Optimizing plant phosphoproteomic data independent acquisition: micro-pillar array columns and traditional packed bed technology

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Abstract

Purpose: Compare DIA results of traditional packed bed columns with microfabricated pillar array columns to develop a best approach for phosphoproteomics.

Methods: An enriched phosphoproteomic extract and total protein extract from *Arabidopsis thaliana* was run using four different columns with 15 minute DIA methods on a Thermo Scientific™ Orbitrap™ Astral™ mass spectrometer.

Results: The traditional packed bed column showed more coverage for the total proteome sample and phosphoproteome enrichment; the pillar array column showed higher peak heights for lower intensity peaks.

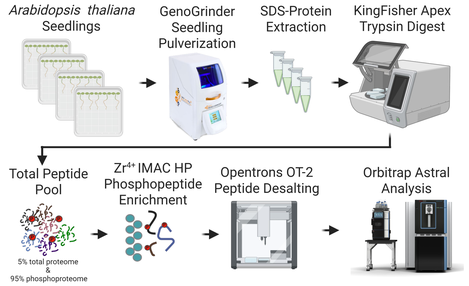
Introduction

Plant research has come to the forefront of modern science as crop production efficiency, environmental impacts, and efficient use of land resources have been required to be balanced with economic output. *Arabidopsis thaliana*, a model plant system, provides the ideal set of characteristics for the study of plant proteomics: short lifecycle, sequenced genome, ease of cultivation, and proven methods of genetic modification. Just as mammals undergo natural cycles of life, plants experience cycles such as seasonal weather changes, day and night photoperiods and changes in temperature or growth media conditions. Many of these changes are mediated by phosphorylation, often challenging due to low phosphoprotein levels which can be enhanced with phosphopeptide enrichment coupled with data independent acquisition using modern chromatography systems such as microfabricated pillar array columns.

Materials and methods

Sample Preparation

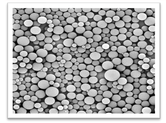
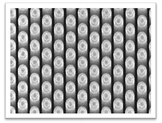
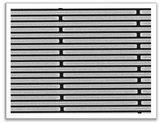
Soil grown *Arabidopsis thaliana* Col-0 plants were grown under a 12h light : 12h dark photoperiod and extracted using an SDS-based SP3 bead method (Leutert et al 2019; Mehta et al 2022), with phosphoproteins enriched using MagReSyn® Zr^{4+}IMAC HP particles (ReSyn Biosciences) as previously described (Leutert et al 2019), using a Thermo Scientific™ KingFisher™ Apex System. Plant rosette tissue was harvested 21 days post-imbibition, with 400 mg of tissue extracted to generate 500 ug of total trypsin peptide used for phosphopeptide enrichment.



Chromatography Methods and Mass Spectrometry

Six replicate samples of total protein and enriched phosphopeptides were run online using the Thermo Scientific™ Vanquish™ Neo UHPLC System with column compartment. The mass spectrometry was acquired using data independent analysis (DIA) on an Orbitrap Astral Mass Spectrometer. Nano chromatography was performed using a trap-and-elute workflow onto: a Thermo Scientific™ Double nanoViper™ PepMap™ Neo UHPLC 75 µm x 15 cm column (DNV75150PN), a Double nanoViper PepMap Neo UHPLC 150 µm x 15 cm column (DNV150150PN), a 50 cm µPAC Neo HPLC column (COL-NANO050NEOB), and a 5.5 cm High Throughput µPAC™ Neo HPLC column (COL-CAPHTNEOB); all columns were run at 50 ºC using a 15 µm Thermo Scientific™ EASY-Spray™ ES994 capillary emitter. Trapping of the peptides was done using a 300 um, 0.5 cm Thermo Scientific™ PepMap™ Neo Trap Cartridge using combined control at 60 uL/min or 1500 bar. The trap was washed in parallel with the analytical column using the Zebra Wash with four cycles. An 11.8 minute gradient of 4 to 40% (0.1% formic acid in mobile phase A and 0.1% formic acid in acetonitrile in mobile phase B) was used to elute the peptides with a linear ramp to 99% to wash the analytical column clean. The flow rate for all columns except the 50 cm was 1.5 uL/min. The 50 cm was run at 0.8 uL/min; limited by the pressure maximum of pillar array columns (450 bar).

Mass spectrometry was done using two scan events: full scan at 240,000 resolution (*m/z* 200) and DIA using two Thompson windows covering 380 – 980 *m/z*. The full scan was run with an AGC setting of $5e^6$ and a maximum injection time of 3 ms. Astral DIA was run with an AGC of 5e4 and a maximum injection time of 3 ms. The ion transfer tube temperature was three-hundred five degrees celsius with run start EASY-IC internal calibration.

DNV75150PN DNV150150PN	50 cm µPAC Neo	High Throughput µPAC Neo
		
• Packed bed • 2 um particle • 75 µm or 150 µm ID • 15 cm length • 1500 bar max	• Circular pillars • Pillar size: 2.5 m • Interpillar distance: 1.25 µm • Pillar height: 16 µm • Bed width: 180 µm • Pore size:100-300 Å • 450 bar max	• Radial Elongated Pillars • Pillar size:75 µm x 3 µm • Interpillar distance: 2 µm • Porous layer: 500 nm • Pore size: 100-300 Å • 5.5 cm • 450 bar max

Data Analysis

HPLC data was analyzed using Thermo Scientific™ FreeStyle™ 1.8 SP2 QF1 with Thermo Scientific™ Proteome Discoverer™ software 3.2. CHIMERYS™ intelligent search algorithm and Spectronaut™ software were used for searching and DIA quantification. CHIMERYS was searched using Araport11_pep_20250214.fasta with the Inferys_4.7.0 fragmentation model using cysteine static modification for carbamidomethylation and dynamic modifications of methionine oxidation and phosphorylation of serine, threonine, and tyrosine. Spectronaut was used to search the files with appropriate parameters.

