Article

Nanoflow LC-MS for High-Performance Chemical Isotope Labeling Quantitative Metabolomics

Zhendong Li, Jaspaul Tatlay, and Liang Li

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.5b03209 • Publication Date (Web): 19 Oct 2015

(Manuscript submitted to *Anal. Chem.* as an Article on August 20, 2015; Revised on October 16, 2015)

Nanoflow LC-MS for High-Performance Chemical Isotope Labeling Quantitative Metabolomics

Zhendong Li[†], Jaspaul Tatlay[†], and Liang Li*

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

†Authors contributed equally.

* Corresponding author. E-mail: Liang.Li@ualberta.ca.

Abstract

Nanoflow liquid chromatography mass spectrometry (nLC-MS) is prevalent in the proteomics field to analyze a small amount of protein and peptide samples. However, this technique is currently not widespread in the metabolomics field. We report a detailed investigation on the development of an nLC-MS system equipped with a trap column for high-performance chemical isotope labeling (CIL) metabolomic profiling with deep coverage and high sensitivity. Experimental conditions were optimized for profiling the amine/phenol submetabolome with ^{13}C-/^{12}C-dansylation labeling. Comparison of analytical results from nLC-MS and microbore LC-MS (mLC-MS) was made in the analysis of metabolite standards and labeled human urine and sweat samples. It is shown that, with a 5-µL loop injection, 7 labeled amino acid standards could be detected with S/N ranging from 7 to 150 by nLC-MS with an injection of 5 nM solution containing a total of 25 fmol labeled analyte. For urine metabolome profiling where the sample amount was not limited, nLC-MS detected 13% more metabolites than mLC-MS under optimal conditions (i.e., 4524±37 peak pairs from 26 nmol injection in triplicate vs. 4019±40 peak pairs from 52 nmol injection). This gain was attributed to the increased dynamic range of peak detection in nLC-MS. In the analysis of human sweat where the sample amount could be limited, nLC-MS offered the advantage of providing much higher coverage than mLC-MS. Injecting 5 nmol of dansylated sweat, 3908±62 peak pairs or metabolites were detected by nLC-MS, while only 1064±6 peak pairs were detected by mLC-MS. Because labeled metabolites can be captured on a reversed phase (RP) trap column for large volume injection and are well separated by RPLC, the CIL platform can be readily implemented in existing nLC-MS instruments such as those widely used in shotgun proteomics.

Introduction

The growth of metabolomics in the past decade has been directly linked to the development of modern analytical techniques that are able to quantitatively profile a wide range of metabolites in a sample. Liquid chromatography mass spectrometry (LC-MS) has become a powerful tool for metabolomic profiling.[1, 2] To increase the sensitivity of the LC-MS platform, researchers are continually developing more sensitive mass spectrometers, new LC techniques and improving ionization efficiency of metabolites. The latter can be done using chemical labeling such as isotope encoded chemical derivatization or chemical isotope labeling (CIL).[3-10] In CIL, one isotopic form of a reagent is used to target a broad submetabolome (e.g., all amines and phenols when using dansyl chloride,[4] or all carboxylic acids using DmPA[5]). In parallel, a reference sample of very similar composition but distinct from the sample which is most commonly made by pooling all available samples is labeled with another isotopic form of the reagent.[11, 12] The derivatized sample and reference are then mixed together and injected into LC-MS for analysis. Peak pairs detected from differentially labeled metabolites are used for metabolite quantification and identification. By using a proper labeling reagent,[3-5] CIL LC-MS allows concomitant improvement in LC separation and MS detection. Accurate relative and absolute quantification of thousands of metabolites can be obtained from a single experiment.[12]

