Performance characteristics of the Agilent 1100 Series Nanoflow LC system for MS

Technical Overview

Abstract

In the field of proteom research, unknown sample proteins are identified after tryptic digestion by LC/MS/MS analysis of their peptides followed by database search with the obtained MS/MS data. In this Technical Overview the performance of the Agilent 1100 Series LC system for MS for protein identification is demonstrated.
The Agilent 1100 Series nanoflow pump and the Agilent 1100 Series micro well-plate autosampler are discussed in detail.

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Introduction

The genome of many organisms and human beings has been elucidated. The next challenge is to understand how the proteins, the translation product of the genome, fulfill their biological role in a living organism. This new aspect of life science in analogy to genomics is called proteomics[1,2]. The target of research in this new scientific discipline is the identification of complex protein patterns and to understand their interaction. This is important in order to understand the pathogenesis of diseases such as cancer, and hence for the development of new drugs in the pharmaceutical industry. For this purpose several advanced techniques such as 2D-gel electrophoresis[3], mass spectrometry[4] and bioinformatic[5] are available today.

In this Technical Overview the performance of the Agilent 1100 Series nanoflow LC system for MS is demonstrated. This nanoflow LC system was especially developed for the use in proteomics research to analyze complex digested protein samples. The Agilent 1100 Series nanoflow pump, which delivers the low nanoliter flow rate and the Agilent 1100 Series micro well-plate autosampler are discussed in detail.

Equipment

The Agilent 1100 Series nanoflow LC system for MS included the following:

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent 1100 Series thermostatted column compartment (TCC) with micro 6-port/2-position valve or Agilent 6-port/2-position micro switching valve box
- Agilent 1100 Series LC/MS Trap XCT equipped with the Agilent Orthogonal Nanospray Source
- Agilent ChemStation A09.03 and Ion Trap software 4.1

Second pump:

- Agilent 1100 Series quaternary pump with micro vacuum degasser

Software used for database search:

- Agilent Spectrum Mill MS Proteomics Workbench

With the HPLC equipment it is possible to work in two different sample injection modes: the direct injection mode, and the typically used enrichment injection mode, in which peptides can be trapped from highly diluted samples on an enrichment column prior to analysis. Figure 1A shows the recommended stack including the thermostatted column compartment with micro 6-port/2-position valve. Figure 1B shows the stack with the 6-port/2-position micro switching valve box. This set-up has the advantage that a shift from one mode to the other is possible by reconnecting only two capillaries. To work in the enrichment mode (figure 2) a second pump is connected to the Agilent 1100 Series micro well-plate autosampler. This module is connected with the micro 2-position/6-port valve in the thermostatted column compartment or to the valve box where the enrichment column is located. Prior to the analytical run the sample peptides are injected on

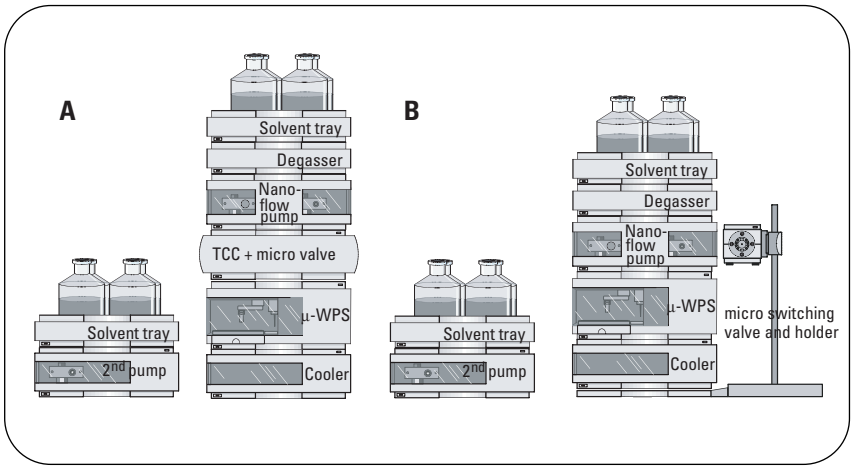

Figure 1
Recommended LC stacks for direct injection and enrichment column injection with TCC or micro switching valve box. In A the micro valve in the TCC and in B the separate micro valve is connected to the MS.

the enrichment column with the high flow rate delivered by the Agilent 1100 Series quaternary pump (figure 2A). After enrichment of the peptides the enrichment column is switched into the analytical nanoflow path and the Agilent 1100 Series nanoflow pump starts to deliver the elution gradient (figure 2B). To work in the direct injection mode the Agilent 1100 Series nanoflow pump is connected directly to the Agilent 1100 Series micro well-plate autosampler (figure 3A). After the sample volume is injected the micro injection valve in the Agilent 1100 Series micro well-plate autosampler is switched to bypass and the Agilent 1100 Series nanoflow pump starts to deliver the gradient for the elution of the sample peptides from the analytical column (figure 3B). Advantages and disadvantages of both modes are summarized in table 1. A typical base peak chromatogram of a direct injection of 100 fmol BSA is shown in figure 5. In both cases the nanocolumn is located directly in the nano electrospray ionization chamber and is connected directly without any dead volume to the nanospray needle (New Objective, PicoTip™).