Results

Chromatography Comparison

Figure 1. The same separation of the four chromatograms is shown. The flow rate of 1.5 uL/min was chosen to minimize the dead volume of the system and associated columns. The shortest runs were using the 75 um DNV column, with a gradient delay estimated at three minutes and a run time overhead of 4.5 minutes (sample pickup, loading time, and equilibration). The 50 cm µPAC showed the longest gradient delay at 8 minutes due to the lower maximum flow rate.

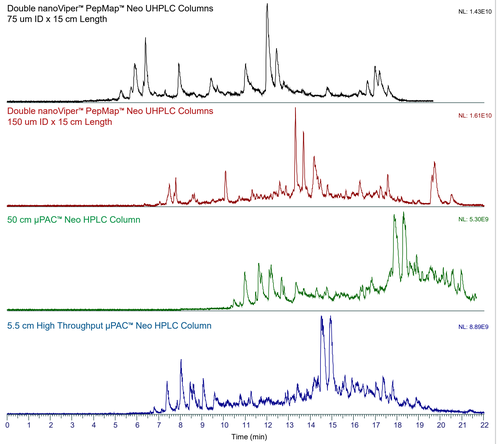


Table 1. Coverage results for phosphopeptide enriched samples. 'Identified Phosphoprotein Groups' and 'Identified Phosphopeptides' only required an ID in one of the six biological replicates; 'Quantified Phosphopeptides' required a computed value in at least four out of six replicates. Search results are shown from Spectronaut; CHIMERYS showed different actual numbers but similar trends.

	DNV75150PN	DNV150150PN	µPAC 50 cm	µPAC HT
Identified Phosphoprotein Groups	2888	3518	2737	2925
Identified Phosphopeptides	8499	10458	7863	8413
Identified Phospho-sites	8780	10892	8106	8642
Quantified Phosphopeptides	3839	5790	3614	3887
Quantified Phosphosites	3967	5981	3725	4004

Figure 2. The peptide, FGFGtKK, from 'Light harvesting complex photosystem I', with a doubly charged phosphothreonine, is plotted as an XIC (the fourth chromatogram is not shown to save space; the peak widths and asymmetry were nearly identical to the other DNV displayed). The double nanoViper column showed a smaller peak width and a lower asymmetry. The pillar array columns, with their lower surface area may be overloading for high intensity peptides. The high loads in these samples may favor traditional packed bed technologies.

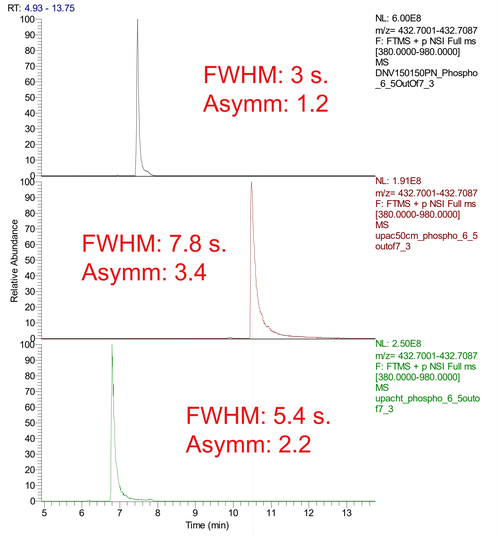
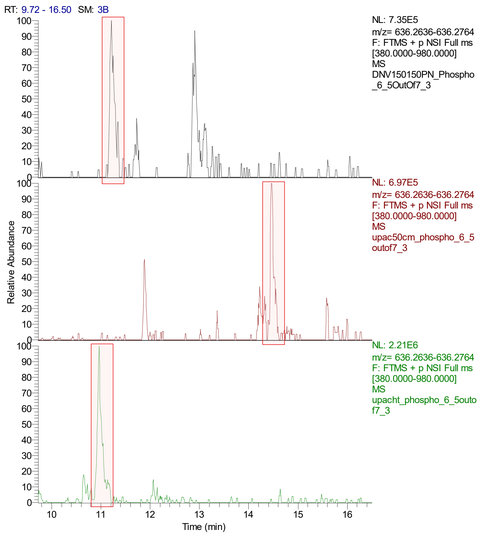


Table 2. Protein coverage results for total protein samples. 'Quantified Protein Groups' required a computed value in at least five of the six replicates. Search results are shown from Spectronaut; CHIMERYS showed different actual numbers but similar trends.

	DNV75150PN	DNV150150PN	µPAC 50 cm	µPAC HT
Identified Protein Groups	6991	8241	6628	7725
Identified Peptides	58387	75111	59574	74472
Quantified Protein Groups	6751	7890	6507	7578
Quantified Peptides	55004	68396	52894	67609

Figure 3. The peptide, STsFKDEIEDEEELR, from 'Permeable Cuticle I', with a triply charged phosphoserine, is plotted as an XIC (the fourth chromatogram is not shown to save space). The High Throughput µPAC column showed higher sensitivity as was observed in many cases for peptides approaching the limits of quantification.



Conclusions

- Higher intensity peaks show better peak shapes on the traditional packed bed column technology, while the microfabricated pillar array columns show higher peak heights for the smaller intensity peaks
- Capillary columns showed better peak shape with lower asymmetry then the nano-scale columns at the flow rates used
- Short gradient high throughput runs are ideal for DIA methods

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