Further sensitivity increase in LC-MS is still highly desirable in handling samples of limited amounts, particularly those requiring multiple analyses. For example, in CIL LC-MS, each labeling reagent covers a selected submetabolome. Therefore, multiple labeling of the same sample using different aliquots needs to be carried out in order to increase the coverage of the overall metabolome. If multidimensional separation of a metabolome or submetabolome is used, the amount of metabolites in individual pre-fractionated aliquots for LC-MS analysis may be

very limited,[13-15] requiring a sensitive detection technique. In this regard, there exists a high sensitivity platform that is already widely used in proteomics,[16] but less common in metabolomics: the nanoflow-LC MS. Only a few studies were reported using nLC-MS for metabolomic analysis.[17-22] This can be attributed to several reasons including technical challenges. In untargeted metabolomic profiling, four modes of LC-MS experiments using two different stationary columns (e.g., reversed phase (RP) and hydrophilic interaction (HILIC) columns) with each operated at positive and negative ion MS detection are often performed on a sample to detect both polar and nonpolar metabolites.[23-27] In nLC-MS, it is a relatively time-consuming process to switch different capillary columns and then optimize their performances thereafter. In addition, injecting a large volume of sample to increase sample loading to nLC is a major challenge.[18] nLC-MS systems used for shotgun proteome analysis is often equipped with a trap column to capture peptides in several microliters of volume prior to nLC separation. However, high efficiency trapping of all metabolites with wide variations in chemical and physical properties is very difficult in metabolome analysis.

CIL metabolomic profiling using a rationally designed labeling reagent can overcome these technical challenges, because chemical labeling such as dansylation increases hydrophobicity of a labeled metabolite to a great extent so that polar or even ionic metabolites can retain on RP columns after labeling.[4, 5] Both RP trap column and analytical column can be used. There is no need to switch columns to handle different classes of metabolites. CIL also reduces the impact of larger retention time shifting in nLC than conventional LC, because quantification is not reliant on accurate chromatographic alignment between different samples and each metabolite is quantified with its own isotopic counterpart as a peak pair in a mass spectrum.[11, 12] We note that there were reports of using nLC-MS for quantifying a limited

number of metabolites using chemical isotope labeling.[9, 21, 22] However, no trapping was used and the labeling reagents used in these reported studies are expected not to alter the metabolite hydrophobicity to such an extent that would allow efficient trapping using an RP column.

In this work, we report a workflow based on nLC-MS equipped with a RP trapping column for routine analysis of chemical isotope labeled metabolomic samples with coverage of a few thousands of metabolites and describe its performance, particularly in comparison with microbore LC-MS (mLC-MS) commonly used in metabolomics. Dansylation labeling was used for analyzing metabolite standards and the amine/phenol submetabolome of human urine and sweat to demonstrate the improvement of detection sensitivity and metabolome coverage by using nLC-MS.

Experimental Section

nLC-MS. All nLC-MS experiments were performed on a Waters nanoACQUITY UPLC (Milford, MA, USA) connected to a Waters Q-TOF Premier quadrupole time-of-flight (QTOF) mass spectrometer (Milford, MA, USA) equipped with a nano-ESI source. Mass spectrometer settings were: capillary voltage 3.5 kV, sampling cone 30 V, extraction cone 3.0 V, source temperature 110°C, and collision gas 0.45 mL/min. A 5 μm I.D. PicoTip by New Objective (Woburn, MA, USA) was used with the nano-ESI source. Chromatographic separations were performed on an Acclaim PepMap RSLC C18 (75 μm x 150 mm, 2 μm) and Acclaim PepMap 100 trap column (75 μm x 20 mm, 3 μm). A Waters nanoAcquity C18 (75 μm x 200 mm, 1.7 μm) column and nanoAcquity Atlantis trap column (180 μm x 20 mm, 5.0 μm) was also evaluated. Mobile phase A was 0.1 % LC-MS formic acid in LC-MS water and mobile phase B was 0.1 % LC-MS formic acid in LC-MS acetonitrile. The 45 minute gradient conditions were; 0

min (15% B), 0-2.0 min (15% B), 2.0-4.0 min (15-25% B), 4.0-24 min (25-60% B), 24-28 min (60-90% B), and 28-45 min (90% B). A wash and equilibration injection was run between samples; the gradient was: 0-10 min (90% B), 10-25 min (15% B). The flow rate was 350 nL/min and the injection volume was 5 μL (the maximum volume of the sample loop used) in most cases except that of studying the trapping efficiency.

