

Capillary-flow LC-MS: combining high sensitivity, robustness, and throughput

Authors

Alexander Boychenko, Stephan Meding, Wim Decrop, Martin Ruehl, Remco Swart
Thermo Fisher Scientific,
Germering, Germany

Keywords

Capillary-flow LC-MS, capLC-MS, sensitivity, throughput, direct injection, preconcentration onto trap cartridge, robustness, proteomics, targeted, shotgun, ESI source, EASY-Spray

Goal

Provide an introduction to the advantages of capillary-flow LC-MS (capLC-MS) for various application areas as well as a guide to set up such an application connected to MS instruments. Describe a robust and sensitive capLC solution that can be used for high-throughput targeted analysis or profiling of complex samples in large sample cohorts.

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1. Introduction

The sensitivity of liquid chromatography-mass spectrometric (LC-MS) analysis with electrospray ionization (ESI) sources can be measured in numerous ways, e.g. using the peak area, the peak height, the signal-to-noise ratio, or the slope of the calibration curve. Independent of the selected metrics, the sensitivity of LC-MS increases significantly with decreasing column internal diameter and flow rate.^{1,2} In this respect, typical ESI-MS detectors behave as concentration-sensitive detectors, i.e. the signal is proportional to the analyte concentration in the eluent, and not mass-sensitive detectors, where the signal is proportional to the amount of sample introduced to the detector per unit time. The comprehensive explanation of the experimentally observed sensitivity gain is complicated.³ The main factors that improve sensitivity are more efficient ESI processes and better ion sampling into the mass spectrometer with decreasing flow rates. Recently, a comprehensive study was completed that compared sensitivity using columns with different internal diameters (IDs) at different flow rates.⁴ The clear experimental relationship between flow rate and sensitivity proved that the sensitivity gain can be described by a power law function (Figure 1). Thus, major improvements in sensitivity can be achieved at capillary and nano flow rates compared to analytical flow rates (Figure 1).

In recent years, the reliability of low-flow LC-MS systems was significantly improved through the introduction of tool-free near-zero dead volume Thermo Scientific™ nanoViper™ fingertight fittings and integrated plug-and-spray Thermo Scientific™ EASY-Spray™ consumables.⁵ However, sensitivity and throughput often counteract each other in low-flow separations. NanoLC-MS with long separation columns allows for the identification of more than five thousand proteins in a single run and quantitative profiling of complex biological samples.⁶ However, the length of a nanoLC-MS run normally exceeds one hour. There are two main reasons for long gradients in nanoLC-MS: (i) the requirement to obtain high peak capacity to improve separation and reduce signal suppression in complex samples; (ii) the physical limitations associated with using nano-flow rates, e.g. the increased impact of gradient delay volumes and sample loading volumes on the total analysis time. In many cases, a compromise capable of delivering high sensitivity as well as acceptable throughput is desired. In this technical note, a capillary-flow LC-MS (capLC-MS) solution is

described that can be used to harness the sensitivity provided by nanoLC-MS while maintaining the high throughput of analytical flow LC-MS.

2. Nano, capillary, micro, and analytical flow rates

The nomenclature used to define different flow ranges differs from publication to publication or from instrument vendor to vendor. The situation becomes even more complex when the flow range is linked to the column internal diameter. The wide number of available column chemistries and structures (e.g. fully porous, solid core, silica, or polymer monolith) results in different flow ranges that are most suited for a particular column ID. In this work, the naming convention shown in Figure 2 is used to differentiate flow rate ranges. As can be seen, the flow rates and column IDs for the respective ranges overlap.

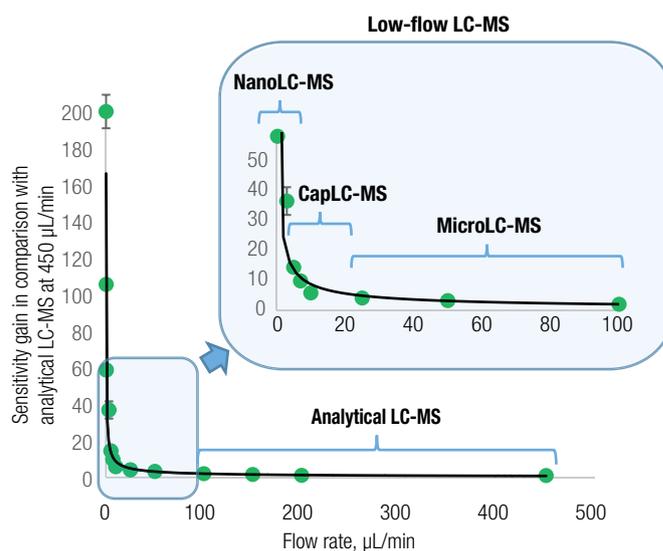


Figure 1. The increase in sensitivity observed with low-flow LC-MS compared to analytical flow LC-MS. The sensitivity gains were measured as relative peak areas averaged for two peptides from cytochrome c digest (KGEREDLIAYLK, m/z 478.93, charge +3 and EDLIAYLK m/z 482.77, charge +2). In all experiments, 1 pmol of cytochrome c digest was injected onto the column. The experimental data could be described by a power law function “Sensitivity gain” = $38.168 \times [\text{Flow rate, } \mu\text{L/min}]^{-0.638}$, $R^2 = 0.976$.

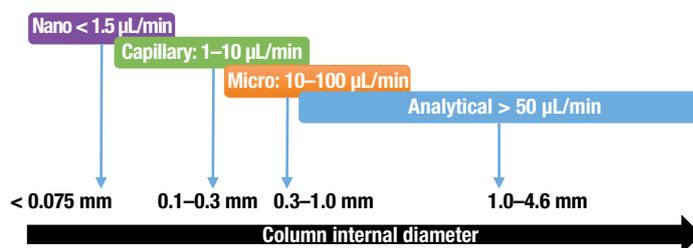


Figure 2. Different flow rate ranges in chromatographic separations and corresponding typical column IDs. Note, the ranges are overlapping.

3. Capillary-flow LC-MS solution

CapLC-MS with 100–300 μm ID columns and flow rates from 1 to 15 $\mu\text{L}/\text{min}$ provides increased MS sensitivity, lower solvent consumption, and minimized source contamination.⁷ Additionally, capLC-MS can give higher throughput in comparison with nanoLC-MS, while maintaining similar sensitivity to nanoLC-MS as higher sample amounts can be loaded. Here, a reliable and easy to set up capLC-MS solution is described. It combines the following:

- An optimized low-flow UHPLC system
- Dedicated consumables, including separation column, trap cartridge, and post-column connections
- ESI source and emitter to interface with the MS detector

The described capLC-MS solution can be used for sensitive targeted analysis as well as profiling of complex samples. The capLC-MS results were compared with nano and analytical flow LC-MS data to demonstrate the advantages and limitations of capLC-MS.

3.1. Electrospray ionization source and emitter for capLC-MS solution

CapLC-MS is positioned between nano and analytical flow LC-MS. Different options for coupling the capLC system with mass spectrometers were thoroughly investigated and an optimal ESI interface for different flow rates was proposed.⁴ The schematics of the recommended (based on the EASY-Spray source) and alternative setups (based on the Thermo Scientific™ Ion Max or Ion Max NG sources) are shown in Figure 3. Detailed guidelines and a description of each setup together with typical gradients that can be used for targeted analysis or profiling of proteomics samples and test data can be found in the following sections.

