A nanoLC-MS-based platform for peptide analysis

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Chapter 5

RAM-based albumin depletion coupled on-line to nanoLC-MS for the analysis of complex proteomics samples

5.1 Introduction

Liquid chromatography-mass spectrometry (LC-MS) is an important analysis technique in proteomics. In order to increase the concentration sensitivity of LC-MS for low-molecular-weight peptides, it is often necessary to enrich them prior to analysis and to remove high-abundance proteins like albumin. Notably, body fluids like serum [1] or cerebrospinal fluid (CSF) [2] often contain considerable amounts of albumin, making the analysis of constituents at nM concentrations or below difficult. Removal of albumin and other high-abundance proteins is often achieved by immunoaffinity chromatography based on a panel of immobilised antibodies [1–3]. However, highly abundant proteins can bind lower-abundance proteins or peptides, which leads to the loss of the less-abundant species upon removal of the high-abundance species. This is especially true when non-denaturing conditions have to be used as in the case of immunoaffinity chromatography [4]. Consequently, improved methodology based on efficient sample pretreatment is often necessary to study the low-abundance peptides in complex biological samples containing albumin.

Restricted access material (RAM) are porous silica materials used in chromatogra-
phy for the separation of low-molecular-weight analytes from matrix components like albumin by a combination of size exclusion and conventional adsorptive chromatography. Though the applicability of RAM for the analysis of peptides has been demonstrated [5], RAM chromatography is seldom used in proteomics [6]. It has been applied to the analysis of cyanobacterial peptides after off-line sample preparation [7] and for the quantitation of neuropeptide Y in porcine plasma. In the latter case, the RAM column was coupled to reversed-phase LC-MS in a forward-flush system, enabling a limit of detection (LOD) of 5 µM [8]. Unfortunately, no data about the efficiency of albumin removal were provided [5, 8]. A more complex set-up for the analysis of highly complex biofluids was described by Wagner et al. [9]. This multidimensional system made use of RAM, coupled on-line with ion-exchange and reversed-phase chromatography. All the columns were connected in a back-flush fashion. Using this system, 92% of albumin was depleted from the sample matrix.

In this study, a novel set-up for multidimensional nanoLC-MS featuring three columns coupled on-line was developed and characterised. In particular, we were concerned with the analysis of Substance P (SP) in albumin-rich matrices. The columns were coupled using a single gradient pump and switching valve to minimise extracolumn dead volumes. Though the flow direction was controlled by a switching valve, the analytes never flowed through valves except during injection. For the first time, a RAM cartridge was coupled to nanoLC-MS in a forward-flush mode and used for the analysis of microdialysis perfusates as model matrix and SP as model peptide. BSA, which was added to reduce non-specific peptide adsorption to the microdialysis membrane and connecting silica capillaries, could be removed efficiently with the RAM cartridge while SP was quantitatively retained. The set-up described here can be used reproducibly with fast gradients (RSD of retention time ≈ 3% over a week), without requiring an extensive time period to stabilise the flow or the pressure. Separation efficiency was high (peak width at half-height of 10s) and samples up to 100 µL could be injected. Perfusates containing up to 4.0 µM BSA were analysed without any off-line sample pretreatment. The total cycle time, including sample pre-treatment, separation, washing and equilibration, was about 40 min. Such rapid, reproducible and robust separations by nanoLC-MS are expected to be of interest for the comparative analysis of complex biofluids.
5.2 Materials and methods

