Enhanced chromatography with the Agilent Polaris-HR-Chip-3C18 improved LC/MS/MS proteomics results

Technical Overview

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Abstract

Typical proteomics workflows involve the chromatographic separation of a protein digest and detection by tandem mass spectrometry (MS/MS). Subsequently, proteins are identified by performing a database search or alternatively quantified using specific target peptides. While a myriad of factors may affect the success of these experiments, critical instrumental factors are: (A) sensitivity and acquisition speed of the mass spectrometer, (B) intelligent use of the available time during the LC/MS/MS analysis, and (C) efficient and reproducible liquid chromatography separation. These factors gain in importance when the complexity of the sample increases, as for instance in the shotgun approach, where the sample may contain hundreds of thousands of peptides. We have recently demonstrated substantial progress in (A) and (B)[1,2]. This Technical Overview describes how these achievements, combined with chromatography improvements (C), impact the success of proteomics experiments.

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Introduction

Performing nano scale separations, which are routinely applied in the proteomics field is not a trivial task because of difficulties in setting-up the system, short maintenance intervals, a lack in robustness, and thus, the challenge to generate reproducible results. The microfluidic HPLC-Chip/MS technology, first commercialized in 2005[3,4], has proven to be a powerful solution to these challenges. HPLC-Chips provide simplicity of use and robustness allowing for enhanced productivity and the generation of consistent quality results. This has been confirmed with many different applications ranging from peptide and small molecule analyses to rather complex workflows such as the automated analysis of *N*-glycans following on-chip enzymatic cleavage from monoclonal antibodies[5-7].

To improve the chromatographic performance of the HPLC-Chip for peptide separations, the stationary phase was the first parameter investigated. Pilot studies compared the performance of a range of commercially available stationary phases that were packed into custom HPLC-Chips and results indicated that Agilent Polaris C18A HPLC columns with 3-µm particle size gave the best peak shapes (narrow, least tailing) for peptides in *E. coli* digests[8,9]. Subsequently, a new chip design was developed that is capable of housing smaller particle sizes; up till now HPLC-Chips contained 5-µm particles.

Here, we present the design of the novel Polaris-HR-Chip-3C18. Data demonstrates robustness, RT-precision, and chip-to-chip reproducibility as well as loading capacity. Further, we show the peak capacity that can be obtained with the new Polaris chip and how this produces enhanced peptide and protein identification results. As a benchmark, we used our Large Capacity Chip (G4240-62010) in addition to a custom chip packed with 5-µm Polaris C18A. Finally, results show the influence of gradient length on the number of identifications and the benefit for proteomic identification experiments gained from the combination of (A), (B), and (C).

Experimental

Instrumental setup

All analyses were run on the 1260 Infinity HPLC-Chip/MS System comprised of a micro autosampler with the thermostat (set to 4 °C), capillary and nanoflow pump with micro degasser, and the Chip-Cube that interfaces LC modules and the MS instrument[3,4,10]. HPLC-grade H_2O (0.1% formic acid (FA)) and ACN (0.1% FA) were used as mobile phases A and B, respectively (unless specified otherwise). ACN was from Merck (Germany), water from a Milli-Q water purification system, FA and trifluoroacetic acid (TFA) from Sigma (USA). Sample analysis used three different gradients (Table 1).

Gradient	1		2		3	
	Time [min]	% B	Time [min]	% B	Time [min]	% B
	0	3	0	3	0	3
	15	40	0.1	4.5	90	40
	16	70	2	9	95	90
	17	70	40	31.5	96	90
	17.5	3	45	36	100	3
			50	81		
			51	3		
Stop	27.5		60		110	
Flow	0.45 µL/min		0.30 µL/min		0.30 µL/min	
Sample loading	3.00 µL at 3% B		2.00 µL at 3% B		2.00 µL at 3% B	
IFV	3 µL		5 µL		5 µL	

Table 1
Gradients, flows and injection flush volumes (IFV).

Mass detection occurred with an Agilent 6410B Triple Quadrupole LC/MS System, an Agilent 6530 Accurate-Mass Q-TOF LC/MS System or an Agilent 6550 iFunnel Q-TOF LC/MS System operated in positive ion mode with:

V_{cap}:	1,800–1,900 V,
Drying gas flow :	5.0 L/minute at T = 350 °C (Triple Quadrupole), 250 °C (6530 Q-TOF), and 280 °C (6550 Q-TOF)
Fragmentor voltage:	130 V (Triple Quadrupole), 175 V (6530 Q-TOF), and 360 V (6550 Q-TOF)

Triple Quadrupole data were acquired in MRM-mode. Transitions were determined using the Skyline software[11].

The 6530 and 6550 QTOF were operated in 2 GHz Extended Dynamic Range mode with:

Mass range:	Set to narrow (Low 1,700 *m/z*)
AutoMS/MS:	4 MS (range 290–1700 *m/z*), 3 MS/MS (range 50–1,700 *m/z*) per/s (6530 Q-TOF) or 8 MS and 3 MS/MS per/s (6550 Q-TOF)

Narrow isolation width (1.3 *m/z*), with collision energy determined on the fly using a slope of 3.6 and intercept of –4.8.

Precursor selection:	10 precursors/cycle
Threshold:	500 counts abs. (6530 Q-TOF) and 1,000 counts abs (6550 Q-TOF) and 0.001% rel
Active exclusion:	After 1 spectrum
Release:	After 0.25 minutes

Scan speed was set to vary with precursor abundance with a target of 25,000 counts/spectrum.

