

The Importance of High Precision Retention Times in LC/MS of Biomarkers

Peter Kent, Lori Ann Upton, Eric Kemp and Kerry Nugent
Michrom Bioresources, Auburn, CA

INTRODUCTION

Many life science researchers are involved in the identification, validation and quantitation of biomarkers to aid in clinical diagnostics, therapeutic drug development and health monitoring. These scientists face many challenges including complexity of samples, variability in the sample population and dynamic sample states.

With the great advances over the past decade, LC/MS has emerged as a major technique for metabolomic and proteomic analysis of biomarkers. Although MS is a powerful tool for the identification and quantitation of biomarkers, the front end sample preparation and separation of complex samples by LC helps improve the confidence of the MS results.

Due to the complexity of biomarker samples, the requirement for precise LC retention times is extremely important in comparative studies. As biomarker discovery moves from identification to validation and quantitation, the need for precise LC retention times will become even more important.

EXPERIMENTAL

Autosampler

Paradigm Bio-Cool HTC PAL

HPLC

Paradigm MS4N MDLC or
Prototype Paradigm VP LC

ESI

Michrom Nano-Capspray

MS/MS

Thermo LCQ Deca ITMS

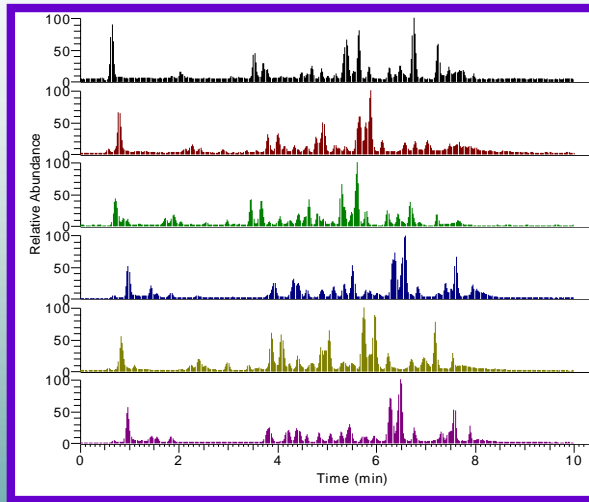
METHODOLOGY

A prototype LC platform was constructed to test all of the factors which influence LC retention time precision. A variety of pumping systems were used to test isocratic and gradient flow accuracy and flow precision at rates from 0.1 – 5000 ul/min over a pressure range from 100-10,000 PSI. Pump variables tested include pump design, pump materials, flow feedback control, split flow vs splitless flow and gradient formation.

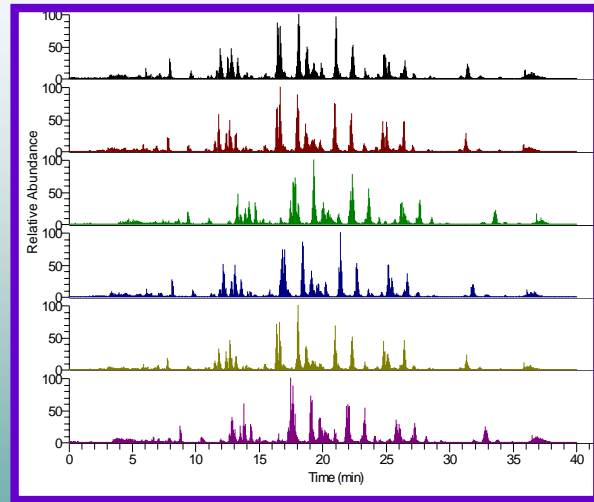
Additional LC factors studied include mobile phase (composition, degassing, mixing, temperature, etc) sample (components, concentration, injection volume, etc), column (size, packing material, temperature, stability, equilibration etc) and system (plumbing, fluid dynamics, software, validation, etc).

Using a series of samples with increasing complexity, the above parameters were tested to determine the optimum conditions to maximize retention time precision for complex samples.

