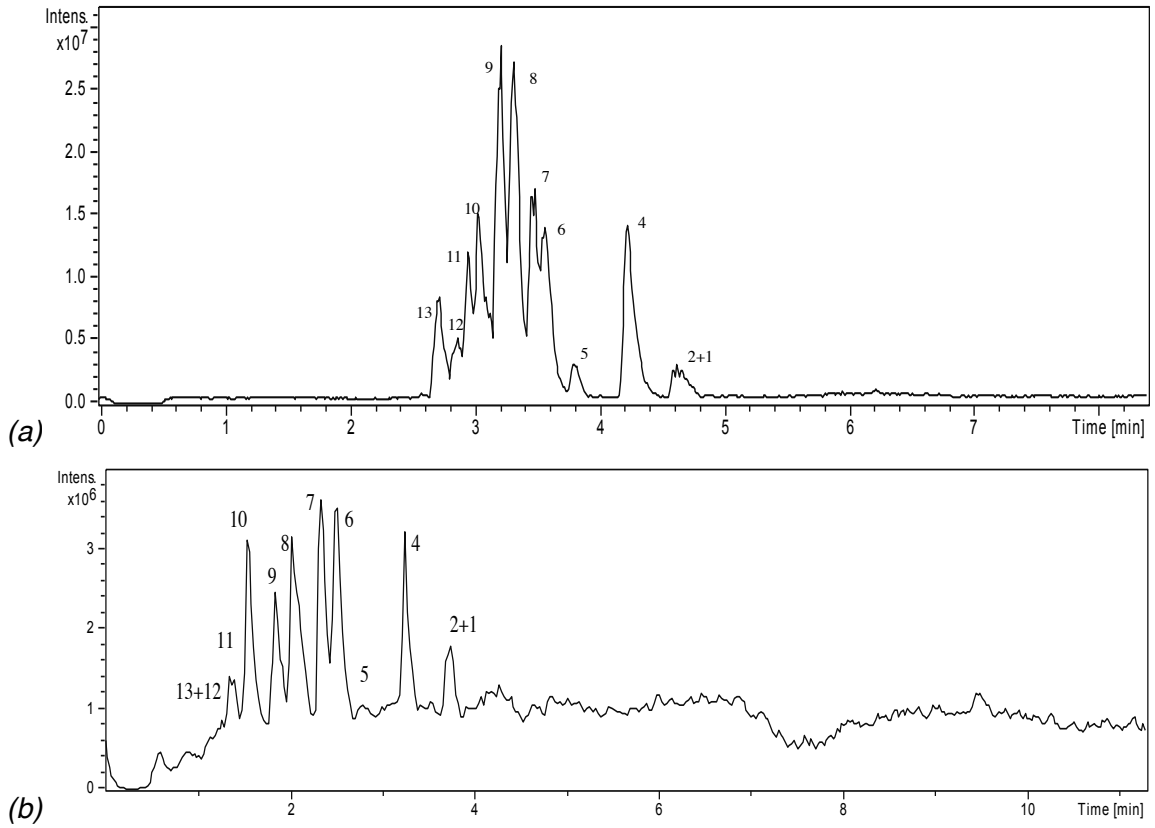


Fig. 3.2. Base peak chromatogram of a Myoglobin digest obtained using a flow-rate of 2.8 μL/min with a capillary Chromolith CapRod RP-18 column and (a) B_0=0% and (b) B_0=20%. For gradient schemes and experimental conditions, see text.

Table 3.2. R_s values for the peaks obtained for the monolithic column with a flow-rate of 2.8 µL/min and with two different starting percentages employed for the gradient

Peak pair	*B_0=0%*	*B_0=20%*
13 - 12	1.1	n.d.
12 - 11	0.9	n.d.
11 - 10	0.6	1.4
10 - 9	1.3	1.4
9 -8	0.8	0.8
8 - 7	1.1	1.5
7 - 6	0.8	1.0
6 - 5	1.7	1.3
5 - 4	2.2	2.3
4 -3	n.d.	n.d.
3 - 2	n.d.	n.d.
2 - 1	n.d.	n.d.

n.d.: not determined

3.2 Composition of the gradient

During the first 2.5 min of the chromatogram in Fig. 3.2[a] no compounds were eluted. This means that the gradient could be changed in order to elute the compounds in the digest faster. Therefore, the starting percentage of B (B_0) was increased from 0 to 20%. For 2.8 µL/min and B_0=20%, the Myoglobin digest was analyzed and the chromatogram is shown in Fig. 3.2[b]. In Table 3.2, the R_s values for B_0=0% and B_0=20% (different starting points for the gradient) were compared. Compared to Fig. 3.2[a], the number of peaks decreased from 11 to 9. Fig. 3.2[b] shows that the most rapidly eluting peaks (peaks 13, 12 and 11) eluted from the column at about the same time. This was quite obvious, since the higher B_0 value had the strongest influence in the first part of the chromatogram. This also explained the change in R_s for peak pair 10–11. For the other peak pairs, the R_s values for both starting percentages of B were about the same. Some of the deviations may be explained by the lower accuracy of the R_s calculated for Fig. 3.2[a] which was due to the peaks not being baseline separated. The asymmetry factors (A_s) for peaks 4 and 10 of Fig. 3.2[b] are 1.3 and 1.5 respectively. The analysis time decreased to 1 min upon using a B_0 of 20%, and so the total analysis of the digest was less than 4 min.

Performing MS–MS under the above mentioned fast conditions (B_0=20% and a flow-rate of 2.8 µL/min) gave a Mascot identification score for the Myoglobin digest of 115. Fig. 3.3 shows the MS-MS spectrum of peak 10 in Fig. 3.2[b], and this

peptide was identified by Mascot as LFTGHPETLEK. Other peaks identified by Mascot, contributing to the identification score, were peak 8 (VEADIAGHGQEVLIR) and peak 11 (HPGDFGADAQGAMTK). The Mascot identification score was 203 for a slower analysis (B_0=0%, 1.0 µL/min), but in this case it took almost 11 min to analyze the digest. This latter score is higher, but the score of 115 from the faster analysis was sufficient to make a reliable identification in a short time.

To study the influence of the gradient steepness on the R_s and PC**, the digest was analyzed using gradient steepnesses of 4, 8, 12, 16 and 20%/min. Since it is our goal to analyze the digest in a short time, the flow-rate used was high (2.8 µL/min) and the starting percentage B_0 was 20%. The R_s values are shown for five peak pairs in Fig. 3.4. The R_s values for peak pairs 5–4 and 6–5 were high, and when a steeper gradient was used the peaks eluted closer to each other and the R_s values decreased. For the other peak pairs, the R_s values were almost the same. Because of the shorter analysis time, the distances between the peaks were smaller, but the peak widths also decreased, resulting in similar R_s values.

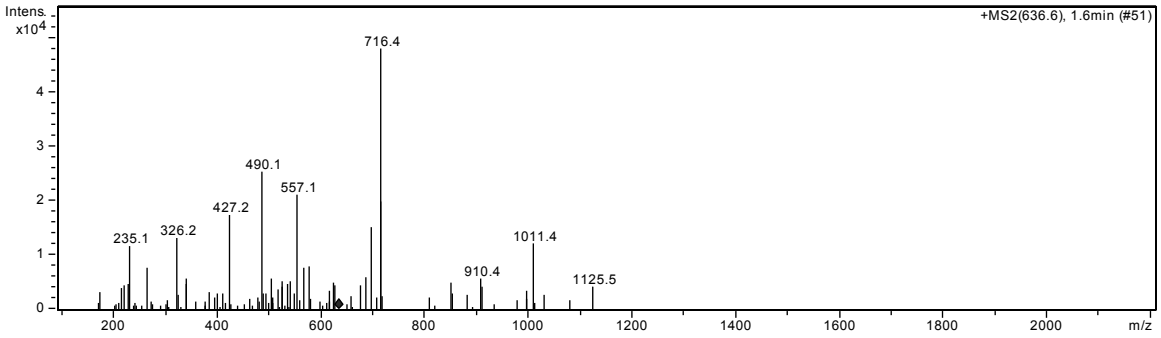


Fig. 3.3. MS-MS spectrum of m/z 636.6 (doubly charged precursor ion) corresponding to the chromatographic peak 10 of Fig. 3.2[b] (LFTGHPETLEK).

Table 3.3 shows the PC** for four peaks using the same conditions as mentioned above. As shown in Eqn (3.2), the PC** is the ratio of the Δt_r to the peak width. The Δt_r is the time between the first eluting peak (coelution of compounds 13, 12 and 11) and peak 4. Peaks 3, 2 and 1 were not always observed, so it was decided to use peak 4 as the last eluting peak. For 4%/min, the Δt_r was the highest and the PC** was also the highest. Jilge *et al.* [20] also showed that the

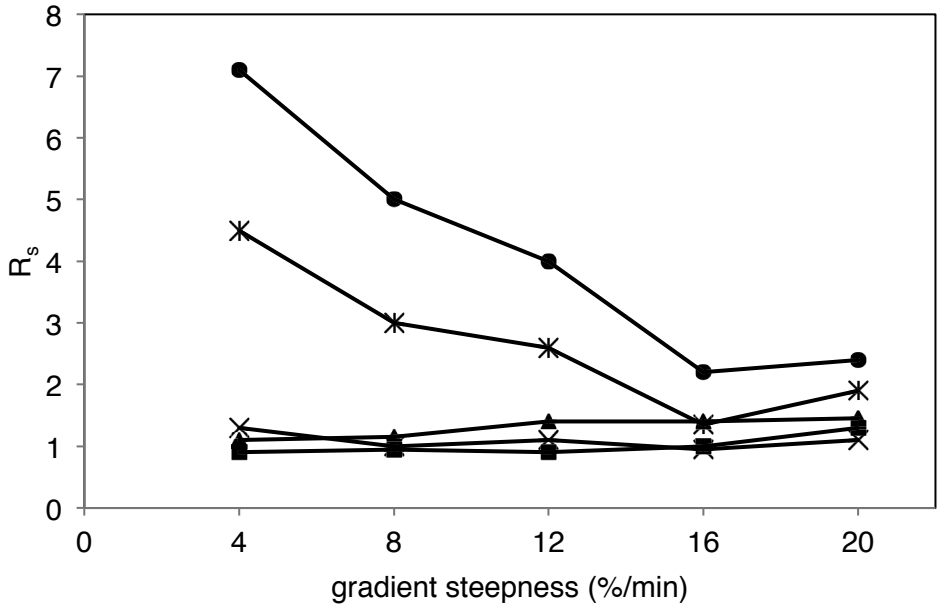


Fig. 3.4. R_s vs gradient steepness for five peak pairs (squares, peak 9-8; triangles, peak 8-7; crosses, peak 7-6, asterisks, peak 6-5, circles, peak 5-4) from the Myoglobin digest obtained using a flow-rate of 2.8 µL/min. B_0=20% and a capillary Chromolith CapRod RP-18 column. For gradient schemes and experimental conditions, see text.

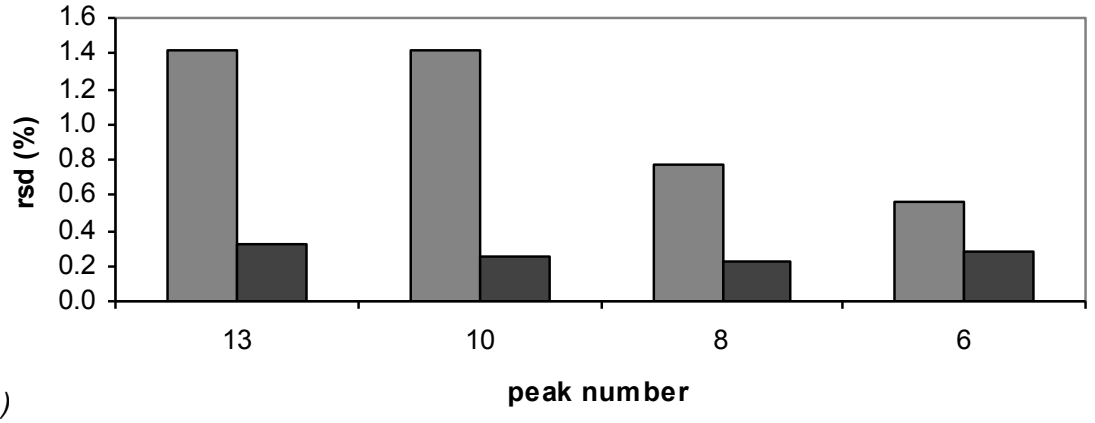
peak capacity increases at a larger gradient time. The gradient time decreased for steeper gradients, resulting in lower peak capacities and finally in a constant value, because the peak width also decreased. For the present system, a gradient steepness of 16 or 20%/min appeared to be optimum. The R_s values of the peak pairs 5–4 and 6–5 were still sufficient and the R_s values and PC** of the other peak pairs were more or less independent of the steepness. Fig. 3.2[b] shows the chromatogram obtained using the "high speed" conditions: a gradient steepness of 16%/min and a B_0 of 20%. An analysis time of about 30 min is commonly employed when applying a particulate capillary column to a Myoglobin digest [21, 22], so our system is about a factor of seven faster.

*Table 3.3. PC** of the monolithic column for various gradient steepnesses*

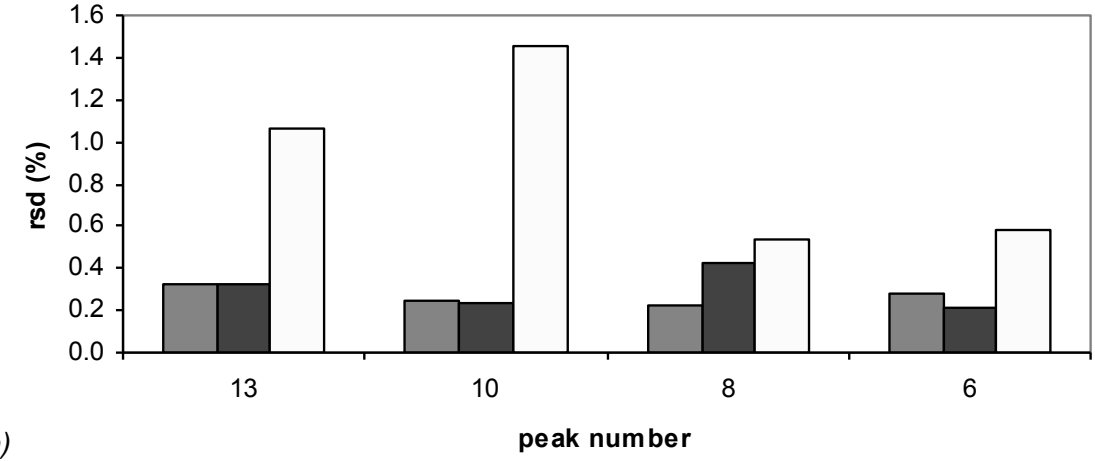
Peak	*4%/min*	*8%/min*	*12%/min*	*16%/min*	*20%/min*
9	20	13.5	11	10	13
8	13	10	8	8	11
7	12	10	11	11	12
6	18	14	13	7	13

3.3 Reproducibility

The *intraday* repeatabilities for both the particulate and the monolithic columns and the *interday* reproducibility for the monolithic column were tested by measuring the retention times of the different compounds in the Myoglobin digest. Fig. 3.5[a] shows the *intraday* relative standard deviation for the particulate column in comparison to that for the monolithic column at a flow-rate of 0.6 µL/min. For the monolithic column, the standard deviation was less than 0.4%, and for the particulate column it was between 0.5 and 1.4%. The pressure obtained when using the particulate column was much higher compared to that obtained when using the monolithic column: 130 vs 22 bar. For this higher but instable pressure, the flow-rate was also unstable, resulting in a higher relative standard deviation for the retention time. Fig. 3.5[b] shows the *intraday* relative standard deviations for the retention time of the monolithic column when using flow-rates of 0.6, 1.0 and 2.8 µL/min. For the two lowest flow-rates, the back-pressures were only 22 and 38 bar, respectively, resulting in relatively low standard deviations (less than 0.5%). For a flow-rate of 2.8 µL/min, the back-pressure of the monolithic column was much higher (about 120 bar), and again it was unstable, resulting in a higher standard deviation for the retention time (between 0.5 and 1.5%). The *interday* relative standard deviation for the retention time, obtained using a flow-rate of 1.0 µL/min, when it was measured over three days, was less than 0.9%.



(a)



(b)

Fig. 3.5. Intraday repeatability for the retention time obtained using a Myoglobin digest and (a) the capillary Chromolith CapRod RP-18 (n=6, black) and the capillary Aqua C18 particulate column (n=5, gray) with a flow-rate of 0.6 µL/min, and (b) the capillary Chromolith CapRod RP-18 column at a flow-rate of 0.6 (n=6, gray), 1.0 (n=7, black) and 2.8 µL/min (n=7, white). For gradient schemes and experimental conditions, see text.

4 Conclusions

The analysis time of the Myoglobin digest was decreased to 4 min by using a capillary monolithic silica column and optimizing the gradient composition (the starting conditions and gradient steepness) and by increasing the flow-rate to 2.8 µL/min. This is considerably faster than the analysis time of about 30 min required for conventional nanoLC. The PC** and R_s values were still sufficient and protein identification via Mascot was still possible. The maximum data acquisition rate of the MS may potentially present a problem with this short analysis time. The *intraday* and *interday* reproducibilities for the retention time of the monolithic column was still good. The potential of this approach should be demonstrated by the analysis of digests of larger proteins and real protein samples. Optimization of

the gradient conditions will be needed and the compromise between speed of analysis and the information obtained from the MS data is always crucial.

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Chapter 4

Comments on the implementation of a simple peak capacity optimization procedure for model peptides using a monolithic RP-18 silica capillary column

Abstract

For the analysis of protein digests, the peak capacity in reversed-phase liquid chromatography is of paramount importance. A univariate method to maximize the peak capacity as developed by Wang *et al.* (*Anal. Chem.* 2006, *78*, 3406-3416) has been applied and tested for a monolithic RP-18 silica capillary column. In their method, using model peptides representing a bovine serum albumin (BSA) digest, the gradient time and temperature are kept constant while the flow-rate and eluent strength are varied. Despite our criticism on the fixed starting conditions, a long gradient time leading to an unnecessary long analysis time and a high temperature leading to possible degradation products in the chromatogram, and the peak capacity as the only optimization parameter this fast and simple optimization strategy turns out to be applicable to capillary monolithic columns. Furthermore, the influence of the peak capacity on a second optimization parameter, the MS protein identification score, is discussed.

1 Introduction

The main goal in proteomics is to identify proteins. This can be achieved by the combination of separation and detection/identification of proteins and peptides. Usually, after a prefractionation of proteins by two-dimensional gel electrophoresis (2-DE) the proteins of selected spots are digested by a proteolytic enzyme *e.g.* trypsin. Each protein or protein mixture may produce many peptides and thus a high separation power in combination with a reliable identification is important. The peptides are typically separated by reversed-phase liquid chromatography (RP-LC) with mass spectrometric (MS) detection for quantification and identification. To decrease the amount of sample needed and to increase the MS sensitivity, frequently a capillary LC or nanoLC system is used at flow-rates of microliters or nanoliters per minute, respectively.