The Agilent orthogonal nanospray source with the special column- and needle-holder (figure 4) offers several advantages. The column and needle is easy to assemble and disassemble without any extra tools (figure 4 A). The need for fragile springs for electrical

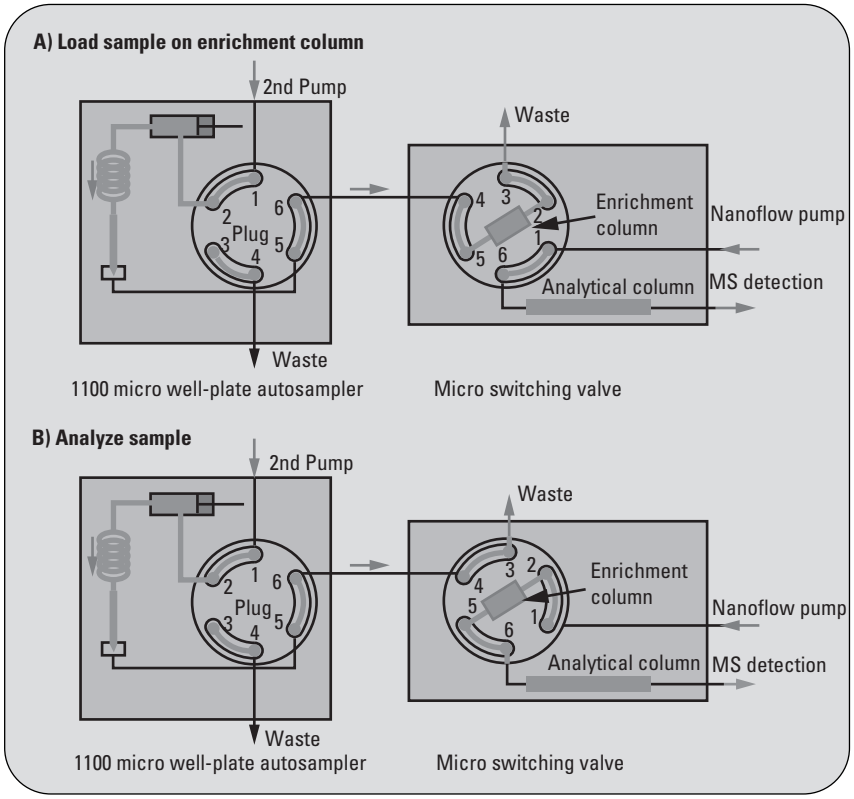

Figure 2
Flow diagram for nanoflow chromatography with enrichment column

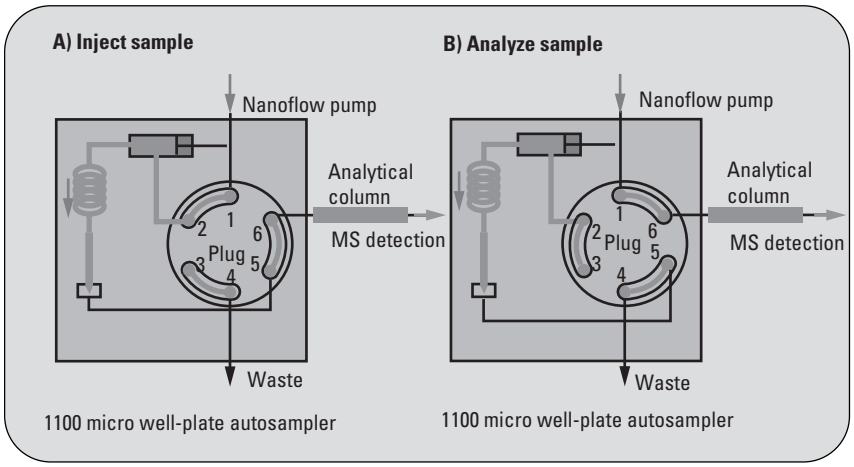

Figure 3
Flow diagram for nanoflow chromatography with direct injection

grounding is also eliminated. The grounding is achieved with an electrical conductive ferrule and conductive pads with less than one Ohm electrical contact resistance. To assemble the sprayer needle into the column fitting no separate reducing sleeves or additional connectors are necessary. The holder is a simple clam shell holder for quick and easy column and needle change-out during the analysis without electrical danger. The clam shell holder can be removed and reinserted while maintaining the original needle position even during acquisition, and when the spray chamber is closed. The needle tip is automatically positioned between the high voltage MS inlet electrode and the special counter electrode. Adjustment is accomplished by turning one or both adjustors alng the x- and y-axes (figure 4B). The counter electrode is operated at 500 V below the MS inlet electrode. The 2nd electrode stabilizes the spray and draws the liquid stream to the opposed direction of the MS inlet (figure 4 C), causing less background and chemical noise. Positioning the nanospray emitter in the ortho position and increasing the spacing eliminates electron avalanches and high voltage discharges, allowing for successful ion polarity switching with the acquisition of a full MS scan each 1.25 seconds. This spray chamber is fully sealed and vented for safety for the operator eliminating any possibility of aerosol particle escape.

Direct injection mode	Enrichment injection mode
