A nanoLC-MS-based platform for peptide analysis
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CHAPTER 1

Introduction

1.1 Proteomic analysis: state-of-the-art

The identification and quantification of the complete set of proteins from an organ, a cell or a body fluid at any one time requires powerful analytical techniques. Much like genomics in the 1990’s, proteomics is a rapidly expanding field of research which requires sensitive and selective technologies. The following set of techniques, each with its own advantages and disadvantages, has been used to analyse proteomics samples:

- Surface-enhanced laser desorption/ionisation (SELDI) coupled to mass spectrometry (MS)
- Protein arrays
- Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)
- Capillary electrophoresis (CE) coupled to MS
- Liquid chromatography (LC) coupled to MS

Surface-enhanced laser desorption/ionisation (SELDI) is a modified version of matrix-assisted laser desorption/ionisation (MALDI). SELDI is an ionisation technique often coupled to mass spectrometry (MS) for the analysis of proteins. It
has the potential to fractionate complex protein mixtures without much sample manipulation prior to MS analysis. However, sensitivity is limited by the small amount of sample that can be deposited on a chip [1]. In addition, non-specific binding of proteins [1] and low reproducibility of signal-to-noise ratio (S/N) and normalised intensity [2] remain an issue with this technique.

Protein arrays consist of biomolecules (e.g. oligonucleotides, proteins, small molecules) immobilised on substrates such as microscope glass slides or microwells on/in which samples are applied. Proteins with affinity for the molecules immobilised are retained on the array and other matrix components are removed by washing. The array is then subjected to detection either by MS, surface plasmon resonance (SPR), fluorescence or electroluminescence. For reasons of selectivity, non-specific binding of biomolecules to the surface must be minimised. In this context, the surface chemistry of these microarrays is still a major research subject [3]. Furthermore, the wide range of protein concentrations observed in proteomics samples complicates their use, and ligand choice in relation to selectivity presents additional limitations.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has long been the method of choice for the analysis of complex protein mixtures, due to its very high resolving power. It enables the separation of thousands of proteins in a single run according to their isoelectric point (pI) in the first dimension and to their molecular weight in the second [4]. 2D-PAGE requires numerous steps (including sample handling and preparation of the gel) to identify protein spots, and the resolution of polypeptides with an extreme size, pI or hydrophobicity is difficult. Reproducibility, sensitivity and dynamic range [5] as well as automation are still problematic.

Capillary electrophoresis (CE) is a technique with high separation efficiency and speed and is suitable to separate a wide range of analytes. CE requires low injection volumes, which can be regarded as both an advantage (low sample consumption) and a drawback (limited sensitivity). An important parameter to be considered in CE is the large influence of the sample matrix on the electrophoretic separation. Peptides and proteins tend to adsorb to the silica surface of the capillary, resulting in band broadening, tailing and memory effects. In addition, adsorption to the capillary wall influences the electro-osmotic flow (EOF) and, consequently, migration times and separation efficiency. As a result, much research has been undertaken to coat silica capillaries [6–8]. Selectivity is enhanced by coupling size-exclusion chromatography (SEC) to CE for the analysis of tryptic peptides [9], and further improved by combining SEC, reversed-phase chromatography (RP) and CE in a multidimensional system [10]. However, coupling of LC
1.2 Miniaturisation of LC systems. Theory and practice

and CE remains difficult, as demonstrated by the off-line RPLC-CE system developed by Issaq et al. [11]. Though CE is a powerful separation technique that has been frequently used in proteome analysis [12], coupling of CE to other separation techniques or to MS, and loading capacity, sensitivity and reproducibility of this technique remain important issues. Therefore, LC-based separation schemes seem more promising alternatives.

With LC, separation is accomplished through a partitioning or adsorption equilibrium between a liquid mobile phase and a stationary phase. The nature of the stationary phase determines the mode of LC. An overview of these different modes is presented later in this Introduction. The composition of the mobile phase influences the state of equilibrium and thereby the selectivity of the separation. Elution can be performed under either isocratic (constant mobile phase composition) or gradient conditions (changing mobile phase composition). Peptide analysis is almost exclusively performed under gradient elution conditions. LC coupled to MS (LC-MS) presents several advantages over the aforementioned techniques. On-line coupling to MS is relatively easily achieved, though the LC-MS interface needs to be thoroughly characterised to enable quantitative analysis. Miniaturised versions of LC-MS allow the sensitive analysis of peptides. Contrary to CE, which is limited by its loadability, nanoLC set-ups making use of a trap column tolerate the injection of large sample volumes. The development of novel LC separation media permits very fast and efficient separation to be performed. LC is a very versatile and highly selective technique. In its multidimensional forms, it offers potential to achieve separation efficiencies approaching those of 2D-PAGE. It is easily automated and coupled to MS, unlike CE. Additionally, sample pretreatment steps can be included in the automated analytical set-up.

1.2 Miniaturisation of LC systems. Theory and practice

1.2.1 Theory

When a sample is injected onto a LC system under isocratic elution conditions, it will be subjected to chromatographic dilution ($D$), which is expressed by the following equation [13]:

$$D = \frac{C_o}{C_{max}} = \frac{\epsilon \pi r^2 (1 + k) \sqrt{2 \pi LH}}{V_{inj}}$$

(1.1)
where $C_o$ is the initial compound concentration in a sample (before injection into the LC system), $C_{max}$ the final compound concentration at the peak maximum, $r$ the column radius, $k$ the retention factor, $L$ the column length, $H$ the column plate height, $\epsilon$ the column porosity and $V_{inj}$ the sample volume injected. $D$ increases proportionally with the square of the column radius and with the square root of the length of the column. Thus, a reduction in column dimensions results in lower chromatographic dilution, whereby $C_{max}$ increases and a lower limit of detection (LOD) can be achieved. Though this reasoning is only strictly valid under isocratic elution conditions, the consequences are commonly extrapolated to gradient elution conditions as well. Under gradient elution conditions, dilution is partly counteracted by increasing the strength of the mobile phase over time. This approach already results in an initial gain in sensitivity. However, a reduction in column dimensions provides an additional gain. Such a reduction in size is also beneficial for analyte detectability when a miniaturised electrospray interface is employed, as is discussed below. The gain in sensitivity due to the use of an LC column of smaller internal diameter (ID) can be approximated by the following relation [13, 14]:

$$f \approx \frac{d_1^2}{d_2^2}, d_1 > d_2$$  \hspace{1cm} (1.2)

where $d_1$ and $d_2$ are the diameters of the conventional and nano HPLC columns, respectively. Therefore, downscaling the column used in an analytical method from 4 mm ID to 50 µm ID should result in a 6400-fold gain in sensitivity. However, direct comparison of the sensitivity of two systems is only possible if every separation parameter apart from columns inner diameter is kept constant.

