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Review

Highly efficient peptide separations in proteomics Part 1. Unidimensional high performance liquid chromatography☆

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Abstract

Sample complexity and dynamic range constitute enormous challenges in proteome analysis. The back-end technology in typical proteomics platforms, namely mass spectrometry (MS), can only tolerate a certain complexity, has a limited dynamic range per spectrum and is very sensitive towards ion suppression. Therefore, component overlap has to be minimized for successful mass spectrometric analysis and subsequent protein identification and quantification. The present review describes the advances that have been made in liquid-based separation techniques with focus on the recent developments to boost the resolving power. The review is divided in two parts; the first part deals with unidimensional liquid chromatography and the second part with bi- and multidimensional liquid-based separation techniques. Part 1 mainly focuses on reversed-phase HPLC due to the fact that it is and will, in the near future, remain the technique of choice to be hyphenated with MS. The impact of increasing the column length, decreasing the particle diameter, replacing the traditional packed beds by monolithics, amongst others, is described. The review is complemented with data obtained in the laboratories of the authors.

Keywords: Proteomics; Peptides; Liquid chromatography; High efficiency; Ultra-high pressure; Elevated temperature; Column formats

Contents

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1. Introduction

The sequencing of many genomes, including the human genome [1,2], has paved the way for the global exploration of the proteome, comprising the true functional entities. Compared to the genome, which is nearly identical in all cells and tissues, the proteome is much more variable and the protein expression level varies among cell type, tissue and largely depends on physiological and environmental conditions. In addition, post-translational modifications, processing and alternative splicing events that are taking place along the path from gene to protein result in unanticipated sample complexities, not even to mention the wide range of protein concentrations that are encountered. The enormous variability, complexity and dynamic range are especially pronounced in the clinically valuable human blood plasma proteome, which comprises next to the classical plasma proteins, other tissue proteomes, viral and bacterial proteins and which is known to have a dynamic range of at least 10^{10} [3,4].

Despite, the enormous challenges that have to be tackled and the rather "immature" technology compared to DNA and RNA analysis, proteome analysis holds great potential. Proteomics will allow to better understand the functioning of organisms in health and disease and will ultimately lead to the identification of new targets for therapeutic intervention and to the development of new biomarkers for diagnosis, prediction of drug response and early detection of diseases [5].

The most popular proteomics approach to date is based on two-dimensional polyacrylamide gel-electrophoresis (2D-PAGE) [6,7]. In the first dimension, proteins are separated according to their isoelectric point (p*I*), followed by separation according to size in the second dimension. The visualized protein spots of interest are subsequently digested using a protease such as trypsin and the resulting peptides are analyzed by MS or MS/MS followed by database searching for protein identification [8,9].

Due to the limitations in dynamic range and difficult analysis of certain protein classes (e.g. membrane proteins, very acidic or basic proteins, large proteins, etc.), amongst others [10,11], the trend in proteomics is toward the development of non-gel based technologies, which allow a more comprehensive and less biased analysis. Since efficient handling and separation of intact proteins in high performance liquid chromatography (HPLC) is difficult and identification of proteins by MS(/MS) is challenging, these procedures often begin with the proteolytic digestion of proteins into peptides, so-called bottom-up approaches. This, of course, drastically increases sample complexity. In plasma, considering 30,000 proteins, with an average of 30 tryptic peptides per protein, this can result in 900,000 peptides, not taking any processing or modification into account. Separation and subsequent mass spectrometric analysis of all peptides present in such a complex mixture, represents a tremendous challenge. The present review describes the advances that have been made in recent years at the level of liquid-based separations to maximize the resolution of the peptides present and, hence, reduce the peptide complexity for successful and comprehensive analysis by MS. In this way, the overall dynamic range can be improved (since low abundance species can be resolved from high abundant components) and typical mass spectrometric issues such as ion suppression and under sampling can be addressed. Both one-dimensional and multidimensional liquid-based separations will be reviewed. In the first part unidimensional HPLC will be addressed. The second part, will deal with multidimensional approaches.

2. Peptides and liquid-based separations: the perfect marriage

The physico-chemical diversity of peptides (charge, isoelectric point, hydrophobicity, size) makes them well suited to be separated by nearly every liquid-based separation mode. The first attempts to separate peptide mixtures via HPLC appeared in the mid 70s, approximately 10 years after the first reports on HPLC [12,13]. It immediately came clear that the separation on non-polar stationary phases held great potential [14–18]. The power of reversed-phase HPLC (RPLC) led to the wide use of the technique for peptide mapping by the mid 1980s [19]. Thanks to column miniaturization efforts, which already started early on in the development of HPLC [20–23], and the introduction of soft ionization techniques, such as matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) [24–27], RPLC in combination with mass spectrometry (MS) evolved into the principal analytical technique in the field of proteomics. Peptides can now routinely be identified and quantified at high sensitivity from limited sample amounts. Dozens of reversed-phase columns are commercially available, specifically designed to separate peptides, differing in length, particle diameter, internal diameter, pore size, hydrophobicity, pH stability, support material, etc. Nevertheless, a substantial number of groups still construct their own columns. Using RPLC, peptides are most often separated under gradient conditions due to the huge difference in capacity (retention) factors (k) typically present in tryptic digests. Acetonitrile is the organic modifier of choice and the mobile phase further contains trifluoroacetic acid (TFA), formic acid, etc. as ion-pair reagent [18].

Notwithstanding its enormous power, reversed-phase LC is only one of several HPLC modes that can be applied to resolve peptide mixtures. Different selectivities are being offered by ion exchange chromatography (IEC) [28,29], size exclusion chromatography (SEC) [30] and hydrophilic interaction chromatography (HILIC) [31,32] only to mention the most important ones [33,34].

Capillary electrophoresis (CE) introduced in the early 80s [35] was rapidly utilized to separate peptides as well. Although, all CE modes can be applied in the field of proteomics, capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIEF) and the hybrid between HPLC and CE termed capillary electrochromatography (CEC) appear to be the most promising [36–38].

All these HPLC and CE methodologies can be used as stand alone; however, very often they are combined to increase the overall resolving power [39,40]. For example, the well-known multidimensional protein identification technology (MudPIT) combines strong cation exchange (SCX) with RPLC in one cap-

illary [41]. Because of the huge amount of information out there, the latter will be covered in a separate part (Part 2).

3. One-dimensional liquid-based separations

It is generally postulated that no single chromatographic or electrophoretic separation is capable of resolving the complex mixture of peptides that results from a global proteolytic digest of a complex proteome. Over the years, however, significant efforts have been conducted to increase the resolving power of unidimensional separations [42] and these efforts have increased the number of measurable analytes in ever more complex samples. Under gradient conditions, needed for efficient peptide separations, peak capacity (P_c) is the most common metric to assess resolving power [43,44]. The peak capacity can be defined as the number of peaks that can be separated within a retention window at unit resolution. It can thus be calculated by dividing the total timeframe in which the peptides elute by the average peak width at 4σ. A one-dimensional RPLC set-up typically provides a peak capacity of several hundreds [45]. Objective calculation appears to be challenging and P_c values can easily be over- or underestimated; the latter case being scarcer. As a guideline, average peak width at 4σ is preferably determined on relative simple mixtures, constituting up to 10 different compounds (preferably peptides) occupying the entire elution window. Using this strategy, the contribution of overlapping peaks is excluded and by using average peak widths, the extremes are leveled out. When using MS detection, peak widths can in principle be derived in complex mixtures using extracted ion chromatograms of several peptides at different retention times.

Factors that improve the isocratic efficiency N also affect the gradient peak capacity. In LC, the plate number N, and hence P_c, can be improved by increasing the column length (L) or by reducing the plate height (H). For packed columns, the latter can be achieved by reducing the particle diameter (d_p):

$$N = \frac{L}{H} \approx \frac{L}{2d_p} \quad (1)$$

Both pathways, however, are limited by the pressure drop over the column. The pressure drop across a packed column depends on the linear mobile phase velocity u, the column length L, the mobile phase viscosity η, the column resistance factor ϕ and the particle size d_p:

$$\Delta P = \frac{(u \times L \times \eta \times \phi)}{d_p^2} \quad (2)$$

An increase in column length or a reduction in particle diameter with a factor 2, results in a pressure increase with a factor 2 and 4, respectively (same linear velocity conditions). The optimal linear velocity for smaller particles, however, is higher compared to larger particles, and is inversely proportional to the particle diameter. Hence, under optimal linear velocity conditions, a reduction in d_p with a factor 2 results in a pressure increase with a factor 8 [46,47]. In both cases, efficiency is expected to increase with a factor 2, this associated with an increase in analysis time when the length is doubled, and a decrease in analysis time when d_p is halved. Since P_c is proportional to the square root of N [45], gradient peak capacity potentially increases with 40% in both cases. The key question here is whether a reduction in d_p or an increase in column length is more beneficial? It is generally accepted that maximum peak capacities are obtained on longer columns and that productivity (peaks separated per time unit) is better on columns packed with small particles [45,47,48]. The latter results from both a smaller H-value and a shift of the optimal linear velocity towards higher values. In addition, compared to larger particles, a smaller increase in H or decrease in N is noticed when the flow rate is further increased above optimal. The pressure constraints associated with small particles, however, results in the finding that, at the pumps pressure limit, highest efficiencies are obtained on long columns packed with large particles, of course at the expense of time. This holds true for separations under isocratic [49] and gradient conditions [48]. This is illustrated in Table 1 partly taken from Ref. [50]. In the end, the use of extremely long columns and very large particles also has its limitations due to the increased peak widths and lower sensitivity which results in a mismatch due to the sensitivity requirements in the field of proteomics. A balance has to be found between resolving power and sensitivity. Longer columns are typically chosen for non-targeted discovery approaches where time is not the limiting factor; while short columns packed with small particles appear to be very attractive for validation purposes which require the processing of a large number of samples in a targeted fashion or for comprehensive 2-dimensional set-ups requiring fast second dimension separations.