Advantage: Good sensitivity and chromatography **Disadvantages:** Salts and other impurities go into MS/nanospray needle. Long delay times for large volume injections.	**Advantages:** Best for samples containing impurities. Best for large volume injections (higher flow to enrichment column enables shortened sample loading time). **Disadvantages:** Requires second pump. Sensitivity and chromatography somewhat compromised, because of the enrichement column.

Table 1
Advantages vs. disadvantages of the direct and enrichment injection mode

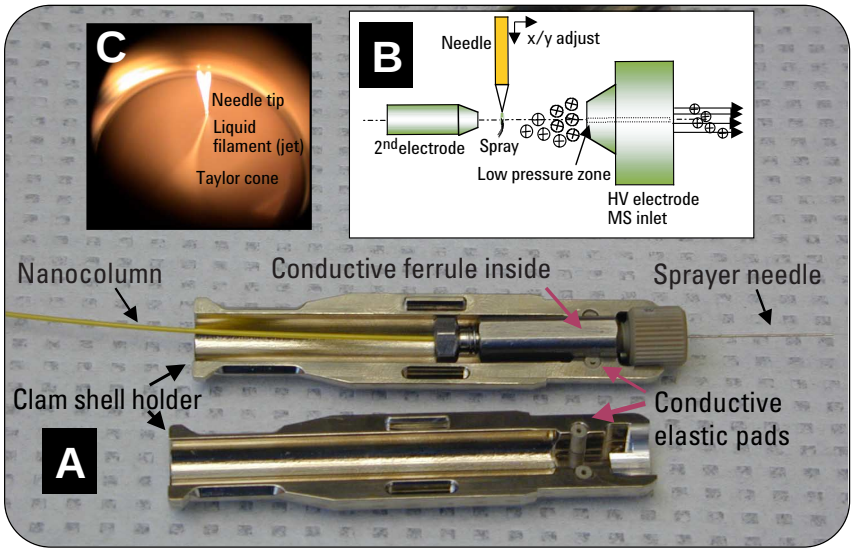

Figure 4
Agilent orthogonal nanospray source and column- and needle holder

Performance and robustness of the Agilent 1100 Series nanoflow pump

To deliver a very stable and robust liquid flow in the low nanoliter flow rate range, the Agilent 1100 Series nanoflow pump is equipped with a special Electronic Flow Control (EFC) instead of a non-regulated passive splitter. With the EFC the primary flow rate is divided into the nanoliter column flow and a waste flow. To control this active splitting ratio the nanoliter column flow is monitored with a special nanoflow sensor. For this sensor calibration curves for all common HPLC solvents and their mixtures are included in the ChemStation software. This sensor signal drives the split ratio at the electromagnetic proportioning valve (EMPV) (figure 6A). From the EMPV to the nanoflow sensor's end the delay volume is only 300 nL, 160 nL for the flow sensor and 140 nL for the capillary from EMPV to the nanoflow sensor. In the primary flow path from the mixing point of the solvents to the EMPV the pump delay volume at 90 bar pressure is 398 µL. This volume consists of 28 µL for the capillaries, 180 µL and 1 µL/bar for the pressure damper and 100 µL for the solvent filter. For the primary flow of 800 µL/min and a nanocolumn flow of 450 nL/min this leads to a gradient delay time of about 2 minutes, which is not relevant for the gradient times used for proteomics applications. The nanoflow sensor[6] (figure 6B) consists of a stainless steel capillary, two temperature sensors and a heater around the capillary.
If there is no flow through the capillary the temperature profile around the heater is symmetric.

