

# Accelerating Metabolomic Analyses by Combining Micro-scale Liquid Chromatography with Time-of-Flight Mass Spectrometry

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## Abstract

Genomics has given way to Proteomics and now Metabolomics appears to have become the next frontier for the drug discovery chemist. Metabolomics studies of complex physiological fluids involve the tracking of large numbers of compounds in moderate to low concentrations. In identifying these compounds, sensitivity and selectivity are of paramount importance. However, speed is also an important factor in that it directly affects overall analytical productivity. By combining the speed, selectivity and sensitivity attributes of micro-scale liquid chromatography with the speed, resolution and mass accuracy of time-of-flight mass spectrometry, we have been able to produce information-rich data while doubling the number of analyses achievable within a given period of time.

## The Promise of Micro-scale HPLC

For over a decade, micro-scale HPLC has promised an inherent increase in resolution, sensitivity and speed. Drawbacks that have prevented this realization include non-optimized instrumentation and a lack of robust nano-spray MS sources. The experimental goal of this project was to adapt an analytical-scale metabolomics method to micro-scale HPLC and use it to analyze real-life human physiological fluids.

The first step was to interface the Eksigent ExpressLC-100 micro-scale HPLC system to the time-of-flight mass spectrometer. The Bruker MicroTOF was chosen for its high scan rate (needed to detect narrow, fast eluting peaks) as well as its high resolution and mass accuracy. The MicroTOF mass spectrometer employs a micro-scale electrospray nebulizer capable of handling flow rates from 0.5 to 50  $\mu\text{L}/\text{minute}$ . A 30  $\mu\text{m}$  ID fused silica capillary was used to transfer flow from the UV detector's flow cell outlet (figure 1) to the ESI source (figure 2).

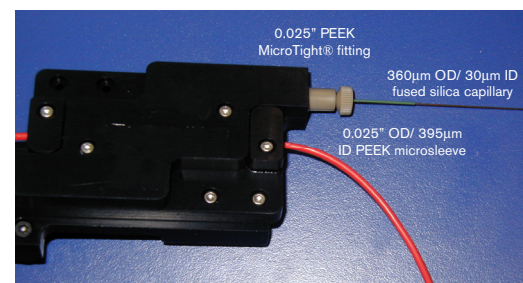


Figure 1 – Micro-scale HPLC flow cell connection

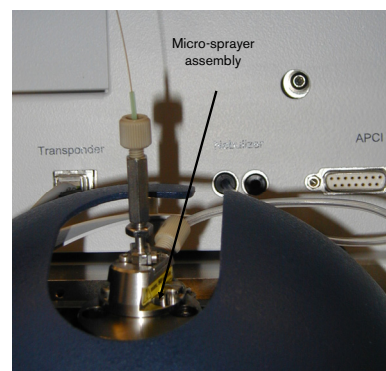


Figure 2 – TOF ESI source connection

Sample:	phthalate standards								
Injection:	100nL								
Column:	Zorbax 300SB-C18, 0.3 x 50mm, 3.5 $\mu\text{m}$								
Flow rate:	10 $\mu\text{L}/\text{min}$								
Solvent A:	Water w/ 0.1% formic acid								
Solvent B:	Acetonitrile								
Gradient:									
	<table border="1"> <tr> <th>Time</th> <th>%B</th> </tr> <tr> <td>0.0</td> <td>2</td> </tr> <tr> <td>1.0</td> <td>2</td> </tr> <tr> <td>2.5</td> <td>98</td> </tr> </table>	Time	%B	0.0	2	1.0	2	2.5	98
Time	%B								
0.0	2								
1.0	2								
2.5	98								

Peak	Conc ( $\mu\text{g}/\text{mL}$ )	UV Detector		MS Detector	
		RT (s)	PW <sub>b</sub> (s)	RT (s)	PW <sub>b</sub> (s)
Diethyl phthalate	112	60.5	1.3	64.5	2.7
Dipentyl phthalate	128	77.8	1.2	82.0	2.5
Diocetyl phthalate	123	96.5	2.3	100.8	3.3

Table 1 – Micro-scale method and results for phthalate standards

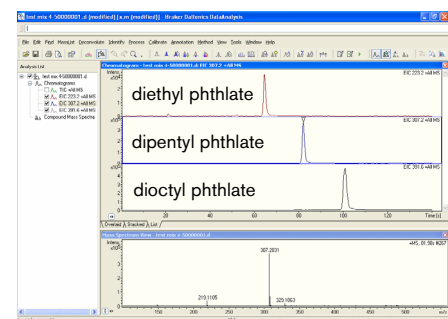


Figure 3 – EIC of phthalate standards

To validate the performance of the interface between the Eksigent ExpressLC-100 and the Bruker MicroTOF, a sample of phthalate esters was analyzed. The results of this test are shown in table 1 and figure 3. The chromatographic dispersion occurring between the detector flow cell and the ESI source appears to be minimal with peak widths, at base, ranging from 2.5 to 3.3 seconds.