5.2.1 Materials

Formic acid (FA) (98-100% pure), formamide, sodium chloride, potassium chloride (all >99.5% pure) and magnesium chloride hexahydrate (99-102% pure) were purchased from Merck KGaA (Darmstadt, Germany), and acetonitrile (ACN) (HPLC Supra-Gradient grade) from Biosolve B.V. (Valkenswaard, The Netherlands). Ethyl acetate (99.9% pure) and 1/16” stainless steel blank nuts were obtained from VWR International B.V. (Amsterdam, The Netherlands) and electrodag PF-407A (carbon ink) from Acheson Industries Europe Ltd. (Scheemda, The Netherlands). Substance P acetate salt (SP) (>98% pure), bovine serum albumin (BSA) (>96% electrophoretically pure) and calcium chloride dihydrate (>99% pure) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands), D(+)-glucose anhydrous from J.T. Baker Chemicals B.V. (Deventer, The Netherlands) and dimethyl sulfoxide (DMSO) (> 99.5% pure) from Fluka (Zwijndrecht, The Netherlands). Fused-silica capillaries (50-200 µm ID, 360 µm OD) were purchased from Composite Metal Service (Ilkley, UK) and microtight PEEK sleeve (0.0155” ID, 0.025” OD) and microT PEEK assembly (0.25” bore) from Upchurch Scientific (Oak Harbor, WA, USA). PEEK cartridges and their stainless steel holder were obtained from SP ARK Holland (Emmen, The Netherlands). Restricted-access chromatographic media of varying selectivity, C_18, C_8 and GFF-II (glycine-phenylalanine-phenylalanine-based material) (C_18-RAM, C_8-RAM & GFF), were a gift from Regis Technologies (Morton Grove, IL, USA) and the potassium silicate solution (Kasil 1) a gift from PQ Europe (Winschoten, The Netherlands).

5.2.2 Preparation of frits, columns and cartridges

Column frits were prepared based on a method described by Meiring et al. [10], which involves polymerisation of potassium silicate in the solution at the end of the capillary. Briefly, 50 µL of formamide were pipetted into a 1.5-mL Eppendorf vial and 150 µL of Kasil 1 were added. Promptly after, the mixture was briefly vortexed, and 10-to-15-cm long silica capillaries were immersed in the polymerising mixture at a right angle with the surface of the liquid to a depth of approximately 5 mm. In this way, the silicate solution was passively introduced to the ends of the capillaries by capillary forces. Typically, 10 frits were prepared at the same time. Polymerisation was completed by heating the capillaries at 100°C in an oven for 4h. The resulting frits were cut to a length of approximately
0.5 mm. Trap columns and cartridges were packed using a pneumatic pump (Knauer K-1900, Berlin, Germany) able to deliver flows at pressures up to 1000 bar. The pump was connected to an empty 3-mm-ID stainless steel LC column acting as a reservoir for the slurry of packing material. The capillary to be packed was connected to the 3-mm-ID column by the end opposite to the frit. The slurry (10 mg/mL in acetone) was introduced in the empty reservoir and the pump was turned on until the chromatographic bed had the desired length. Cartridges with a stainless-steel frit at one end were prepared according to the same procedure. They were positioned in a cartridge holder during both packing and chromatographic separation.

### 5.2.3 NanoLC-MS set-ups

All experiments were performed using an 1100 LC system consisting of a vacuum degasser and a high pressure-mixing binary pump without static mixer (Agilent Technologies, Waldbronn, Germany). The damper was positioned in-line between one of the pump heads and the T-piece used to mix the solvents. This set-up minimised delay in gradient delivery. 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile (ACN) were the two solvents (respectively solvents A & B) making up the mobile phase. A Midas autosampler (SPARK Holland, Emmen, The Netherlands) equipped with a 250-µL syringe and either a 10- or 100-µL injection loop was used in conjunction with an LCQ Classic ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). The nanoelectrospray ion source was built in-house. The gold-coated nanoelectrospray tip and the analytical column were butt-connected using a Teflon sleeve of 360-µm-ID to minimise post-column dead volumes (3-4 nL), and placed in the stainless steel holder on the source table to ensure electrical contact. The nanospray tip was positioned 1 mm away from the opening of the heated MS capillary. The spray voltage was normally set at 1.6 or 1.7 kV.