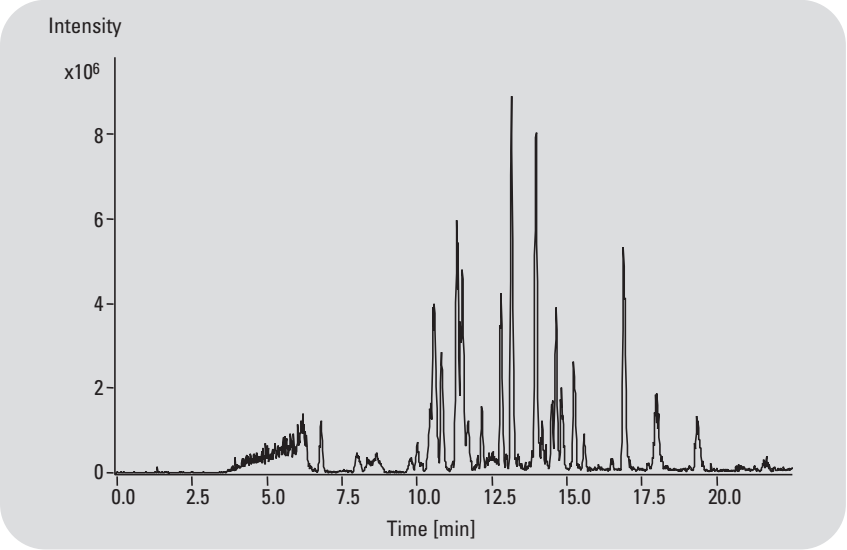

Figure 5
Base peak chromatogram of 100-fmol tryptic BSA digest by direct injection method