Table 1
Theoretical calculation of peak capacities obtained with different particle sizes at the pump's pressure limit (reprinted with permission from [50])

d_p (μm)	L (cm)	Gradient time (min)	Pressure drop (bar)	P_c
1.8	7.5	60	400	377
3.5	28.5	228	400	543
5.0	58.0	464	400	603

Another important parameter that affects the peak capacity is the gradient slope. In general, longer gradient times produce higher peak capacities although peak capacity tends to reach a limit at longer gradient times. Gilar et al. showed that the peak capacity on a 150 mm $L \times 4.6$ mm ID $\times$ 5 μm d_p C_{18} column increased from 157 for a 25 min gradient to 351 for a 100 min gradient [45]. Marchetti et al. noticed a 79.5% loss in P_c on a 50 mm $L \times 4.6$ mm ID $\times$ 2.7 μm d_p C_{18} porous shell column upon increasing the gradient slope with a factor 10 [51]. Fig. 1a shows the impact of the gradient time on the peak capacity on a C_{18} packed capillary column and on the MALDI-TOF/TOF identification of peptides/proteins from human serum and cellular (Jurkat cells) tryptic digests. An increase in the number of peptide and, especially in the case of the cellular digest, protein identifications is noticed, however, it levels off at a certain stage. Despite the increase in peptide hits, the impact of the gradient on the number of unique peptide and protein identifications in serum is smaller compared to cells. This can be explained by the

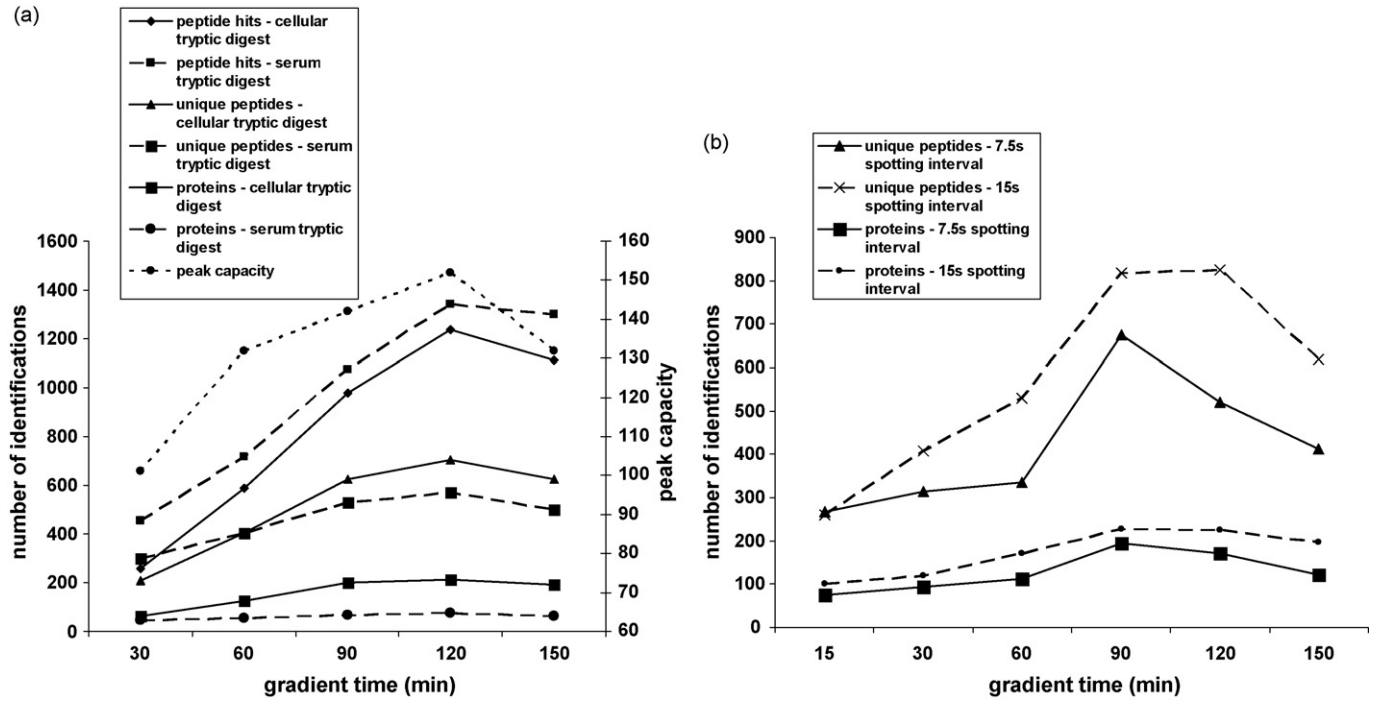

Fig. 1. (a) Dependence of the gradient slope on peak capacity and number of peptide and protein identifications resulting from the LC-MALDI-TOF/TOF analyses of 100 ng of a cellular (Jurkat cells) and human serum tryptic digest. An Ultimate 3000 HPLC system (Dionex, Amsterdam, The Netherlands) and a 4800 MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) were used. Mixtures were separated on a commercially available packed capillary (15 cm $L \times 75$ μm ID × 3 μm Pepmap 100 Å C_{18} particles—Dionex). Mobile phases consisted of 0.05% TFA (solvent A) and 80% acetonitrile, 0.04% TFA (solvent B). A linear gradient was applied between 4% and 55% during the period of time specified in the graphs. Samples were loaded (0.05% TFA) onto a short C_{18} precolumn (5 mm $L \times 300$ μm ID × 5 μm Pepmap C_{18} particles) at 20 μL/min and were subsequently injected in the back-flush mode onto the analytical column at a flow rate of 300 nL/min. Peptides eluting from the column were mixed with a matrix solution (4 mg/mL α-cyano-hydroxy-cinnamic acid in 70% ACN, 0.1% TFA) at a microtee and directly deposited onto MALDI targets. The spotting interval was 15 s and fractions were collected over the entire peptide elution window. The programmed mixing ratio of 1:4 (mobile phase:matrix solution) resulted in the deposition of 375 nL every 15 s. MALDI-MS and MS/MS measurements were performed in the positive reflectron mode using default calibration. The scan range for the MS spectra stretched from 800 to 3500. A list of the top 30 signals, per MS spectrum was generated and MS/MS experiments were performed under "metastable precursor on" conditions, without the use of CID (collision induced dissociation) and at 1 keV. The precursor mass window was set at a resolution of 200 FWHM (full width half maximum). Unfiltered MASCOT generic files (mgf) were subsequently searched against both standard and ragged human Sprot databases using MASCOT as search engine. The latter database was used to detect N-terminally ragged peptides which are abundantly present in serum. Only peptides ranking #1 with scores above the 95% probability threshold were withheld. Spectra that had multiple peptide hits above the probability threshold were regarded as unidentified. Random hits were determined to be around 5% by searching the data against randomized databases. Proteins were reported if they had at least one peptide that unequivocally defines it. (b) Dependence of the spotting interval on the number of identifications resulting from the LC-MALDI-TOF/TOF analyses of 100 ng cellular tryptic digest. A commercially available silica-based monolithic capillary was used (15 cm $L \times 100$ μm ID—Phenomenex, Torrence, CA, USA). LC conditions were similar as described above except that the column was operated at 1 μL/min in the direct injection mode and that the mobile phases contained twice the amount of TFA. Column effluent was mixed in a 1:1 ratio with the matrix solution. Spot volumes resulting from the 7.5 s and 15 s spotting interval were 250 nL and 500 nL. MALDI-MS settings and search strategy were identical as described above.

huge dynamic range that is encountered (same peptides and peptides originating from the same abundant proteins are identified). Also interesting to note, is the fact that the number of peptide hits, are larger in the case of serum, however, the uniquely identified peptides are smaller compared to the cellular tryptic digest, once more illustrating the contribution of the dynamic range. Fig. 1b further shows the dependence of the MALDI target spotting interval on the number of identifications. In that particular study, a silica-based monolithic capillary was used. Important to note is the impact of the sample load on the number of identifications. As shown in Table 2, this impact is huge and sample loadability is thus of utmost importance and unrelated to the chromatographic performance of a specific column format. Again the impact on cells is more pronounced. Using on-line LC–MS/MS, Smith and coworkers also recognized that longer gradients result in an increased number of peptide and protein identifications and a similar logarithmic trend was obtained [52,53]. However, it was also shown that the identifications can further increase once the peak capacity has reached a plateau [53]. For a *Shewanella oneidensis* protein tryptic digest, the number of identified peptides and proteins approximately doubled when the separation

Table 2
Impact of sample amount on peptide and protein identifications in a LC-MALDI-TOF/TOF set-up. For experimental conditions see Fig. 1

Gradient		Jurkat cells		Depleted human serum	
		100 ng	500 ng	100 ng	500 ng
30 min	Peptide hits	258	1023	456	1065
	Unique peptides	208	766	300	641
	Proteins	64	257	47	84
150 min	Peptide hits	1111	2808	1299	2244
	Unique peptides	625	1464	498	876
	Proteins	191	418	62	104

time was extended from 200 to 600 min. The peak capacity only increased slightly (from 420 to 450) illustrating that the relationship between peak capacity and identification rate is very complex. However, the signal to noise ratio decreased by 50% when the separation time was extended from 200 to 600 min (due to the increased peak width). Apparently, in that particular study, the sample load was sufficient to compensate for the intensity loss.

