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ANALYSIS OF COMPLEX PROTEIN DIGEST SAMPLES BY OFF-LINE STRONG CATION EXCHANGE CHROMATOGRAPHY IN COMBINATION WITH LC/MS/MS ON A HYBRID QUADRUPOLE ORTHOGONAL ACCELERATION TIME-OF-FLIGHT (Q-TOF) MASS SPECTROMETER

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INTRODUCTION

Advances in both LC and mass spectrometry instrumentation have allowed the analysis of protein complexes which have not been separated on a two dimensional gel. These experiments involve separation of a complex digest mixture by nanoscale liquid chromatography connected to an instrument capable of data directed switching between the MS and MS/MS modes. Protein identification is then achieved via databank searching of the ESI/MS/MS spectra, providing qualitative information on the proteins that are present. Hundreds of MS/MS spectra can be acquired in a fully automated fashion, resulting in the identification of significant numbers of proteins, including low copy number proteins, from a single LC/MS/MS experiment.

If, however, a complex protein mixture is to be investigated then a fractionation step prior to separation of the peptides on the basis of their hydrophobicity is advantageous. This has resulted in 2*D* LC approaches being adopted for the analysis of extremely complex tryptic digest samples. By placing a strong cation exchange (SCX) cartridge in front of a C_{18} trap cartridge, or by using a bi-phasic analytical column it is possible to pre-fractionate the peptides on-line before separation and analysis by reversed phase LC/MS/MS. In this paper, the optimization of an off-line SCX fractionation step, in combination with automated fraction collection, prior to analysis by reversed phase LC/MS/MS is described. This will be compared to off-line reverse phase fractionation of the sample, followed by automated NanoSpray and on-line 2*D* LC. Data will be presented on a global tryptic digest of a K12 strain derived *Escherichia coli* sample.

METHODS

Preparation of an *E. coli* Cytosolic Protein Digest

An aliquot of 500 µL of K12 strain derived *Escherichia coli* cytosol was diluted in 0.1% RapiGest™ in 25 mM ammonium bicarbonate, 0.5 M DTT, 1 M $CaCl_2$ and digested by adding 400 µg of Promega sequencing grade trypsin in resuspension buffer. After digestion at 37 °C for 14 hours, a second aliquot of 100 µg of trypsin was added and the digestion continued for an additional 4 hours. Finally, the sample was centrifuged at 12,000 g for 10 min. The supernatant was collected and diluted five times with off-line SCX mobile phase A buffer and directly injected on the multidimensional LC/MS/MS system. The final concentration of E. coli cytosolic protein digest equaled 1 µg/µL.

Off-line 2D LC/MS/MS System

The off-line setup comprises a modular ternary Waters® CapLC® pump equipped with a dual wavelength UV absorbance detector and a 15 cm x 1.0 mm i.d. PolySULFOETHYL SCX column (PolyLC, Columbia, MD, USA). The SCX mobile phases consisted of:

SCX mobile phase A: 0.5% acetic acid in water

SCX mobile phase B: 0.5% acetic acid in water/acetonitrile (65:35, v/v) + 250 mM KCl

SCX mobile phase C: 0.5% acetic acid in water/acetonitrile (65:35, v/v) + 500 mM KCl

The flow rate equaled 10 µL/min and the detection wavelength was 214 nm. The gradient was from 0 to 100% solvent B in 45 min, from 100% solvent B to 100% solvent C from 45 to 60 min and kept at 100% C for an additional 5 min. Fractions were collected for every 2 min with a Waters 2700 Sample Manager in a 96-well PCR plate for the complete duration of the first dimension SCX

separation experiment. 20 µL of an aqueous 0.1% formic acid solution was added to each of the fractions.

