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# Application of Displacement Chromatography to Online Two-Dimensional Liquid Chromatography Coupled to Tandem Mass Spectrometry Improves Peptide Separation Efficiency and Detectability for the Analysis of Complex Proteomes

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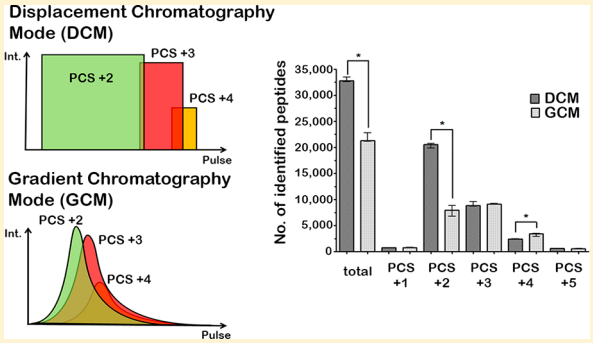
Supporting Information

**ABSTRACT:** The complexity of mammalian proteomes is a challenge in bottom-up proteomics. For a comprehensive proteome analysis, multidimensional separation strategies are necessary. Online two-dimensional liquid chromatography−tandem mass spectrometry (2D-LC-MS/MS) combining strong cation exchange (SCX) in the first dimension with reversed-phase (RP) chromatography in the second dimension provides a powerful approach to analyze complex proteomes. Although the combination of SCX with RP chromatography provides a good orthogonality, only a moderate separation is achieved in the first dimension for peptides with two (+2) or three (+3) positive charges. The aim of this study was to improve the performance of online SCX-RP-MS/MS by applying displacement chromatography to the first separation dimension. Compared to gradient chromatography mode (GCM), displacement chromatography mode (DCM) was expected to improve the separation of +2-peptides and +3-peptides, thus reducing complexity and increasing ionization and detectability. The results show that DCM provided a separation of +2-peptides and +3-peptides in remarkably sharp zones with a low degree of coelution, thus providing fractions with significantly higher purities compared to GCM. In particular, +2-peptides were separated over several fractions, which was not possible to achieve in GCM. The better separation in DCM resulted in a higher reproducibility and significantly higher identification rates for both peptides and proteins including a 2.6-fold increase for +2-peptides. The higher number of identified peptides in DCM resulted in significantly higher protein sequence coverages and a considerably higher number of unique peptides per protein. Compared to conventionally used salt-based GCM, DCM increased the performance of online SCX-RP-MS/MS and enabled comprehensive proteome profiling in the low microgram range.




Despite recent technological advances in mass spectrometry, which have led to increased speed, sensitivity, resolution, and mass accuracy, the complexity of mammalian proteomes remains a major challenge in bottom-up proteomics. Although high proteome coverages can be obtained with one-dimensional liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis using reversed-phase (RP) ultrahigh performance liquid chromatography (UHPLC) and modern high resolution orbitrap and Q-TOF instruments,[1,2] deep proteome profiling requires multiple dimensions of separation.[3] Two-dimensional liquid chromatography (2D-LC) separation is most often used with low pH RP chromatography directly coupled to MS analysis in the second dimension. For the first separation dimension, several techniques exist such as high pH RP chromatography,[4] hydrophilic interaction liquid chromatography (HILIC),[5] and strong cation exchange (SCX) chromatography.[6,7] Multidimensional peptide separation methods can be used either offline or online, having their own advantages and disadvantages.[8] Offline separation strategies offer a higher degree of freedom in optimizing parameters for each separation

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dimension. In addition, fractions collected in the first dimension can be reanalyzed and sample size can be reduced by combining different fractions. Online approaches are more easily automatable, offer an improved recovery, and reduce sample losses and contamination.[9]

One of these online approaches is the multidimensional protein identification technology (MudPIT) pioneered by Yates and coworkers, which combined SCX with RP-LC-MS/MS analysis and became the template for many online 2D-LC-MS/MS approaches.[6,7] In conventionally used online SCX-RP-MS/MS, the peptides are first loaded onto a SCX column, where they bind to the SCX material via electrostatic interactions. The peptides are usually eluted from the SCX column onto the RP column by injecting increasing concentrations of a volatile salt. In between the salt injections, the peptides are separated by an ACN gradient applied to the RP column and analyzed by tandem mass spectrometry. The SCX-RP-LC-MS/MS approach is the most frequently applied online 2D-LC-MS/MS approach in bottom-up proteomics. Under the acidic condition of the eluent with formic acid as an additive, which is mainly used for conventional online SCX-RP-MS/MS analysis, most tryptic peptides are doubly positive charged (+2). One charge is based on a basic residue such as lysine or arginine at the C-terminus. The second charge is due to the N-terminal amino group. Tryptic peptides with a three (+3) or even a higher number of positive charges are expected due to internal histidine, lysine, or arginine residues.[10] The combination of SCX with RP chromatography provides a good orthogonality but only a moderate separation efficiency for tryptic peptides in the first dimension (SCX) because peptides with two (+2) and three (+3) positive charges tend to elute in clusters.[11,12]