While miniaturisation of an LC system essentially involves the reduction of the column ID of the column, extracolumn peak-broadening effects must be reduced accordingly in order to preserve optimal performance. Excessive extracolumn band-broadening causes considerable loss of separation efficiency and, thereby, sensitivity. Ideally, the contribution of extracolumn peak broadening should be negligible compared to the peak broadening caused by the separation process on the analytical column. Generally, a 5-10% reduction in chromatographic performance is considered as acceptable [13]. Extracolumn band broadening can be divided into two categories, pre-column band broadening and post-column band-broadening. The former, also the most trivial, causes gradient delay. The latter can cause eluting peaks to broaden significantly, and result in loss of resolution and sensitivity. The following equation can be used to approximate the effect of
post-column dead volumes on the volume of a peak [14]:

\[
V_{p,obs} = V_p \sqrt{1 + \frac{V_{ex}}{V_p}}
\]

(1.3)

where \( V_{p,obs} \) is the volume of a peak as observed in the detector, \( V_{ex} \) corresponds to the post-column dead volumes and \( V_p \) is the volume of a peak eluting off a column.

### 1.2.2 Practical aspects of miniaturisation

Miniaturisation of an LC system implies that all system components should be downscaled, including column, connecting tubing, injector and detection interface. LC columns of 75 µm ID or less require flow rates of 200 nL/min or less. To reach the very high sensitivity often required in proteomics analysis, it is necessary to deliver a stable and reproducible flow at rates at which miniaturised electrospray interfaces perform best. Flow-splitting systems, in which the flow is split between the pump and the injector, are often used because they allow the use of conventional HPLC pumps in combination with an easily constructed flow-splitting device. The simplest system splits both the flow and the sample (i.e. splitting after the injector). However, this type of set-up results in great sample losses [15]. More recently reported systems allow injection of the whole sample onto the LC column by splitting the flow in front of the injection device [16] (cf. Chapter 4). A ”split/splitless” scheme with a splitter after the injector is also possible [14] (cf. Chapter 5). The main drawback of flow-splitting systems is related to the fact that the split ratio is defined by the resistance-to-flow of the two paths. As a result, the split ratio varies during gradient elution or when flow resistance in the column increases, for example due to partial clogging of the column. In the worse case, complete clogging of the column is not noticed, because most of the flow is directed through the split line anyway. To overcome these problems, systems integrating flow-feedback have been developed [16, 17]. An alternative to flow splitting is the use of dual syringe pumps that can generate low-flow gradients and accommodate high backpressures. As the flow is not split with this technique, solvent consumption is very low, allowing for continuous automated analysis even if the syringes can only contain a limited volume. Nevertheless, it is necessary to stop the analysis to refill the syringes from time to time. The use of a single pump, either a syringe pump [18] or pneumatic pump [19], in combination with a relatively large mixing chamber has been shown to generate exponential gradient profiles [18, 19]. Exponential and sigmoidal gradients were
generated using two syringe pumps, a relatively large mixing chamber and two 4-port valves [20]. Recently, gradients were generated using two reservoirs under equal gas pressure, which are interconnected with PEEK tubing (Fig. 1.1). The difference in solvent height between the reservoirs ensures dilution of the solvent in reservoir A by that contained in reservoir B, which is pushed out by the gas pressure applied. The inner diameter and length of the PEEK tubing are parameters that can be adjusted to modify the gradient profile [21]. Alternatively, various gradient generators have been reported in which eluents of different compositions were supplied through the loops of switching valves [22–26]. Ericson [27] described an approach based on the thermal expansion of the LC solvent inside a tubular spiral when heated in a water bath. This pump was shown to achieve very low volumetric flow (0.01-10 µL/min) and high pressures (>20000 bars). It is, however, difficult to manipulate the gradient because of solvent diffusion inside the loop. Hence, most of the systems described above are not as versatile for gradient profile control or as practical as binary/quaternary piston pumps. Therefore, flow-splitting set-ups are most commonly used.

Figure 1.1: Schematic diagram of a nanoLC pump based on the difference in solvent height in two reservoirs under equal pressure.
Miniaturised LC systems can only accommodate nL injection volumes [17,28]. However, sensitivity is proportional to the absolute amount of sample loaded on the column. Therefore, a lower injection volume results in diminished sensitivity and thus a higher LOD (see Equation 1.1). The first miniaturised LC separations were performed using conventional LC pumps with flow splitting between the injection loop and the column, thus leading to great sample losses [15]. To overcome this problem, different methodologies were investigated, such as off-line preconcentration, large-volume injection directly onto the analytical column, and on-line preconcentration (using a relatively short and wide LC column). Off-line preconcentration is usually performed either using a vacuum centrifuge, ultrafiltration or an LC column of different selectivity. Any of these techniques can be used to greatly reduce the sample volume and allow the injection of the whole sample onto a nanoLC column. However, sample loss due to peptide adsorption on the wall of sample vials (cf. Chapter 6) or other parts of an analytical system [29] may still reduce the sensitivity and reproducibility of the system. As an alternative, it has been shown that large sample volumes (1.8 µL) can be loaded directly onto miniaturised LC columns (25 µm ID) in 5 min at high flow rate, while elution is performed at low flow rates [30]. This methodology, however, requires the sample to be dissolved in a solvent significantly weaker (20-30% less organic solvent) than the solvent necessary to elute the analytes off the LC column [13]. As a result, large direct on-column injections are only possible when the analyte is strongly retained, such as in the case of a neuropeptide on a reversed phase (RP) stationary phase material [31]. Apart from the sample volume and solvent, the nature of the analyte plays an important role. The maximum amount to be injected is determined by the analyte in a mixture that is least retained [31]. For example, it was not possible to inject more than 100 nL of a nucleotide solution on a 75-µm-ID RP column without a loss in separation efficiency [28]. Even much smaller sample volumes were shown to be deleterious to the quality of the separation [15]. Therefore, the need arose to develop a column-switching technique that allowed the injection of large volumes (>1 µL) on a trap column (also known as preconcentration column) without overloading the analytical column. Trap columns enable fast sample loading through use of high flowrates compared to the separation. While loading 1.8 µL directly onto a 25-µm-ID column took 5 min, systems incorporating a trap column could load 10-100 µL in 5 min or less [32] (cf. Chapter 5). A trap column also provides efficient protection of the analytical column against clogging. Two different column-switching procedures are possible; namely the back-flush [16,33] (cf. Chapter 4) and forward-flush elution [14] (cf. Chapter 5 & 6).
A back-flush procedure requires a set-up (Fig. 1.2) incorporating two pumps, with a trap column mounted on an additional switching valve. The sample plug is injected and concentrated on the trap column using the loading pump and the flow is further directed to waste during sample loading. The nanoflow pump is also connected to this switching valve, in order to elute the analytes off the trap column onto the analytical column. Though back-flush set-ups require two pumps, they also present clear advantages. Back-flush elution prevents exces-

