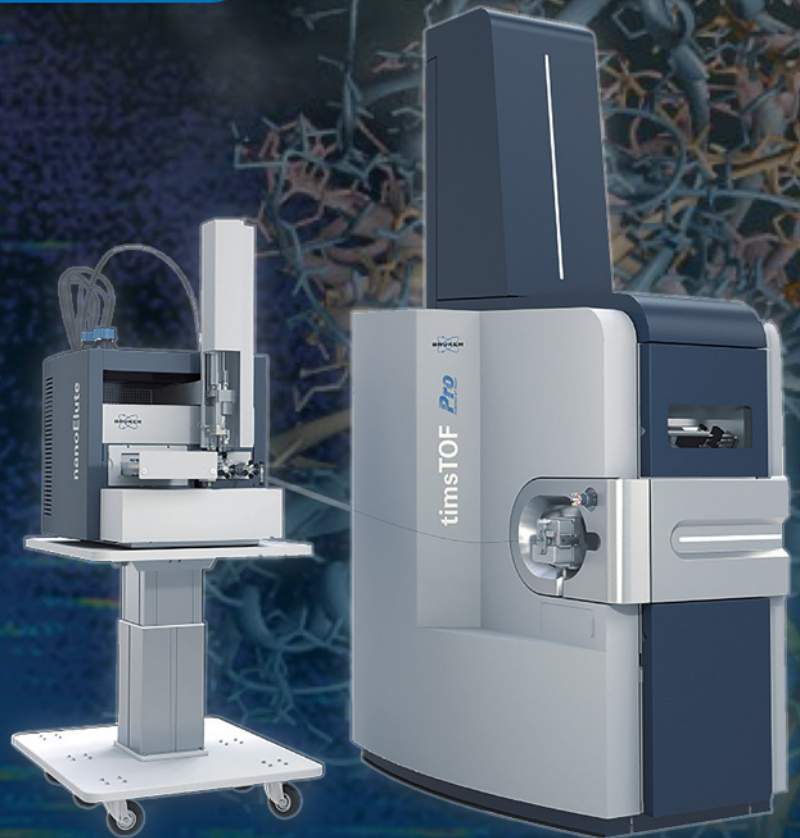




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**SPECIAL ISSUE RESEARCH ARTICLE**

A positive pressure workstation for semi-automated peptide purification of complex proteomic samples

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Rationale: High-throughput reliable data generation has become a substantial requirement in many “omics” investigations. In proteomics the sample preparation workflow consists of multiple steps adding more bias to the sample with each additional manual step. Especially for label-free quantification experiments, this drastically impedes reproducible quantification of proteins in replicates. Here, a positive pressure workstation was evaluated to increase automation of sample preparation and reduce workload as well as consumables.

Methods: Digested peptide samples were purified utilizing a new semi-automated sample preparation device, the Resolvex A200, followed by nanospray liquid chromatography/electrospray ionization (nLC/ESI) Orbitrap tandem mass spectrometry (MS/MS) measurements. In addition, the sorbents Maestro and WWP2 (available in conventional cartridge and dual-chamber narrow-bore extraction columns) were compared with Sep-Pak C18 cartridges. Raw data was analyzed by MaxQuant and Perseus software.

Results: The semi-automated workflow with the Resolvex A200 workstation and both new sorbents produced highly reproducible results within 10–300 µg of peptide starting material. The new workflow performed equally as well as the routinely conducted manual workflow with similar technical variability in MS/MS-based identifications of peptides and proteins. A first application of the system to a biological question contributed to highly reliable results, where time-resolved proteomic data was separated by principal component analysis (PCA) and hierarchical clustering.

Conclusions: The new workstation was successfully established for proteolytic peptide purification in our proteomic workflow without any drawbacks. Highly reproducible results were obtained in decreased time per sample, which will facilitate further large-scale proteomic investigations.

