DECREASING CYCLE TIME WHILE MAINTAINING ANALYTICAL SENSITIVITY IN MICROFLOW LC/MS UTILIZING A NOVEL VALVE SWITCHING ALGORITHM

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INTRODUCTION

- **Microflow LC/MS has garnered attention for applications requiring maximal sensitivity**
- **Sensitivity gain is a consequence of the reduced flow rate which also results in undesirably longer cycle times**
- **Adopting a dual pump trapping configuration utilizing a novel valve switching algorithm preserves sensitivity while also drastically reducing cycle time (Figure 2 and 3)**
- **For a digested mixture of 4 protein standards cycle times were reduced between 37 and 56% with no loss in sensitivity compared to a conventional microflow method (Table 1)**
- **Quantitation results for thyroglobulin in plasma using this optimized microflow method were compared to standard flow:**
  1. **Cycle times were on par with the standard flow method[1]**
  2. **Excellent linear correlation of 0.998 between the methods (Figure 7)**
  3. **Better precision at all quantitation levels for the optimized microflow method (Figure 7)**

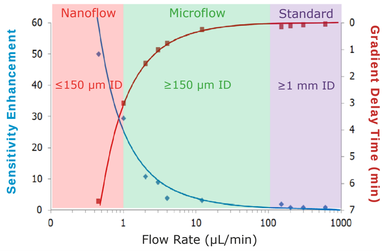


Figure 1. Sensitivity enhancement and gradient delay increase normalized to a 2.1mm separation as one scales down the chromatographic separation for a mix of small molecules.

METHODS AND RESULTS

50 fmol/µL Mixture of 4 Digested Proteins

- LC: ACQUITY MClass
- Column: 150 µm x 50 mm PST BEH C18 iKey
- Trap: 300 µm x 50 mm Symmetry C18
- MS: Xevo TQ-S operated in MRM mode
- Injection: 2 µL partial loop in a 5 µL loop
- iKey Temp: 45º C
- Gradient: 5-45%B in 4 min @ 3µL/min + 2 min wash
- Analytes: 7 peptides across elution range

The novel valve switching algorithm was developed, tested, and refined. The algorithm allows for rapid and easy method development as it only requires the retention time of the analyte of interest from one scouting run to fully calculate the optimized high throughput method.

The valve switching algorithm was applied and the decoupling time for each of the 7 analytes determined. Using this approach we can heart-cut the gradient for sets of the analytes, reducing cycle time by 37 to 56% compared to the conventional configuration **(Figure 1)**. Furthermore, sensitivity is conserved due to the analytical flow rate remaining the same, a benefit over other approaches **(Table 1)**.

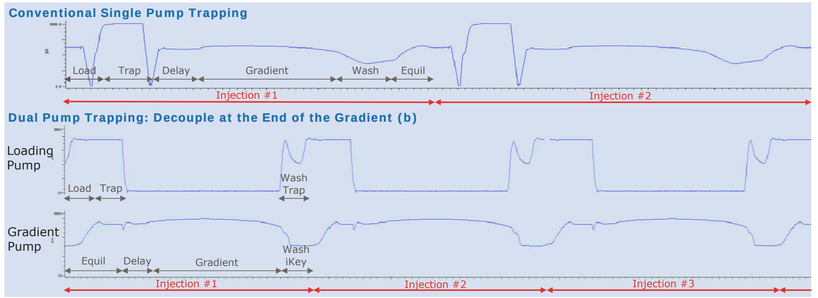
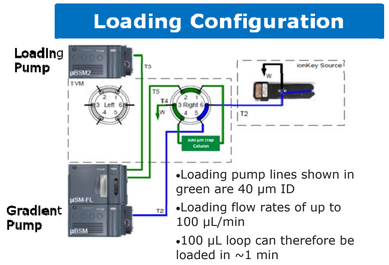


*Figure 2. Comparison of pump activity. A **reduction of cycle time of ~37% is achieved** by employing a dual pump trapping configuration and switching the trapping valve at the end of the gradient as calculated by the algorithm. The valve switch decouples the trap column from the iKey and saves cycle time by allowing fast and independent washing and equilibration of the trap column, overlapping of the loading of the next sample on the trap with the slow equilibration of the iKey, and elimination of injection ramps between trap and gradient flow rates as seen above. **Additional cycle time can be saved by decoupling or heart-cutting portions of the gradient not used for analyte elution as seen in Figure 4 and Table 1.***



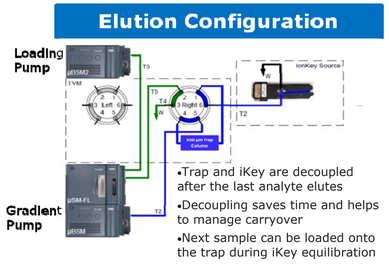


*Figure 3. Dual pump trapping configuration. The use of a dedicated loading and gradient pump allows for **fast sample loading** and **high throughput operation** through the ability to decouple the trap and iKey fluidically.*

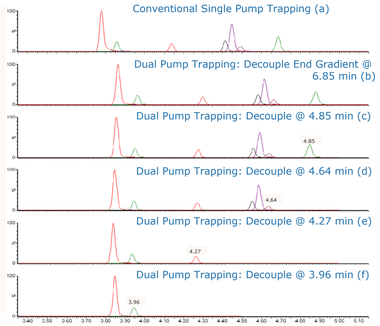
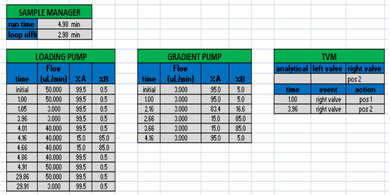


Figure 4. Chromatographic comparison for methods predicted by the valve switching algorithm to a conventional single pump trapping method. Retention times between (a) and (b) are not expected to be the same.