To improve the peptide separation of the LC system the parameters for defining the chromatographic performance have to be considered. These parameters are the column efficiency, resolution (R_s) and peak capacity (PC). Column efficiency expressed by plate height H or plate number N, is only applicable for isocratic elution. Therefore, since protein digests are separated with gradient elution, the H

and N cannot be used [1]. For samples containing many components, like a protein digest, the PC is a useful and better parameter for defining the chromatographic separation performance of systems with gradient elution [2, 3]. The PC is initially defined by Giddings for isocratic chromatography [4]. Horváth and Lipsky [5] showed that the PC increases by using gradient elution because of the smaller peak width of all compounds. For 95% separation (R_s=1), the sample PC (PC**), according to Dolan *et al*. [6], can be calculated using Eqn (4.1), where $t_{r,1}$ is the retention time of the first, $t_{r,n}$ is the retention time of the last eluting peptide and W_b is the mean baseline peak width.

$$PC^{**} = (t_{r,n}-t_{r,1})/W_b = \Delta t_r/W_b \qquad \text{Eqn (4.1)}$$

Some authors use the total gradient time t_g [2], the time that the composition of the solvent changes, or the gradient time minus the dead time [4] instead of Δt_r and obtain a system PC. These definitions increase the t-term compared to the equation above and improve the peak capacity. Usually peptides elute in a smaller time window, depending on the elution behavior of the specific analytes in the sample, so the definition above gives a more realistic value of the peak capacity[a].
For narrow bore particulate columns Wren [2] and Wang *et al*. [7] showed that the peak capacity increases non-linearly with t_g. The initial rapid increase of the peak capacity flattens out soon with time working at a non-optimized flow-rate. These authors also notice that by increasing the gradient time the maximum peak capacity is dependent on the flow-rate. Wang *et al.* [7] have found for a 50 mmx2.1 mm i.d. (d_p=3.5 μm) column that at an optimum flow-rate the PC** increases with longer gradient times and higher temperatures. For a constant gradient time and temperature the PC** has an optimum at an intermediate flow-rate. Therefore they suggest to set the gradient time and temperature to the highest values and then optimize the flow-rate using a reasonable but arbitrary value of the final eluent strength (*i.e*. composition of the eluent at the end of the gradient). In the last part

[a]The PC is defined as the gradient time (t_g) divided by the peak width ($PC = 1 + t_g/W_b \approx t_g/W_b$ when $t_g/W_b >> 1$); because the sample occupies only a fraction of the gradient time it is better to use a time window (Δt_r) based on the first and last real peak resulting in the above mentioned PC** [6].

of their procedure the final eluent strength is adjusted so that the last analyte elutes as close as possible towards the end of the gradient. Concerning the starting conditions (long gradient time and elevated temperature) of this procedure we have some comments. Due to the definition of the peak capacity a longer gradient time always leads to a higher peak capacity. But the analysis of a sample is always a compromise between chromatographic performance and total analysis time, as studied before [8]. In the Wang procedure the gradient time can easily be set too high leading to an unnecessary long analysis time. An increase of the temperature leads to a lower solvent viscosity, to an increase of the diffusion rate and to reduced plate heights and to a higher PC** [9]. Due to this lower viscosity the column back-pressure is also diminished leading to a wider range of flow-rates applicable. The higher temperature might be a problem for the analysis of peptides. For some peptides, depending on the protein digestion procedure, the intra-molecular disulfide bonds can be broken and the amide backbone might be hydrolyzed leading to extra peaks, degradation products, in the chromatogram [9]. Despite these drawbacks the main advantage of the Wang optimization procedure, compared to a conventional multidimensional optimization method, like ANOVA (analysis of variance) or Simplex, is the quick and easy process. They theoretically checked their optimization method by a Monte Carlo simulation. 5000 sets of conditions were generated using a random number generator with ranges chosen with care of flow-rate, gradient time, final eluent strength and temperature. The PC** under each condition was then predicted. Conditions with pressures exceeding 380 bar or in which the last peptide was eluted after the gradient time were eliminated. The remaining conditions were sorted to locate the maximum PC**. The best five set of conditions are consistent with their findings using the univariate approach, *i.e.* a relative long gradient time, high temperature, an intermediate flow-rate and a low final eluent strength.

Next to the discussed LC-optimization the MS-MS protein identification score is important for the recognition of peptides (and eventually the proteins). Partly separated peptides, with a low PC**, can have the same identification score as peptides measured with baseline separation. These peptides are sufficiently recognized by the MS. For example, the identification score for a BSA digest using

a 15- and 75-cm column is the same, but the PC** for the longest column is much higher [10]. Thus for analyses in proteomics a low PC** can still be sufficient. So, next to the PC** the protein identification score should also be included in the total optimization procedure. For example the flow-rate for a particulate column to obtain the highest identification score is optimized [11]. In the present paper this aspect will be studied using data obtained by reference [11] and [12]. But first the LC optimization strategy, according to Wang *et al.* [7] for a narrow bore particulate column, is tested if this approach is also applicable for a monolithic RP-18 silica capillary column. Only the chromatographic performance is optimized so a UV detector is sufficient. This study was a preliminary survey for the LC-optimization conditions (gradient and flow-rate) of 5, 15 and 64-cm monolithic columns [11, 12].

2 Experimental

2.1 Materials

The monolithic column (Chromolith CapRod RP-18 endcapped, 350x0.1 mm i.d.) was obtained from Merck (Darmstad, Germany). Acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid (TFA, biochemical grade) was from Acros (Geel, Belgium), iodoacetamide (99%) was from Sigma Aldrich (Zwijndrecht, The Netherlands) and ammonium bicarbonate (>99%) and dithiothreitol (DTT, >98%) were from Fluka (Zwijndrecht, The Netherlands). A Sartorius Arium 611 system (Göttingen, Germany) was used to prepare deionized water (>18 MΩcm). Samples: Neurotensin fragment 1-8, 100%, 7.1 ng/μL; Phe-Phe, 100%, 12.9 ng/μL; Angiotensin II, 100%, 0.78 ng/μL; [Val^5]-Angiotensin I, 99%, 1.4 ng/μL; LHRH, 100%, 0.61 ng/μL; Gly-Phe, 100%, 129.2 ng/μL; Insulin chain B oxidized, 85%, 7.5 ng/μL; substance P, 98%, 0.93 ng/μL; Renin substrate, 100%, 5.7 mg/μL and Melittin, 93%, 10.6 ng/μL were all obtained from Sigma (St. Louis, MO, USA).

2.2 Methods

Water with 5% (m/m) acetonitrile and 0.1% TFA (solvent A) and acetonitrile with 5% (m/m) water and 0.1% TFA (solvent B) were used for the mobile phases. Gradient: t_g=60 min; gradient: 0 min 0%B, 5 min 0%B, 65 min 100%B, 70 min

100%B, 80 min 0%B, 110 min 0%B. Flow-rate: 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0 µL/min.

All chromatographic separations were performed with a Dionex/LC Packings Ultimate 2000 (Amsterdam, The Netherlands) nanoLC system with UV detection (214 nm, detector cell: 8 mm light path, 30 nL). 1 µL of the peptide sample was loaded on a C18 trapping column (5x0.30 mm, 5 µm particles; Dionex/LC Packings) using a flow-rate of 8 µL/min of eluent A; after 5 min the valve is switched and the sample is desorbed by the mobile phase and transferred to the monolithic column.

3 Results and discussion

The 10 model peptides used were selected to span a range in retention time similar to that of the tryptic peptides from a BSA digest [7, 13]. According to Wang's guidelines [7] the gradient time should be set to the longest acceptable time. Since it usually takes about 15 to 80 min to analyze a BSA or Myoglobin digest, depending on the column length [10, 14], it was decided to use t_g=60 min for a medium-length column. A capillary monolithic column of 35-cm was used and the flow-rate was varied between 0.8 and 2.0 µL/min (maximum back-pressure of 300 bar reached) to optimize the PC**. The final eluent strength was first set to 100%B and in a later stage varied (see below). Fig. 4.1 shows a representative chromatogram of the mixture at a flow-rate of 1.0 µL/min. As can be seen in this

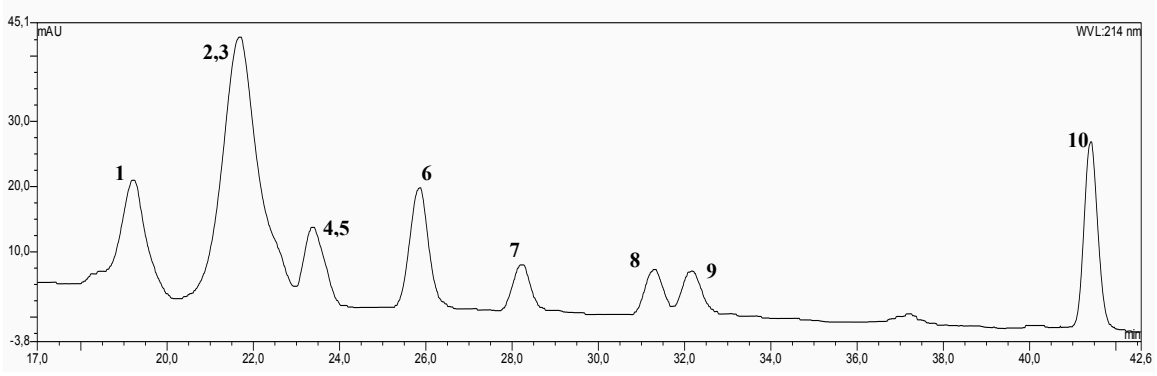

Fig. 4.1. UV (214 nm) chromatogram of model peptides using a 35-cm capillary Chromolith CapRod RP-18 column and a flow-rate of 1.0 µL/min; (1) Neurotensin fragment 1-8, (2) Phe-Phe, (3) Angiotensin II, (4) [Val^5]-Angiotensin I, (5) LHRH, (6) Gly-Phe, (7) Insulin chain B oxidized, (8) Substance P, (9) Renin substrate, (10) Melittin. For gradient scheme and further conditions, see text.

chromatogram not all peptides were baseline separated, as in a real BSA digest sample. Peptides 2, 3 and 4, 5 co-elute.

Fig. 4.2 shows the PC** of the baseline separated peptides at various flow-rates. For these peptides the PC** reaches a maximum at a flow-rate of about 1.2 to 1.5 µL/min and decreases at higher flow-rates. Liu *et al*. [1] found an optimum flow-rate between 0.75 and 1.0 µL/min for their 10-cm 75 *µ*m i.d. column. Because of the higher back-pressure at 1.2 to 1.5 µL/min and the increase using a longer column (the aim in a next study [11]), it was decided to use 1.0 µL/min as the operational flow-rate. For this flow-rate the final eluent strength (Φ_{final}) was adjusted so that the last peptides elute as closely as possible to the end of the gradient. For a final eluent strength of 50%B the last peptide eluted at about 60 min. So the operational conditions are a flow-rate of 1.0 µL/min and a gradient of 0%B to 50%B in 60 min.

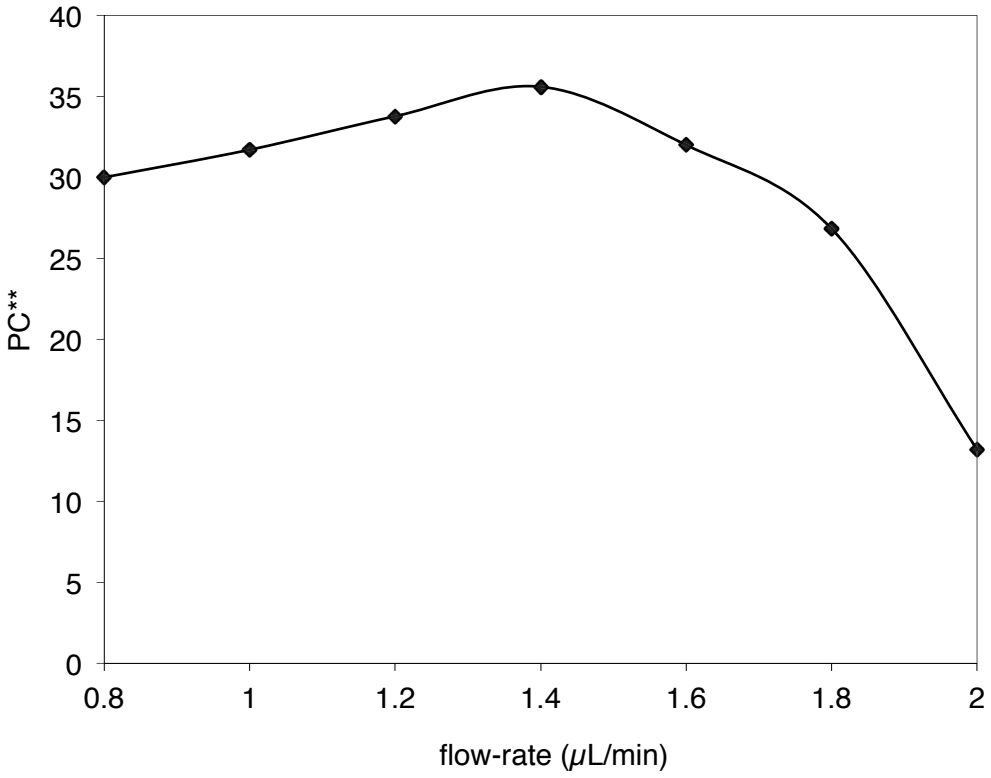

*Fig. 4.2. Average PC** vs flow-rate based on the separated peptides of Fig. 4.1 ((1) Neurotensin fragment 1-8, (6) Gly-Phe, (7) Insulin chain B oxidized, (8) Substance P, (9) Renin substrate, (10) Melittin). For gradient scheme and further conditions, see text.*

From reference [11] and [12] data are extracted to show the influence of the PC** on the sequence coverage as visualized in Fig. 4.3. These were obtained by calculating the PC** of a Myoglobin and BSA digest using monolithic RP-18 silica capillary columns of 5-, 15- and 64 cm and a 15-cm particulate column. The sequence coverage for PC**=0 was determined by direct infusion of the digest sample into the MS. The figure clearly shows that increment of the sequence coverage is not linear with the PC** and that for a higher PC** the slope of the curve decreases (Myoglobin) or starts to decrease (BSA). For Myoglobin, a simple protein, a plateau is already reached from PC**=100. For BSA, a more complex protein, it is still beneficial to increase the PC** to obtain a higher identification score.

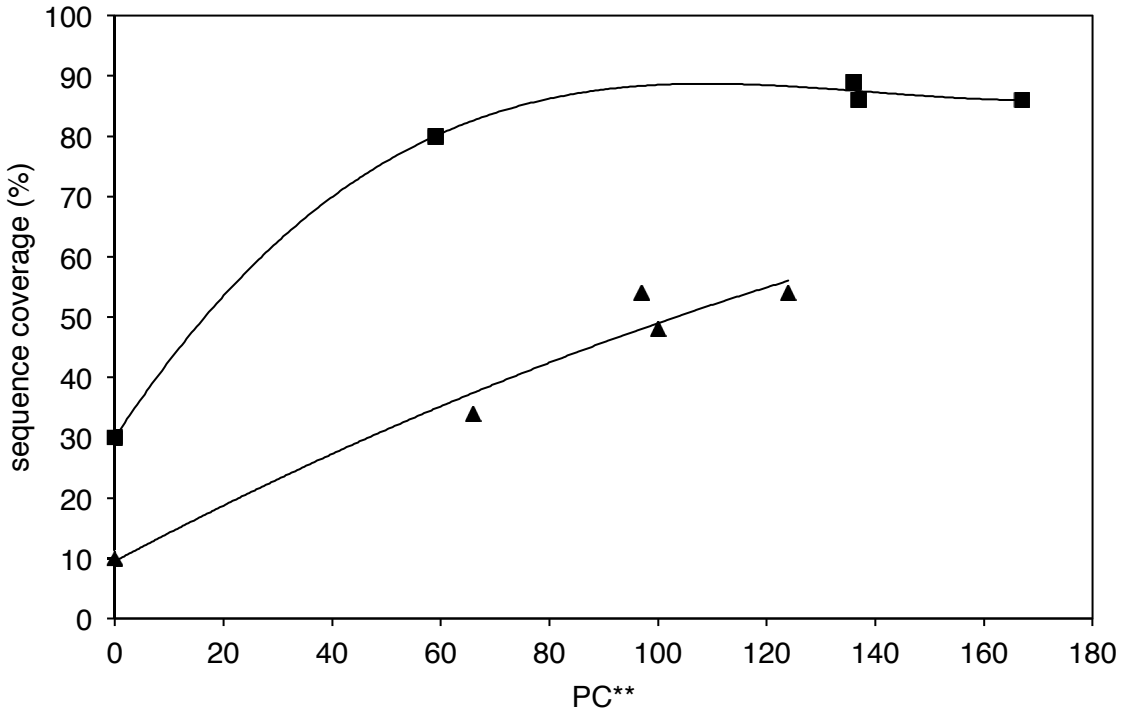

*Fig. 4.3. Sequence coverage vs PC** for Myoglobin (squares) and BSA (triangles). For experimental conditions, see text.*

4 Conclusions

Also for capillary monolithic columns the optimization strategy of Wang *et al*. [7] is a fast and useful method to optimize the peak capacity for the LC separation of a protein digest. The gradient time for the 35-cm column is set at 60 min. The operational conditions using UV detection and a BSA digest are a flow-rate of 1.0

µL/min and a final eluent strength of 50%. A substantial increment of the sample peak capacity (>100) hardly increases the MS-MS protein identification score for a simple digest, for complex proteins this increase is more beneficial.

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Chapter 5

Modifying a commercial ultra pressure LC system to a capillary one for the analysis of a protein digest

Abstract

Capillary LC-MS for the identification of digested proteins is a major tool in proteomics. In this study we describe a simple, fast and cost-effective method to change a conventional ultra pressure LC system into a capillary one. The system is tested by analyzing a Myoglobin digest and using a monolithic and a sub-micrometer particulate column. The chromatographic and MS identification results for this system are comparable with a commercially available capillary HPLC system.

1 Introduction

In the last decades miniaturization of high performance liquid chromatography (HPLC) has gained popularity, in particular in the field of proteomics. An increased signal-to-noise ratio for concentration sensitive detectors, the direct coupling of LC and mass spectrometry (MS) and the limited amount of sample needed are the main benefits [1, 2]. The typical internal diameter (i.d.) of capillary or nanoLC columns is in the μm range and flow-rates of μL/min to nL/min are applied, respectively. Other developments deal with the packing material. Monolithic materials [3] and sub 2-μm particles [4] contribute and further improve the throughput and chromatographic performance of these miniaturized systems, respectively. However, special characteristics of the equipment for the latter material are required.

