Improving Protein Identification on a Microfluidic HPLC-MS Chip

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Introduction

The analysis and identification of complex peptide mixtures is the domain of nano-LC/MS as this technology can handle smallest sample amounts and delivers superior MS sensitivity. An optimized chromatographic system, including minimal delay and dispersive volume, high sample enrichment capacity and high performance separation columns are contributing to the number of identified peptides and proteins. HPLC-chip technology allows minimizing the delay and dispersive volume, due to the integration of the enrichment column, separation column and nanospray emitter in a single microfluidic structure (Figure 1). In this study we investigate the importance of the column geometry , column packing density and column packing material on the chromatographic performance of a microfluidic HPLC/MS chip.

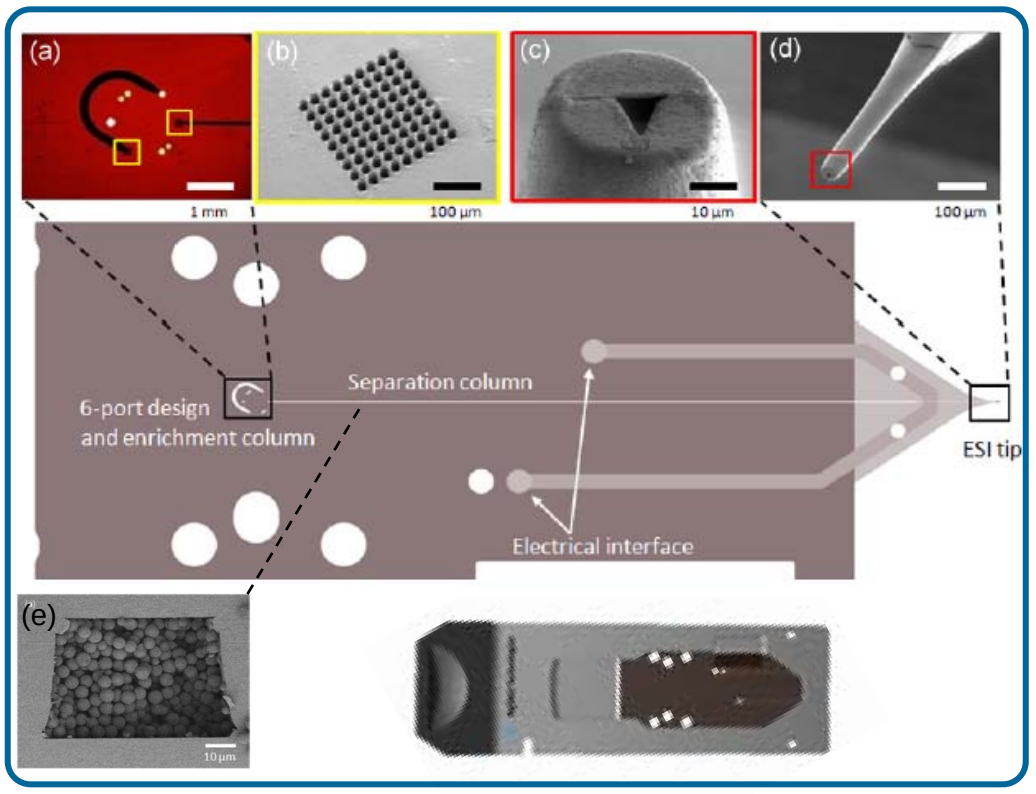

Figure 1: HPLC-chip: (a)enrichment column, (b) filter, frit, (c) tip channel (d)spray tip, (e) channel cross-section.

To systematically investigate the column contribution, HPLC-chips were compared to fused silica packed capillary columns and the impact of channel geometry under multiple packing conditions compared to theoretical values. In order to reduce extra column band broadening, a 5 nL loop (including port volume) for isocratic experiments was integrated into the chip as well as on chip UV detection (post-column volume 2.5 nL) was employed. Additional experiments were performed with standard chips (gradient elution) with 40 nL trap and various stationary phases in order to investigate the loading capacity as a function of the packing material used.