Precursor purity:	Stringency 100%, purity cut-off at 30%

Internal reference mass calibration used *m/z* 1221.9906 and 299.2945.

The Agilent MassHunter Workstation software (B04.00 or B.05.00) was used for data acquisition and processing. Spectrum Mill MS Proteomics Workbench (B04.00 or B.05.00) was used for database search using SwissProt.ecoli and a Peptide Global FDR set to 1%.

Samples

HSA Peptides Standard Mix (G2455-85001) was dissolved in 50 µL of a 50% B solution and aliquots (10 pmol/µL) were stored at –20 °C. On the day of the experiment, stock solutions were diluted in 10% B to give a working solution (100 fmol/µL). From this working solution, 0.1-µL injections were made.

An *E. coli* protein sample (BioRad) was digested using sequencing grade modified trypsin (Promega). The *E. coli* protein sample (2.7 mg protein) was dissolved in 2 mL of a 50:50 v/v solution of 2,2,2-trifluoroethanol/ammonium bicarbonate (100 mM). Aliquots of 100 µL were transferred into reaction vessels (1.5-mL Eppendorf tubes). After 2.5 µL dithiothreitol (DTT) (200 mM) was added, the solution was mixed and then heated to 60 °C for 45 minutes. After cooling to room temperature, 10 µL of an iodoacetamide solution (200 mM) was added, and the vessels were vortexed and stored in the dark at room temperature for 45 minutes. After adding 2.5 µL DTT (200 mM) to quench excess alkylating reagent, the samples were incubated in the dark at room temperature for 45 minutes. This was followed by addition of 800 µL ammonium bicarbonate solution (25 mM) and 6.0 µL trypsin stock solution (6 µg), then incubation at 37 °C overnight. Solutions were then pooled and the reaction quenched by adding 72 µL of concentrated FA. Aliquots of this solution (150 ng total protein/µL) were stored at –80°C. Samples were defrosted on the day of the experiment. 600-ng samples were prepared by evaporating the 150-ng/µL sample to dryness and reconstituting in ¼ of the original volume. Injections were made between 0.5 and 8.0 µL. All reagents were from Sigma-Aldrich (USA) and solutions were prepared in Milli-Q H_2O.

Polaris-HR-Chip-3C18 layout and operation

Figure 1A illustrates the design of the Polaris-HR-Chip-3C18, which is made from inert, biocompatible polyimide. It integrates: (A) a 360-nL enrichment column (EC) and (B) a 150 mm × 0.075 mm id separation column (SC) that directly connects to (C) the metalized nano electrospray tip.

Both columns are packed with the Polaris C18A stationary phase that has a 3-µm particle-diameter, 180Å pore size, and a surface area of 200 m^2/g. Polaris C18A is a polar-modified material with hydrogen bond-accepting end-capping. Figure 1B shows a schematic. Both, the chip design and the manufacturing process have been improved to provide chip robustness at the higher back pressures that result from smaller stationary phase particles.

Benchmark HPLC-Chips

Large capacity Chip (II), 150 mm × 0.075 mm id analytical and 160-nL trap columns packed with Agilent ZORBAX SB-C18, 5 µm, 300Å (G4240-62010).

Custom Chip, 150 mm × 0.075 mm id analytical and 360 nL trap columns packed with Polaris C18A, 5 µm, 180Å; custom chips can be ordered through the custom chip program[9].

Chip operation has been described in detail[3,4,10]. Briefly, the Chip-Cube interface automatically positions the chip orthogonal to the MS inlet and makes the necessary electrical and hydraulic connections to the chip. The stator-chip-rotor sandwich creates a 6-port valve. In the loading position, the autosampler is connected to the EC,

where the sample becomes enriched while salts are flushed out to waste. The automated valve switch to analysis position sets the EC in line with the separation column. The reversed phase gradient elutes the trapped peptides onto the SC where chromatographic separation occurs prior to transmission of the analytes to the mass spectrometer.

Results and discussion

Retention time precision, inter-chip variability, robustness

Retention time (RT) precision was determined using the seven HSA peptide mix employing the 15 minutes gradient 1 (Table 1). Table 2 shows the results from the assessment of four chips. RT RSD values calculated for 20 consecutive injections were typically below 0.2%. Figure 2A shows a representative MRM chromatogram with 20 consecutive injections superimposed.