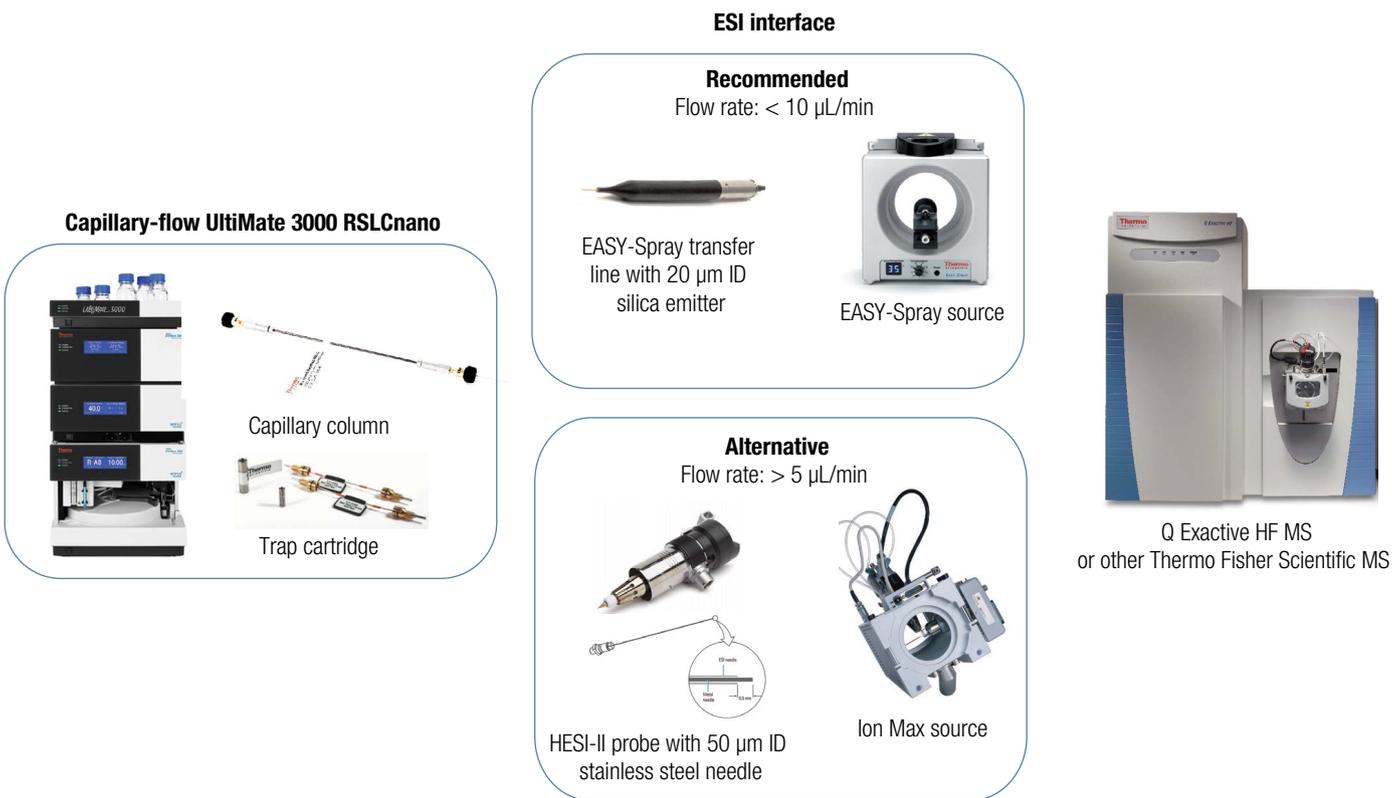


Figure 3. Recommended (top) and alternative (bottom) capLC-MS solution.

3.1.1. EASY-Spray source with EASY-Spray transfer line

The EASY-Spray source is designed to interface low-flow LC with MS, yielding highly reproducible results. The EASY-Spray transfer line is equipped with a 20 μm silica emitter that is connected to a transfer capillary (which has a diameter of 75 μm and a length of 500 mm) that finishes with a nanoViper fingertight fitting. This fitting allows for a quick, simple, and reproducible connection between the EASY-Spray transfer line and the column outlet, which does not require any tools (Appendix A, Table A2). Near-zero dead volume post-column connections ensure high quality separations. The assembly with the emitter is positioned in the EASY-Spray source and the column is positioned inside the column compartment of the Thermo Scientific™ UltiMate™ 3000 RSLCnano system. The EASY-Spray source with EASY-Spray transfer line can be successfully used for flow rates up to 10 $\mu\text{L}/\text{min}$ and does not require sheath or nebulizing gas to obtain stable spray.

3.1.2. Ion Max (NG) source with HESI-II probe and 50 μm ID needle insert

The Ion Max source with HESI-II probe is a universal solution that provides excellent ionization efficiency for analytical flow LC-MS and allows optimization of the signal by changing the temperature of the ion transfer tube, HESI vaporizer temperature, sheath gas pressure, sweep gas flow, auxiliary gas flow, and spray voltage. Additionally, the probe depth and horizontal position can be adjusted. The Ion Max source with HESI-II probe can also be used for capLC-MS analysis after optimization of the fluidic flow path to minimize post-column dispersion.

Good peak intensity and signal stability for capLC-MS with flow rates from 5 to 10 $\mu\text{L}/\text{min}$ were observed when the HESI probe depth was set to the “A” position and the sheath and auxiliary gases were set to zero. It should be noted that the low-flow needle with 50 μm ID gives low backpressure compared to the column, and therefore mobile phases need to be properly degassed to avoid post-column outgassing. The parts required to reduce peak dispersion post-column are described in Appendix A, Table A1. In general, for all connections in the capLC-MS solution, using near-zero dead volume nanoViper fingertight fittings with 50 μm ID is recommended.

3.1.3. Sensitivity of capLC-MS using EASY-Spray and Ion Max source

The highest sensitivity and best peak shape for capLC-MS analysis were obtained using a 20 μm ID silica emitter EASY-Spray transfer line with the EASY-Spray source. As an alternative setup, the Ion Max source with a Thermo Scientific™ HESI probe and low-flow needle insert (50 μm ID, stainless steel) can be used for capLC-MS analysis. However, it should be noted that the latter setup is more suited to micro-flow rates and wider column IDs. The sensitivity of capLC-MS analysis with the recommended EASY-Spray transfer line with the 20 μm ID silica emitter and the EASY-Spray source is two to four times higher in comparison to optimized for low-flow rates HESI probe (Figure 4). This is probably related to the close proximity of the EASY-Spray emitter to the transfer capillary, which allows for better ion sampling.

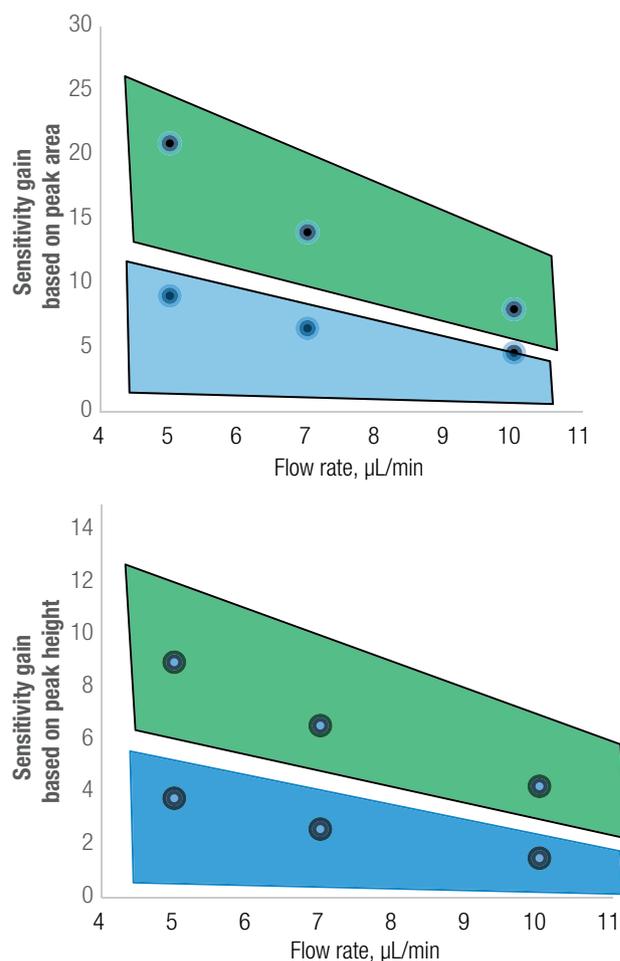


Figure 4. The relative sensitivity gain observed at different flow rates using the EASY-Spray transfer line with 20 μm ID silica emitter (green) and HESI-II probe with 50 μm ID stainless steel needle (blue) compared to analytical LC-MS at 450 $\mu\text{L}/\text{min}$.

The sensitivity gain was measured as the relative peak area or relative peak height of EDLIAYLK (m/z 482.77, charge +2) peptide from the cytochrome c tryptic digest with capillary-flow LC-MS compared to results obtained using analytical flow LC-MS. In all experiments, 1 pmol of cytochrome c digest (1 pmol/ μ L) was injected onto the column. CapLC-MS experiments were conducted using a Thermo Scientific™ Acclaim™ PepMap™ 100 C18 LC column (0.3 × 150 mm, 2 μ m) at flow rates 10, 7, and 5 μ L/min. Analytical flow experiments were carried out using a Thermo Scientific™ Vanquish™ Horizon UHPLC system with a 2.1 × 100 mm Thermo Scientific™ Accucore™ aQ C18 column (2.1 mm ID × 100 mm, 2.6 μ m) at a flow rate of 450 μ L/min. Both the capLC and analytical LC were coupled to a Thermo Scientific™ Q Exactive™ HF high-resolution, accurate-mass mass spectrometer. Data were acquired in Full MS mode.

3.2. Capillary-flow UHPLC system

The UltiMate 3000 RSLCnano system is a versatile platform that can be used for both routine and advanced low-flow LC-MS applications.⁸ The system can be configured to provide precise flow rates under gradient conditions for three recommended flow ranges: nano (50 nL/min to 1.5 μ L/min), capillary (0.5 to 10 μ L/min), and micro (5 to 50 μ L/min). The dedicated nano/capillary/micro flow meters can be easily interchanged by the user, and the system can be used for a wide range of low-flow applications with different sensitivity and throughput requirements. The NCS-3500RS pump module in the UltiMate 3000 RSLCnano system contains (i) a binary, low-flow high-pressure gradient mixing (HPG) pump with very low delay volume (< 25 nL), (ii) a ternary micro-flow low-pressure gradient mixing (LPG) pump capable of delivering flow rates up to 2500 μ L/min at 620 bar pressure, and (iii) a column compartment that provides high temperature stability (0.1 °C). The capLC system can be configured for direct injections or preconcentration

onto a trap cartridge (Appendix A, Table A3). The WPS-3000TPL RS autosampler provides precise injections and near-zero sample loss in μ L pick-up mode.

3.2.1. Direct injection onto analytical column

The capLC with direct injection is recommended for clean and desalted samples with injection volumes up to 5 μ L to avoid significant gradient delays due to the impact of the sample loop volume. Also, the direct injection configuration is useful for analysis of very hydrophilic analytes that are poorly retained on reversed-phase column packing materials.