Experimental

Setup for measuring chromatographic performance using on-chip UV detection

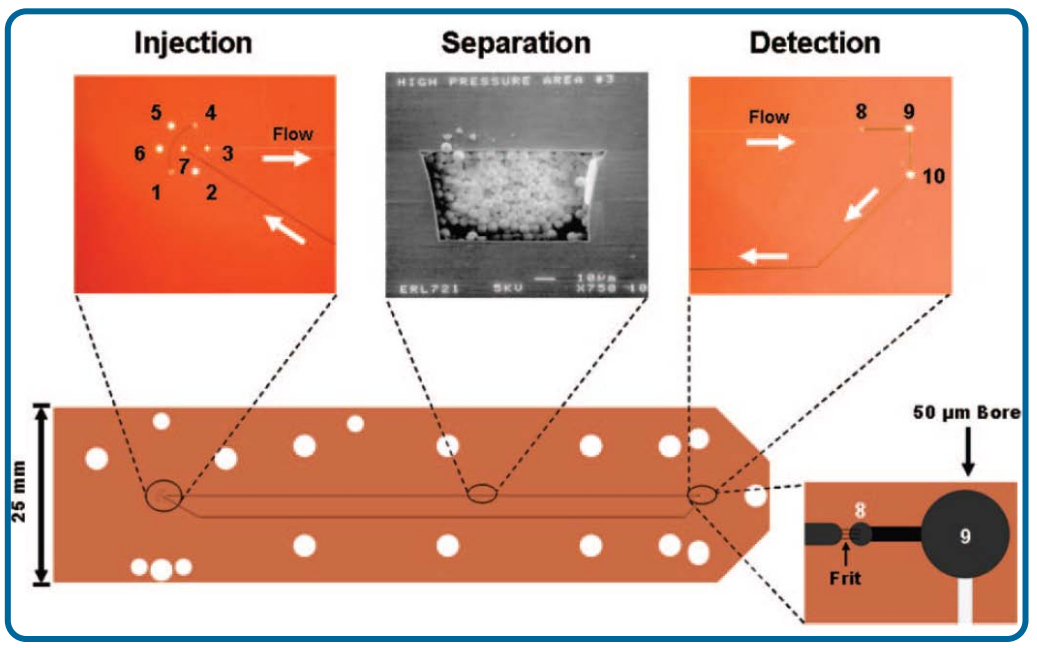

Figure 2: Layout of a chip with fixed loop injection and on-chip UV detection. Sample loop between ports 1 and 4: 0.7 nL. Postcolumn volume between ports 8 and 9: 2.5 nL. Empty column volume between port 3 and 8: 260 nL. (75x50 um x 75 mm.); Detection cell: 50 um ID x 300 um length.

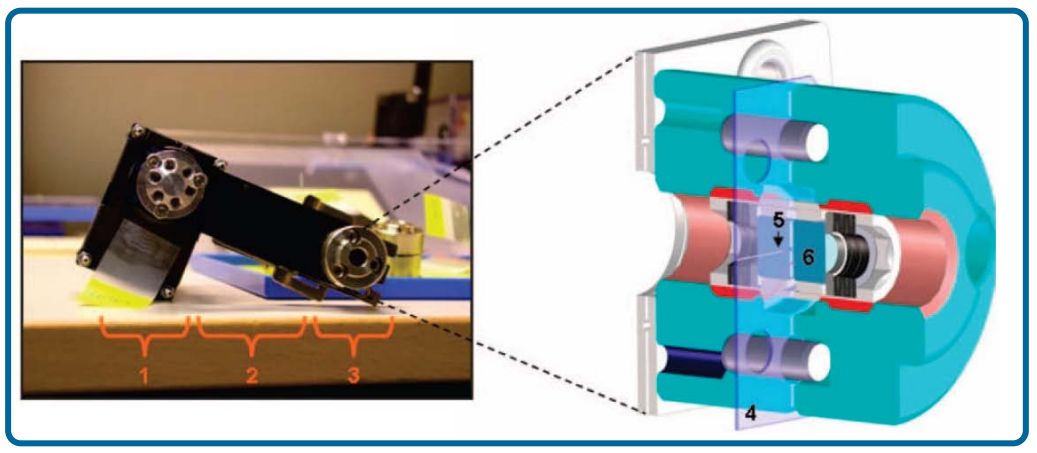

Figure 3: Chip UV setup: 1: sample injector, 2: separation, 3: Detector interface, 4: chip, 5: separation channel, 6: sapphire window.

Chip packing procedure
50 mg. (5um) or 75 mg. (3.5um) of dry particles (Zorbax SB C18) were prepared in 1 mL THF. Push solvent used was MeOH. Chips were packed in a holder at different pressures with and without the addition of ultrasonication.