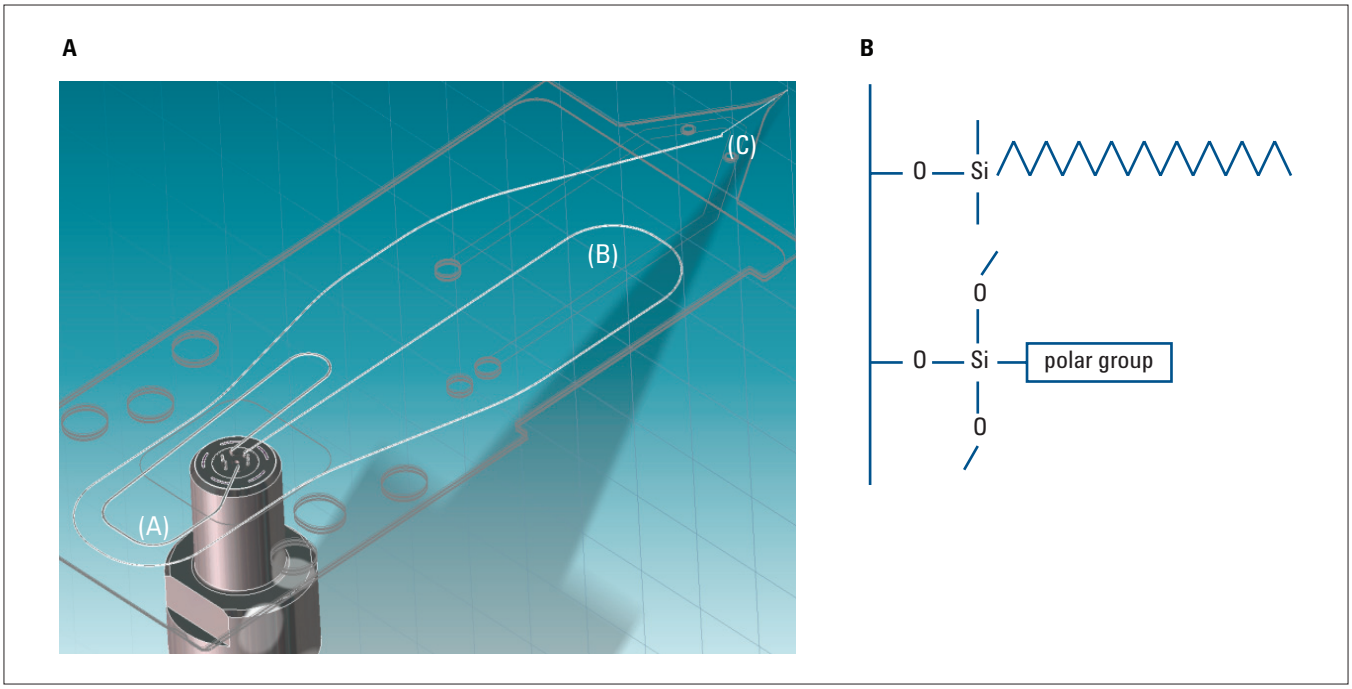

Figure 1
A: Schematic of Agilent Polaris-HR-Chip-3C18: (A) enrichment column (EC), (B) separation column (SC), (C) nanoelectrospray tip.
B: Schematic of Agilent Polaris C18-A material.

	Precision								Reproducibility	
	Chip 1		Chip 2		Chip 3		Chip 4		38 chips	
Peptide	Avg RT [min]	RSD [%]	Avg RT [min]	RSD [%]	Avg RT [min]	RSD [%]	Avg RT [min]	RSD [%]	Avg RT [min]	RSD [%]
1	6.70	0.18	6.74	0.29	6.61	0.15	6.69	0.15	8.07	0.88
2	9.37	0.08	9.43	0.13	9.28	0.10	9.34	0.12	10.94	0.73
3	9.62	0.07	9.67	0.08	9.54	0.09	9.60	0.10	11.02	0.72
4	9.72	0.08	9.77	0.08	9.65	0.08	9.70	0.06	11.34	0.67
5	10.83	0.07	10.89	0.07	10.76	0.10	10.80	0.10	12.41	0.60
6	12.76	0.06	12.82	0.08	12.70	0.08	12.74	0.08	13.85	0.58
7	14.47	0.08	14.54	0.09	14.42	0.18	14.40	0.07	N/D	N/D
Average		**0.09**		**0.12**		**0.11**		**0.10**		**0.70**

Table 2
Precision: Average retention time (Avg RT) for seven peptides over 20 consecutive injections on four chips and corresponding precision values [%RSD]. Reproducibility: Average RT for six peptides calculated over 38 chips which were from five manufacturing batches and the corresponding inter-chip variability [%RSD].

RT reproducibility (comparing chip-to-chip) was determined using 38 Polaris chips selected from five different manufacturing batches. Figure 2B shows the RT of six peptides versus the number of chips studied. Table 2 (far right column) summarizes the corresponding average RT and %RSD values demonstrating the exceptional chip-to-chip reproducibility (< 1%). These experiments used TFA instead of FA in the mobile phases, which is the reason for longer RT's compared with the previous precision measurements; the response of peptide 7 was greatly suppressed by TFA, thus, this peptide is not shown in the results.

These results are particularly important for quantitative peptide work, especially in combination with complex samples that may require precise, stable, and reproducible retention times during MRM experiments to ensure collection of sufficient numbers of data points across the chromatographic peak[12].

The robustness of HPLC-Chips (just as with any HPLC column) depends on the quality of the sample injected or in other words the clean-up of real-life samples prior to injection. With the HSA peptide mix (standard), no marked changes in the chromatographic performance measured in RT, resolution, peak width and symmetry were observed after 1,000 injections; (two chips studied).

Note that in order to achieve best RT-stability with polar bonded phases, the columns must be conditioned properly. Reference 13 describes a procedure which is provided with every chip.