The UltiMate 3000 RSLCnano capillary-flow bundle (Appendix A, Table A3) contains all consumables and columns required to configure a new capLC system for direct injection, i.e. injection of the sample onto the column. To convert the fluidics of an existing UltiMate 3000 RSLCnano system to capillary flow, a dedicated “Direct Injection Capillary LC Kit” (Table 1) that contains capillaries, an Acclaim PepMap C18 separation column, a 5 μ L sample loop, and cytochrome c digest is available. Additionally, the capillary-flow flow meter needs to be installed in the pump module (Appendix B, Table B1).

The setup comprises the connection of the low-flow HPG pump with the injection valve in the WPS-3000TPL RS autosampler (Figure 5). The injection valve is connected using a nanoViper capillary to the separation column that is positioned in the column compartment of the NCS-3500RS module (Figure 5).

The outlet of the separation column can be connected to an EASY-Spray or Ion Max source on Thermo Scientific MS instruments using an EASY-Spray transfer line or a nanoViper capillary (see Section 3.1). To check the capLC-MS solution, dedicated capLC-MS methods were developed and are described in Section 4.

Table 1. UltiMate 3000 RSLCnano Direct Injection Capillary LC Kit (P/N 6720.0305) contents.

Item	Replacement P/N	Location in Figure 5
300 μ m ID × 15 cm, packed with Acclaim PepMap RSLC C18, 2 μ m, 100 Å, nanoViper	164537	a
nanoViper capillary FS/PEEK sheathed ID × L 50 μ m × 750 mm	6041.5580	1
nanoViper capillary FS/PEEK sheathed ID × L 50 μ m × 550 mm	6041.5560	2
nanoViper sample loop 5 μ L, FS/PEEK sheathed ID × L 200 μ m × 159 mm	6826.2405	
Polypropylene vials for WPS with glass insert, 250 μ L, 100 pcs	6820.0027	
Polypropylene caps for WPS vials, 1000 pcs	6820.0028	
Cytochrome C digest, 1.6 nmol, lyophilized	161089	

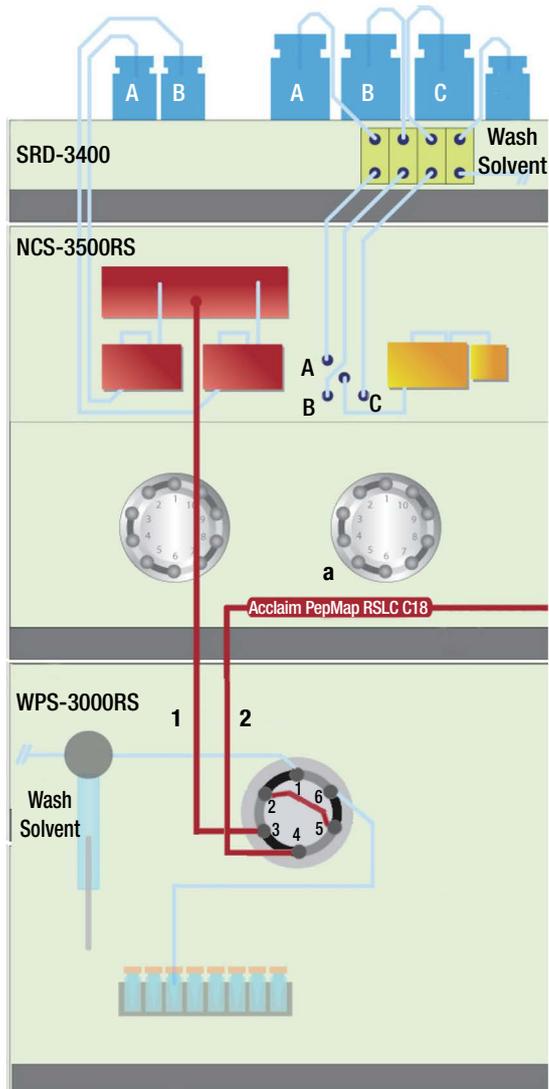


Figure 5. Fluidic connections for a direct injection experiment onto a capillary column. Details for numbered items in Table 1. Red: low-flow HPG pump and corresponding fluidic lines; Yellow: micro-flow LPG pump.

The general steps of direct injection are as follows:

1. The sample is loaded into the sample loop of the WPS-3000TPL RS autosampler with one of three preprogrammed injection modes: full loop injection for maximum precision, partial loop injection for sample volume accuracy, or the unique μL pick-up injection for near-zero sample loss. Additionally, user-defined programs are available for specific applications. During this step, the low-flow HPG pump bypasses the sample loop and delivers the flow directly to the separation column.

2. The injection valve is switched to the “inject” position. The flow from the low-flow HPG pump pushes the sample out of the loop onto the separation column and analytes are separated with a predefined gradient. After the run is finished, the process starts from the beginning.

3.2.2. Preconcentration onto trap cartridge

Preconcentration onto a trap cartridge is recommended for the analysis of large cohorts of complex samples that require clean up/salt removal prior to MS detection, concentrating samples and minimizing injection time for large sample volumes. This setup is often used to analyze peptides after enzymatic digestion of proteins. The typical injection mode for the preconcentration setup is μL pick-up into a 20 μL injection loop to minimize sample losses. The volume that is used to transfer sample onto the trap cartridge is usually below 100 μL to avoid breakthrough of hydrophilic peptides.

Preconcentration onto a trap cartridge (trap-and-elute setup) serves several functions:

- Concentrates sample
- Cleans up sample by removing salts and other non-retained compounds on reversed-phase material
- Acts as a guard column in forward-flush elution configuration
- Loads sample quickly onto the trap cartridge with the micro-flow loading pump integrated in the NCS-3500RS module
- Reduces the gradient delay volume by excluding the sample loop from the flow path after the sample has been transferred onto the trap cartridge

To configure a capLC system for preconcentration onto a trap cartridge, the additional low-dispersion switching valve needs to be installed in the column compartment of the NCS-3500RS module (Table 2) as well as dedicated fluidics (Table 3). The installation can be done in seconds using the tool-free, snap-in design for valves. NanoViper fingertight fittings allow for the setup of all required fluidics with ease.

Two main preconcentration fluidic setups exist: forward-flush and back-flush. These terms are used to indicate whether the mobile phase flows in the same or opposite direction during sample loading onto the trap compared to the mobile phase flow direction during gradient elution. Figure 6 shows the different fluidic setups for the forward- and back-flush fluidic paths.

It is generally believed that in back-flush elution the peaks are slightly sharper than in forward-flush elution. In contrast, the forward-flush setup of the trap cartridge prevents contamination of the separation column with any particulates from the sample. In this work, the back-flush setup was used for all preconcentration experiments.

The hardware layout and required fluidic connections for the preconcentration onto a trap cartridge setup are shown in Figure 7.

The general principle of trap-and-elute injections can be described in the following steps:

1. The sample is loaded into the sample loop of the WPS-3000TPL RS autosampler.
2. The injection valve is switched to the “inject” position and the sample is transferred onto the trap cartridge using the ternary micro-flow LPG loading pump. The prolonged sample loading (normally up to five volumes of the injection loop) can be used to simultaneously clean up sample and concentrate analytes onto the trap cartridge. During sample loading onto the trap cartridge, the low-flow HPG pump delivers flow to the separation column to equilibrate the column.
3. The low-dispersion valve in the column compartment switches the trap column in-line with the separation column and analytes are separated with a predefined gradient.

Table 2. Low-dispersion switching valve required for preconcentration onto trap column setup.

Item	Replacement P/N
Low-dispersion 2-position, 10-port valve	6041.0001

Note: Alternatively, low-dispersion 2-position, 6-port valve can be used for preconcentration setup (P/N 6041.0004).

Table 3. The UltiMate 3000 RSLCnano Preconcentration Capillary LC kit (P/N 6720.0315) contents.

Item	Replacement P/N	Location in Figure 7
300 µm ID x 15 cm, packed with Acclaim PepMap RSLC C18, 2 µm, 100 Å, nanoViper	164537	a
300 µm ID x 5 mm, packed with Acclaim PepMap100 C18, 5 µm, 100 Å (set of 5 cartridges)	160454	b
µ-Precolumn holder, 5 mm, with 30 µm ID connecting tubing, nanoViper fittings	164649	
nanoViper capillary FS/PEEK sheathed ID x L 50 µm x 350 mm	6041.5540	1
nanoViper capillary FS/PEEK sheathed ID x L 75 µm x 650 mm	6041.5775	2
nanoViper capillary FS/PEEK sheathed ID x L 75 µm x 550 mm	6041.5760	3
nanoViper capillary FS/PEEK sheathed ID x L 50 µm x 750 mm	6041.5580	
nanoViper sample loop 20 µL, FS/PEEK sheathed	6826.2420	
PTFE tubing, 500 µm ID, 100 cm, used as waste tubing	6720.0077	4
1/16" Universal fingertight fitting, one-piece design, extra-long thread, 4 pieces	6720.0015	
Polypropylene vials for WPS with glass insert, 250 µL, 100 pieces	6820.0027	
Polypropylene caps for WPS vials, 25 pieces	6820.0028	
Cytochrome c digest, 1.6 nmol, lyophilized	161089	