The reversed phase LC/MS/MS system consisted of a Waters® CapLC pump equipped with a Stream Select Module for on-line desalting and preconcentration of the first dimension fractions onto a 5 mm x 300 µm i.d. PepMap precolumn (LC Packings—A Dionex Company, Amsterdam, The Netherlands), which was conducted at a flow rate of 20 µL/min for 5 min with an aqueous 0.1% formic acid solution. The sample was directed from the precolumn onto a 15 cm x 75 µm ID C_{18} Waters Atlantis™ NanoEase™ analytical column at a flow rate of 200 nl/min. The sample was eluted from the C_{18} analytical column using an increasing organic solvent concentration from 5 to 50% B in 60 min. Solvent A consisted of 0.1% formic acid in acetonitrile/water (2:98, v/v) and solvent B of 0.1% formic acid in acetonitrile/water (80:20, v/v).

Electrospray MS and MS/MS data were acquired on a Waters Micromass® Q-Tof Ultima™ API mass spectrometer fitted with a NanoLockSpray™ ion source. The mass spectrometer was operated in the positive ion mode with a potential of 2200 V applied to the spray tip. All LC/MS data were acquired with the mass spectrometer operating in Data Directed Analysis (DDA™) MS/MS mode. Somatostatin and erythromycin were used for the lock mass correction of the precursor and product ions, respectively.

On-line 2D LC/MS/MS System

The on-line set up is as described previously (Hughes *et al.*, The Analysis of Complex Tryptic Digest Mixtures by Multidimensional LC/MS/MS on a Hybrid Quadrupole Orthogonal Acceleration Time-of-Flight Mass Spectrometer, ABRF 2001 poster presentation) with minor modifications, which include the use of two 17 mm x 1.0 mm i.d. SCX Opti-PAK precolumns (Waters Corporation) and different SCX step gradient conditions. On overview of the valve detail is shown in Figure. 1. Discrete fractions are sequentially eluted from the SCX precolumn by injecting solvent plugs comprising 0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0 and 25 mM KCl in acetonitrile/water (5:95, v/v) and 50.0, 75.0, 100, 125, 150, 200, 250 and 500 mM KCl in acetonitrile/water (10:90, v/v). After the injection of a certain salt concentration, a fraction of the peptide population is eluted from the SCX precolumn and sequentially retained on the reversed phase C_{18} trap column where they are desalted and preconcentrated. Finally, the peptides are eluted from the C_{18} precolumn by running a reversed phase gradient for separation and elution into the mass spectrometer.

The reversed LC/MS/MS system and conditions are as described above in the section Off-line 2*D* LC/MS/MS system.

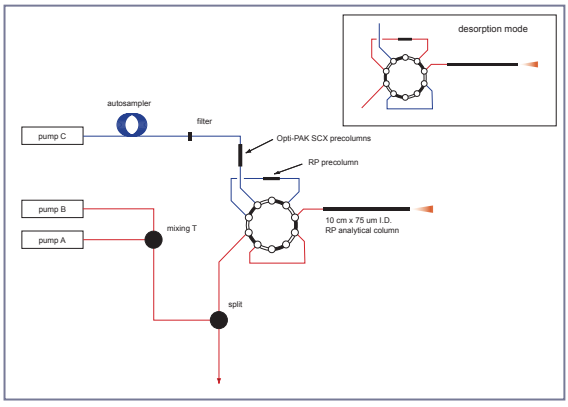

Figure. 1. Schematic representation of the valve and column configuration of the on-line 2D LC/MS/MS system.

RESULTS

SCX Off-Line Separation Optimization

The SCX off-line dimension separation performance and robustness was evaluated by means of injections of a two-peptide mixture comprising Oxytocin and Angiotension I—representing the extremes of the type of peptides to be expected from a complex tryptic mixture with respect to charge—and a tryptic digest of Bovine Serum Albumin. The results for the two-peptide mixture are shown in Figure. 2. The peak widths at base of the two components were respectively 0.7 and 1.4 min and the elution window about 40 to 50 min. Hence, a total number of 20 to 25 SCX fractions of 2 min each, that is, 20 µL elution volume—could be collected.

The repeatability of the system was tested with repetitive injections a tryptic digest of BSA of which the results are shown in Figure. 3. The average retention time reproducibility was found to be < 0.05%.