**Figure 1.2:** Schematic diagram of back-flush (top) and forward-flush (bottom) nanoLC-MS set-ups. More details can be found in Chapter 4 about a back-flush set-up used for the characterisation of a 50-µm reversed-phase monolith. Chapter 5 describes a novel forward-flush set-up we developed. Depending on the connection scheme, a back-flush set-up can be turned into a forward-flush set-up using the exact same components.
1.3 Nanospray. Coupling nanoLC to MS using electrospray ionisation

Since the introduction of electrospray ionisation (ESI) [35] and MALDI [36] for the analysis of large biomolecules, these techniques have become the most powerful tools for protein identification and characterisation. ESI is a soft ionisation technique allowing the transfer of liquid-phase ions to the gas-phase through a gentle process that facilitates the sensitive analysis of non-volatile and thermolabile compounds [35]. ESI is currently the most popular interface for coupling LC to MS in proteomic analysis.
1.3.1 Theory

When a high voltage difference is applied between an electrode and a counter electrode, a strong electric field is created. In the case of ESI, the high voltage is applied to the end of a capillary (also referred to as tip or emitter) delivering the analyte solution. This capillary is often connected to an LC column for analyte separation. The solvent extends from the tip of the emitter in the shape of a cone, known as Taylor cone [37]. When the coulombic (electrostatic) repulsion between charged molecules at the surface of the Taylor cone is stronger than the surface tension of the solution (or Rayleigh limit), charged droplets are expelled from the cone. Droplet disintegration, assisted by solvent evaporation, results in ever smaller offspring droplets. As the solvent evaporates, the droplets shrink further and the coulombic repulsion increases. Finally, ions are expelled from the remaining solvent and travel toward the counter electrode along the lines of the electric field [38]. The mechanism by which the Taylor cone is established is to date not fully understood.

Most conventional ESI interfaces operate with maximum sensitivity at flow rates in the low-to-mid $\mu$L/min range, whereas nanoESI interfaces perform best at flow rates in the low nL/min range. Solvent flow rate [37, 39], emitter ID, solvent composition and electrolyte concentration can all influence the quality of the spray [40]. The size of the droplets sprayed from the Taylor cone is highly dependent on flow rate and emitter ID and thus critical to efficient ionisation of the analytes. Lowering the flow rate from the $\mu$L/min range to the nL/min range results in the formation of smaller droplets (100-1000 times) [39]. Since smaller droplets contain less solvent, ionisation is more efficient; because lesser fission events are required to produce gas-phase ions [37–39]. With fewer, smaller droplets, more analyte enters the mass spectrometer and thus higher sensitivity is achieved [37]. Thus, coupling of nanoLC with MS has greatly benefited from the design of novel low-volume ESI interfaces - referred to as nanoelectrospray ionisation (nanoESI).

1.3.2 Fabrication

The term nanoelectrospray ionisation (nanoESI) initially referred to off-line spraying [39]. The importance of nanoLC in proteomic analysis and the technological progress in this area resulted in nanoESI now being used for both on-line and off-line techniques applying nL/min flows.

When coupling nanoLC to MS, the spray voltage can be either applied on the outside of the emitter or directly to the liquid to be sprayed. The former requires a conductive layer on the outside of the emitter [39, 41] while the latter, referred
to as a "liquid junction", makes use of uncoated silica emitters [41]. The emitters used in liquid-junction interfacing are, therefore, easier and cheaper to prepare. However, they require a higher voltage to obtain a stable spray as a result of the significant voltage drop through the solvent. In some cases, LC columns were prepared inside nanoESI tips, thereby dramatically minimising post-column dead volumes [42]. Similarly, several groups sprayed directly from the LC column, especially when columns were of the monolithic type [43, 44]. NanoESI can also be performed with capillaries packed without need of a frit. In this case, silica beads arrange themselves in a manner like stones in an arch [45]. However, these set-ups still require electric contact to be made with the LC eluent and are not commonly used.

The fabrication of nanoESI emitters is often a two-step process, making and coating the tip. The first nanoESI emitter was made from a borosilicate capillary heated using a laser and pulled at the same time to a fine tip with a capillary puller. It was then coated with gold by vapour deposition [37]. However, the difficulties associated with making nanoESI emitters this way, including their fragility and their low reproducibility, led to the development of other procedures. Stainless steel (SS) emitters were employed with varying success. While untapered emitters did not give a satisfactory spray [41], tapered tips did, with very stable sprays ultimately [46]. Emitters with a tapered end of smaller ID and with thinner walls gave the best results in terms of sensitivity and stability at low flow rates [46]. No adsorption of peptides to the stainless steel walls was detected.

However, this might become problematic at very low flow rates, as observed with glass and fused-silica capillaries [46]. Moreover, peptides are likely to form adducts with alkali-metal ions when peptides are allowed to make contact with metal during spraying [47]. Microfabrication has been used to produce nanospray emitters from different materials including: glass [48], silicon [49] and polymers, such as polydimethylsiloxane (PDMS) [50, 51], polyimide [52, 53], cycloolefin [54], polycarbonate [55, 56] and poly(methylmethacrylate) [55, 57].

Initially, gold was the material of choice for coating nanoESI emitters, whether it was vapour-deposited [39] or sputtered [58]. However, gold coatings are very sensitive to electrical discharge. Part of the coating peels off with every discharge and the emitter performs less effectively with increasing loss of coating [59]. Thus, more stable coatings were necessary. Silver and gold with or without an intermediate chromium layer to improve adhesion have been investigated, but have not yielded a stable coating. Silver was probably unsuccessful due to oxidation, changing it into an insulator. More robust tips were obtained by vapour-depositing a thin film of gold on the outer surface of the capillary following treatment with
an organosilane [60]. Graphite has been used as an intermediate coating to make gold adhere more strongly to silica capillaries. Coatings made of graphite [61], a mixture of polyimide and graphite [62], or polypropylene and graphite [63] were successfully applied to nanoESI using silica-based emitters. Graphite-based coatings show much greater mechanical stability than gold coatings, and stable spraying may be performed for a week or more [62]. Moreover, graphite-coated tips are electrochemically more stable than metal-coated tips [61]. Polyaniline-coated nanotips showed high sensitivity and durability and were resistant to electrical discharge (probably because of the thick polyaniline coating) [64]. Whereas novel methodologies have addressed, sensitivity and ruggedness issues, reproducibility is still problematic with most approaches.