## Typical Metabolomic LC/MS Method

A gradient HPLC method currently being used for metabolomic studies was chosen to be scaled-down for the comparison with the micro-scale HPLC system. Figures 4 and 5 show both the method and a typical data analysis of a control human urine sample separated on a 2.1 mm ID Waters Atlantis C18 column. Icoria's proprietary peak picking and alignment software was used to generate the data for the plots.

Injection:	10 $\mu\text{L}$																
Column:	Atlantis dC18, 2.1 x 100mm, 3 $\mu\text{m}$																
Flow rate:	250 $\mu\text{L}/\text{min}$																
Solvent A:	0.5 mM Ammonium acetate, pH 5.5																
Solvent B:	Acetonitrile																
Gradient:	<table border="1"> <tr> <th>Time</th> <th>%B</th> </tr> <tr> <td>0.0</td> <td>0</td> </tr> <tr> <td>0.2</td> <td>0</td> </tr> <tr> <td>8.0</td> <td>50</td> </tr> <tr> <td>9.0</td> <td>50</td> </tr> <tr> <td>10.0</td> <td>75</td> </tr> <tr> <td>11.0</td> <td>0</td> </tr> <tr> <td>16.0</td> <td>0</td> </tr> </table>	Time	%B	0.0	0	0.2	0	8.0	50	9.0	50	10.0	75	11.0	0	16.0	0
Time	%B																
0.0	0																
0.2	0																
8.0	50																
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10.0	75																
11.0	0																
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Figure 4 – 2.1 mm column method

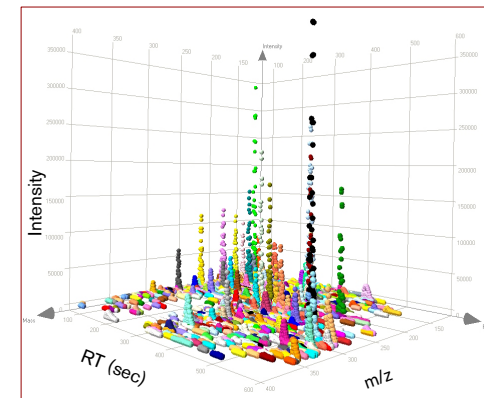


Figure 5 – Spotfire® plot of typical human urine sample

## Analysis of Gradient Performance

However, before modifying the gradient, we first needed to correlate the gradient profile to the mobile phase composition which the column actually "sees". Figure 6 allows visualization of the gradient profile on the 2.1 mm column system by plotting %B which is water containing a small amount of thiourea, a strong UV absorber. What we noticed in particular was that the system required a very long gradient delay and re-equilibration time. Running the same gradient profile on the ExpressLC-100 system produced the plot shown in figure 7. Knowing how the micro-scale system responds to gradient changes allowed us to modify the existing method to achieve the same separation without the extra time associated with the gradient delay and column re-equilibration.

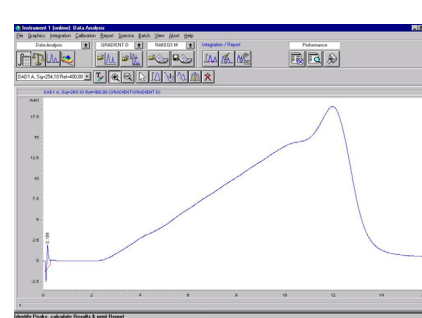


Figure 6 – Gradient on 2.1 mm column system

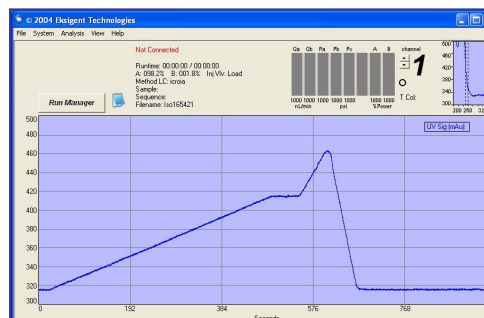


Figure 7 – Gradient micro-scale system

## Modified Micro-scale Method and Results

Figure 8 lists the modified micro-scale method used to separate the same human urine sample on the ExpressLC-100 micro-scale HPLC system. The reduction in delay volume and re-equilibration time allowed us to successfully shorten the analysis by 7 minutes from its typical length of 16 minutes (see figure 9).