It has recently been shown that the optimal operating conditions (flow rate, etc.) for the peak capacity of a peptide mixture in gradient elution deviates substantially from the optimal conditions for isocratic efficiency [54]. In their paper focusing on peak capacity optimization of peptide separations in RPLC on narrow-bore columns, Carr et al. showed that the optimum flow rate varies substantially with gradient time on a fixed column format. They stated that one should not attempt to reach the highest peak capacity simply by using a flow rate that minimizes the isocratic plate height H. They further demonstrated that temperature has a positive impact on P_c. Gilar et al. [45] also showed that, for a fixed gradient time, peak capacity on 4.6 mm columns with different lengths and particle sizes maximizes at different flow rates. In a recent study on 75 μm columns, Liu et al. [55] observed that the peak capacity maximizes at a flow rate of 750 nL/min for a 3 μm particle column and at 1000 nL/min for a 1.7 μm particle column ($L = 10$ cm). Based on H–u curves one would typically operate these columns at much lower flow rates. The authors, however, are correct in their statement that peak capacity is only one of several important factors to be considered. A factor against the use of higher flow rates is definitely ESI-MS sensitivity. Sensitivity might also suffer from the use of longer gradients since the peaks become broader and peak heights lower. Marchetti et al. demonstrated the dependence of both the flow rate and the gradient time on the peak capacity using a short 4.6 mm column packed with 2.7 μm porous shell particles [51]. In that study, the optimum flow rate appeared to be smaller with peptides than with low molecular weight chemicals due to their lower molecular diffusivity. At relative low flow rates, there is a region where high peak capacities can be achieved by increasing the gradient time. The positive influence of long gradient time on P_c decreases at higher flow rates. It can thus be stated that maximizing peak capacity for peptide separations is a challenging task since a lot of variables, which interact with one another, need to be taken into account. For example, if one increases the length of the column without adjusting the gradient, the outcome might be rather disappointing [45].

An often overlooked parameter is the mobile phase additive. Formic acid as ion pairing reagent decreases P_c compared to TFA. Unfortunately the hyphenation with ESI-MS favors the use of the former. The group of Smith demonstrated good ESI efficiency while limiting the loss in separation efficiency by adding a small amount of acetic acid to mobile phase A and maintaining 0.1% TFA throughout the separation [56].

Other strategies that have been used for obtaining highly efficient peptide separations are the use of monolithic columns [57,58] and of a hybrid between HPLC and CE known as capillary electrochromatography (CEC) [38]. Implementation of monolithic columns has an effect similar as reducing the particle diameter. The H-value is reduced and the van Deemter curve tends to flatten out at higher velocities. Hence, fast efficient separations can be achieved. Compared with packed columns, however, permeability is much higher favoring the use of high flow rates or very long columns without the requirement for specialized instrumentation. The principle of CEC is analogous to LC except that the mobile phase is driven by electro-osmosis instead of pressure. Due to the plug-like flow profile generated, higher efficiencies are expected when compared to HPLC. In addition, the lack of pressure limitation in CEC, allows the use of smaller stationary phase particles and/or longer columns which further increases efficiency.

The following parts describe the most important achievements in HPLC on both packed columns and monolithic columns. As already pointed out, a wide variety of modes are available for resolving the complex mixtures of peptides. This review only considers RPLC since it is the final peptide separation technique in most proteomics approaches. This can be attributed to its compatibility with MS, its robustness and because, in case of peptide separations, it outperforms all other HPLC modes in terms of resolving power [59]. However, future developments might see increased efficiencies for other modes as well. As an example, Lee et al. recently reported a peak capacity of 167 on monolithic SCX capillaries [60]. Other HPLC modes will be detailed in the second part of this review describing multidimensional set-ups.

3.1. Packed columns

3.1.1. Ultra-high pressure HPLC (>600 bar)

A substantial number of reports appeared continuing the ultra-high pressure HPLC work of Jorgenson and coworkers [46]. They showed that plate numbers greater than 200,000 and as high as 330,000 could be obtained in less than 35 min on a 66 cm long capillary column (30 μm) packed with 1.5 μm nonporous octadecyl silica particles. To operate the column near the optimal flow rate, an inlet pressure of 1300 bar was required which was delivered by specialized instrumentation. Analysis time could be reduced to less than 10 min by running at flow rates above optimum requiring pressures as high as 4100 bar. The use of small internal diameter columns permits efficient dissipation of the heat that is generated at extremely high pressures. Later, they extended this work to gradient elution LC and demonstrated the separation of an ovalbumin tryptic digest on a 27 cm long capillary column packed with 1 μm particles and operated at a pressure of 2550 bar [61]. A peak capacity of 300 was demonstrated for a 30 min analysis time. The group of Smith has particularly been successful in translating and fine tuning this approach to further address the needs in comprehensive proteomic analysis, i.e. high efficiency, sensitivity and throughput. They initially reported separations on 85 cm long capillaries with inner diameters of 150 μm packed with 3 μm porous C_{18} particles [56]. Peak capacities up to 1000 were achieved for cellular proteolytic peptides in 3 h at back-pressures of 10,000 psi (690 bar). Efficiency was not jeopardized when performing these separations in a multiple capillary format for high-throughput proteome analysis [62]. By combining these

highly efficient separations on-line with high resolution ESI-FTICR-MS (11.5 Tesla) more than 6×10^7 polypeptides could potentially be resolved [62]. For a yeast tryptic digest, >100,000 putative peptides were detected. In comparison with low efficient separations (30 cm packed with 5 μm particles), the number of detected polypeptides was three-fold greater for the same sample loading. It was also demonstrated that long capillary columns packed with porous particles can tolerate large sample loadings and increasing the load with a factor 10 (5–50 μg) resulted in a nearly seven-fold increase in detected peptides [56]. The authors stated that the ability to inject a significant amount of sample onto the column while retaining separation quality, improves the detection of low-abundance peptides thereby widening the dynamic range. In a later study, the same group demonstrated that decreasing the inner diameter of these highly efficient separations (nanoscale) has an effect equivalent to increasing sample loading [63]. Upon increasing the sample amount from 25 to 1000 ng on a 29.7 μm packed capillary (separation was only compromised above loadings of 1000 ng), the number of detected species increased 14 fold. For the same sample load, decreasing the inner diameter from 74.5 to 14.9 μm increased the number of detected species ~200 fold (Fig. 2). This is a direct consequence of the lower flow rates that are associated with these narrow-bore columns. A linear relationship was noticed between flow rate and MS response. Nanospray emitter orifice diameters were adjusted accordingly. The 14.9 μm column was operated at flow rates of 20 nL/min (near optimal linear velocity) and it is believed that ion suppression is no longer present at these flow rates. In that particular study, the authors did not report on a change in peak capacity by changing the internal diameter. Using reversed-phase isocratic ultra-high pressure LC, however, it has been shown that, for capillary columns (10–150 μm), efficiency increases with decreasing internal diameter [64–66]. The group of Smith later extended their set-up with on-line solid-phase extraction (SPE) (column switching set-up) to allow a more efficient injection of samples without compromising peak capacity. They demonstrated ultrasensitive protein identifications from as little as 0.5 pg of a complex proteome digest [67,68]. An ultra-high pressure dual on-line SPE/capillary RPLC system employing two SPE columns and two capillary columns in a single LC system was described by another group allowing high throughput and highly sensitive proteomic analyses [69].

More recently, peak capacities between 1000 and 1500 were reported on 40–200 cm fused silica capillaries (50 μm) packed with 1.4–3 μm porous C_{18} bonded silica particles operated at 20 kpsi (1380 bar) [52]. It was noted that smaller particles does not provide higher peak capacity (within the pressure limit of 20 kpsi) but improve the productivity (peak capacity/time). This separation quality enabled the reproducible identification of over 12,000 different tryptic peptides from more than 2000 distinct *Shewanella oneidensis* proteins in 12 h using a linear ion trap. For such a slow analysis, porous particles were favored over non-