Two nanoLC-MS set-ups were evaluated. These are schematically presented in Figure 5.1. One consisted of a 50-µm-ID analytical column (15 cm long) and a 100- or 200-µm-ID trap column (2 or 5 cm long). This set-up will be referred to as Set-up 1. The other set-up is based on Set-up 1, but contained an additional 1-mm-ID cartridge (1 cm long) between the injection assembly and the trap column and is referred to as Set-up 2. The analytical column was packed with Biosphere C$_{18}$ material (5 µm $d_p$, 100 Åpore size). The trap column was either packed with a
5.2. Materials and methods

regular RP stationary phase (Biosphere C\textsubscript{18}, 5 \textmu{}m \textit{d}_p, 100 Å pore size), or a RAM phase (Regis C\textsubscript{8}, C\textsubscript{18} or GFF-II; 5 \textmu{}m \textit{d}_p, 100 Å pore size). The cartridge was only used with Regis C\textsubscript{8} RAM packing material. Columns packed with Biosphere C\textsubscript{18} were purchased from Nanoseparations (Nieuwkoop, The Netherlands). In both set-ups, the 6-port switching valve of the MS was used to control the flow direction.

\textbf{Figure 5.1:} Schematic representation of the nanoLC-MS set-ups. A: Set-up 1. The trap column was either packed with C\textsubscript{18} or different RAMs (C\textsubscript{8}, C\textsubscript{18} or GFF) (20-50 mm long, 0.1-0.2-mm ID, 5 \textmu{}m \textit{d}_p, 100 Å pore size). B: Set-up 2. The RAM cartridge was packed with C\textsubscript{8}-RAM particles (10*1.0 mm ID, 5 \textmu{}m \textit{d}_p, 100 Å pore size) and the trap column consisted of a bed of C\textsubscript{18} particles (20*0.1-mm ID, 5 \textmu{}m \textit{d}_p, 100 Å pore size). The arrows indicate the direction of the LC eluent in the system depending on the position of the switching valve (P1: split flow & P2: unsplit flow). The full arrows indicate how the flow is split in front of the trap column. Only a small portion (1:3000 to 1:4000 depending on mobile phase composition) of the eluent flows through the trap and analytical columns. The dotted arrows show the path of the flow prior to splitting that is directed through the trap column to waste.
5.2.4 NanoLC Procedures

Set-up 1 has been extensively described by Meiring et al. [10]. Briefly, the sample plug was concentrated on either a C$_{18}$ (20 * 0.1-mm-ID) or a RAM (50 * 0.2-mm-ID) trap column. The flow (10 to 50 µL/min, 1 to 10% ACN) was directed through the trap column by switching the valve so as to connect the split line (300 * 0.05 mm ID) to a blank nut. Elution of the analytes from the trap column onto the analytical column was performed by switching the valve so as to connect the split line to the waste line. The flowrate was concomitantly raised to 400 µL/min, which was split down to 100-150 nL/min, and the gradient was started. The gradient ran up to 65% ACN at 3%/min. Subsequently, the solvent composition was returned to the starting composition and the system was equilibrated for 10 min.

Set-up 2 is based on Set-up 1 and Figure 5.1 illustrates its operation. An additional RAM C$_{8}$ cartridge, in a cartridge holder, was positioned between the injection assembly and the split line. Programming of both the gradient pump and the switching valve is described in Table 5.1. Briefly, the principle of the set-up is the following. During the first 10 minutes, the flow was split between the RAM cartridge and the nanoLC part of the system discarding the BSA that is not retained on the RAM to waste. The transfer of SP from the RAM cartridge to the trap column of the nanoLC was performed by directing the flow through the trap column without splitting it. After loading of the trap column, the valve was switched back to its original position, thereby splitting the flow again. At the same time, the flow was raised and the gradient started, simultaneously washing the RAM cartridge of the remaining BSA to waste and eluting SP off the nanoLC.

In the following discussion, the term “trap” refers to the type of column used in Set-up 1 (100- or 200-µm ID). The term ”cartridge” applies to the column (1-mm ID) at the front-end of Set-up 2.

5.2.5 Sample preparation

The perfusate was composed of 140 mM NaCl, 3 mM KCl, 1.25 mM CaCl$_2$, 1 mM MgCl$_2$, 3 mM glucose and either 0.025 or 0.0025% BSA (w/v) (≈ 4.0 or 0.4 µM).