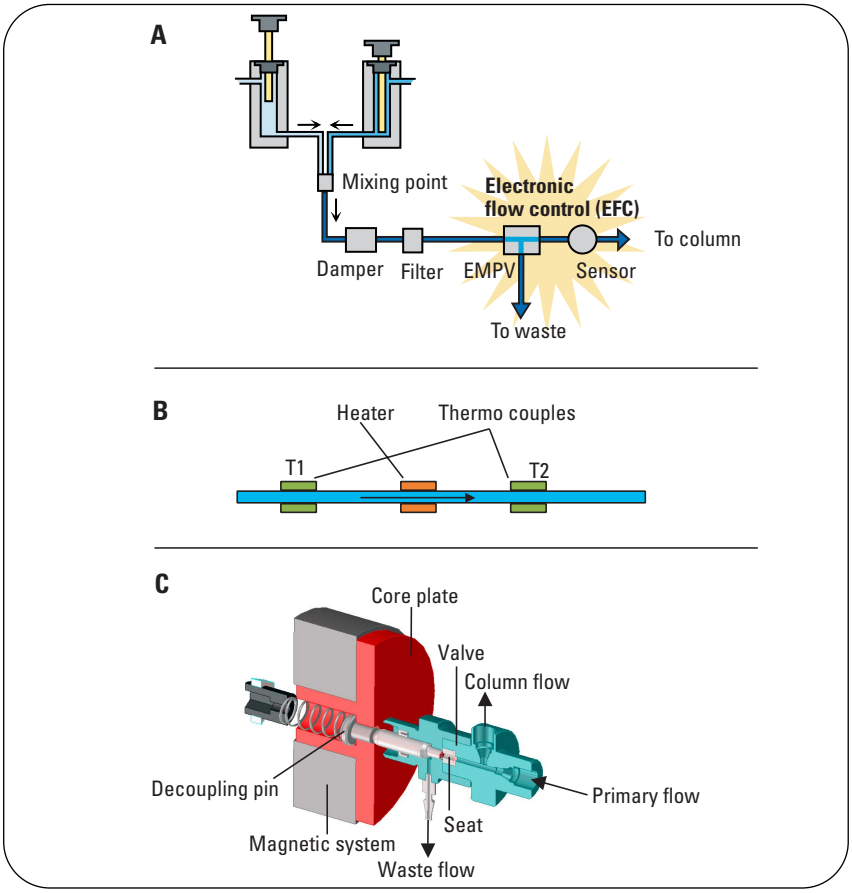

Figure 6
The electronic flow control of the Agilent 1100 Series nanoflow pump

If there is a flow though the capillary the temperature profile shifts downstream. The shift in the temperature profile represents a temperature difference caused by the heat transport of the flowing fluid. This heat transport is proportional to the flow rate. Therefore, this sensor measures the flow of the fluid and delivers a feedback to the EMPV (figure 6C). This ensures an outstanding flow stability independent from system backpressure fluctuations.
The Agilent 1100 Series nanoflow pump is able to deliver the gradient with a high step height accuracy, step height precision and low mixing noise (figure 7). The step height accuracy with an RSD of 0.047 % and the step height precision with an RSD of 0.07 % shows the excellent adjustment of the gradient steps based on the unique EFC of the pump. The mixing noise RSD of 0.07 % proofs the high performance of the solvent mixing in the primary pump flow. The calculation methods for these values are mentioned previously[7].
For flow rates from 1000 nL/min down to 100 nL/min the very precise and stable flow and the corresponding pressure is shown in figure 8 in a normalized overlay of three runs. Corresponding flow rates and pressures are also indicated. To establish the new flow rate after a step increase typically takes 0.2 minutes (primary flow rate setting: high solvent consumption mode). Even after a weekend shut down the typical nanoflow performance is established within one minute after switching on the nanoflow pump. In addition, the ChemStation software provides test routines to detect leaks in the

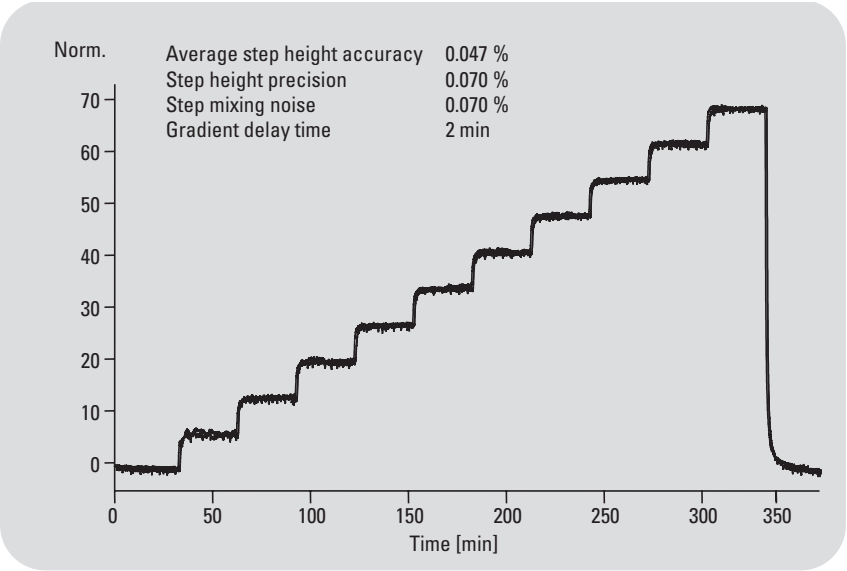

Figure 7
Composition accuracy, composition precision and mixing noise of a gradient delivered from the Agilent 1100 Series nanoflow pump. Overlay of three runs. (Solvent: A=water, B=water+0.5 % acetone; Gradient: from 0 % to 10 % in steps of 1 %, each step was held for 30 min; Column flow: 800 nL/min; Column: 100 µm x 150 mm, 3.5 µm; DAD: 267/10 nm, ref. 360/10 nm: Cell: 80 nL)

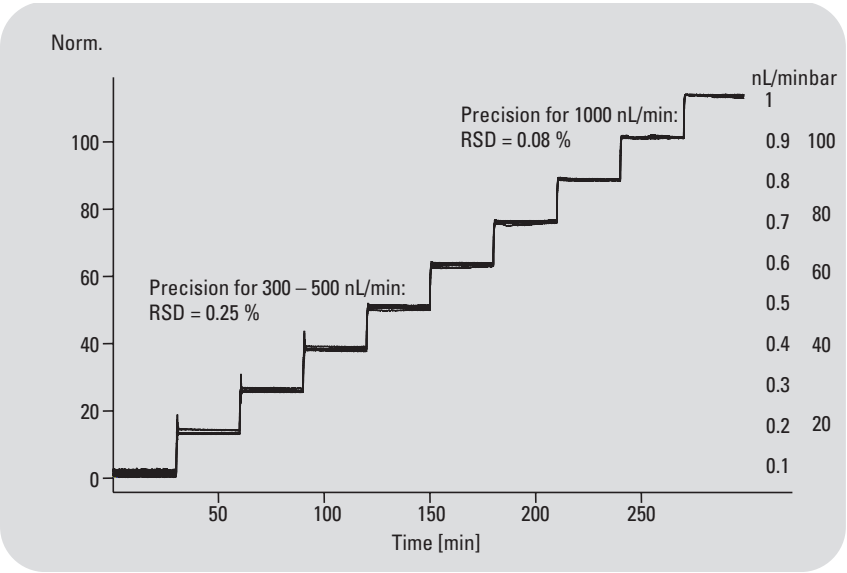

Figure 8
Typical flow rate performance of the Agilent 1100 Series nanoflow pump. Overlay of three runs. (Solvent: A=water; flow gradient: from 100 nL/min to 1000 nL/min in steps of 100 nl, each step was held for 30 min; column: ZORBAX 300 SB C18 100 µm x 150 mm, 3.5 µm; system pressure was measured)