In the early 1940s, Tiselius defined an alternative chromatography mode called displacement chromatography (DC).[13] DC is based on competitive binding of the components themselves and the displacer molecule. First, the column is equilibrated with a carrier solution that is also used as the sample application buffer. The carrier solution must support a high binding affinity for the analytes toward the stationary phase.[14] During sample loading, the analytes arrange themselves according to their affinity in a process described as sample self-displacement.[15,16] The analyte with the highest affinity toward the stationary phase is binding to the chromatographic material at the top of the column, displacing analytes with lower affinities from their binding sites. Compared to conventionally used gradient chromatography (GC), 50−100% of the column binding capacity is used for sample loading in DC.[10,14] The analytes are eluted in DC by loading a molecule onto the column called "displacer", which is usually dissolved in the carrier solution. It is mandatory that the displacer has a very high affinity toward the stationary phase. Ideally, the displacer has a higher affinity toward the stationary phase than any of the sample analytes. Upon adsorption of the displacer to the stationary phase, it displaces the analyte with the highest affinity bound to the top of the column. Each displaced analyte itself acts as a displacer for the adjacent analyte with a lower affinity toward the column. This leads to the formation of a "displacement train".[14,17,18] In a displacement train, the analytes move down the column in a system of contiguous zones with rectangular shapes. The zones will keep their rectangular shape even if mass transfer resistances and slight kinetic or flow misdistributions are present, while these effects are responsible for bandspreading in elution chromatography.[14,19] The self-sharpening effect of the boundaries between the zones during the displacement process increases the effectiveness of the separation. Provided the column is sufficiently long and the analytes are present in sufficient quantities, each zone contains only one analyte in a high purity.[14] Thus, displacement chromatography mode (DCM) offers several advantages for peptide separation in SCX such as an improved separation of peptides in accordance to their charge state compared to gradient chromatography mode.[10,20] However, DCM has been so far only applied to proteomics in a few cases.

The aim of this study was to investigate whether DCM applied to the first separation dimension improves the performance of online SCX-RP-MS/MS compared to conventionally used GCM due to a better separation of peptides according to their charge states.

## EXPERIMENTAL SECTION

A detailed description of the materials and methods used for this study is included in the Supporting Information, whereas concise descriptions of the materials and methods are presented below.

**Online SCX-RP-MS/MS Setup.** An OPTI-PAK SCX trap column (5 $\mu$m particle size, 120 nL bed volume, Dichrome, Marl, Germany) was used for the online SCX-RP-MS/MS analysis. The SCX trap column was installed directly behind the injection valve (nanoACQUITY, Waters, Manchester, UK) or the autosampler valve (UltiMate 3000 RSLCnano, Dionex, Thermo Fisher Scientific, Bremen, Germany) (Figure S-1). The SCX trap column was connected to a RP trapping and a RP separation column that was directly coupled to a ESI-Q-TOF[21] (Q-TOF Premier, Micromass/Waters, Manchester, UK) or a ESI-Q-IT-OT[22] (Orbitrap Fusion, Thermo Fisher Scientific, Bremen, Germany). A detailed description of the online SCX-RP-MS/MS setup is included in the Supporting Information.

**Determination of SCX Binding Capacity and Displacer Pulses.** Binding capacity of the SCX trap column was determined by repeated injections of 400 ng tryptic peptides (HeLa digest, $c$ = 1 $\mu$g/$\mu$L, 0.1% FA dissolved in HPLC-$H_2O$, buffer A). Peptides were loaded on the SCX column with a flow-rate of 3 $\mu$L/min with 2% buffer B (0.1% FA, dissolved in ACN) and separated in the second dimension by RP chromatography using a linear gradient from 2 to 30% buffer B in 30 min (300 nL/min). Peptides were analyzed by ESI-Q-TOF-MS/MS as described in the Supporting Information.

Displacer pulses were determined by loading 5 $\mu$g of tryptic peptides (tryptic HeLa digest, $c$ = 1 $\mu$g/$\mu$L, dissolved in buffer A) on the SCX column (2% buffer B, 3 $\mu$L/min). Peptides were eluted from the SCX column by repeated injections of the displacer molecule spermine ($m$ = 25 ng, dissolved in buffer A), separated by RP chromatography and analyzed by ESI-Q-TOF-MS/MS as described above. For separation with optimized displacer pulses, 5 $\mu$g tryptic peptides (tryptic HeLa digest, $c$ = 1 $\mu$g/$\mu$L, dissolved in buffer A) were loaded on the SCX column (2% buffer B, 3 $\mu$L/min). Elution was achieved by injection of the following displacer pulses: pulse 1:150 ng spermine, pulse 2−5:25 ng spermine, pulse 6−8:50 ng spermine, pulse 9:1000 mM $NH_4Ac$ (dissolved in buffer A).

