UvA-DARE (Digital Academic Repository)

Comprehensive Two-Dimensional Liquid Chromatography with Stationary-Phase-Assisted Modulation Coupled to High-Resolution Mass Spectrometry Applied to Proteome Analysis of *Saccharomyces cerevisiae*

Vonk, R.J.; Gargano, A.F.G.; Davydova, E.; Dekker, H.L.; Eeltink, S.; de Koning, L.J.; Schoenmakers, P.J.

DOI

[10.1021/acs.analchem.5b00708](https://doi.org/10.1021/acs.analchem.5b00708)

Publication date

2015

Document Version

Final published version

Published in

Analytical Chemistry

License

Article 25fa Dutch Copyright Act

[Link to publication](#)

Citation for published version (APA):

Vonk, R. J., Gargano, A. F. G., Davydova, E., Dekker, H. L., Eeltink, S., de Koning, L. J., & Schoenmakers, P. J. (2015). Comprehensive Two-Dimensional Liquid Chromatography with Stationary-Phase-Assisted Modulation Coupled to High-Resolution Mass Spectrometry Applied to Proteome Analysis of *Saccharomyces cerevisiae*. *Analytical Chemistry*, 87(10), 5387-5394. <https://doi.org/10.1021/acs.analchem.5b00708>

General rights

It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations

If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: <https://uba.uva.nl/en/contact>, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 CP Amsterdam, The Netherlands. You will be contacted as soon as possible.

Comprehensive Two-Dimensional Liquid Chromatography with Stationary-Phase-Assisted Modulation Coupled to High-Resolution Mass Spectrometry Applied to Proteome Analysis of *Saccharomyces cerevisiae*

Rudy J. Vonk,^{*,†} Andrea F. G. Gargano,^{†,‡} Ekaterina Davydova,[†] Henk L. Dekker,[§] Sebastiaan Eeltink,^{||} Leo J. de Koning,[§] and Peter J. Schoenmakers[†]

University of Amsterdam, [†]van't Hoff Institute for Molecular Sci, Analytical-Chemistry Group, and [§]Swammerdam Institute for Life Sciences, Mass Spectrometry of Biomacromolecules, Science Park 904, 1098 XH Amsterdam, The Netherlands

[‡]TI-COAST, Science Park 904, 1098 XH Amsterdam, The Netherlands

^{||}Vrije Universiteit Brussel, Department of Chemical Engineering, Pleinlaan 2, B-1050 Brussels, Belgium

Supporting Information

ABSTRACT: Stationary-phase-assisted modulation is used to overcome one of the limitations of contemporary comprehensive two-dimensional liquid chromatography, which arises from the combination of a first-dimension column that is typically narrow and long and a second-dimension column that is wide and short. Shallow gradients at low flow rates are applied in the first dimension, whereas fast analyses (at high flow rates) are required in the second dimension. Limitations of this approach include a low sample capacity of the first-dimension column and a high dilution of the sample in the complete system. Moreover, the relatively high flow rates used for the second dimension make direct (splitless) hyphenation to mass spectrometry difficult. In the present study we demonstrate that stationary-phase-assisted modulation can be implemented in an online comprehensive two-dimensional LC (LC $\times$ LC) setup to shift this paradigm. The proposed active modulation makes it possible to choose virtually any combination of first- and second-dimension column diameters without loss in system performance. In the current setup, a 0.30 mm internal diameter first-dimension column with a relatively high loadability is coupled to a 0.075 mm internal diameter second-dimension column. This actively modulated system is coupled to a nanoelectrospray high-resolution mass spectrometer and applied for the separation of the tryptic peptides of a six-protein mixture and for the proteome-wide analyses of yeast from *Saccharomyces cerevisiae*. In the latter application, about 20000 MS/MS spectra are generated within 24 h analysis time, resulting in the identification of 701 proteins.

For the analyses of complex samples, a separation technique with a high peak capacity is required. The increasing complexity of mixtures encountered in biomedical and environmental samples often exceeds the limits of peak capacity (n_c) achievable using traditional one-dimensional separations. The n_c obtained with one-dimensional gradient-elution liquid chromatography (LC) in relatively short analyses times is quite limited.

Recently, organic monolithic columns have been developed that yielded an impressive n_c of 1000, but at the cost of a 10 h gradient

Received: February 20, 2015

Accepted: April 20, 2015

Published: April 20, 2015

time.¹ The online combination of two very different (“orthogonal”) separation techniques creates attractive comprehensive two-dimensional (2D) separation devices that offer high peak capacities ($^{2D}n_c$) in relatively short analyses times. Examples are the combination of strong cation-exchange LC with reversed-phase LC (SCX $\times$ RP)^{2–5} and RP-LC coupled to capillary electrophoresis (CZE).⁶ Online comprehensive SCX $\times$ RP-LC, using pulsed elution in the first dimension, is widely applied in proteomics. It is at the heart of the multidimensional protein-identification technology (MudPIT), first described by Yates et al.^{7–9} Recent improvements have resulted in the identification of 4488 proteins with a microscale MudPIT.¹⁰ With this latter setup 39 ammonium-acetate (NH₄Ac) concentration steps were used to collect SCX fractions, each of which was subsequently analyzed by gradient-elution RP-LC coupled to mass spectrometry. A reduction of about 40% in total analyses time was obtained in comparison with conventional MudPIT.^{8,10} Nevertheless, the analysis time exceeded 16 h, with a protein identification rate of about 5 proteins per min. Other attempts have been made to identify more proteins, but at the expense of very long analyses times (>5 days).¹¹ An additional drawback of MudPIT derives from the pulsed nature of the first-dimension (¹D), which results in reduced first-dimension resolution and carryover of peptides in subsequent SCX fractions.¹² Up to 58% of the early eluting peptides have been reported to occur in several SCX fractions.^{13,14}

To make full use of the increased peak capacities when combining two or more separation mechanisms, it is essential that these are as orthogonal as possible, that is, that the retention times in one dimension are independent of those in another dimension.¹⁵ The highest n_c in the shortest analysis time can eventually be achieved by applying a spatial separation,¹⁶ for example by using a high-resolution version of thin-layer liquid chromatography.^{17,18} However, the practical requirements for such spatial separations are still demanding.

