Optimized two-dimensional nano-liquid chromatography tandem mass spectrometry protocols for proteomics applications: on-line or off-line coupling?

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Nanochromatography tandem mass spectrometry (nanoLC-MS/MS) has become a standard analysis of complex proteolytic peptides mixtures in proteomics. The peptides are sequentially sequenced in tandem mass spectrometry after a prior separation on a reversed phase (RP) column at nanoflow rates. For highly complex samples, like whole cells lysates, the total number of peptides widely exceeds the peak capacity of conventional columns. Co-elution of tens of peptides decreases the percentage of peptides that can be sequenced and limits the proteome coverage. In this case, two-dimensional nanoLC (2D nanoLC) offers an additional separation dimension that is necessary to overcome this bottleneck.
Strong Cation Exchange (SCX) LC followed by RP nanoLC is the default coupling in proteomics and different protocols can be found in the literature. However these protocols need to be evaluated and compared. Total performance of this 2D LC depends on the resolution of both separations. Indeed if both types of separations are orthogonal (based on totally different physicochemical characteristics, here pI and hydrophobicity) and optimized, the global peak capacity of the system is the product of the peak capacities of each separation. In the present work, on-line and off-line 2D nanoLC are systematically compared and optimized. In both case, MS data are acquired on a hybrid linear ion trap-FT-ICR mass spectrometer (LTQ-FT, ThermoFisher, CA, USA).

METHODS

Samples : LCP = tryptic digest of 6 proteins mix from 11 kDa to 135 kDa (Cytochrome C, Lysozyme, Alcohol dehydrogenase, Bovine serum albumin, Serotransferrin and ß-Galactosidase, Dionex, Netherlands). Eyelash sample : tryptic digest of 50 eyelashes, total volume 150µL ; 5µL/injection.

Mass spectrometry : LTQ-FT (ThermoFisher Scientific).
Automatic high dynamic mode : alternate acquisitions in FTMS full scan survey mode (m/z range 400-2000), 3 FTMS SIM scan mode for exact mass and charge state determination of peptide and 3 LIT MS/MS mode for sequencing [2].

Protein identification : Search with Mascot 2.2 (Matrixscience) against Swissprot from UniProtKB release 9.6; criteria: trypsin, up to 2 misscleavages; partial modifications for LCP: carbamidomethylation, carboxymethylation and deprotonation of Cys, Nter carbamylation, deamidation of Asn and Gln, oxidation and dioxydation of Met, oxidation of His and Trp; for eyelash Nter acetylation, deamidation of Asn, Gln and Arg, deprotonation and carbamidomethylation of Cys, oxidation of His, Trp, Lys and Met, phosphorylation of Ser and Thr.

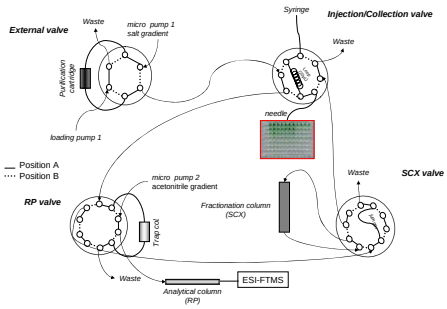
Validation [3] : At least 2 different peptide sequences identified as first candidates for the protein with a minimal ion score of 30. Mass tolerance was set to 10 ppm in MS mode, and 1 Da in MS/MS mode.

Solvents : solvent A: H20/AcN/AF, 98/2/0,1, v/v/v, solvent B: H20/AcN/AF, 10/90/0,1, v/v/v, solvent B': see below

LC 1D: Reversed-phase separation (RP-LC) on Ultimate 3000 (Dionex).
Trap-column: C18 PepMap100, 3 µm, 300µm i.d., length 5 mm (Dionex) loading with a 15µL/min flow rate of solvent A.
Analytical column: C18 PepMap100, 3 µm, 100 Å, 75 µm i.d., length 15 cm, Dionex.
Linear gradient: from 0 to 50% of B in 35min, flow rate of 220nL/min.
To eliminate polymeric contaminants a purification cartridge (C18 PepMap100, 5µm, 100 Å, 1 mm i.d., longueur 15 mm, Dionex) is inserted between the loading pump and the injection valve. After sample injection, this cartridge is flushed with 40µL/min of solvent B during 25min [1].