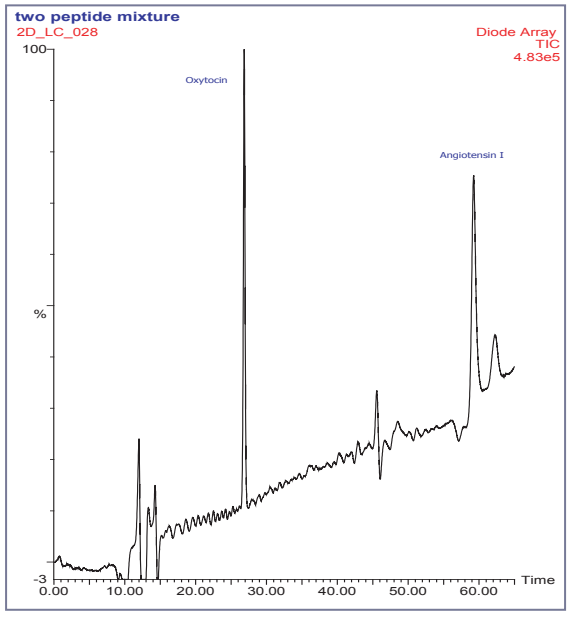

Figure. 2. SCX separation of a two-peptide mixture comprising Oxytocin (charge 0.1+ at pH 3) and Angiotension I (charge 3.0+ at pH 3). The gradient conditions are described in detail in the section Methods.

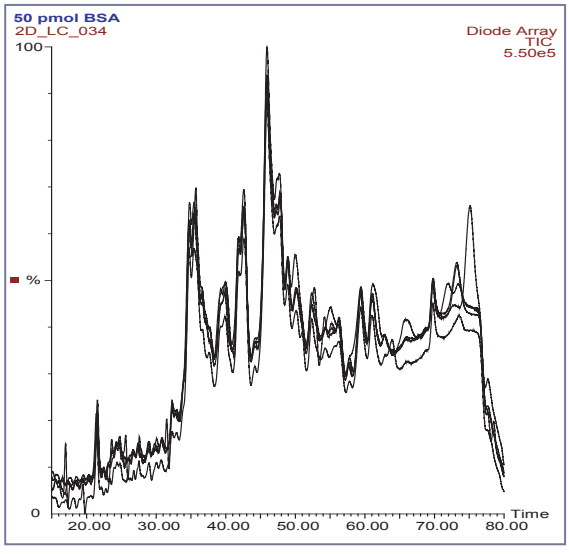

Figure. 3. Five consecutive SCX separations of 50 pmol of tryptic digested BSA illustrating the repeatability and robustness of the developed first dimension separation. UV absorption detection was at 214 nm and the gradient conditions as described in the section Methods.

SCX Off-Line Fractionation of an *Escherichia coli* Sample

50 µL of the diluted global digest—corresponding to an effective load of 50 µg—of the cytosolic K12 strain derived *Escherichia coli* sample was injected onto the SCX column. An optimized bimodal gradient—that is, KCl salt concentration and organic solvent percentage—was used for the separation of the tryptic peptides. The larger, more highly charged peptides elute normally later in the SCX gradient. The UV absorption chromatogram of the SCX separation of the *E. coli* digest is shown in Figure 4. The dashed lines in Figure 4 represent the collected fractions that were selected for the second dimension separation and identification by nanoscale reversed phase LC/MS/MS.