1.3.3 Practical considerations

An additional advantage of the reduced number of disintegration events is the higher tolerance of nanoESI toward salts, resulting in less spectral background noise [40]. At very low flow rates (low nL/min), signal suppression can be greatly reduced as compared to that at higher flow rates (>50 nL/min) [65]. LC separations are often performed in the reversed-phase mode, where the mobile phase is generally a mixture of acidified water and an organic modifier. The lower surface tension and boiling points of the organic solvent facilitate the formation of gas-phase ion [40, 66]. Since solvent evaporation plays an important role in the mechanism of electrospray ionisation, it is not surprising that a low flow rate, together with a high percentage of organic modifier, improves the efficiency of droplet size reduction and, thus, the release of ions from droplets. It was found that, at certain solvent compositions, the signal intensity decreased dramatically [41]. Such a drop in intensity is likely to happen at different mobile phase compositions in nanoLC-ESI-MS. Eluent flow rate and composition, emitter ID and geometry, as well as analyte concentration, are all likely to influence the stability of the spray [40] and its morphology [67]. Moreover, a shift in the charge state of peptides has been observed as a result of changing experimental parameters in nanoESI [68]. Therefore, it can be difficult to obtain a stable and reproducible spray and an optimal signal when the mobile phase composition changes. Consequently, coupling nanoLC to MS by means of a nanoESI interface requires a careful investigation of the influence of all the aforementioned parameters on the spray stability and peptide spectra.
1.4 Column technology

Since 2D-PAGE has difficulties resolving either very small or very large proteins as well as polypeptides with extreme pI, size and/or hydrophobicity [4], chromatographic alternatives have emerged. In chromatography, the analytical column is the heart of the technique. Its performance will be of paramount importance to achieve efficient and rapid separation. Consequently, much research has focused on the synthesis of more suitable chromatographic stationary phases (SP). The characteristics of the solid support that bears the stationary phase play an immense role in the separation efficiency of proteins and peptides and thus, much attention must be paid to parameters such as particle and/or pore size as well as to the porosity of the stationary phase. Moreover, the material from which the stationary phase is made will determine the boundary conditions under which separations can be carried out.

The Van Deemter equation (cf. Chapter 3) summarises the different terms contributing to band broadening on a chromatographic column. These are the ”Eddy diffusion term” \(A\)-term), the ”axial diffusion term” \(B\)-term) and the ”resistance-to-mass-transfer term” \(C\)-term). These terms are influenced by the aforementioned structural parameters (particle and pore size). The understanding of the relation between the structural parameters of a column and its chromatographic performance drove the development of more efficient stationary phases. Additionally, stationary phases must be chemically and physically stable. They need to be stable at extreme pH values and at the high pressures at which they are operated (up to 5000 bars in extreme cases [69]). Furthermore, they should not swell when in contact with organic solvents. In order to achieve the analysis of low-level peptides and proteins, a large loading capacity is also required. Using stationary phase supports with different physico-chemical properties might be a way to vary the selectivity of a chromatographic separation (i.e. by using different pHs). Materials used for the preparation of chromatographic SP support materials include silica, organic and inorganic polymers, as well as materials with enhanced thermal, chemical and physical stability such as zirconia and titanium. Naturally-occurring organic polymers exhibit a porosity adequate for protein analysis, and are stable over a large pH range \(3<pH<13\) [70]. Unfortunately, they are mechanically weak and swell easily [70]. Moreover, such particles are usually rather large \((10-60 \, \mu m \, dp)\) and often exhibit large size distribution, thereby contributing to larger \(A\) and \(C\) terms in the Van Deemter equation. Silica is the most commonly used stationary phase support material in HPLC, due to its resistance to high pressures and its well-known chemistry. Commonly, porous silica beads
in the 3-10 µm size range are used to separate biomacromolecules. Peptides are usually analysed on supports with a mean pore diameter of 300 Å, while bigger proteins are analysed on supports with pores up to 1000 Å [70]. Though efficient separations are possible with such materials, intra-particle mass transfer is still an issue. 1-µm particles resulted in more efficient separations at the expense of much greater backpressures [71]. Jorgenson introduced injectors, pumps and chromatographic stationary phase support materials able to cope with the pressures (up to 5000 bars) required to perform chromatography with such small particles [69,72], accordingly termed ultra-high pressure liquid chromatography (UHPLC). Only recently, an LC system working at up to 1000 bars with 1.7-µm particles was commercialised [73]. In open-tubular columns, the walls of silica capillaries have been derivatised with e.g. carbon chains to obtain a reversed-phase retention mechanism [74–76]. Very high efficiencies were achieved, but loading capacity was too low to achieve satisfactory sensitivity. Non-porous particles were developed to avoid band broadening due to stagnant mobile phase transfer [77], but these also exhibited limited loading capacity. To avoid the problems related to the need for very high pressures associated with 1-µm ID particles and the low capacity of open-tubular columns and non-porous media, perfusion chromatography was introduced at the beginning of the 90’s [78]. However, at low pressures, the mobile phase and analytes tended to go around the particles without penetrating them [79]. Shortly after, the first monolithic columns were synthesised. These stationary phase support materials, based on polymer or silica, are characterised by high porosity and low flow resistance (only low pressures are required) leading to efficient and fast separations. The synthesis, characterisation and application of silica-based monoliths are described in more detail in Chapter 3 and 4. Though silica-based stationary phases are stable when used with organic liquid phases, silica hydrolyses rapidly at extreme pHs [80]. This greatly stimulated research on chromatographic supports based on other inorganic materials that are chemically and physically more stable than silica- and organic-based SP support materials. Monoliths synthesised from metal oxides such as zirconium [81,82] and hafnium [81] were successfully applied to chromatographic separations. The surface of these monoliths was either derivatised with C_{18} chains or left unaltered. The latter offers different selectivities as compared to conventional silica materials. A monolithic column was prepared from a graphitised phenolic resin [83]. Unfortunately, it has not yet been used for the separation of proteins and peptides.
1.5 Multidimensional chromatography

The proteome of an organism is generally too complex to be analysed by applying only a single separation step without considerable loss of information. Therefore, multiple-step analyses are required. A multidimensional separation system like 2D-PAGE subjects analytes to two or more orthogonal (based on different partitioning mechanisms) separations, which results in greater selectivity and peak capacity. This is explained by the fact that during separation, multiple compounds may show similar properties and thus interfere with the analysis. When different separation mechanisms are used, chances of co-elution diminish.