The total n_c of various multidimensional setups can be optimized using the Pareto-optimality approach.^{16,19,20} The Pareto-optimal front is formed by those points that cannot be bettered both in terms of analysis time and in terms of peak capacity. There is not one single optimum, but a series of Pareto-optimal points. The conditions at the obtained Pareto-optimal front correspond to combinations of narrow ¹D columns (small 1d_c) with intermediate particle sizes (3–5 μm) and short and wide ²D columns (large 2d_c) packed with the smallest possible particles to allow fast second-dimension analysis without exceeding the maximum backpressure of the system. Thus, optimum configurations are characterized by a high $^2d_c/^1d_c$ ratio.¹⁹ In Figure 1 the dashed line (a; partly obscured by the drawn line representing ideal modulation) represents such a combination ($^2d_c/^1d_c$ of 7). The gradual decrease in highest possible peak capacity ($^{2D}n_{c,th}$) with decreasing $^2d_c/^1d_c$ ratio is illustrated in Figure 1 by lines (b) to (f). Additional details are reported in the Supporting Information (section S.1).

So far, we have assumed that the fractions collected from the ¹D effluent are injected in the ²D in their entirety, without changing the volume or the concentrations of the analytes. This common situation is referred to as “passive modulation”. In contrast, in “active modulation” the volume is reduced and the concentrations are increased between the first- and second-dimension columns. Ideal modulation implies that the volume of the collected fractions is reduced to a value such that the second-dimension band broadening is not significantly affected, without losing any of the analytes. Thus, in a modulated system the

Figure 1. Implementation of modulation can be used to achieve the highest possible $^{2D}n_{c,th}$ in LC $\times$ LC with free choice of column dimensions. Pareto-optimal fronts are shown for the $^2d_c/^1d_c$ ratios of (a) 7 (partly obscured by the drawn line representing ideal modulation), (b) 4, (c) 2, (d) 1, (e) 0.5, (f) 0.25. In the present paper, a ratio of 0.25 is used with (nearly) ideal modulation.

$^2d_c/^1d_c$ ratio can be very small without affecting $^{2D}n_{c,th}$. The solid line in Figure 1 represents the case of ideal modulation, which is independent of the $^2d_c/^1d_c$ ratio. One aspect of LC $\times$ LC in proteomics is that the detection of low-abundant analytes necessitates a relative large first-dimension column with a high loadability.^{21,22} The sensitivity of MS, on the other hand, increases when reducing the LC effluent flow to the nanolitre-per-minute range.²³ These opposing requirements for the two dimensions imply a compromise is needed for hyphenation. By implementing stationary-phase-assisted modulation (SPAM), a form of active modulation, a relatively large capillary-scale ¹D column (0.3 mm internal diameter; i.d.) can be combined with a “nano-scale” ²D column (0.075 mm i.d.), which is fully compatible with nanospray interfaces for high-resolution MS. The apparent mismatch between the ¹D flow rate (1F ; typically $4 \mu\text{L}\cdot\text{min}^{-1}$) and the ²D flow rate (2F ; typically $400\text{--}500 \text{ nL}\cdot\text{min}^{-1}$) can be overcome by implementing SPAM. Other forms of active modulation are thermal modulation^{24,25} or solvent-assisted modulation.^{26,27} In the current study SPAM was achieved by substituting the empty loops that are typically present in LC $\times$ LC interfaces, with small traps that retain the peptides eluting from the first dimension. Salts from the ¹D effluent are washed from the trap prior to desorption of the analytes in a small volume to the analytical column. The basic principle of SPAM has been already demonstrated in the late 1970s for injecting large sample volumes in conventional one-dimensional LC.²⁸ This approach has later been adapted for the online collection of pulsed ¹D-fractions in an online SCX $\times$ RP setup^{3,4,13,29,30} operated with very long (>75 min) ²D RP analyses. When incorporating intermediate trapping (SPAM) in 2D analyses, the retentive properties of the trap need to exceed that of the ¹D column, but the desorption solvent should not be so strong as to cause breakthrough of the analytes from the ²D column.³¹ Trapping of analytes has been previously used to overcome mismatches in mobile-phase composition, for example for the combination of size-exclusion chromatography and RP-LC (SEC $\times$ RP),³² SCX $\times$ RP,^{3,4,14,29} and when using porous graphitic carbon (PGC) in RP $\times$ RP.³³ SPAM was also used for sample enrichment in an RP $\times$ RP setup.^{31,34}

In the current study active modulation was implemented to incorporate online desalting, to concentrate the analytes prior to

injection in the second dimension and to perform a relatively large number of short ^2D gradients to increase the overall peak capacity and the peak-production rate (i.e., peak capacity per unit time). The performance of in-house prepared organic-monolithic stationary phases was lower than that of commercially available silica-based packed-bed columns (see Supporting Information, section S.4). The applicability of SPAM is demonstrated in an LC $\times$ LC setup using a capillary-scale ^1D SCX-column in combination with a nanoscale ^2D RP-column hyphenated to a high-resolution Fourier-transform ion-cyclotron-resonance mass-spectrometer (FTICR-MS) for the analyses of a tryptic digest of a mixture of six proteins and the digested proteome of *Saccharomyces cerevisiae*.

To suppress the hydrophobic effects on the SCX column (and thus to increase the orthogonality of the two separation methods) organic modifier was added to the first-dimension mobile phase. To avoid the loss of hydrophilic peptides a makeup flow was introduced that was mixed with the first-dimension effluent to reduce the concentration of organic modifier prior to trapping in the modulator.³⁵ Additionally, in the proposed setup we performed online desalting of the collected fractions prior to the reversed-phase separation coupled to the FTICR-MS.

■ EXPERIMENTAL SECTION

Chemicals and Materials. For a detailed list of chemicals used, we refer to the Supporting Information, section S.2.