**Online SCX-RP-MS/MS Analysis in DCM and GCM.** For separation in DCM and GCM, 5 $\mu$g tryptic peptides (tryptic HeLa digest, $c$ = 1 $\mu$g/$\mu$L, dissolved in buffer A) were loaded on the SCX column (2% buffer B, 3 $\mu$L/min). Elution in DCM was achieved by injection of the displacer molecule spermine (dissolved in buffer A) and in GCM by injection of increasing $NH_4Ac$-concentrations (dissolved in buffer A). Details of


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displacer and $NH_4Ac$ injections are shown in Table 1. Peptides eluting from the SCX column were separated by RP chromatography with a linear gradient from 2 to 30% buffer B in 30 min (300 nL/min) and analyzed by ESI-Q-IT-OT-MS/MS as described in the Supporting Information.

**Table 1. Displacer (Spermine) and $NH_4Ac$ Pulses (Both Dissolved in 0.1% FA) Used for the SCX Separation in DCM and GCM**

| pulse | DCM | GCM |
|---|---|---|
| 1 | 150 ng spermine | 25 mM $NH_4Ac$ |
| 2 | 25 ng spermine | 50 mM $NH_4Ac$ |
| 3 | 25 ng spermine | 100 mM $NH_4Ac$ |
| 4 | 25 ng spermine | 150 mM $NH_4Ac$ |
| 5 | 25 ng spermine | 200 mM $NH_4Ac$ |
| 6 | 50 ng spermine | 250 mM $NH_4Ac$ |
| 7 | 50 ng spermine | 300 mM $NH_4Ac$ |
| 8 | 50 ng spermine | 500 mM $NH_4Ac$ |
| 9 | 1000 mM $NH_4Ac$ | 1000 mM $NH_4Ac$ |

**Data Analysis.** MS raw data were processed with MaxQuant (version 1.5.2.8). Peptide and protein identification was carried out with Andromeda against a human SwissProt database (UP000005640, downloaded November 10, 2016, 20 161 entries) and a contaminant database (298 entries). The searches were performed using the following parameters: precursor mass tolerance was set to 35 ppm (Q-TOF MS analysis) or 8 ppm (OT MS analysis) and fragment mass tolerance was set to 0.1 Da (Q-TOF MS/MS analysis) or 0.5 Da (IT MS/MS analysis). For peptide identification, two missed cleavages were allowed, a carbamidomethylation on cysteine residues (static modification), an oxidation of methionine residues, and acetylation of protein N-terminus (variable modifications). Peptides and proteins were identified with a false discovery rate (FDR) of 1%. Proteins were quantified with the MaxLFQ algorithm[23] considering only unique peptides and a minimum number of two unique peptides per protein.

For generating peptide charge state (PCS) plots, the charge of peptides at pH 2.3, the pH of the mobile phase of the SCX in DCM and GCM, of each identified peptide in relationship to its amino acid sequence was calculated. The calculation was performed with a Mathematica script (Wolfram research, version: 11.1.1.0) as follows: +1 for each basic amino acid (H, K, R), +1 for the N-terminus, unless the N-terminus was acetylated as described.[10]

**Data Availability.** The mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD008562.

## RESULTS AND DISCUSSION

**Determination of Binding Capacity and Displacer Pulses.** A mandatory step for a successful chromatographic separation in displacement chromatography mode is the determination of the total binding capacity of the sample and the displacer for the chosen column. The column should be saturated with the sample, and appropriate amounts of the displacer have to be applied. The binding capacity of the SCX column was experimentally determined by repeated injections of tryptic peptide aliquots ($m$ = 400 ng) onto the SCX column in the online SCX-RP-MS/MS setup (Figure S-1 A). Base peak chromatograms (BPCs) of the first 12 injections showed almost identical peak patterns with no considerable differences from the first to the twelfth injection (Figure S-2). The peaks in the BPC were mainly caused by peptides with a peptide charge state (PCS) of +1, such as peptides from both the N-terminal and C-terminal region of the proteins (Figure S-3 A). The affinity of these peptides toward the SCX material was not high enough to be retained under the loading conditions. The term PCS used in this manuscript describes the charge states of individual peptides in the mobile phase of the SCX at a pH of 2.3 and is not to be confused with the charge state $z$ of peptides in the gas phase of the mass spectrometer. With the 13th injection, a change in the BPC was observed (Figure S-2) and the number of emerging peaks was caused by peptides with a PCS of +2 (Figure S-3 B). These peptides eluted from the SCX column due to low affinities and/or sample displacement.[24] According to the amount of injected peptides necessary to cause a change in the BPC, the binding capacity of the SCX column was between 4.8 and 5.2 $\mu$g.