1.5.1 Interaction of proteins with chromatographic stationary phase

Protein retention in chromatography is determined by the physico-chemical properties of the proteins (charge, hydrophobicity and conformation) and the system parameters, namely retention mode and nature of the chromatographic support and mobile phase [70]. In the RP retention mode, the distribution of hydrophobic groups on a protein surface has been shown to influence protein retention. Following studies of digestion of proteins in solution and adsorbed onto RP stationary phases, it was suggested that adsorption of a protein might occur through several amino acids [84]. Assuming that digested regions of proteins were in contact with the solvent and non-digested regions contacted the RP stationary phase, the regions that were inaccessible to tryptic digestion were found to correspond to hydrophobic domains on the protein surface [85]. Protein retention in RPLC has been modelled by Regnier and Geng [82]. In their model, retention is a function of the number of moles (Z) of solvent required to displace the protein from the stationary phase and of the displacing agent concentration. Z was found to be directly proportional to the molecular weight of a series of proteins when 60% formic acid was used as additive. Retention also depends on other characteristics of mobile phase additives (e.g. buffer type and concentration). Study of the retention mechanism in ion-exchange chromatography (IEC) showed that the 3-D structure of a protein was strongly related to its retention. Areas with especially high charge density, rather than the total protein charge, seem to dictate the behaviour of a given protein [86, 87]. Though only protein separation mechanisms in RPLC and IEC have been thoroughly investigated, it seems that peptides and proteins almost always interact with the stationary phase through superficial adsorption sites.
Ideally, a stationary phase should not display non-specific adsorption of proteins and peptides. Non-specific interactions of peptides and proteins with the stationary phase cause peaks to tail, leading to deterioration of chromatographic resolution. Residual silanol groups on silica support materials are especially notorious for their influence on chromatographic efficiency. By decreasing the surface concentration of accessible silanol groups through derivatisation, chromatographic resolution can be improved. This process is referred to as end-capping. System performance (efficiency and selectivity) is greatly related to the nature of the derivatising agent as well as its structure. With small molecule derivatising agents, a high degree of coverage of residual silanol groups on a solid support and/or capillary wall can be achieved. An alternative to end-capping is the encapsulation of the stationary phase (e.g. silica particles) with polymers. The resulting particles had mechanical properties similar to those of common inorganic supports and chemical behaviour similar to polymeric beads [88]. The selectivity and physico-chemical properties (i.e. resistance to pH) of stationary phases can be drastically changed following coating. While silica hydrolyses rapidly at extreme pHs [80], inorganic materials such as titania or zirconia are chemically and physically more stable than silica- and organic-based stationary phases. Silica supports have been coated with titanium for the analysis of nucleotides [89] and phosphopeptides [90], or with zirconium [91] for the analysis of a range of small molecules.

1.5.2 Separation mechanisms

1.5.2.1 Reversed-phase

Reversed-phase (RP) LC is the most commonly used mechanism for the separation of peptides and proteins. Separation is achieved on the basis of differences in hydrophobicity between analytes. In RP mode, elution is achieved with mobile phases based on aqueous buffers to which organic modifiers such as acetonitrile (ACN) and methanol (MeOH) are added. In multidimensional systems, RP is the favourite mode of separation for the last LC dimension. This is because coupling of RPLC to MS is facilitated by the volatility of the organic modifiers traditionally used to perform RPLC separations. On a few occasions, 2D-LC set-ups applying the RP separation mechanism for both dimensions, have even been used. Variation of the selectivity between the first and second dimensions was achieved by using different organic solvents [92] or pHs [93]. Since each organic modifier or buffer has a different influence on solute-solvent interactions, 2D-RP-RP-LC using different mobile phases exhibits orthogonal selectivity [94].
1.5.2.2 Ion-exchange

Ion exchange (IEX) is the favourite mode of separation for the purification of intact proteins and the prefractionation of peptides. In IEX, the charges and their distribution over the surface of a protein determine the interactions between the protein and the charged surface groups on the packing material, thus dictating protein retention [86,87]. Which amino acid residues are charged is mainly dependent on pH. In IEX, controlling the pH of the mobile phase is therefore of utmost importance for successful peptide separation. At a pH $\leq 3$, the carboxyl groups of peptides are neutral, while basic amino acids (arginine, lysine and histidine) as well as the peptide N-terminus contribute to a net positive charge of the peptide.

![Figure 1.3: Elution profile of some of cytochrome c tryptic peptides using a strong cation-exchanger coupled on-line with RP nanoLC-MS. Columns with different patterns represent different peptides of cytochrome c having mid-range hydrophobicity. The SCX column was a BioSCX II (Agilent, Waldbronn, Germany), and elution was performed in steps of increasing concentration of ammonium formate. The nanoLC-MS set-up was as described in Chapter 6. Every peptide elutes over 2-4 salt fractions.](image)

The fully protonated peptides can be fractionated by cation-exchange chromatography at the same pH as for a possible second-dimension RP separation. In anion-exchange chromatography, a high pH is required. However, completely deprotonated basic residues may require a pH $>12$, which will lead to hydrolysis.
of the silica support material. Novel stationary phases (i.e. based on zirconium and titanium) are therefore required for the analysis of highly basic proteins and peptides. IEX is a general term that encompasses four types of ion-exchangers, namely weak anion-exchanger (WAX), weak cation-exchanger (WCX), strong anion-exchanger (SAX) and strong cation-exchanger (SCX). While weak ion-exchangers exhibit their ion-exchanging capabilities over a relatively narrow pH range, strong ion-exchangers are charged at most pH values. Elution is performed by varying the mobile phase pH or the concentration of a salt added to the mobile phase to displace peptides from the stationary phase. In most cases, elution is performed by increasing the salt concentration in steps. As the strength of the eluting solvent does not increase continuously when performing step gradient elution, desorption of a peptide often takes place over several steps or fractions (Fig. 1.3).

Applying a continuous salt gradient will solve this problem, but is practically more difficult to implement [95]. With respect to the nature of the salt displacer, ammonium acetate [96] or formate are often preferred to alkali salts. This is because, in contrast to Na$^+$ and K$^+$ (Fig. 1.4), ammonium ions do not show adduct formation with the proteins to be separated.