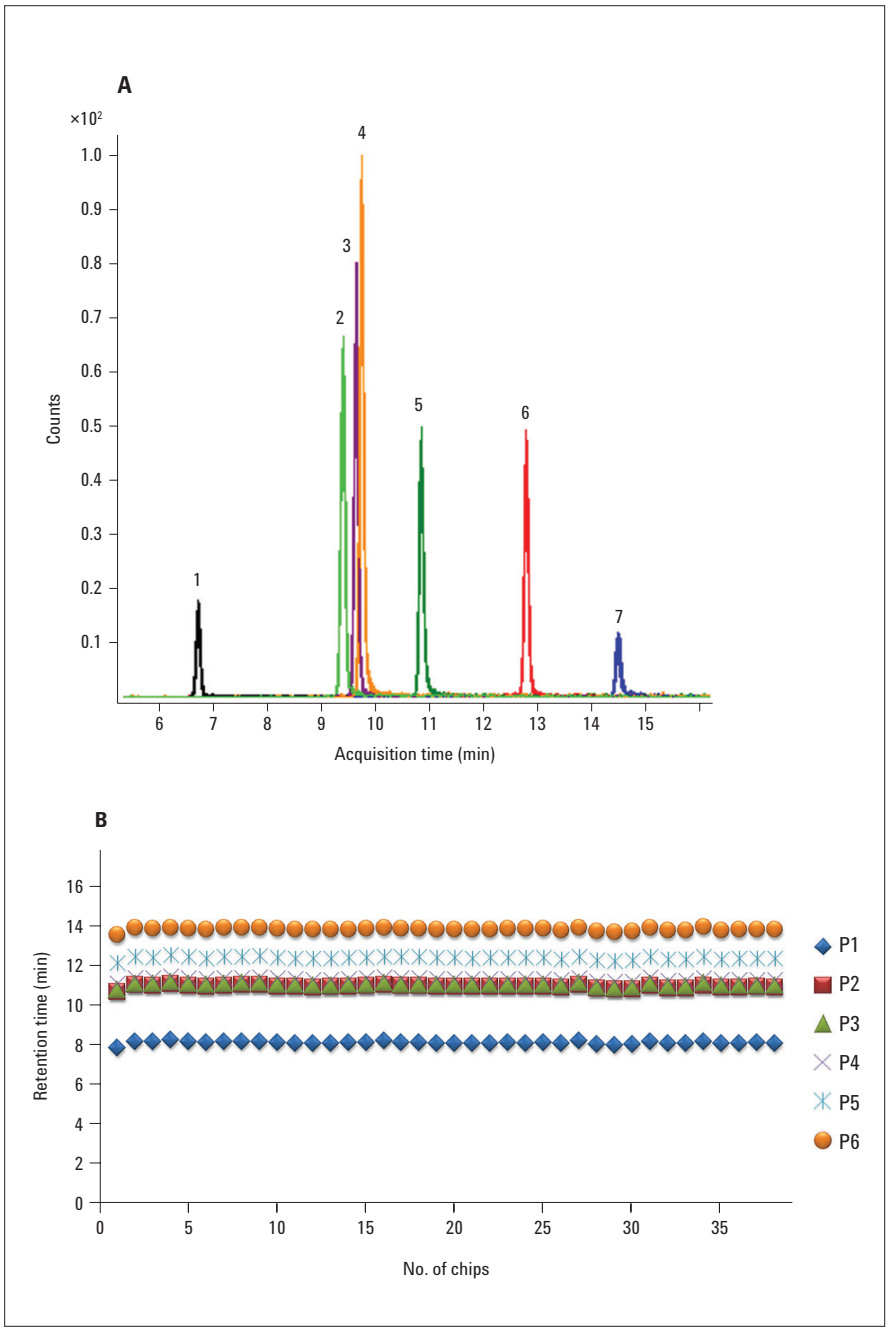

Figure 2
A: MRM chromatograms obtained from 20 consecutive injections of the seven HSA peptide mix superimposed.
B: Inter-chip variability (reproducibility): RT for six peptides obtained with 38 chips (five different manufacturing batches).

Loading capacity

Loading capacity is an important parameter because higher loads generally increase the number of peptides and proteins identified, especially for low abundant species. Loading capacity was determined using the *E.coli* digest employing the 45 minutes gradient-2 (Table 1). Samples contained 150 or 600 ng total protein/µL with injection volumes ranging from 0.5 to 8 µL. Figure 3A shows a total ion current (TIC) chromatogram (black). Ten representative peptides across the elution range were used to assess loading capacity. Extracted ion chromatograms are shown overlaid in Figure 3A (blue) with the y-axis set to %/%-scale for better illustration.