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1 | INTRODUCTION

Purification of proteolytic peptides prior to sensitive nanospray liquid chromatography/tandem mass spectrometry (nLC/MS/MS) analysis is one important technique in bottom-up proteomics.¹ Especially for preparing and interpreting quantitative data, highly reproducible methods are required.^{2–4} However, the high manual workload in a proteomic sample preparation workflow has several drawbacks in terms of contamination risk, time needed, costs and introduced bias to the sample. Routinely, the peptide purification is performed via reversed-phase solid-phase extraction (SPE) chromatography^{5,6} in a manual tip or cartridge format. To overcome challenges at this step, SPE technology together with a positive pressure workstation turned out to be a powerful tool in proteomic sample preparation. Positive pressure systems are well known and suitable for simultaneous preparations.⁷ In addition, workstations including solvent dispensing and computer-controlled pressure gradients are highly valuable in saving time, consumables and costs while maintaining reproducible parallel preparation of up to 96 samples. As several deep proteome investigations have been published recently,^{8,9} it is evident that the general need for high-throughput methods has greatly increased also for proteomic investigations over recent years.

Here, the positive pressure solid-phase extraction (ppSPE) technology was evaluated by using a new semi-automated sample preparation device with silica (Maestro) and polymeric (WWP2) reversed-phase sorbents. For comparison, a routinely performed manual peptide purification workflow using Sep-Pak C18 cartridges was conducted. Sep-Pak cartridges contain C18-modified, silica-based sorbent, which is additionally endcapped – thus supporting hydrophobic characteristics.¹⁰ In contrast to this endcapped chemistry, the Maestro sorbent is unendcapped and characterized by a much smaller particle size of 10 μm than Sep-Pak, with a particle size specified in a range of 55–105 μm . Another type of sorbent is polymeric WWP2 with a particle size of approx. 30 μm . Both sorbents tested here are available in conventional column design (similar to Sep-Pak cartridges) and in a special dual-chamber design, called Narrow Bore Extraction™ (NBE™). NBE columns are characterized by an airlock technology for in-cartridge-based sample preparations, which is similar to the well-established in-StageTip method.¹¹ The use of this feature possibly reduces the need for consumables and at the same time should minimize sample loss through decreased sample transfer.

Whenever the workflow of sensitive nLC/MS/MS analysis is changed, several aspects need to be considered, for example, the introduction of leaching and/or interfering substances. Leachables are a known problem during reversed-phase purification of pharmaceutically relevant proteins.¹² However, in addition to contamination of industrial-scale preparative workflows, leachables should also be considered for proteomic workflows in terms of potential damage to expensive column material, ion suppression or contamination of sensitive MS components.^{13,14} Therefore, the two new sorbents were tested with blank samples to allow assessments about any leaching of nLC/MS/MS-interfering substances.

Two different set-ups were used to evaluate the advantages and limitations of the workstation and the two sorbents, Maestro and WWP2, in the proteomic sample preparation workflow. First, the manual workflow with Sep-Pak cartridges was performed in three technical replicates and the automated workflow was carried out in comparison using two different input samples for the two new sorbents: Maestro and WWP2 (Figure 1A). In a second set-up, varying amounts of peptides were purified using the semi-automated Resolvex A200 workstation (Tecan Group Ltd, Männedorf, Switzerland) using the different sorbent and column combinations: Maestro NBE, WWP2 NBE and, in conventional SPE cartridges, WWP2 Cerex as well as Sep-Pak C18 (Figure 1B).

The optimized workflow was then used to answer a question important to the application of proteomics in industrial cell culture technology: Does a cellular proteome reproducibly change within three individual and independent fed-batch cultivation processes or does cell heterogeneity predominate?

2 | MATERIALS AND METHODS

2.1 | Protein extraction

Cells were lysed with glass beads and 1 mL 10 mM Tris-HCl, 1 mM EDTA, pH 8.8, were added to a pellet of $2 \cdot 10^7$ Chinese hamster ovary (CHO) K1 cells. The suspension was mixed in three cycles for a period of 15 s, where samples were allowed to cool down between the cycles. Proteins were separated from cell debris using a 20 min centrifugation step at 16,000 g and 4°C. The supernatant was transferred to a new reaction tube and protein concentration was measured via the biconchonic acid (BCA) method.