Although the major property governing peptide retention during IEX involves ionic interactions, it has been noted that IEX sorbents suffer from non-specific interaction due to the hydrophobicity of the IEX stationary phase, which often results in incomplete recovery, poor peak shape or elution of peptides over several fractions [97]. Adding an organic solvent to the IEX mobile phase alleviates these problems but may interfere with the separation in a second-dimension separation step if RPLC is chosen for that dimension [93]. The lack of stability of silica support materials, and the need to exchange buffers before entering a possible second-dimension RP separation when working a high pH, explains why SCX is the preferred fractionation mode for multidimensional analysis of peptides. An efficient SCX-RP system using direct injections of fractions from the SCX column onto the RP column allowed the identification of 480 proteins from a cell lysate in 20 h [98]. Yates et al. combined SCX with RP LC-MS/MS using a single, biphasic column [96, 99]. About 25% of the complete proteome of S. cerevisiae was identified in a single run using this approach [96]. Further improvements made this set-up more reproducible, with variation on retention times about 0.5% between analyse [99].
1.5. Multidimensional chromatography

Figure 1.4: MS spectrum (between m/z 620 and 790) of Na$^+$ and K$^+$ adducts of the oxidised form of substance P, a very hydrophobic, 11-amino acid peptide. The doubly-protonated ion of SP is the most intense at 682.6. The following Na adducts were found: SP-Na and SP-Na$_2$ at m/z of respectively 693.6 and 704.6. Other peaks were observed at 733.4 and 762.2 and might be attributable to the K-adducts: SP-Na-K$_2$ and SP-K$_4$, respectively. Adducts complicate the understanding of MS spectra and result in a decrease in intensity, which can prove extremely deleterious to quantitation.

1.5.2.3 Affinity chromatography and high-abundance protein depletion

The presence of particular structural features such as phosphorylation, glycosylation, nitration or histidine residues can be exploited for the selective enrichment of proteins and peptides using affinity chromatography (AC). AC is based on the selective interaction between two molecules, one of them being, in our case, a protein or peptide to be retained. For the second molecule immobilised on a solid support material, the following options have been applied:
• an antibody (immunoaffinity chromatography (IAC))
• a metal ion (immobilised metal affinity chromatography (IMAC))
• a ligand; e.g. lectin, dye or a small molecule

In IAC, antibodies are generally immobilised on stationary phases with limited pressure stability (e.g. agarose-based materials), and more rarely on silica and vinyl polymers [100]. IAC owes its selectivity to the specificity of antibodies, which define the performance of the method. Selectivity can be explained by the precise 3D arrangement of amino acids within an antibody’s binding site, which can undergo various non-covalent interactions (H bonding, ionic, dipole-dipole and nonpolar interactions) [101]. The probability of an antibody cross-reacting with more than just a single protein determines the specificity of the interaction. In other words, specificity is determined by the uniqueness of the steric (tridimensional) match of antigen and antibody, as well as by the number of molecular interactions taking place between both molecules. In a specific case, where 11 antibodies were tested against 5000 proteins of the yeast proteome, every antibody exhibited a certain degree of cross-reactivity, in addition to binding its respective antigen. Comparing the amino acid sequence of antibody and antigens showed similarities that could partially explain cross-reactivity, but was not able to predict it a priori [102]. In order to preserve the tridimensional structure of the immobilised antibodies, buffers with a composition and pH close to physiological conditions are used during equilibration, loading and washing of the column. In contrast, elution is generally performed using acidic buffers [103–105] that disrupt the 3D structure of antibodies significantly to release the antigen. IAC has been used for the extraction of structurally-related amino acids and proteins. Antibodies specific for nitrotyrosine have been used for immunoprecipitation [106]. Such antibodies have also been immobilised on chromatographic media [105], extending the seminal work by Helman and Givol [104]. Protein A and Protein G form complexes with immunoglobulins. This feature is exploited in the depletion of proteomic samples of very abundant proteins, and is detailed in a later paragraph.

IMAC is a technique making use of the formation of complexes between metal ions and specific amino acids or functional groups. In principle, the side-chains of lysine, methionine, asparagine, arginine, tyrosine and histidine, as well as the N- and C-termini of peptides and proteins, complex with metal ions. However, it has been demonstrated that interactions mainly take place through surface histidine groups [107]. Cu^{2+}-IMAC is often used in proteomics analysis for the selective retention of histidine-containing proteins and peptides [107]. Other metal ions can
be applied as well, however. Recombinant ovine growth hormone was purified using both Ni\(^{2+}\) and Cu\(^{2+}\)-IMAC [108]. The Ni\(^{2+}\) column was found to retain more protein and result in a purer fraction. Phospho-proteins and amino acids bind to immobilised Fe\(^{3+}\) ions [109–111]. Ga\(^{3+}\) [112], Lu\(^{3+}\), Sc\(^{3+}\) and Th\(^{4+}\) ions [110] were found to have similar properties to Fe\(^{3+}\) ions. Metal oxides based on aluminium [113], zirconium [114] and titanium [114, 115] were used to selectively retain phosphopeptides from complex mixtures. Column preparation is more straightforward with these metal oxides than with other IMAC materials, since the ion chelation and washing steps required for preparation of IMAC columns can be avoided. Elution of IMAC columns usually takes place by increasing pH or by introducing imidazole [108] or phosphate ions [110]. The main problem of IMAC lies in its rather low selectivity when the sample contains peptides with multiple aspartate and glutamate residues. Moreover, current methods are often limited to the identification of phosphoserine and phosphothreonine residues. This retention mode provides the advantages of low cost and high stability of the support media.

Among the many types of post-translational modifications, glycosylation is the most common and the most complex [116]. Lectins are proteins that target oligosaccharide structures recognising specific glycosylation motifs, though overlap in specificity has been observed [117, 118]. It has been shown that concanavalin A (Con A) mainly recognizes α-mannose [118] whereas wheat germ agglutinin (WGA) exhibits affinity for N-acetylglucosamine (GlcNAc) [118] and sialic acid [117]. Jacalin specifically recognises galactose-N-acetylgalactosamine (Gal-GalNAc) and more generally O-type glycopeptides [119]. Lotus tetragonolobus agglutinin (LTA) has been used for its high affinity for fucose-containing glycoconjugates [120, 121]. Sambucus nigra agglutinin (SNA) has been employed to select and compare the concentration of sialic acid-containing glycopeptides [122]. The buffers used during loading and elution of lectin columns contain a Tris buffer and NaCl; Ca\(^{2+}\) and Mn\(^{2+}\) may also be added. Elution buffers generally contain a higher NaCl concentration and 0.2 to 0.8 M sugar. The eluting glyco-peptides or proteins are enzymatically deglycosylated prior to second dimension RPLC-MS analysis, as the presence of oligosaccharide moieties complicates MS determination of molecular mass and sequence [120].