nanoflow path, which cannot be seen with the naked eye. How independent the column flow rate is from system backpressure fluctuation is demonstrated when a temporary or permanent partial blockade in the sprayer needle occurs (figure 9). During blockade the pressure
raises significantly, however the flow rate is kept constant which is not possible with a nonregulated passive splitter. In figure 9 a temporary needle blockage occurs at eight minutes. The flow rate drops and the loss is compensated by the electronic flow control which increases the pressure to maintain the original flow rate. After 20 minutes the blockage passes the needle and releases the needle tip again. Immediately the flow is kept stable by the electronic flow control. The electronic flow control is even capable to compensate fast changes in the flow rate as obtained in the system during a valve switch. Because temporary and permanent blockades occur during the lifetime of a sprayer needle, this long constancy of the flow rate is important for the reliability of the retention time. This is demonstrated by measuring the retention time RSD for some ions, extracted from ten TICs of a tryptic BSA digest (figure 10). The determined RT-RSD is < 0.2 %. The Agilent 1100 Series nanoflow pump was operated with standard settings.

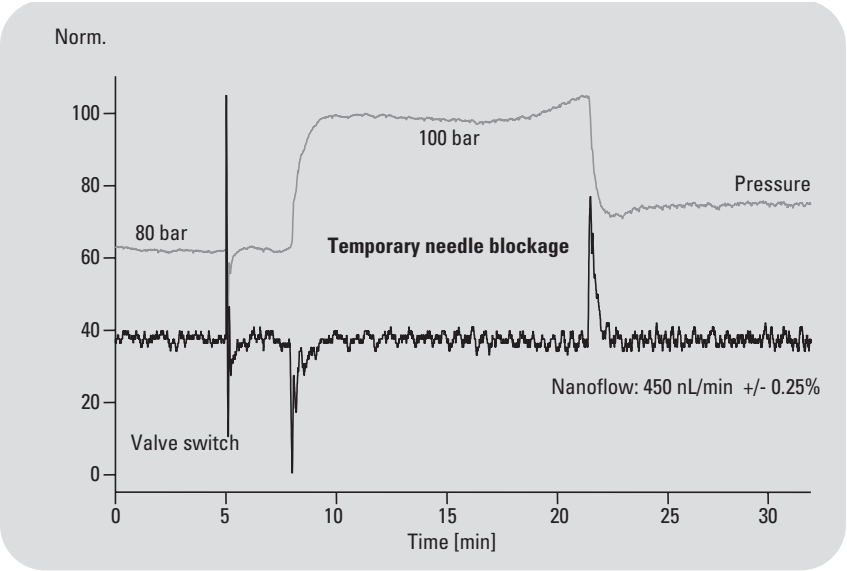

Figure 9
Robust nanoflow independent from back pressure

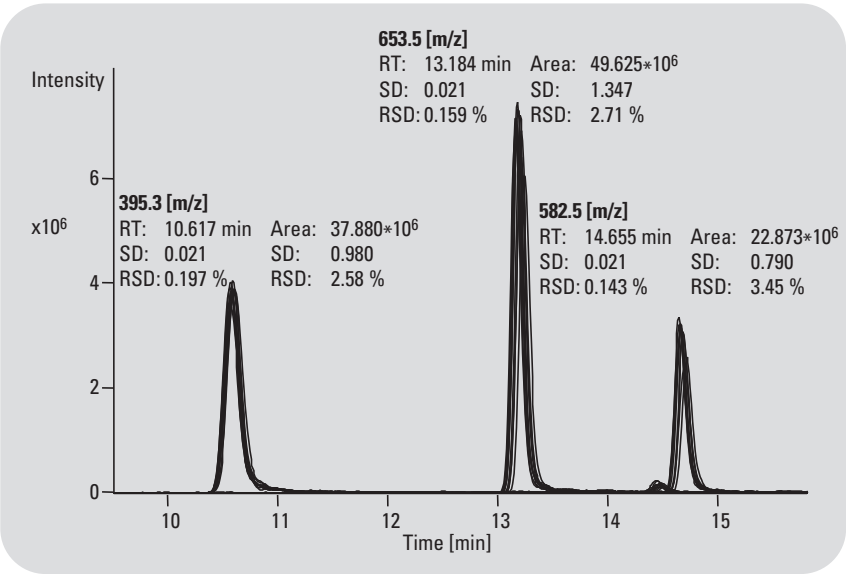

Figure 10
Retention time precision of the Agilent 1100 Series nanoflow pump and area precision for injections with the Agilent 1100 Series micro well-plate autosampler. (Overlay of ten EICs for each peak; RT=retention time; SD=standard deviation, RSD = relative standard deviation).