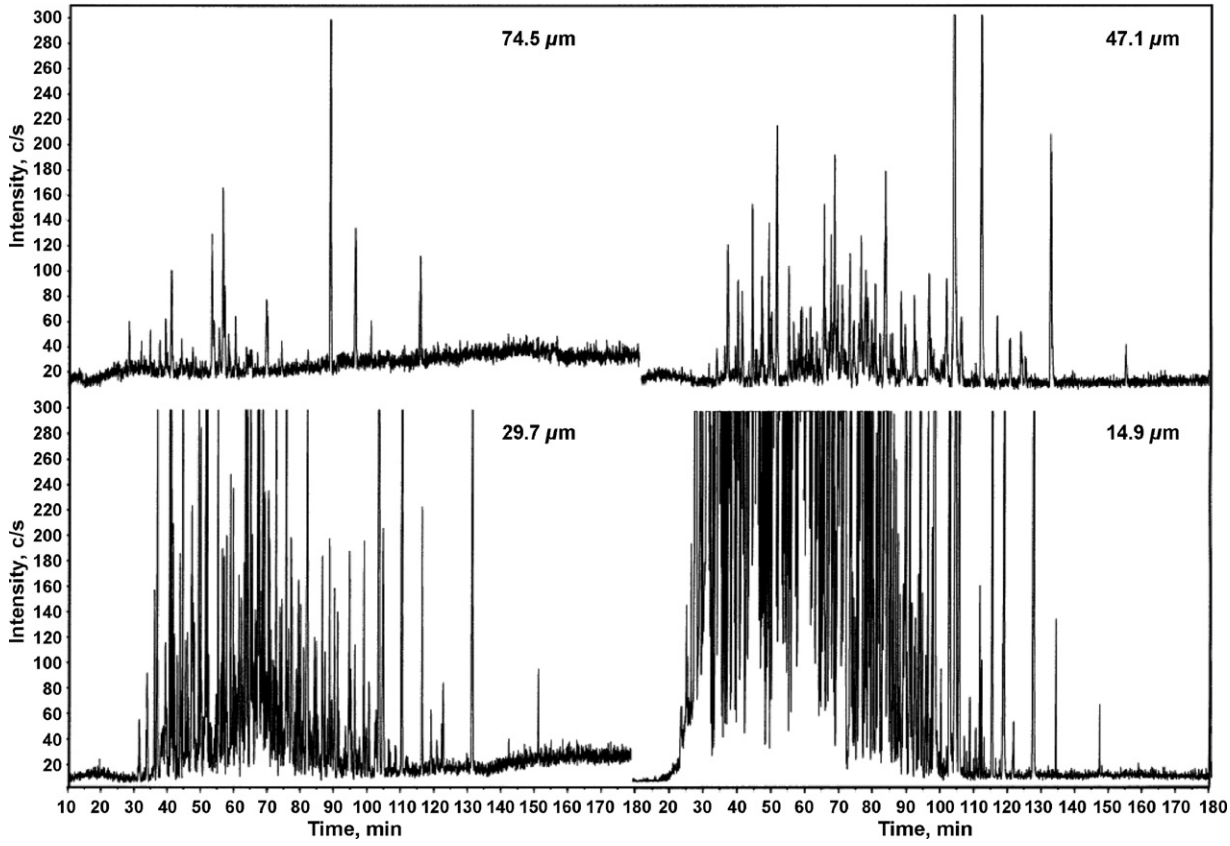

Fig. 2. Effect of downscaling the internal diameter on the ESI-MS response. The LC–MS base peak chromatograms represent the analyses of 100 ng of a yeast soluble protein tryptic digest on 87 cm long capillaries packed with 3.6 μm C_{18} silica-based particles. Capillaries were operated at 10,000 psi (690 bar) and the resulting flow rates were 393, 155, 76 and 20 nL/min for the 75, 50, 30 and 15 μm capillaries, respectively (reprinted with permission from [63]).

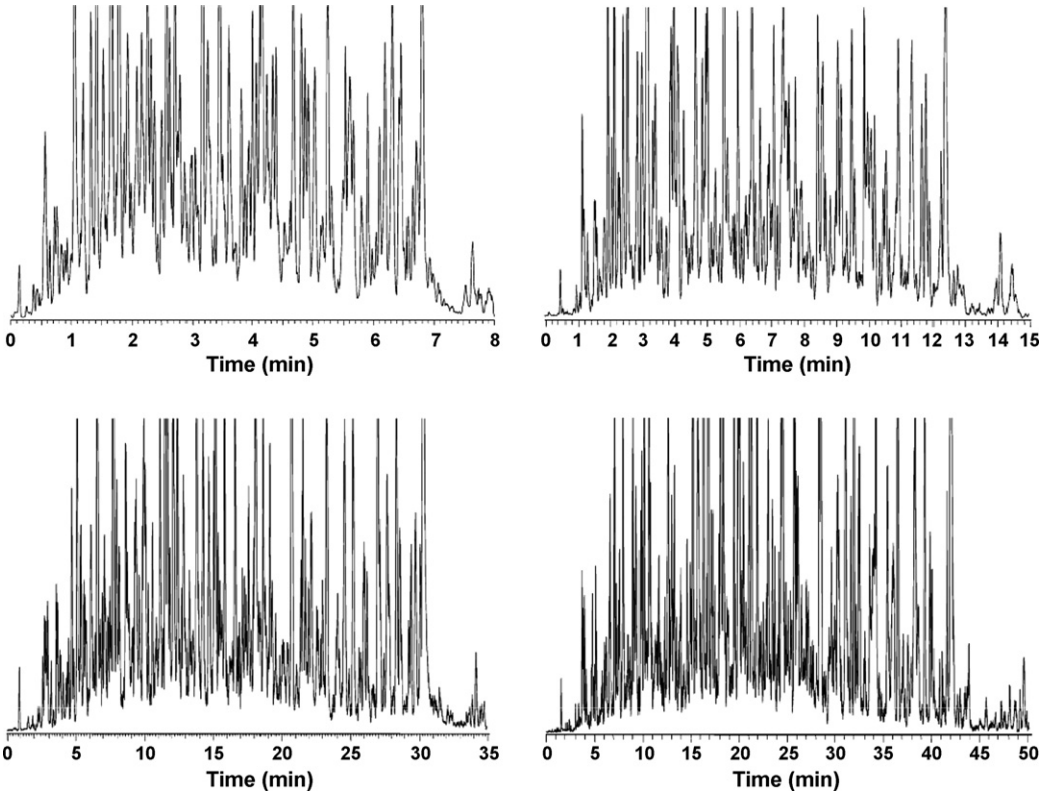

Fig. 3. Fast, highly efficient LC–MS of 1 μg of a *S. oneidensis* tryptic digest. The 20 cm $L \times 50$ μm ID capillary, packed with 0.8 μm porous particles was operated at 20,000 psi. Peak capacities were calculated to be between 130 (8 min analysis) and 420 (50 min analysis) (reprinted with permission from [71]).

porous particles due to the smaller separation window (which affects P_c) and limited loadability of the latter. Based on the analysis of a non-depleted human blood plasma sample, for which 835 distinct proteins were identified with high confidence, the dynamic range of the set-up was estimated to be 10^6. The number of identified proteins appeared to be seven-fold greater than that obtained using an 85 cm $L \times 30$ μm ID $\times$ 3 μm d_p packed capillary and equivalent to an ultra-high efficiency 2D (SCX-RPLC) separation combining SCX and RPLC using the 10 kpsi set-up [70]. The performance of the improved 20 kpsi separations will in part be due to the upgrade from a 3D ion trap (LCQ) to a linear ion trap (LTQ). The same instrumentation was subsequently used for fast efficient RPLC separations using sub micrometer (0.8 μm) C_{18} bonded porous particles packed in 20 cm $\times$ 50 μm fused silica capillaries [71]. Peak capacities of 130–420 were obtained in short analysis times (8–50 min) due to the improved mass transfer in and out of the stationary phase and to the small contribution of the Eddy diffusion term (Fig. 3). Approximately 1000 *Shewanella oneidensis* proteins could be identified in 50 min from ~4000 tryptic peptides; ~550 proteins in 20 min from ~1800 peptides; and ~250 proteins in 8 min from ~700 peptides. Upon comparison of direct infusion ESI-MS/MS with RPLC–MS/MS for high-throughput proteomics, it could be concluded that, for the same sample load, the latter approach identified 10 times more peptides and proteins, with a higher confidence, in a similar analysis time (8 min). This can be explained by a reduction in complexity, combined with a concentration effect. The authors noted that these faster separations are particularly useful for targeted analysis. Dynamic range measurements were performed using human plasma and were determined to be 3–4 orders of magnitude. Proteins present at ng/mL levels in plasma were not detected by the fast RPLC–MS/MS analysis. The ability to obtain such results, is in part due to the improvements at the level of the mass spectrometer (the LTQ ion trap was able to acquire 25 high quality MS/MS spectra in 6.5 s). Even greater throughput, however, is achievable when the MS/MS stage is omitted and the accurate mass measurements provided by a high resolution MS instrument are exploited [72]. RPLC-ESI-TOF- and RPLC-ESI-FTICR-MS using 10 cm $L \times 50$ μm ID $\times$ 0.8 μm d_p C_{18} porous particles, allowed the identification of ~2000 different peptides from ~600 different *S. oneidensis* proteins in 2–3 min using the accurate mass and time (AMT) tag approach.

Yates and coworkers recently reported RPLC separations on a long column (50 cm $L \times 150$ μm ID $\times$ 3 μm d_p) as part of their MudPIT approach [73]. They used 20 kpsi instrumentation to drive a triphasic MudPIT capillary and, using a 350 min gradient, the long RPLC column could generate peak capacities up to 400. The authors stated that the use of highly efficient second dimension separations greatly reduces the number of salt steps. If the second dimension separation has insufficient separation power for the sample analyzed, less salt steps result in less identifications. When comparing their long 50 cm RPLC column MudPIT approach, with a short 10 cm RPLC column MudPIT set-up, ~30% more yeast protein identifications where achieved using the former with the same total run time. The MudPIT pro-

cedure will be described in more detail in the second part of this review.

The above described reports require specialized high pressure pumping equipment which is not accessible to every lab. In the meantime, however, several companies introduced ultra-high pressure instrumentation allowing operation up to 1034 bar (15,000 psi). Capillary columns packed with small particles (sub 2 μm) and longer columns packed with larger particles are now commercially available and highly efficient peptide separations based on ultra-high pressure may begin to find applications in routine environments. Plumb et al. [74] reported high peak capacities (~1000) on 15 cm $L \times 2.1$ mm ID $\times$ 1.7 μm d_p Bridged Ethyl Hybrid (BEH) C_{18} particles in 1 h using ultra-high pressures (11,000 psi), elevated temperatures (90 °C) and high flow rates (800 μL/min). This is probably the productivity (peak capacity/time) record to date. The authors stress that such high resolution separations might find applications in proteomics although smaller diameter columns need to be implemented not to sacrifice ESI sensitivity. Using a nanoAQCUITY, Liu et al. [55] reported peak capacities as high as 600 on 30 cm $\times$ 75 μm RPLC columns packed with 1.7 μm BEH C_{18} particles using a 432 min gradient. The same gradient on a 15 cm column provided a P_c of 450 and on a 30 cm column packed with 3 μm particles a P_c of 370. This P_c improvement was not constant over the gradient range (24–432 min) tested but varied between 11 and 36% when doubling the column length. They further observed that, with the same back-pressure, a shorter (15 cm) 1.7 μm particle column outperformed a longer column (50 cm) packed with 3 μm particles over gradient lengths ranging between 24 and 432 min. This is in contrast with the general finding that longer columns packed with larger particles yield higher peak capacities. The slope of the peak capacity vs. gradient time of the latter, however, appeared to be higher. For extremely long gradients, the 3 μm packed long column might outperform the 1.7 μm column which is consistent with literature data [48,50]. Yang et al. reported on the analyses of a complex mouse brain proteomic sample on 15 cm and 100 cm $L \times 75$ μm ID capillaries packed with 3.5 μm d_p C_{18} particles [75]. A splitless ultra-high pressure nano-LC system operated up to 15,000 psi (Micro-Tech Scientific) was used. It was demonstrated that the 100 cm column showed an improvement with a factor 1.4 in the number of identified peptides when using the same gradient and an improvement with a factor of 2.7 when the gradient time was extended.