LC-MS. All LC-MS experiments were performed on an Agilent 1100 Series Binary LC System (Santa Clara, CA, USA) connected to the same Q-TOF Premier mass spectrometer used in the nLC-MS experiment, with the nESI source swapped out for an ESI source. Mass spectrometer settings were: capillary voltage 3.5 kV, sampling cone 30, extraction cone 3.0, source temperature 110°C, desolvation temperature 220°C, desolvation gas 800 L/hr, and collision gas 0.45 mL/min. Chromatographic separations were performed on a Waters Acquity BEH C18 column (2.1 mm x 100 mm, 1.7 μm) with the same mobile phases as the nano-LC. The 45 minute gradient conditions were; 0 mins (20% B), 0-3.5 min (20-35% B), 3.5-18 min (35-65% B), 18-24 min (65-99% B), 24-37 min (99% B), and 37.1-45 min (20% B). The flow rate was 180 μL/min.

nLC-MS Trapping Efficiency. A mixture of amino acids at a concentration of 1 mM each was dansylated[4] (see Supplemental Note N1). The dansylation efficiencies for these amino acids have been determined previously by comparing the signal intensities of labeled product and any remaining unlabeled metabolite using LC-MS.[4] $^{12}C_2$ and $^{13}C_2$-dansyl labeled amino acids were mixed 1:1 by volume and diluted to 1000, 2000, 4000, 6000, 8000, and 10000 fold using serial dilution. Injection volume was varied for each diluted sample to ensure 120 fmol of dansylated amino acids are loaded onto the column for each injection. Data was de-noised, smoothed, centered and peak areas extracted using Waters QuanLynx software.

Dynamic Range of Peak Pair Detection. $^{12}C_2$-dansylated amino acids were diluted by half and mixed with undiluted $^{13}C_2$-dansylated amino acids in a 1:1 volume ratio. The theoretical peak ratio of $^{12}C_2$- to $^{13}C_2$-labeled amino acid should be 1:2. The sample was then diluted using serial dilution and increasing sample amounts were injected into the nLC-MS and mLC-MS. Ratios were calculated by dividing the $^{12}C_2$-labeled amino acid peak area by the $^{13}C_2$-labeled amino acid peak area.

Urine and Sweat Analysis. A human urine sample was split into two vials; one was $^{12}C_2$-dansyl labeled and the other was $^{13}C_2$-dansyl labeled. The $^{12}C_2$-dansyl urine was quantified to be 48.2 mM using the LC-UV method[28] (see Supplemental Note N1). The $^{12}C_2$-dansyl urine and $^{13}C_2$-dansyl urine were mixed 1:1 by volume then diluted using serial dilution. These diluted samples were injected at increasing concentrations into the nLC-MS and LC-MS. Peak pairs were then extracted from the processed data using IsoMS.[11]

A human sweat sample was treated the same way as the urine sample. The concentration of the sweat was determined to be 8.4 mM using the LC-UV method. The $^{12}C_2$-dansyl sweat and $^{13}C_2$-dansyl sweat were mixed 1:1 (v/v) for injection into nLC-MS and mLC-MS for analysis.

Results and Discussion

Column Selection. We recognize that some users may re-purpose an existing nLC-MS system used for shotgun proteomic analysis to analyze metabolomic samples for metabolomics. Various factors need to be considered to make such a switch including column selection. We initially used a set of Waters trap column and analytical column used for proteomic analysis to analyze the dansyl labeled urine samples. The resulting chromatogram showed wide peak widths of around 0.8 min with tailing (an example is shown in Supplemental Figure S1A), compared to

widths of ~0.06 min for peptides. This problem was found to be caused by the Waters Symmetry C18 trap which uses high purity silica with end capping. Peak broadening was not observed when the sample was directly loaded onto the analytical column which uses a polymeric bonded phase. It is very likely that a significant amount of residual silanol activity existed in the trap column that caused broadening for the basic dansylated metabolites but not the peptides. We then switched the trap and column set to the Thermo Scientific PepMap 100 C18 set which also used high purity silica. However, the PepMap 100 set was found to give adequate peak widths of 0.2 minutes with reduced tailing (see Supplemental Figure S1B). This set was thus selected for the subsequent experiments. This example illustrates the importance of selecting a proper column and trap combination for profiling labeled metabolites.