The perfusate was used, pure or as a mixture with 0.1% FA in DMSO in the following ratios, perfusate: DMSO (19:1, 3:1 and 1:1), to dilute standards [11]. SP standards were also prepared in 0.1% FA in water:DMSO at the same ratios. Concentrations ranged from 0.5 up to 50 nM. The volumes injected were either 10 or 100 µL depending on the experiment.
### Table 5.1: Programming of both the gradient pump and the switching valve in Set-up 2.
The terms split flow and unsplit flow are related to the position of the switching valve (P1 & P2) and its action on the flow (see Figure 5.1). During loading of the sample and elution of the nanoLC, the flow is split (P1) in front of the trap column with a large fraction going to waste and a small one flowing through the trap and the analytical columns. The split ratio is 1:3000 to 1:4000, depending on the mobile phase composition. Conversely, the flow is unsplit (P2) when the switching valve directs the flow through the trap column to waste (during transfer of the analytes from the RAM cartridge to the trap column) (cf. Figure 5.1).

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5.3 Results and discussion

#### 5.3.1 NanoLC-MS: Set-up 1

Initially, Set-up 1 was used to analyse microdialysis perfusates containing large amounts of BSA. The need for adding BSA to the perfusate to increase recovery of SP has a three-fold negative effect on the analysis by nanoLC-MS. First, the elution peak of SP overlaps with a very large peak of BSA (Figure 5.2) causing severe ionisation suppression. Moreover, an excess of BSA overloads the system and its only partial elution from the trap column makes the enrichment of SP difficult if not impossible after a few runs. Additionally, BSA eluting off the column crystallises at the inlet of the MS and obstructs it. Though SP could be analysed in the presence of a large excess of BSA, retention time varied greatly. After only 3 injections, the retention time already varied by almost 6%. Altogether, this resulted in a non-robust nanoLC-MS system that cannot be used for the routine analysis of microdialysis perfusates.
Figure 5.2: Evaluation of the efficiency of the albumin depletion step by RAM chromatography (TIC chromatograms obtained in full scan mode between m/z 300 and 1500). Top: 10 µL of perfusate on Set-up 1 equipped with a C18-trap column. The flow (10 µL/min, 1% ACN) was directed through the trap column for 5 min. Elution of the analytes was performed using a gradient that increased to 65% ACN in 20 min. The solvent composition was returned to 1% ACN and the system was equilibrated for 10 min. Bottom: 100 µL of perfusate on Set-up 2 after approximately 750 injections. Programming of the gradient pump and MS valve as indicated in Table 5.1.

To reduce the load of albumin on the nanoLC-MS system, the C18 trap column was replaced by trap columns packed with C18-RAM, C8-RAM or GFF-RAM. The quality of the resulting chromatographic separations was assessed by injecting (in triplicate) 10 µL of solutions of increasing concentrations of SP in different matrices (i.e. containing 25 or 50% DMSO to increase recovery [11]). The results showed no significant differences in selectivity and signal intensity between the different trap columns (data not shown). Peak shapes were comparable for all restricted-access packing materials, though it was expected that peak focusing on the C18 analytical column would provide narrower peaks in combination with the C8-RAM as compared to the C18-RAM.
The use of a trap column packed with RAM not only improved the reproducibility of retention time (1.5 <RSD (%) <4.3 depending on sample matrix) but also increased the period during which the system could be operated reproducibly. However, this system still did not enable the analysis of both unknown samples and standard solutions under routine conditions. BSA was still found
to crystallise at the MS opening, so the system had to be cleaned on a daily basis. The presence of BSA crystals on the MS endplate indicated that a significant portion of the injected BSA was not eliminated by the RAM column. To better characterise BSA retention in the system, the C$_8$-RAM trap column was coupled directly to the ion trap using a 10-µm-ID gold coated nanospray emitter mounted on the nanospray interface and 10-µL injections of SP in perfusate were performed. Interestingly, BSA eluted in two fractions, the first one in the flowthrough and the second after the elution of SP (Figure 5.3). Preliminary experiments showed that linear flowrate and mobile phase composition were factors of major importance for efficient removal of BSA and retention of SP. As much as 80% of BSA remained on the RAM trap at higher flowrates (10-20 µL/min) during sample loading (data not shown). Using a C$_8$-RAM trap column (20*0.1-mm ID) and a flow of 2.5µL/min, improved BSA depletion was observed; 75% BSA could be discarded in the flowthrough. Lower flowrates were expected to result in even more efficient depletion of BSA. However, such low flowrates are highly impractical with the system used. Firstly, such low flows are very unstable if delivered without flow splitting. Moreover, sample volumes as large as 100 µL were expected to be brought onto the trap column, which would result in extremely long loading times. As an alternative, 0.2-mm-ID trap columns were considered but, when coupled to a 50-µm ID analytical column, this resulted in broad chromatographic peaks. Therefore, a change in set-up was necessary, incorporating an on-line albumin depletion step using a larger ID C$_8$-RAM cartridge coupled to the original reversed-phase nanoLC system.