UNOPTIMIZED SYSTEM REPRODUCIBILITY



Manual Injections of 50fm HSA over 12 hr
0.2 x 50 mm 5u 300A Magic C8
5-35% ACN/0.1%FA in 6 min @ 4 ul/min
Undegassed Solvents and Tee Splitflow
Room Temperature (25 ± 5 °C)



Manual Injections of 50fm HSA over 12 hr
75u x 150 mm 5u 200A Magic C18AQ
5-35% ACN/0.1%FA in 30 min @ 400 nl/min
Undegassed Solvents and Tee Splitflow
Room Temperature (25 ± 5 °C)

FACTORS THAT AFFECT REPRODUCIBILITY

LC MOBILE PHASES

- Degassing
- Stability
- Mixing
- Equilibration
- Temperature

SAMPLE INJECTION

- Timing
- Mass
- Volume
- Diluant
- Temperature

LC PUMPS

- Split - Splitless
- Accuracy
- Resolution
- Flow Sensors
- Temperature

LC COLUMN

- Stability
- Robustness
- Capacity
- Equilibration
- Temperature

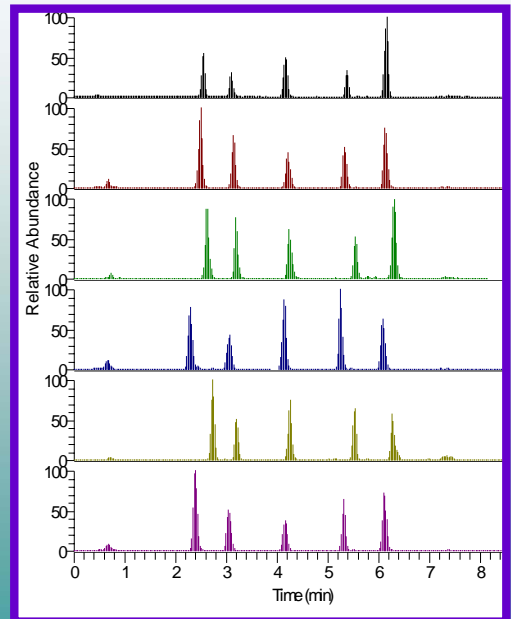
HIGH THROUGHPUT LC/MS TEST SYSTEM

A high throughput LC/MS protocol was developed to test all of the different factors that influence LC retention times.

Five peptides which differed in MW, pI and hydrophobicity were selected for this screening process because their relative retention times were greatly influenced by changes in the various LC parameters.

Using the new Michrom Plug and Play Nano-Capspray Interface, we were able to achieve a 10 minute total analysis time which allowed us to test each variable with six replicates in one hour.

The LC/MS runs at the right show that when conditions are not controlled, the retention time variability of these five peptides ranges from 2-8% RSD.



0.2 x 50 mm 5u 300A Magic C8
5-65% ACN in 6 min @ 4 ul/min

OPTIMIZATION OF LC MOBILE PHASES

DEGASSING

Solvents hold different amounts of gases, altering the refill of low volume LC pumps, impacting retention times. A helium sparge and blanket system insures complete refill of LC pumps.

STABILITY

A helium sparge and blanket system minimizes solvent decomposition and differential volatilization of mixed solvents, which adversely affect long term retention time repeatability.

MIXING

Inadequate mixing results in micro oscillations in solvent composition during gradient LC, which increases RT RSDs. Preblended mobile phases and mixers designed for LC pumps minimize these effects.

EQUILIBRATION

Inadequate equilibration of system/column to initial mobile phase adversely impacts RT RSDs, especially early in the runs. Careful optimization of system fluid dynamics and adequate re-equilibration volume (5-10 column volumes) improves RSDs.

TEMPERATURE

Variable mobile phase temperature may impact composition, stability, pump refill volume, flow rate, flow sensors and column temperature, all of which adversely impact RT RSD's.