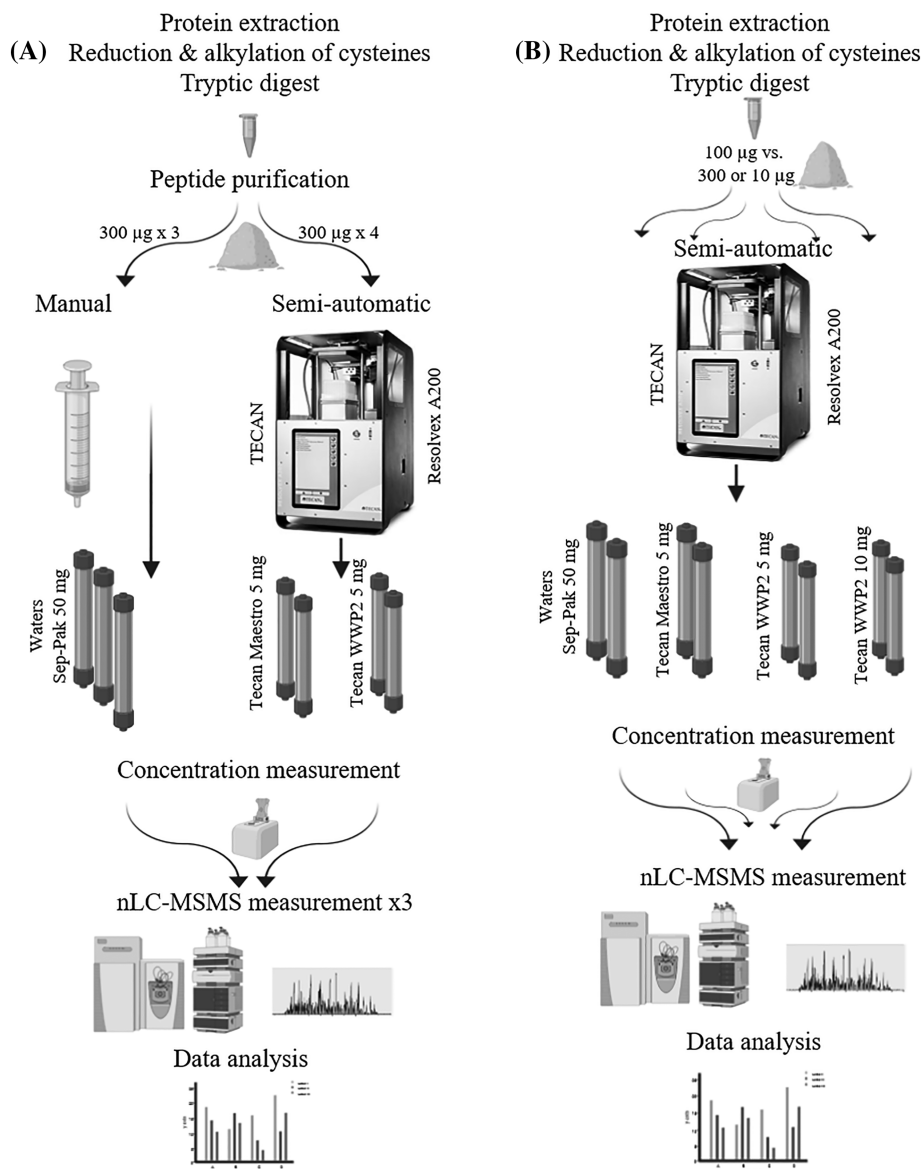
2.2 | Tryptic digest

The reduction and alkylation of cysteines were performed with the appropriate amount of starting material (10–300 μg protein) and initiated by adding 7 mM dithiothreitol (DTT) for 30 min and centrifugation at 200 rpm at 56°C. Proteins were allowed to cool down and were further incubated with 20 mM indole acetic acid (IAA) for 30 min at room temperature in the dark. The reaction was stopped by adjusting the DTT concentration to 20 mM for 40 min. Proteins were then enzymatically digested with Trypsin Gold (Promega, Madison, WI, USA) in a protein:enzyme ratio of 1:100 overnight at 37°C.

2.3 | Peptide purification

The reversed-phase (RP)-based automatic purification of digested proteins was evaluated and compared with the routinely performed manual cleanup with 50 mg Sep-Pak C18 Vac cartridges (Waters, Milford, MA, USA). For the examination of the Tecan cartridges the

