High resolution online two-dimensional nano LC/MS for the analysis of proteome samples – Implementation of an effective continuous salt gradient in the first dimension

Application Note

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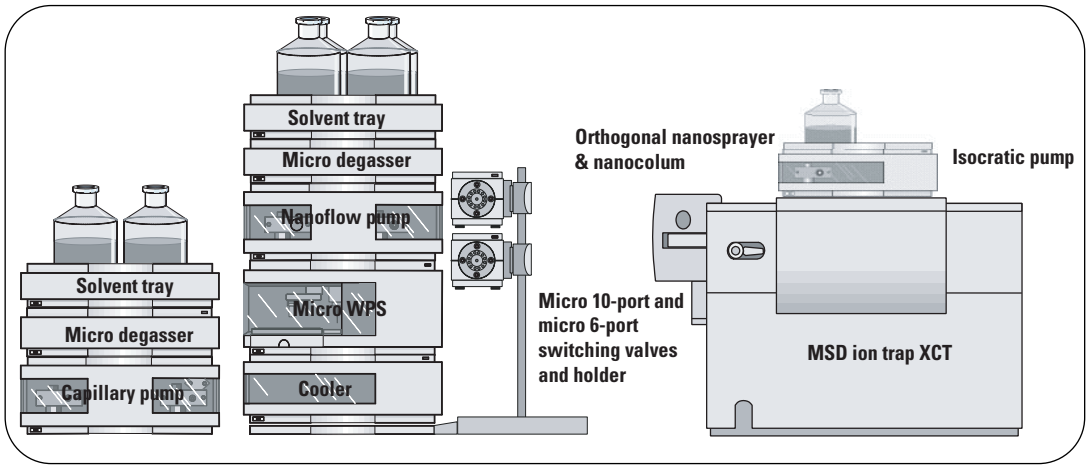

Abstract

In online two-dimensional LC/MS applications for the analysis of proteome samples a strong cation exchange (SCX) column is typically used for the first dimension and a reversed phase (RP) column for the second one. Usually the peptides are gradually eluted from the SCX column and separated in a gradient prior to MS analysis in the second dimension. Unfortunately, the resolution of the sample peptides suffer from these eluting conditions, which directly influences the number of identified proteins. It is necessary to elute the sample peptides with a continuous salt solution gradient from the SCX column to get the highest possible resolution. This Application Note describes an LC system for online 2D LC/MS, which uses an effective continuous salt solution gradient in the first dimension. The results will be compared to previously obtained results.

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Introduction

In order to identify proteins from a complex mixture of 5 x 10^3 to 5 x 10^4 with a dynamic range of at least 10^5 [1] it will be crucial to develop technologies with extremely good resolving power on one hand and with extraordinary sensitivity on the other hand. The main approach, which has been addressed in order to separate and resolve complex protein expression patterns is the combination of different techniques adapted for separation on the peptide level[2]. The entire workflow for this approach can be easily automated since the digestion of the complete protein content of a sample takes place in the early stages of the workflow. Multiplying the total amount of different compounds by one to two orders of magnitude increases the initial complexity of the sample. It is obvious that these challenging tasks will not be achieved with a single analytical technology, but with a combination of separation and detection techniques. In order to achieve this goal, liquid chromatography based multidimensional separation techniques directly coupled to MS detection were developed. The combination of different orthogonal HPLC separation techniques for peptide mixtures has been described in a variety of publications:

- Reversed phase chromatography RP – capillary electrophoresis[3,4]
- Size exclusion chromatography (SEC) – RP[5]
- Strong cation exchange chromatography (SCX) – RP[6,7], and
- Affinity chromatography (AC) – RP[8,9].

The latter is mainly applied on more specific functional approaches like phosphopeptide analysis[10].