### 5.3.2 Set-up 2: RAM cartridge coupled to nanoLC-MS

In order to remove BSA prior to nanoLC-MS in a more efficient manner, Set-up 2 was used (Figure 5.1). In this set-up, a RAM cartridge (10 * 1.0-mm-ID) was positioned in-between the injection valve and the trap column. During the first 10 minutes, the flow was split between the RAM cartridge and the nanoLC part of the system, discarding the BSA that was not retained on the RAM to waste. The transfer of SP from the RAM cartridge to the trap column of the nanoLC was performed by directing the flow through the trap column without splitting. After loading of the trap column, the valve was switched back to its original position, thereby splitting the flow again. At the same time, the flow was raised and the gradient started, simultaneously washing the RAM cartridge of the remaining BSA to waste and eluting SP off the nanoLC. In this set-up a RAM cartridge was coupled to nanoLC-MS in a forward-flush mode, an approach
that has, to our knowledge, not been described before. This set-up has the advantage that no additional switching valve or pump are required, whereas most nanoLC designs making use of a trapping column do require a second "loading" pump [12, 13]. Commonly, in the elution step, the sample is back-flushed off the RAM cartridge and flows through the switching valve before reaching the analytical column. Back-flush set-ups minimise the contact time between analyte and trap column. However, positioning the switching valve on the sample path increases non-specific interactions between analytes and metal parts of the valve. The set-up described here can be used reproducibly with very fast gradients and without requiring an extensive period to stabilise both the flow and the pressure [13]. Moreover, the use of fewer parts is likely to reduce down-times of the instrumental set-up, though this remains to be seen in further long-term studies. Additionally, large sample volumes (10 to 100 µL) could be injected at high flowrates (50 µL/min) on Set-up 2. Haskins described a sensitive nanoLC-MS system for the analysis of neuropeptides in microdialysates in the absence of BSA in which no trap column was used. 5 min were necessary to inject 1.8 µL of sample on a 25-µm-ID column [14]. On-line coupling of trap and analytical columns enabled faster loading (<2 min) of larger sample volumes (10 µL) while separation was performed at nL/min flowrates [12]. The design of the nanoLC set-up described here allows for even larger sample volumes (up to 100 µL) to be loaded in a short time. Moreover, analytes can be efficiently separated from the BSA-containing sample matrix during loading before being further separated on the nanoLC-MS part of the system.

This set-up has great potential for on-line sample preparation coupled to nanoLC-MS. Its selectivity can be tailored to suit the needs of a particular application by varying the selectivity of the cartridge and/or of the trap column. To demonstrate the feasibility for on-line albumin depletion coupled to nanoLC-MS, a C8-RAM cartridge was characterised and coupled on-line with a C18 nanoLC system.