Separation Parameters. Several nLC parameters were optimized to achieve an optimal coverage of the amine/phenol submetabolome profile within the shortest run time. First, analytical flow rates of 500, 350, 150 nL/min were tested. With the three different flow rates, we found no significant differences in the number of peak pairs or metabolites detected. However, while using 500 nL/min gave the shortest run time, it could increase the backpressure significantly, resulting in popping the fused-silica capillaries out of their fittings. At the lowest flow rate of 150 nL/min, the analysis time was increased by 10 min. Thus the flow rate of 350 nL/min was chosen as a compromise for the work.

Next we optimized the gradient separation condition. It was found that the majority of the labeled metabolites eluted between 15% and 60% mobile phase B (acetonitrile 0.1% formic acid). Thus, a shallow gradient from 25% to 60% over 20 min was used to improve separation.

The solvent composition of the diluent used to prepare the dansylated samples was also optimized. Initially, the samples were diluted using the same solvent composition as that used for

the dansylation labeling reaction, i.e., 1:1 acetonitrile:water (v/v) 0.1% formic acid, to prevent any potential precipitation of highly non-polar dansylated metabolites. This sample plug with a high organic composition greatly reduced the retention of metabolites on the trap column, causing metabolites to be flushed out of the trap column and into the waste. As a result, a significant amount of early eluting peaks were reduced in intensity (see Supplemental Figure S2A). After testing a number of different diluents, a diluent composed of 1:9 acetonitrile:water (v/v) 0.1% formic acid was found to give no sample loss or precipitation for the urine samples studied (see Supplemental Figure S2B).

Trapping Optimization and Efficiency. A trapping column is an integral part of nLC-MS for injecting a relatively large volume of samples. It is not commonly used in mLC-MS, as injection of several microliters of sample is compatible with the high flow rate. In nLC-MS, prior to separating on the analytical column, the sample is first pushed through a short trap column, usually at a higher flow rate compared to the analytical flow rate. Analytes are retained on the trap while extra diluent and other non-retaining matrix components are flushed into the waste. This serves two functions: the first is to remove salts and other interfering chemicals and the second is to reduce the time it takes for samples to reach the column. As a result, a large volume of sample can be loaded onto the column in a short time. Figure 1 shows the chromatograms of the separation of a mixture of dansylated amino acids using a 5 μL injection loop at a flow rate of 350 nL/min. Without the use of the trap column, there was a dead time of 16.67 min and the first retained analyte eluted in 23.63 min (see Figure 1A). With the trap column, at a trapping flow rate of 7.0 μL/min, the dead time of the sample loop was reduced to 0.71 min leaving only the dead time of the gradient delay which was 7.10 min (see Figure 1B). Overall, there was a

reduction of 16.53 min in run time when the trap was used. Therefore, analyte trapping is essential for reducing the dead time of nLC-MS operating at nanoliter flow rates.

In using the trap, the goal is to have metabolites completely retained on the trap while mobile phase is pushed through at the highest flow rate possible to wash out salts and other non-analytes. The concern is that with higher flow rate there will be more metabolites that are flushed into the waste. Therefore, the trapping flow rate, trapping mobile phase composition and trapping time need to be carefully balanced. Several trapping flow rates ranging from 1 μL/min to 20 μL/min was tested; 20 μL/min was the highest flow rate possible without over pressuring the trap column. By increasing the trap flow rate, the number of peak pairs detected was reduced due to sample loss. Decreasing the flow rate caused a longer dead time and longer overall run time with no significant increase in peak pair number. The optimal flow rate was found to be 7 μL/min which was the highest flow rate without significant sample loss. The trapping mobile phase composition was optimized to be 2% acetonitrile in water. Increasing the organic composition washed away the sample. Finally, the shortest trapping time to wash the entire sample out of the 5 μL sample loop and onto the trap, in addition to washing out salts, was set at 1 min.