3.1.2. Conventional HPLC (<600 bar)

Recently several papers have been published reporting on highly efficient peptide separations on long columns using conventional HPLC instrumentation (up to 600 bar). Temperature was fully exploited to reduce the mobile phase viscosity, hence, column back pressure. Carr et al. demonstrated that comparable peak capacities to those in ultra-high pressure LC can be obtained at much lower pressures by using a 60 cm long set of narrow-bore columns packed with 5 μm pellicular (superficially porous) particles [50]. They showed that, when time is not the limiting factor, best peak capacities in gradient elution are obtained by using large particles and the longest column

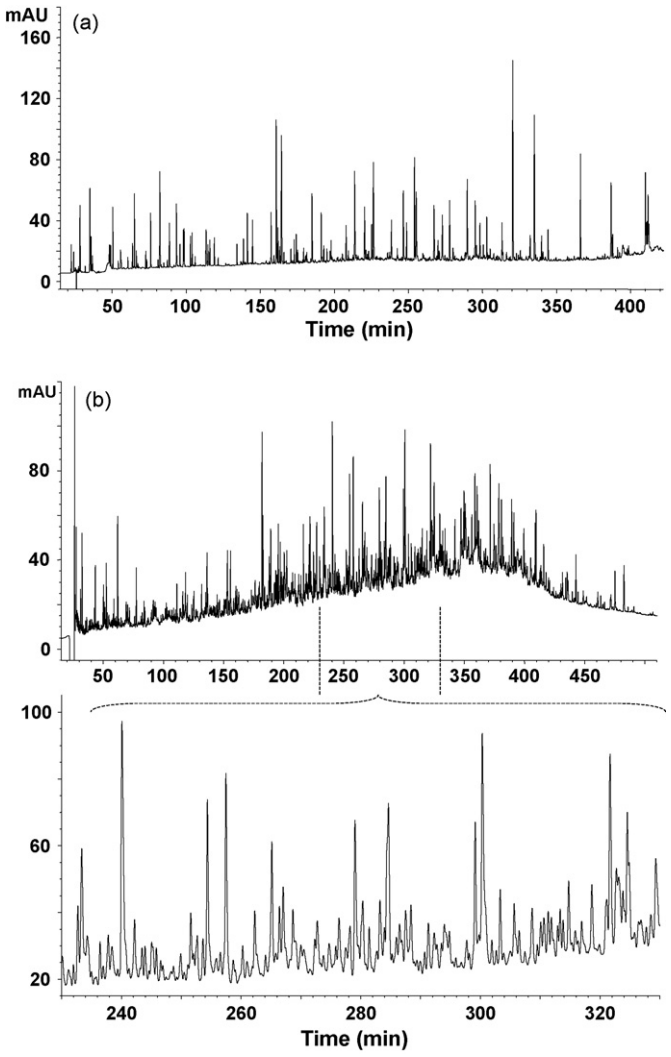

Fig. 4. Separation of a BSA (a) and a depleted human serum tryptic digest (b) on a 8 × 25 cm $L \times 2.1$ mm ID × 5 μm d_p Zorbax SB300-C_{18} column-set. Columns were operated at 60 °C. Mobile phase A consisted of 2% ACN, 0.1% TFA and mobile phase B of 70% ACN, 0.1% TFA. The acetonitrile gradient proceeded at 0.135% B/min and the flow rate was set at 200 μL/min. Detection was performed at 214 nm (reprinted with permission from [77]).

that can be operated at the pump's pressure limit. A peak capacity of 460 was obtained in 4 h at room temperature. Compared to a 7.5 cm column, P_c is about 2.8 times larger (for the same gradient steepness) corresponding well with theory (square root of the ratio of the column lengths). By increasing the column temperature to 70 °C, the analysis time was reduced to 2 h without compromising P_c. The higher solute diffusivity at higher temperatures allows one to operate at higher flow rates while maintaining the gradient steepness. Their results were compared with peak capacities obtained in ultra-high pressure liquid chromatography; more in particular with references [52,53,56] and they concluded that the results are comparable. In that same study, it was also reported that pellicular particles gave about 50% higher peak capacities compared to the analogous totally porous material. Apparently, these porous shell particles are gaining much interest in the field of peptide separations. Marchetti et al. [51] showed good peptide sepa-

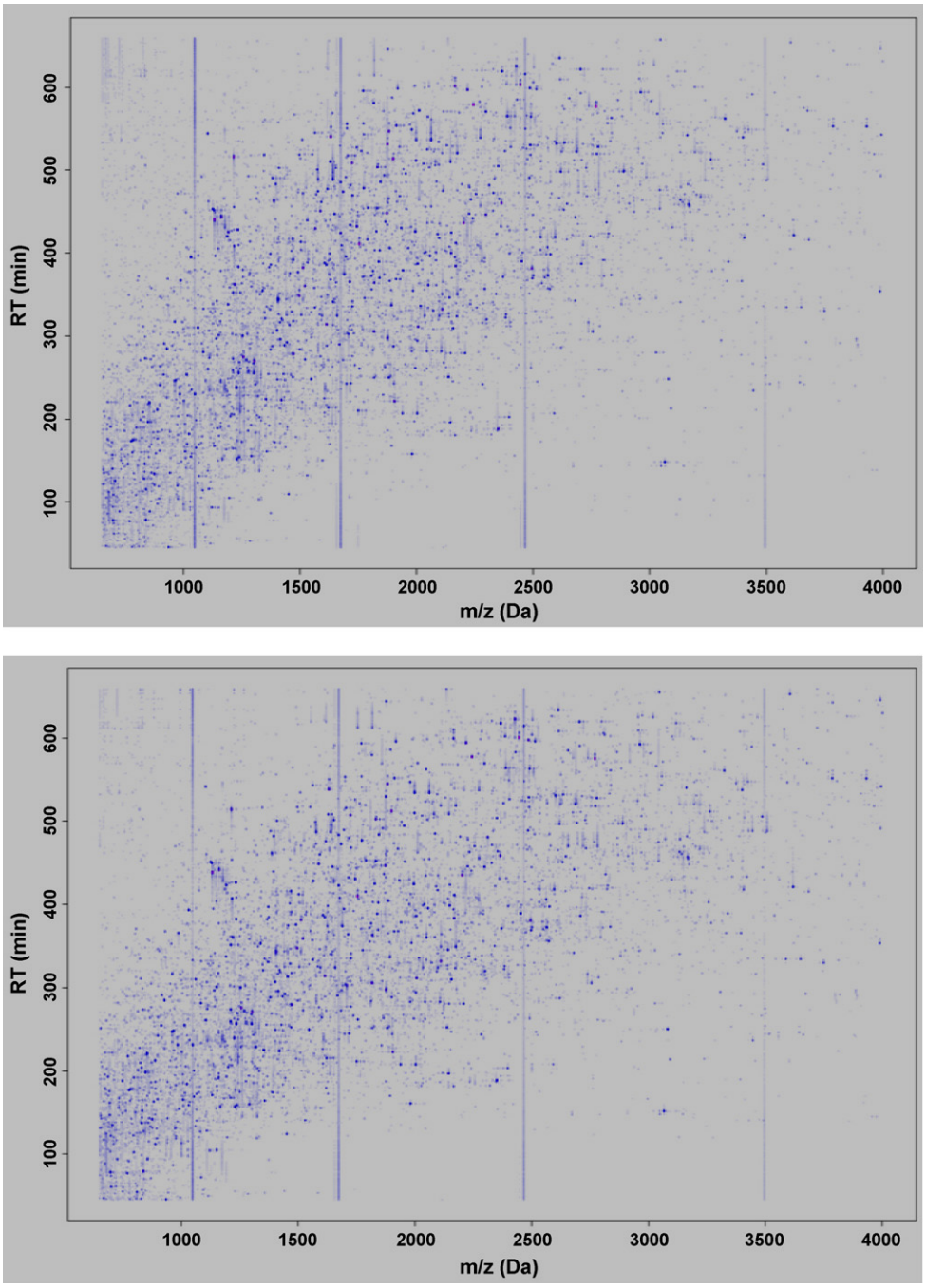

Fig. 5. Duplicate 3 m LC-MALDI analysis of a healthy human serum tryptic digest. An Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany) and a 4800 MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) were used. Sample was depleted from the six most abundant serum proteins (albumin, alpha-1-antitrypsin, haptoglobin, IgG, IgA and transferrin) using a multiple affinity removal system (MARS—Agilent Technologies). The sample was split in two equal parts following depletion, and both parts were reduced and alkylated prior to overnight trypsin digestion. 1 mg of each sample was injected onto a 12 × 25 cm L × 2.1 mm ID × 5 μm d_p Zorbax SB300-C_{18} column-set (Agilent Technologies). Columns were operated at 70 °C (using a Polaratherm 9000 series—SandraSelerity Technologies, Kortrijk, Belgium) and mobile phases (identical to those described in Fig. 4) were delivered at a flow rate of 200 μL/min. A 700 min acetonitrile gradient was applied (0–70% B, 0.1% B/min). Initial column pressure stabilized at 460 bar and reached its maximum value at 500 bar during the gradient. Relatively large sample volumes (500 μL) were injected to prevent precipitation of the material present. Sample was focused at the inlet of the column, prior to application of the acetonitrile gradient. Fractions were collected every 12 s in 384 well plates. In total 3072 fractions (8 × 384 well plates) were collected, spanning an elution window of 614.4 min. Since the 1200 series fraction collectors can only accommodate four well plates, two fraction collectors had to be combined in a serial fashion in order to collect fractions over the entire elution window. This could easily be achieved using a switching valve. Following fraction collection, well plates were dried and fractions were spotted on MALDI targets using a 384 head robot (Sciclone ALH 3000 Caliper Life Sciences, Mountain View, CA, USA). To every fraction, 4.5 μL of matrix solution (4 mg/mL α-cyano-hydroxy-cinnamic acid in 50% ACN, 0.1% TFA) was added and 2 μL was spotted on MALDI targets. The matrix solution contained an internal standard consisting of five peptides used for internal calibration of the acquired spectra. The internal standard peptides are clearly visible in the 2D plot at m/z 1046.54, 1672.92, 2465.19, 3494.65. MS measurements were performed in the positive reflectron mode and consisted of 3000 laser shots per spot.

rations (peak capacities of 400) in a relative short period of time (1 h) on 15 cm $L \times 4.6$ mm ID columns packed with a new type of C_{18} shell particles consisting of a 1.7 μm core and a 0.5 μm porous shell (2.7 μm d_p). The high efficiencies result from the low Eddy diffusion and the small mass transfer contribution. Upon comparison with similar sized columns packed with 3 μm fully porous particles, an improvement in P_c with a factor of 1.4 was noted. A long set of columns can potentially be operated at very high efficiency because the pressure required to operate the columns around their maximum efficiency remains moderate (permeability comparable with 3 μm fully porous particles [76]). The potential of such a short column to provide very fast, still efficient, separations was further demonstrated.