### 5.3.3 Characterisation of RAM material

Using Set-up 2, it is of great importance to avoid that SP elutes in the flowthrough containing the majority of BSA and to maximise resolution between SP and the remainder of BSA that is retained on the RAM cartridge. To this end, loading and elution conditions and the %DMSO in the sample were optimised. A cartridge (10*1 mm) packed with C8 RAM was coupled directly to the ion trap mass spectrometer using a commercially available interface.
5.3.3.1 Sample loading conditions

The impact of the mobile phase composition (1 to 15% ACN) during loading of the sample was investigated. In addition, the influence of the flowrate during loading of the RAM cartridge was investigated (50 to 250 µL/min). The influence of the ACN percentage was investigated at a flow of 250 µL/min, while for the investigation of flowrate an ACN percentage of 1% during loading of the sample was used. Prior to injection, the RAM cartridge was equilibrated for 2 min (at 250 µL/min) at the mobile phase composition used for sample loading. After sample loading, the gradient was started at this percentage. The ACN percentage increased to 95%, the change in volume fraction of acetonitrile per gradient volume was held constant, i.e., the volume of the column effluent from the start to the end of the gradient was constant. Every experiment was performed with perfusates containing 0.1% FA in DMSO at the following ratios (19:1, 3:1 and 1:1).

Two BSA fractions were observed, one in the flowthrough and the other resulting from retention of a part of BSA on the RAM column. Retention of BSA on the RAM may have resulted from either one or a combination of three distinct phenomena. First, BSA may interact with the polyethylene glycol chains present on the outer surface of the RAM silica particles in a hydrophilic interaction chromatography (HILIC) retention mode. However, HILIC using the C₈-RAM cartridge was only observed for SP at very high percentages of ACN (>60% ACN, data not shown). HILIC can therefore be ruled out at the mobile phase composition (1% ACN) used during loading of the sample on the trap column. Second, BSA may bind to the remaining silanol groups at the surface of the RAM as suggested by Wagner et al. who used cation-exchange RAM particles [9]. Most likely, however, is that some of the BSA passes through the polyethylene glycol network and enters the pores, where it interacts with the C₈ groups.

SP eluted between the two BSA fractions. 30 to 60% BSA were retained on the C₈-RAM cartridge depending on the mobile phase composition and the %DMSO in the sample. Increasing the %ACN in the mobile phase during loading from 1 to 15% resulted in an increase of 5 to 10% of BSA (at fixed %DMSO in the sample) in the flowthrough. It indicated that, indeed, BSA apparently interacts with the octyl groups inside the pores of the silica particles. As expected, retention of SP was also negatively influenced by an increased %ACN in the loading buffer. Loading at 1% ACN offered the best compromise. SP was strongly retained on the RAM phase and well separated from the second BSA fraction. Increasing the flowrate only resulted in minor changes. Resolution between SP and the second BSA fraction and the %BSA in the flowthrough were barely in-
fluenced. However, the time window to switch the valve and direct BSA to waste and SP to the nanoLC logically decreased with increasing flowrate. Increasing the %DMSO in the sample increased the intensity of the MS signal for SP indicating that DMSO probably prevents interactions between SP and BSA. It also resulted in a greater proportion of BSA in the flowthrough on the RAM. However, the SP peaks showed a better reproducibility in terms of peak shape and peak width when the samples contained only a limited amount of DMSO. Therefore, the flowrate was set at 50 µL/min and 25% DMSO in the sample and 1% ACN in the loading buffer were selected as the best compromise in terms of signal intensity, reproducibility (MS signal and retention time) and depletion of BSA.

5.3.3.2 Percentage ACN during elution of the RAM cartridge

To attain efficient elution of SP from the RAM cartridge while avoiding co-elution with the fraction of BSA also retained on the RAM cartridge, the %ACN during the elution step was investigated. The sample (containing 25% DMSO) was loaded on the RAM cartridge at 1%ACN. After 5 min, SP was eluted applying a step gradient up to 10 or 30%ACN for 5 min. The gradient was then linearly increased up to 95% ACN at a flow of 50 µL/min. The RAM cartridge was equilibrated at 1 %ACN for 10 min prior to the next injection.