Capillary and nanoLC systems have been developed by several instrument manufacturers, like Waters (using 1.7 μm silica particles), Agilent (with 1.8 μm silica particles) and Dionex (with monolithic polymeric materials), while companies like Merck mainly produce columns as well as column materials.

We have a conventional ultra pressure LC system (Agilent 1200 LC Series) and have tested if it is possible to modify this system into a capillary one. The chromatographic performance (expressed as the sample peak capacity, PC**) as well as the protein identification scores (obtained by MS-MS) of the modified ultra pressure LC and a Dionex capillary HPLC are compared. A low back-pressure monolithic column and a Myoglobin digest are used. The modified system is also

tested using the sub 2-μm particulate column. Due to the extreme high pressures the Dionex capillary HPLC can not be used any longer.

2 Experimental

The monolithic column (Chromolith CapRod RP-18 endcapped, 150x0.1 mm i.d.) was obtained from Merck (Darmstad, Germany). The particulate column (C18, 1.8 μm, 150x0.15 mm i.d.) was acquired from Micro-Tech Scientific (Vista, CA, USA). Acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid (TFA, biochemical grade) was from Acros (Geel, Belgium). A Sartorius Arium 611 system (Göttingen, Germany) was used to prepare deionized water (>18 MΩcm). Myoglobin, 95-100%, was obtained from Sigma (St. Louis, MO, USA).

Digestion procedure: 1 mg of Myoglobin was dissolved in 300 µL 100 mM ammonium bicarbonate (>99%, Fluka, Zwijndrecht, The Netherlands), pH=8.2. 18 µL of the trypsin solution (750 µg/mL; Sigma Aldrich, Zwijndrecht, The Netherlands) were added and incubated overnight at 37^{o}C.

The chromatographic separations were performed with a Dionex/LC Packings Ultimate 2000 (Amsterdam, The Netherlands) nanoLC system and a modified Agilent 1200 Series LC (Amstelveen, The Netherlands). The injection volume was 1.0 µL (300 ng) for the monolithic column and 2.2 µL (660 ng) for the particulate column. When using the Dionex LC the peptide sample was loaded on a C18 trapping column (5x0.30 mm, 5 µm particles; Dionex/LC Packings) using a flow-rate of 8 μL/min of eluent A; after 5 min the valve is switched and the sample is desorbed by the mobile phase and transferred to the monolithic column. Water with 5% (m/m) acetonitrile and 0.1% TFA (eluent A) and acetonitrile with 5% (m/m) water and 0.1% TFA (eluent B) were used for the mobile phase. Gradient for 15-cm monolithic column: t_g=26 min; 0 min 0%B, 5 min 0%B, 31 min 50%B, 31.1 min 100%B, 61 min 100%B, 61.1 min 0%B, 91 min 0%B. Flow-rate: 1.0 μL/min. For the modified Agilent 1200 Series LC the t_g=26 min; 0 min 0%B, 30 min 0%B, 56 min 50%B, 56.1 min 100%B, 86 min 100%B, 86.1 min 0%B, 116 min 0%B. Flow-rate: 1.0 μL/min (monolithic column) and 1.5 μL/min (particulate column).

The LC systems were coupled with an Agilent LC/MSD iontrap XCT (Agilent, Palo Alto, CA, USA) in the positive ion mode. The applied voltage of the MSD was -4 kV. The flow-rate of the drying gas was 4 L/min and the temperature was kept at 325°C. LC-MS-MS data were converted to the Mascot generic format using the data-analysis software. These .mgf files were searched against the MSDB database with Mascot's MS-MS ion search module [5]. The database was searched for tryptic peptides allowing one missed cleavage per peptide. The peptide mass tolerance was 2.0 Da and the MS-MS tolerance 0.8 Da.

3 Results and discussion

A schematic view of the modified Agilent LC system when using a monolithic or 1.8-μm particulate column is shown in Fig. 5.1. Zero-dead-volume connections and

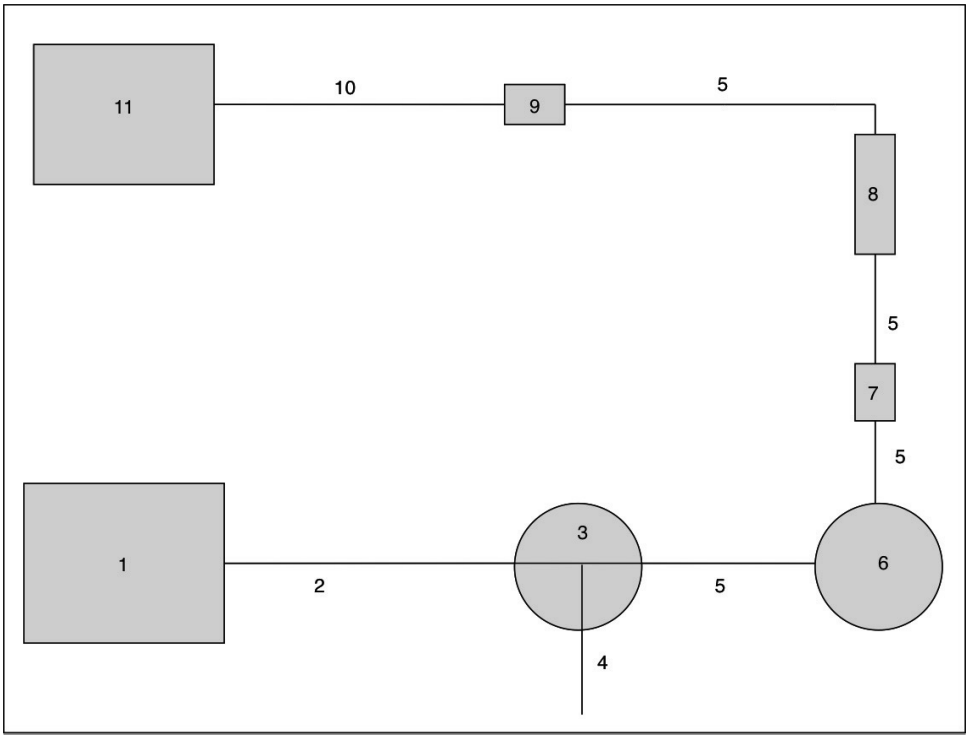

Fig. 5.1. Schematic view of the modified Agilent LC system; 1: HPLC pump, 2: stainless steel capillary (170 µm i.d.), 3: Valco T-junction, 4: restrictor, 40 cmx64 µm i.d. (monolithic column) or PEEKsil tubing, 20 cmx25 µm i.d (particulate column), 5: PEEK tubing, 64 µm i.d. (monolithic column) or PEEKsil tubing, 25 µm i.d (particulate column), 6: injection valve, 7: pre-column filter (frit 0.5 µm), 8: capillary column, 9: union, 10: Electrospray Ionization (ESI) needle, 11: MS.

tubing with an i.d. in the μm range are used. For the low back-pressure monolithic column PEEK tubing of 64 μm i.d. is required to obtain a flow-rate of a few μL/min. For the particulate column PEEKsil tubing (PEEK tubing containing a silica capillary of 25 μm i.d.) was applied. The latter can resist pressures up to 1000 bar. The flow from the HPLC pump was split (1:100 for the monolithic and 1:200 for the particulate column) using a simple T-junction and the appropriate tubing. Due to the large volume (100 μL) of the injector the conventional injection procedure has additionally to be altered.

The needle is part of the injector system; in order to inject a sample the needle is moved from the injector, filled with the desired volume of analyte and reinstalled in the injection system (Fig. 5.2[a]; position A). Eluent A flows directly, port 1 and 6 of the injection valve are connected, to the column. Then for 30 min eluent A flushes through the loop (already filled with eluent A), by switching the injection valve and connecting port 1 and 2, and propelling the injection plug onto the top of the analytical column (Fig. 5.2[b], position B). Port 1 and 6 of the valve are connected again (Fig. 5.2[a], position A) and eluent A enters the column through this shortcut avoiding again the internal volume of the injector. Then the gradient starts and the chromatographic process begins.

Fig. 5.3 shows a representative base peak chromatogram (bpc) of a Myoglobin digest using the above mentioned injection technique and the modified Agilent LC. Fig. 5.4 shows the same digest using the Dionex capillary LC. A monolithic column (back-pressure of about 50 bar) was used so the pressure limit of the Dionex LC is not the restrictive factor. The peptides of Fig. 5.3 are more distributed in the chromatogram leading to a larger Δt_r (*i.e.* time difference between the first and last eluting peptide) as shown in Table 5.1. The difference in Δt_r between the studied systems is probably caused by a gradually decrease of the flow-rate of the modified system during the analysis. The flow was split in the static mode (by using a simple T-junction). During the analysis the viscosity of the gradient decreases, more eluent leaves the splitter leading to a lower flow-rate to the column. To avoid this, a dynamic splitter that corrects the flow-rate to a constant

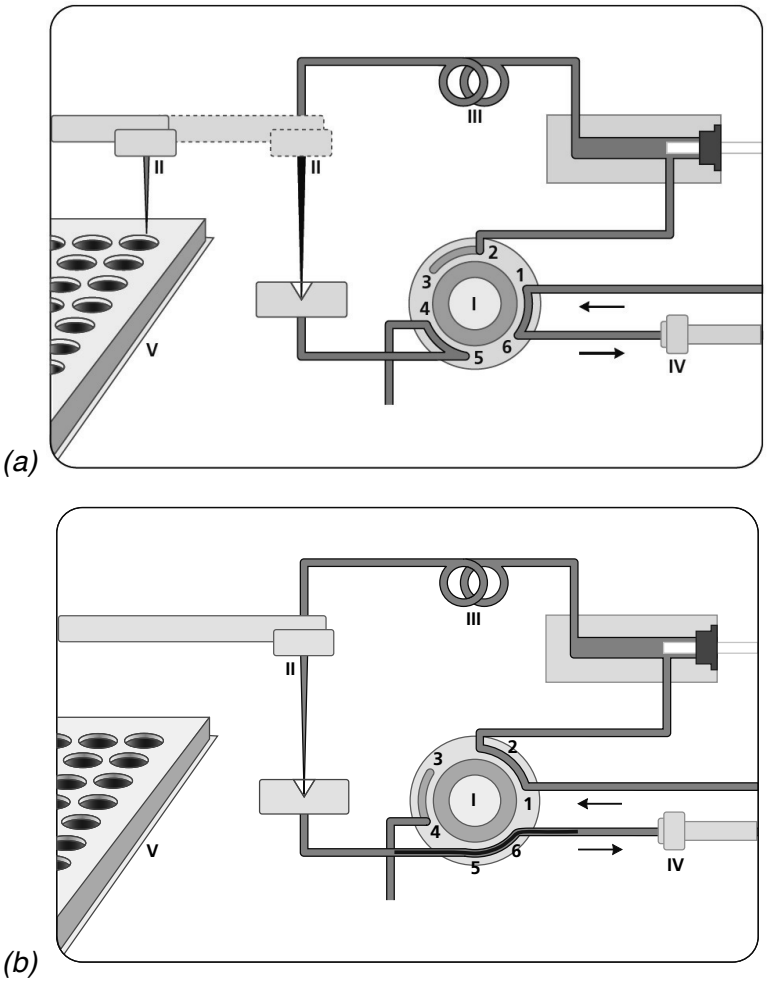

Fig. 5.2. Injection system of the modified Agilent LC system, (a) position A and (b) position B. I: injection valve, II: injection needle, III: injection loop, IV: capillary column, V: sample tray. For further details: see text [6].

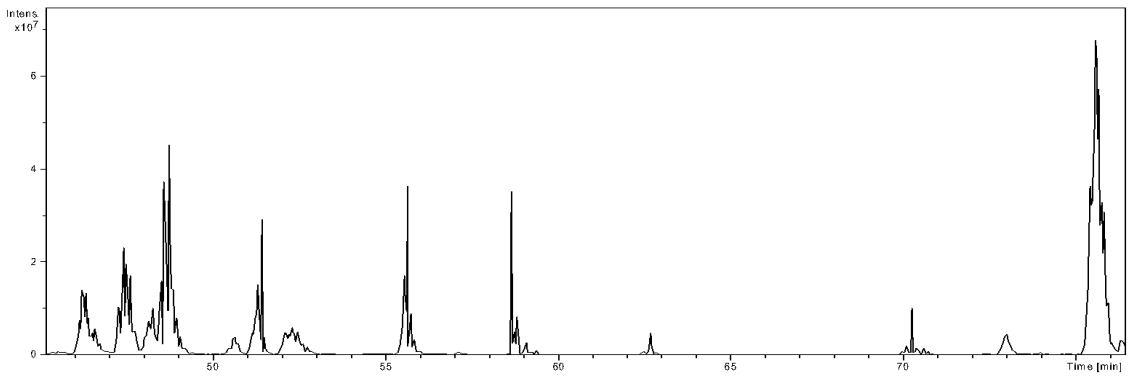

Fig. 5.3. Base peak chromatogram of a Myoglobin digest using the 15-cm capillary Chromolith CapRod RP-18 column with the modified Agilent LC system. For gradient scheme and further conditions, see text.

level, should be used. For the Dionex LC the peptide sample was loaded on a C18 particle trapping column. Probably the peptides undergo extra chromatographic interactions on this column so the chromatograms of Fig. 5.3 and 5.4 do not match perfectly. Due to the larger Δt_r the sample peak capacity ($PC^{**} = \Delta t_r/W_b$) of the modified LC system is higher. The peak width (W_b) for the peptides using the modified LC system has slightly increased because the peptides elute at a longer gradient time. The sequence coverage for both systems is almost identical. The Myoglobin digest was also analyzed using the 1.8-μm particulate column and the modified system. Due to the high pressures up to 500 bar it is not possible to use the Dionex LC. Figure 5.5 shows the bpc. Also the set-up of this system (Fig. 5.1) is successful. The peptides are much better separated compared to the monolithic

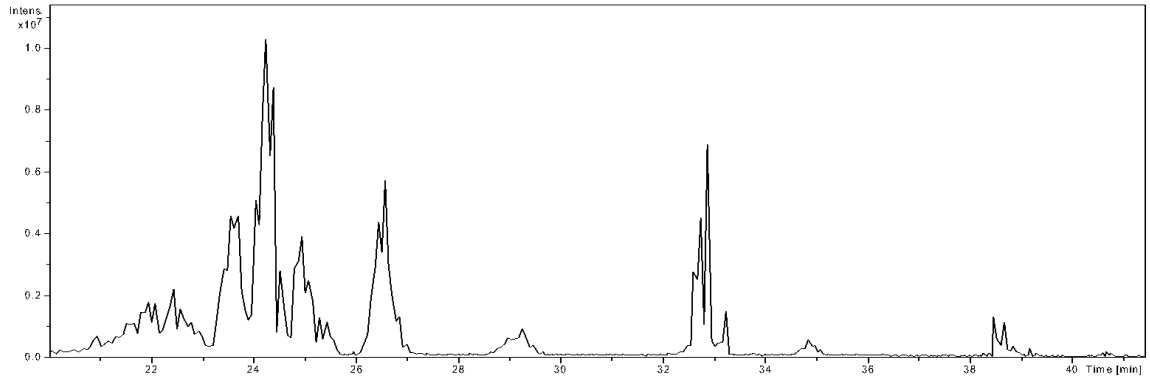

Fig. 5.4. Base peak chromatogram of a Myoglobin digest using the 15-cm capillary Chromolith CapRod RP-18 column with the Dionex LC system. For gradient scheme and further conditions, see text.

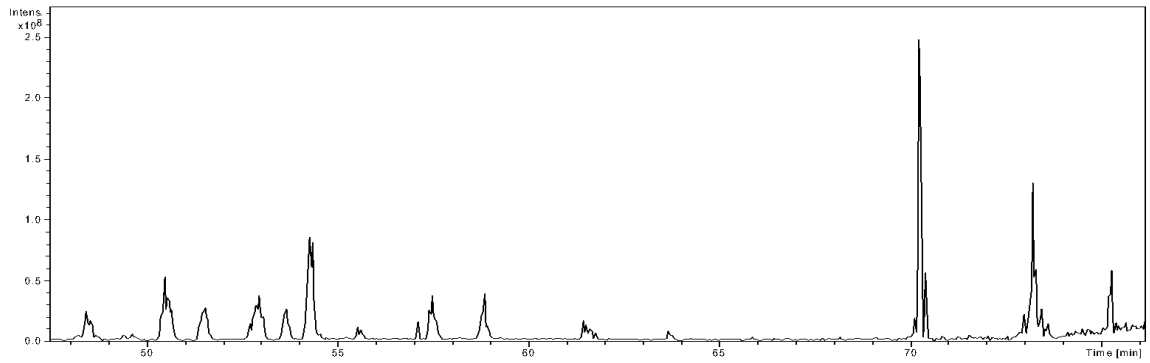

Fig. 5.5. Base peak chromatogram of a Myoglobin digest using the 15-cm capillary C18 1.8-µm column with the modified Agilent LC system. For gradient scheme and further conditions, see text.

column (peak width of 0.20 min) and so more peptides are identified resulting in a sequence coverage of nearly 90%. We will discuss these results more thoroughly in a next paper [7].

Table 5.1. Chromatographic data and identification score of a Myoglobin digest using a 15-cm monolithic column

	Dionex LC	*modified Agilent LC*
W_b (min)	0.58	0.64
Δt_r (min)	20.1	29.1
PC**	35	45
Sequence coverage (%)	75	78

4 Conclusions

A simple, fast and cost-effective method has been developed to modify a conventional Agilent ultra pressure LC to a capillary one. The chromatographic and MS-MS identification results of the modified LC system are compared to a Dionex capillary LC by using a monolithic column. The peak width and Mascot identification score are comparable for both systems when using a Myoglobin digest. Applying a sub 2-μm particulate column for the modified LC system was also possible. This system will, in the future, be used to study the influence of the PC** on the protein identification score [7, 8].

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Chapter 6

Comparison of monolithic and 1.8-µm RP-18 silica capillary columns using chromatographic data and mass spectrometric identification scores for proteins

Rozenbrand, J., De Jong, G. J., Van Bennekom, W. P., *J. Sep. Sci.* 2011, *34,* 2199-2205

Abstract

A goal in proteomics is the analysis of proteins by LC-MS. The proteins are enzymatically digested and the resulting peptides are chromatographically separated and introduced into a tandem MS. The obtained MS data are used for a search in sequence databases, providing identification scores for the proteins. A method to improve that score is to increase the chromatographic separation and peak capacity. In this study the chromatographic conditions were optimized for a relatively large gradient time by varying flow-rate and gradient composition. The influence of the monolithic column length (15 and 64 cm) and particle diameter (1.8 μm; 15-cm length) on the sample peak capacity, productivity and identification score was studied. For comparison of gradient systems a scaling factor was introduced to normalize the properties/performance of columns for material, diameter and length. As model proteins/digests, a simple (Myoglobin) and a larger (BSA) protein were used. The smallest peak width, highest sequence coverage (54% and 89% for BSA and Myoglobin, respectively) and productivity (5.0 and 4.0 respectively) were obtained for the 15-cm particulate column. The study also demonstrates that a further increase of the chromatographic performance is beneficial for BSA but hardly increases the identification score for the relatively small Myoglobin.