Next, amounts for displacer pulses were determined. For this, 5 $\mu$g of a tryptic HeLa digest were loaded onto the SCX column. For elution, the polyamine spermine was used as a displacer. At pH 2.3, spermine has a charge state of +4. Peptides were eluted from the SCX by pulsed injections of 25 ng spermine. To visualize the efficiency of the SCX separation, the number of identified peptides with PCSs ranging from +1 to +5 was plotted against the displacer pulses to obtain a PCS plot (Figure S-4). Sample loading (pulse 0) resulted mainly in the detection of peptides with a PCS of +1 that had the lowest affinity toward the SCX column. The pulsed displacer injections resulted in a good separation of peptides according to their PCSs. Peptides with a PCS of +2 (pulse 4−13) eluted first followed by peptides with PCS of +3 (pulse 14−20) and +4 (pulse 18−20). Sharp boarders between the different PCS zones with a low degree of overlapping highlights the separation power of displacement chromatography mode. These results are consistent with the results obtained by Ahrends et al.[10] and Trusch et al.[20] for SCX separation of tryptic peptides in DCM using spermine as a displacer. For optimizing both separation and analysis time, the amount of the displacer was fitted to the observed elution behavior (Figure S-4). The separation of 5 $\mu$g of a tryptic HeLa digest with the defined displacer pulses resulted in a good separation of the peptides according to their PCSs (Figure S-5). Peptides with a PCS of +2 mainly eluted within the first 5 displacer pulses, followed by peptides with a PCS of +3 (displacer pulse 6−9) and +4 (displacer pulse 8 and pulse 9 (1000 mM $NH_4Acet$)). Compared to the approach of Trusch et al.,[20] the total analysis time was reduced from almost 120 h, and a sample amount of more than one milligram to 8.5 h and a sample amount of 5 $\mu$g. Once the binding capacities and displacer pulses are defined and optimized, these parameters can be used for online SCX-RP-MS/MS analysis in displacement chromatography mode for complex proteomes after tryptic digestion because the peptide charge state distribution of tryptic peptides is similar for human, mammals, yeast, and bacteria (Table S-1).

**Comparison of Displacement Chromatography Mode with Gradient Chromatography Mode.** *Separation Space.* To investigate whether DCM improves the performance of online SCX-RP-MS/MS compared to conventionally used salt-based GCM in terms of a better separation of peptides according to their PCSs, 5 $\mu$g of a tryptic HeLa digest were separated either by DCM or GCM. Visualization of the SCX separation showed that with both DCM and GCM, a separation of peptides according to their PCSs was achieved (Figure 1). However, the



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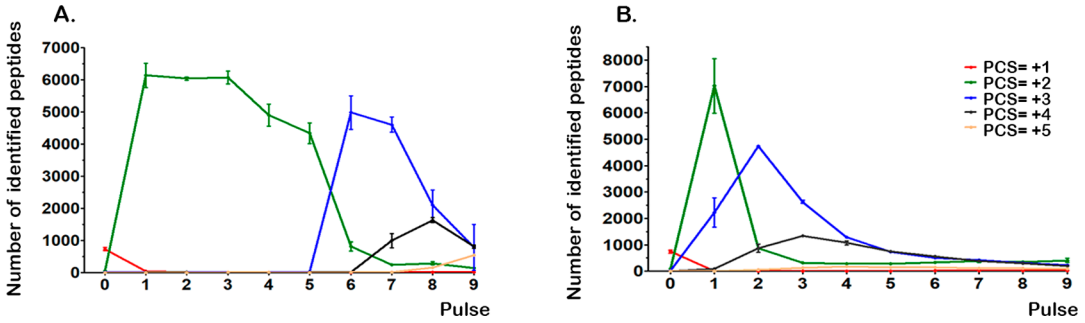


**Figure 1.** SCX separation of tryptic peptides in displacement chromatography mode (A) and gradient chromatography mode (B) in online SCX-RP-MS/MS analysis. For visualization of the SCX separation, the number of identified peptides with peptide charge-states (PCS) ranging from +1 to +5 were applied against the pulse number (median with SD). The PCS of the peptides were calculated based on their amino acid sequence at pH of 2.3, the pH of the mobile phase during SCX.

elution profiles were considerably different. While in GCM most of the peptides with a PCS of +2 eluted within the first two ammonium acetate ($NH_4Ac$) pulses (Figure 1B), peptides with a PCS of +2 were separated over 5 displacer pulses in DCM (Figure 1A). This observation is consistent with the theory of displacement chromatography, where analytes with a low affinity toward the stationary phase, such as peptides with a PCS of +2, elute in broad zones.[14] With increasing affinity, analytes elute in zones with decreasing width and higher concentrations, which is consistent with the results observed for the majority of peptides with a PCS of +3 and +4 that eluted within three displacer-pulses each. In GCM, peptides with a PCS of +2 and +3 showed a distinct coelution upon the first pulse at a low $NH_4Ac$ concentration ($c$ = 25 mM). Furthermore, a broad elution width and coelution of peptides with PCS of +3 and +4 was observed in GCM. This observation made in GCM is consistent with the results of Gilar et al.,[11] who investigated the orthogonality of separation in 2D-LC, and revealed that in SCX-RP peptides with a PCS of +2 and +3 cannot be effectively separated from each other in the first dimension of separation (SCX) using GCM. In contrast, DCM provided an almost complete separation of peptides with a PCS of +2 and +3 in remarkably sharp zones with an extremely low degree of coelution (Figure 1A).