Sample Preparation of *Saccharomyces cerevisiae*. A tryptic digest of *Saccharomyces cerevisiae* (strain ATCC 204508/288c) was prepared in-house by the trichloroacetic acid-method as described in the Clontech Yeast Protocols Handbook.³⁶ In short, a pellet was extracted, corresponding to about 2–3 mg of proteins. The extract was transferred to a poly acrylamide gel-electrophoresis (PAGE) plate and the proteins were separated based on their size. The distance covered by the proteins in the gel was subdivided in eight (equally spaced) fractions. Each fraction was in-gel digested using trypsin.³⁷ The eluted peptide digests were dried in a vacuum centrifuge and dissolved in 30 μL of 2% FA in 50% ACN. Of each of the seven trypsin-digested fractions, discarding the fraction containing the smallest proteins, 1 μL or 2 μL of each fraction was pooled prior to analyses in matrix containing 0.1% TFA and 10% ACN in water.

Instrumentation and Chromatographic Conditions.
Instrumentation. All 1D analyses were performed on an Ultimate 3000 RSLCnano system (Thermo Scientific, Breda, The Netherlands). The system is essentially identical to that described previously.³⁸

Column Characterization. The performance of the commercially available RP column (Acclaim PepMap) and trap columns was compared to that of the in-house prepared organic-based monolithic columns. The RP columns were characterized using mobile phase A consisting of 0.1% (v/v) FA in H_2O and mobile phase B consisting of 20:80% (v/v) H_2O /ACN with 0.1% (v/v) FA. Prior to use, the eluent was degassed by sonication for 20 min. All gradient separations were performed at 30 $^\circ\text{C}$ using 1 μL full-loop injection, unless otherwise specified. The gradient times and compositions were adapted to obtain a maximal effective gradient window.

Online Desalting. The efficiency of the online desalting (removing the IEX-buffer from the ^1D effluent) in the LC $\times$ LC modulator was investigated using two methods. In the first method a UV-detector was used together with a 500 mM KBr solution (UV active, unlike KCl) containing 15% ACN as ^1D effluent, pumped through the traps at a flow rate of 1 $\mu\text{L}\cdot\text{min}^{-1}$.

The UV absorbance of bromide at 214 nm was studied at various desalting times. In the second method a quantitative estimate of the salt residue was obtained by analyzing the potassium (KCl) levels in the total ^2D effluent collected from a 4 h modulated LC $\times$ LC run using inductively coupled-plasma MS (ICP-MS). The desalting efficiency was estimated by comparing the ^2D effluent of a nondesalted setup to that of a setup in which 30 s desalting was incorporated.

LC $\times$ LC-FTICR-MS/MS Analyses with Modulation. To couple a capillary-scale ^1D column online to a nanoscale ^2D column, running fast ^2D gradients, the ^1D effluent volumes must be greatly reduced. In our setup this was achieved by implementing SPAM to trap the peptides eluting from the first-dimension column and to wash out salts prior to desorption of the analytes in small volumes to the nanoscale second-dimension column. The ^1D effluent was diluted with a makeup flow containing 0.1% heptafluorobutyric acid (HFBA) to reduce the ACN concentration and to enhance the trapping.³⁹ Desalting is essential to avoid suppression of the electrospray ionization. Online desalting was achieved using an additional pump, delivering a wash-solvent (0.1% FA in 2% ACN in water; 10 $\mu\text{L}\cdot\text{min}^{-1}$). This allowed the traps to be desalted prior to ^2D injection and equilibrated after injection, while the nanopump maintained a stable flow to the ^2D column and, therefore, to the mass spectrometer. In this configuration the trap and ^2D RP column were simultaneously equilibrated, reducing the overall modulation time. The composition of the wash solvent was identical to the starting composition for the ^2D gradient elution.

The setup used an Agilent HP100 micro pump (Agilent Technologies, Amstelveen, The Netherlands) as ^1D pump. A microtee was used to mix the effluent from the ^1D with the aqueous makeup flow delivered by a 10A_{VP} pump module (Shimadzu, 's Hertogenbosch, The Netherlands). Wash solvent for desalting and equilibration of the traps was delivered by the loading pump of the Ultimate 3000 and the ^2D gradient was delivered by the nanopump of the Ultimate 3000. To maximize the compatibility with electrospray ionization, a volatile ammonium formate (AF) SCX buffer was used to moderate the desalting step.^{4,40} Throughout the gradient, the mobile phase contained 15% ACN to reduce hydrophobic interactions.

Optimization of the LC $\times$ LC-FTICR-MS/MS Analyses. To investigate the various modulators the valve setup shown in Figure S.3A was used. To investigate the applicability of the modulation LC $\times$ LC system a tryptic digested six-protein mixture was analyzed for the sequence recovery and the occupation of the separation space. This was performed by using a 2-h ^1D gradient from 0 mM AF to 300 mM AF at $^1F = 1 \mu\text{L}\cdot\text{min}^{-1}$. The ^2D gradient was programmed from $\phi_{\text{ACN}} = 0.008$ to $\phi_{\text{ACN}} = 0.36$ in 7 min at $^2F = 500 \text{ nL}\cdot\text{min}^{-1}$.

LC $\times$ LC-MS/MS data were acquired with a Bruker ApexUltra FTICR-MS (Bruker Daltonics, Bremen, Germany) equipped with a 7 T magnet and a nanoelectrospray Apollo II DualSource. An absolute amount of 0.5 pmol of the protein mixture tryptic digest was injected. LC $\times$ LC-FTICR-MS/MS spectra were recorded as in our previous study.³⁸ The processed MS/MS data were searched with the MASCOT server program 2.3.02 (MATRIX science, London, U.K.) against the Swissprot protein database (release 2014–07).⁴¹ The oxidation of methionine and carboxy-methylation of cysteine were allowed as variable modifications. The peptide tolerance was set to 10 ppm and the peptide-fragment mass tolerance was set to 0.03 Da. The cutoff for MASCOT peptide identification was set to 20. The exported

mascot generic files (mgf) of the processed MS/MS peak lists were processed to create the 3D plot as shown.