Method 1: Determination of interstitial porosity ϵ_{inter}
Packing porosity was determined by inverse size exclusion chromatography. Small polystyrene spheres in Methylene Chloride, that were excluded from the pores using their measured elution volume. Detection with UV was done at 230 nm.

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Method 2: Determination of specific Darcy permeability k_D
Darcy's Law describes the resistance to flow in a packed bed.

$$u_{sf} = \frac{F_v}{A} = \frac{\kappa_D \Delta p}{\eta L_{bed}}$$

u_{sf} is the superficial velocity which is proportional to the volumetric Flow Fv over the cross-sectional area A. The viscosity η under the experimental conditions (80:20 ACN/water could be calculated. By measuring ΔP as a function of velocity, k_D can be determined.

Method 3: Determination of separation efficiency using isocratic elution chromatography UV
A mixture of alkylbenzenes (benzene, ethyl-benzene, propyl-benzene, butyl-benzene and pentyl-benzene) were separated using an 80:20 ACN/water mixture. Uracil was used as the t_0 marker. Flow rates were set using an Agilent nano-pump and additionally measured using an external flow sensor located at the exit of port 7. Peak-widths were measured using Chemstation SW.

Method 4: Determination of separation efficiency using isocratic elution chromatography UV and MS (fixed loop)
A mixture of phenones (pentano-, hexano-, heptano- and octano-phenone) were separated using a varying 50:50 ACN/water mixture with 0.1 % TFA. MS measurements were performed with HPLC-chip cube. As the temperature in the MS experiments was higher (38 C) as compared to the UV setup, linear velocity values are shifted. Peak-width were measured using Chemstation SW.

Method 5: Determination of separation efficiency using gradient chromatography MS (trapping)
Although true chromatographic performance can only be measured isocratically, most nano-flow LC applications are performed using gradient conditions. A mixture of 4 peptides (Met-enkephalin, Angiotensin III, Neurotensin and substance P) were separated using an ACN/water gradient (2-40%) using the HPLC-chip cube. Peak-width were measured using Chemstation SW.

Method 6: Loading capacity of stationary phases using gradient chromatography MS (trapping)
Increasing amounts of BSA digest were loaded onto a standard chip and analyzed using a 500 nL/min 2-40% ACN/water gradient with 0.1% TFA. Peak width for peptide m/z (2+) was monitored as function of the stationary phase.

Results and Discussion

Packing density characterization using porosity and permeability measurements (Methods 1 and 2)

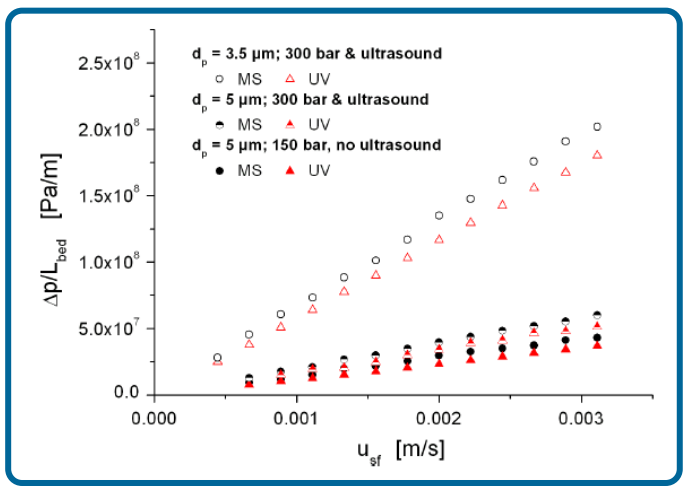

Figure 4: ΔP/L vs. u_{sf} for HPLC-chips packed with 5 and 3.5 um particles under varying packing conditions.

Results for the specific Darcy permeability, k_D and the interstitial porosity are shown in the table below. As can be seen increasing packing pressure and the application of ultrasound during packing result in a lower interstitial porosity and a reduced permeability, indicating a better packed bed. For comparison ϵ_{inter} for capillaries packed under the same conditions was about 0.42

Particle size and packing conditions	HPLC/UV κ_D (m^2)	HPLC/MS κ_D (m^2)
5 µm; 150 bar, no ultrasound	4.49×10^{-14}	3.77×10^{-14}
	$\varepsilon_{inter} = 0.48$	$\varepsilon_{inter} \approx 0.46$
5 µm; 300 bar and ultrasound	3.17×10^{-14}	2.79×10^{-14}
	$\varepsilon_{inter} = 0.42$	$\varepsilon_{inter} \approx 0.41$
3.5 µm; 300 bar and ultrasound	0.920×10^{-14}	0.823×10^{-14}
	$\varepsilon_{inter} = 0.40$	$\varepsilon_{inter} \approx 0.39$

Comparing separation efficiency of chips vs. capillaries using isocratic method 3 (fixed loop)
3 UV chips were packed with 5um particles at 300 bar with ultrasound and separation efficiency compared to 50 and 75 um capillaries packed under the same conditions. The chips showed slightly better performance indicating that when densely packed the geometry is not a factor.