While trapping can increase the detection sensitivity by allowing for the injection of a large volume of dilute sample, there is a greater chance that the analytes might be washed out with larger loading volume. After optimizing the trapping conditions, we investigated the trapping efficiency by injecting a series of diluted dansylated amino acid mixtures where the injection amount was kept constant at 120 fmol by adjusting the injection volume and concentration. There was no observed trend that indicated sample loss from 1000 to 10000 fold diluted samples when looking at the measured peak area for selected dansylated amino acids as shown in Figure 2. This shows that the nLC-MS trapping condition used was efficient at trapping

low-concentration and high-injection-volume dansylated samples without affecting chromatographic separation or incurring significant sample loss. It should be noted that we chose the amino acid standards for this trapping efficiency study, because they represent some of the most polar compounds found in urine, serum or other biological samples. Thus the results of dansyl labeled amino acids on a C18 trap represent the extreme cases of otherwise unretained polar metabolites without labeling. Other metabolites in a biological sample will be more hydrophobic and should be retained on the trap after dansylation even more efficiently.

Chromatographic Reproducibility. In untargeted metabolomic studies, reproducible retention time is required for data file alignment to generate accurate abundance information across hundreds of samples that are run on different days or even different weeks. Supplemental Table T1 shows the intraday retention time reproducibility of dansylated amino acids measured using the nLC and mLC. The average relative standard deviation (%RSD) of the nLC retention times was 0.48%, which was significantly worse than the %RSD of the mLC at 0.06%. This confirms reports by other groups that nLC retention time is not as stable as mLC.[29] The lower retention time reproducibility may be due to the reduced quality in stationary phase packing in preparing the nLC columns and a larger flow rate variation with nLC pumps vs. mLC pumps. Retention time stability has a negative effect on the quantification of unlabeled metabolites between different samples and several peak alignment methods have been reported to reduce the effect.[30] However, with CIL, each $^{12}C_2$ dansylated metabolite in a sample is quantified relative to the $^{13}C_2$ dansylated metabolite in a control and thus precise alignment is not required for relative quantification.

In addition to retention time, the intensity of the metabolites needs to be stable between sample runs for accurate quantification. Supplemental Table T2 shows the intensity %RSDs of

the dansylated amino acids. The average %RSD of nLC intensities was found to be similar to that of mLC (3.6% vs. 3.3%). Thus, the quantitative precision of the two systems is similar.

Sensitivity Improvement. Figure 3A shows the plots of peak areas as a function of sample injection amount for nLC- and mLC-MS using dansyl alanine as an example. Signal saturation was observed for nLC-MS when the analyte concentration was over 48 µM, corresponding to 240 pmol with 5 µL injection. In contrast, even at 238 µM, the peak area obtained by mLC-MS was not very high. In fact, it was slightly lower than that obtained using the solution of 0.5 µM in nLC-MS. This result demonstrates a more than 476-fold increase in mass-detection sensitivity at the high concentration region. At the low limit, as Figure 3B shows, mass spectral signals were still detectable at S/N 61 with the injection of the 0.005 µM or 5 nM solution, corresponding to 0.025 pmol or 25 fmol amount. The limit of detection (LOD) for dansyl alanine was 1.2 nM in mLC-MS and 0.25 nM in nLC-MS. This sensitivity enhancement for detecting dansyl labeled metabolites using nLC-MS is consistent with what others observed for nano-ESI of other types of molecules due to improvement in ionization efficiency, reduced ion suppression and more efficient ion acceptance to MS.[31, 32]

For other labeled amino acids tested (see Figure 4 and Supplemental Figure S3), injections of 5 nM of dansylated glycine, glutamic acid, asparagine, phenylalanine, leucine and tryptophan gave signals with S/N 130, 120, 11, 30, 150 and 7, respectively, and their corresponding LODs are 0.12 nM in nLC-MS (0.52 nM in mLC-MS; same below), 0.13 nM (0.95 nM), 1.4 nM (>20 nM), 0.5 nM (6.0 nM), 0.10 nM (3.3 nM) and 2.1 nM (8.6 nM). These LODs are significantly lower than those reported using nLC-MS without a trap and with other labeling reagents. LODs of isobaric *N*,*N*-dimethyl leucine labeled alanine, phenylalanine, leucine and tryptophan were found to be 110 nM, 7 nM, 30 nM and 10 nM, respectively.[21] LODs of

isobaric *N*-hydroxysuccinimide ester labeled alanine, glycine, glutamic acid, phenylalanine, leucine and tryptophan were determined to be 19 nM, 21 nM, 2 nM, 1 nM, 16 nM and 2 nM, respectively.[22] Our results illustrate that with a 5-µL loop injection we can now analyze metabolites at <5 nM concentrations with an analyte amount of <25 fmol.