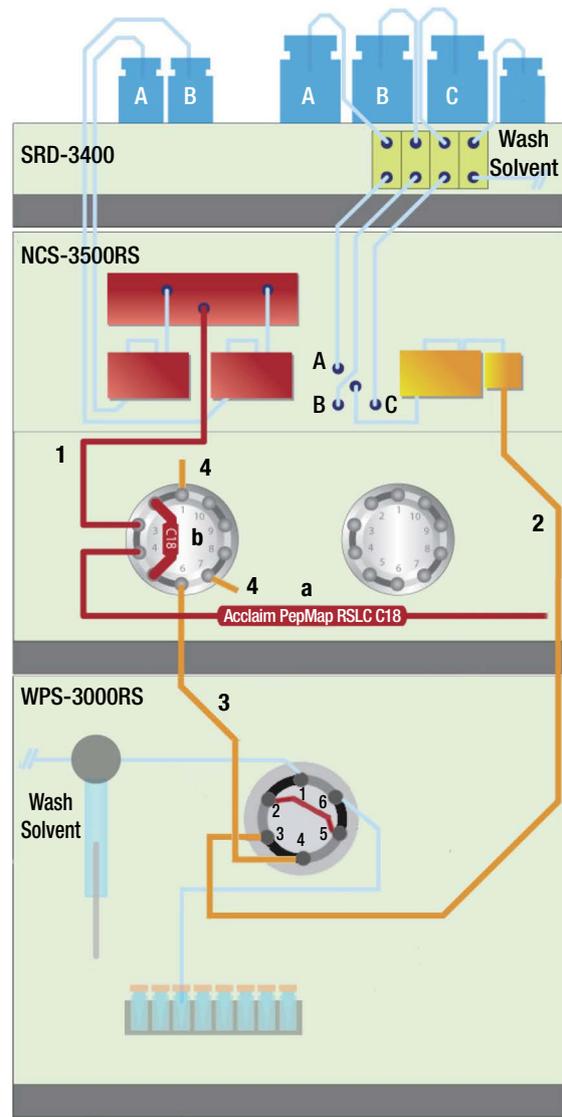
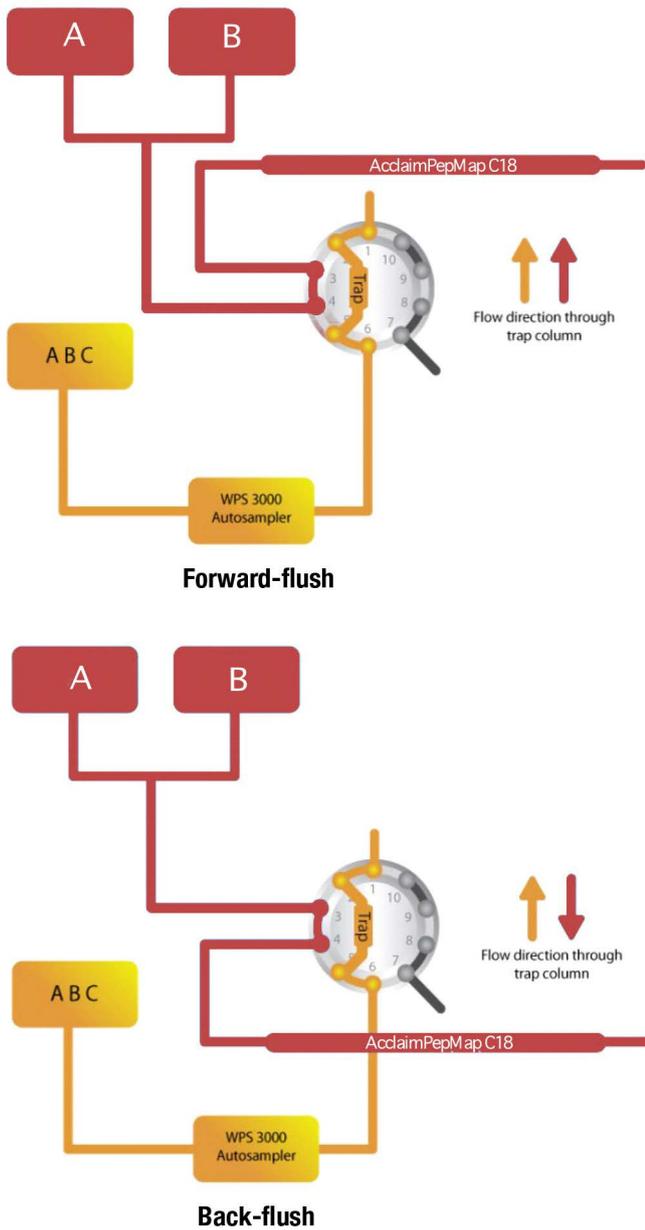


Figure 7. Fluidic connections for a preconcentration experiment onto a capillary column, hardware layout. Red: low-flow HPG pump and corresponding fluidic connections; Yellow: micro-flow LPG pump and corresponding fluidic connections.

Figure 6. Difference between forward-flush (top) and back-flush (bottom) configuration. Red: low-flow HPG pump and corresponding fluidic connections; Yellow: micro-flow LPG pump and corresponding fluidic connections.

3.3. Capillary separation columns and trap cartridges

All experiments with the capLC-MS solution were carried out using an Acclaim PepMap RSLC C18, 2 μm , 100 \AA separation column and an Acclaim PepMap trap cartridge. This setup is ideal for capLC-MS analysis of peptides because the silica-based Acclaim PepMap stationary phases have virtually no silanophilic activity, resulting in minimal peak tailing. The Acclaim PepMap columns can be operated with mobile phases without adding trifluoroacetic acid (TFA) and provide excellent peak shape, making them ideal for LC-MS based targeted and shotgun proteomics. Acclaim PepMap stationary phases can also be used for the analysis of small molecules, e.g. metabolites or pesticides. Additionally, a number of other fully porous and monolithic capillary columns and trap cartridges are available from Thermo Fisher Scientific for capLC-MS analysis (Appendix A, Tables A4 and A5). (Please also consult the current “Chromatography Columns and Consumables Catalog” at <https://www.thermofisher.com>)

4. CapLC-MS system suitability test

To verify the basic functionality and performance of the capLC-MS solution, two simple methods were developed. These methods reflect the two most common fluidic setups for capLC-MS solution: direct injection and preconcentration onto the trap cartridge (see details in Section 3.2).

4.1. Direct injection

The capillary-flow UltiMate 3000 RSLCnano system was configured in direct injection mode (see Section 3.2.1) with a Q Exactive HF MS. The separation column was coupled with an EASY-Spray source using an EASY-Spray transfer line (see Section 3.1.1). The details of the LC conditions and separation gradient are described in Table 4. The parameters of the ESI source and MS settings are shown in Table 5.

Representative total ion current chromatograms of cytochrome c protein digest are shown in Figure 8 and the characteristics of three peptide peaks are shown in Table 6.

Table 4. LC conditions for the direct injection cap-flow LC-MS system suitability test.

Parameters / Components	Settings / Details		
Column	Acclaim PepMap RSLC C18, (300 μm ID \times 15 cm, 2 μm , 100 \AA), nanoViper		
Mobile phase	A: 0.1 % formic acid (FA) in H_2O B: 0.1 % FA in acetonitrile		
Column temperature	40 $^\circ\text{C}$		
Injection mode / sample loop volume	Full loop / 5 μL sample loop		
Injection volume	5 μL		
Sample amount on column	1 pmol of cytochrome c digest (0.2 pmol/ μL)		
Separation gradient (low-flow HPG pump)	Time (min)	%B	Flow Rate ($\mu\text{L}/\text{min}$)
	0	5	5
	1.0	5	5
	11.0	35	5
	12.0	90	5
	12.1	90	10
	13.0	90	10
	13.1	5	10
	15.5	5	10
	15.6	5	5
16.0	5	5	
Loading conditions (micro LPG pump)	Not used		

Table 5. MS and source settings for the capLC-MS system suitability test.

Parameters / Components	Settings / Details
Source settings	
ESI source	EASY-Spray
Polarity	Positive
Ion transfer tube temperature	310 °C
Spray voltage positive ion	1750 V
MS settings	
MS instrument	Q Exactive HF
Acquisition mode	Full MS
Resolution	15,000
AGC target	3 × 10 ⁶
Maximum IT	20 ms
Scan range	350–1600 <i>m/z</i>

A good separation of major cytochrome c protein digest peptides was obtained within a 10 min gradient using capLC-MS in direct injection configuration. The flow rate ramping during the wash and equilibration step (Table 4) allows thorough and high speed washing of the analytical column and prevents carryover. Carryover from three selected peptides (Table 6) was not detected in consecutive blank injections of cytochrome c protein digest.

4.2. Preconcentration onto trap cartridge

The capillary-flow UltiMate 3000 RSLCnano system was configured for preconcentration onto a trap cartridge (see Section 3.2.2) with a Q Exactive HF MS. The separation column was coupled with an EASY-Spray source using an EASY-Spray transfer line (see Section 3.1.1). The details of the LC conditions and separation gradient are described in Table 7. The parameters of the ESI source and MS settings were the same as for the direct injection setup and are described in Table 5. In trap-and-elute experiments, the sample is transferred quickly onto the trap cartridge using the micro-flow loading pump. At the same time, the low-flow HPG pump continuously delivers mobile phase to the separation column and silica emitter. This prevents drying of the emitter in the EASY-Spray transfer line during the loading phase and ensures a longer lifetime compared to a preconcentration setup without flow through the emitter (vented setup). Preconcentration onto a trap cartridge decreases the gradient delay times by excluding the sample loop from the flow path. This results in faster gradient formation and shifts the eluted peaks to earlier retention times (compare Figure 8 and Figure 9). This setup allows rapid cleanup/desalting of the sample on the trap cartridge; however, some very hydrophilic analytes might break through the the

Table 6. Typical characteristics of peptide peaks from cytochrome c protein digest obtained with capLC-MS and direct injection.