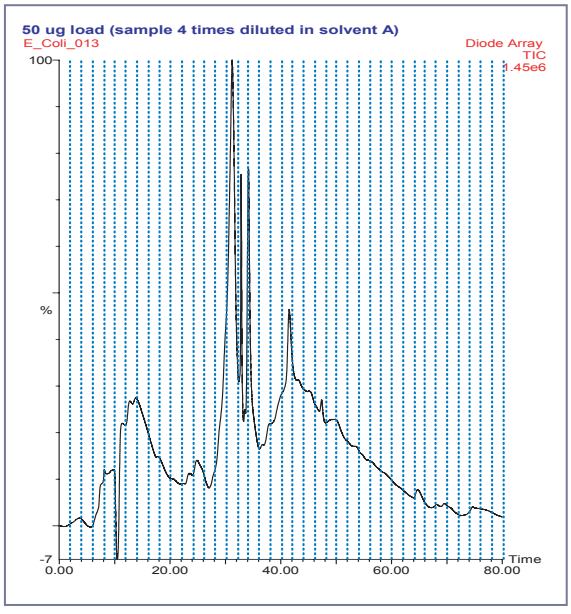

Figure. 4. SCX first dimension off-line separation of a global tryptic digested K12 strain derived Escherichia coli sample. The chromatographic conditions are as described before.

Off-line 2D LC/MS/MS Results

20 µL of an aqueous 0.1% formic acid solution was added to each of the fractions prior to injecting the SCX fractions onto the second dimension reversed phase separation system. 5 µL of final fraction solutions was introduced to the nanoscale LC/MS/MS system resulting in an effective consumption of 4 µg of the cytosolic *E. coli* sample. As an example, the results for one of the fractions are shown in Figure 5a and Figure 5b, respectively. Charge state recognition was used to select doubly, triply and quadruply charges precursor ions for the MS/MS experiments, which also includes the automated selection of the collision energy based on charge and mass. A maximum of eight precursors was defined for concurrent MS/MS acquisitions from one single MS survey scan and a 3 s acquisition time per MS/MS experiment.

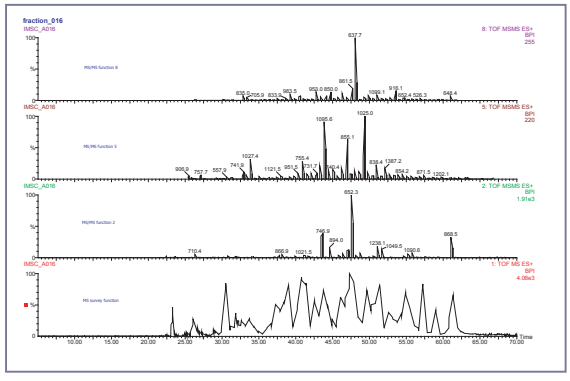

Figure. 5a. Base peak intensity chromatograms of the survey MS and MS/MS functions - 4 out of 9 shown - from off-line fraction 16.

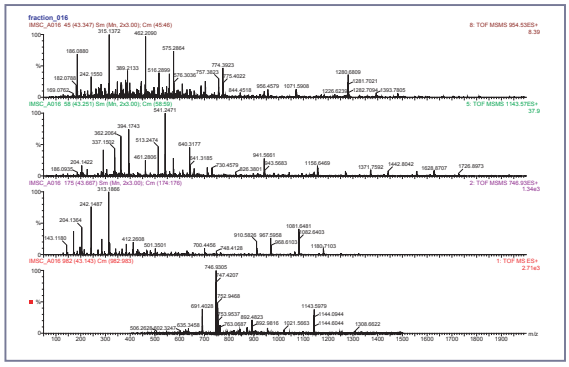

Figure. 5b. MS survey MS and MS/MS spectra - 4 out of 9 shown - of the components eluting at time 43 min from off-line fraction 16.

Each individual LC/MS/MS dataset—that is, salt fraction from the first dimension off-line SCX separation—was searched against a species-specific database using ProteinLynx™ Global SERVER v2.0.5. An example of the database search result for one of the fractions is shown in Figure 6.

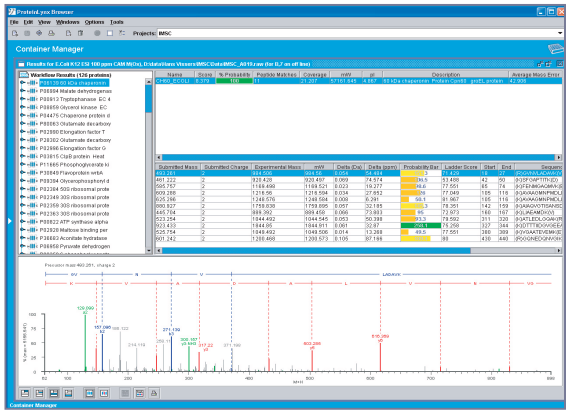

Figure. 6. Protein identification of validated search results - that is, only the top scoring peptides and MS/MS spectra that exhibit a y-ion sequence stretch of a least three amino acids are reported - of off-line fraction 19 with ProteinLynx Global SERVER v2.0.5.