The average purity of peptides with a PCS of +2 within the first 5 displacer-pulses was 99.41% (±0.24%, Figure S-6), whereas purity of peptides with a PCS of +2 in the first $NH_4Ac$-pulse of GCM was 75.28% (±2.35%). Even for peptides with a PCS of +3 and +4, a good separation was achieved in DCM resulting in a purity for peptides with a PCS of +3 of 85.56% (±2.69%, pulse 6), 78.73% (±3.2%, pulse 9), and 51.22% (±4.39%, pulse 8). Altogether, elution in DCM provided significantly higher purities of the most abundant PCSs in each fraction compared to GCM except for the last pulse, here, for both DCM and GCM $NH_4$−Ac injections were used. This result can be explained by the different elution processes in DCM and GCM. In DCM, the peptides elute from the column by pulsed injection of the displacer molecule. Due to its high affinity toward the stationary phase the displacer forces peptides, which have a lower affinity than the displacer to move down the column. These peptides displace peptides with an even lower affinity, and a displacement train is formed.[14,18] When the displacement train moves down the column, a self-sharpening effect increases the purity within the bands, namely of peptides with the same PCS. In GCM, peptides elute from the SCX column by suppression of electrostatic interactions between negatively charged groups of the stationary phase and positively charged groups of the peptide. Elution is achieved by injection of increasing $NH_4Ac$ concentrations, but the affinity of the $NH_4^+$-ions is not high enough to induce a displacement train like in DCM. A self-sharpening effect does not occur in GCM and peptides with a PCS of +2 and +3 coelute and are not separated from each other in sharp-zones as in DCM, thus application of DCM in the first separation dimension significantly improves the performance of online SCX-RP-MS/MS.

This was further confirmed by investigating the amount of information obtained in DCM and GCM computed with Shannon entropy (SE) (Table S-2). The degree of information obtained in DCM is higher than in GCM. Computation of SE showed that there were higher probabilities to find a peptide for all the pulses in DCM (Table S-2, Figure S-7). The reproducibility of DCM and GCM was further investigated at the level of reported peptide intensities (Figure 2, Figure S-8).

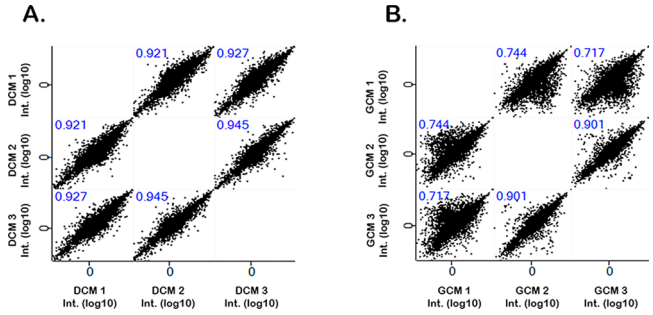


**Figure 2.** Scatterplot indicating Pearson's correlation between replicates of detected peptide intensities. DCM: displacement chromatography mode. GCM: gradient chromatography mode. Only peptides identified in all replicates were considered for the analysis.

DCM showed a considerably higher degree of reproducibility compared to GCM (DCM: Pearson's $r$ = 0.93 ± 0.007; GCM: Pearson's $r$ = 0.79 ±0.057). A possible explanation for the higher degree of reproducibility can be the better separation in DCM. This reduces sample complexity and may reduce ion suppression effects during MS analysis.[25]

*Peptide Identification Rates.* Investigation of peptide identification rates at the PCS-level revealed no significant differences for peptides with a PCS of +1, +3, and +5 (Figure 3). For peptides with a PCS of +4 a significantly higher number of peptides was identified using GCM ($n$ = 3,268 ± 336) compared to DCM ($n$ = 2,406 ± 80). This was expected, since spermine as a displacer with a charge state of +4 efficiently displaces peptides with PCSs of +1, + 2 and +3. For peptides with a PCS of +5, no


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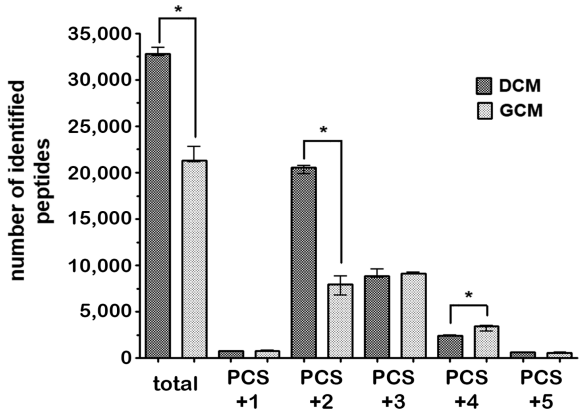


**Figure 3.** Comparison of peptide identification rates obtained in DCM and GCM. Bar chart (median with SD) showing the number of identified peptides (total) and the number identified peptides per peptide charge state (PCS: +1, +2, +3, +4, and +5) calculated from the peptide amino acid sequences at pH of 2.3, the pH of the mobile phase during SCX. *: $p < 0.05$ (Kruskal–Wallis).