Increasing the %ACN during step elution barely influenced the retention time of SP as 10% ACN was already sufficient for SP to start eluting. However, the retention of the retained BSA fraction was strongly influenced by the higher percentage of ACN during elution (Figure 5.3).

Step elution of SP using 10% ACN ensured baseline resolution between SP and the BSA fraction retained on the cartridge whereas step elution using 30% ACN resulted in co-elution of SP and BSA. The principle of Set-up 2 ensures complete albumin depletion while SP was retained on the cartridge and chromatographically well-resolved from the second BSA fraction. Therefore elution of SP was performed by step elution using 10% ACN.

5.3.3.3 Recovery

%ACN and flowrate during loading and %DMSO in the sample were expected to affect SP recovery. However, with respect to flowrate, no decrease in recovery was observed over the range of flowrates investigated. Breakthrough of SP was observed when more than 5%ACN were used during loading of the sample on the RAM cartridge or when the sample contained more than 25% DMSO.
Additionally, the recovery of SP was expected to be influenced by the presence of BSA and the time for which the RAM column was washed. The recovery of SP in the presence (or absence) of BSA and with varying loading/washing times (1 or 10 min) of the RAM cartridge was investigated by injecting 100 µL of sample. Loading was performed using 1% ACN during either 1 or 10 min. Subsequently, the gradient went up to 95% ACN in 6 min at a flowrate of 250 µL/min. Finally, the cartridge was subsequently re-equilibrated for 2 min at the loading buffer composition (1% ACN). The sample solvent was either perfusate (4.0 µM BSA):0.1% FA in DMSO (3:1) or 0.1% FA in water:DMSO (3:1). Samples containing 14.8, 148 & 1480 nM SP were sequentially injected onto the system. The resulting calibration curves showed different sensitivities that can be directly related to the respective recovery. The four different experiments showed decreasing recovery in the following order: samples without BSA and 1 min wash >samples with BSA and 1 min wash >samples without BSA and 10 min wash >samples with BSA and 10 min wash. The washing time of the RAM cartridge clearly led to great losses of SP. Recovery decreased by as much as 50% when the washing time was increased from 1 to 10 min. Part of the SP was probably washed away by the large excess of mobile phase used to wash the RAM cartridge. Sensitivity was a few percent lower in the presence than in the absence of BSA, indicating that only a small percentage of SP remained bound to BSA and was discarded to waste together with the eliminated BSA. Though only repeated gel filtration could dissociate the complex formed between SP and the high-molecular weight plasma components [15], it was possible with our set-up to efficiently enrich SP and discard BSA using a short RAM cartridge and a relatively high flowrate.

5.3.4 Set-up 2 under optimised conditions

The optimised chromatographic conditions for coupling the RAM cartridge with nanoLC-MS in Set-up 2 (Figure 5.1, Table 5.1) were the following. 100 µL of perfusate (containing 0.4 or 4.0µM BSA and 25% acidified DMSO) was loaded on the RAM cartridge using 1 %ACN at 50µL/min. A 50µL/min flowrate during elution made it possible to couple the RAM cartridge directly to the reversed-phase trap column without operating pressures exceeding 160 bar. A step gradient to 10% ACN allowed the transfer of SP from the RAM cartridge to the C<sub>18</sub> trap column. No breakthrough of SP on the C<sub>18</sub> trap column (20 * 0.1-mm ID) was observed even up to an ACN concentration of 30 %ACN in the elution step (data not shown). The RAM cartridge was washed during elution of SP over the nanoLC system using a linear gradient up to 65% ACN. The programming of
the gradient pump and switching valve is described in Table 5.1. Separations were very efficient with peak widths of 10s at half height. Such peak widths are consistent with other high-efficiency nanoLC systems [12, 14]. These set-ups, however, did not integrate an on-line sample pretreatment step. The total analysis time including sample pretreatment, separation of the analytes, washing and equilibration of the system, was about 40 min.