In general, peak capacity for multidimensional separation is calculated by multiplying the peak capacity of the single separation steps[11]. By combining two different orthogonal LC techniques with individual peak capacities between 50 and 100 it is theoretically possible to achieve total capacities of 2500 to 10000 peaks. For the separation of complex peptide mixtures the combination of orthogonal separation techniques like strong cation exchange chromatography (SCX) and reversed phase chromatography (RP)[6,7] has been used successfully for the analysis of comprehensive protein expression profiles from yeast *Saccharomyces cervisiae* or *Plasmodium falciparum*[12,13,14]. In the course of the analysis, tryptic peptides are gradually eluted by injecting salt plugs of increasing ionic strength from the SCX column in the first dimension. In the second dimension these peptides are first trapped on a reversed phase (RP) enrichment column and finally separated on an analytical RP column. It has been shown that this method, especially in nano scale[15], is capable of resolving large proteomes as well as identifying a subset of proteins expressed under special conditions[16,17]. In contrast to this well established online method, offline 2D LC-MS/MS significantly increases chromatographic resolution of highly complex proteome samples[18]. This improvement is attributed to continuous gradient SCX chromatography in the first dimension without intermitting RP chromatography intervals. As a consequence a higher resolution and peak capacity, and therefore a higher number of identified proteins is associated with the offline 2D LC-MS/MS approach[19]. However, the offline 2D LC approach is not fully automated and requires additional handling to transfer the collected fractions from the first to the second dimension. In this Application Note an LC system for online 2D LC/MS, which uses an effective continuous salt solution gradient in the first dimension, will be introduced. The results will be compared to previously obtained results.

Experimental

Equipment

- Agilent 1100 Series nanoflow pump with micro vacuum degasser
- Agilent 1100 Series capillary pump with micro vacuum degasser
- Agilent 11100 Series isocratic pump
- Agilent 1100 Series thermostatted micro well-plate autosampler
- Agilent 1100 Series micro 2-position/6-port valve
- Agilent 1100 Series micro 2-position/10-port valve
- Agilent 1100 Series MSD Ion Trap XCT

Columns

- Agilent BioSCX Series II, 0.3 x 35 mm, 3.5-µm particles
- ZORBAX 300SB C18, 75 µm x 150 mm, 3.5-µm particles
- ZORBAX 300SB C18, 0.3 x 5 mm, 5-µm particles

Software

- ChemStation A10.02
- Trap software 4.2
- Spectrum Mill MS Proteomics Workbench

Valve configuration

The nano LC system (see schematic on title page) for online 2D LC with a continuous salt solution gradient includes a micro 2-position/6-port valve and a washing pump to clean the enrichment columns from salt residues prior to the reversed phase separation of the second dimension. The micro 10-port valve with its two enrichment columns is directly connected to the 6-port valve. Depending on its position, the 6-port valve either connects the washing pump or the nanoflow pump and the nanocolumn to the peptide charged enrichment column (figure 1).