LC $\times$ LC-FTICR-MS/MS Analyses of *Saccharomyces cerevisiae*. From the evaluation of the LC $\times$ LC-MS data we observed differences in performance between traps operated in forward-flush and back-flush modes (forward-flush yielded a lower efficiency; see Supporting Information, section S.4). Therefore, we adopted a different configuration for the analyses of the *Saccharomyces cerevisiae*. The new valve configuration is shown later in Figure 5. The 2D gradient was set from 20 mM AF

Figure 2. Estimated peak capacity as a function of the total analyses time. For 2 (◆), 4 (■), or 16 h (●) with in the Acclaim PepMap column. $^2F = 500 \text{ nL} \cdot \text{min}^{-1}$ at a $^2\varphi_{\text{ACN}}$ programmed from 0.008 to 0.32; $^2T = 30^\circ \text{C}$; $^2t_{\text{dwell}} = 3 \text{ min}$ for each modulation. Solid symbols are experimental data obtained with a 50 mm long column, the open symbols represent the extrapolated performance of a 150 mm long column. Lines are drawn for clarity and cannot be used for interpolation. Gradient conditions in the gray shaded area cannot be used due to the time needed for the MS/MS duty cycle. Data based on the average peak widths at-half-height ($W_{1/2}$) of angiotensin, neurotensin, ACTH_{18-39} , and bovine insulin chain B.

(pH 2.9) to 400 mM AF (pH 3.3) at $^1F = 1 \mu\text{L} \cdot \text{min}^{-1}$. The 2D gradient was either programmed from $\varphi_{\text{ACN}} = 0.008$ to 0.40 in 7 min or from $\varphi_{\text{ACN}} = 0.008$ to 0.36 in 6 min, followed by an instantaneous increase to $\varphi_{\text{ACN}} = 0.58$ for 1 min at $^2F = 400 \text{ nL} \cdot \text{min}^{-1}$.

LC $\times$ LC-MS/MS data were recorded during the 12 and 24 h first-dimension gradients. In order to limit the raw-data-file size,

the 12 and 24 h runs were segmented in individual acquisitions of 110 min with a time-gap of about 10 s. The segmented chromatograms were recorded using the Bruker DataAnalysis 4.0 program. Data-dependent Q-selected peptide ions were fragmented in the hexapole collision cell at an argon pressure of $6 \times 10^{-6} \text{ mbar}$ (measured at the ion gauge) and the fragment ions were detected in the ICR cell at a resolution of up to 60000. In the MS/MS duty cycle, three different precursor peptide ions were selected from each survey MS. After performing the MS/MS acquisitions the MS precursor ions were not selected for fragmentation for 1 min. The MS/MS duty cycle time for one survey MS and three MS/MS acquisitions was about 3 s. Instrument mass calibration was better than 2 ppm over an m/z range of 250 to 1500. Raw FTICR-MS/MS data were processed with the MASCOT DISTILLER program, version 2.4.3.1 (64 bits), MDRO 2.4.3.0 (MATRIX science, London, U.K.). This program allows the corresponding data set segments for the 12 and 24 h runs to be processed as multfiles which results in a combined processed data set for each run. Peak picking for both MS and MS/MS spectra was optimized for the mass resolution of up to 60000. Peaks were fitted to a simulated isotope distribution with a correlation threshold of 0.7, with minimum signal-to-noise of 2. For each chromatographic run the segment-combined processed data were searched in a MudPIT scoring approach with the MASCOT server program 2.3.02 (MATRIX science) against the *Saccharomyces cerevisiae* Yeast strain ATCC 204508 ORF translation database (Uniprot 2011 update, downloaded from <http://www.uniprot.org/uniprot>). The database was complemented with its corresponding decoy database for statistical analyses of peptide false discovery rate (FDR). Trypsin was used as enzyme and one missed cleavage was allowed. Carbamidomethylation of cysteine was used as a fixed modification and oxidation of methionine as variable modification. The peptide-mass tolerance was set to 10 ppm and the peptide-fragment-mass tolerance was set to 0.03 Da. MASCOT MudPIT peptide-identification score was set to a cutoff of 20. At this cutoff and based on the number of assigned decoy peptide sequences, a peptide FDR of less than 1% for all analyses was obtained. The MASCOT protein-identification reports were exported as XML and then imported in a custom-made VBA-software program running in Microsoft Excel. The program facilitated organization and data mining of large sets of proteomics data.

Figure 3. Effect on retention times by implementation of stationary-phase-assisted modulation for large volume injections. A $1 \mu\text{L}$ sample injection (solid) compared to a large volume ($10 \mu\text{L}$) injection (dashed) and large volume injection using modulation (dotted). Without active modulation the 2t_c is much too long due to the low 2F .

RESULTS AND DISCUSSION

Online coupling of LC $\times$ LC puts restrictions on the column combinations used due to the limited sample volumes that can be injected in the second dimension, detector considerations (e.g., only volatile buffers in ^1D or ^2D when hyphenating with MS), or flow-rate mismatch (e.g., when coupled to nanospray MS). To overcome these limitations we developed a modulation system in which trap columns replaced the empty loops typically used to couple the dimensions of an LC $\times$ LC system. Analytes eluting from the ^1D are actively concentrated on the stationary phase present in the loop, reducing both the fraction volume and the gradient-delay volume to the ^2D separation. This modulation allows online desalting of the SCX fractions collected prior to the ^2D analyses without jeopardizing MS compatibility.

Active Modulation. To evaluate the efficiency obtained using active modulation we compared a direct injection (small-volume injection; 1 μL), large-volume injection (mimicking the injection of large loops for ^1D fractions; 10 μL) and modulated injection of a large volume (10 μL) on a nanoscale ^2D column as shown in Figure 3. All peptides were eluted using a 2F of 500 nL $\cdot$ min $^{-1}$ with a 7 min ^2D gradient (ϕ_{ACN} from 0.04 to 0.40). The large-volume injection (without modulation) resulted in long ^2D analyses times and a dramatic decrease in the peak capacity, while the modulated large-volume injection showed the same gradient dwell times on the ^2D column as the small-volume injection, without loss in peak capacity. This clearly shows the ability to trap the samples during relative long ^1D fraction-collection times and to desorb them from the traps without loss in ^2D efficiency.