We recently obtained high efficiency separations (up to 200,000 plates) on conventional LC equipment by coupling eight 25 cm $L \times 2.1$ mm ID columns packed with 5 μm Zorbax 300SB C_{18} particles (total length 2 m) and operation at 60 °C using a dedicated LC oven [77]. The reduced mobile phase viscosity at the elevated temperature allowed us to operate the coupled column set-up at optimal linear velocity. The separations of a BSA and a depleted human serum tryptic digest are shown in Fig. 4. The peak capacity in this 1D set-up was calculated to be 900. The same analyses were repeated on conventional 4.6 mm columns and the peak capacity was 1090. Efficient separations can thus be performed in conventional column formats without the problem of heat generation as is typically encountered in ultra-high pressure LC [46,47]. The replicate LC-MALDI analysis of a healthy serum sample, treated in parallel from depletion, on a 3 m coupled column set (12 × 25 cm $L \times 2.1$ mm ID × 5 μm d_p Zorbax SB300-C_{18}), is presented in Fig. 5. Temperature was further increased to 70 °C. Higher temperatures, combined with low pH mobile phases (<pH 2) have limitations due to increased incidence of peptide bond cleavages next to aspartic acid residues. However, if these cleavages appear to be reproducible this does not necessarily exclude the use of even higher temperatures. The high flow rates (200 μL/min) originating from the narrow-bore columns were not compatible with direct spotting on MALDI-targets. Therefore, fractions were collected in 384 well plates and dried, prior to spotting on MALDI targets using a 384 head robot. Thousands of species could be discriminated and very similar 2D patterns (*m/z* in *x*-axis, chromatographic retention time in *y*-axis) could be obtained.

Such highly efficient separations were subsequently combined with the COFRADIC procedure for protein biomarker discovery purposes [78]. COFRADIC (COmbined FRActional DIagonal Chromatography) relies on a (diagonal) chromatography-based isolation of subsets of peptides, which are representative for their parent proteins [79]. The extremely powerful combination of this peptide-centric technology with 1 m highly efficient separations (operated at 60 °C) resulted, for the analysis of serum, in an increase in the uniquely identified peptide sequences by a factor of 2.6, compared to the COFRADIC procedure on a standard 25 cm column. This is a reflection of the increased peak capacity obtained on the 1 m column, which was calculated to be a factor 2.7 higher than on the 25 cm column. Besides more efficient peptide sorting, less ion suppression was noticed. An additional advantage is the increased loadability of these coupled columns. The improved separations appeared to be highly reproducible (also see Fig. 5) which is of course the principal requirement in diagonal chromatography but is further critical for building a solid label-free methodology for differential analysis. Upon replacing the mobile phase additive TFA with formic acid or ammonium acetate, the described approach becomes compatible with $^{16}O/^{18}O$ labeling (unpublished results).

The highly efficient narrow-bore separations described above were subsequently miniaturized to allow the coupling with highly sensitive nanospray without the requirement for splitting and to allow direct spotting on MALDI targets (K. Sandra unpublished results). Fig. 6 shows the replicate separation of a depleted human serum tryptic digest on a 1.2 m L × 100 μm ID × 5 μm Zorbax 300SB C_{18} particles column. Peak capacities in excess of 400 could be obtained. Conventional instrumentation was used and column back-pressure was reduced by working at elevated temperatures (60 °C). The sample was first injected on a commercially available μSPE column which consisted of the same packing material and, hence, could also be operated at elevated temperatures. In contrast with the above described set-ups, the column consisted of one piece, to limit the dead volume, and was coiled to be placed in the oven. The impact of the coiling on the efficiency was not investigated although, this might be negligible in the case of miniaturized columns. The construction of long miniaturized columns by connecting different commercially available nano-columns appeared to be detrimental due to

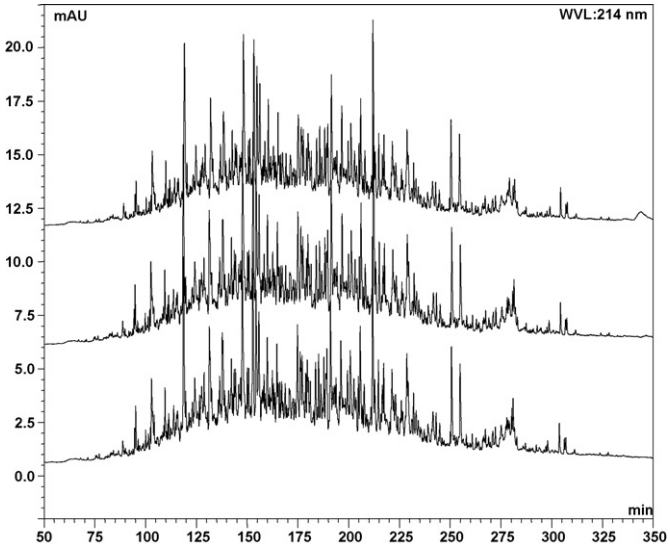

Fig. 6. Replicate injection of 1 μg of a healthy serum tryptic digest onto a long miniaturized column (1.2 m $L \times 100$ μm ID × 5 μm Zorbax 300SB C_{18} particles). The capillary was coiled and placed in the column compartment of the LC system (Ultimate 3000) which was thermostated at 60 °C. Solvent systems consisted of 0.05% TFA (solvent A) and 0.04% TFA, 80% ACN (solvent B). The sample was injected onto a short commercially available μSPE column, consisting of identical pH and temperature resistant particles (5 mm $L \times 300$ μm ID × 5 μm 300SB Zorbax C_{18}), at a flow rate of 20 μL/min (0.05% TFA). After sample loading, sample was back-flushed at a flow rate of 450 nL/min and a 360 min gradient (4–65% B, 0.17% B/min) was applied. Peptide elution was monitored at 214 nm. A silica sintered outlet frit positioned the packed bed in place and no inlet frit was constructed.