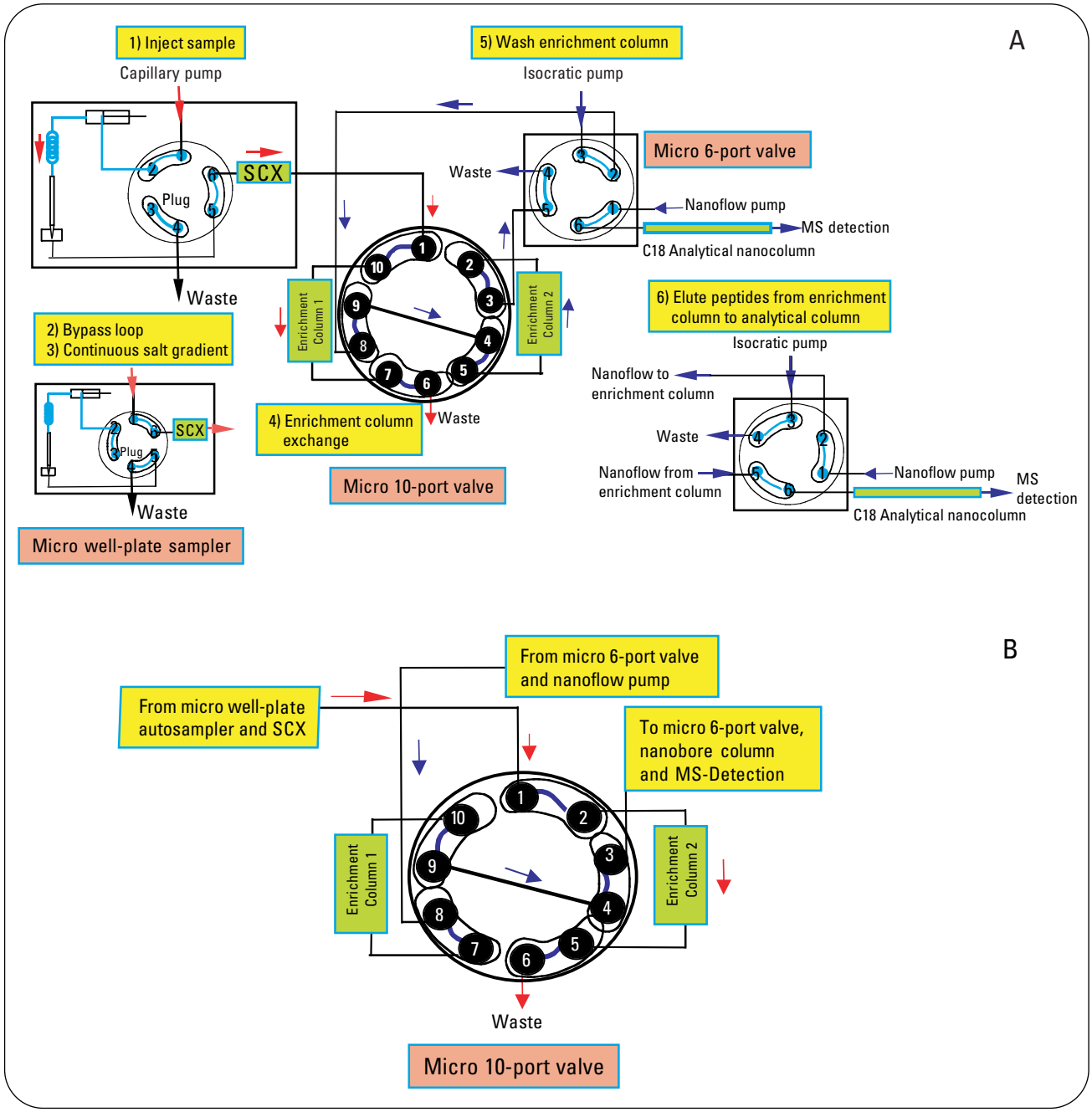

Figure 1
Flow diagram for online nano 2D LC with effective continuous salt solution gradient SCX chromatography.

In the Agilent 1100 Series micro well-plate autosampler the capillary pump is connected to another micro 2-position/6-port valve, which is also connected with the micro 10-port valve. After sample injection (figure 1A – 1) the autosampler loop is switched to bypass to avoid its dead volume (figure 1A – 2). The sample peptides retained on the SCX column are continuously eluted by means of the salt solution gradient pumped by the capillary pump (figure 1A – 3) and subsequently trapped on the enrichment column which is serially connected to the SCX column (figure 1A – 4). After the enrichment column exchange the charged enrichment column is connected to the washing pump via the micro 6-port valve (figure 1A – 5) to remove salt residues. The enrichment column is then switched into the nanoflow path via the 6-port valve (figure 1A – 6) and the nanoflow pump starts to deliver the gradient for the reversed phase separation. Simultaneously the other enrichment column is serially connected with the SCX column and the peptides released in the continuous salt solution gradient from the SCX column are captured on this column and the cycle is repeated (figure 1B). The workflow principle of the online 2D LC using a continuous salt solution gradient for the elution of the tryptic proteome sample peptides is shown in figure 2.

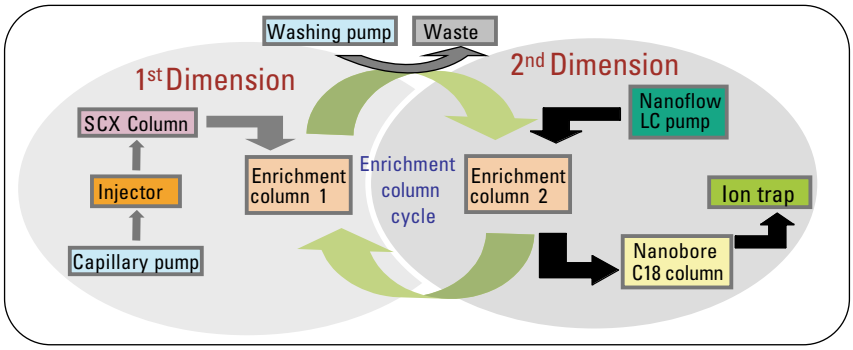

Figure 2
Principle of online 2D LC with continuous salt solution gradient.