The database results for all the off-line collected SCX fractions were merged with ProteinLynx Global SERVER v2.0.5 to provide a comprehensive overview of the identified proteins from a cytosolic K12 strain derived *E. coli* sample of which the results are shown in Figure 7. The results shown in Figure 7 represent the number of unique proteins identified in each SCX fractions. Furthermore, the number of cumulative proteins is presented after removal of the redundant cumulative identifications and isoforms. This because tryptic peptides of the same protein are likely to be distributed over and identified in multiple SCX fractions.

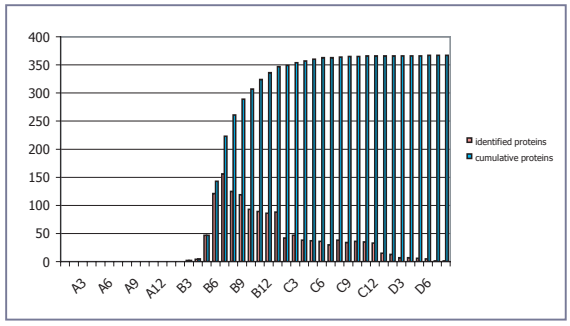

Figure. 7. Distribution of the non-redundant identified proteins with off-line 2D LC/MS/MS for the cytosolic K12 strain derived Escherichia coli sample.

On-line 2D LC/MS/MS Results

5 µL of the final digest solution was introduced onto the on-line 2D nanoscale LC/MS/MS system resulting in an effective consumption of 5 µg of the cytosolic *E. coli* sample. The first dimension step gradient conditions are described in the section Methods and the reversed phase nanoscale LC/MS/MS conditions are as described above.

Each individual LC/MS/MS dataset was processed and database searched as described for the off-line multidimensional experiment.
A comprehensive overview of the identified proteins from the cytosolic *E. coli* sample with an on-line approach is shown in Figure 8.

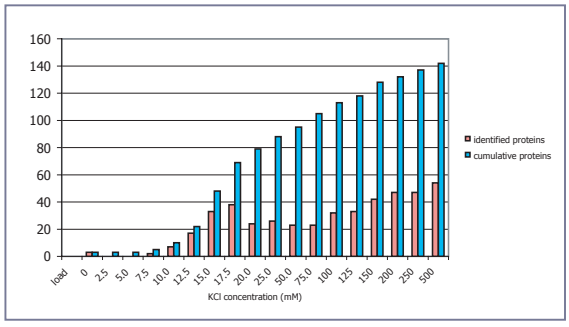

Figure. 8. Distribution of the non-redundant identified proteins with on-line 2D LC/MS/MS for the cytosolic K12 strain derived Escherichia coli sample.

CONCLUSIONS

- An optimized SCX gradient elution method using a 1.0 mm i.d. SCX column resulted in efficient peptide separation addressing issues such as required sample quantity load and dynamic range
- Off-line approaches do not require compromises with respect to the first dimension gradient conditions
- Greater number of identified peptides and proteins with off-line vs. on-line
- Improved amino acid sequence coverage with off-line vs. on-line (data not shown)
- The developed off-line multidimensional LC/MS/MS method provided improved sensitivity compared to the adopted on-line approach

ACKNOWLEDGEMENT

Dr. Rob van der Hoeven of DSM Food Specialties, Delft, Netherlands is greatly acknowledged for providing globally digested Saccharomyces cerevisiae cell lysate that was used for the initial optimization experiments of the first dimension SCX separation.

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Dr. Scott Berger of Waters Corporation, Milford, USA is thanked for supplying a global tryptic digested K12 strain derived *Escherichia coli* sample.

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