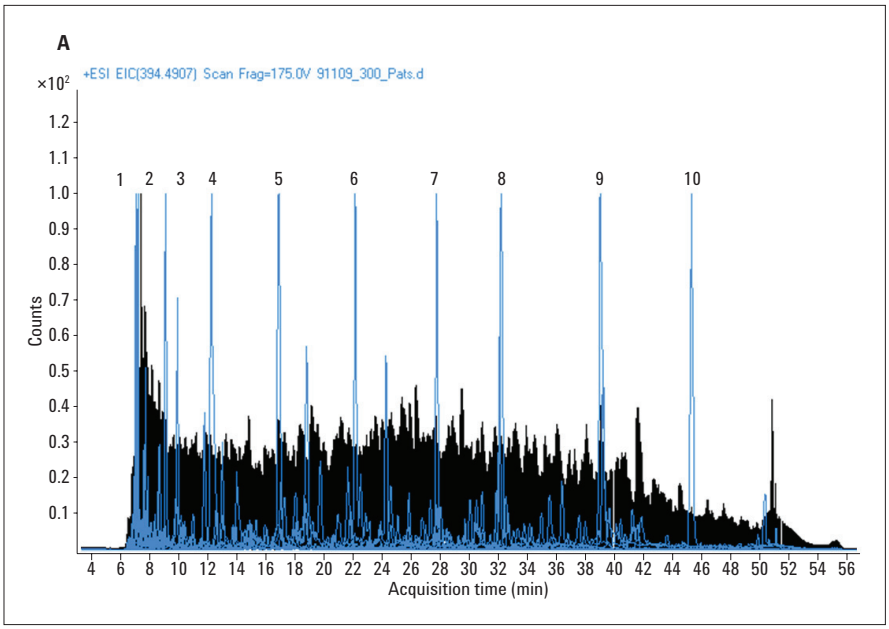

Figure 3A
TIC chromatogram (black trace) for an injection of 500 ng total protein in the *E. coli* digest. EIC's of peptides taken for evaluation of loading capacity overlaid (blue). Y-axis = %/%-scale for better illustration.

Figure 3B(a) shows a normalized chart of peak area obtained at a given sample load divided by peak area obtained at highest load demonstrating reasonable linearity up to 1,200 ng total protein injected (8 µL of the 150 ng/µL sample). To the right (c) it is seen that higher loads, for example, up to 4,200 ng total protein (7 µL injection of the 600 ng/µL sample) are possible, if outside the linearity range.

Figure 3B (b) shows the observed increase in identification of peptides and proteins with load. For this shorter 45 minutes gradient, the curve %-increase versus sample load levels off above 600 ng.

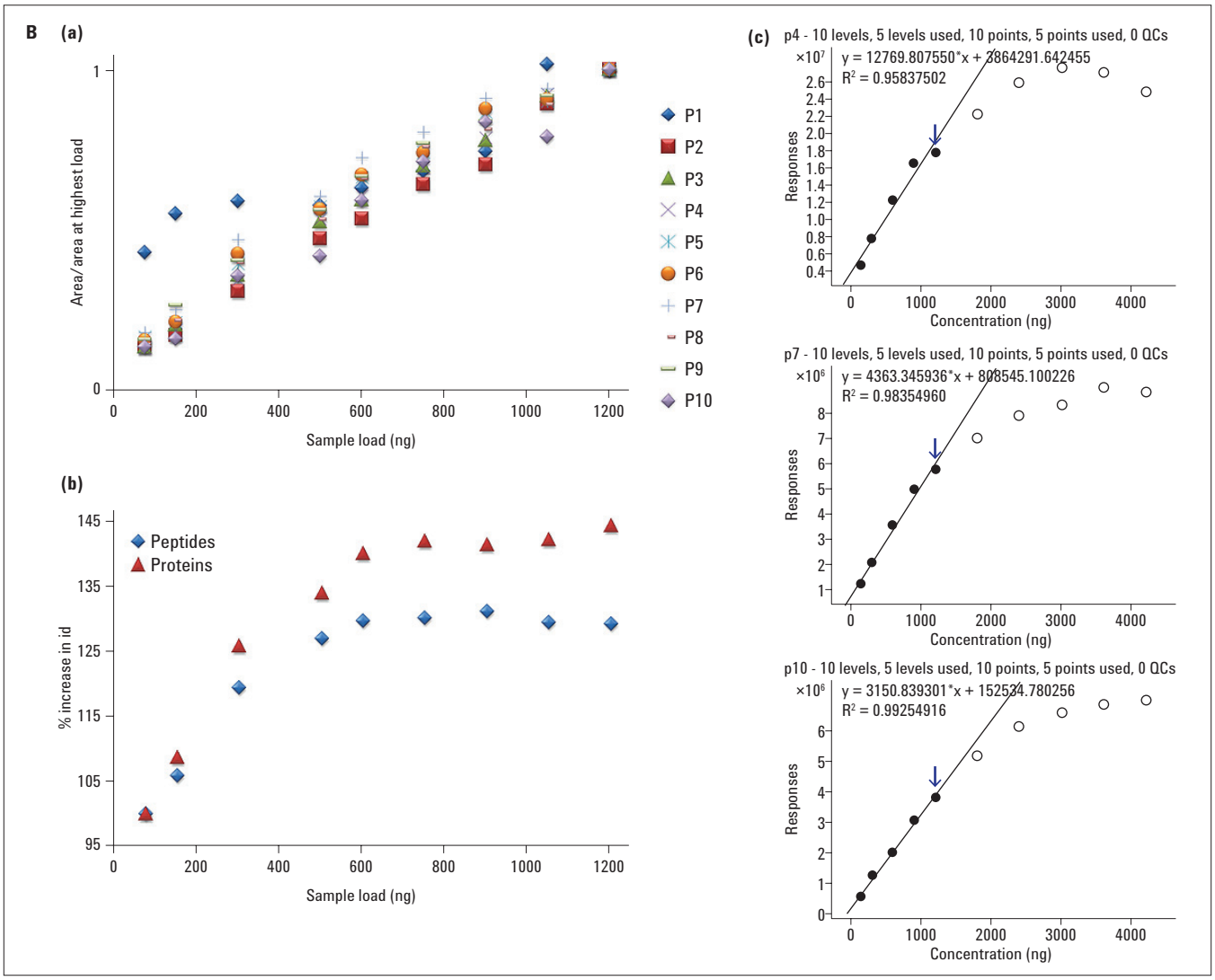

Figure 3B

(a): Peak area (normalized) versus sample load of *E. coli* digest in nanogramm abs. on column.