FIGURE 1 A, The first set-up was used to compare two NBE sorbents (Tecan) within the automated device against manual purification of peptides with Sep-Pak cartridges (Waters). B, In the second set-up two NBE sorbents (Tecan) were directly compared with Sep-Pak cartridges (Waters) within the automated device using an extra cartridge holder. In parallel, conventional SPE (10 mg bed mass) and NBE (5 mg) WWP2 cartridges were compared



following different sorbents and sizes were used: 5 mg Maestro NBE columns, 5 mg WWP2 NBE columns, and 10 mg Cerex® WWP2 SPE columns (Table 1). For semi-automatic peptide purification the Resolvex™ A200 positive pressure workstation (Tecan Group Ltd) with an integrated solvent dispenser was used. The columns were conditioned with 900 µL 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) and then equilibrated with 900 µL 0.1% TFA, each in LiChroSolv water (Merck, Darmstadt, Germany). The samples

(pH <4) were loaded in a volume of max. 300 µL and washed with 900 µL 0.1% TFA. A deepwell plate was placed below the columns and 300 µL 80% ACN in 0.1% TFA was added to elute the bound peptides. Samples were dried in a Concentrator plus vacuum centrifuge (Eppendorf AG, Hamburg, Germany) and peptides were dissolved in 2.5% ACN in 0.1% TFA. A peptide concentration measurement for all samples was performed with a NanoDrop One photometer (Thermo Fisher Scientific, Waltham, MA, USA).

TABLE 1 Characterization of different sorbents used for peptide purification

	Sorbent substrate	Particle size (µm)	Chemistry	Column design
Tecan NBE Maestro	Silica	10	C18, unendcapped	Dual-chamber
Tecan NBE WWP2	Polymer	10	Reversed phase	Dual-chamber
Tecan Cerex WWP2	Polymer	10	Reversed phase	Dual-chamber
Waters Sep-Pak	Silica	55–105	C18, endcapped	Conventional

[Correction added on 15 September 2020, after first online publication: The 2nd – 4th rows of Table 1 were omitted and have been restored.]

2.4 | nLC/MS and data analysis

Peptides were injected into an Ultimate 3000 nanoLC system (Thermo Fisher Scientific, Dreieich, Germany) equipped with an Acclaim™ PepMap™ 100 C18-based prepurification column (300 μm ID × 5 mm) and a Acclaim™ PepMap™ 100 C18 analytical column (2 μm, 75 μm × 250 mm). The nLC system was coupled to an Orbitrap tandem mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Dreieich, Germany), where peptides were measured online by electrospray ionization (ESI) in positive ion mode over a mass range of m/z 350 to 2000. In full MS scanning mode the resolution was set to 70,000 with an AGC target of $3e6$ and 64 ms maximum IT. For dd-MS² a resolution of 17,500 was configured with an AGC target of $2e5$ and 100 ms maximum IT. In a top10 measuring mode the normalized collision energy was set to 28 and precursor ions were isolated with a window of 1.6 m/z units. In addition, selected precursors required a minimum AGC target of $8e2$ and an intensity threshold of $8e3$. The workflow test samples were measured within a 40 min LC gradient from 4% B (80% ACN in 0.1% FA) to 50% B with a configured dynamic exclusion of 15 s. The fed-batch cultivation samples were separated and measured within an effective LC gradient of 60 min.

Data was analyzed using MaxQuant (1.6.10.43) software with integrated Andromeda search engine and further evaluated with Perseus (1.6.10.43). For database searches the two UniProt TrEMBL protein databases of *Cricetulus griseus* and *Mus musculus* were used. The set-ups were analyzed separately without the match-between-runs function. Unlike the default parameters only unique peptides were used for quantification. The proteinGroups and peptides table were loaded into Perseus software and the data was filtered for contaminants, reverse hits and proteins, which are only identified by peptides carrying a modification. The raw data was log₂-transformed to convert zero values into "NaN" and multi-scatter plot and Venn diagram function was used to inspect the data. The fed-batch

cultivation data was statistically evaluated by performing two sample t-tests (permutation-based false discovery rate (FDR) <0.05, SO: 0.1) and clustered by principal component analysis (PCA), which made an additional filter step for 100% valid values necessary.

Detailed RSLCnano and Q Exactive Plus parameters are available as an online resource table (see supporting information). The MS proteomics data have been deposited to the ProteomeXchange consortium via the PRIDE¹⁵ partner repository with the dataset identifiers PXD018444 (workflow tests) and PXD018439 (fed-batch cultivations).