The Agilent 1100 Series micro well-plate autosampler

For proteomics applications it is a common problem that only a small amount of a complex and valuable sample is available. Therefore, it is essential that the autosampler included in the LC system is able to draw very low sample volumes with high precision. The Agilent 1100 Series micro well-plate autosampler offers two different sample loops: an 8-µL and a 40-

µL loop. The injection volumes are set in 10-nL steps beginning at 20 nL. The recommended minimum injection volume is 200 nL. To determine the injection precision 1 µL of a tryptic BSA digest with a concentration of 100 fmol/µL was injected 10 times (direct injection method) and the peak area of three selected ions were detected with MS (figure 10). Under these conditions the RSD of the peak area is between 2.58 % and 3.45 %. This is excellent for quantification tasks in proteomics wirth, for example, ICAT and GIST[8,9].

The relative standard deviation of response factors which is in the range of 2 % to 4 %, and the injection volume linearity over the whole injection volume area are discussed detail in another note[7]. Due to the high value and limited volume of proteomics samples it is also very important that the autosampler is able to draw as much sample volume as possible from a given volume out of the sample vial. In this case, with a vial-optimized method the Agilent 1100 Series micro well-plate autosampler is able to draw nearly the complete sample volume out of the vial. This can be accomplished using the "find bottom" feature of the autosampler. For peptide and protein samples it is recommended to use plastic vials or well-plates. To analyze low volume samples 300-µL wide open conical polypropylene vials (Agilent part number 9301-0978) and 100-µL polypropylene inserts (Agilent part number 5182-0549) for 2-mL glass vials are recommended. From these vials the micro well-plate auto-sampler is able to perform 4 full 2-µL injections out of 10 µL[7]. Therefore, for the next full 2-µL injection 4-µL sample volume is required. An application for the large 40-µL injection loop is enrichment of highly diluted samples. For this application the large volume of diluted sample is in-jected into the C-18 enrichment column, which is used in the enrichment method (vide supra). To demonstrate the performance of enrichment a highly diluted solution of a tryptic BSA digest with a concentration of 2.5 fmol/µL was injected with increasing volumes. The obtained increase in score, number of detected peptides and sequence coverage clearly indicates the enrichment effect (table 2). The scores increased by a factor of about 6.5 and the sequence coverage went up went up from 8 % to 29 %. For proteomics research it is very important to detect low abundant proteins because they are often important for biological functions. For their correct qualitative, and even more quantitative detection, it is important that there is no carry over between different samples caused by the autosampler when high concentrated samples are analyzed in a sequence with highly diluted samples and mass spectrometric detection is possible even in the low fmol range. Therefore, the following experiment was performed to determine the effect of carry over of the autosampler especially for peptide samples. A high concentrated sample of a tryptic Serotransferrin digest (1 pmol/µL) was injected without overloading the ion trap mass spectrometer. After the analysis a blank run with a water injection was performed. In the first injection Serotransferrin was found in a database search with a score of 627 with 30 peptides and with a sequence coverage of 30 %. From the blank run MS/MS spectra were isolated from the noise and subjected to the database search with no significant hit. To look more specific, the peptide TSDANINWNNLK, which gives the highest ion score from the database search was extracted from the base peak chromatograms of both analysis (figure 11). The comparison of the corresponding peak areas indicated for a carry

Injected volume [µL]	Score	Peptides	Sequence coverage [%]
1	53	7	8
10	118	9	18
20	184	9	17
30	327	17	29

Table 2
Enrichment experiment of a tryptic BSA digest dilution with 2.5 fmol/µL

over for this peptide of 0.035 %. This value, which was obtained without any special cleaning procedure is absolutely neglectable for all applications and database searches. An outside needle wash using a 50 % solution of methanol in water with 1 % formic acid is typically sufficient for peptide samples of high viscosity that stick to the injector needle. It is clear that special hydrophobic peptides may stick to the inner surface of silica capillaries and valves. To remove these peptides a wash pulse of organic solvent (85 % acetonitrile and 0.1 % formic acid) can be introduced by the second pump through the autosampler valve. In addition, it is possible to program a valve cleaning procedure by repeated fast valve switching for the autosampler valve as described for highly problematic compounds[7].