Highly abundant proteins often mask proteins and peptides of lower abundance and thus prevent or hinder their detection and identification. Albumin for example constitutes \(\approx 60\%\) of the total human serum protein content [123]. As in serum [124], the high albumin concentration in urine interferes with protein separation [125]. Several approaches are available to lower the level of highly
abundant proteins based on either specific antibodies, dye ligands or protein A and G. Albumin depletion is performed using either a dye such as Cibacron-Blue [126] or Affi-Gel Blue [125], or serum albumin antibodies. Removal of some IgG is performed using immobilised protein A [127] and even better using protein G [127, 128] columns. Effective depletion of human serum with respect to albumin and/or \( \gamma \)-globulins was achieved using both dyes and antibodies. A column designed to bind both albumin and \( \gamma \)-globulins yielded the highest protein concentration in the flow-through, maybe due to its proven lower efficiency in albumin removal [103]. Most depletion columns (if not all) also exhibit non-specific binding of proteins and peptides of lower abundance. As a result, combining several columns might lead to considerable loss of information, which may well be the main limitation in applying depletion columns to proteomics studies.

1.5.2.4 Miscellaneous retention modes

Other retention mechanisms have been used for the analysis of proteins and peptides, though their use is much less common than the other aforementioned mechanisms. Size-exclusion chromatography (SEC) makes use of a matrix with different pore sizes that the protein and peptide analytes can enter or not, depending on their size. The large proteins can enter a lower number of pores than the small peptides and therefore elute first. Restricted-access media (RAM) are porous silica materials used in chromatography for the separation of low-molecular-weight analytes from matrix components like albumin, by a combination of size exclusion and conventional adsorption chromatography. The outside of the silica particles is often coated with a hydrophilic layer while the inside of the pores is derivatised with RP or IEX chains. RAMs are often used in pharmaceutical analysis to simplify and automate sample preparation. Though the applicability of RAM materials for the analysis of peptides has been demonstrated [129], RAM chromatography is seldom used in proteomics. Normal-Phase chromatography (NP), hydrophilic interaction liquid chromatography (HILIC) and hydrophobic interaction chromatography (HIC) are other alternatives that have been seldom (or not) used in proteomics applications. NP was used as a clean-up step in conjunction with ethylacetate extraction of a peptide before further analysis by LC-MS [130]. To our knowledge, this is the only application of NP LC to proteomic studies. Hydrophilic interaction liquid chromatography (HILIC) exploits the same separation mechanism as NP-LC. However, the stationary phases are different. Whereas bare-silica particles are normally used in NP LC, silica particles are coated with hydrophilic moieties in HILIC. Applications of NP LC and HILIC to proteomics
are limited due to the poor peptide solubility in mobile phases containing large amounts of ACN or MeOH. Hydrophobic interaction chromatography (HIC) is often used for the separation of proteins because of its mild non-denaturing properties. Even if HIC is based on the same retention principle as RPLC, the mobile phases are very different. In HIC, elution is done with aqueous buffers of decreasing ionic strength. Though HIC is commonly applied to the separation of proteins [131], to our knowledge, it has not been used for the multidimensional analysis of peptides by LC.

1.6 Sample pretreatment

1.6.1 Digestion

The enzymatic digestion of the sample (a set of proteins) is a key step in proteomic studies. The resulting mixture of peptides can be analysed using MS-based technologies to generate amino-acid-dependent data. Algorithms were developed to match the observed tandem mass spectrum of a peptide to theoretical mass spectra from protein databases, allowing the identification of proteins present in mixtures. Without prior digestion into sufficiently small fragments, further fragmentation using MS/MS is not possible.

The most commonly used proteolytic enzyme is trypsin, a serine endoprotease with well-defined substrate specificity. Digestion is generally performed in solution, which presents a number of limitations in increasing the efficiency and throughput of the process. Digestion times are long (typically >12 h) due to the necessity to work with low trypsin-to-substrate ratios to prevent autodigestion of trypsin and limit the number of interfering autolytic peptides. Moreover, at low substrate concentrations, obtaining a sufficient number of peptides to allow protein identification is problematic, since the digestion rate is limited by substrate concentration [132]. The secondary and tertiary structure of proteins (i.e. disulphide bridges, hydrophobic cores) can further reduce the digestion rate. Disulphide bridges are usually reduced and alkylated [132, 133] in order to prevent adduct formation during digestion and to improve accessibility to the cleavage sites. To increase the speed and robustness of digestion steps, proteolytic enzymes have been immobilised on solid supports. Immobilisation strongly reduces digestion times because the effective protease concentration on the solid support is very high. Digestion times have been reduced from several hours down to between 1 [134] to 5 min [135] and even 5s [136] for certain proteins using immobilised-enzyme reactors. Moreover, immobilisation stabilises proteases.
against denaturation due to organic solvents and chaotropic agents. Last but not least, immobilisation facilitates the incorporation of the digestion step in on-line multidimensional LC systems. Immobilised-enzyme reactors have been prepared in cartridges based on beads [85,136–138] and monoliths [139,140]. They have also been made in pipette tips using monoliths [141]. Increased protease activity resulted from the acetylation of lysine residues of trypsin immobilised on a solid support (Fig. 1.5), which also helped in reducing autolytic products [142].

![Figure 1.5](image)

**Figure 1.5:** Effect of acetylation on the trypsin digestion rate of 4 µM cytochrome c, analyzed by LC-MS. Digestion was performed in 1-mm cartridges at a flow rate of 40 µL/min (residence time 4 s). Without acetylation, most of cytochrome c is still intact after 4s whereas acetylated trypsin does not show any residual cytochrome c [142].

### 1.6.2 Protein precipitation

Protein precipitation is a sample-preparation technique that has only seldom been used in automated/on-line systems. It is more likely to be incorporated in off-line or at-line schemes. Precipitation is performed under harsh conditions and is the consequence of denaturation. After precipitation, samples are usually centrifuged, and the supernatant is further evaporated and reconstituted before LC-MS analysis. The whole procedure normally takes between 5 min [143] and 1h [144]. Precipitation of serum or plasma is performed by mixing it with a large volume of an organic solvent such as ACN [143], ethanol [145], MeOH [144] or acetone [146].
A combination of organic solvent and acid has also been used, in which case the addition of as little as 1% trifluoroacetic acid (TFA) to ACN was necessary for proteins to precipitate [143]. Solvents can be cooled to less than 4°C [144] to limit protein solubility, or heated to 82°C [146] to denature proteins. Precipitation is also performed using salts [147]; the use of ammonium sulphate is still widely reported [148]. The lack of selectivity of most precipitation methodologies can lead to great losses as a result of coprecipitation.