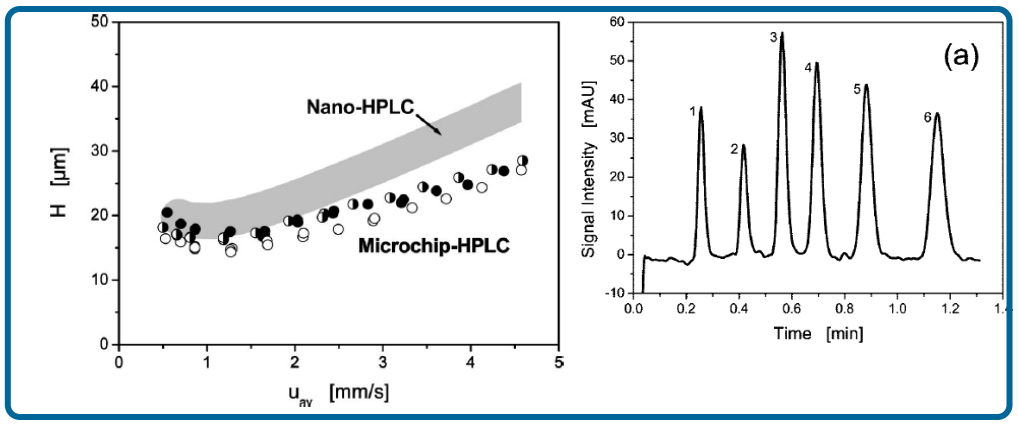

Figure 5: Plate height vs. mobile phase velocity for pentyl-benzene on HPLC chips and capillaries. 5a) Isocratic separation of the alkyl-benzene mixture at 4.6 mm/sec.

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Results and Discussion

Comparing UV chips to MS chips using isocratic method 4
The chromatographic performance of octano-phenone is measured by the isocratic method 4 and shown as a function of the linear velocity as well the k'. In order to achieve the desired k' the ACN/water ratio was gradually increased to 80:20.

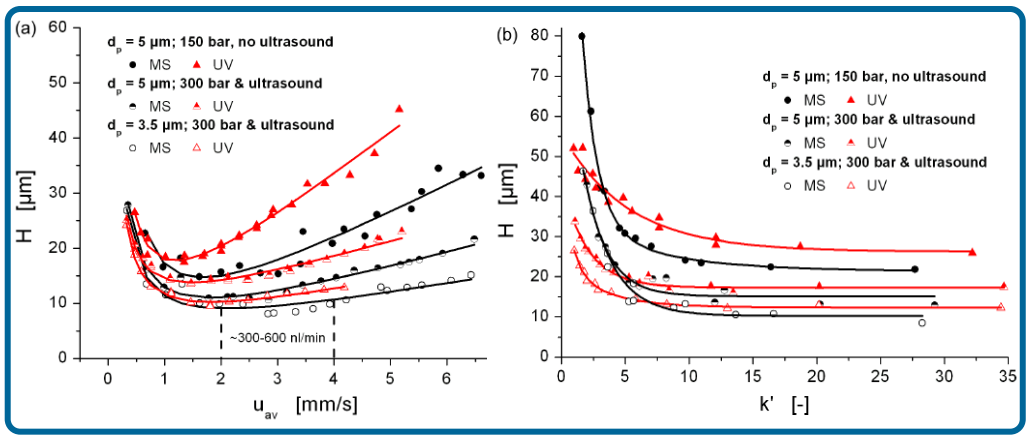

Figure 6: Plate height vs velocity and k' for ocatanophenone

Again column performance improved when higher pressure and ultrasound was used during packing. Also shown is that MS chips with 43 mm. columns can be more efficiently packed than UV chips with 75 mm. columns. However for small k' values UV chips show better performance, possibly due to post column dispersive effects. The minimum reduced plate height for the different conditions is shown below.