3 | RESULTS AND DISCUSSION

3.1 | Establishment of semi-automated peptide purification and general comparison with manual workflow

Peptides purified with both new sorbents, Maestro and WWP2, in NBE column format using the Resolvex A200 workstation showed high Pearson correlation of 99.3% in nLC/MS/MS measurements (triplicates each, Figure 2A). In addition, the manual workflow with Sep-Pak cartridges (triplicates on two levels: purification and measurement) showed equally good Pearson correlation of 99.6%. Differences in numbers of identified proteins were relatively small with 1104 proteins for Sep-Pak (mean of three replicates), 969 proteins for Maestro and 1029 proteins for WWP2-based purification, each resulting from 1/100 injected peptides of 300 μg digested CHO whole cell lysate.

The parallel executed blank samples for the Maestro and WWP2 sorbents did not show any absorption in the UV trace and only normal background spectra in TIC (data not shown). Consequently, no indications for any leaching of LC- or MS-interfering substances are given.

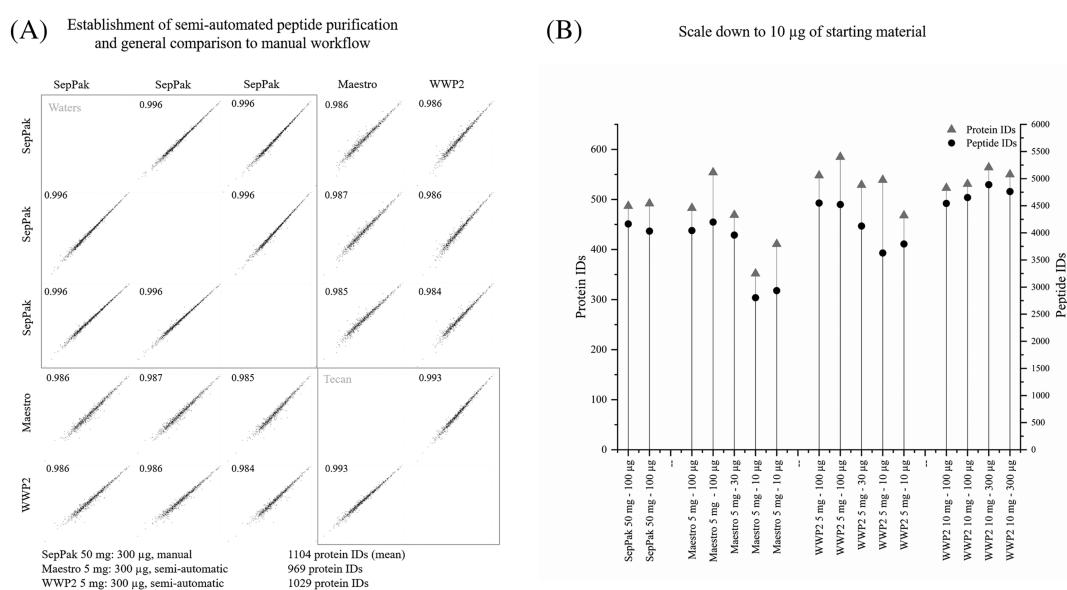


FIGURE 2 A, Label-free quantified proteins of the first experimental set-up in a multi-scatter plot including Pearson correlation coefficient. B, Identified peptides and proteins for the second experimental set-up with varying amounts of starting material

With the semi-automatic workflow in 96-well format it is possible to process 4.8-fold more samples in parallel than when using a standard vacuum manifold. The integrated solvent dispenser allows a reduction from five manual pipetting steps to one step – the sample load. Eight samples are automatically filled in parallel and filling up 96 samples takes less than 40 s. Both advantages represent considerable savings in time, down to around 20 min total time needed for 96 samples.

3.2 | Scale down to 10 μg of starting material

In a scale-down approach the recovery of samples was evaluated, which is of particular importance for low-input clinical proteomics.^{8,16} Here, Sep-Pak cartridges were tested for the first time directly within the Resolvex A200 workstation (100 μg of starting material, Figure 2B). Within a 40 min nLC gradient online coupled to the Orbitrap mass spectrometer *ca* 4100 peptides were identified. In parallel, the 5 mg Maestro columns yielded almost similar identification numbers of *ca* 4120 peptides (duplicate purification). Compared with this, in the 30 μg -based sample almost no losses were detectable (3959 peptides). The 10 μg -based samples only resulted in identification of *ca* 2900 peptides (duplicate purification). However, the injected peptide amount was not normalized for 10 μg compared with the others (1 μg), which may lead to incorrect assessments about the scalability for this sorbent. Generally, similar findings were recorded for the parallel-performed 5 mg WWP2 sorbent-based purifications, although the identification rates were slightly increased (*ca* 4500 for 100 μg , 4126 for 30 μg and *ca* 3700 for 10 μg of starting material). For WWP2, the injected peptide amount of the 10 μg -based sample was adjusted to the same peptide amount as for 100 μg - and 30 μg -based injections, leading to more consistent results. In addition, another available type of WWP2 cartridges containing 10 mg of sorbent without dual-chamber design was tested. Here, 100 and 300 μg of starting material performed equally as well as the dual-chamber version of WWP2 and Maestro (*ca* 4600 for 100 μg and 4800 for 300 μg of starting material).