OPTIMIZATION OF LC PUMPS

SPLIT – SPLITLESS	Although splitless nanoflow pumps have the advantage of running at the desired flow rates and not wasting solvents, these advantages may be outweighed by split flow pumps that offer better performance at higher flows.
PRE - POST MIX	One pump low pressure gradient LC provides precise flows but suffers from poor fluid dynamics, while multi pump high pressure gradient LC requires flow resolution to 1% of total flow rate but can have very good fluid dynamics.
ACCURACY	Flow rate accuracy depends on the electro-mechanical properties of the pumps, but is also affected by compressibility, temperature and trace leaks or blockages in the LC fluid path.
FLOW SENSORS	Flow sensors with feedback help compensate for flow related problems in nano/cap LC, but can be impacted by solvent composition, temperature and leaks after the flow sensors.
TEMPERATURE	Temperature has minimal impact on the electro-mechanical properties of most LC pumps, but can impact flows by changing compressibility, viscosity and system pressures.

OPTIMIZATION OF SAMPLE INJECTION

TIMING	Since LC columns are difficult to completely equilibrate, it is important to inject samples at exactly the same time in the LC re-equilibration process to insure reproducible retention times (best done using a LC autosampler).
MASS	Variability in the mass of samples injected can cause differential overloading, which results in changes to the retention times of the compounds being overloaded.
VOLUME	Variability in the injection volume from sample to sample can alter the sample loading time (which affects equilibration) and changes the loading characteristic of the sample components with respect to the sample diluants, mobile phase and column.
DILUANT	The diluant should completely solubilize all sample components while delivering the sample to the column in the tightest band possible. Variability in modifiers used for solubility or retentivity may also contribute to greater RT RSD's.
TEMPERATURE	Although biological samples are more stable at low temperatures, care must be taken to minimize precipitation or other changes to the sample if run at higher temperatures.

OPTIMIZATION OF LC COLUMN

STABILITY

LC columns must be well packed with materials that can withstand the pressures, temperatures, solvents and samples to which it will be subjected. An inadequately packed column may initially offer high resolution, but may deteriorate rapidly.

ROBUSTNESS

The column packing must be robust enough to withstand attacks from solvents (pH, temp) and samples (irreversible adsorption, aggregation), while offering rapid equilibration.

CAPACITY

Column overloading can cause peak fronting and adversely impact retention time reproducibility, as well as more rapid column deterioration. Always use the largest column ID possible that gives the desired sensitivity.

EQUILIBRATION

100% equilibration of a LC column can take > 100 column volumes (CV), but 5-10 CV will give >95% equilibration in a well designed LC (adequate for good RT RSDs if consistent).

TEMPERATURE

The LC column is the primary component impacted by changes in temperature, with RSDs increasing about 0.5% per °C change in the LC column temperature.

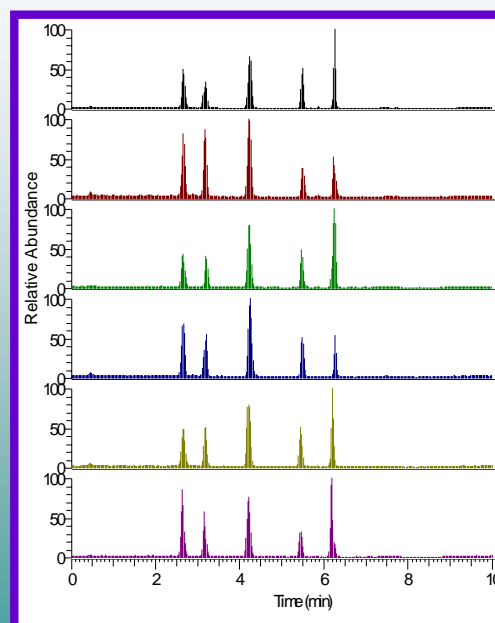
OPTIMIZED HT LC/MS SYSTEM

This high throughput LC/MS protocol allowed us to run 144 gradient separations per day to test all of the different factors that influence LC retention times.

Using this protocol with the new Michrom Plug and Play Nano-Capspray Interface, we were able to run 24/7 for over 30 days, with no change in column or interface performance (> 5000 runs).

As expected, the different factors were inter-related and often had to be optimized in parallel to obtain the best results.

The LC/MS runs at the right show that when conditions are well controlled, the retention time variability of these five peptides ranges from 0.1-0.4% RSD.



