From analytical to nano-flow LC-MS: high robustness and sensitivity to answer complex biological questions

2017-03-26 Alexander Boychenko, Remco Swart
• LC-MS sensitivity gains and tailored method selection
• Capillary and micro-flow LC-MS: what to expect?
• Nano-flow LC-MS: the gold standard in proteomics
• Why chromatography is important for LC-MS proteomics
• Nano-flow and sub-nano flow rates
LC-MS method development

Sensitivity

Throughput

Selectivity

Reproducibility

Precision

Linearity

Accuracy
When do you need to downscale your chromatography?

- Analytical flow LC-MS on 2.1 mm ID column
- Micro-flow LC-MS on 1.0 mm ID column
- Capillary-flow LC-MS on 0.30-0.15 mm ID column
- Nano-flow LC-MS method on 75-50 µm mm ID column
- Sub-nano-flow LC-MS

Throughput:
- ~450 µL/min
- ~100 µL/min
- ~1-15 µL/min
- ~300 nL/min
- <100 nL/min

Sensitivity

Can you confidently detect and/or quantify a target?
LC-MS with ESI: flow rate and sensitivity gain

ESI-MS exhibits a mixed behavior of both concentration and mass-sensitive detectors

Sensitivity gains (experimental, relative to 2.1 mm ID)

- microLC-MS: 2-4
- capLC-MS: 4-50
- nanoLC-MS: > 50
- sub-nanoLC-MS: > 100

Reasons

- Increase of analyte concentration with decrease of column ID
- Improved ionization efficiency

The sensitivity gains were measured as a relative peak area averaged for Cytochrome C tryptic peptides

Flow rate, µL/min

Sensitivity gain in comparison with analytical LC-MS at 450 µL/min
From analytical to micro-flow LC-MS

- 255 pesticides separated on Accucore aQ \(100 \times 2.1\) mm, 2.6 \(\mu\)m

  - Ultrafast UHPLC-MS SRM method
  - > 500 SRMs with positive/negative
  - Retention time standard deviation < 0.3 sec

- 255 pesticides separated on Hypersil GOLD \(100 \times 1.0\) mm, 1.9 \(\mu\)m

  - Same throughput and robustness as for 2.1 mm ID
  - 2 times sensitivity gain on average

- 20 pesticides separated on Hypersil GOLD \(100 \times 0.5\) mm, 1.9 \(\mu\)m

  - 7 times sensitivity gain on average
  - Increased analysis time (15 min)

Sensitivity gains:

- 2.1 mm ID
- 1 mm ID
- 0.5 mm ID

- 7 times sensitivity gain on average
Why do we need nanoLC-MS?

- Extremely low amount of sample (e.g. laser micro-dissected cells, small animal bio-fluids)
- Tumor biopsies
- Low analyte concentration in a complex matrix (biomarkers)
- High sample complexity and wide dynamic range
- Single cell -omics

LMD captured cells

Patel et al. doi: 10.1158/1078-0432.CCR-07-1497

mRNA expression of GAPDH from individual Jurkat cells

Toriello et al. doi: 10.1073/pnas.0806355106
NanoLC-MS is a gold standard in proteomics

Base peak chromatograms of HeLa cell lysate digest (1 µg)
analytical flow LC-MS: blue
capLC-MS: green
nanoLC-MS: purple

High LC-MS sensitivity is essential for deep proteomics

~ 3000 Protein groups

1 µg HeLa digest
NanoLC-MS proteomics: the effect of column length

Protein ID: blue line
Peptide ID: green bars

✓ Deeper proteome coverage with longer columns
Why chromatography is important

✓ Longer gradients give more peak capacity
✓ The peak width increases proportionally to the gradient time
✓ Longer columns should be used with long gradients

Thermo Scientific™ EASY™-spray, ES805 and ES802
Increased protein identification rates with 75 cm columns

- Comparison of 75 cm and 50 cm EASY-Spray column
- Sample: HeLa Digest (1 µg)
- Gradient: 120 min or 240 min

Application Note AN639
Increased protein identification rates with 75 cm columns

Peak Width

75 cm long columns give:
✓ Narrower peak width and higher peaks
✓ Higher peak capacity
✓ ~10% more protein identifications than 50 cm long column
NanoLC HRAM MS targeted quantification

### FSGSGSGT SYS LTISR

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Average: 23.03
STD: 0.03
RSD, %: 0.12

1:10⁴ rituximab to HeLa total protein amount in a sample
- 7 consecutive replicates
- 30 min gradient
- EASY-Spray 75 µm x 50 cm, 2 µm
- 4 sec peak width
- 30 min gradient

✓ LOD at amol level for PRM quantification in matrix
✓ High RT reproducibility permits PRM scheduling

Multiplexed scheduled HRAM analysis of rituximab in human matrix

**Heavy chain:**
- 5 unique peptides

**Light chain:**
- 3 unique peptides

Thermo Scientific™ Q Exactive™ HF hybrid quadrupole-Orbitrap mass spectrometer
Thermo Scientific™ UltiMate™ 3000 RSLCnano system
Enhanced sensitivity with low nano-flow rates

Sample loading
300 nL/min

Column washing
and re-equilibration
300 nL/min

Peptides separation
100, 150, 200 nL/min

- 90 min gradient
- Acclaim PepMap 75 µm x 15 cm, 2 µm

Protein IDs
20 ng injection

- 1755
- 1929
- 1545

< 1 % false discovery rate (FDR)

- R.GC[CAM]HLLVATPGR.L
- O00571 (DDX3X_HUMAN)

More sensitivity for limited sample amounts
Same LC-MS setup and gradient length
Ultralow-flow nanoLC (100-10 nL/min)

Protein IDs
10 ng injection

20 nL / min

Unique Proteins (grouped)

Gradient Time / [h]

0 2 4 6 8 10

500 1000 1500 2000 2500

Analytical Column (prototype): 25 µm ID x 25 cm, Acclaim PepMap RSLC C18, 2 µm, 100 Å
Trap: 50 µm ID x 7 cm (2 cm packed), Acclaim PepMap C18, 3 µm, 100 Å
Emitter: 10 µm ID, tip opening 5 µm
Connections: 10 µm ID nanoViper

No flow splitting (prototype)
Vented column setup

Increased sensitivity in comparison with regular nanoLC-MS
Good retention time precision, RSD < 0.2%

Conclusions

• Downscaling LC separations results in significant increases in LC-MS sensitivity, permits the analysis of complex samples and facilitates very low level quantification

• The chromatographic scale adopted for an LC-MS method should be chosen according to the relative importance of sensitivity and throughput

• Nano LC-MS using long separation columns is now routine in proteomics

• Ready-to-use capillary connections, integrated plug-and-spray consumables and easy-to-operate LC systems have made nano LC-MS accessible to people with limited LC experience

• Extremely high sensitivity can be obtained using ultra-low nano flow rates. However, a great deal of expertise is required for such setups