1 Introduction

A major goal in proteomics is the identification of proteins of interest. Typically, two-dimensional gel electrophoresis is used to separate the proteins based on charge by isoelectric focusing and on their molecular weight (MW) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The spots are transferred and processed further for identification using analytical techniques including liquid chromatography (LC) and mass spectrometry (MS).

Two different ways for protein identification have been developed: the ‘top-down’ and ‘bottom-up’ approach. In the first method the intact protein is fragmented inside a high resolution MS (*e.g.* Electrospray Ionization – Fourier transform MS (ESI-FTMS)) to create a ladder of ions indicative for the amino acid sequence. Computer algorithms to use these data for the identification of protein sequences

from databases have been developed. In the second method the proteins are enzymatically digested, the formed peptides are chromatographically separated and introduced into a tandem MS (*e.g.* ESI-Iontrap). The fragmentation pattern derived of each peptide is indicative for the amino acid sequence of the peptide/protein and can be used to search in sequence databases. Several algorithms (like Mascot) have been developed for this process of protein identification [1, 2].

A method to improve the score in the 'bottom-up' approach is to increase the chromatographic performance. The peptides will then be separated with a higher resolution, thus providing possibilities for a more reliable identification of the peptides and thus the proteins. Since protein digests are separated with gradient elution this performance is expressed by the peak capacity PC [3-7] or sample peak capacity PC** [6, 7]. The PC is defined as the ratio of the gradient time and peak width and the PC** as the ratio of the retention time difference of the first and last peptide (Δt_r) and peak width. Both parameters can be calculated using Eqn ($6.1^{a\text{-}c}$) where t_g is the gradient time, $t_{r,1}$ is the retention time of the first, $t_{r,n}$ is the retention time of the last eluting peptide and W_b is the average baseline peak width. When $t_g/W_b >> 1$ or $t_g >> W_b$ Eqn (6.1^a) approaches Eqn (6.1^b).

$$PC = 1+(t_g/W_b) \qquad \text{Eqn } (6.1^a)$$

$$PC \approx t_g/W_b \qquad \text{Eqn } (6.1^b)$$

$$PC^{**} = (t_{r,n}-t_{r,1})/W_b = \Delta t_r/W_b \qquad \text{Eqn } (6.1^c)$$

The separation throughput can be described by the productivity or production rate P, defined as the quotient of PC and t_g or PC** and Δt_r (see below) [1].

$$P = PC/t_g = PC^{**}/\Delta t_r \qquad \text{Eqn } (6.2)$$

P describes the number of compounds per minute and systems with different gradient times (or different Δt_r) can be compared. Combining Eqn (6.1^b) or (6.1^c)

and (6.2) shows that P can be determined by calculating the reciprocal value of W_b (Eqn (6.3)).

$$P = 1/W_b \qquad \text{Eqn (6.3)}$$

The peak capacity is dependent on the column length, the temperature and the particle size [1]. Wang *et al*. [8] have demonstrated that the peak capacity increases linearly with the square root of the column length. Van de Meent and de Jong have shown that a five-times longer capillary monolithic column increases the PC by a factor 1.6-2.4 [9] or 2.7-4.0 [10]. Working at higher temperatures reduces the viscosity of the mobile phase and lowers the back-pressure over the column. The increased diffusion coefficients give a higher mass transfer and as a consequence smaller peak widths, and simultaneously shorter retention times are obtained [11-13]. Therefore the overall effect on the separation performance (including PC) depends on the conditions. For proteins and peptides the choice of temperature is limited by the possible degradation of these compounds. Small particles are favorable for both the A (eddy diffusion) and C (resistance to mass transfer) term in the Van Deemter equation and so decrease the peak width [14-16] and, according to Eqn (6.1^{a-c}), increase the PC. Several examples for peptides have been described. Decreasing the particle diameter from 3 μm to 1.7 μm increases the PC 1.5 times [3], from 3.5 μm to 1.7 μm doubles the PC [1]. Wu *et al.* [17] showed total ion chromatograms of the analysis of a Cytochrome C digest using ultra performance (UP) LC (1.7 μm) and LC (4 μm). A homemade capillary column with a particle diameter of even 0.8 μm for the analysis of protein digests has been reported [18].

Next to the parameters described above the gradient and flow-rate also influence the peak capacity. For a fixed column length Wren [4] and Wang *et al*. [11] showed that the peak capacity increases non-linearly with the gradient time. For a non-optimized flow-rate the initial increase of the peak capacity flattens out with longer gradient times. Wang *et al.* [11] reported that for an optimum flow-rate the peak capacity increases with larger gradient times and higher temperatures. Therefore they suggest to optimize the flow-rate using the longest allowable gradient time

and the highest temperature possible. Finally the composition of the eluent at the end of the gradient is adjusted so that the last peptide elutes in the final part of the gradient.

In the present paper the influence of the P and PC** on the Mascot identification score is investigated for two monolithic columns of different length and a 1.8-μm particulate column. To better compare different gradient systems a scaling factor (SF) will be introduced. The chromatographic performance, including PC**, W_b and P, and MS performance (sequence coverage) of the monolithic columns and the particulate column are compared. As model compounds a relatively simple (Myoglobin) and a more complex (Bovine Serum Albumin, BSA) protein are used.

2 Experimental

2.1 Materials

Acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid (TFA, biochemical grade) was from Acros (Geel, Belgium), iodoacetamide (99%) and trypsin were from Sigma Aldrich (Zwijndrecht, The Netherlands) and ammonium bicarbonate (>99%) and dithiothreitol (DTT, >98%) were from Fluka (Zwijndrecht, The Netherlands). A Sartorius Arium 611 system (Göttingen, Germany) was used to prepare deionized water (>18 MΩ cm). Bovine Serum Albumin (BSA), 96%, 0.25 μg/μL and Myoglobin, 95-100%, 0.30 μg/μL were obtained from Sigma (St. Louis, MO, USA).

The chromatographic separation with the 64-cm monolithic column was performed with a Dionex/LC Packings Ultimate 2000 (Amsterdam, The Netherlands) nanoLC system. For the 15-cm monolithic and particulate column a modified Agilent 1200 Series LC system was applied. This LC system was operated in the capillary mode by using PEEKsil tubing (25 μm i.d.) as connection and splitter tubing. Due to the large volume of the injector it took 30 min to load the injection volume (2.25 μL for the particulate and 1.0 μL for the monolithic column) of the peptide sample on the top of the analytical column. Subsequently, the separation process was started by directing the mobile phase to the analytical column without passing it through the injector.

Both systems were coupled with an Agilent LC/MSD iontrap XCT (Agilent, Palo Alto, CA, USA). The monolithic columns (Chromolith CapRod RP-18 endcapped, 150x0.1 mm internal diameter (i.d.), 350x0.1 mm i.d. and 640x0.1 mm i.d.) were obtained from Merck (Darmstad, Germany). The particulate column (C18, 1.8 μm, 150x0.15 mm i.d.) was acquired from Micro-Tech Scientific (Vista, CA, USA).

2.2 Methods

For the 64-cm monolithic column LC solvent A was water with 5% (m/m) acetonitrile and 0.1% TFA, solvent B was acetonitrile with 5% (m/m) water and 0.1% TFA. The injection volume was 1.0 µL. Gradient: t_g=110 min; 0 min 0%B, 5 min 0%B, 115 min 50%B, 115.1 min 100%B, 145 min 100%B, 145.1 min 0%B, 175 min 0%B. For the particulate and 15-cm monolithic column LC solvent C was water with 0.1% TFA, solvent D was acetonitrile with 0.1% TFA. Gradient: t_g=26 min, 0 min 5%D, 30 min 5%D, 56 min 50%D, 56.1 min 95%D, 70 min 95%D, 70.1 min 5%D, 90 min 5%D.

The voltage of the MS was -4 kV. The flow-rate of the drying gas was 4 L/min and the temperature was kept at 325^{o}C. LC-MS-MS data were converted to the Mascot generic format using the data-analysis software. These .mgf files were searched against the MSDB database with Mascot's MS-MS ion search module [19]. The database was explored for tryptic peptides allowing one missed cleavage per peptide and containing (for BSA) carbamidomethyl cysteine as modification. The peptide mass tolerance was 2.0 Da and the MS-MS tolerance 0.8 Da.

Digestion procedures: 1 mg of Myoglobin was dissolved in 300 µL 100 mM ammonium bicarbonate, pH=8.2. 18 µL of the trypsin solution (750 µg/mL) were added and incubated overnight at 37^{o}C. 1 mg of BSA was dissolved in 200 µL 200 mM ammonium bicarbonate (pH=8.2) and 12.5 µL 130 mM DTT. The mixture was incubated for 30 min at room temperature. 25 µL 200 mM iodoacetamide were added and incubated for 45 min in the dark at room temperature. Excess DTT and iodoacetamide were removed by washing with ammonium bicarbonate and centrifuging (7 min/10 000 rpm) the solution thrice. The protein solution was diluted to 400 µL with the ammonium bicarbonate solution and 18 μL trypsin (750 µg/mL) were added and incubated overnight at 37^{o}C.

3 Results and discussion

3.1 Optimization and normalization of the LC conditions

The flow-rate and gradient were optimized using the fast, simple and easy univariate method for a set of model peptides as proposed by Wang *et al.* [11]. The model peptides were selected to span a range in retention time similar to that of the tryptic peptides from a BSA digest. Since it usually takes about 15 to 80 min to analyze a BSA or Myoglobin digest, depending on the column length [9, 20], it was decided to use a t_g=60 min for a 35-cm monolithic column and varying the flow-rate from 0.8 to 2.0 μL/min. The highest PC** was found at an operational flow-rate of 1.0 μL/min. For a final eluent composition of 50%B the last peptides eluted at about 60 min. These (normalized) conditions were applied for all columns. For the particulate column the flow-rate was separately optimized in the range from 1.0 to 1.5 μL/min. Most separated peptides were found at the highest flow-rate. Due to the low porosity of the column the flow-rate is limited by the maximum back-pressure of the system.

For a correct comparison of the monolithic columns it is important to scale the gradient from one column to the other. Therefore we used Eqn (6.4) describing the scaling factor (SF). In this equation, $\Delta\phi/V$ is the gradient slope (difference between modifier percentage ϕ at the beginning and at the end of the gradient divided by the liquid column volume V), t_g is the gradient time, v is the flow-rate, ε is the porosity, L is the length and r the radius of the column.

$$SF = (\Delta\phi/V)*t_g*v = (\Delta\phi/(\varepsilon L\pi r^2))*t_g*v \qquad \text{Eqn (6.4)}$$

For a correct scaling of columns the SF, *i.e.* the product of gradient slope, gradient time and flow-rate, should be kept constant. This can be done in some ways, most logically by changing the gradient time proportionally with the column length while keeping the flow-rate constant [8] or by changing the flow-rate proportionally with the column length and keeping the gradient time constant [21]. A disadvantage of the latter procedure is that the plate height is not constant when the flow-rate is varied, so we have chosen the first method. Moreover, it should be noted that for

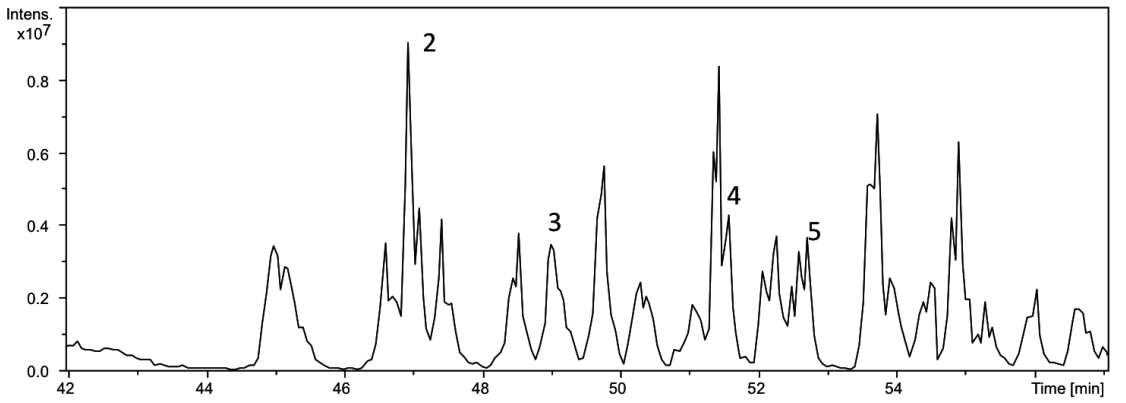
comparison of different column diameters also the injection volume should be adjusted with the ratio of the square of the internal diameter.

3.2 LC-MS-MS of protein digests

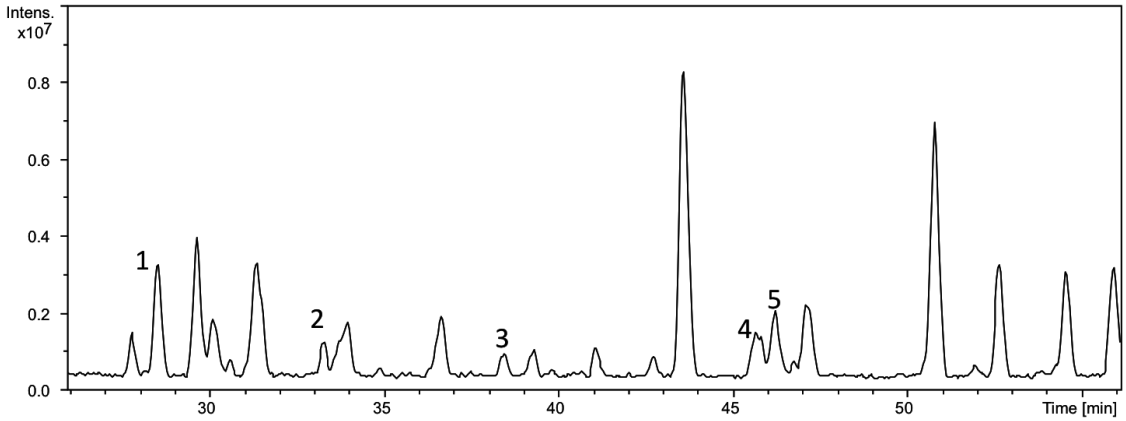
Monolithic columns

Figs 6.1[a-b] show the base peak chromatograms (bpc's) of a BSA digest using the 15-cm and 64-cm monolithic column. The gradient time was normalized to 26 and 110 min, respectively, by using Eqn (6.4). Some of the identified peaks are numbered and their identity is shown in Table 6.1. Table 6.2 shows chromatographic data and Table 6.3 identification scores (or sequence coverage) for Myoglobin and BSA. The longer gradient time for the 64-cm column leads to larger peak widths, a higher Δt_r and a higher PC (values not shown) and PC** (a factor of 1.5 to 3). If chromatograms with the same gradient slopes are compared the ratio of the peak capacity should be close to the square root of the column length ratio (=2.0) [8]. For our system a ratio of 1.5 has been found. Van de Meent *et al.* [9] noticed the same deviation using relative long gradient times. Due to the smaller peak width of peptides in the 15-cm column the productivity P is almost doubled.

The protein identification scores for the longer column are higher. Due to this longer length the peptides are better separated (higher plate number) and more peptides have been identified (*e.g.* peptide 1 is not identified in Fig. 6.1[a]). However, a longer column does not always lead to a higher identification score. For a BSA digest Miyamoto *et al*. [22] reported an increase of the PC comparing a 28-cm and 300-cm monolithic silica capillary column. The PC for the latter column was the highest. Despite the different length both bpc's show the same number of peaks. For the longer column they only elute in a wider time frame resulting in the same protein identification score. Van de Meent and De Jong have observed the same behaviour for a BSA digest. The sequence coverage for a 15-cm (100 μm i.d., t_g: 15 min) and 75-cm (200 μm i.d., t_g: 75 min) monolithic capillary column is 29% for both columns despite the higher PC found for the longer column [9]. They also analyzed a mixture of four digested proteins using 15- and 75-cm columns with the same internal diameters (100 μm). The PC for the long column was again



(a)



(b)

Fig. 6.1. Base peak chromatogram of a BSA digest obtained with a 15-cm capillary monolithic column (a) and a 64-cm monolithic capillary column (b). Peak numbers correspond to peptides listed in Table 6.1. Flow-rate: 1.0 µL/min; for gradient scheme and further conditions, see text.

higher. For long (normalized) gradient times (45 vs 225 min) the sequence coverage for BSA was about the same (27%). However, for short (normalized) gradient times (9 vs 45 min) they obtained a sequence coverage of 19% and 33% for the short and long column, respectively [10].

Particulate column

As the i.d. of the particulate column is 150 µm, the injection volume has been increased with a factor 2.25 compared to the monolithic columns. Fig. 6.2 shows the chromatogram of a BSA digest using the 15-cm 1.8-*µ*m particulate column. Some of the identified peaks are again numbered and their identity is shown in Table 6.1. Table 6.2 shows chromatographic data and Table 6.3 identification

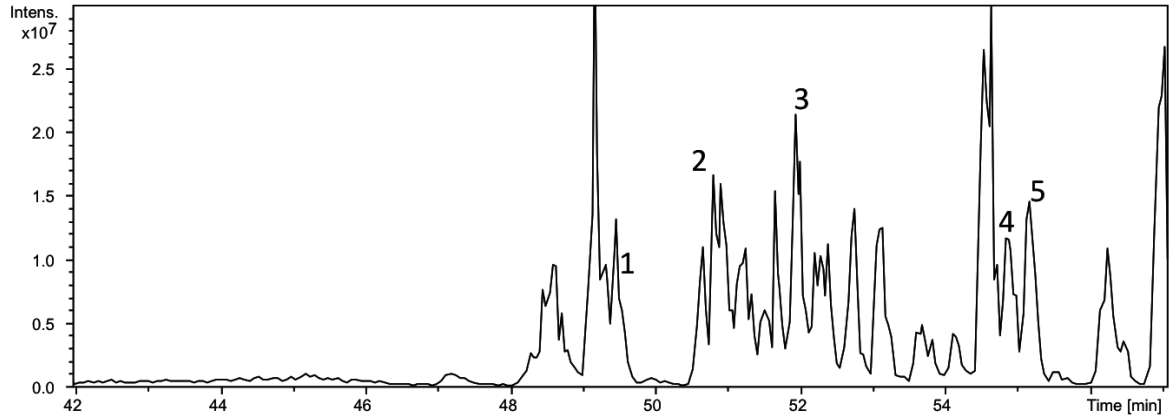


Fig. 6.2. Base peak chromatogram of a BSA digest obtained with a 15-cm capillary 1.8-µm particulate column. Peak numbers correspond to peptides listed in Table 6.1. Flow-rate: 1.5 µL/min; for gradient scheme and further conditions, see text.

scores for Myoglobin and BSA. As mentioned before due to the column characteristics the smallest peak width (see Table 6.2) is obtained for the 1.8-μm particle column. Compared to the monolithic column with the same length higher P and PC** values have been obtained. For Myoglobin the P and PC** increases from 4.5 to 5.0 and from 59 to 136 and for BSA from 3.4 to 4.0 and from 66 to 124, respectively. The increment of P is caused by the smaller peak width (see Eqn (6.3)). Due to the different selectivity and lower linear flow-rate Δt_r in Eqn (6.1^c) is higher. In combination with the smaller peak width this also improves PC**. The PC values (not in Table 6.2) are less increased as t_g is used instead of Δt_r (Eqn (6.1^b)). For both columns t_g is the same and the difference in PC is only caused by W_b. The peak intensity (see the figures) for the particulate column is about a factor 3 higher compared to the monolithic columns. The higher sequence coverage, 89% vs 80% and 54% vs 34% for Myoglobin and BSA respectively, is not caused by the larger injection volume used for the particulate column. Earlier studies revealed that doubling the injection volume from 1.0 to 2.0 µL do not result in a significantly higher sequence coverage. So, due to the smaller peak width more peptides are separated and identified leading to a higher sequence coverage.