Particle size and packing conditions	HPLC/UV h_{min}	HPLC/MS h_{min}
5 µm; 150 bar, no ultrasound	3.5	2.9
5 µm; 300 bar and ultrasound	2.6	2.1
3.5 µm; 300 bar and ultrasound	2.5	2.1

Efficiency for MS chips using gradient elution method 5
As most applications are run under gradient conditions, a separation of 4 peptides is shown. Also here the improvements are clear though not a pronounced as in isocratic runs due to the effect of the gradient elution.

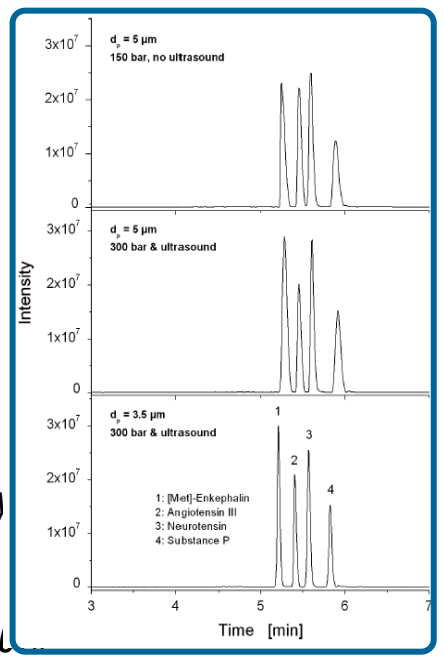

Figure 7: Comparing packing quality in gradient elution. (100 fmol each, 1 uL injection onto a 40 nL trap). Even with steep gradients, about 4[illegible] improvement can be achieved

Impact of stationary phase on peak performance at increasing loading amount.
Different standard MS chips (40nL trap, 43 mm. column) were prepared with a range of stationary phases. Ever increasing amounts of BSA digest were injected and peak width of a specific peptide monitored.

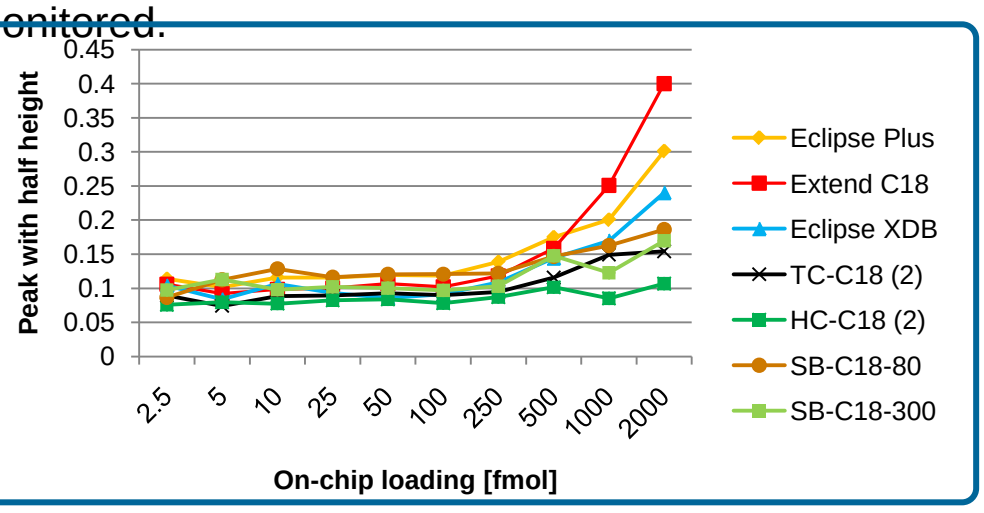

Figure 8: Loading study using different stationary phases.

As can be seen the HC material clearly showed the best chromatographic performance at high loading. This correlates directly to the very high specific surface area of this material (over 300 m^2/g). Optimizing stationary phase for Proteomics applications is important to maintain good chromatographic performance.

Conclusions

In this study we have shown several aspects related to chromatographic performance for nanoflow LC/MS.

- *Using microfluidic technology delay and dispersive volumes can be minimized.*
- *Column performance can be accurately measured using isocratic elution with on chip sample loop and UV detection.*
- *With optimized packing conditions, no impact of the separation column geometry can be found.*
- *Optimized packing conditions result in column packing densities near the theoretical limit.*
- *Packing materials with a high specific surface area maintain good chromatographic performance at higher sample loading.*

References:
1. *Ehlert et al. Anal. Chem. 80 (2008) 5945.*
2. *Ehlert et al. JMS 45 (2010) 313.*