(b): %-increase in peptides (blue diamonds) and protein (red triangles) identification with increasing sample load; number of identifications at lowest load was taken as baseline = 100%

(c): Extended load (using the 600 ng *E. coli* sample).

Peak capacity

Peak capacity (Pc) is the most commonly used metric to measure separation performance in reversed-phase chromatography when gradients are used. It defines the number of ideal Gaussian peaks that can be resolved relative to the gradient time (tG) or alternatively within the time window the peaks elute in, which is then called the conditional Pc (nc)[14].

The peak capacity of the chips was determined using the *E.coli* digest employing the linear 90 minutes gradient-3 (Table 1). Total protein load was 500 ng (3.3 µL of a 150 ng/µL sample). Figure 4 shows a typical TIC chromatogram (black). Equations 1 and 2 were used respectively to calculate *Pc* and *nc* from 4σ peak widths (width at 13.4% peak height[14]) of a number of EIC-peaks across the elution range. Figure 4 depicts the EIC's (orange) laid over the TIC trace along with corresponding values for Pc and nc; y-axis set to %/%-scale for better illustration.

$$\text{Eqn. 1:} \qquad Pc = 1 + \frac{tG}{w4\sigma}$$

$$\text{Eqn. 2:} \qquad nc = 1 + \frac{Rt(last) - RT(first)}{w4\sigma}$$

Peak capacities of 14 Polaris chips packed with 3-µm particles (PS-3 µm) were compared with those obtained from four chips packed with 5-µm particles, three of which were the Large Capacity Chip packed with ZORBAX SB-C18 (ZX-5 µm) and one custom chip packed with Polaris C18-A (PS-5 µm). Results obtained for Pc are summarized in Figure 5 showing a graph of Pc versus chip type. Peak capacities for ZX-5 µm ranged between 122 and 148 (average: 138, %RSD 8.5), that for PS-5 µm was 160, and those calculated for PS-3 µm ranged between 178 and 210 (average: 191, %RSD 5.5).

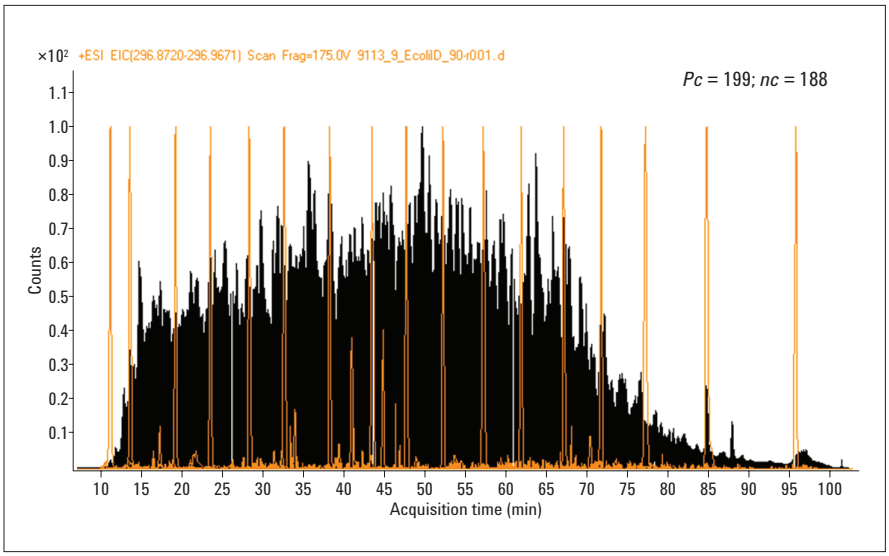

Figure 4
TIC chromatogram (black trace) for an injection of 500 ng total protein of *E.coli* digest. EIC's of peptides taken for evaluation of peak capacity overlaid (orange). Y-axis = %/%-scale for better illustration.

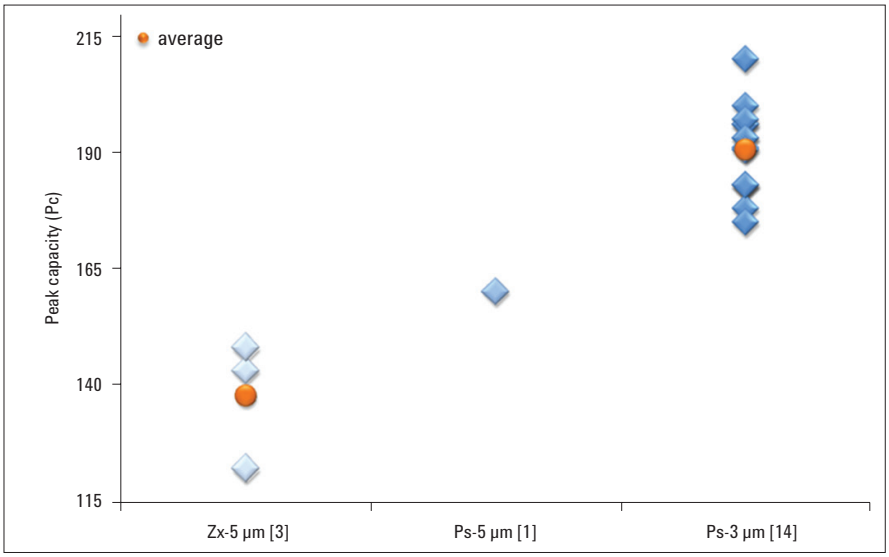

Figure 5
Peak capacity obtained from HPLC-Chips packed with Agilent ZORBAX SB-C18, 5 µm [ZX-5 µm], Agilent Polaris C18A, 5 µm [PS-5 µm], and Agilent Polaris C18A, 3 µm [PS-3 µm]. The value in brackets indicates the number of chips tested.