Comparing the particulate column with the longer monolithic column the PC** (BSA only) and P (for both digests) are the highest using the first column. For BSA the P and PC** increases from 2.0 to 4.0 and 100 to 124, respectively. For Myoglobin, because of the relatively large Δt_r, the long monolithic column shows a

Table 6.1. Examples of identified peptides in a BSA digest (see Fig. 6.1 and 6.2)

	Peptide
1	YICDNQDTISSK
2	EYEATLEECCAK
3	ECCHGDLLECADDR
4	LKPDPNTLCDEFK
5	KVPQVSTPTLVEVSR

Table 6.2. Chromatographic data of Myoglobin and BSA tryptic digests

	15 cm x 100 µm i.d. (monolithic)		*64 cm x 100 µm i.d. (monolithic)*		*15 cm x 150 µm i.d. (1.8 µm)*	
	Myoglobin	*BSA*	*Myoglobin*	*BSA*	*Myoglobin*	*BSA*
W_b (min)	0.22	0.29	0.47	0.51	0.20	0.25
Δt_r (min)	13	19	78.3	51.0	27.2	30.9
t_g (min)	26	26	110	110	26	26
PC**	59	66	167	100	136	124
P (min^{-1})	4.5	3.4	2.1	2.0	5.0	4.0

Table 6.3. MS data of Myoglobin and BSA tryptic digests

	15 cm x 100 µm i.d. (monolithic)		*64 cm x 100 µm i.d. (monolithic)*		*15 cm x 150 µm i.d. (1.8 µm)*	
	Myoglobin	*BSA*	*Myoglobin*	*BSA*	*Myoglobin*	*BSA*
Mascot score	712	1137	727	1368	800	1772
Sequence coverage (%)	80	34	86	48	89	54

higher PC**. In contrast, the PC (values not in Table 6.2) for the longer monolithic columns is the highest due to the very long t_g (factor 1.5 to 2 compared to the Δt_r). The sequence coverage for the small Myoglobin is only slighty increased using the particulate column (from 86% to 89%); for BSA the increment is larger (from 48% to 54%). Probably the maximum score for the small protein is almost reached with the longer monolithic column.

Fig. 6.3 shows the identified peptides of BSA using the 15-cm monolithic column (top) and the particulate column (bottom). More peptides, cut by trypsin on the carboxyl side of Arginine (R) and Lysine (K), are identified using the particulate column. However, due to the different type of stationary phase not all identified peptides using the monolithic column are also identified using the particulate column (as marked in Fig. 6.3, EACFAVEGPK).

```
  1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
 51 FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
201 LLPKIETMRE KVLTSSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
601 STQTALA
```

```
  1 MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EHFKGLVLIA
 51 FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL HTLFGDELCK
101 VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF
151 KADEKKFWGK YLYEIARRHP YFYAPELLYY ANKYNGVFQE CCQAEDKGAC
201 LLPKIETMRE KVLTSSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE
251 FVEVTKLVTD LTKVHKECCH GDLLECADDR ADLAKYICDN QDTISSKLKE
301 CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL
351 GSFLYEYSRR HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL
401 KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
451 RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC
501 TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP DTEKQIKKQT
551 ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV
601 STQTALA
```

Fig. 6.3. BSA sequence showing identified peptides (in bold) by using a 15-cm capillary monolithic column (top) and a 15-cm capillary 1.8-µm particulate column (bottom).

The above-discussed results show that decreasing the particle diameter to 1.8 μm is more beneficial for the productivity P and protein identification score than increasing the monolith column length with a factor 4. Comparison of the results from our system with literature data is rather difficult (despite the SF introduced) because different gradient times, column lengths, particles sizes and peptide samples are used. P was calculated using Eqn (6.2) from the available data in the references. Liu *et al*. [3] measured a PC of 150 and a P of 6 using a 15-cm capillary column and a t_g of 25 min. This is comparable with our PC (for Myoglobin 130) and P. Neue [7] presents a P of 8.5 with a 15-cm (2.1 mm i.d.) column and a gradient time of 23 min. Tolley *et al*. [23] determined the sequence coverage for a BSA digest using a capillary particulate column. For a 22-cm capillary column they obtained a BSA protein coverage of 73% using a high resolution MS (Q-TOF). The productivity (P=3) was lower compared to our system. Finally, Shen *et al.* [18] used a capillary column with 0.8 μm particles and a t_g of 25 min and found a PC of 250 and a high productivity of 10 for a tryptic digest.

4 Conclusions

The sample peak capacity and identification score using a long monolithic column are higher than for a small column mainly because of the higher plate number and the longer gradient time. For the short column the productivity (peak capacity per unit time) is the highest. Smaller peak widths, a higher productivity and protein identification scores are obtained for the 1.8-μm particulate column. For BSA, the sequence coverage increases from 34% (short monolithic column), 48% (longer monolithic column) to 54% (particulate column) and for Myoglobin from 80%, 86% to 89%, respectively. So further increase of the chromatographic performance for Myoglobin will hardly increase the score. For BSA, other large proteins or mixtures of proteins the higher chromatographic performance will be more advantageous. Better separation can be obtained by use of a longer particulate column containing relatively small particles. However, the limiting factor for such a column will be the lower permeability. In conclusion monolithic and UPLC columns are very promising for the LC analysis of protein digests.

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Chapter 7

Comparison of poly(styrene-divinylbenzene) and RP-18 silica monolithic capillary columns of 5-cm for the analysis of protein digests

Abstract

The comparison of two popular types of monolithic capillary LC columns (silica-C18 and poly(styrene-divinylbenzene)) of the same length for the analysis of protein digests is reported. After optimizing flow-rate and gradient steepness simple protein digests were analyzed. The calculated chromatographic parameters, like productivity and peak capacity, and identification score for both columns are about the same. For a more complicated nine protein digest the performance of the silica monolith is slightly better.

1 Introduction

Before the introduction of ultra small particle columns with a high chromatographic performance [1], monolithic columns were launched in the late 1980s as newest type of stationary phase for liquid chromatography (LC). These monolithic (‘*one stone*’) materials consist of a continuous support, prepared directly in the separation column, filling it completely thus avoiding interparticular spaces. Other benefits, compared to particulate columns, are the absence of retaining frits, high permeability and low resistance to mass transfer [2-4]. Recently the properties, preparation and proteomic applications using capillary monolithic materials focusing on silica, poly(styrene-divinylbenzene) (PS/DVB) and methacrylate have been reviewed [4]. Tao *et al*. [5] reviewed micro/nano-scale LC for proteome research paying attention on multidimensional LC including the use of monolithic columns. Nischang *et al.* [6] published an interesting review on the preparation of polymer monoliths.

Monolithic columns can be divided in two main categories, *i.e*. silica-based and polymer-based materials. The first inorganic types are commercialized by Merck (Darmstadt, Germany); the commercially available polymer-based columns PS/DVB can be obtained from LC Packings (Sunnyvale, CA, USA). The morphology of the silica monolith is completely different from that of the organic polymeric monolith. The latter consist of little organized microglobules with large pores located among them (low surface area). In contrast, the silica-based material has a well-ordered structure of through-pores and mesopores providing a lower back-pressure and larger surface area [2]. Therefore the separation

efficiency for small molecules (*e.g.* peptides) of polymer-based monolithic columns remains low compared to C18 silica monoliths of the same length [2]. However, according to Saito *et al*. [7] the polymer-based monoliths are in general better suited for separation of proteins because the silanol interactions of the silica-based materials are avoided. A remedy can be the use of endcapped columns (see the 'materials and methods' section).

Both PS/DVB and silica materials are used for the separation of peptides. For example, Premstaller *et al.* [8] and Walcher *et al.* [9] showed the potential of PS/DVB columns in proteomics for the mass fingerprinting of peptides obtained via tryptic digestion. Yue *et al*. [10] identified 4400 peptides within an analysis time of 4.4 h using a capillary column (10 μm i.d.). For silica monoliths the applications are numerous, for example the fast separation of a Myoglobin digest [11], a BSA digest [1], a mixture of digested proteins [12] and a human serum digest [13]. Luo *et al.* [14] even analyzed 2400 peptides in an 10 h run using a 70 cmx20 μm i.d. column. Finally, Tao *et al.* [5] showed some recent applications in their review, like the development of silica based columns with integrated MS (mass spectrometry) tips.

In the present paper the performance of a PS/DVB and silica capillary monolithic column for the analysis of tryptic protein digests is compared. Gurcya *et al.* [3] compared a 15-cm silica monolith with a 5-cm PS/DVB monolith with the same i.d. and linear mobile-phase velocity but different gradient steepness and temperature. For the latter column they calculated a sample peak capacity (PC**, see below) of only 65 (inferior peak shapes) and about 140 for the silica monolith for a Cytochrome C digest. The number of identified proteins for the PS/DVB column is lower compared with the Chromolith column (197 vs 270) for an *Arabidopsis thaliana* digest. In contrast, Van de Meent *et al.* [15] conclude that the silica monoliths do not outperform the PS/DVB monoliths. They found a much higher sample peak capacity (170) for their PS/DVB column when working at a higher flow-rate and slightly higher temperature. The elevated temperature increases the separation efficiency due to the reduction of the resistance of mass transfer by higher diffusivity of the peptides. In the current paper a simple (Myoglobin, BSA) and a more complex (mixture of 9 proteins) digest is analyzed using ambient

temperature and 5-cm column length, the only commercially available PS/DVB column length. The peak capacity (PC), sample peak capacity (PC**), productivity (P; *e.g.* peak capacity per unit time) and the MS sequence coverage is compared for both systems. These chromatographic performance parameters were calculated using Eqn (7.1) – (7.3) where t_g is the gradient time, $t_{r,1}$ is the retention time of the first, $t_{r,n}$ is the retention time of the last eluting peptide and W_b is the baseline peak width. For a more detailed explanation of these equations we refer to reference [1].

$$PC = 1+(t_g/W_b) \approx t_g/W_b \text{ } (when\ t_g/W_b >> 1) \quad \text{Eqn (7.1)}$$

$$PC^{**} = (t_{r,n} - t_{r,1})/W_b = \Delta t_r/W_b \quad \text{Eqn (7.2)}$$

$$P = PC/t_g = PC^{**}/\Delta t_r = 1/W_b \quad \text{Eqn (7.3)}$$

A different approach by evaluating different column materials is suggested by Eeltink *et al*. [16, 17]. They use the kinetic-plot method taking the effect of column structure on peak width and permeability into account. In this process the gradient time to column dead time ratio (t_g/t_0) is maintained constant when applying different flow-rates. The separation performance of the different column materials is visualized via kinetic plots depicting the gradient time required to achieve a certain PC when operating at the maximum system pressure. In contrast to our approach they only focus on the chromatographic performance and do not take the protein identification score as a parameter into account.

2 Experimental

2.1 Materials

Acetonitrile (gradient grade) was purchased from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid (TFA, biochemical grade) was from Acros (Geel, Belgium), iodoacetamide (99%) and trypsin were from Sigma Aldrich (Zwijndrecht, The Netherlands) and ammonium bicarbonate (>99%) and dithiothreitol (DTT, >98%) were from Fluka (Zwijndrecht, The Netherlands). A Sartorius Arium 611

system (Göttingen, Germany) was used to prepare deionized water (>18 MΩcm). Samples (0.3 µg/µL): Cytochrome C, Bovine Serum Albumine (BSA), β-Lacotoglobuline A, Carbonic Anhydrase, Catalase, Lysozyme, Myoglobin, Ribonuclease A and α-Lactalbumine were all obtained from Sigma (St. Louis, MO, USA).

The silica column (Chromolith CapRod RP-18 endcapped, 150x0.1 mm i.d.) was obtained from Merck (Darmstad, Germany). The length of this column was reduced to 50 mm by cutting 100 mm. The poly(styrene-divinylbenzene) column (50x0.2 mm i.d.) was acquired from Dionex/LC Packings.

2.2 Methods

Solvent A was water with 5% (m/m) acetonitrile and 0.1% TFA, solvent B was acetonitrile with 5% (m/m) water and 0.1% TFA. The injection volume was 1.25 µL for the silica-based column and 5.0 µL for the PS/DVB column (adjusted for the ratio of the square of the i.d. [1]).

The chromatographic separations were performed at ambient temperature with a Dionex/LC Packings Ultimate 2000 (Amsterdam, The Netherlands) nanoLC system. The system was coupled with an Agilent LC/MSD iontrap XCT (Agilent, Palo Alto, CA, USA).

Gradient steepness of 0.5%/min: t_g=100 min; 0 min 0%B, 5 min 0%B, 105 min 50%B, 105.1 min 100%B, 135 min 100%B, 135.1 min 0%B, 165 min 0%B.

Gradient steepness of 1.0%/min: t_g=50 min; 0 min 0%B, 5 min 0%B, 55 min 50%B, 55.1 min 100%B, 85 min 100%B, 85.1 min 0%B, 115 min 0%B.

Gradient steepness of 1.9%/min: t_g=26.3 min; 0 min 0%B, 5 min 0%B, 31.3 min 50%B, 31.4 min 100%B, 61.3 min 100%B, 61.4 min 0%B, 91.3 min 0%B.

Gradient steepness of 2.5%/min: t_g=20 min; 0 min 0%B, 5 min 0%B, 25 min 50%B, 25.1 min 100%B, 55 min 100%B, 55.1 min 0%B, 85 min 0%B.

Gradient steepness of 4%/min: t_g=12.5 min; 0 min 0%B, 5 min 0%B, 17.5 min 50%B, 17.6 min 100%B, 47.5 min 100%B, 47.6 min 0%B, 77.5 min 0%B.

Gradient steepness of 5%/min: t_g=10 min; 0 min 0%B, 5 min 0%B, 15 min 50%B, 20.1 min 100%B, 45 min 100%B, 45.1 min 0%B, 75 min 0%B.

The applied voltage of the MS was -4 kV. The flow-rate of the drying gas was 4 L/min and the temperature was kept at 325°C. LC-MS-MS data were converted to the Mascot generic format using the data-analysis software. These .mgf files were searched against the MSDB database with Mascot's MS-MS ion search module [18]. The database was explored for tryptic peptides allowing one missed cleavage per peptide and containing (for BSA) carbamidomethyl cysteine and M-oxidization as modifications. The peptide mass tolerance was 2.0 Da and the MS-MS tolerance 0.8 Da.

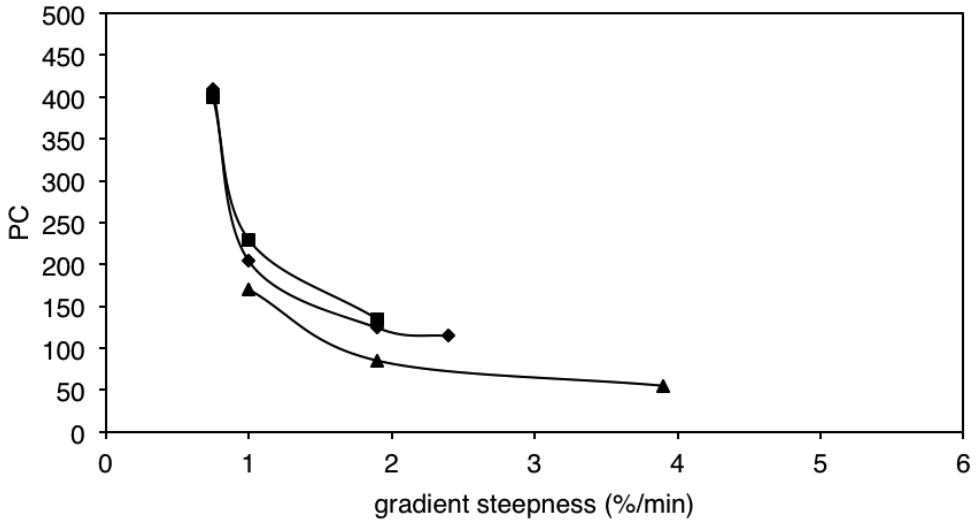
Digestion procedures: 1 mg of each protein was dissolved in 200 µL 200 mM ammonium bicarbonate (pH=8.2) and 12.5 µL 130 mM dithiothreitol (DTT). The mixture was incubated for 30 min at room temperature. 25 µL 200 mM iodoacetamide were added and incubated for 45 min in the dark at room temperature. Excess DTT and iodoacetamide were removed by washing with ammonium bicarbonate and centrifuging (7 min/10,000 rpm) the solution for three times. The protein solution was diluted to 400 µL with the ammonium bicarbonate solution and 18 *µ*L trypsin (750 µg/mL) were added and incubated overnight at 37°C. For the digested protein mixture the individually digested proteins (10 µL) were collected in one sample.