Injection size:	200nL												
Column:	Atlantis dC18, 0.3 x 100mm, 3 $\mu\text{m}$												
Flow rate:	5 $\mu\text{L}/\text{min}$												
Solvent A:	0.5mM NH <sub>4</sub> OAc, pH 5.5												
Solvent B:	Acetonitrile												
Gradient:	<table border="1"> <tr> <th>Time</th> <th>%B</th> </tr> <tr> <td>0.0</td> <td>0</td> </tr> <tr> <td>0.3</td> <td>0</td> </tr> <tr> <td>7.5</td> <td>50</td> </tr> <tr> <td>8.5</td> <td>75</td> </tr> <tr> <td>9.0</td> <td>0</td> </tr> </table>	Time	%B	0.0	0	0.3	0	7.5	50	8.5	75	9.0	0
Time	%B												
0.0	0												
0.3	0												
7.5	50												
8.5	75												
9.0	0												

Figure 8 – 300  $\mu\text{m}$  column method

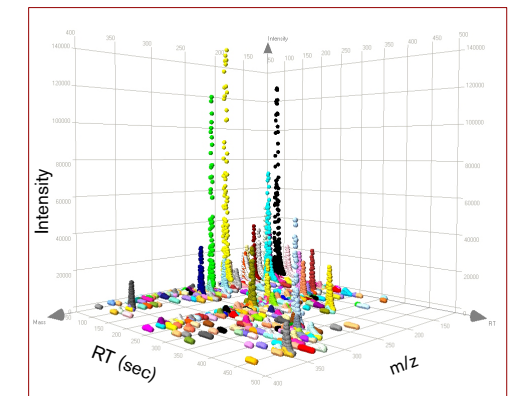


Figure 9 – Spotfire® plot of MicroTOF data

## PCA Results

Principal component analysis (PCA) is a common tool used to visualize differences between highly complex data sets, and is often employed in metabolomics studies. Four human urine samples from the same individual at different pre- and post-dose time points were diluted with 1:4 Milli-Q water containing several internal standards. The samples were injected in triplicate in a randomized sequence and the data processed using Icoria's peak picking and alignment software. The entire metabolic profile of each injection was then used to generate the PCA plots shown in figures 10 and 11 (Spotfire®). Both plots show similar grouping of samples with a slight better separation of the 24-hour and 72-hour post-treatment samples on the ExpressLC-100/MicroTOF system.

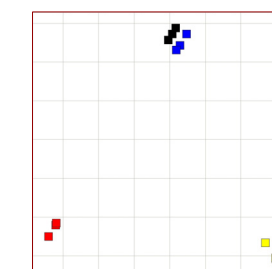


Figure 10 – PCA from 2.1 mm column system

Key	
■	0-hour control
■	6-hour treated
■	24-hour treated
■	72-hour treated

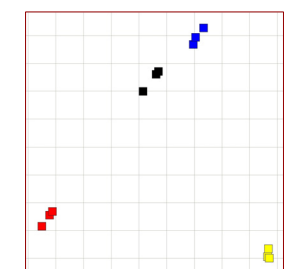


Figure 11 – PCA from micro-scale system

## Conclusion

Micro-scale high performance liquid chromatography has gained a lot of attention recently in the separation and analysis of materials of biological relevance. Instrumentation now available provides micro-scale separations with increased separation efficiencies and speed while maintaining the quantitative accuracy and robustness. By combining recent advances in micro-scale fluid delivery and state-of-the-art time-of-flight mass spectrometry, analytical throughput can be significantly increased while maintaining high resolution with peak widths of less than 4 seconds. In addition we demonstrated that global metabolic profiles obtained with our micro-scale LC/MS system were comparable to those obtained with a conventional system, but with a significant savings in time.