LC 2D: on Dual Ultimate 3000 (Dionex)
First dimension: Strong Cation Exchange separation
Column: BioBasic SCX 5µm, 300 Å, 0,32mm i.d., length 15cm (ThermoFisher Scientific) ; For details on on-line and off-line protocols see next section.
To eliminate polymeric contaminants, purification cartridges (C18 PepMap100, 5µm, 100 Å, 1 mm i.d., length 15 mm, Dionex) are inserted after loading pump 2 and micropump. These cartridges are regularly regenerated by a flushing with 40µL/min of solvent B [1].
Second dimension : As described above.

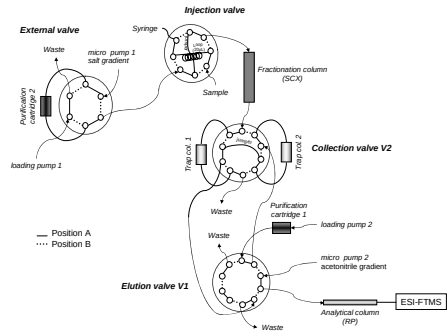
Off-line coupling



Peptides are first trapped on the SCX column. A rapid gradient of salt buffer (20 min) elutes peptides from the column. They are automatically collected into a multi-well plate in the autosampler. Fractions are dried, reconstituted in A and reinjected for a RP-HPLC separation.

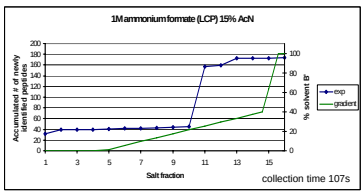
Salt buffers B' used : from 100mM to 1M ammonium formate pH=3 with acetonitrile (5% to 25%). Different gradients and different collection times have been tested.

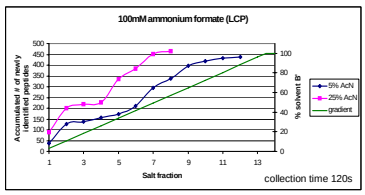
On-line coupling

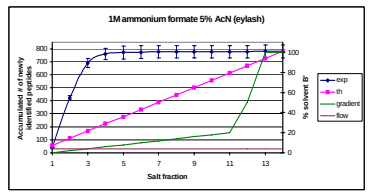


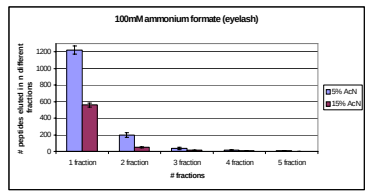
Peptides are first trapped on the SCX column. A linear gradient of salt buffer (15 h) elutes peptides from the column. They are alternatively trapped on the C18 trap1 or on the C18 trap2 and are desalted with A. Valve V2 is then switched and the trapped peptides are flushed towards the analytical column for a RP-HPLC separation.

Salt buffers B' used : from 100mM to 1M ammonium formate pH=3 with acetonitrile (5% to 25%). Different gradients have been tested.







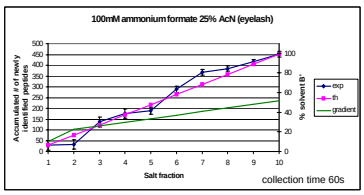


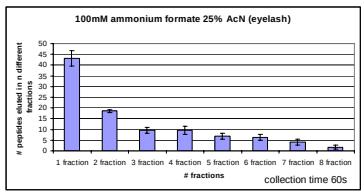
Salt buffer concentration: Starting with 1M ammonium formate in B', nearly all peptides are eluted in one single fraction after 10 empty fractions. To evenly distribute the peptides in every fractions, we decrease salt concentration in B' to 100mM. To elute peptides faster in the very first fractions, we inject sample at 10% B' and gradient begins with 20% B'.