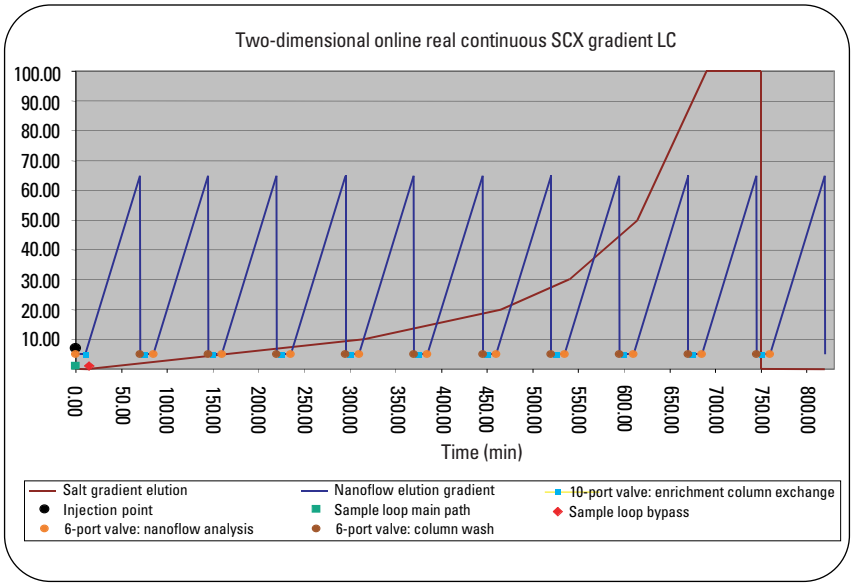

Figure 3
SCX and PR gradients. and valve switching times for online 2D LC with effective continuous salt solution gradient.

Chromatographic method

It is necessary to set up two different gradients for chromatographic separation in the first and second dimension, a continuous gradient for SCX chromatography and one for RP separation, which alternates between two limits of organic solvent. The continuous salt solution gradient for the elution of the peptides from the SCX column is delivered from the Agilent 1100 Series capillary pump and the gradient for the RP separation is delivered from the Agilent 1100 Series nanoflow pump (figure 3). The nanoflow gradient starts with 5 % acetonitrile and increases up to 65 % acetonitrile with a slope of 1 %/min for each RP analysis.

The salt gradient starts at 0 % and proceeds up to 50 mM NaCl in 300 min with a slope of 0.34 %/min. After the initial phase the slope of the salt solution gradient increases with higher salt concentrations because the majority of the peptides are eluted from the SCX columns with a salt concentration up to about 150 mM NaCl. At the starting point of each RP analysis cycle the charged enrichment column is exchanged for the empty one by switching the micro 10-port valve (figure 3).

Nanoflow pump																	
Time	0.00	10.00	70.00	70.01	85.00	145.00	145.01	160.00	220.00	220.01	235.00	295.00	295.01	310.00	370.00	370.01	385.00
% Solvent B	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00
Time	445.00	445.01	460.00	520.00	520.01	535.00	595.00	595.01	610.00	670.00	670.01	685.00	745.00	745.01	760.00	820.00	820.01
% Solvent B	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00	5.00	65.00	5.00
Capillary pump																	
Time		0,00	15.00	15.01	315.00	465.00	540.00	615.00	690.00	750.00	750.01	820.01					
% Solvent B		0.00	0.00	0.00	10.00	20.00	30.00	50.00	100.00	100.00	0.00	0.00					
10-port valve																	
Switch position		1	2	1	2	1	2	1	2	1	2	1	2				
Time		0,00	10,00	75,00	150,00	225,00	300,00	375,00	450,00	525,00	600,00	675,00	750,00				
6-port valve																	
Nanoflow position		0,00	85,00	160,00	235,00	310,00	385,00	460,00	535,00	610,00	685,00	760,00					
Wash position		70,00	145,00	220,00	295,00	370,00	445,00	520,00	595,00	670,00	745,00						

Table 1A
Detailed settings for SCX and RP chromatography and valve switching points.