More selective methods require the use of antibodies (immunoprecipitation) [149, 150], in which case the compounds precipitated are the compounds of interest. Alternative methods use metal ions [146] to form a complex with the analyte following the same mechanism as that used in IMAC. Affinity precipitation makes use of an affinity macroligand (AML), generally a polymer to which one or more types of affinity ligand are bound. Proteins or peptides bind to their corresponding affinity ligands and thus are indirectly bound to the polymer backbone. Precipitation using AMLs is reversible and triggered by a change in pH or temperature [151]. It results in rather high recovery (≥ 75%) of the target molecule. High specificity is also ensured by the use of affinity ligands.

The advantages of precipitation include their ease of use and the low cost of chemicals and reagents (with the exception of immunoprecipitation). Automation is possible using a pipetting robot. However, the centrifugation step remains difficult to automate.

1.6.3 Solubility and adsorption of peptides

Proteins and peptides interact with the solvent and walls of the container in which they are held. The physico-chemical properties of a peptide will determine its solubility in a given solvent in a given container. If a peptide does not show sufficient ”affinity” for the solvent in which it is dissolved, it will tend to adsorb to the walls of the container [152], leading to a lower peptide concentration than in the original matrix. However, this fact is overlooked in most proteomic analytical methods, even those designed to allow quantification. Adsorption has been shown to present an important limitation in the quantitative analysis of various peptides [153–155]. In the previous sections, the chemical nature of peptides was demonstrated to be of great importance to their solubility. The material of which containers are made also has a great impact on the solubility of peptides [156]. The influence of pH, container material, surfactants and organic modifiers on adsorptive behaviour has been studied [152, 155, 156]. Calcitonin adsorbed to the wall of a glass vial according to a Langmuir isotherm at pH ≈ 4 suggesting that
calcitonin formed a monolayer at the glass/solution interface. On the other hand, it adsorbed according to a Freundlich equation at neutral to basic pHs, suggesting the formation of aggregates and/or multilayers on the glass wall [152]. The addition of 1% TFA to the solvent significantly improved the solubility of a relatively large peptide [143], but no such effect was observed in the case of the analysis of microcystsins [155]. The addition of salt (NaCl) also did not improve peptide recovery [155]. Surfactants were successfully used in the analysis of membrane proteins [143], in which they proved to minimise adsorption effects and improve quantitation. However, surfactants would interfere with MS detection. The addition of 1% n-nonyl-\(\beta\)-D-glucopyranoside to the solvent decreased the adsorption of a 36-amino-acid peptide during extraction from animal and human plasma [143] but, like surfactants, sugars are also likely to suppress the ionisation of analytes. Adding ACN [143] and MeOH [154, 155] greatly improved the solubility of hydrophobic peptides. Depending on the peptide to be quantified, the optimal percentage of organic solvent varies. Unfortunately, the presence of an organic solvent can also interfere with RPLC separation. Therefore, selection of the organic solvents used is important. Dimethylsulfoxide (DMSO) proved to be a better solvent than ACN or MeOH for improving the solubility of hydrophobic peptides due to its weaker eluting power in RPLC (cf. Chapter 6).

1.7 Plan of the thesis

In order to study the biochemical processes underlying pharmacological and medical events, a sensitive and selective analytical method is necessary. The miniaturisation of LC-MS allows detection of amol and even zmol LOD levels and seems to be the most promising technique to tackle these challenges. To achieve the high throughput and ease necessary in routine comparative proteomic studies, several limitations of nanoLC-MS need to be overcome. Systems must become even more sensitive, more selective, more reproducible, more robust, faster and easier to use. This thesis presents several studies in which various aspects and components of a nanoLC-MS system have been optimised, with the aim to overcome the limitations listed. In Chapter 2, the characterisation of the nanoESI interface we built is presented. Working toward quantitative analysis of low-level peptides using nanoLC-MS, it is necessary to know how a nanospray interface responds to changes in the mobile phase composition, spray voltage and morphology and position of the nanoESI emitter. The morphology of the nanospray tip - ID and geometry of the tip opening, the presence (or absence) of a conductive coating - was shown to influence the optimal working conditions and the resulting
spectra. Parameters such as tip diameter, flow rate, analyte concentration and solvent composition can all affect the observed ions and the charge states. In Chapter 3, the state of the art in silica-based monolith synthesis, characterisation and application is presented, while in Chapter 4, the detailed evaluation of the performance of a new 50-µm-ID monolithic column and its application to peptide analysis is demonstrated. Traditionally, chromatographic stationary phases are prepared from naturally-occurring organic polymers (e.g. agarose) or silica. Silica beads are the most commonly used solid support materials in the LC-MS analysis of peptides. The smaller the silica beads, the higher the back-pressure. The larger the beads, the lower the separation efficiency and flow resistance. Moreover, highly porous beads have a higher capacity. Thus, the structure of the stationary phase will be of paramount importance to achieve efficient, rapid and sensitive separation. Much research has been focused on the synthesis of higher-performance chromatographic stationary phases. The latest advance in column technology was the synthesis of monolithic phases, first organic phases followed later by silica phases. A number of groups are working to prepare better monolithic columns and understand the underlying principles of their working. In Chapter 5, a novel set-up allowing on-line albumin depletion and sample fractionation is presented. By increasing the efficiency, selectivity and loading capacity of an analytical system, multidimensional approaches increase the number of peptides that can be analysed in a complex sample. One of the many advantages offered by multidimensional chromatography is the possibility to deplete samples of high-abundance proteins (e.g. albumin). In Chapter 6, a study of the repeatability issues in LC-MS is presented. Poor repeatability of peak areas is a problem frequently encountered in peptide analysis with nanoLC-MS. As a result, quantitative analysis is seriously hampered, unless the observed variability can be corrected for. Currently, labeling techniques or addition of internal standards are often applied to this end. However, these procedures are often elaborate and error prone, and do not always improve repeatability. The addition of salt (NaCl) did not improve peptide recovery [155] and is likely to result in the formation of adducts and/or ionisation suppression of the peptides. Surfactants were proven to minimise adsorption effects [143] and improve quantitation. However, surfactants interfere with MS detection and are not the most favourable solution to improve solubility in proteomics studies. Adding organic solvents [143,154,155] can greatly improved the solubility of hydrophobic peptides. Unfortunately, the presence of an organic solvent can interfere with RPLC separation. Therefore, selection of the organic solvents is important. We used dimethylsulfoxide (DMSO) to increase peptide solubility and thereby pep-
tide recovery (cf. Chapter 6). Depending on the peptide to be quantified, the optimal percentage of organic solvent varies.
Finally, in Chapter 7, an outlook for the further development of this nano-LC-MS platform will be provided.
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