significant differences were observed since these peptides were detected in DCM in the last pulse representing the $NH_4Ac$ injection. For peptides with a PCS of +1, no differences were observed as they were mainly detected after sample loading (pulse 0). For peptides with a PCS of +2, a remarkable and significantly higher number of identifications were achieved using DCM ($n = 20{,}360 \pm 266$) compared to GCM ($n = 7{,}858 \pm 607$, Figure 3). This is of particular relevance since peptides with a PCS of +2 represents the majority of tryptic peptides in human proteome samples after tryptic digestion (Table S-1). DCM led to a 2.6-fold increase for the most prevalent PCS (+2) due to the better separation efficiency for peptides with a low affinity like peptides with a PCS of +2, which cannot be achieved in GCM (Figure 1). Notably, the number of identified peptides with a PCS of +2 in DCM was almost as high as the total number of identified peptides in GCM (Figure 3). In general, DCM provided a 1.5-fold increase and a significantly higher number of peptide identifications compared to GCM. With DCM, 32,930 (±261) different peptides were identified compared to 21,740 (±526) different peptides in GCM.

The number of identified peptides in GCM ($21\,740 \pm 526$) is consistent with the number of identified peptides in the study from Krisp et al. (14 021).[26] To the best of our knowledge, the 5 $\mu$g sample amount, used in this study, represents together with the study of Krisp et al. the lowest sample amount used for online SCX-RP-MS/MS up to now. Compared to the study of Krisp et al., who analyzed 5 $\mu$g of a tryptic thyroid cancer cell line digest, a 2.3-fold increase in peptide identifications was achieved in this study, applying DCM to the first dimension of separation.

Magdeldin et al. used an online SCX-RP-MS/MS approach in GCM with similar $NH_4Ac$ pulses that were also used in this study to analyze a tryptic digest of HEK293 cells.[9] In their study, they used a sample amount of 100 $\mu$g and identified 24 771 peptides. Compared to the results obtained by Magdeldin al., the application of DCM to the first separation dimension of online SCX-RP-MS/MS revealed a higher number of identified peptides with a 20-fold lower sample amount.

A comparison of the peptide identification reproducibility based on the peptides identified in all replicates revealed that with DCM, a considerably higher degree of reproducibility was achieved compared to GCM. The application of GCM resulted in a peptide identification reproducibility of 59.4%, whereas DCM provided a reproducibility of 71.5% (Figure 4A). With DCM 23 533 different peptides were reproducibly identified,

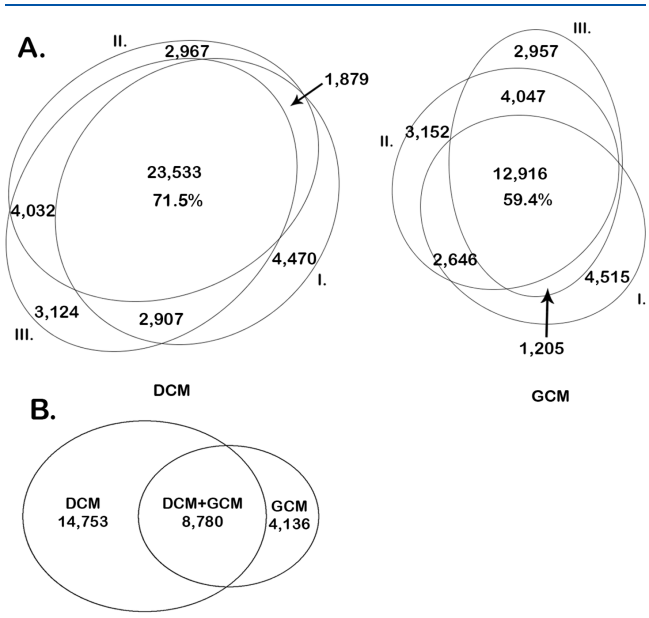


**Figure 4.** Comparison of peptide identifications obtained in DCM and GCM. A: Venn diagrams showing the reproducibility of DCM or GCM within three replicates (I, II, III). B: Venn diagram showing the number of peptides reproducibly identified in both (DCM + GCM) and exclusively in DCM or GCM.

and with GCM, 12 916 different peptides. A possible explanation for this observation is the better separation of the peptides over several fractions in DCM, thus reducing complexity and the effect of "undersampling".[27] A more detailed investigation of the reproducibly revealed that 8780 peptides were identified in both DCM and GCM (Figure 4B). However, the number of exclusively and reproducibly identified peptides was considerably higher in DCM ($n$ = 14 753) compared to GCM ($n$ = 4136). Interestingly, the number of peptides, exclusively identified in DCM, was even higher than the number of peptides identified with both elution modes ($n$ = 8780). These results reveal that DCM and GCM show a certain degree of orthogonality and clearly highlight the strength of DCM in the first dimension of an online SCX-RP-MS/MS approach to analyze complex peptide mixtures.