Thus, on average, a %-increase in Pc was obtained with PS-3 µm of 19 and 39% compared to PS-5 µm and ZX-5 µm, respectively. Clearly, such increase was expected since Pc is inversely proportional to the plate height of the column that is typically reduced with decreasing particle size, provided columns are well packed.

Figure 6 shows a portion of the chromatograms (EIC's) obtained with ZX-5 µm (blue) and PS-3 µm (purple) (44 to 90 minutes) illustrating the difference in selectivity obtained. Some peptides (peaks 1–4, and 7) elute at a somewhat later time with PS-3 µm and some others appear later with ZX-5 µm (5 and 6). Earlier elution on ZX-5 µm may be partly explained by the lower volume of the enrichment phase (160 nL compared to 360 nL on the PS-3 µm chip), which means a somewhat smaller volume of a specific gradient composition has to pass to reach the chip's electrospray tip. However, RT differences between the pairs (for example, 1/1, 3/3 and especially 4/4 respectively on ZX-5 µm/PS-3 µm) are different too, which clearly points towards the influence of selectivity. The literature[15] has described this as polar selectivity that originates in interactions of embedded polar groups (that is, amide, carbamate, or urea) with carbonyl groups of the solute in addition to those the solute undergoes with the C18 ligands of the phase.

Indeed, the most obvious difference can be observed with peak 4 that is just about resolved from peak 3 on the ZORBAX material (retention time difference, ΔRT ~ 1 minutes) whereas, with Polaris, these peaks elute much further apart from each other (ΔRT ~ 5 minutes). The corresponding peptide no. 4 contains 19 amino acids (EMNIADYDAELWQAMEQEK), nine of which bear a carbonyl group in their side chain. In contrast, peptide no. 5 (VKPVLLGPVTWLWLGK) does not include such amino acids and elutes slightly later on ZX-5 µm than on PS-3 µm.

Although probably less important in the analysis of digests produced from whole cell lysates (where such selectivity changes could probably also be shown in favor of the pure C18 phase), this finding clearly illustrates the potential of Polaris to resolve separation problems which may exist when using other materials containing exclusively C18 ligands. Note: This polar selectivity of polar groups can be amplified with protic mobile phase modifiers such as methanol[15].

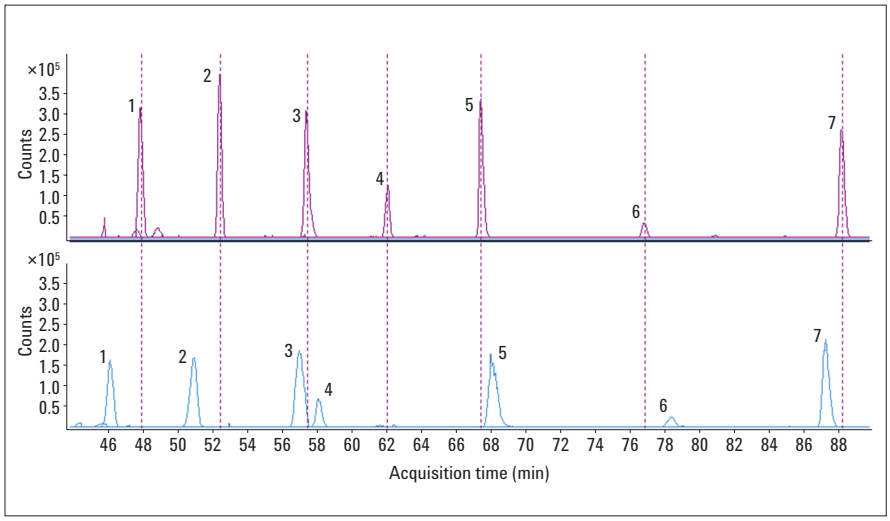

Figure 6
EIC's of obtained from *E.coli* anlyses with PS-3 µm (top, purple) and ZX-5 µm (bottom, blue) illustrating the polar selectivity of Agilent Polaris C18A.

Peptide and protein identification

The improvement in Pc was accompanied by improved peptide/protein identification results as is exhibited in Figure 7 A-C. Panel-A shows the number of peptides identified with the 45 minutes gradient-2 (Table 1) and the 6530 QTOF LC/MS System with each data point being the average of at least two runs. Using four ZX-5 µm chips, between 3,455 and 3,913 peptides (average: 3742) were found, with the PS-5 µm, 3970, and with the 12 PS-3 µm chips we were able to identify between 4,288 and 5,035 peptides (average: 4,509). On average this translates into a percentage improvement in peptide identification obtained with PS-3 µm of 13 and 20% over PS-5 µm and ZX-5 µm, respectively, as exhibited in Panel-C. Panel-B shows the results for proteins: ZX-5 µm: 613 to 666 (average: 649), PS-5 µm: 637, PS-3µm: 700 to 785 (average: 730). Similarly as for peptides, panel-C shows the percentage improvement for proteins obtained with PS-3 µm, which was 15 and 13% over PS-5 µm and ZX-5 µm, respectively. Clearly, this was an expected result since improved separation decreases the number of peptides presented to the mass spectrometer at any given time and increases the instantaneous signal for the same amount injected, also yielding better detection of lower abundance species.