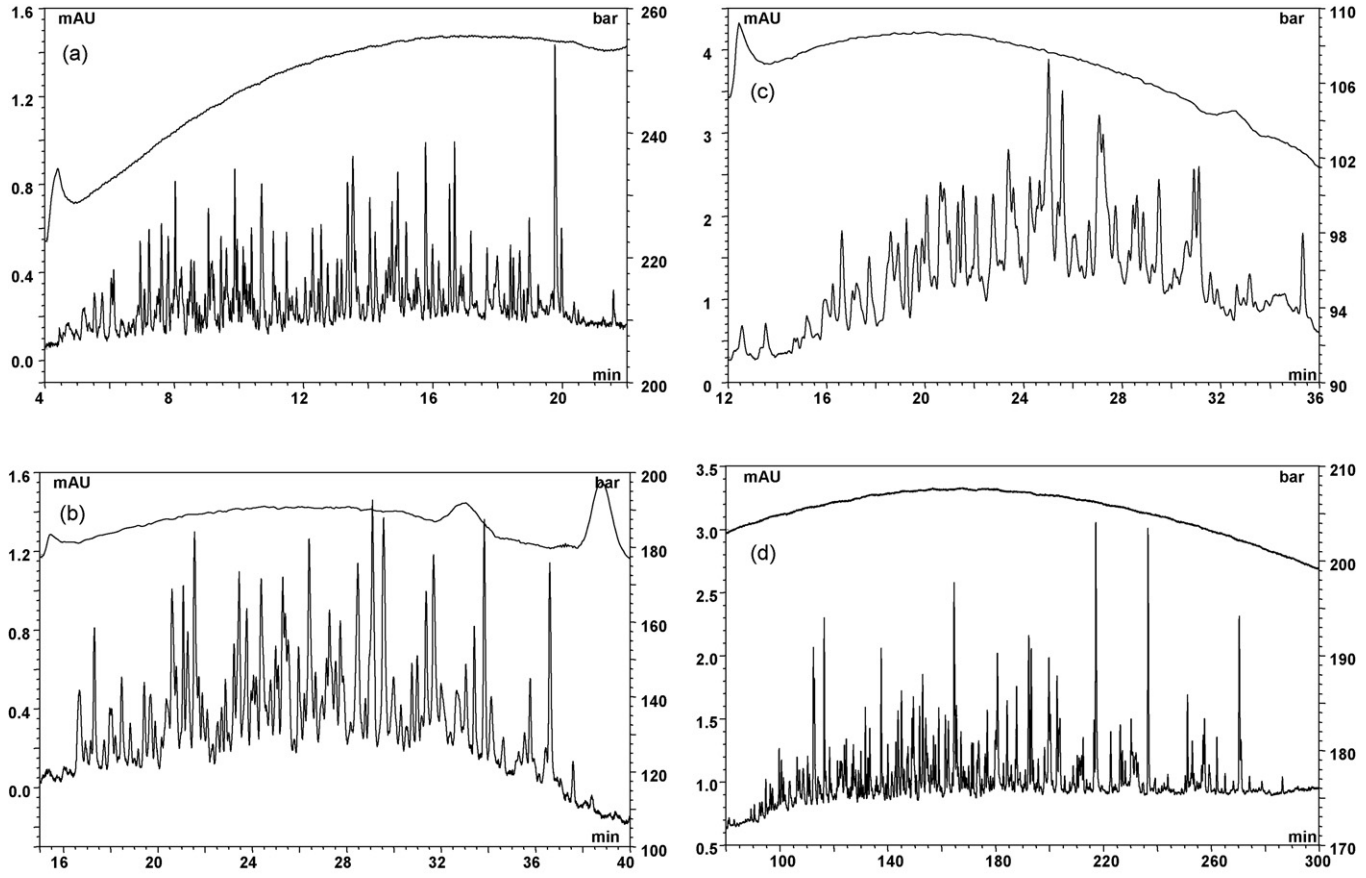

Fig. 7. Separation of a tryptic digest of 10 reduced and alkylated proteins (bovine albumin, human alpha-1-acid-glycoprotein, human alpha-1-antitrypsin, human alpha-2-macroglobulin, bovine catalase, bovine cytochrome *c*, human haptoglobin, bovine hemoglobin, bovine beta-lactoglobulin and human transferrin) on three different commercially available capillary columns and one in-house packed long capillary: (a) PS-DVB monolith (5 cm $L \times 200$ μm ID—Dionex), (b) C_{18} silica based monolith (15 cm $L \times 100$ μm ID – Phenomenex), (c) packed C_{18} capillary (15 cm $L \times 75$ μm ID $\times 3$ μm d_p—Dionex), (d) long in-house packed capillary (1.2 m $L \times 100$ μm ID $\times 5$ μm d_p). Both UV-traces (214 nm) and column pressure profiles are displayed. The PS-DVB monolith was operated at a flow rate of 2.5 μL/min and thermostated at 60 °C. Mobile phases consisted of 0.05% TFA (solvent A) and 50% ACN, 0.04% TFA (solvent B). A 20 min gradient ranging from 0 to 70% solvent B was applied. Despite the short column length and the high operating temperature, column back pressure was substantial. The silica-based monolithic and the short packed column (b) and (c) were operated at 2 μL/min and 300 nL/min, respectively, (25 °C) using a 30 min gradient. Other operational conditions, including solvents, were similar as those described in Fig. 1. The 1.2 m long packed capillary (d) was run at a gradient of 300 min from 4 to 55% ACN at a flow rate of 450 nL/min. Other conditions were identical to those described in Fig. 6. Interesting to note is that column back-pressure is lower on the 1.2 m long capillary than on the 5 cm PS-DVB monolith. Both capillaries were operated at 60 °C.

the dead volumes that are inevitably present at the connection points. Guzetta et al. packed a 60 cm $L \times 150$ μm ID capillary with identical 5 μm Zorbax 300SB C_{18} particles for use in a triphasic MudPIT approach and demonstrated that long capillary columns are significantly better than standard length columns at handling complex mixtures [80].

Separations on such long columns actually take us back in time. It was already in 1988 that Novotny and Karlsson obtained an isocratic efficiency of 200,000 in 30 min time on a 1.95 m $L \times 44$ μm ID $\times 5$ μm d_p column [64]. Twenty years earlier, at the early stages of the development of HPLC, Horvath and Lipsky already separated compounds on long capillaries [20]. It was nicely quoted by Guzetta and Chien: If ever there was a chromatographic problem that needed long column chromatography due to the inherent complexity of the sample, it is proteomics [80]. Despite this, it has to be stressed that the gain in P_c upon switching from a 1 m column to a 2 m column is, potentially, only a factor 1.4.

3.2. Monolithic columns

In the ever ongoing quest for better peptide separations, monolithic columns appear to be very promising. Monolithic columns, consisting of a single piece of separation media instead of a cluster of packed particles, are characterized by an inherent high permeability and low mass transfer resistance. This allows highly efficient separations of large molecules (with low diffusion constants) with low resistance to flow. Monolithic columns can be divided into two groups: the organic monoliths (polymer-based, e.g. polystyrene-divinylbenzene and polymethacrylate) [81–85] and the inorganic monoliths (silica-based) [86–87]. It has been reported that silica monolithic columns are better suited for the separation of small molecules, whereas organic monolithic columns are favored for the separation of macromolecules [57]. Both types, however, have been successfully used for peptide separations [88,89]. The separations of a tryptic digest of 10 proteins on commercially available PS-DVB, C_{18} silica mono-

lithic and short (15 cm) microparticular capillaries and on a long (1.2 m) in-house packed capillary are presented in Fig. 7. Upon visual inspection of the chromatograms it becomes clear that the monolithic columns possess a higher resolving power than the commercially available packed capillary. This holds especially true for the PS-DVB monolithic column which was operated at 60 °C. The latter operating temperature was suggested to be optimal for peptide separations on PS-DVB capillaries by Huber and coworkers [90].

The same group compared commercially available PS-DVB monolithic capillaries and packed capillaries in an LC-ESI-MS set-up [91]. They demonstrated that a 100 μm PS-DVB monolithic capillary operated at 500 nL/min yields better results, in terms of identified peptides and cumulative MOWSE scores, over a 75 μm packed capillary operated at 200 nL/min. The high performance of the 100 μm monolithic column can be explained by a combination of the high chromatographic efficiency with a small column ID leading to sharp peaks and a relative high concentration of the eluting analytes. Moreover, the monolithic column appeared to be more reproducible for peptide identification in three consecutive runs. Karger and coworkers [92] demonstrated that PS-DVB monolithic capillaries (50 μm ID × 10 cm *L*) deliver ∼30% higher separation performance than granular (5 μm) packed capillaries as well as providing an increased recovery of larger peptides. Earlier studies by Huber et al. revealed that the time to generate similar peak capacities is 2–4 times higher with conventional peptide separation columns packed with porous silica-based particles compared to PS-DVB capillaries [89,93]. Typical peak widths at half height on a 60 mm *L* × 0.2 mm ID PS-DVB monolith ranged from 3 to 5 s in a 40 min run translating into a peak capacity of several hundreds. An additional claimed advantage of monolithic columns over packed columns is their resistance to fouling by analysis of complex, real samples. This is particularly useful when analyzing samples of biological origin such as serum or plasma [58]. The impact that the multiple injection of a serum tryptic digest has on the performance of a long narrow-bore packed column is shown in Fig. 8. A systematic increase in peak width of a mixture of five peptides is noticed after injection of large amounts (1 mg) of serum tryptic digests on long narrow-bore columns. Performing a solid-phase extraction (SPE) step using a C_{18} sorbent prior to injection prevented this column fouling.

Monolithic columns have been successfully used to provide fast, highly efficient peptide separations, analogous to columns packed with smaller particles. It has been shown by Regnier and coworkers that little loss in peptide resolution is noticed on conventional silica monolithic columns (4.6 mm ID) as mobile phase flow rate is increased from 1 to 10 mL/min [94]. Gradient volume was kept constant which means a 10 times shorter gradient time and, hence analysis time, in the latter case. One negative feature of the very fast gradient elution associated with the high flow rates, was that some analytes carried over in later fractions which can be problematic for complex sample analysis because high abundant peptides from earlier fractions can obscure the MS detection of lower abundant peptides. Bischoff et al. [95,96] demonstrated similar results on capillary silica monolithic columns and a 50 μm capillary column could be operated

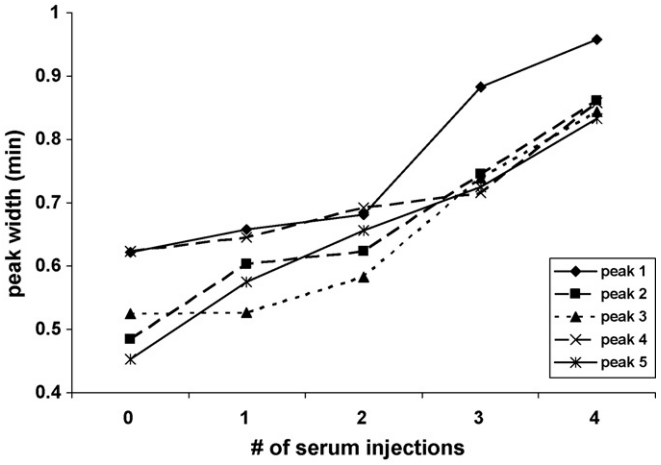

Fig. 8. Column fouling upon injection of multiple depleted human serum tryptic digests (1 mg—500 μL injection) onto a long narrow-bore HPLC column (see Fig. 5 for additional chromatographic information). The peak widths of a mixture of five peptides (Bradykinin fragment 1–5, Angiotensin II, Neurotensin, ACTH clip (18–39) and Insulin B-chain oxidized), analyzed following the injection of 0–4 serum digests, are plotted in function of the number of serum injections. Following depletion, the sample was reduced, alkylated, digested by adding trypsin, acidified, centrifuged and injected as such without any additional sample clean-up.

at flow rates up to 2 μL/min, which is more than 10 times the flow rate typically used with a packed column of the same dimension. Leinweber et al. further demonstrated that silica monoliths showed superior results over packed capillaries with respect to both analysis time and separation efficiency [97]. Very fast separations (9 min gradients) in combination with ultra fast tandem MS/MS have also been shown on short 5 cm *L* × 200 μm ID PS-DVB columns. In comparison with packed columns traditionally used in proteomics, a three fold gain in time could be obtained [98]. Karger and coworkers also demonstrated high-speed and high-resolution separations on commercially available PS-DVB capillaries [99]. The LC effluent was mixed with matrix and continuously deposited onto a MALDI target. Due to this form of sample deposition, the high resolution delivered by the monolithic column could be preserved. Over 2000 components could be discriminated using a 10 min gradient and up to 400 peptides could be identified, following MALDI-MS and -MS/MS analysis. According to the authors, a much longer gradient (up to 60 min) would be required to analyze the same sample using conventional nano-LC-MALDI-MS. It was further stated that, in comparison to ESI-MS, the off-line coupling of LC to MALDI-MS removes the time constraint for precursor selection. The ability to provide fast efficient separations without the requirement for specialized instrumentation makes these monolithic columns extremely attractive to use in a multidimensional set-up. Several authors, however, stated that the performance of monolithic columns for fast second dimension separations is currently lower than that of a particulate column packed with 2 μm particles, but higher than that of a column packed with 5 μm particles [48,58,100–102]. From kinetic plots, it can be concluded that monolithic columns are especially attractive to obtain high efficiency separations when time is not the limiting factor [101].