Peptide	Monoisotopic Mass	<i>m/z</i>	Charge	Retention Time (min)	PWHM (s)	Asymmetry
IFVQK	633.385	634.392	1	6.7	4.8	1.0
MIFAGIK	778.441	779.448	1	9.6	3.6	1.2
EDLIAYLK	963.528	482.771	2	10.6	4.4	1.1

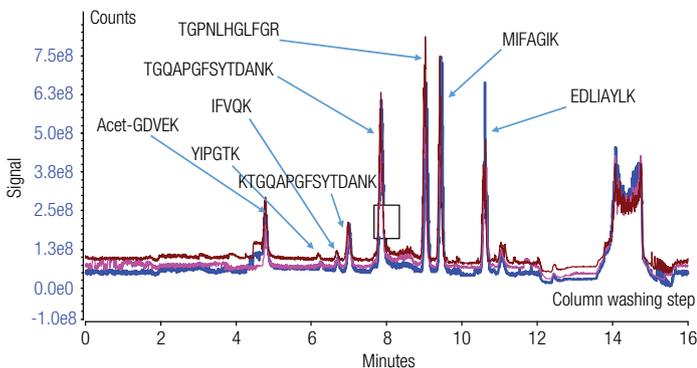


Figure 8. Overlay of three consecutive capLC-MS measurements with direct injection of 1 pmol cytochrome c protein digest. Total ion current (TIC) chromatograms are displayed with an offset between runs, and selected peaks are annotated with peptide sequences.

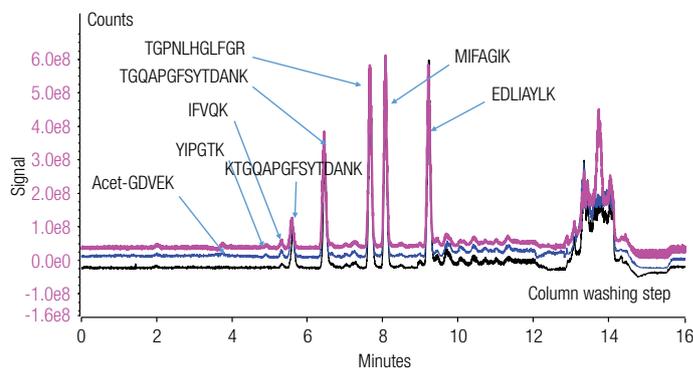


Figure 9. Overlay of three consecutive capLC-MS measurements with preconcentration onto the trap cartridge of 1 pmol cytochrome c protein digest. Total ion current (TIC) chromatograms are displayed with an offset between runs, and selected peaks are annotated with peptide sequences.

trapping cartridge due to their low retentivity. In order to improve the retentivity of hydrophilic analytes on the trap cartridge, mobile phase additives, e.g. trifluoroacetic acid, can be used.

Table 7. LC conditions for the preconcentration onto trap cartridge in the capLC-MS system suitability test.

Parameters / Components	Settings / Details		
Column	Acclaim PepMap RSLC C18, (300 µm ID × 15 cm, 2 µm, 100 Å), nanoViper		
Mobile phase	A: 0.1 % FA in H ₂ O B: 0.1 % FA in acetonitrile		
Column temperature	40 °C		
Injection mode / sample loop volume	µL pick-up / 20 µL sample loop		
Injection volume	5 µL		
Sample amount on column	1 pmol of cytochrome c digest (0.2 pmol/µL)		
Separation gradient (low-flow HPG pump)	Time (min)	%B	Flow Rate (µL/min)
	0	5	5
	1.0	5	5
	11.0	35	5
	12.0	90	5
	12.1	90	10
	13.0	90	10
	13.1	5	10
	15.5	5	10
	15.6	5	5
16.0	5	5	
Mobile phase (micro LPG pump)	A: 0.1 % FA in H ₂ O		
Loading conditions (micro LPG pump)	Time (min)	%A	Flow Rate (µL/min)
	0	100	100
	0.5	100	100
	0.51	100	10
	15.0	100	10
	15.1	100	100
	16.0	100	100
Switching program for low-dispersion valve with installed trap cartridge:	Time (min)	Valve Position	
Position 1-2: trap cartridge is in-line with micro loading pump	0	1-2	
	0.25	10-1	
Position 10-1: trap cartridge is in-line with low-flow HPG pump and separation column	15.25	1-2	

The peak characteristics for cytochrome c peptides were similar for direct injection and preconcentration onto trap cartridge setups (Tables 6 and 8). Thus, the choice of preferred capLC-MS configuration should be made on a case-by-case basis considering the quality of samples, analyte properties, and throughput requirements bearing in mind that sample loading onto a trap cartridge using a micro-flow pump can be done in less than a minute.

5. Robustness of capLC-MS analysis

To check the robustness of the capLC-MS solution, the stability of retention times and MS signal were evaluated over an extended injection sequence. The capLC system

was configured for preconcentration onto a trap cartridge as described in Section 3.2.2. The capLC system was coupled to a Thermo Scientific™ TSQ Quantiva™ MS.

A low concentration of cytochrome c digest was used to evaluate retention time and peak area stability. Four peptides eluting throughout the gradient were detected by selected-reaction monitoring (SRM). The peptide sequences and transitions used for quantification are given in Table 9. For confirmation, one additional transition was used per peptide. The parameters of the ESI source and MS settings are shown in Table 10.

Table 8. Typical characteristics of peptide peaks from cytochrome c protein digest obtained with capLC-MS solution configured for preconcentration onto trap cartridge injections.

Peptide	Monoisotopic Mass (Da)	m/z	Charge	Retention Time (min)	PWHM (s)	Asymmetry
IFVQK	633.385	634.392	1	5.3	4.6	1.0
MIFAGIK	778.441	779.448	1	8.0	4.0	1.1
EDLIAYLK	963.528	482.771	2	9.1	4.8	1.1

Table 9. MRM settings for quantification of four peptides from cytochrome c digest.

Peptide	MS1	Collision Energy (V)	MS2, Quantification Ion	MS2, Conformation Ion
IFVQK	317.7	12.4	374.2	521.3
TGQAPGFSYTDANK	728.8	24.8	1099.5	945.4
MIFAGIK	390.2	14.6	535.3	648.4
EDLIAYLK	482.8	20.4	494.3	607.3

Table 10. MS and source settings for the capLC-MS system robustness testing.

Parameters / Components	Settings / Details
Source settings	
ESI source	EASY-Spray
Polarity	Positive
Ion transfer tube temperature	275 °C
Spray voltage positive ion	1750 V
MS settings	
MS instrument	TSQ Quantiva MS
Acquisition mode	MRM
Cycle time	1 s
Chrom filter	3.0 s
Collision gas pressure	1.5 mTorr
Q1 resolution (FWHM)	0.7
Q2 resolution (FWHM)	0.7
Source fragmentation	0 V

To determine the retention reproducibility with the capLC-MS setup, 500 injections of cytochrome c were acquired. The retention time stability over six days of operation is shown in Figure 10. The RSD values of retention time were $\leq 1\%$ over 500 injections, indicating excellent run-to-run repeatability for capLC-MS measurements.

In addition, the peak area reproducibility for the capLC-MS setup in complex matrix analysis was investigated. Therefore, cytochrome c digest, was spiked into *E. Coli* digest and 100 consecutive measurements were carried out. During each injection, 500 ng of *E. Coli* digest and 50 fmol of cytochrome c digest were loaded onto the column. The peak area precision for four peptides over 100 injections is shown in Figure 11. A peak area precision of approximately 5% RSD at very low levels was achieved even without using an internal standard to correct for MS signal variability.

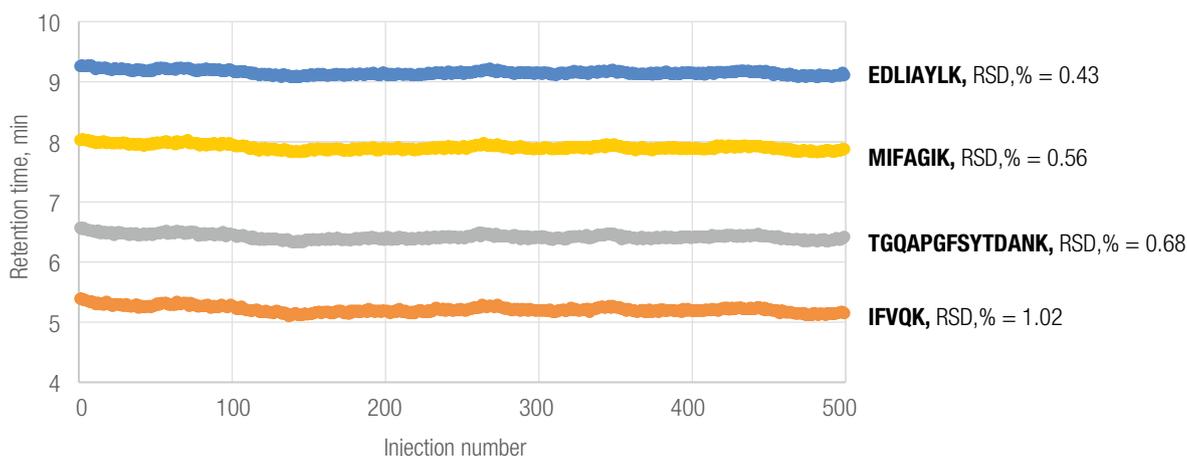


Figure 10. Retention time stability with targeted capLC-MS analysis of peptides in MRM mode. Note: performance may vary depending on matrix and analyte properties.