Finally, we investigated the effect of gradient time on protein identification with the Polaris-HR-Chip-3C18 using 45, 90, 120, and 150 minutes gradients (gradient-3, Table 1). This study was conducted using the 6550 iFunnel Q-TOF. As expected, peptide and protein identifications increased considerably with gradient time as depicted in Figure 8 panels A and B. The 6550 Q-TOF with iFunnel technology and hexabore capillary inlet has greatly improved sensitivity compared to the 6530 Q-TOF, and this led to significantly larger numbers of peptides (7,644 to 8,290) and proteins (1,154 to 1,211) identified using a 45 minutes gradient. Outstanding results were obtained using a 120 minutes gradient with the largest number for peptides, 9,604, and for proteins, 1,401, identified from *E. coli* in a single run.

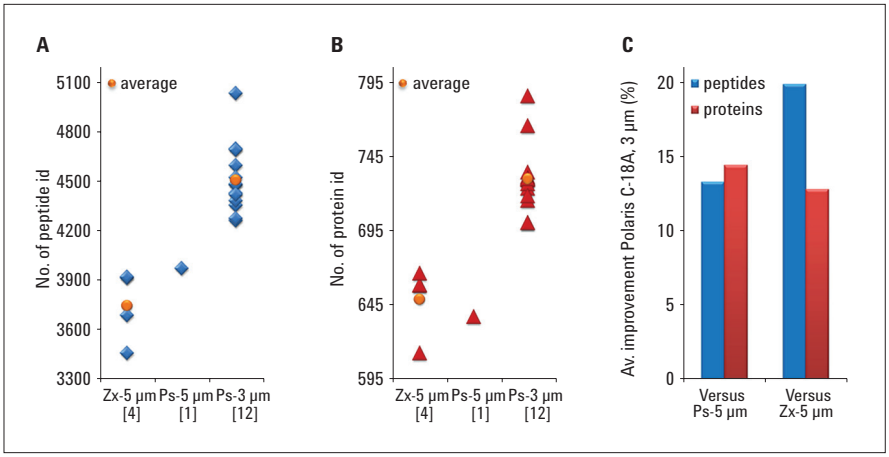

Figure 7
A: Number of peptides identified
B: Number of proteins identified
C: %- improvement in identified peptides (blue bar) and proteins (red bar) obtained with Agilent Polaris C18A, 3 µm [PS-3 µm] over Agilent Polaris C18A, 5 µm [PS-5 µm], and Agilent ZORBAX SB-C18, 5 µm [ZX-5 µm]. The value in brackets indicates the number of chips tested.

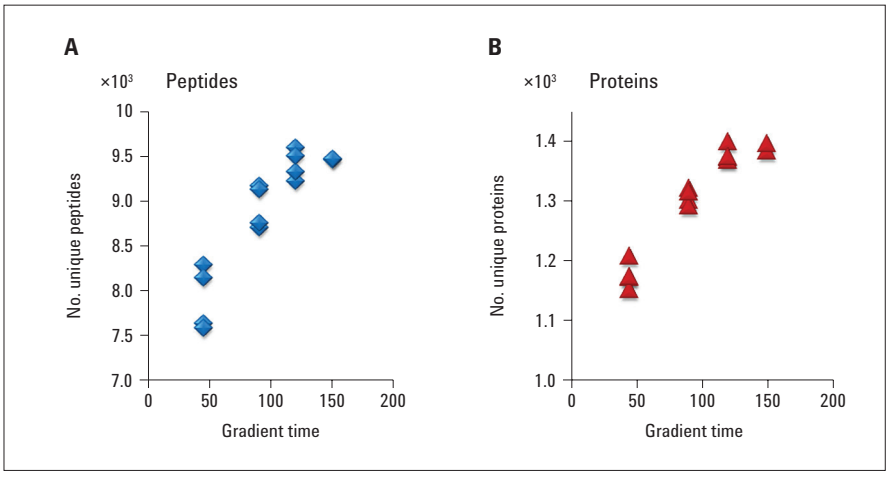

Figure 8
Effect of gradient length in:
A: peptide identifications
B: protein identifications. Sample: 500 ng *E.coli*,
Chip: Agilent Polaris-HR-Chip-3C18, Mass spectrometer: Agilent 6550 iFunnel QTOF.

Conclusion

The new Agilent Polaris-HR-Chip-3C18 was developed with the goal to further improve the quality of peptide separation in proteomics experiments. Data demonstrate its excellent RT-precision, chip-to-chip reproducibility and robustness, as well as loading capacity. Higher peak capacities obtained with the new Polaris chip result in a considerably larger numbers of peptides and proteins identified, demonstrating the impact enhanced chromatography can have on results for protein identification experiments.

Finally, we reported outstanding identification results obtained from combinatorial instrument improvements implemented[1,2]: (A) sensitivity and acquisition speed of the mass spectrometer, (B) the intelligent use of the time that is available during the LC-MS/MS run, and (C) the enhanced chromatography.

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