N=3 Replicates	(a)	(b)	(c)	(d)	(e)	(f)
Avg RT Deviation	-	-	-0.41%	-1.20%	-1.40%	-0.68%
Avg Area Recovery	-	108%	101%	92%	98%	93%
Avg Change PW@10%	-	+5.7%	+2.0%	+2.9%	+2.9%	-0.6%
Cycle Time (min)	15.23	9.58	7.67	7.42	7.00	6.73
Time Savings (min)	-	-5.65	-7.57	-7.82	-8.23	-8.50
Change Cycle Time	-	-37%	-50%	-51%	-54%	-56%

*Table 1. Comparison of metrics of value for methods. The high recoveries, small RT deviations, and reproducible peak resolutions prove the **algorithm is well correlated to reality** and the **dual pump methodology is sound**.*



SAMPLE MANAGER

run time	4.98 min
loop o/ln	2.98 min

LOADING PUMP

time	Flow (uL/min)	%A	%B
initial	50.000	99.5	0.5
1.00	50.000	99.5	0.5
1.05	3.000	99.5	0.5
3.96	3.000	99.5	0.5
4.01	40.000	99.5	0.5
4.16	40.000	15.0	85.0
4.66	40.000	15.0	85.0
4.86	40.000	99.5	0.5
4.91	50.000	99.5	0.5
29.86	50.000	99.5	0.5
29.91	3.000	99.5	0.5

GRADIENT PUMP

time	Flow (uL/min)	%A	%B
initial	3.000	95.0	5.0
1.00	3.000	95.0	5.0
2.35	3.000	82.4	16.6
2.66	3.000	15.0	85.0
3.66	3.000	15.0	85.0
4.16	3.000	95.0	5.0

TVM

analytical	left valve	right valve
		pos 2
time	event	action
1.00	right valve	pos 1
3.96	right valve	pos 2

Figure 5. Valve switching algorithm output for method (f).

SISCAPA Enriched Thyroglobulin in Plasma[3]

- Gradient: 9.9-27.5%B in 2.2 min @ 3µL/min
- Trap Loading: 50µL/min for 0.8 min in 99.5% A
- Injection: 20 µL partial loop in a 22.8 µL loop
- **Cycle Time: 6.75 minutes**

The algorithm was also used to optimize a method for the quantitation of of thyroglobulin in varying amounts of pooled human plasma **(Figure 6)**. The trap and iKey were decoupled at 4.2 minutes or just after the signature peptide for thyroglobulin elutes.

After optimization, the quantitation results were compared to those determined on a standard flow system utilizing the recommended method parameters for the instrument **(Figure 7)**.

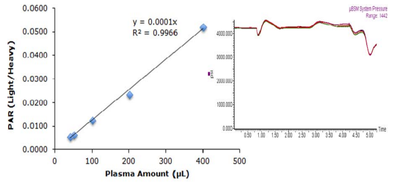
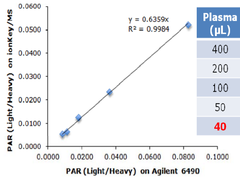


*Figure 6. A linear response was achieved for pooled human **plasma amounts down to 40 µL** with no observed backpressure change demonstrating the optimized dual pump trapping methodology is compatible, robust, and sensitive for plasma extracts.*



Plasma (µL)	ionKey/MS CV	Agilent 6490 CV
400	6.8%	17.2%
200	6.5%	9.7%
100	9.6%	12.5%
50	13.9%	17.7%
40	6.0%	36.2%

*Figure 7. The experiment was replicated on a standard flow Agilent 1290/6490 and an **excellent correlation of 0.998** was obtained. The high correlation and better precision across 4 replicates proves the optimized dual pump trapping methodology is well suited for analyzing plasma extracts.*

References

1. Kushnir, M.M., et al. Measurement of Thyroglobulin by Liquid Chromatography-Tandem Mass Spectrometry in Serum and Plasma in the Presence of Antithyroglobulin Autoantibodies. *Clin. Chem.* (2013) 59(6), 982.
2. Roman, G.T., et al. Achieving Greater Sensitivity, Throughput, and Robustness with ionKey/MS and Trapping. LITR134894058. PN 720005610EN.
3. Johnson, J.S., et al. Improving the Detection of Thyroglobulin in Human Plasma for Clinical Research by Combining SISCAPA Enrichment and Microflow LC/MS. APNT134866663. PN 720005509EN.

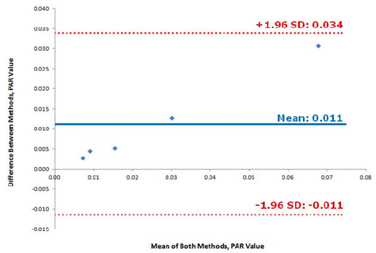


*Figure 8. Bland-Altman Plot showing all differences between the standard flow and dual pump method measurements lie within the upper and lower 95% confidence interval. **Agreement is therefore expected for 95% of the samples.***

CONCLUSION

- **Dual pump trapping preserves the sensitivity of microflow LC/MS while drastically reducing cycle time (Figure 2,3,4)**
- **A novel valve switching algorithm allows for rapid and easy method development with only a single scouting run (Figure 5)**
- **The methodology and algorithm was tested and found to reduce cycle times between 37 and 56% minutes for a set of peptides with no adverse effect on data quality (Table 1)**
- **The quantitation of thyroglobulin was optimized and compared to standard flow (Figures 6,7,8):**
  1. **Cycle times were on par with the standard flow method**
  2. **Excellent linear correlation of 0.998 and agreement w/i 95% CI**
  3. **Better precision at all quantitation levels**

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