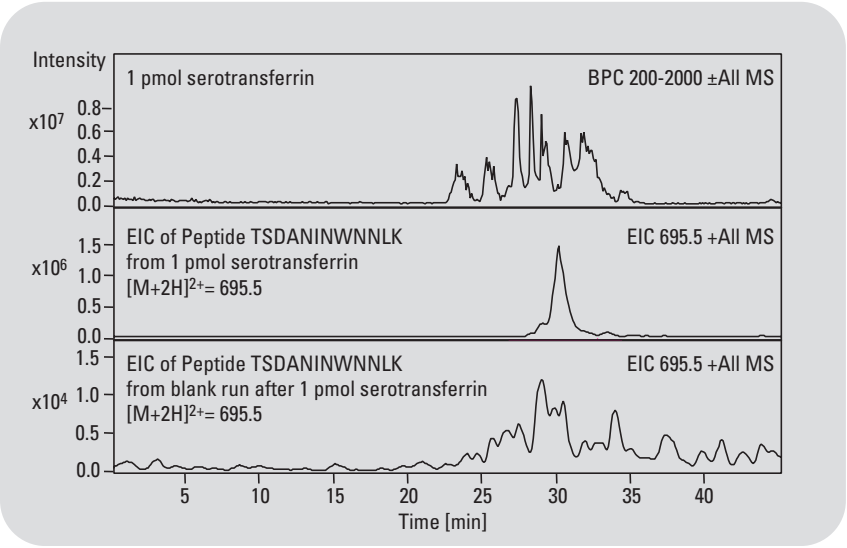

Figure 11
Carry over of 0.035 % of the peptide TSDANINWNNLK from a tryptic digest of 1pmol Serotransferrin

Conditions: Nano LC/MS method

Nanoflow pump

Solvent: A = H_2O + 0.1% FA; B = AcN + 0.1% FA	
Nanoflow pump gradient	6 min 3% B, 66 min 60% B, 75 min 60% B
Stop time:	75 min
Post time:	15 min
Flow:	300 nL/min for 150 mm column, 450 nL/min for 50 mm column
Column:	ZORBAX 300 SB C-18, 75 µm x 150 mm, 3.5 µm or 75 µm x 50 mm, 3.5 µm
Enrichment column:	ZORBAX 300 SB C-18, 0.3 mm x 5 mm, 3.5 µm
Column temperature:	ambient
Column switch (enrichment method): 5 min	
Injector program (Direct injection method):	Switch valve bypass after 5 min
Injection volume:	1 µL
Injection loop size:	8 µL

Quaternary pump (enrichment method)

Stop time:	none
Post time:	none
Flow:	20 µL
Solvent:	water + 3 % AcN + 0.1 % FA

Change to conditions for LC/MSD Trap XCT

Source: positve orthogonal nanoelectrospray
Drying gas flow: 5 L/min
Drying gas temperature: 225 °C
Skim 1: 35 V
Cap exit offset: 115 V
Octopole 1: 12 V
Octopole 2: 3.5 V
Trap drive: 80 V
ICC: On
  Averages: 4
  Max. accu time: 150 ms
  Target: 125.000
Ion mode: positive
Automatic MS/MS:
  Number of precursors: 2
  Isolation width:1.15V
  Preferred charge state: +2
  SmartFrag: On, 30-200 %

Conclusion

The Agilent 1100 Series nanoflow pump used in the system delivers the required nanoliter flow rates and eluent gradients with high precision and accuracy over a wide nanoliter flow rate range. The electronic flow control, used in the Agilent 1100 Series nanoflow pump with its active splitter makes the flow independent from any system backpressure variations, such as a permanent or partially blocked MS sprayer needle. With the new easy-to-use Agilent column and needle holder it is simple to install the nanocolumn and sprayer needle in the orthogonal nanospray source. With the Agilent 1100 Series micro well-plate autosampler it is possible to inject samples from nanoliters up to microliters with high precision. With its capability to inject up to 40 µL it is possible to enrich peptides from highly diluted samples on an enrichment column prior to the separation on the RP nanocolumn and MS/MS analysis. Peptide samples originating from digested proteins can be handled with the Agilent 1100 Series micro well-plate autosampler with neglectable carry over. The outstanding performance of the Agilent 1100 Series nanoflow LC system for MS make it the ideal system for the analysis of highly complex proteomics samples, with methods such as two-dimensional LC/MS/MS[10]. For data analysis of these highly complex samples Agilent recently introduced the Spectrum Mill MS Proteomics Workbench software.

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