3 Results and discussion

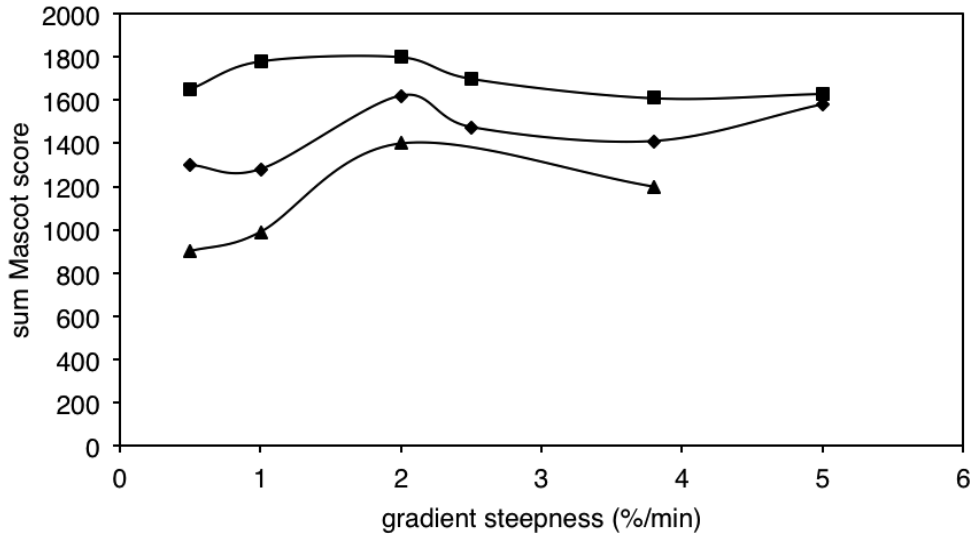
Optimization of the LC conditions

The LC conditions for the analysis of the digested protein mixture using the polymeric column were at ambient temperature optimized, by varying flow-rate and gradient steepness, to obtain the best chromatographic performance (expressed as the PC) and largest MS-MS protein identification score. The flow-rate was varied between 0.8 and 2.2 µL/min, limited by the maximum backpressure of about 300 bar, and the gradient steepness between 0.5 %/min (t_g=100 min) to 5 %/min (t_g=10 min). Fig. 7.1[a] shows the PC vs the gradient steepness at various flow-rates. According to the optimization theory of Wang *et al*. [19] the best PC is obtained at the highest gradient time and a medium flow-rate. This highest gradient time was 100 min, corresponding with a gradient steepness of 0.5%/min. The highest flow-rate (2.2 µL/min) shows the lowest PC. Fig. 7.1[b] compares the

MS-MS protein identification score (sum of the nine individually protein Mascot scores) using different gradient time/steepness and flow-rates. The highest score is obtained at a gradient steepness between 1 and 2%/min (respectively a gradient time of 50 and 26 min) at a flow-rate of about 1.5 µL/min. Combining the optimization results of both figures we decided to use a relatively short gradient time (26 min; 1.9%/min) and a flow-rate of 1.5 µL/min. A representative base peak chromatogram (bpc) of the digested protein mixture using these optimized conditions is shown in Fig. 7.2.



(a)



(b)

Fig. 7.1. (a) PC vs gradient steepness and (b) sum Mascot score vs gradient steepness for the nine digested protein sample using the PS/DVB column: 0.8 µL/min (diamonds), 1.5 µL/min (squares) and 2.2 µL/min (triangles). For further conditions, see text.

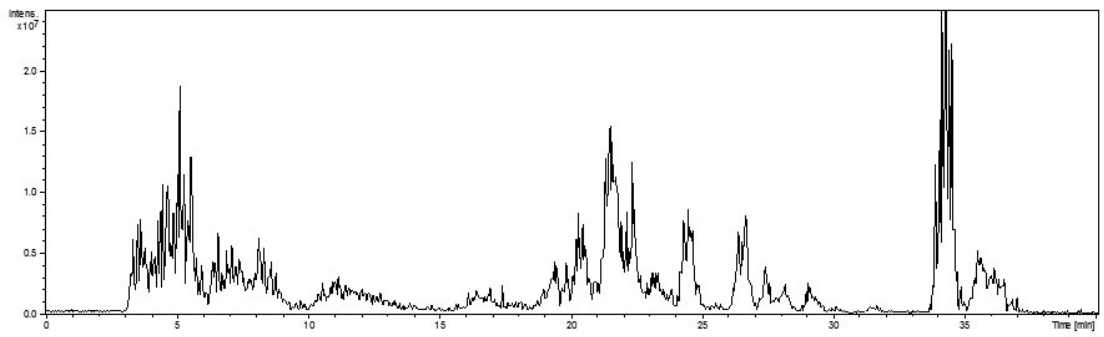


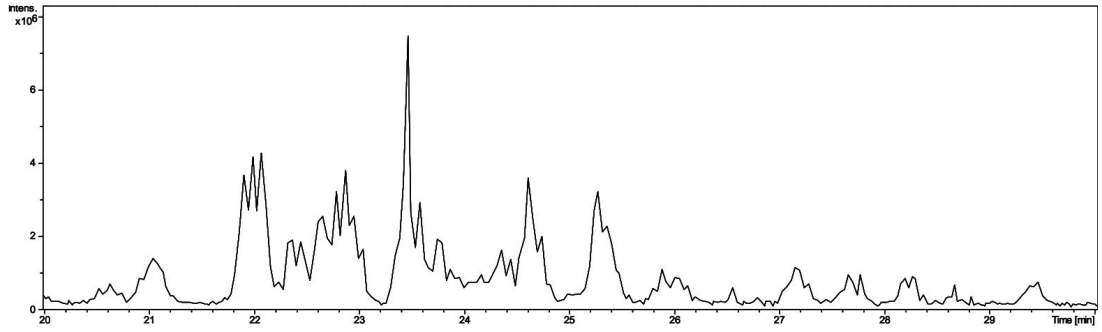
Fig. 7.2. Base peak chromatogram of the nine digested protein sample using the PS/DVB column and a flow-rate of 1.5 µL/min and a gradient steepness of 1.9 %/min.

The LC conditions for the analysis of a protein digest using the silica column were already optimized in a previous study [20]. For a 35-cm column, using the same composition of solvent A and B and also working at ambient temperature, the optimum flow-rate lies between 1.2 – 1.5 µL/min and a gradient time of 60 min (0.8%/min). Normalizing for the 5-cm column will result in a gradient time of only 9 min (5.6%/min). As noticed earlier [11] such a short gradient time will probably lead to coeluting peaks and a lower protein identification score. Therefore we decided to use the same gradient time as for the polymeric column, *i.e.* 26 min (1.9%/min), and an optimum flow-rate of 1.5 µL/min.

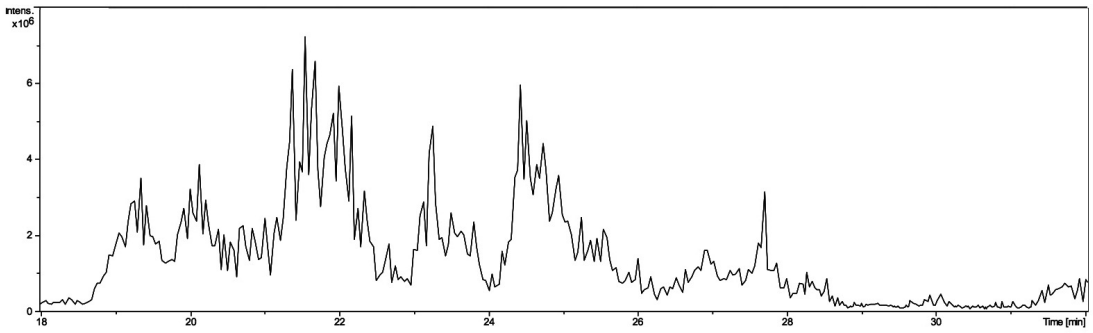
Comparison of a monolithic PS/DVB and silica column

For a correct comparison the injection volume was tuned by the ratio of the square of the internal diameter [1]. Fig. 7.3[a-b] show the bpc of a BSA digest using the silica and PS/DVB column, respectively. Due to the different selectivity the chromatograms show a different retention behavior of the eluting peptides. Table 7.1 shows chromatographic data and Mascot scores and sequence coverages of the studied proteins. As noticed earlier [1], for both columns the peak width for the peptides of the BSA digest is slightly wider compared to the peptides of the Myoglobin digest. The Δt_r of the Myoglobin digest is larger than the Δt_r of the BSA digest due to the different identity and retention time of the first and last eluting peptide. The PC for BSA and Myoglobin is for both columns about the same. The PC** for the silica column for these two digests is higher because of the larger Δt_r.

Due to the constant peak width the productivity P for these two columns is the same; because of the same chromatographic efficiencies of both columns, the



(a)



(b)

Fig. 7.3. Base peak chromatogram of a BSA digest obtained using (a) 5-cm silica RP-18 and (b) a 5-cm PS/DVB monolithic capillary column. For gradient scheme and further conditions, see text.

Table 7.1. Chromatographic and MS data of several protein digests

	5 cm x 100 µm i.d. silica column			*5 cm x 200 µm i.d. PS/DVB column*		
	Myoglobin	*BSA*	*Mixture*	*Myoglobin*	*BSA*	*Mixture*
W_b (min)	0.21	0.27	0.25	0.21	0.26	0.30
Δt_r (min)	28.7	26.2	37.3	25.6	20.6	35
PC	124	96	104	124	100	87
PC**	137	97	149	122	79	117
P (min^{-1})	4.8	3.7	4	4.8	3.9	3.3
Mascot score			1811			1800
Sequence coverage (%)	86	54		90	49	

sequence coverage is almost the same. The chromatographic performance for the silica monolith regarding the digested protein mixture is at its maximum. The smaller peak width and higher Δt_r leads to a higher PC, PC** and P but not to an increased identification score. Probably the smaller chromatographic performance of the PS/DVB column is sufficient for the relatively high protein identification score.

According to Guryca *et al.* [3] the C18 silica monolith is superior compared to the PS/DVB monolith due to a better peak profile and a superior sample peak capacity. However, they compared different column lengths, gradient steepness and temperatures. Van de Meent *et al.* [15] disagree with Guryca's [3] conclusion. They found a good performance for their studied polymer columns using a higher flow-rate (3 μL/min) and a slightly higher temperature (60^{o}C instead of 50^{o}C). Our comparison of *5-cm* columns at ambient temperature show that for simple proteins, like BSA and Myoglobin, the chromatographic efficiency (W_b, PC and P) and sequence coverage is about the same. For a more complex mixture of several digested proteins the chromatographic performance for the silica monolith is a little bit higher but does not lead to an increased identification score.

4 Conclusions

The comparison of two main types of commercially available monolithic capillary LC columns, of the same length, used in reversed-phase liquid chromatography is carried out. Under the conditions used the silica monolith does not outperform the PS/DVB one for simple digests. The peak capacity, productivity (peak capacity per unit time) and sequence coverage using 5-cm columns are about the same. For Myoglobin their mean values are 124, 4.8 and 88% and for BSA 98, 3.8 and 52%, respectively. The sample peak capacity for the silica column is slightly higher due to a larger time difference between the first and last eluting peptide. For a mixture of a nine protein digest this monolith shows better chromatographic results but hardly a higher protein identification score.

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Chapter 8

Off-gel isoelectric focusing for prefractionation of peptides and proteins

Abstract

High resolution peptide or protein separation is crucial for the identification of proteins in proteomics. Due to some well known drawbacks, like the low sensitivity and the difficulty with the direct connection to the mass spectrometer due to the presence of carrier ampholytes, the traditional separation technique, two-dimensional electrophoresis, is gradually replaced by other prefractionation methods. A powerful substitute is the off-gel electrophoresis-isoelectric focusing (OGE-IEF) system in combination with LC-MS. In this manuscript these techniques are shortly described and compared, including benefits and shortcomings. For the off-gel electrophoresis technique a number of selected applications are studied, like the analysis of proteins/protein digests in different biological matrices and the comparison of this technique with (cation exchange) LC. Some preliminary experiments have been performed, like the analysis of model peptides/proteins and digests with OGE-IEF in the first and LC-UV, LC-MS and SDS-PAGE in the second dimension.

1 Introduction

Since 1975 two-dimensional electrophoresis (2-DE) is the major tool for studying proteins [1]. The proteins are separated based on their isoelectric point (pI) by isoelectric focusing (IEF) in the first and on their molecular weight (MW) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension [1, 2]. In 1982 the technique is further refined for the first dimension by using immobilized pH gradients (IPG) strips [3]. This orthogonal method separates thousands of proteins in a single run and with high resolution. Small differences in pI or MW *e.g.* due to phosphorylation or glycosulation or metabolism, can be separated [1]. Typically, the proteins are made visible as spots and can be transferred to *e.g.* the mass spectrometer (MS) for further analysis. Also proteolysis with *e.g.* trypsin or pepsine on the spot is possible. The major drawbacks are the limited applicability to hydrophobic proteins, extreme MW or pI [4], the low sensitivity (in the μM range) so only high abundant proteins are made visible [5, 6], the difficulty to automate [6] and the presence of carrier ampholytes makes the direct connection with the MS complicated. Due to these limitations

other prefractionation techniques have been developed based on chromatography and electrophoreses [5, 7]. As discussed by Righetti *et al.* [5, 8] examples of the first practice are affinity, ion-exchange and reversed-phase (RP) chromatography, examples of the latter are multi-compartment electrolyzers, like the off-gel (Agilent) and Zoom IEF fractionator (Invitrogen) or the Rotofor (BioRad) [8, 9]. The latter two methods use carrier ampholytes which need to be removed for subsequent steps (like MS) [9]. Also the pI accuracy of the Rotofor is quite poor [5]. So the off-gel method seems to be the most promising.

For the off-gel isoelectric focusing (OGE-IEF) technique, commercialized in 2006 [1], analyte separation takes place in a two-phase system with an upper liquid phase that is divided in compartments (wells) and a lower phase that is an IPG gel strip. See Fig. 8.1. The sample is diluted and loaded into all wells and charged according to their pI and the pH imposed by the gel. An electric field is applied between two electrodes located on the beginning and end of the gel. There is no open fluidic connection between the wells so the analytes are forced to migrate through the gel where the actual separation takes place. Once the analytes reach the well in which $pH_{well} = pH_{strip} = pI_{analyte}$ the compounds will lose their charge and can be recovered from that particular compartment for further processing [7, 10]. The resolution of the technique depends on the number of wells and pH range of the strip. The analytical throughput is high because multiple samples can be run simultaneously and in parallel [11]. It is fast, repeatable and the preconcentrated low volume fractions (about 150 μL or less [10]) can be directly analyzed [12], for example by MS due to the already mentioned absence of carrier ampholytes [9]. Finally, the pI of the analytes can easily be determined [10].

The OGE-IEF technique can be used both for the separation of proteins and peptides. Intact proteins can be fractionated first and then further analyzed and identified or they can be digested into peptides first, fractionated, analyzed and identified. This latter ‘shot-gun’ approach has the advantage of peptides being easier to keep in solution, so proteins are not lost, but the context to parent proteins is lost and sample complexity increased. The first method has the benefit to monitor distributions of intact protein isoforms and protein fragments [13].

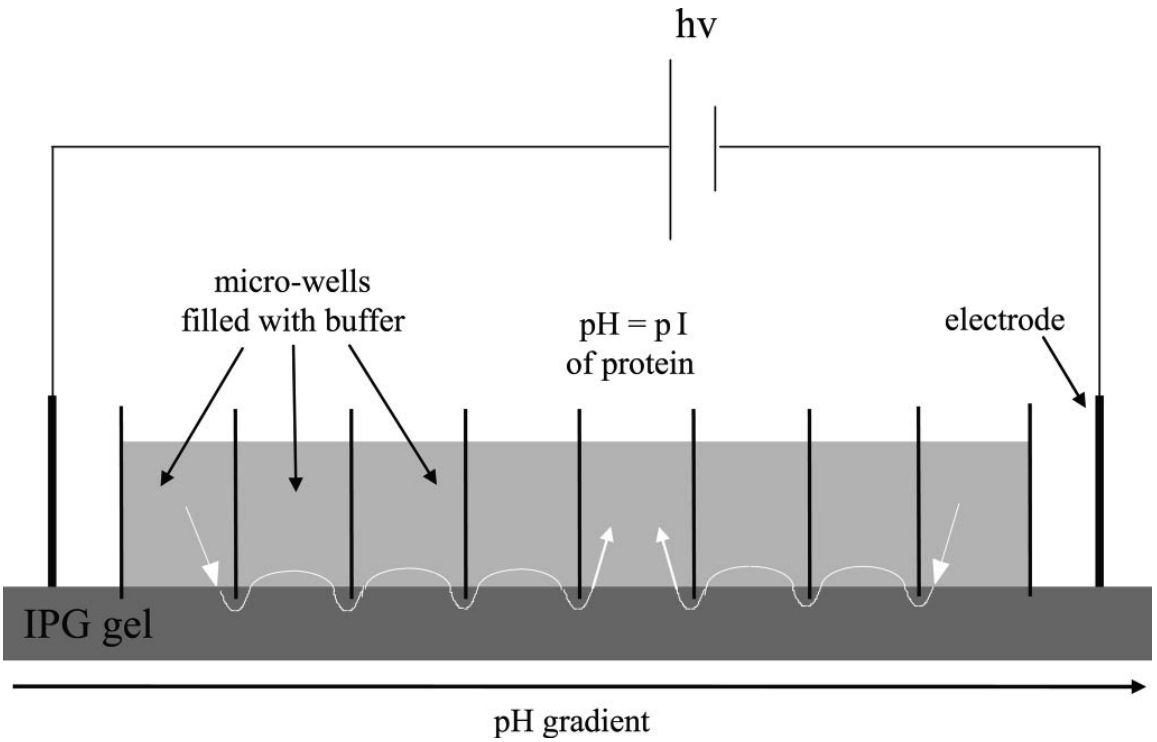

Fig. 8.1. Schematic presentation of the setup used for OGE-IEF of proteins (or peptides) [10].

Usually both approaches are combined with LC-MS-MS. See [2] and [14] for the analysis of peptides. Already in 2003 the group of Girault [6] shows an experimental setup to perform some initial experiments. They demonstrated the gradually distribution of β-Lactoglobulin B into one well after 0, 4, 8 and 24 h and of an 11-proteins sample into a limited number of wells after 17 h.

For the separation of peptides in the first dimension some groups compare the performance of LC vs OGE-IEF. In the second dimension (nano) RPLC-MS-MS was used. Yang *et al.* [4] compared high pH reversed phase-LC (hpRP) vs OGE-IEF. They prefer the hpRP-LC technique due to more unique peptide/protein identifications and a higher sequence coverage. Elschenbroich *et al.* [7] and Waller *et al.* [9] compare both OGE-IEF with strong-cation exchange (SCX) LC. The first group concludes that both techniques are equally capable of high quality peptide separations in terms of peptide/protein identification and reproducibility of a sample with medium complexity. Waller *et al.* [9] prefer the OGE-IEF technique (156 identified proteins instead of 115 when using SCX-LC) for the analysis of a more complex sample, a cerebrospinal fluid (CSF) digest. An *E. coli* tryptic digest

is separated using OGE-IEF followed by LC-MS-MS. About 90% of the identified peptides were found in only 2 of the 24 available wells [10].

For the separation of proteins Aziz *et al*. [15] and Bonzon-Kulichenko *et al*. [16] combine SDS-PAGE of proteins with in-gel digestion joint with OGE-IEF and (nano)LC-MS-MS. The first group was able to identify 130 proteins from hydatid cyst fluid, a complex biological mixture containing a wide range of proteins of parasite and host origin [15]. Surowiec *et al* [12] and Burgess *et al* [17] combine OGE-IEF with SDS-PAGE and the identification of certain spots by nanoLC-MS-MS to analyze biomarkers to prove if chicken meat is mechanically recovered (cheaper in price) or hand-deboned [12] or any form of brain insult in CSF [17]. In the latter application high-abundance proteins were first removed by immunodepletion so odd proteins are better detectable. In CSF and serum these large quantity proteins, Albumin and IgG's, represent more than 50% of the total protein mass [8].