Counted back the 1 μg which was injected into the nLC/Orbitrap mass spectrometer of the 10 μg -based Maestro sample originates from around 6500 CHO cells. However, for the same instrumental set-up and parameters the injection of 200 ng HeLa protein digest results in the identification of three times more proteins. The discrepancy in identification levels of less input and higher output is based to a great extent on the availability of high-quality CHO-specific databases. Experiments for decreasing the amount of input cells to low-input clinical ranges of less than 1000 cells should be performed with human material to exclude protein database-based limitations.

In summary, the Maestro and WWP2 sorbents showed highly reproducible results within a range of 10–300 μg of CHO peptide starting material. No differences were evaluated between the NBE dual-chamber design and the conventional SPE design of WWP2 sorbent in the tested range of peptide amounts.

3.3 | Application of the workflow for proteomic analysis of CHO fed-batch cultivations

Samples from a CHO cell fed-batch cultivation process were used to apply the workflow to the question of whether the cellular proteome reproducibly changes during independent fed-batch cultivation processes. The cellular cultivations were started from three vials of one working cell bank and cultivated for three passages (each three to four days and doublings) prior to inoculation of the bioreactors. Daily sampling started in the exponential growth phase at day 3 going on until day 11, when the viability dropped below 70% (data not shown).

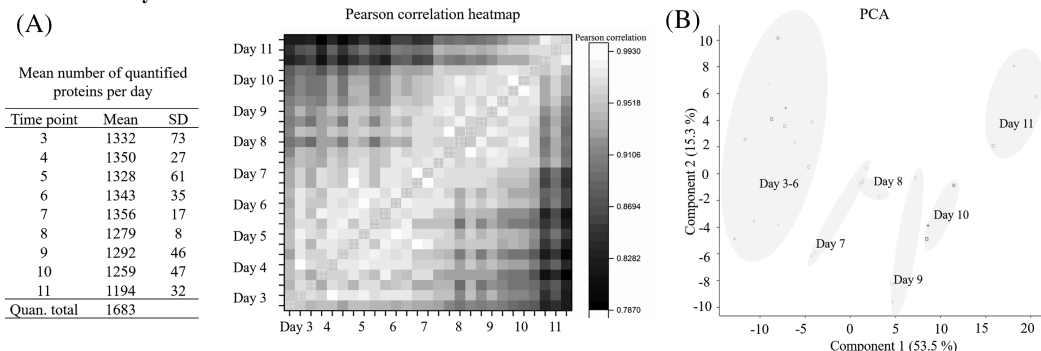
Proteomic samples were prepared, and peptides were purified, within the Resolvex system with WWP2 sorbent and measured in one batch to reduce bias through potentially different laboratory conditions.

The acquired data of nine time points for three cultivations (27 nLC/MS measurements) resulted in the identification of *ca* 2500 proteins, quantification of *ca* 1700 proteins and a mean Pearson correlation of $93.7 \pm 4\%$ (following filtering for at least three valid values), with higher correlation between biological replicates than distant time points (Figure 3A). For PCA a filter for 100% valid quantitative values was applied. The PCA 'component 1' (53.5%) separates the different time points in largely chronological order, whereas for the exponential growth phase only (days 3 to 6) no trend is obvious. In general, biological replicate three shows higher difference than the other two biological replicates, where e.g. the day 7 sample clusters together with day 8 samples of biological replicates one and two (Figure 3B).