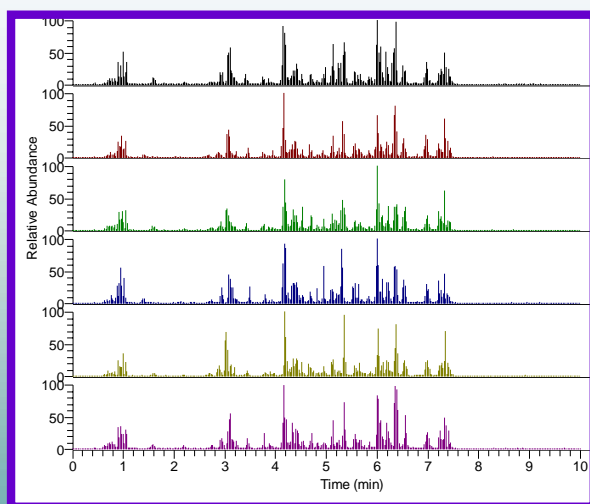
0.2 x 50 mm 5u 300A Magic C8
5-65% ACN in 6 min @ 4 ul/min

SUMMARY

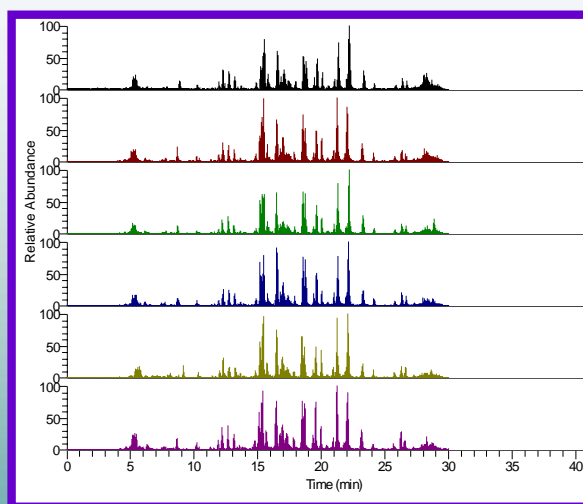
Using the prototype Paradigm VP LC platform, coupled with the Michrom Nano-Capspray Interface and a high throughput LC/MS protocol, we were able to test all of the factors which influence LC retention time reproducibility. The major factors which influenced retention time RSD's were pump flow reproducibility (which requires a flow sensor with pump control feedback for gradient flows from 50nl/min to 50 ul/min), LC/MS system temperature ($\pm 1^\circ\text{C}$), column temperature ($\pm 0.1^\circ\text{C}$), column quality and total system integration and automation.

As shown on the slide that follows, these optimized conditions were then applied to a tryptic digest of human serum albumin (the major background in screening serum samples for biomarkers) at flow rates of 400 nl/min and 4ul/min, with results identical to our 5 peptide model system. Since the Nano-Capspray interface provides nanospray sensitivity at capillary flow rates, this protocol offers a great solution to researchers who want to increase proteomics LC/MS sample throughput, reproducibility and robustness without sacrificing detection sensitivity.

NANO/CAP LC/MS FOR HT PROTEOMICS



Auto Injections of 10 fmol HSA over 24 hr
0.2 x 50 mm 5u 300A Magic C8
5-35% ACN/0.1%FA in 6 min @ 4 ul/min
He Degas/Pressurized Solvents
System Temperature ($40 \pm 0.1^\circ\text{C}$)



Auto Injections of 10 fmol HSA over 24 hr
75u x 150 mm 5u 200A Magic C18AQ
5-35% ACN/0.1%FA in 30 min @ 400 nl/min
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CONCLUSIONS

- A Wide Range of Factors Impact LC/MS RT RSD's
- Nano LC Pumps (Split or Splitless) Require Flow Sensors
- Temperature Variation (Column & System) Impact RT RSD's
- LC Column Robustness is Essential for Good RT RSD's
- LC/MS System Integration & Automation Improves RT RSD's
- Nano-Capspray Allows HT Proteomics at High Sensitivity
- 0.1-0.4% RT RSD Can be Achieved for Biomarker Analyses