Online Desalting. Besides reducing the fraction volume, active modulation allows online desalting of the ^1D fraction prior to the ^2D analyses. Desalting was investigated by filling the traps with 500-mM KBr solution and measuring the UV absorbance (at 214 nm) as a function of the desalting times, until the salt concentration dropped below the detection limit. A time dependent decrease of the UV absorbance was observed. A desalting time of 30 s (at 10 $\mu\text{L} \cdot \text{min}^{-1}$) was sufficient to wash all salt away. In a separate experiment, ICP-MS was used to measure the potassium content of the combined total effluent from a 4 h SCX $\times$ RP-LC run (24 fractions), using 500 mM KCl solution in the first dimension. With 30 s desalting less than 1 ppm of potassium was observed, which implied that more than 99.4% of all potassium had been removed. Clearly, the 30 s desalting sufficed to eliminate potassium from the traps and, therefore, this desalting time was used in further experiments. The total second-dimension cycle time for each ^1D fraction ($^2t_{\text{c}}$) consisted of the second-dimension system dwell time ($^2t_{\text{sys}}$), the desalting time of the traps ($t_{\text{desalting}}$), the equilibration time of the ^2D column ($^2t_{\text{reeq}}$), and the ^2D gradient time ($^2t_{\text{G}}$). The combination of all nonproductive times can be defined as the total dwell time for the current setup ($^2t_{\text{dwell}} = ^2t_{\text{sys}} + t_{\text{desalting}} + ^2t_{\text{reeq}}$), which was 3 min. For a detailed overview of the obtained results see section S.5, Supporting Information.

Characterization of ^1D and ^2D Columns. The hydrophobic character of the polysulfethyl aspartamide (PSE-ASP) columns was investigated in a manner similar to that described in literature⁴² (see Supporting Information, section S.6). To minimize hydrophobic interaction on the SCX PSE-ASP column, while efficiently trapping hydrophilic peptides on the modulation traps, all further SCX analyses were performed using 15% ACN in the mobile phase.

The total peak capacity of the comprehensive two-dimensional setup ($^{2\text{D}}n_{\text{c}}$) can be estimated by multiplying the peak capacity of

the first dimension ($^1n_{\text{c}}$; equal to the number of fractions collected, because the modulation time is longer than the ^1D bandwidth, 1W) with the peak capacity of the second dimension ($^2n_{\text{c}}$).¹⁹ A longer ^2D analysis time decreases the former, but increases the latter. Thus, there is an optimal second-dimension gradient time ($^2t_{\text{G}}$). For each fraction $^2t_{\text{dwell}}$ (3.0 min) was taken into account, independent of $^2t_{\text{G}}$. The ^2D peak capacity ($^2n_{\text{c}}$) can be calculated by dividing $^2t_{\text{G}}$ by the average (4σ) bandwidth (2W) and adding 1.⁴³ In Figure 2, the estimated $^{2\text{D}}n_{\text{c}}$ is shown as a function of the number of ^1D fractions collected in either 2 h (diamonds), 4 h (squares), or 16 h (spheres) total analyses time for the Acclaim Pepmap column (operated at 500 nL $\cdot$ min $^{-1}$ to ensure MS compatibility). $^{2\text{D}}n_{\text{c}}$ decreases for shorter analyses times ($^2t_{\text{G}} < 3$ min) due to increased contribution of $^2t_{\text{dwell}}$. For longer gradients ($^2t_{\text{G}} > 30$ min) the peak capacity may be increased by implementing a longer column. The performance of the 50 mm long column was extrapolated using the kinetic-performance approach⁴⁴ to estimate the performance of a 150 mm long ^2D column ($^2t_{\text{G}} > 30$ min). The estimated performance is illustrated in Figure 2 with open symbols, revealing that $^{2\text{D}}n_{\text{c}}$ increases with a longer ^2D column. Nevertheless, $^{2\text{D}}n_{\text{c}}$ did not exceed the value found for short modulation times with a short ^2D column. The best S-co-DVB monolithic column yielded an approximately 30% lower performance than the packed C₁₈ column under the same gradient conditions. Therefore, the latter was used in further analyses.

As can be seen in Figure 2, the maximum system performance could be obtained using a $^2t_{\text{G}}$ of 5 min collecting a total 30 SCX fractions in 4 h analyses time, resulting in a $^{2\text{D}}n_{\text{c}}$ of 1461 and a peak production rate of 6.1 peaks/min. To further demonstrate the power of the proposed separation approach we coupled the chromatographic setup to an FTICR-MS. For obtaining the max 60000 resolution the acquisition time was relative long, resulting in a duty cycle with one MS and three MS/MS spectra in 3 s, hence, 60 MS/MS spectra per min. This required a $^2t_{\text{G}}$ of at least 5 min to maintain 2W above 15 s. Otherwise, coeluting peptides might not be isolated for fragmentation. The MS would then be occupied in the duty cycle of a previous precursor ion and a partially resolved peptide might no longer be available for fragmentation in a subsequent cycle. The shaded area in Figure 2 represents ^2D gradient settings resulting in too narrow peptide peaks for the current MS/MS data acquisition.

For further analyses a $^2t_{\text{G}}$ of 7 min was selected, collecting 25 SCX fractions in 4 h, resulting in a $^{2\text{D}}n_{\text{c}}$ of 1432, which is 2% less than the maximum n_{c} achieved with the current setup in the selected analyses time. To achieve a similar peak capacity as was obtained with MudPit ($n_{\text{c}} = 3240$ in about 30 h),⁸ the current setup would require about 9 h analyses time, which implies a 3-fold reduction in total analyses time.