For statistical analysis, grouping of biological replicates was applied and time point comparison via two-sample t-test (permutation-based FDR <0.05, SO: 0.1) was performed to extract differential expressed proteins. The numbers of statistically significant hits were visualized in a heatmap (Figure 3C). Overall, increasing differences in the CHO cellular proteomes from day to day with progressing cultivation time were observed, where, for example, only 11 significant changes were calculated between days 7 and 8, but 126 significantly different expressed proteins for a day 10 to day 11 comparison. In addition, an analysis of variance (ANOVA) (permutation-based FDR <0.05, SO: 0.1) was calculated and significant hits were filtered, normalized and clustered based on Euclidean distance. A distance adjustment factor was calculated to prominent clusters were visualized as profile plots (Figure 3D). Cluster 1 holds 322 proteins with decreasing protein expression values during CHO cell fed-batch cultivation, while cluster 2 holds only 180 proteins with increasing expression of respective proteins. Proteins in cluster 3 (75 proteins) were initially up-regulated but then again down-regulated over time, whereas cluster 4 (11 proteins) holds proteins with an opposite trend.

To evaluate affected biological processes Fisher's exact enrichment test (Benjamini-Hochberg FDR <0.02) for gene ontology (GO) and KEGG annotations was performed on the extracted clusters. For up-regulated proteins the annotations *hydrogen transport*, *tricarboxylic acid cycle* and *cofactor catabolic process* were significantly enriched. The terms *Splicosome* and *mRNA processing* were calculated

Raw data analysis



Statistical analysis

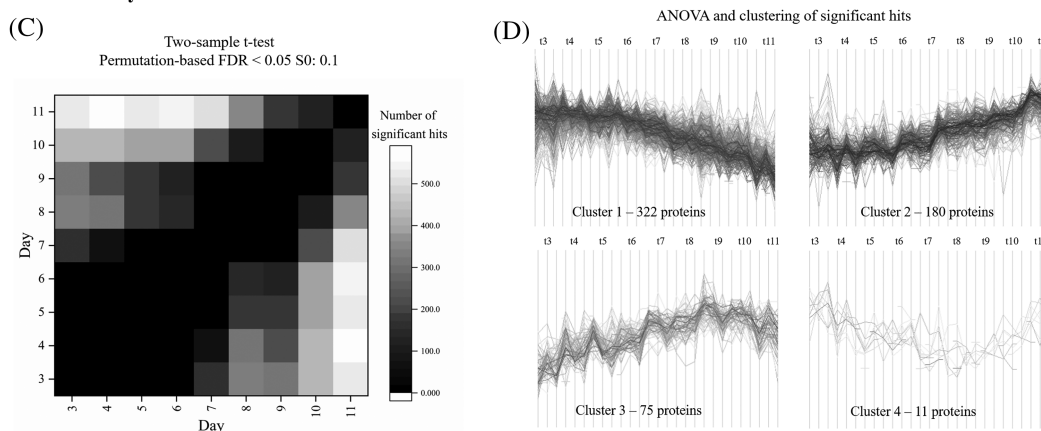


FIGURE 3 A, Overview of quantified proteins per investigated cultivation day and Pearson correlation heatmap of 27 analyzed nLC/MS measurements (filtered for at least three valid values in total). B, Principal component analysis filtered for proteins holding quantitative values in all samples (100% valid). C, Compiled results of significant differences between time points calculated by two-sample t-tests (permutation-based FDR < 0.05, S0: 0.1). D, ANOVA (permutation-based FDR < 0.05, S0: 0.1) based significant differential expressed proteins clustered via Euclidean distance and average linkage method. The four (out of six) most prominent clusters were visualized in profile plots

to be significantly enriched for down-regulated proteins. For the curved profile cluster 3 the term with the highest enrichment factor was calculated to be *tRNA aminoacylation for protein translation*. Hence, this new data revealed interesting insights into cell altering during fed-batch cultivation with increasing nutrient limitations. With a reproducible sample preparation utilizing the positive pressure workstation it was possible to precisely analyze time-resolved proteomic changes.

4 | CONCLUSIONS

The new semi-automated positive pressure workstation Resolvex[®] A200 is of great benefit for proteomic sample preparation approaches such as the peptide purification techniques tested here. The use of the workstation drastically reduces the time required per sample and the amount of consumables. The evident benefits in combination with the investigated high reproducibility and ease of use make the workstation a optimal solution for most (proteomic) laboratories with increased sample throughput for sample preparations.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/rcm.8873>

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SUPPORTING INFORMATION

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