As a control of the efficiency of BSA removal by RAM sample pretreatment using Set-up 2, 100 \( \mu \text{L} \) of perfusate (containing 4.0\( \mu \text{M} \) BSA) were injected on Set-up 2 and the eluent of the nanoLC was monitored by full-scan mass spectrometry. The area of the BSA peak was compared to the area of the BSA peak resulting from the injection of 10 \( \mu \text{L} \) of perfusate on Set-up 1 equipped with a C\(_{18}\)-trap column. The peak area due to remaining BSA on Set-up 2 was estimated to be approximately 2-3\% of that resulting from a ten-time smaller direct injection on the C\(_{18}\)-nanoLC system (Set-up 1). Therefore, only about 0.2-0.3\% of the injected BSA on Set-up 2 reached the MS (Figure 5.2). With the system described by Wagner et al., as much as 8\% of the original albumin content remained on the system after the RAM step [9]. Although antibody-based depletion approaches were specifically designed to selectively remove albumin and other high-abundance proteins [3], depletion did not reach the level achieved by our system. In the same study, other depletion (antibody- or dye-based) columns apparently achieved better results than our system but were not tested with a system as sensitive as a nanoLC-MS. Moreover, anti-HSA columns require exchanging solvents before LC-MS analysis.

An advantage of RAM materials over antibody-based columns is that non-specific binding of SP to BSA was reduced by adding 25\% acidified DMSO (0.1\% FA) to the sample, which is not possible for antibody-based systems.

Based on the optimised conditions, Set-up 2 was operated for more than a week (\( \approx \) 250 injections) without the need for washing either the nanoLC or the MS endplate. Retention times were much more reproducible (RSD \( \approx \) 3\% over a week) than for Set-up 1 (RSD \( \approx \) 6\% over 3 runs) even though samples with two different matrices (0.4 or 4.0\( \mu \text{M} \) BSA) were injected. After approximately 750 injections of perfusate (0.4 or 4.0\( \mu \text{M} \) BSA:0.1\%FA in DMSO (3:1)) on Set-up 2, 100 \( \mu \text{L} \) of perfusate were injected and the elution of BSA followed by full scan mass spectrometry. The peak area of BSA was comparable to the area observed after only a few injections. We therefore conclude that the system still operated reproducibly after 750 injections.
5.4 Conclusion

In this study, a novel set-up for multidimensional nanoLC-MS featuring three columns coupled on-line was developed and characterised. For the first time, a RAM cartridge was coupled on-line to nanoLC-MS in the forward-flush mode. No additional switching valve or pump are required to couple the three different columns. The set-up described here can be used reproducibly with very fast gradients without requiring an extensive period to stabilise either flow or pressure [13]. Moreover, its simplicity makes it very robust and cheap. After optimisation, all SP in the perfusate was brought on the nanoLC part and virtually all BSA (99.7-99.8%) was discarded. While analysing the sample by nanoLC-MS, the RAM cartridge was washed using the eluent of the nanoLC. The RAM cartridge and the nanoLC were re-equilibrated using the same mobile phase. Separations were very efficient with peak widths (at half height) of 10s. The total analysis time including sample pretreatment, separation of the analytes, washing and re-equilibration of the system, was about 40 min. Moreover, the separation was highly reproducible (RSD on retention time $\approx 3\%$ over a week) and robust (750 injections without any change of RAM cartridge or nanoLC columns) compared to a trap-nanoLC system such as Set-up 1. It was used almost continuously for a couple of months before the different columns and the RAM cartridge were exchanged as a precautionary measure. The set-up is particularly suitable for peptides and other low-molecular weight analytes in matrices containing large amounts of high-molecular weight proteins like BSA. Importantly, it can be used for the analysis of relatively large volumes (10-100 $\mu$L) for nanoLC-MS. Our rapid, reproducible and robust RAM-nanoLC-MS set-up is expected to be of great interest for comparative analysis of large sets of samples. Further developments of this modular system involve the use of stationary phases of different selectivity and the coupling to a more sensitive triple quadrupole or linear ion trap mass spectrometer. We consider that this system holds promise in the field of biomarker discovery, where limited sample is available (e.g. CSF from animal studies) and concentrations of interesting molecules is rather low.
References