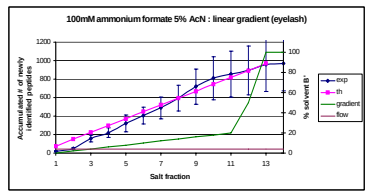
Acetonitrile concentration: Increasing acetonitrile concentration allows to elute peptides faster ; 25% AcN avoids unspecific hydrophobic interactions on the SCX phase. However even with 25% AcN, 38% of the peptides are still eluted in three or more fractions. This is probably due to the injection volume (20µL in µL-pickup) incompatible with a punctual injection (analytical conditions).

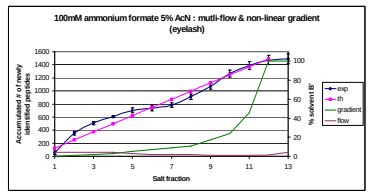
Salt buffer concentration: Starting with 1M ammonium formate in B', all peptides are eluted after only 4-5 fractions. As each salt fraction last 71min, the total amount of salt is a very important parameter because peptides are eluted in isocratic mode.
→ It was necessary to drastically reduce salt concentration to 100mM in the buffer B' in order to regularly distribute peptides along the separation time.

Acetonitrile concentration: Increasing acetonitrile concentration allows to decrease the proportion of peptides eluted in more than one fraction: almost 100% of peptides are eluted in a single SCX fraction at 25% of acetonitrile. However, increasing acetonitrile concentration decreases the retention of peptides on C18 trap-columns for on-line 2DLC. At 15 % of acetonitrile, 55 % of peptides are lost and at 25 % of acetonitrile, 85 % of peptides are lost!





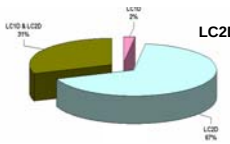




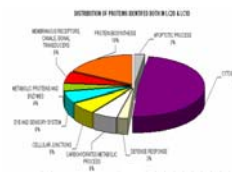
Assuming that between two successive fractions, peptides are linearly eluted, the salt amount and the corresponding flow and salt buffer % required for every fraction can be calculated in isocratic mode. The optimized gradient allows to elute peptides linearly in an very reproducible way.

Comparison between LC1D and LC2D

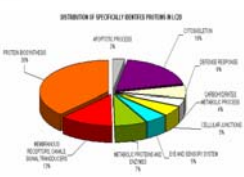
Whatever the chosen protocol (off-line or on-line), LC2D allows to multiply the number of peptides and proteins identified by approximately 3.



LC2D : An undoubtedly successful tool !!



With LC1D, nearly 50% of identified proteins in eyelash sample are cytoskeleton proteins like keratins. With LC2D, proportion of proteins from this category is widely reduced to the benefit of the others like the protein biosynthesis group.

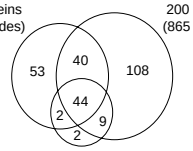


CONCLUSION : Comparison between off-line and on-line

Results of both on-line and off-line modes remain comparable. The number of peptides identified in off-line is slightly lower probably because of the dispersion of peptides between diverse fractions but also because of the drying step. Isocratic step gradient in on-line configuration allows to compensate the peak broadening induced by the non punctual injection of our system. Large volume injection is specific to biological studies. The amount of material is too low to afford any prior concentration step. It would induce non negligible sample losses and prevent any final detection. To correct this peak broadening we intend to evaluate an on-line pre-concentration step on the SCX, similar to the classical RP-HPLC one.

LC2D Off-line
139 identified proteins
(749 different peptides)

LC2D On-line
200 identified proteins
(865 different peptides)



LC 1D
57 identified proteins
(266 different peptides)

References :
1 Hesse A.M. *et al.* (2008), J. Chrom. A **1189** (1-2) 2 Olsen J. V. *et al.* (2004). Mol Cell Proteomics. **3** (6) 3 Kapp, E. A. *et al.* (2005). Proteomics **5** (13)

Acknowledgments : We thank L'Oréal for Anne-Marie Hesse PhD fellowship ; RNG and FRRT for fundings