This is further underlined by the fact that the majority of tryptic peptides, derived from human samples, have a PCS of +2 under common low pH conditions used in online online SCX-RP-MS/MS (62.8%, Table S-3). This means that most peptides have a low affinity toward the SCX column under acidic conditions that are typically used in online SCX-RP-MS/MS approaches. In GCM the majority of identified peptides had a PCS of +3 (42.24%, +2-PCS: 36.35%), whereas the majority of identified peptides in DCM had a PCS of +2 (61.24%), followed by a PCS of +3 (27.3%), matching extremely well to the expected distribution of PCSs after in-silico digestion of the human proteome (Table S-3). Thus, DCM is very well suited to analyze complex proteomes after tryptic digestion by online SCX-RP-MS/MS due to a significantly improved separation and identification of peptides with a PCS of +2. These results showed that the application of DCM to the first separation dimension of online SCX-RP-MS/MS improves their performance in terms of separation efficiency and detectability, resulting


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in significantly higher peptide identification rates and a better reproducibility compared to GCM. Furthermore, the use of DCM represented an almost salt-free online SCX-RP-MS/MS approach compared to GCM. With the exception of the last pulse, which was used to elute the displacer molecule and to recondition the SCX column, no salt additives were necessary to elute the peptides from the SCX column. While the injection of increasing concentrations of $NH_4Ac$ in GCM led to an increase in pressure over time during the loading process, no pressure increase was observed in DCM. Thus, the application of DCM to the first separation dimension provided excellent compatibility with downstream RP-LC-MS analysis.

*Protein Identification Rates.* The performance of displacement chromatography mode (DCM) and gradient chromatography mode (GCM) was further compared at the protein level. Based on reported protein intensities, DCM showed a higher degree of reproducibility compared to GCM (DCM: Pearson's $r$ = 0.94 ± 0.006; GCM: Pearson's $r$ = 0.86 ± 0.028, Figure 5, Figure S-9). A comparison of the number of identified proteins, where at least two unique peptides had to be identified per protein, showed that a significantly higher number of proteins could be identified in case of DCM ($n$ = 3853 ± 36) compared to GCM ($n$ = 3266 ± 24, Figure 6 A). Compared to the study of Krisp et al.,[26] who identified 2850 proteins, a 1.4-fold increase in protein identifications was achieved ion our study using DCM. Considering proteins identified with at least one unique peptide per protein ($n$ = 4801 ± 79, Figure S-10), even a 1.7-fold increase in protein identification was achieved in our study using DCM. Compared to the study of Magdeldin et al.,[9] who identified 4636 proteins, a higher number of protein identifications was achieved in DCM ($n$ = 4801 ± 79) with a 20-fold lower sample amount. In addition, the number of identified proteins achieved in this study using DCM ($n$ = 4801 ± 79) was higher compared to the number of proteins identified by Rauniyar et al.[28] ($n$ = 4415 ± 69), who analyzed 50 $\mu$g of digested human bronchial epithelial cells by online SCX-RP-MS/MS in GCM with similar $NH_4Ac$ pulses also used in this study. The application of DCM to the first separation dimension improves the performance of online SCX-RP-MS/MS in terms sensitivity at the protein level due to a significantly higher identification rate compared to GCM.

The degree of reproducibility was considerably higher in case of DCM. While 79.5% of the proteins were identified in all replicates using GCM, DCM provided a reproducibility rate of 87.1% (Figure S-11 A), and a higher number of proteins were reproducibly and exclusively identified in DCM ($n$ = 991) compared to GCM ($n$ = 254, Figure S-11 B). A comparison of

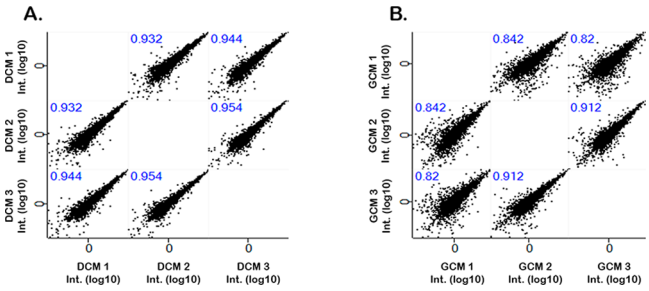


**Figure 5.** Scatterplot indicating Pearson's correlation between replicates of detected protein intensities. DCM: displacement chromatography mode. GCM: gradient chromatography mode. Only proteins identified in all replicates were considered for the analysis.