Vaezzadeh *et al.* [11] use a one-step sample concentration, purification, Albumin depletion by means of a Vivaspin filter with an anti-HSA resin. The remaining proteins are digested, separated and analyzed using an OGE-IEF – LC-MS-MS system. Stalder *et al*. [13] and Michel *et al.* [18] combine immunodepletion of the most abundant proteins in human serum [13] or plasma [18], followed by OGE-IEF, tryptic digestion and (nano)LC-MS-MS

Heller *et al.* [19] combined two-stage OGE-IEF: protein separation in the first stage followed by tryptic digestion of the contents of one well followed by a second OGE-IEF step of the obtained peptides. The separated peptides were analyzed using LC-MS-MS. 53 proteins in human plasma were identified.

In the present paper our aim is to separate peptides or proteins by two dimensions; OGE-IEF in the first and LC in the second dimension. Preliminary experiments using the above discussed approaches are performed. Peptides and proteins are analyzed by OGE-IEF - LC-UV. Proteins are separated by OGE-IEF and visually detected or off-line digested and further separated using LC-MS-MS. Finally a standard serum solution was spiked with proteins and after depletion of the most abundant proteins analyzed by OGE-IEF and PAGE in the second dimension.

2 Experimental

2.1 Materials

Immobiline Drystrips, linear pH range 3.0–10.0, 24-cm length and IPG buffer pH 3.0-10.0 (carrier ampholyte mixture) were purchased from General Electric Health Care (Diegem, Belgium). The PlusOne Drystrip Cover Fluid was obtained from Amersham Biosciences (Piscataway, NJ, USA). The centriplus centrifugal filters with a MW cutoff of 30 kDa were acquired from Millipore (Bedford, MA, USA). Acetonitrile (gradient-grade) was purchased from Biosolve (Valkenswaard, The Netherlands), trifluoroacetic acid (TFA, biochemical-grade) was from Acros (Geel, Belgium), iodoacetamide (99%) and trypsin were from Sigma-Aldrich (Zwijndrecht, The Netherlands) and ammonium bicarbonate (>99%) and dithiothreitiol (DTT, >98%) were from Fluka (Zwijndrecht, The Netherlands). A Sartorius Arium 611 system (Göttingen, Germany) was used to prepare deionized water (>18 MΩcm). Ammonium bicarbonate and all proteins were obtained from Sigma (St. Louis, MO, USA).

2.2 Methods

OGE-IEF and SDS-PAGE experiments

The OGE-IEF separations were performed with a prototype Agilent OGE G3100A (Agilent, Palo Alto, CA, USA). This apparatus is composed of a linear raw of 24 adjacent polyurethane wells separated by vertical walls. Each OGE fraction contains peptides or proteins spanning a pI range of approximately 0.3 units. The top and bottom of each well are opened to enable sample introduction and uptake and direct contact with the immobilized pH gradient gel (IPG). The multiwall device was placed on top a Immobiline DryStrip. An electrode was placed near each extreme compartment (lowest and highest pH) containing respectively 10 mM H_3PO_4 and 20 mM NaOH. The separations were run by adding 40 μL of 1% IPG buffer, to allow the DryStrip to swell for one hour, and 150 μL sample (0.1 to 0.15 mg/mL) in each well. Starting voltage was set to 100 V for 15 min, followed by 500 V to 6000 V (45 min) and 6000 V (9 h) with a current limit of 20 μA. The temperature was maintained below 10 ^{o}C.

A 0.71 mm thick 12.5% gel (BioRad Laboratories, Hercules, CA, USA) for SDS-PAGE was prepared according to standard procedures [20]. Electrophoresis was carried out and proteins were stained using Coomassie Brilliant Blue.

HPLC-UV and HPLC-MS experiments

The chromatographic separation was performed with a Dionex/LC Packings Ultimate 2000 (Amsterdam, The Netherlands) nanoLC system coupled with a UV detector (214 nm, detector cell: 8 mm light path, 30 nL) or an Agilent LC/MSD iontrap XCT. The monolithic column (Chromolith CapRod RP-18 endcapped, 150x0.1 mm i.d.) was obtained from Merck (Darmstadt, Germany). Solvent A was water with 5% (m/m) acetonitrile and 0.1% TFA, solvent B was acetonitrile with 5% (m/m) water and 0.1% TFA. The injection volume was 1.0 μL. Gradient: 0 min 5%B, 30 min 5%B, 56 min 50%B, 56.1 min 95%B, 70 min 95%B, 70.1 min 5%B, 90 min 5%B.

The voltage of the MS was -4 kV. The flow rate of the drying gas was 4 L/min and the temperature was kept at 325^{o}C. LC-MS-MS data were converted into the Mascot generic format using the data-analysis software. These .mgf files were searched against the MSDB database with Mascot's MS-MS ion search module [21].

Digestion procedure

The protein was diluted with 200 mM ammonium bicarbonate (pH=8.2) and 12.5 μL 130 mM DTT was added. The mixture was incubated for 30 min at room temperature. About 25 μL 200 mM iodoacetamide was added and incubated for 45 min in the dark at room temperature. Excess DTT and iodoacetamide were removed by washing with ammonium bicarbonate and centrifuging (7 min/10 000 rpm) the solution thrice. The protein solution was diluted to 400 μL with the ammonium bicarbonate solution and 18 μL trypin (750 μg/mL) was added and incubated overnight at 37^{o}C.

Centrifugal serum ultrafiltration

The filter membranes were rinsed and used according to the manufacturer's specifications. To 5 mL of the solution mimicking serum 40 mL 25 mM ammonium bicarbonate (pH=8.2) and 10 mL of acetonitrile were added and applied onto a centrifugal membrane (30 kDa). The sample was centrifuged (10 min/7 500 rpm) and the filtrate lyophilized to dryness.

3 Results and discussion

For the separation of peptides and proteins IEF-OGE in the first dimension is combined with visually detection, LC-UV, LC-MS-MS or SDS-PAGE in the second dimension. Three types of samples were applied, *i.e.* two simple digests, intact proteins, serum and a solution mimicking a serum solution and spiked with proteins.

OGE-IEF of peptides and proteins followed by LC-UV

The peptides of a Myoglobin and a BSA (Bovine Serum Albumin) digest were per digest separated using OGE-IEF in the first dimension. The samples from the 24 wells were further analyzed using capillary LC combined with UV detection (the MS detector was not available at that time). The chromatographic peak patterns were visually compared with chromatograms of the same digests obtained without OGE-IEF. For the 2-D separated peptides of a Myoglobin digest the peak patterns in each well are more or less the same, also compared with the LC separation of the same digest without OGE-IEF. Two representative chromatograms are shown in Fig. 8.2[a-b], respectively without and with OGE-IEF. The exceptions are chromatograms obtained from wells 5, 6, 7, 12 and 24. In these samples hardly any (peptide) peaks were detectable. The chromatograms of the 2-D separated BSA digest samples of wells 1 to 8 show the same peak pattern and are comparable to the 1-D separation (LC separation without OGE-IEF), the chromatograms of well 9 to 24 show the same pattern but less peaks compared to the 1-D separation.

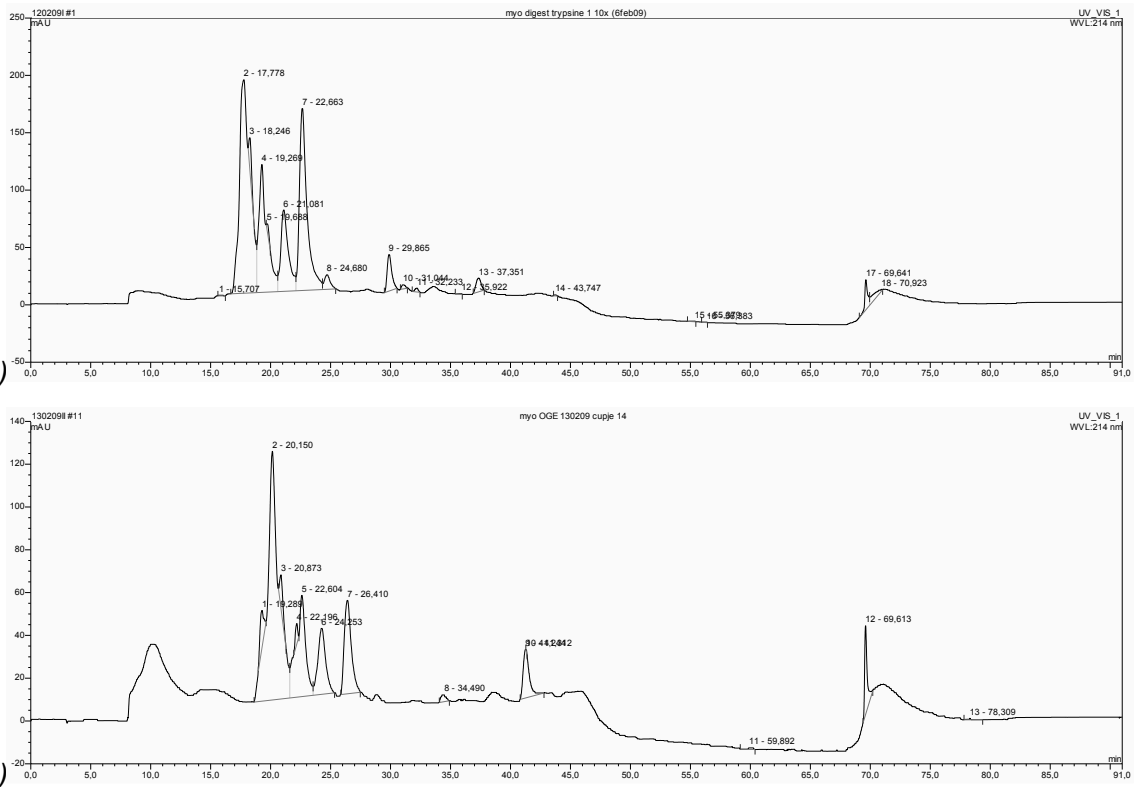

Fig. 8.2. (a) LC-UV chromatogram of a Myoglobin digest and (b) OGE-IEF - LC-UV chromatogram (well 14) of a Myoglobin digest. For experimental details: see text.

This OGE-IEF method is not very successful. The peptides should migrate, according to their charge, to the different wells and each well should contain zero, one or a few peptides. Most of the wells contain several peptides and the peak pattern in the 2-D chromatogram is even comparable with the 1-D chromatogram. A disadvantage of the used UV detection method is the unavailability to identify the different peptides. One peak in the chromatogram might be composed of several coeluting peptides. After 2-D separation the sample can contain one peptide eluting at the same retention time as the mixture after the 1-D separation. So it is difficult to see the difference of one or more peptides in a sample and it is not possible to draw correct conclusions from these UV chromatograms. Therefore a discrete number of proteins, with a limited number of peaks in the chromatogram, will be separated using OGE-IEF.

Five intact proteins, HSA, Lactoglobulin B, Carbonic Anhydrase, Myoglobin and Ribonuclease, (see Table 8.1) were analyzed using OGE-IEF – LC-UV. Except for wells 1 and 2 (one peak) all wells show 2 or 3 peaks in the corresponding UV chromatogram. According to their pI (see Table 8.1) a single protein is expected in

certain wells, like HSA in well 6, Lactoglobulin B in well 8, Carbonic Anhydrase in well 10, Myoglobin in well 14 and Ribonuclease in well 22. Again, it is difficult to draw conclusions from these experiments because we could not use the MS to identify the different proteins. Therefore in further research the MS is used. For a better identification the separated proteins in each well were digested prior to the LC-MS analysis.

Table 8.1. Properties of some proteins

Protein	*pI*	*MW (kDa)*
HSA	4.6	67
Lactoglobulin B	5.3	18.4
Carbonic Anhydrase	5.9	34.4
Myoglobin	7.2	16.7
Ribonuclease A	9.5	13.7
Glucose Oxidase	4.2	160
Cytochrome C	10.2	12
β-Lactoglobulin A	5.1	18.4
Lysozyme	9.3	14.7
α-Lactalbumine	4.2 - 4.5	14.2

OGE-IEF of proteins followed by off-line digestion and LC-MS-MS or visually detection

The proteins of a diluted serum solution were separated using OGE-IEF. All the samples (1 to 24) were off-line digested and analyzed using LC-MS-MS. These data were converted to the Mascot generic format and searched against the MSDB database. Like Stalder *et al*. [13] it was tried to isolate the disturbing abundant proteins in a limited number of wells and the remaining less abundant proteins in the other fractions. Unfortunately, every sample was identified as BSA. A representative base peak chromatogram is shown in Fig. 8.3. The content of BSA in the serum is so high that in all the samples BSA was identified and the OGE-IEF system did not work properly. In future, prior to the analysis of serum the BSA should be removed (for example by immunodepletion or centrifugal ultrafiltration [22]).

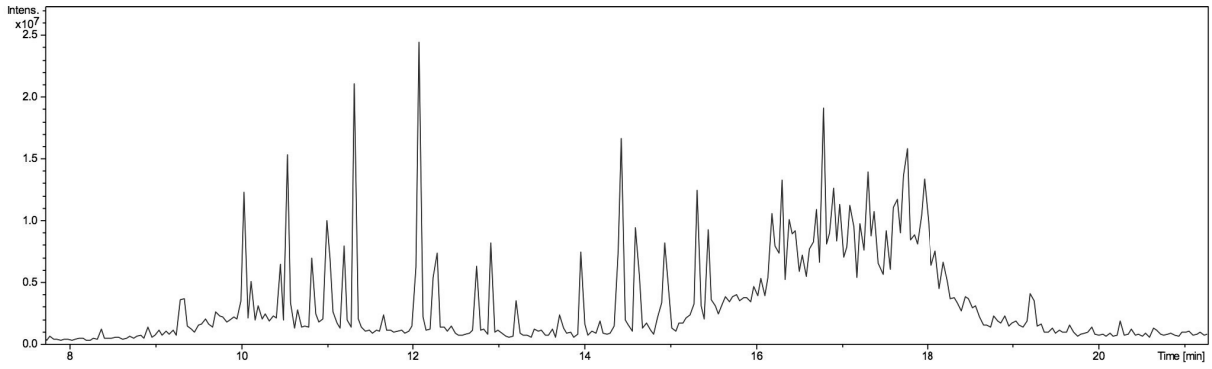

Fig. 8.3. OGE-IEF - LC–MS-MS base peak chromatogram (well 10) of a serum solution digest. For experimental details: see text.

Because the OGE-IEF systems does not work properly it was decided to separate three coloured proteins, *i.e.* Cytochrome C (red), Myoglobin (brown) and Glucose Oxidase (yellow) and detect them visually. According to their iso-electric point Glucose Oxidase is expected in well 4 or 5, Myoglobin in well 14 and Cytochrome C in well 24. Well 13, 14 and 23, 24 are colored brown/red. Except for Glucose Oxidase the system seems to work.

OGE-IEF of proteins spiked in a serum solution followed by SDS-PAGE

Due to the presence of large amounts of Albumin in serum we decided to remove these abundant protein by centrifugal serum ultrafiltration, as described by Tirumlai [22], instead of immunodepletion.

A solution, mimicking serum containing 91% H_2O, 6% Serum Albumin and 2% NaCl, was prepared and spiked with six proteins (0.016%): Myoglobin, Cytochrome C, β-Lactoglobulin A, Lysozyme, Ribonuclease A and α-Lactalbumin. After sample pretreatment by serum ultrafiltration the remaining sample was separated by OGE-IEF. The separated proteins were brought on a poly(acryl)amide gel for further identification. The spots were visualized by blue staining. Despite the OGE-IEF and sample treatment in all the samples Albumin is still visible. Probably the concentration of Albumin was too high so the ultrafiltration filter was overloaded. β-Lactoglobulin A and Cytochrome C show no spot at all. The other proteins are visible in all the samples. The OGE-IEF was not performed successfully.

4 Conclusions and future perspectives

Recent literature shows that OGE-IEF in combination with LC is a promising alternative technique for the traditional two-dimensional gel electrophoresis. It lacks some major disadvantages like the low sensitivity and the presence of carrier ampholytes. Unfortunately, in the executed experiments the used prototype OGE-IEF apparatus did not work properly. Efforts should be made to solve this technical problem first. A simple method to test if the system works well is the separation of a limited number of coloured proteins with visual detection. So the separation problem is focused only on the prefraction technique. When the system works fine we suggest to separate intact proteins with OGE-IEF in the first dimension and then digest the separated proteins. The reverse order is not recommended because the context of the peptides with the parent protein can be lost when using the MS. For the second dimension Ultra Pressure LC (UPLC) in combination with MS-MS is advised for extra resolution of the obtained peptides. Finally proteins can be spiked in a biological matrix, *e.g.* serum, and after immunodepletion separated with OGE-IEF and after digestion with UPLC-MS-MS in the second dimension.

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Chapter 9

Conclusions and future perspectives

1 Introduction

In this thesis the influence of the LC separation on the identification of protein digests is studied. In the first part the chromatographic parameters for silica monolithic columns were optimized to obtain a high throughput or a high separation performance. In the second part different column materials, monolithic and ultra small particulate columns, were compared. In the last part trends and suggestions for future research will be given.

2 Optimization for silica monolithic columns focused on high-throughput and chromatographic performance

One of the benefits of monolithic columns is the high-throughput. Due to the open structure and the corresponding low back-pressure a high flow-rate is possible. In *Chapter 3* is proven, that by optimizing eluent composition and flow-rate (for a 15-cm column up to almost 3 μL/min and a gradient steepness of 16%/min), it is feasible to analyze a Myoglobin digest within 4 min and still obtain a sufficient PC** and protein identification score. This is about seven times faster than the analysis time of about 30 min when applying a particulate capillary column. With this high flow-rate and short gradient time the maximum data acquisition rate of the MS may present a problem. Also for the analysis of a digest of large proteins or mixture of proteins this time will be too short but the potential of monolithic columns for fast separations is clearly demonstrated.

Next to the high-throughput optimization the chromatographic conditions are optimized in *Chapter 4* to gain the highest separation power in terms of PC**. The adapted univariate optimization method [1], by varying flow-rate and gradient steepness and keeping the temperature and gradient time fixed and as high as possible, shows to be applicable for capillary monolithic columns. The operational chromatographic conditions for the studied 35-cm column are a flow-rate of 1.0 μL/min and a gradient steepness of almost 1%/min. Some remarks concerning the starting conditions of this method. Higher temperatures may lead to degradation products/extra unexpected peaks in the chromatogram and problems in the interpretation of the MS spectrum. A long gradient time may lead to an unnecessary long analysis time and decreases the sample throughput. Next to the

PC** also the protein identification score should be included as an optimization parameter. If the sequencing speed of the MS is high enough, the partly separated peptides, even with a low PC**, can have the same identification score as peptides measured with baseline separation. So a relatively low PC** does not necessary lead to a low protein identification score.