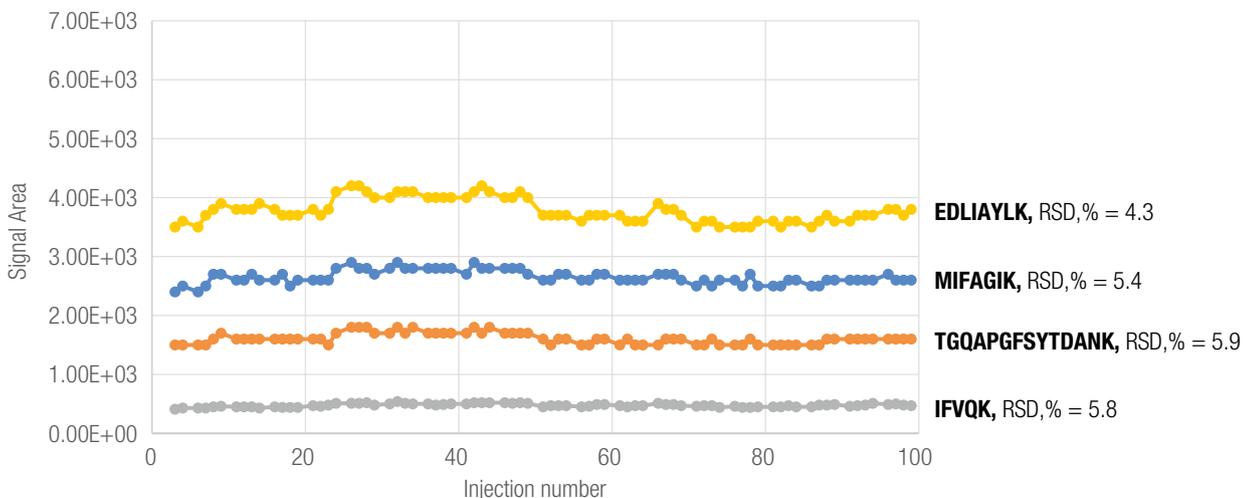


Figure 11. Peak area stability with targeted capLC-MS analysis of peptides in a complex matrix. Note: performance may vary depending on matrix and analyte properties.

6. Proof-of-principle capLC-MS studies

6.1. Targeted protein quantification

The capLC-MS solution has attracted increased interest for bioanalytical applications, where high sensitivity and high throughput are crucial factors. Thus, as a proof-of-principle study, a method for targeted capLC-MS quantification of infliximab with a Q Exactive HF high-resolution, accurate-mass MS was developed. The separation method consisted of fast sample loading onto the trap column (< 30 s) using the integrated micro

loading pump and peptide separation with the low-flow HPG pump (Table 11).⁹ At the end of the gradient, the trap and analytical columns were thoroughly washed at an increased flow rate (10 $\mu\text{L}/\text{min}$). Three unique peptides DILLTQSPAILSVPGER, SINSATHYAESVK, and DSTYLSSTLTLSK were selected for parallel reaction monitoring (PRM) quantification of infliximab and showed good ESI MS response. MS settings used for PRM quantification are shown in Table 12.

Table 11. CapLC conditions for targeted analysis of infliximab.

Parameters / Components	Settings / Details		
Column	Acclaim PepMap RSLC C18, (300 μm ID \times 15 cm, 2 μm , 100 \AA), nanoViper		
Mobile phase (low-flow HPG pump)	A: 0.1 % FA in H_2O B: 0.1 % FA in acetonitrile		
Column temperature	40 $^\circ\text{C}$		
Injection mode / sample loop volume / inject volume	μL pick-up / 20 μL sample loop / 5 μL		
	Time (min)	%B	Flow Rate ($\mu\text{L}/\text{min}$)
	0	10	5
	0.25	10	5
	7.0	35	5
Separation gradient (low-flow HPG pump)	7.0	35	10
	7.5	60	10
	7.5	99	10
	10.5	99	10
	10.5	10	10
	12.0	10	10
Mobile phase (micro LPG pump)	A: 0.1 % FA in H_2O		
	Time (min)	%A	Flow Rate ($\mu\text{L}/\text{min}$)
Loading conditions (micro LPG pump)	0	100	100
	1	100	100
	1	100	10
	12	100	10
Switching program for the low-dispersion valve holding the trap cartridge	Time (min)	Valve Position	
Position 1-2: trap cartridge is in-line with micro loading pump	0	1-2	
Position 6-1: trap cartridge is in-line with HPG pump and separation column*	1	6-1	

* 2-position, 6-port valve low dispersion valve was used in this setup (P/N 6041.0004)

Table 12. MS and source settings for capLC-MS targeted analysis of infliximab.

Parameters / Components	Settings / Details
Source settings	
ESI source	EASY-Spray
Polarity	Positive
Ion transfer tube temperature	250 °C
Spray voltage positive ion	1700 V
MS settings	
MS instrument	Q Exactive HF
Acquisition mode	PRM and Full MS SIM
PRM settings	
Resolution	60,000
AGC target	2e5
Maximum IT	150 ms
MSX count	1
Isolation window	2 <i>m/z</i>
NCE	30
Inclusion list	
SINSATHYAESVK	469.60 <i>m/z</i> , charge 3
DILLTQSPAILSVPGER	632.70 <i>m/z</i> , charge 3
DSTYLSSTLTLSK	751.88 <i>m/z</i> , charge 2
Full MS - SIM	
Resolution	120,000
AGC target	3e6
Maximum IT	50 ms
Scan range	375-1500 <i>m/z</i>

The highest sensitivity was observed for DILLTQSPAILSVPGER (*m/z* 632.68, charge +3). The quantification was done using the *y*7 ion of DILLTQSPAILSVPGER with *m/z* 731.38. The lowest calibration point and LOQ was 16 amol and the linearity range covered five orders of magnitude in “pure” solutions of infliximab digest (Figure 12). In the spiked HeLa digest (500 ng), the lowest detectable amount (LOD) of infliximab was approximately 3 fmol.

6.2. Profiling of proteomics samples

The capLC-MS solution is an ideal technique for routine and robust proteome profiling of complex samples, especially when large sample cohorts should be analyzed and the available sample amount is not strictly

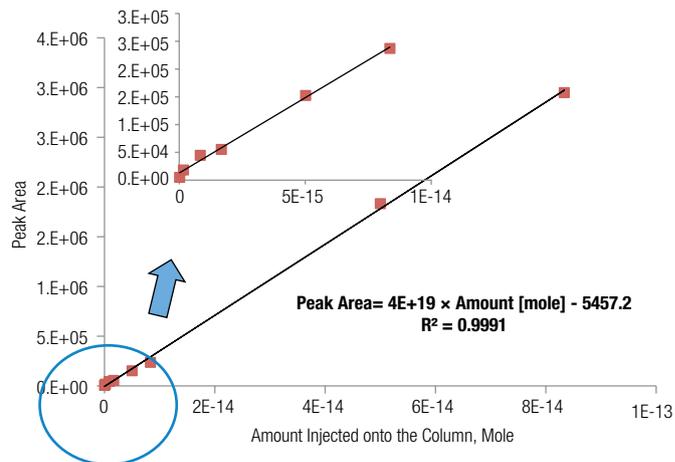


Figure 12. The calibration plot for PRM quantification of infliximab based on DILLTQSPAILSVPGER peptide in “pure” solution of digested protein.

limited. To evaluate the performance of the capLC-MS solution for shotgun proteomics with data dependent acquisition (DDA), HeLa cell lysate digest was analyzed with nano- and capLC-MS solutions.

In Table 13, a capLC-MS gradient for HeLa cell lysate profiling is given. MS conditions for shotgun proteomics experiments in DDA mode are described in Table 14.

The obtained base peak chromatograms (BPCs) confirmed that sensitivity for capLC-MS at flow rate 5 $\mu\text{L}/\text{min}$ is 5 to 30 times higher than analytical flow LC-MS analysis and 5 to 10 times lower in comparison with nanoLC-MS (Figure 13), when the same sample amount of 1 μg is injected onto the column.

However, it should be noted that a 300 μm ID column has around 15 times higher sample capacity in comparison to a conventional nanoLC column with 75 μm ID and the same length. The increase of loadable sample cannot completely eliminate the difference in results between nano- and capLC-MS, but can significantly reduce it. The number of identified proteins with capLC-MS solution reached ~70% of nanoLC-MS results with the same loading amount of 1 μg HeLa digest and ~80% with four times greater amount of HeLa digest loaded onto the column. A similar trend was also observed for peptides identified with capLC-MS (Figure 14).

Table 13. LC conditions for capLC-MS shotgun proteomics (direct injection).