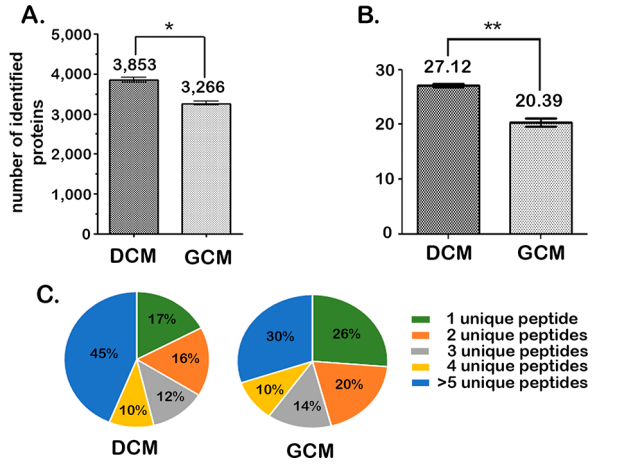


**Figure 6.** Comparison of DCM and GCM at the protein level. At least two unique peptides had to be identified for a protein to be taken into account. (A) Bar chart (median with SD) showing the total number of proteins identified in all replicates. *: $p < 0.05$ (Kruskal–Wallis). (B) Bar chart (median with SD) showing the average protein sequence coverage achieved in DCM and GCM. **: $p < 0.01$ (two-way ANOVA). (C) Distribution of the number of unique peptides identified per protein in percentage. Only proteins identified in all replicates were taken into account.

the number of identified unique peptides per identified protein revealed that in DCM proteins were identified with a higher number of unique peptides per protein compared to GCM (Figure 6C). In DCM, for 45% of the identified proteins, 5 or more unique peptides were identified, and only 17% of the proteins were identified based on one unique peptide. In GCM, 26% of the proteins were identified based on 1 unique peptide, and 30% of the identified proteins were identified based on 5 or more unique peptides. The higher number of identified unique peptides per protein can explain the higher level of reproducibility observed for reported protein intensities in DCM compared to those in GCM (Figure 5). The reported protein intensities were calculated based on the intensities of the identified unique peptides. Thus, a higher number of identified unique peptides per protein provides a more accurate and reproducible calculation of protein intensities, which is beneficial and of great importance for differential proteomics in general.

The better peptide separation in DCM and the associated increase in peptide identifications resulted in a significantly higher average protein sequence coverage in DCM (27.12%, ±0.2) compared to GCM (20.39%, ±0.44, Figure 6B). The number of proteins, for which a higher sequence coverage was obtained, was significantly higher in case of DCM compared to GCM (DCM>: $n$ = 1877 ± 47, GCM>: $n$ = 376 ± 38, Figure S-12).

The average protein sequence coverage obtained in this study using GCM is consistent with the results obtained by Rauniyar et al.,[28] who obtained an average protein sequence coverage of 22.02% for human bronchial epithelial cells using online SCX-RP-MS/MS in GCM with similar $NH_4Ac$ pulses also used in this study. The significantly higher protein sequence coverages obtained in DCM can be of interest for applications such as proteogenomics, where it is important to have high protein sequence coverages to identify genetic variability leading to protein sequence changes such as single amino acid variants and splice-junction peptides.[29,30]



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## CONCLUSIONS

This work reports on an improved online SCX-RP-MS/MS approach for the analysis of complex proteomes. The online SCX-RP-MS/MS system combines SCX chromatography in displacement chromatography mode (DCM) in the first dimension of separation and RP chromatography in the second dimension of separation. Applying DCM to the first dimension of separation appeared to have several advantages for analyzing complex peptide mixtures compared to conventionally used salt-based gradient chromatography mode (GCM).

First, DCM provided a better separation of peptides according to their charge states. Due to the formation of a displacement train during elution in DCM, peptides were separated from each other in remarkably sharp zones with a low degree of coelution. In particular, peptides with a charge state of +2 were separated over several fractions, which was not possible to achieve in GCM because the elution strength of ammonium acetate was insufficient to form a displacement train. This is of particular relevance for proteome analysis because peptides with a charge state of +2 represents the majority of complex proteomes after tryptic digestion. The better separation in DCM provided significantly higher identification rates at the peptide level and at the protein level, thus improving the performance of online SCX-RP-MS/MS in terms of detectability. Especially for peptides with a charge state of +2, a 2.6-fold increase in identifications was achieved in DCM.

Second, the higher number of identified peptides in DCM significantly increased the sequence coverage of the identified proteins and the number of identified unique peptides per protein. These results are beneficial for both differential proteomics and application such as proteogenomics. For differential proteomics, because the higher number of identified unique peptides per protein will provide more accurate and reproducible quantitative results. For proteogenomics, because high protein sequence coverages are necessary to identify genetic variability leading to changes within the protein sequence.

Third, the use of DCM in the first dimension of separation is an almost salt-free online SCX-RP-MS/MS approach, with the exception of the last pulse, which is used to recondition the SCX column. Thus, the application of DCM to the first separation dimension provides an excellent compatibility with downstream RP-LC-MS/MS analysis. Altogether, the reported online SCX-RP-MS/MS approach applying DCM to the first dimension of separation provided remarkably high peptide separation efficiencies, allowing for a sensitive and comprehensive analysis of complex proteomes in the range of a few micrograms.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02189.

Supplementary figures, tables, and a supplementary experimental section (PDF)

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### Notes

The authors declare no competing financial interest.

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