3 Comparison of monolithic and ultra small particulate columns

In *Chapter 5* a commercial conventional ultra pressure LC system is modified to a capillary one in a simple, fast and cost-effective way by applying the appropriate connections and an alternative injection procedure. Compared to a commercial capillary LC system, when using a 15-cm silica monolith and a Myoglobin digest, the obtained chromatographic performance and protein identification scores for both systems are about the same. The modified system is also (successfully) used to compare the performance of silica monolithic columns of different lengths (15- and 64 cm) and a 15-cm ultra small particulate (d_p=1.8 μm) column (*Chapter 6*). A scaling factor is introduced to correctly match columns with different dimensions. Compared to the smaller monolithic column the longer one shows a higher PC** and protein identification score mainly because of the longer gradient time and higher plate number. However, the shorter column shows the highest P. Smaller peak widths, a higher P and protein identification scores are obtained for the ultra small particulate column. For BSA, the sequence coverage increases from 34% (short monolithic column), 48% (longer monolithic column) to 54% (particulate column) and for Myoglobin from 80%, 86% to 89%, respectively. Thus, further increase in the chromatographic performance for Myoglobin will hardly increase the score. Probably the maximum score is almost reached. For BSA, other large proteins or mixture of proteins the higher chromatographic performance will be more advantageous. A further separation of coeluting peptides will probably lead to a higher score. This better separation can be obtained by the use of a longer particulate column containing the same packing material. However, the limiting factor for such a column will be the lower permeability.

In *Chapter 7* the performance of small 5-cm silica and poly(styrene-divinylbenzene) monolithic columns is compared. After optimization of flow-rate

and gradient time the P and sequence coverage of Myoglobin and BSA for both columns are about the same. For Myoglobin their mean values are 4.8 and 88% and for BSA 3.8 and 52%, respectively. The P and sequence coverage are in the same range as for the 15-cm ultra small particulate column (5.0, 89% and 4.0, 54% for Myoglobin and BSA respectively). However, the gradient time of that column, compared to its length, is much shorter.

4 Future perspectives

Future developments in proteomic research are being considered by focusing on the (pre)fractionation and separation, important steps in the proteomic workflow (see *Chapter 1*). The quality of these steps can be further increased by the development of more sophisticated stationary phases. For the preparation of silica monolithic columns with a constant quality the packing density can be investigated by using laser scanning microscopy. This is a fast and quantitative method as an alternative for the present destructing method (cutting the capillary). So the quality of the column packing material between different batches can be more or less constant. Also the development and applications of (silica) monoliths for sample preparation with a low back-pressure and a high surface area will increase in the near future. For example silica precolumns with ionizable groups and a large surface area are very promising in solid phase extraction (SPE) of polar compounds. Also in the field of efficient chiral separations using silica monolithic columns much progress will be made in the near future [2]. Next to the commercial available monolithic column materials also homemade columns are and will be further developed (an overview of the present state applications is given in *Chapter 2*). For example silica columns of larger lengths, very small internal diameters, equipped with different on-line functionalities, like particles for trapping of the analytes or an integrated ESI emitter. Tailor made organic monolithic methacrylate columns can be used for all kind of applications, like the on-line desalination, preconcentration and separation of peptides or as support for on-line enzyme reactors. Organic continuous bed poly(styrene-divinylbenzene) monolithic columns (PS/DVB) are commercially available but homemade they can be extremely long (porous-layer open-tubular; PLOT) with a very low back-pressure.

For conventional particulate columns a further decrease in particle diameter (Shen *et al*. [3] prepared columns with 0.8 μm i.d.) is a trend although the increasing deviation in particle size uniformity may negatively influence the flow pattern leading to broader peak widths and a lower PC or PC**. The available pumps and connections must be able to handle the accompanying extreme high pressure. The sample throughput for these columns can be high because the column length can be decreased to gain the same chromatographic performance as longer columns with a conventional stationary phase. The development of these ultra small particle columns will probably challenge the monolithic column manufacturers for further research to gain the same separation performance [2].

Columns combining the benefits of the above two types have already been developed, containing a porous shell (like monolithic columns; for high-throughput) and a small internal core (like ultra small particle columns; for high separation power). Also the need for instruments to be able to handle extreme pressures is not necessary anymore. They are commercially available (RP mode) and more types of these columns, containing different stationary phases, will probably be developed in the near future, although a recent study is not very promising. Vaast *et al*. [4] made a comparison between capillary silica monolithic columns, porous particle columns (d_p=3 μm) and a 2.7 μm fused-core column taking the effect of columns structure on peak width and permeability into account. Their calculations demonstrate that the monolithic column shows the lowest contribution in mass transfer and the highest permeability leading to the best chromatographic performance and highest throughput.

Combining the above mentioned columns, preferably on-line, in series, in the same capillary can improve the dynamic range so low abundance proteins can be analyzed among abundant components. In this thesis, as an alternative for 2-DE (two-dimensional (gel) electrophoresis) fractionation, we made our first step (*Chapter 8*) for the off-line combination of off-gel isoelectric focusing (OGE-IEF) in combination with RP-LC-MS-MS using a silica monolithic column.

For the further miniaturization of LC systems organic monolithic materials play an important role in the development of microfluidic devices in proteomics. Due to the easy preparation into a specific area, no need for retaining frits and the possibility

for certain materials to attach all kind of groups make these columns very selective and suitable for microfluidic. Pressure driven LC system will probably replaced by electrokinetic CEC (Capillary Electrochromatography). A promising new technique in microfluidics is spatial chromatography. The sample flows with the first solvent through a monolithic medium and with the second solvent perpendicular on the flow path of the first to obtain a 2-D separation. The obtained spots in the chip are scanned with MALDI.

In this thesis quantitative proteomics was not our goal but the quantitative analysis of the proteome is gaining in importance. Wright *et al.* [5] noticed that the number of quantitative proteomic studies increased largely after 2008. These quantitative studies provide more information on the dynamic processes taking place in cells and on biomarkers, whose concentration indicates the presence of certain diseases, than identification studies alone. Further development of these techniques, is important. Therefore we strongly suggest for further research to take also this aspect in mind.

Another approach by comparing different columns materials, instead of calculating PC, PC** and P is the kinetic-plot method suggested by Eeltink *et al*. [4, 6]. This method takes the effect of column structure on peak width and permeability into account. In this process the gradient time to column dead time ratio (t_g/t_0) is maintained constant when applying different flow-rates. The separation performance of the different column materials is visualized via kinetic plots depicting the gradient time required to achieve a certain PC when operating at the maximum system pressure. This group also advocates the top-down approach instead of the bottom-up approach in combination with PS/DVB monolithic columns coupled to the MS. The protein sample to be separated is a factor of 20 less complex than the corresponding peptide sample. They are further improving these columns for protein separation.

A final remark on new developments in multidimensional LC and MS (like improvements in quadrupole ion traps and quadrupole time-of-flight mass analyzers) is the cost/expertise barrier. It is easier and much cheaper to set-up and run 2-DE than develop multidimensional LC systems [5]. In contrast, for complex protein/peptide samples simple systems are not sufficient anymore.

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Chapter 10

Nederlandse samenvatting

Dankwoord

Curriculum Vitæ

Nederlandse samenvatting

'Proteomics' is de studie van het proteoom: de eiwitten in een organisme waarbij de onderlinge relaties en samenhang worden bestudeerd. De aanwezigheid van een bepaald eiwit, kan duiden op een ziekte of afwijking. Het aantonen van zo'n eiwit, een biomarker, in een vroeg stadium kan cruciaal zijn. In tegenstelling tot 'genomics', de studie van de individuele genen, is het proteoom door diverse posttranslationele biologische processen geen statisch geheel. In een andere 'omics'-studie, 'metabolomics', worden de (eind)producten van cellulaire processen in organen, weefsels en organismen bestudeerd. In 'systems biology' worden bovenstaande onderzoeken gecombineerd om een overzicht te krijgen van de staat van het organisme.

Een deel van de 'proteomics'-studies is gericht op de kwantificering en/of het karakteriseren van de eiwitten. De klassieke techniek hiervoor is twee-dimensionale gel elektroforese. Bij deze techniek worden intacte eiwitten gescheiden op grond van hun iso-electrische punt (eerste dimensie) en hun molecuulmassa (tweede dimensie). De verkregen 'spots' in de gel worden zichtbaar gemaakt door middel van kleuringen (bijvoorbeeld met 'Coomassie Brilliant Blue') en worden uitgesneden en verder opgewerkt (bijvoorbeeld digestie), waarna met massa spectrometrie (MS) de molecuulmassa wordt bepaald. Vanwege de ongevoeligheid (alleen relatief hoge concentraties eiwitten kunnen worden bepaald) van de gel elektroforese, tijdsduur van de scheiding en de bewerkelijkheid wordt dit proces steeds vaker vervangen door alternatieve technieken, zoals bijvoorbeeld vloeistofchromatografie of de in dit proefschrift beschreven 'off-gel isoelectric focusing'.

Een veel gebruikte strategie na de voorscheiding is het digesteren van het intacte eiwit met een geschikt enzym, meestal trypsine, het scheiden van de gevormde peptiden en de identificatie/kwantificatie van deze peptiden met behulp van MS.

Voor eiwitherkenning worden de verkregen MS data vergeleken met bestanden in een internet database, zoals SwissProt of MSDB, waarbij het resultaat van deze vergelijking wordt uitgedrukt in de eiwitidentificatiescore. Bij een betere scheiding worden meer peptiden herkend door de MS waardoor deze score hoger zal liggen. Een scheidingstechniek die hiervoor gebruikt kan worden is hogedruk, soms ook

hoge prestatie genoemd, vloeistofchromatografie (HPLC). De signaal/ruis verhouding voor de LC detectie is sterk verbeterd door miniaturisering. Dit door het verkleinen van de interne diameter van de kolom (van mm naar μm of nm) resulterend in capillaire LC of nanoLC. Bij deze technieken zijn geschikte HPLC pompen nodig om lage volume snelheden (μL of nL/min) van het eluens door de kolom te kunnen leiden. Ook de leidingen en detectorcellen dienen aangepast te worden om de bijdrage aan de piekverbreding te minimaliseren. Met de injector moeten kleine monsterhoeveelheden reproduceerbaar ingebracht kunnen worden.
De scheiding is verder verbeterd door het verkleinen van de deeltjesdiameters van 3-5 μm naar 1,7-1,8 μm. De benodigde pompen moeten bij de kleine-deeltjes kolommen een druk van ruim 1000 bar kunnen leveren om een redelijke flow te krijgen. Dit heeft geleid tot UPLC (ultra hogedruk vloeistofchromatografie) systemen. Een andere ontwikkeling is de aard van het kolommateriaal bestaande uit *één stuk* polymeer of silica materiaal. Deze zogenaamde monolithische kolommen bezitten relatief grote (macro)poriën voor het transport en nauwere (meso)poriën voor de vertraging van de analieten. Een belangrijk voordeel van dit materiaal is dat er met relatief lage drukken gewerkt kan worden. Er kunnen dus langere kolommen (betere scheiding) en/of hogere flows (snellere scheiding) gebruikt worden.
De kwaliteit van de LC scheiding bij gradiënt-elutie van peptiden wordt uitgedrukt in piekcapaciteit (PC), monsterpiekcapaciteit (PC**) en productiviteit (P). De PC is gedefinieerd als het aantal pieken in een chromatogram uitgaande van de gradiënttijd (de tijd tussen de start en het einde van de vloeistofgradiënt), de PC** als het aantal pieken tussen het eerste en laatst eluerende peptide van het digest en de P als het aantal pieken dat de kolom per minuut verlaat.
In dit proefschrift wordt de invloed van de chromatografische scheiding op de kwalitatieve analyse van eiwitdigesten onderzocht door gebruik te maken van capillaire ‘reversed-phase’ LC in combinatie met 1,8 μm en monolithische silica- en poly(styreen-divinylbenzeen) kolommen. De behaalde resultaten, uitgedrukt in PC, PC** en P, de MS resultaten in eiwitidentificatiescores, worden onderling vergeleken.

In *Hoofdstuk 1* worden het doel van het proefschrift, de plaats van dit onderzoek en aanvullende/alternatieve analyse-technieken in proteomics besproken.

Hoofdstuk 2 geeft een literatuuroverzicht van het gebruik van monolithische capillaire silica en polymere kolommen in de eiwitanalyse. Besproken worden recente/toekomstige ontwikkelingen, materialen en diverse toepassingen.

In *Hoofdstuk 3* wordt de snelheid van de analyse en in *Hoofdstuk 4* de chromatografische scheiding gemaximaliseerd. In *Hoofdstuk 3* gebeurt dit door gebruik te maken van de poreuze structuur van monolithische silica kolommen. Door de relatief lage tegendruk kan de eluenssnelheid van een 15-cm kolom verhoogd worden tot bijna 3 μL/min. Na het optimaliseren van de samenstelling van de mobiele fase daalt de analysetijd van 30 naar ongeveer 4 minuten. De PC** en eiwitidentificatiescore zijn dan nog steeds voldoende voor de herkenning van het testeiwit myoglobine.

In *Hoofdstuk 4* wordt een optimalisatie-procedure toegepast voor de chomatografische scheiding van peptiden. Voor een 35-cm capillaire monolithische silica kolom wordt deze procedure gebruikt voor de PC** maximalisatie door optimalisatie van de eluenssamenstelling en snelheid (1 μL/min). Ook wordt de verhoging van de PC** op de eiwitidentificatiescore onderzocht. Voor eenvoudige eiwitten blijft de score ongeveer gelijk, voor complexere eiwitten neemt deze score toe.

In *Hoofdstuk 5* wordt op een eenvoudige en goedkope manier een conventionele UPLC aangepast voor capillair gebruik. Dit gebeurt door geschikte capillaire leidingen te gebruiken en een alternatieve injectieprocedure toe te passen. Het blijkt dat de gemodificeerde LC vergelijkbare resultaten geeft als een commerciële capillaire LC als gelet wordt op de PC** en eiwitidentificatiescores voor een testeiwit.

In *Hoofdstuk 6* wordt onder andere deze aangepaste capillaire UPLC gebruikt voor het vergelijken van monolithische silica kolommen van verschillende lengtes (15 en 64 cm) en een 15-cm kleine-deeltjes kolom (1,8 μm). De langste monolithische kolom geeft de hoogste PC** en eiwitidentificatiescore voor Myoglobine en runderserumalbumine (BSA). De kleinere kolom laat echter een grotere P zien. De beste P en eiwitidentificatiescore worden verkregen met de kleine-deeltjes kolom.

Bijvoorbeeld voor BSA loopt laatstgenoemde score op van 34% (15-cm monolithische kolom), 48% (64-cm monolithische kolom) tot 54% (kleine-deeltjes kolom). Voor wat betreft P en de eiwitidentificatiescore is de kleine-deeltjes kolom superieur aan de monolithische kolommen.

In *Hoofdstuk 7* worden twee monolithische silica en poly(styreen-divinylbenzeen) kolommen, van dezelfde lengte (5 cm), na optimalisatie van de chromatografische condities met elkaar vergeleken. Voor eenvoudige eiwitten geven beide kolommen nagenoeg dezelfde PC, P en eiwitidentificatiescores.

In *Hoofdstuk 8* is een eerste stap gemaakt voor een multidimensionaal scheidingssysteem, namelijk de off-line combinatie van isoelectric focusing met LC. Dit is een snelle, gebruiksvriendelijke en reproduceerbare methode voor een eerste scheiding van eiwit(digesten).

Het proefschrift wordt afgesloten met *Hoofdstuk 9* met algemene conclusies, toekomstperspectieven en aanbevelingen voor verder onderzoek.

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Na een periode van alleen onderwijs gegeven te hebben ben ik me verder inhoudelijk gaan verdiepen in de analytische chemie door te starten met een parttime promotietraject opleverend dit proefschrift.

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JR

Curriculum Vitæ

Johan Rozenbrand werd geboren op zondag 5 maart 1967 te Sprang-Capelle. De Ichthus School voor mavo in zijn geboortedorp werd in 1983 afgerond. Na het volgen van de havo aan het Willem van Oranje College te Waalwijk is hij de analisten opleiding (HLO) gaan doen bij de Hogeschool West-Brabant te Breda. Na afronding van deze opleiding in 1989 viel de keuze voor een vervolgstudie op Scheikunde bij de Vrije Universiteit te Amsterdam. Na het behalen van het doctoraal examen in 1992 werd gestart met een AIO-2 opleiding bij dezelfde universiteit (bul behaald in 1995). Daarna is hij begonnen als junior docent bij de Universiteit Utrecht, Faculteit Farmacie, groep Analyse & Toxicologie en later gestart met een parttime promotietraject. Hij is nog steeds werkzaam als universitair docent bij dezelfde instelling, inmiddels omgedoopt tot Faculteit Bètawetenschappen, departement Farmaceutische Wetenschappen, groep Moleculaire Farmacie, onderdeel Biomoleculaire Analyse.

Abbreviations

1-D	one-dimensional
2-D	two-dimensional
2-DE	two-dimensional electrophoresis
AIBN	2,2'-azobisisobutyronitrile
Ala	Alanine
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
ANOVA	analysis of variance
A_s	asymmetry factor
A-term	eddy diffusion
AX	anion exchange
bpc	base peak chromatogram
BSA	Bovine Serum Albumine
B-term	longitudinal diffusion
CDI	carbonyldiimidazol
CEC	Capillary Electrochromatography
CSF	cerebrospinal fluid
C-term	resistance to mass transfer
DTT	dithiothreitol
EDMA	ethylene dimethacrylate
ESI	Electrospray Ionization
GC	Gas Chromatography
Gly	Glycine
GMA	glycidyl methacrylate
H	plate height
HETP	height equivalent to a theoretical plate
HIC	Hydrophobic Interaction Chromatography
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Pressure Liquid Chromatography
HSA	Human Serum Albumine

i.d.	internal diameter
IEF	isoelectric focusing
IMAC	Immobilized Metal Ion Affinity Chromatography
IPG	immobilized pH gradient
L	column length
LC	Liquid Chromatography
LHRH	Luteinizing Hormone Releasing Hormone
MALDI	Matrix-assisted Laser Deorption/Ionization
MS	mass spectrometer/mass spectroscopy
MudPIT	Multidimensional protein identification technology
MW	molecular weight
N	plate number
n.d.	not determined
ODS	octadecyl silane
OGE	off-gel electrophoresis
P	productivity
p	pressure
PC	peak capacity
PC**	sample peak capacity
PEEK	polyetheretherketone
Phe	Phenylalanine
pI	isoelectric point
PLOT	porous-layer open-tubular
PS/DVB	poly(styrene-divinylbenzene)
Q	Quadrupole
r	column radius
RP	reversed-phase
R_s	resolution
SAX	strong-anion exchange
SCX	strong-cation exchange
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography

SEM	scanning electron microscope
SF	scaling factor
SPE	solid phase extraction
t_0	dead time
TFA	trifluoroacetic acid
t_g	gradient time
TIC	total ion current
TOF	time of flight
t_r	retention time
UPLC	Ultra Pressure Liquid Chromatography
V	liquid column volume
v	flow-rate
Val	Valine
W_b	peak width
WCOT	wall-coated open-tubular
ε	porosity
ϕ	eluent strength