The use of the number of ^1D fractions collected to estimate $^1n_{\text{c}}$ is only valid if the ^2D analyses time (modulation time) is very large (viz. the modulation time is much greater than 1W). Additional band broadening can be calculated as shown in previous studies.¹⁹ Based on experimental data the ^1D peak width when using a 13 h gradient is about 20 min (column band broadening plus external dispersion). The two-dimensional peak capacity ($^{2\text{D}}n_{\text{c,exp}}$) can be estimated from eq 1.

$$^{2\text{D}}n_{\text{c,exp}} = \frac{^1t_{\text{G}}}{4R_s \sqrt{(^1\sigma_{\text{peak}})^2 + [(^2t_{\text{w}})^2 / \delta_{\text{det}}^2]}} \times \left(\frac{^2t_{\text{G}}}{^2t_{\text{w}}} + 1 \right) \quad (1)$$

An experimental value of δ_{det}^2 of 4.76 can be used in the “undersampling” term¹⁹ and $^2t_{\text{G}}$ was equal to $^2t_{\text{G}}$ in the current study. The $^{2\text{D}}n_{\text{c,exp}}$ of the setup was 1880 compared with a $^{2\text{D}}n_{\text{c}}$ of 4925 estimated using the number of ^1D fractions that can be collected in 13 h analyses time as maximum value for $^1n_{\text{c}}$.

Optimization of the Modulator. Trap Column Chemistry and Dimensions. To optimize the modulation a makeup flow can be implemented after the trapping column, prior to the ^2D column. However, this will result in an increase in 2F , which will make it more difficult to meet the constraints of the MS coupling. A post-column splitter could be implemented to overcome this problem but this would result in a loss of sensitivity. When using SPAM the trap-column and the ^2D column cannot contain exactly the same stationary phase because this will cause inadequate refocusing at the top of the ^2D column and an inefficient reduction of the ^2D (injection) band broadening. This may be achieved by ensuring that the trap is less retentive for the analytes than the ^2D column when using the wash solvent (initial ^2D solvent) as eluent. To improve the modulation process the performance of silica-based packed-bed C_{18} traps (TRAP- C_{18} ; 0.3 mm i.d.) was compared to that of in-house prepared organic-polymer monoliths. The effect of the inner diameter of the modulator on the system efficiency was addressed using five standard peptides (chosen to cover a broad range of hydrophobicity index⁴⁵) and a tryptic digest of a protein mixture. Monoliths were prepared in 50 mm long capillaries as described in the Supporting Information, section S.4. As expected, an increase in internal diameter resulted in an increased loading capacity of the modulator (section S.4). We selected the TRAP-75, since it offered the best compromise between a high loading capacity, a low flow resistance and low additional band broadening. The performance of TRAP-75 was then compared with TRAP- C_{18} for the separation and identification of a tryptic digested protein mixture in an LC $\times$ LC-MS/MS setup.

LC $\times$ LC-MS/MS of Digested Six-Protein Mixture. To investigate the performance of the modulated LC $\times$ LC system a tryptic digested protein mixture was analyzed and the chromatographic performance and the sequence coverage were addressed. In Table 1 the sequence coverage obtained with the present

Table 1. Peptide Sequence Coverage in Percentage for Proteins in a Mixture Obtained with Two Different Modulators, Compared to Reported Peptide Sequence Coverages

protein	organism	ref 1	TRAP-75	TRAP- C_{18}
serum albumin	bovine	82	53	80
apo-transferrin	bovine	74	54	74
lysozyme	chicken	76	69	78
β -galactosidase	<i>Escherichia coli</i>	64	27	34
alcohol dehydrogenase	<i>Saccharomyces cerevisiae</i>	66	36	52
cytochrome c	bovine	70	28	70

setup, equipped with either TRAP-75 or TRAP- C_{18} is compared to the sequence coverage reported using a 50 mm long monolithic column with a three times longer gradient time.¹ In the modulated system, the TRAP- C_{18} clearly outperformed the monolithic traps in terms of peptide identification and protein-sequence coverage. The digest of the protein mixture contains a large numbers of small, hydrophilic peptides, which were not sufficiently trapped on the monolithic traps, resulting in a low sequence coverage. The sequence coverage obtained with the

modulated setup with TRAP- C_{18} were similar to those reported in literature,¹ except for β -galactosidase, a protein with a low number of trypsin cleavage points, resulting in high-molecular-weight (hydrophobic) peptides. These are difficult to observe with SCX $\times$ RP-LC,⁴⁶ even if the hydrophobic character of the PSE-ASP SCX column is suppressed by the addition of ACN to the mobile phases.⁴² To investigate the coverage of the available separation space using the current modulated system, BSA was selected as reference protein. The distribution of the total-ion sum originating from BSA is shown in Figure 4. A good coverage of

Figure 4. Coverage of the separation space. The sum of the ion-peaks identified of BSA peptides from the tryptic digested protein mixture obtained with the modulated LC $\times$ LC applied with the TRAP- C_{18} . The $^1t_{\text{G}}$ set to 180 min from 20 to 400 mM AF at 1F of $1 \mu\text{L} \cdot \text{min}^{-1}$ and $^2t_{\text{G}}$ of 7 min with a ϕ_{ACN} from 0.016 to 0.48 at 2F of $500 \text{ nL} \cdot \text{min}^{-1}$.

the separation space was obtained, as well as a good sequence coverage of the proteins. The number of identifications can be increased by increasing the MS/MS data-acquisition rate without sacrificing mass resolution and mass accuracy.

The two traps in the current configuration were not eluted in the same manner to the ^2D . The first trap is filled by the ^1D effluent and emptied in the same direction (“forward-flush”), whereas the second trap is filled and emptied in opposite directions (“back-flush”). To reduce the band broadening resulting from the traps the valve-configuration shown in Figure 5 was used.

LC $\times$ LC-MS/MS Analyses of *Saccharomyces cerevisiae*.

In the new setup (Figure 5) the two traps were both operated in back-flush mode, while, as in the previous configuration, the bypass-tubing attached to the valve on the right ensures a continuous nanoflow to the ^2D column and the MS, regardless of the valve positions. In the Supporting Information (section S.7) a detailed description of the various valve configurations is provided. A drawback of the setup of Figure 5 is that the jumper on the left valve is positioned differently in the two positions, either before or after the trap. The SCX sample contained in this jumper (140 nL) will be lost when switching from trapping on loop II to loop I. This implies a loss of about 2 s of ^1D SCX effluent every other modulation cycle (about every 20 min). Since the peaks eluting from the ^1D are more than 2 min broad this small loss will not result in peptides being overlooked. The lower band broadening observed with this back-flush configuration results in a higher overall peak capacity of the

Figure 5. Schematic overview of the modulated LC $\times$ LC setup used for yeast analysis.