The detailed gradient settings for SCX and RP chromatography as well as the valve switching points for the micro 6-port valve and the micro 10-port valve are outlined in table 1A. Table 1B summarizes the additional instrument parameters.

Sample preparation

Lyophilized Yeast cells (*Saccharomyces cerevisiae*), resuspended in cooled 50 mM NH_4HCO_3 containing 8M urea were disrupted in a bead beater (0.5 mm glass beads). After centrifugation to remove cell debris the proteins in the supernatant liquid were reduced with 1 mM DTT at 37 °C for 1 h, alkylated in the dark with 10 mM iodoacetamide for 30 min at RT, ultrafiltrated for buffer exchange and tryptically digested with TPCK trypsin at 37 °C for 16 h. Finally the sample was lyophilized in a SpeedVac and dissolved in 5 % AcN, 0.03 % formic acid prior to analysis.

Nanoflow pump
Solvents: A = water + 0.1 % formic acid, B = AcN + 0.1 % formic acid.
Primary flow: 200-500 µL/min.
Column flow: 300 nL/min.
Stop time: 825 min.
Post time: 15 min.

Capillary pump
Solvents: A = water + 3 % AcN + 0.1 % formic acid,
B = 500 mM NaCl + 3 % AcN + 0.1 % formic acid.
Primary flow: 500-800 µL/min.
Column flow: 10 µL/min.

Trap XCT MS conditions
Ionization mode: Positive nano electrospray with Agilent orthogonal source
Drying gas flow: 5 L/min
Drying gas temperature: 300 °C
Vcap: typically 1800-2000 V
Skim 1: 30 V
Capillary exit offset: 75 V
Trap Drive: 85 V
Averages: 1 or 2
ICC: On
Maximum accumulation time: 150 ms
Smart Target: 125,000
MS scan range: 300-2200
Automatic MS/MS:
Peptide scan mode
Number of parents: 3 or 4
Averages: 2
Fragmentation amplitude: 1.3 V
SmartFrag: on, 30-200 %
Active exclusion: on, 2 spectra, 1 min
Prefer +2: on
MS/MS scan Range: 100-1800
Ultra scan: on
ICC target: 125,000

Table 1B
Instrument parameters.

Results and discussion

The advantage of offline 2D LC, which uses a continuous salt solution gradient to elute the tryptic sample peptides from the SCX column, is that the SCX column is operating under optimum conditions. This will result in a better separation of the peptides and therefore much sharper elution bands for each specific peptide in comparison to online SCX separations which use salt plugs for peptide elution. This also prevents tryptic peptides from being distributed over more than one RP separation step in the second dimension. Therefore, the probability of detecting low abundant peptides and consequently the corresponding low abundant proteins increases. In mixtures of lower complexity this effect can clearly be demonstrated by the increased sequence coverage due to more detected peptides for each protein. For that purpose an artificial mixture of 10 tryptically digested bovine proteins was analyzed with the continuous gradient online 2D LC/MS method at the same concentration as described earlier (100 fmol for each protein) for an online 2D LC/MS analysis working with the injected salt plug method[15]. After a database search with the MS/MS spectra measured by the continuous salt gradient method, the comparison to the results obtained for the salt solution plug method clearly demonstrates this effect by the resulting sequence coverage obtained (table 2). To demonstrate the full capability of the continuous salt solution gradient method for online 2D LC a whole cell digest sample from yeast (*Saccharomyces cerevisiae*) was used. From the Spectrum Mill data evaluation of the measured MS/MS spectra 130 protein hits were obtained. The highest abundant protein hits as well as the last obtained protein hits are listed in table 3.