Parameters / Components	Settings /Details																														
Column	Acclaim PepMap RSLC C18, (300 μm ID × 15 cm, 2 μm, 100 Å), nanoViper																														
Mobile phase	A: 0.1 % FA in H ₂ O B: 0.1 % FA in acetonitrile																														
Column temperature	40 °C																														
Injection mode / sample loop volume	μL pick-up / 20 μL sample loop																														
Separation gradient (HPG pump)	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>%B</th> <th>Flow Rate (μL/min)</th> </tr> </thead> <tbody> <tr><td>0</td><td>5</td><td>5</td></tr> <tr><td>4</td><td>5</td><td>5</td></tr> <tr><td>44</td><td>25</td><td>5</td></tr> <tr><td>48</td><td>35</td><td>5</td></tr> <tr><td>52</td><td>60</td><td>5</td></tr> <tr><td>54</td><td>99</td><td>5</td></tr> <tr><td>56</td><td>99</td><td>5</td></tr> <tr><td>56</td><td>5</td><td>5</td></tr> <tr><td>60</td><td>5</td><td>5</td></tr> </tbody> </table>	Time (min)	%B	Flow Rate (μL/min)	0	5	5	4	5	5	44	25	5	48	35	5	52	60	5	54	99	5	56	99	5	56	5	5	60	5	5
	Time (min)	%B	Flow Rate (μL/min)																												
	0	5	5																												
	4	5	5																												
	44	25	5																												
	48	35	5																												
	52	60	5																												
	54	99	5																												
56	99	5																													
56	5	5																													
60	5	5																													
Switching program for "Injection Valve"*	<table border="1"> <thead> <tr> <th>Time (min)</th> <th>Valve Position</th> </tr> </thead> <tbody> <tr><td>0</td><td>Inject</td></tr> <tr><td>4</td><td>Loading</td></tr> </tbody> </table>	Time (min)	Valve Position	0	Inject	4	Loading																								
	Time (min)	Valve Position																													
	0	Inject																													
4	Loading																														
Loading conditions (micro LPG pump)	Not used																														

* To switch the Injection valve to the loading position after the sample has been transferred onto the column, the command "Sampler.InjectValveToLoad" should be added in the script editor of the LC program at the corresponding time point. This command will switch the sample loop out of the flow path.

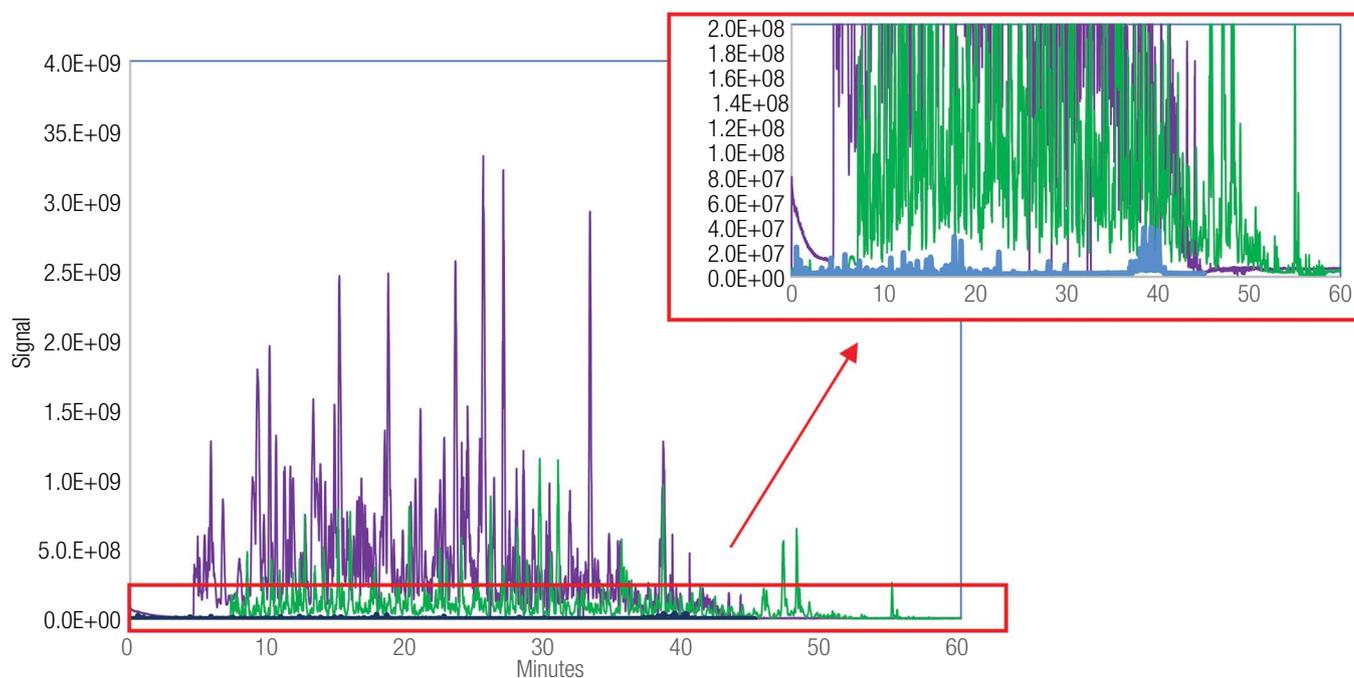


Figure 13. Base peak chromatograms of HeLa cell lysate digest with capillary (green), nano (purple), and analytical flow (blue) LC-MS setups

Table 14. MS and source settings for capLC-MS shotgun proteomics experiments.

Parameters / Components	Settings / Details
Source settings	
ESI source	EASY-Spray
Polarity	Positive
Ion transfer tube temperature	250 °C
Spray voltage positive ion	1800 V
MS settings	
MS instrument	Q Exactive HF
Acquisition mode	Full MS / DDA
Full MS	
Resolution	60,000
AGC target	3e6
Maximum IT	50 ms
Scan range	350–1500 <i>m/z</i>
DDA	
Resolution	15,000
AGC target	2e5
Maximum IT	50 ms
TopN	20
Isolation window	1.4 <i>m/z</i>
Fixed first mass	100 <i>m/z</i>
NCE	27
AGC target	1e3
Charge exclusion	Unassigned, 1, >8
Peptide match	Preferred
Dynamic exclusion	10 s

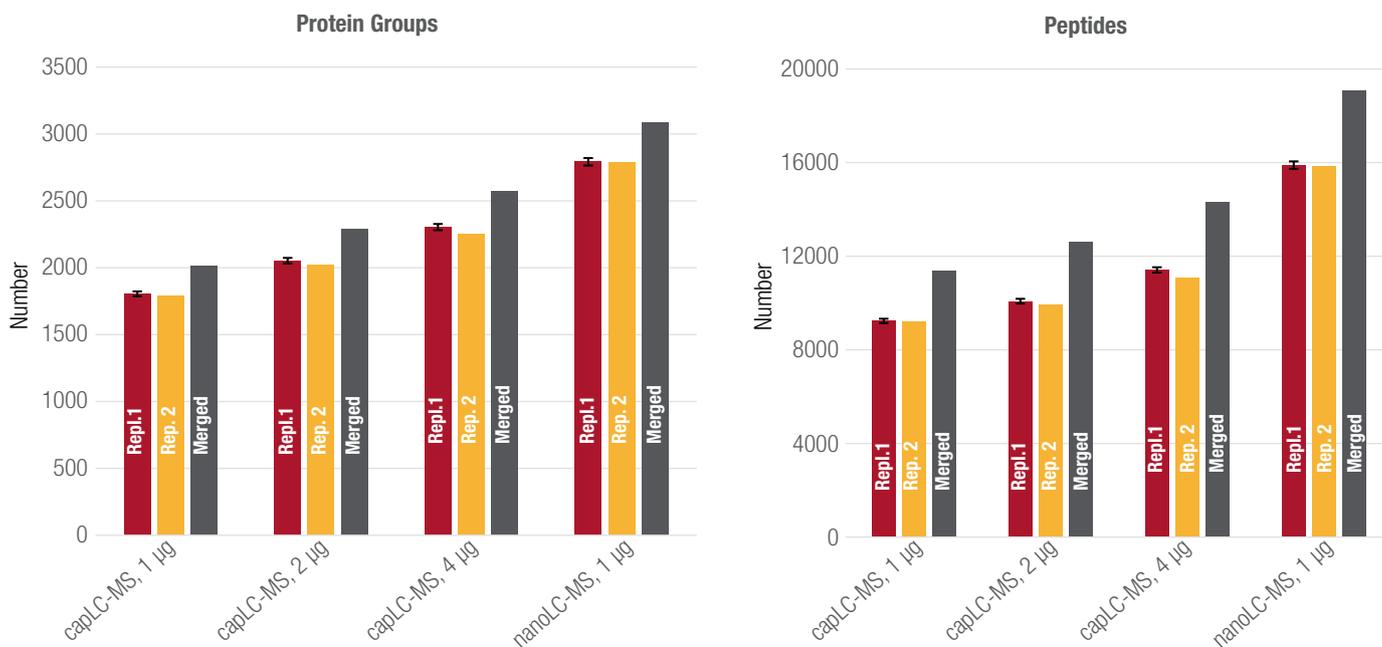


Figure 14. The number of protein groups and peptides identified with nano- and capLC-MS using 60 min total analysis time and 15 cm long separation columns. Repl. stands for replicate. NanoLC-MS: 0.075 × 150 mm, Acclaim PepMap C18, 2 µm column. CapLC-MS: 0.3 × 150 mm Acclaim PepMap C18, 2 µm column.