SCX $\times$ RP-LC system. The modulated LC $\times$ LC instrument shows superior selectivity in comparison with one-dimensional separation techniques. It was used with the TRAP-C₁₈ to analyze digested-proteome of *Saccharomyces cerevisiae*. To achieve a $^{2D}n_c$ of 10000 in 24 h the 2F was slightly adapted to optimize nanospray flow conditions for a long analysis time. 2F was reduced from 500 to 400 nL·min⁻¹ with the 2t_c adapted accordingly. For the first analysis of 1 μ g of digested yeast sample the 1D gradient was ramped from 3 to 200 mM AF in 13 h. The broad dynamic range of the peptide mixture, in combination with the long MS/MS duty-cycle and the peptide intensity threshold for MS/MS analyses of the lowest intensity peptides limited the number of identifications of coeluting peptides. To reduce the number of peptides coeluting and increase the identification rate, the amount of sample injected was doubled on a longer 1D column (300 mm) in combination with a shallower 1D gradient. From the resulting separation 19601 MS/MS spectra were recorded and 701 unique proteins were identified in 24 h analyses time. The gain in newly identified proteins by increasing the analyses time was minimal and many peptides eluted in more than three fractions. This suggests that the 1D selectivity is the limiting factor in the 24 h gradient. Moreover, doubling the amount of sample injected resulted in an accumulation of the hydrophobic peptides in the reversed-phase column. The 2D gradient (up to 50% B) was found not to wash all hydrophobic peptides from the modulator traps and the 2D analytical column. As a result, progressive peak broadening was observed during the second half of the analysis, up to elution times exceeding 1 min for hydrophobic peptides with 2D retention times longer than 3 min. In contrast, no broadening of the peaks of the hydrophilic peptides in the corresponding 1D fraction, were observed. This peak broadening due to accumulation of hydrophobic peptides was prevented by adding a wash-step with 70% B in the final minute of each 2D gradient. The three successive analyses together resulted in the identification of 901 proteins (see Table S.7). Before addressing the repeatability of yeast analyses by applying this approach in more detail some improvements can be made in the stationary-phase-assisted modulation. The 1D selectivity and the 2D gradient performance can be enhanced to reduce the number of peptides coeluting, for example by using a longer 2D column in combination with a longer 2D gradient time. Moreover, the number of identifications can be increased by increasing the MS/MS data-acquisition rate, without sacrificing mass resolution and mass accuracy. Also, highly retentive stationary-phase material may be applied in traps smaller than the current

ones (0.30 mm i.d.) to reduce the additional band broadening observed and to shorten the unproductive (washing and re-equilibration) periods in the modulation cycle. The modulation may be applied to various other separation problems, including the study of intact proteins.

CONCLUDING REMARKS

In most LC $\times$ LC setups, used in proteomics research, a nanoscale 1D separation is combined with a microscale 2D column. The resulting relatively high second-dimension flow rates make the splitless hyphenation with nanoelectrospray mass-spectrometry difficult. By implementing stationary-phase-assisted modulation in the LC $\times$ LC system we could combine a high-loadability 1D column with a nanoflow 2D column, which could be hyphenated with a nanospray MS, losing neither sample, nor efficiency, as is evident from the high tryptic-peptide sequence coverage obtained in the analysis of a six-protein mixture. Other applications of active modulation are the subject of future research.

The modulated LC $\times$ LC proved to be applicable for proteome-wide analyses by hyphenation to a high-resolution MS (HRMS) instrument. The setup can be further optimized to improve proteome-wide identification and quantification by enhancing the 1D selectivity, by miniaturizing the trap volume (reducing modulation times), by upgrading the 2D performance and by increasing the MS/MS data acquisition rate. The method has the potential to replace current methods of proteome analysis, including time-consuming and sample-inefficient off-line protein prefractionation followed by the digestion of individual protein fractions and LC-MS/MS data acquisition.

ASSOCIATED CONTENT

Supporting Information

Additional experimental details. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +31 (0)20 525 6424. Fax: +31 (0)20 525 5604. E-mail: rudy.vonk@hotmail.com.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank the EUREKA organization (C.2332.0227), as well as Thermo Fisher Scientific, for supporting this work. A.F.G.G. received funding from The Netherlands Organisation for Scientific Research (NWO) in the framework of the Technology Area COAST programme (053.21.102). S.E. received funding from Research Foundation Flanders (G.0919.09 and G.0103.11.N.10). A. Barcaru (University of Amsterdam) is kindly acknowledged for his help with the figures. L. de Lange (University of Amsterdam) is kindly acknowledged for his help with the ICP-MS analyses.

REFERENCES

- (1) Eeltink, S.; Dolman, S.; Detobel, F.; Swart, R.; Ursem, M.; Schoenmakers, P. J. *J. Chromatogr. A* **2010**, *1217*, 6610–6615.
- (2) Bushey, M. M.; Jorgenson, J. W. *Anal. Chem.* **1990**, *62*, 161–167.
- (3) Nägele, E.; Vollmer, M.; Hörth, P. *J. Chromatogr. A* **2003**, *1009*, 197–205.
- (4) Liu, H.; Finch, J. W.; Luongo, J. A.; Li, G.; Gebler, J. C. *J. Chromatogr. A* **2006**, *1135*, 43–51.