	Distinct peptides		% AA coverage	
Method	**1**	**2**	**1**	**2**
Identified protein				
Serum albumin	36	6	66	11
Serotransferrin	29	14	44	19
Catalase	17	6	38	13
Lactoperoxidase	14	5	21	10
Glutamate dehydrogenase	10	4	21	8
Chymotrypsinogen A	7	3	35	16
Hemoglobin	6	1	40	6
Carboxypeptidase A	4	4	9	7
Carbonic anhydrase	3	10	12	17
beta Lactoglobulin	1	5	6	19

1) Online 2D LC with continuous salt solution gradient for SCX chromatography

2) Online 2D LC with injected salt solution plug SCX chromatography

Table 2
Comparison of results obtained with online 2D LC/MS by injected salt solution steps and by an effective continuous pumped salt solution gradient.

Hit Number	MS/MS score	Peptides	Spectra Number	% AA Coverage	Protein name
1	203.56	12	50	42	Phosphopyruvate hydratase
2	167.85	11	25	37	Phosphoglycerate kinase
3	138.18	9	9	33	Pyruvate kinase 1
4	121.07	8	10	23	Pyruvate decarboxylase
5	117.85	9	12	22	Heat Shock Protein SSA 1
6	108.42	9	14	41	Alcohol dehydrogenase
7	95.31	7	9	28	Hexokinase A1
8	92.27	6	75	36	Glycerinaldehyde 3-phosphate dehydrogenase
9	80.92	5	5	28	Heat shock protein 26
10	72.79	6	7	50	Triosephosphate isomerase
11	69.95	5	10	15	Elongation factor 1-alpha
12	67.42	4	5	37	Eycariotic translation initation factor 5A-2
13	61.65	5	7	16	Aledhyde dehydrogenase
14	61.38	4	4	17	Fructose bisphosphate aldolase
15	54.33	4	4	40	Superoxide dismutase
16	53.02	4	4	21	Phosphoglycerate mutase 1
17	49.91	3	4	19	Gluco kinase
18	39.31	3	3	20	60S ribosomal protein L5
19	29.80	3	2	8	Glycero-3-phosphate dehydrogenase
20	28.10	5	4	6	Aconitate hydratase
128	9.24	1	1	6	CSG2 protein precursor
129	9.11	1	1	1	DNA helicase type protein
130	9.03	1	1	2	Glutamyl tRNA synthetase

Table 3
Results obtained from the analysis of a yeast proteome sample.

The newly developed online 2D LC method was compared to the previously examined methods:
1) the online 2D LC injected salt solution plug method[15],
2) the online 2D LC pumped semi-continuous salt solution gradient method[19], and

Method	Online 2D LC with injected salt steps	Online 2D LC with pumped semi-continuous salt gradient	Online 2D LC with pumped effective continuous salt gradient	Off-line 2D LC with pumped continuous gradient
Identified proteins	101	122	130	144
Assigned peptides	179	207	228	269

Table 4
Comparison of the performance of different 2D LC/MS methods for protein identification.

3) the offline 2D LC method applying a continuous salt solution gradient in the separate first dimension[18]. For this comparison the same sample, the same LC/MS system, the same columns and conditions were chosen. To compare the performance of the methods, the number of identified proteins and the number of assigned peptides were evaluated (table 4). The results clearly demonstrated the superiority of the new method over methods 1) and 2) mentioned earlier. However, the data also clearly shows the undisputed superiority of the offline 2D LC method for the analysis of highly complex proteome samples. The superiority of the newly developed method as well as the offline 2D LC method also undoubtedly proves the importance of a continuous elution of the peptide from the SCX column, which is then working under optimum conditions, to obtain the best separation performance and therefore the best results in terms of protein identification of highly complex proteome samples. To demonstrate the quality of the acquired data, the doubly charged peptide with the sequence TGVIVGEDVHNLFTYAK 932.15 m/z of the identified protein *Fructose bisphosphate aldolase* (hit number 14, SpectrumMill score 61.38, 4 peptides, 17 % sequence coverage) was considered in detail. Starting from the base peak chromatogram of the entire LC/MS run, (figure 4A) the doubly charged peptide