7. Conclusions

The Thermo Scientific capillary-flow LC-MS solution (consisting of an UltiMate 3000 RSLCnano system, a plug-and-spray EASY-spray ion source, and a transfer line with emitter), a diverse column portfolio, and high-performance MS instrumentation combines the advantages of high-throughput analytical flow LC separations and the high sensitivity of nano-flow LC-MS analysis. This is a robust solution that can be used for routine experiments and analysis of large sample cohorts. Standardized fluidic configurations for direct injection and preconcentration onto a trap cartridge (trap-and-elute) allows for development of capLC-MS methods for a variety of different applications and sample types. CapLC-MS has been shown to be a valuable tool for targeted peptide quantification as well as bottom-up proteomics. Additionally, the described hardware configurations can also be used for small molecule analysis, e.g. metabolomics, or intact protein separation on monolithic or wide porous columns.

The following technological advances make the proposed capLC-MS solution well suited for robust, sensitive, and high-throughput analysis:

- The EASY-Spray transfer line allows the coupling of the capLC system with the MS in seconds without using any tools.
- Flow path containing all nanoViper fingertight fittings minimizes dead volumes and leakages.
- Fingertight nanoViper fittings allow the installation of any capLC configuration within minutes.
- The integrated micro-flow pump allows users to perform preconcentration experiments without any flow interruption at the emitter. This improves reproducibility of capLC-MS results.

- Fast sample loading with the integrated micro-flow pump and column washing at increased flow rates allow for high-throughput separations comparable to analytical flow LC methods.
- High gradient precision of capLC provides high retention time repeatability that is essential for targeted scheduled MS methods.
- The absolute sensitivity of capLC-MS is 5 to 30 times higher at 5 $\mu\text{L}/\text{min}$ than the sensitivity of analytical flow LC-MS analysis.
- The increased sample capacity of capillary columns allows for high protein and peptide identification rates in short analysis times.

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Appendix A

Parts required to set up a capillary-flow UltiMate 3000 RSLCnano system

Table A1. Ion Max or Ion Max NG source with HESI-II probe and low-flow needle insert interface for capLC-MS (alternative solution).

Product	P/N
Capillary from the column to the grounding union 50 µm × 350 mm; nanoViper capillary*	6041.5540
Connection between grounding union and low-flow needle insert 65 µm × 150 mm ID; Viper PEEK capillary	6041.5615
	OPTON-20523 (for first generation sources, e.g. Q Exactive series)
Low-flow metal needle (50 µm ID) for HESI-II probe in Ion Max or Ion Max NG source	OPTON-30139 for NG sources (Thermo Scientific™ Orbitrap Fusion™ Tribrid™ MS, TSQ Quantiva MS, Thermo Scientific™ TSQ Endura™ MS)**

* The capillary length depends on the actual LC and MS position; longer nanoViper capillary can be used to connect the column outlet and grounding union of the MS instrument if needed (e.g. 50 µm ID × 550 mm P/N 6041.5560).

** OPTON-30138 with low-flow needle and 65 µm × 15 cm connection between grounding union and needle can be ordered alternatively.

Table A2. EASY-Spray ESI interface for capLC-MS (recommended solution).

Product	P/N
EASY-Spray emitter, (silica emitter ID 20 µm, transfer line 75 µm ID × 500 mm with nanoViper fittings)	ES792
	ES081 (for first generation sources, e.g. Exactive series)
EASY-Spray source	ES082 for NG sources (Orbitrap Fusion MS and Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ MS, TSQ Quantiva MS, TSQ Endura MS)

Table A3. UltiMate 3000 RSLCnano capillary-flow bundle (P/N 5200.0356) contents.

Item	Replacement P/N
SRD-3400, Solvent Rack with Four Degasser Channels	5035.9245
NCS-3500RS CAP, LC Pump with Capillary-Flow Meter and Column Compartment	5041.0020
WPS-3000TPL RS, Thermostatted Pulled-loop Well Plate Autosampler	5826.0020
Low dispersion, 2-position, 10-port valve	6041.0001
Preconcentration Capillary LC Kit*	6720.0315
Signal-cable, 6-Pin Mini DIN, 5 m	6000.1004
Capillary tubing to connect LC and MS	6041.5294
Capillary tubing to connect LC and MS nanoViper capillary, 50 µm ID × 550 mm	6041.5295
Sample loop, 5 µL	6041.5560
Cytochrome C digest	6826.2405
Instruction manual SRD solvent rack	161089
Instruction manual NCS-3x00 pump	4820.3550
Instruction manual WPS autosampler	4820.4101
	4828.2050

* See content of the Kit in Table 2.

Table A4. Linear capillary separation columns for capLC-MS solution.

Stationary Phase	ID (µm)	Length (cm)	Particle Size (µm)	P/N
Acclaim PepMap	300	5	2	164560
		15		164537
Thermo Scientific™ Acclaim™ PolarAdvantage II (PA2)	300	15	3	164592
Thermo Scientific™ Hypersil GOLD™	300	15	3	25003-150365
Hypersil Gold AQ	300	10	1.9	25302-100365
Hypersil Gold PFP	300	10	3	25403-100365
Thermo Scientific™ Hypercarb™	320	10	3	35003-100365
		15		35003-150365
Thermo Scientific™ PepSwift™	200	5	NA	164557
		25		164542
Thermo Scientific™ ProSwift™ RP-4H	200	25	NA	164923
ProSwift C4 RP-5H	200	25	NA	164930

Table A5. Trap cartridges and trap column holders available for capLC-MS solution.

Product				P/N
Trap Holders				
μ-Precolumn holder, 5 mm, with 30 μm ID connecting tubing, nanoViper fittings				164469
μ-Precolumn holder, 5 mm, with 75 μm ID connecting tubing, nanoViper fittings				164650
Connecting Tubing				
Set of 2 connecting tubing with nanoViper fittings, 30 μm ID x 10 cm 1/32"				164648
Trap Cartridges*				
Stationary phase	ID (μm)	Length (mm)	Particle size (μm)	P/N
Acclaim PepMap C18, 100 Å	320	5	5	160454
	1000	5	5	160434
		15	5	160438
Acclaim PepMap C8, 100 Å	300	5	5	161194
Acclaim PepMap C4, 300 Å	300	5	5	163591
Acclaim PepMap C18, 300 Å	300	5	5	163589
Accucore 150-C18	300	5	5	16126-900379
Accucore 150-C4	300	5	5	16526-900379
Accucore 150-Amide-HILIC	300	5	5	16726-900379

* Set of 5 cartridges

Appendix B

Parts required to upgrade a nano-flow UltiMate 3000 RSLCnano to a capillary-flow system

These parts are sufficient to configure an LC system for direct injections and for preconcentration onto the trap cartridge.

Table B1. Recommended capLC-MS solution with EASY-Spray transfer line.

Product	P/N
Capillary-flow meter for NCS-3500RS	6041.7902A
Preconcentration capillary LC kit*	6720.0315
Low dispersion 2P-10P valve	6041.0001
nanoViper capillary FS/PEEK sheathed ID x L 50 μm x 550 mm	6041.5560
nanoViper sample loop 5 μL, FS/PEEK sheathed ID x L 200 μm x 159 mm	6826.2405
EASY-Spray emitter, (silica emitter ID 20 μm, transfer line 75 μm ID x 500 mm with nanoViper fittings)	ES792
EASY-Spray source	ES081 (for first generation sources, e.g. Exactive series)
	ES082 for NG sources (Orbitrap Fusion MS and Orbitrap Fusion Lumos MS, TSQ Quantiva MS, TSQ Endura MS)

* Includes 1 analytical column (Acclaim PepMap C18, 300 μm ID x 15 cm); 1 trap column cartridges holder and 5 trap cartridges (Acclaim PepMap, 300 μm ID x 5 mm).

Table B2. Alternative capLC-MS solution with low-flow metal needle for HESI-II probe in Ion Max or Ion Max NG source.*

Product	P/N
Capillary-flow meter for NCS-3500RS	6041.7902A
Preconcentration capillary LC kit**	6720.0315
Low dispersion 2P-10P valve	6041.0001
nanoViper capillary FS/PEEK sheathed ID × L 50 µm × 550 mm	6041.5560
nanoViper sample loop 5 µL, FS/PEEK sheathed ID × L 200 µm × 159 mm	6826.2405
Capillary from the column to the grounding union 350 mm × 50 µm ID; nanoViper capillary***	6041.5540
Connection between grounding union and low-flow Needle Insert 150 mm × 65 µm ID; Viper PEEK capillary	6041.5615
Low-flow needle (50 µm ID) for HESI-II probe in Ion Max or Ion Max NG source	OPTON-20523 (for first generation sources, e.g. Exactive series) OPTON-30139 for new generation sources (Orbitrap Fusion MS and Orbitrap Fusion Lumos MS, TSQ Quantiva MS, TSQ Endura MS)

* Ion Max or Ion Max NG source with HESI-II probe for analytical flow is always a part of MS order.

** Includes 1 analytical column (Acclaim PepMap C18, 300 µm ID × 15 cm); 1 trap column cartridges holder and 5 trap cartridges (Acclaim PepMap, 300 µm ID × 5 mm).

*** The length of the capillary depends on the actual LC and MS positioning, a longer nanoViper capillary can be used to connect the column outlet and grounding union of the MS instrument if needed (e.g. 50 µm ID x 550 mm, P/N 6041.5560).

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