- (5) Pepaj, M.; Holm, A.; Fleckenstein, B.; Lundanes, E.; Greibrokk, T. *J. Sep. Sci.* **2006**, *29*, 519–528.
- (6) Bushey, M. M.; Jorgenson, J. W. *Anal. Chem.* **1990**, *62*, 978–984.
- (7) Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. *Nat. Biotechnol.* **1999**, *17*, 676–682.
- (8) Wolters, D. A.; Washburn, M. P.; Yates, J. R. *Anal. Chem.* **2001**, *73*, 5683–5690.
- (9) Washburn, M. P.; Wolters, D.; Yates, J. R. *Nat. Biotechnol.* **2001**, *19*, 242–247.
- (10) Webb, K. J.; Xu, T.; Park, S. K.; Yates, J. R. *J. Proteome Res.* **2013**, *12*, 2177–2184.
- (11) Peng, J.; Elias, J. E.; Thoreen, C. C.; Licklider, L. J.; Gygi, S. P. *J. Proteome Res.* **2003**, *2*, 43–50.
- (12) Lau, E.; Lam, M. P. Y.; Siu, S. O.; Kong, R. P. W.; Chan, W. L.; Zhou, Z.; Huang, J.; Lo, C.; Chu, I. K. *Mol. Biosyst.* **2011**, *7*, 1399–1408.
- (13) Maynard, D. M.; Masuda, J.; Yang, X.; Kowalak, J. A.; Markey, S. P. *J. Chromatogr. B* **2004**, *810*, 69–76.
- (14) Masuda, J.; Maynard, D. M.; Nishimura, M.; Ueda, T.; Kowalak, J. A.; Markey, S. P. *J. Chromatogr. A* **2005**, *1063*, 57–69.
- (15) Giddings, J. C. *J. Chromatogr. A* **1995**, *703*, 3–15.
- (16) Davydova, E.; Schoenmakers, P. J.; Vivó-Truyols, G. *J. Chromatogr. A* **2013**, *1271*, 137–143.
- (17) Han, Y. H.; Levkin, P. A.; Abarientos, I.; Liu, H. W.; Svec, F.; Fréchet, J. M. J. *Anal. Chem.* **2010**, *82*, 2520–2528.
- (18) Vanhoutte, D. J. D.; Eeltink, S.; Kok, W. T.; Schoenmakers, P. J. *Anal. Chim. Acta* **2011**, *701*, 92–97.
- (19) Vivó-Truyols, G.; van der Wal, S.; Schoenmakers, P. J. *Anal. Chem.* **2010**, *82*, 8525–8536.
- (20) Vanhoutte, D. J. D.; Vivó-Truyols, G.; Schoenmakers, P. J. *J. Chromatogr. A* **2012**, *1235*, 39–48.
- (21) Chervet, J. P.; Ursem, M.; Salzmänn, J. P. *Anal. Chem.* **1996**, *68*, 1507–1512.
- (22) Blonder, J.; Goshe, M. B.; Moore, R. J.; Pasa-Tolic, L.; Masselon, C. D.; Lipton, M. S.; Smith, R. D. *J. Proteome Res.* **2002**, *1*, 351–360.
- (23) Emmett, M. R.; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 605–613.
- (24) Eghbali, H.; Sandra, K.; Tienpont, B.; Eeltink, S.; Sandra, P.; Desmet, G. *Anal. Chem.* **2012**, *84*, 2031–2037.
- (25) Verstraeten, M.; Pursch, M.; Eckerle, P.; Luong, J.; Desmet, G. *Anal. Chem.* **2011**, *83*, 7053–7060.
- (26) De Vos, J.; Eeltink, S.; Desmet, G. *J. Chromatogr. A* **2015**, *1381*, 74–86.
- (27) De Vos, J.; Desmet, G.; Eeltink, S. *J. Chromatogr. A* **2014**, *1360*, 164–171.
- (28) Lankelma, J.; Poppe, H. *J. Chromatogr. A* **1978**, *149*, 587–598.
- (29) Nägele, E.; Vollmer, M.; Hörth, P. *J. Biomol. Tech.* **2004**, *15*, 134–143.
- (30) Pepaj, M.; Wilson, S. R.; Novotna, K.; Lundanes, E.; Greibrokk, T. *J. Chromatogr. A* **2006**, *1120*, 132–141.
- (31) Sweeney, A. P.; Shalliker, R. A. *J. Chromatogr. A* **2002**, *968*, 41–52.
- (32) Winther, B.; Reubsaet, J. L. E. *J. Sep. Sci.* **2005**, *28*, 477–482.
- (33) Zhao, Y.; Szeto, S. S. W.; Kong, R. P. W.; Law, C. H.; Li, G.; Quan, Q.; Zhang, Z.; Wang, Y.; Chu, I. K. *Anal. Chem.* **2014**, *86*, 12172–12179.
- (34) Kong, R. P. W.; Siu, S. O.; Lee, S. S. M.; Lo, C.; Chu, I. K. *J. Chromatogr. A* **2011**, *1218*, 3681–3688.
- (35) François, I.; dos Santos Pereira, A.; Lynen, F.; Sandra, P. *J. Sep. Sci.* **2008**, *31*, 3473–3478.
- (36) Clontech. http://www.clontech.com/xxclt_abcGetAttachment.jsp?clntItem=17602, .
- (37) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. *Anal. Chem.* **1996**, *68*, 850–858.
- (38) Vonk, R. J.; Barcaru, A.; Vivó-Truyols, G.; Eeltink, S.; de Koning, L. J.; Schoenmakers, P. J. *Anal. Bioanal. Chem.* **2015**, *407*, 3817–3829.
- (39) Schley, C.; Swart, R.; Huber, C. G. *J. Chromatogr. A* **2006**, *1136*, 210–220.
- (40) Gilar, M.; Olivova, P.; Daly, A. E.; Gebler, J. C. *Anal. Chem.* **2005**, *77*, 6426–6434.
- (41) The UniProt Consortium. *Nucleic Acids Res.* **2014**, *42*, D191–D198.
- (42) Alpert, A. J.; Andrews, P. C. *J. Chromatogr. A* **1988**, *443*, 85–96.
- (43) Dolan, J. W.; Snyder, L. R.; Djordjevic, N. M.; Hill, D. W.; Waeghe, T. J. *J. Chromatogr. A* **1999**, *857*, 1–20.
- (44) Broeckhoven, K.; Cabooter, D.; Lynen, F.; Sandra, P.; Desmet, G. *J. Chromatogr. A* **2010**, *1217*, 2787–2795.
- (45) Guo, D.; Mant, C. T.; Taneja, A. K.; Hodges, R. S. *J. Chromatogr. A* **1986**, *359*, 519–532.
- (46) Gilar, M.; Olivova, P.; Chakraborty, A. B.; Jaworski, A.; Geromanos, S. J.; Gebler, J. C. *Electrophoresis* **2009**, *30*, 1157–1167.