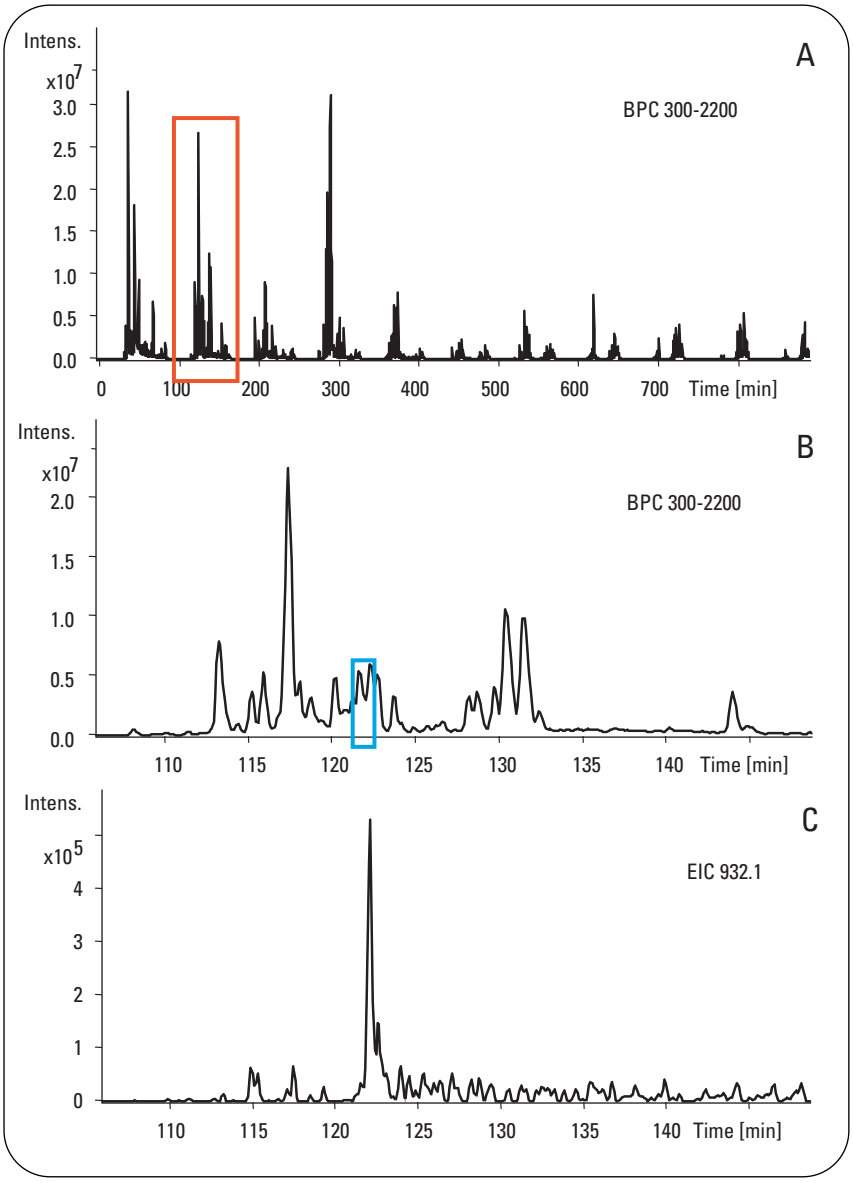

Figure 4
A) Base peak chromatogram of the whole 2D LC/MS run of the yeast cell lysate sample.
B) BPC of the region containing the doubly charged peptide ion m/z 932.1.
C) Extracted ion chromatogram of the doubly charged peptide ion m/z 932.1 at 122.0 min from the identified protein Fructose bisphosphate aldolase.