The use of higher flow rates also has disadvantages due to the concentration sensitive nature of electrospray ionization. A compromise between speed and sensitivity has to be found. We observed that, when using the same gradient, a silica-based monolithic capillary slightly outperformed a similar sized packed capillary in an LC-MALDI-TOF/TOF set-up with direct spotting when the spot size is kept constant (also consider Fig. 1). This can easily be done by adjusting the flow rate of the matrix solution that is added through a microtee prior to deposition on the MALDI-target. However, if the spot size is doubled the number of identifications dropped substantially (factor 1.5–2). In an on-line combination with an ESI-QqTOF instrument, the monolithic capillary performed below its potential (not below its chromatographic performance) and the number of identifications using a packed column was at least two times higher. This is a direct consequence of the flow rate difference between these two (200 nL/min packed capillary vs. 1 μL/min monolithic capillary). Despite the generation of narrower peaks on the monolithic silica capillary, the five times higher flow rate increases the peak volume drastically thereby decreasing the ESI-MS sensitivity.

Next to the capability of these columns to provide fast separations at high linear velocities, the low back-pressure of silica monolithic columns, which is comparable to columns packed with 11 μm particles (although with the separation efficiency of a packed bed with 3 μm particles) [57,87], allow them to be used in long column formats without requiring specialized instrumentation. It is believed that the true benefit of monolithic columns lies in their ability to provide high resolution separations on long columns [48,101]. It has been shown that monolithic silica capillaries measuring 140 cm produce 160,000 theoretical plates [102]. More recently, 1,000,000 plates have even been reported [103]. Enhanced chromatographic resolution and reduced ion suppression were shown in a plant metabolomics study when switching from 30 to 90 cm silica-based monolithic capillaries while maintaining the gradient [104]. The 90 cm column showed 65,000 theoretical plates at 80 cm using on-column UV detection. Barosso et al. [95] and van de Meent and de Jong [105] demonstrated the gain in resolution when going from a 15 cm to a 50 cm and 75 cm capillary C_{18} silica monolithic column, respectively. Luo et al. [53] prepared 70 cm × 20 μm ID silica-based monolithic columns and showed their performance in proteomic analyses. A separation peak capacity of 420 was provided in 200 min in conjunction with on-line SPE. The authors operated the columns at low flow rates (40 nL/min) and the concomitant low amol sensitivity achievable, combined with the high efficiency allowed the identification of 2367 peptides covering 855 distinct *S. oneidensis* proteins from a 2.5 μg tryptic digest sample in 10 h. Xie et al. designed [106] a 60 cm × 75 μm monolithic silica ODS capillary column with integrated nano-electrospray emitter. The authors demonstrated that 20% of the separation efficiency is sacrificed when the monolithic column is interfaced to an external ESI-emitter. In contrast with packed capillaries with integrated emitters, failure of the emitter does not necessarily destroy the column. The analysis of 0.5 μg of a *S. cerevisiae* tryptic digest, resulted in the identification of 5501 unique peptides (1323 proteins) over a 400 min gradient elution.

Long capillary monolithic columns are thus far not commercially available. Column connectors, however, can be purchased to connect multiple conventional silica monoliths (Merck, Phenomenex). The performance of long conventional columns, however, is much lower than that of long capillary columns, most likely due to the column connectors themselves [58,103]. Despite that, Bones et al. obtained peak capacities in excess of 1000 in a retention window of 290 min for typical proteomic samples by using a meter long coupled monolithic silica column [107].

A very remarkable finding is the absence of organic polymer-based monoliths in long column formats. The back-pressure of these columns, however, is surprisingly high as is also presented in Fig. 7. At a column temperature of 60 °C and a flow rate of 2.5 μL/min, a short (5 cm) PS-DVB capillary with an internal diameter of 200 μm exhibited a back-pressure in excess of 200 bars. This value even reached 250 bar during the progress of the acetonitrile gradient. A 15 cm $L \times 100$ μm ID silica monolithic column only generated 180 bar at 25 °C and at a flow rate of 2 μL/min. This finding was also the driving force for Smith and coworkers to construct long silica monolithic columns [53].

Monolithic capillaries, both organic [108] and inorganic [109], have recently proven to be very suitable for ultra sensitive analysis. This stems from the fact that these columns can easily be fabricated in ultra narrow 10–20 μm capillaries. Efficient packing of capillaries with these dimensions is extremely difficult and monolithic capillaries provide a good alternative. The low nL/min flow rates associated with these narrow capillaries, allow the low amol/high zmol ESI-MS and MS/MS analysis of protein tryptic digests [92,108]. Karger and coworkers found a factor of up to 20-fold sensitivity improvement when switching from a 75 μm packed column to a 20 μm PS-DVB capillary and they successfully identified proteins extracted from ∼1000 cells of breast cancer tissue [108]. Smith and coworkers reported greater than 10-fold improvement in sensitivity on 10 μm C_{18} silica monolith compared to the analysis on more conventional capillary LC (150 μm) [109]. This enabled the identification of more than 5000 different peptides by MS/MS from 100 ng of a *S. oneidensis* tryptic digest. They further demonstrated that ion suppression is virtually eliminated, making this effort extremely valuable for relative or absolute quantitative measurements without the need for internal standards or isotopic labeling. More recently, 10 μm ID silica-based monolithic capillary columns with integrated nanoESI emitters have been developed [110]. It is believed that monolithic columns, one day, will become the main workhorse in chromatography [58]. They will probably affect the market of capillary separations at an early stage and this will have a direct impact in the discipline of proteomics. However, they will have to compete with a technology that has a proven reliability and that is in widespread use, namely that of silica-based packing material. There is definitely a lot of movement in the field of monolithic columns and peptide mixtures are the samples of choice to demonstrate the advances that are being made.

3.3. Porous layer open tubular (PLOT) columns

Open tubular columns, although the workhorses in gas chromatography, have found limited utility in LC due to the slower diffusion in liquid mobile phases. In order to achieve efficiencies comparable to those of good packed columns, PLOT columns must have an inner diameter of 10 μm or less [42]. PS-DVB PLOT columns with such dimensions were very recently constructed and utilized in proteomics by Karger and coworkers [111]. The high permeability of these columns allows them to be used in very long formats without requiring specialized instrumentation. A 4.2 m × 10 μm ID PLOT column yielded a peak capacity of 400 for the separation of a complex tryptic digest mixture. According to the authors longer and smaller columns will further boost the efficiency. The low nL/min flow rate associated with the PLOT column allowed to achieve low to sub attomole detection levels. The power of the set-up was further illustrated by the identification of 689 unique peptides covering 238 distinct *Methanosarcina acetivorans* proteins from 4 ng of an in-gel tryptic digest sample of a SDS-PAGE fraction (<70 kDa). The PLOT column was also successfully applied to the separation of larger peptides resulting from Lys C digestion.

4. Conclusions and future directions

Different approaches to achieve highly efficient peptide separations using HPLC have been described. Comparing them, in terms of performance in proteomics applications, is extremely difficult not to say impossible because different samples and sample amounts are utilized. For example, due to the dynamic range and protein processing issues associated with human serum, protein identifications are usually smaller in comparison to cellular samples. Moreover, the chromatographic performances are strongly linked to the MS systems and search strategies applied. Mass spectrometers work optimal when peak volumes are small. High flow rates and large peak widths should be avoided at all times.

On the other hand, a large number of technologies described rely on expertise of the research groups and many times tools and columns are designed in-house and do not become commercially available. For laboratories, less skilled in separation technologies, where the proteomics application dominates above chromatographic development, the availability of columns and robustness of the technology is of utmost importance.

Notwithstanding, requiring a comprehensive protein coverage using 1D chromatography might sometimes be too ambitious because the complexity of a proteome can exceed by far the capacity of the chromatographic set-up. This is especially true in the case of serum. Therefore a reduction of the complexity of the sample, by only analyzing subsets of peptides, upfront chromatography and MS analysis has proven to be extremely powerful [79,112–114].

The development of highly efficient peptide separations in one dimension is also beneficial as back-end in a two dimensional set-up, since it lowers the stress on the first dimension and less primary fractions are needed [73].

In this review, only peptide separations were dealt with and a number of findings is not valuable for protein separations due to the pure adsorption/desorption process associated with the analysis of proteins. The benefit of using extremely long columns might be negligible in this case.

What the future will bring in terms of highly efficient peptide separations is difficult to predict although we expect the introduction of second generation monolithic columns, longer columns and smaller particles, micro-fabricated separation media as part of full integrated systems and further exploration of the performance of open tubular columns. However, major developments at the level of MS will lower the stress at the chromatographic side. This is not anticipated to be for the near future.

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