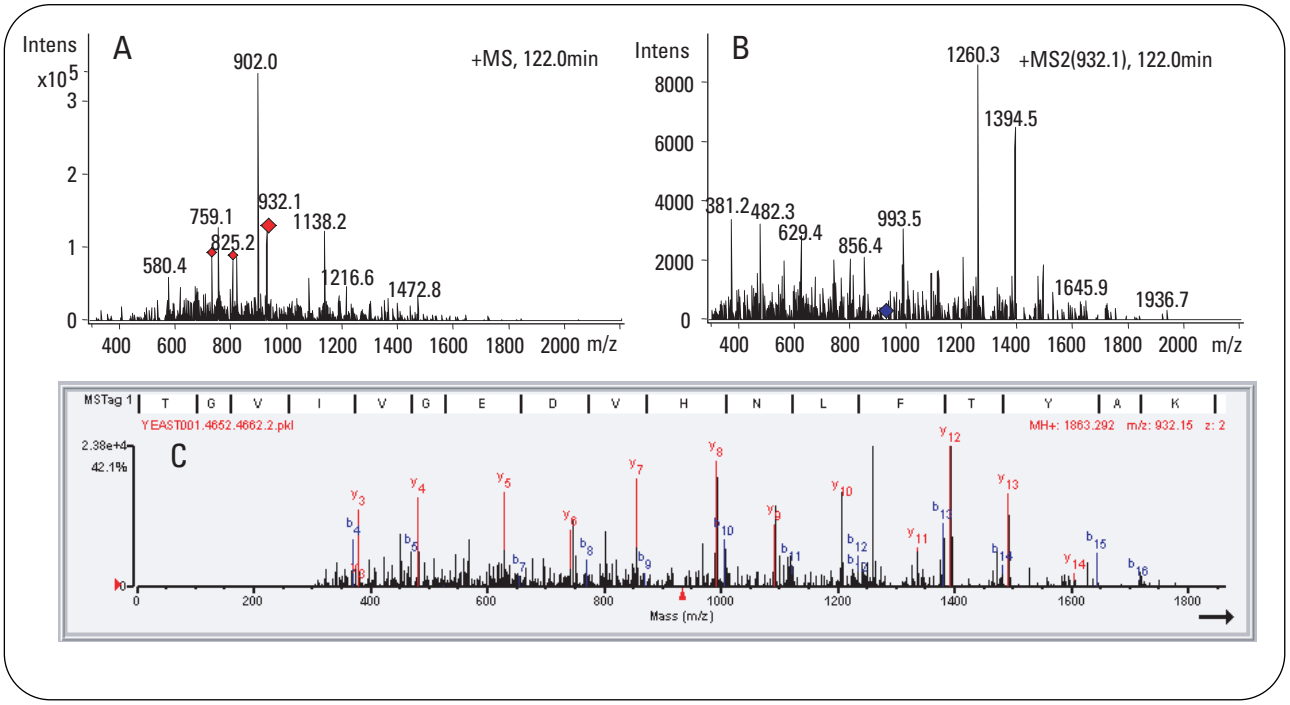

Figure 5
A) MS spectrum from the peak at 122.0 min with the selected precursors for MS/MS.
B) MS/MS spectrum of the peptide ion m/z 932.1.
C) Interpreted MS/MS spectrum with the assigned fragmentation pattern and peptide amino acid sequence.

ion m/z 932.1 was extracted from the region between 110 and 140 minutes (figure 4B) at 122.0 minutes (figure 4C). Three precursor ions were selected for MS/MS (figure 5A) from the MS spectrum, which was acquired from the peak at 122.0 min. The ions are marked with a red rhomb. The MS/MS spectrum in relation to the peptide ion m/z 932.1 is shown in figure 5B and the place of the doubly charged peptide parent ion is marked with a blue rhomb. After database analysis with the Spectrum Mill software and identification of the related protein, the MS/MS spectrum is interpreted with the full fragmentation pattern of the y- and b-series ions and with its assigned amino acid sequence (figure 5C).

Conclusion

The experiments discussed in this Application Note demonstrate the potential improvement of the basic nano 2D LC/MS system for protein identification of proteomics samples by implementing only slight modifications. To improve the performance of the LC system from simply a system working with injected salt solution steps to an effective continuous salt solution gradient system we have introduced a micro 10-port valve as well as a third pump (isocratic) in the first dimension. The increase in performance can be attributed directly to the continuous salt solution gradient for the elution of the sample peptides from the SCX column in the first dimension, which ensures optimum conditions for this column. As shown, the online 2D LC method performed using the continuous salt solution gradient gives higher sequence coverage for proteins identified from a medium complex mixture and increases the number of identified proteins from a highly complex mixture as well as providing high quality data.

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