Dynamic Range for Relative Quantification. Quantitative metabolomics relies on relative quantification of all the metabolites in comparative samples, not just one or a few metabolites. In CIL LC-MS, relative quantification of each metabolite is achieved by calculating the peak ratio of the ^{12}C-labeled metabolite in a sample and the ^{13}C-labeled same metabolite in a control. It is always desirable to detect as many peak pairs as possible in a mass spectrum to quantify the low and high abundance metabolites. However, if a peak pair becomes saturated in a mass spectrum, the highest peak in the pair will become compressed, distorting the measured peak ratio. In our work, the dynamic range for detecting peak pairs was evaluated by analyzing a series of diluted solutions of a 1:2 mixture of a ^{12}C-labeled amino acid and its ^{13}C-labeled counterpart for several amino acids. The theoretical peak intensity ratio should be 0.5. However, the ^{13}C-labeled peak should be saturated first when the concentration of the mixture increases. Thus, the measured peak ratio will be greater than 0.5 when the ^{13}C-peak is saturated.

Figure 4 shows the deviation of the peak ratios of glutamic acid and glycine at different mixture concentrations. Both mLC-MS and nLC-MS showed deviations from the theoretical ratio of 0.5 when the sample concentration increased, showing the effect of detection saturation on the quantification of metabolites. The trends were similar with the other dansyl amino acids (see Supplemental Figure S3). The mLC-MS dansyl peak ratios for glycine and glutamic acid deviated more than 20% at concentrations of > 5 µM, and below 0.5 µM the amino acids were not observed. This means that an accurate peak ratio can only be obtained within 1 order of

magnitude in concentration for this mLC-MS setup. The nLC-MS deviated above 20% at 0.5 µM for glycine and 0.1 µM for glutamic acid. The higher sensitivity allowed the lower end concentration to be reduced down to 0.005 µM, giving a concentration range of 2 and 1.3 orders of magnitude for glycine and glutamic acid, respectively.

To extend the dynamic range when the peaks become saturated, the natural-^{13}C peaks can be used to recover the accurate peak ratio because they are of lower intensity and still reflect the sample ratios.[33] Figure 4 shows that the natural-^{13}C peak ratios were more resistant to deviation caused by detector saturation. In mLC-MS, the natural-^{13}C peak ratios of glycine deviated above 20% for glycine and glutamic acid at 24 µM, instead of 5 µM, when measuring dansyl peak ratios. The nLC-MS deviated past 20% at 2 µM for both amino acids which was between 4 and 20 times higher concentration than using the dansyl peak ratio. Combining the concentrations that deviated less than 20% using both dansyl and natural-^{13}C peak ratios, the nLC-MS had a range of 2.6 orders of magnitude, while mLC-MS had 1.7, for both amino acids.

The above results demonstrate that nLC-MS offers a greater dynamic range for detecting peak pairs with accurate quantification, compared to mLC-MS. This result is not surprising as one would expect that mLC-MS which is less sensitive than nLC-MS would have a lower dynamic range of detection.[31, 32] If we relax the deviation to ~30%, instead of 20%, the quantitative dynamic range becomes 476-fold (i.e., 0.5 to 238 µM) for mLC-MS and 47600-fold (i.e., 0.005 to 238 µM) for nLC-MS for a 1:2 mixture of ^{12}C-/^{13}C-dansyl glycine or ^{12}C-/^{13}C-dansyl glutamic acid.

Urine Submetabolome Profiling. Dansylated human urine was used for the direct comparison of metabolomic analysis sensitivity between nLC-MS and mLC-MS. A urine sample was split and labeled with ^{12}C- and ^{13}C-dansyl chloride, followed by mixing together in a 1:1

ratio. The total concentration of all dansylated metabolites in urine was quantified to be 48.2 mM. The ^{12}C-/^{13}C-labeled urine mixture was diluted up to 10000-fold and injected in triplicate in decreasing metabolite amounts.

Figure 5A shows that the maximum number of detected metabolites in urine using nLC-MS was 4524±37 (n=3) at 26.076 nmol of metabolites injected, while mLC-MS gave a maximum number of 4019±40 at 52.151 nmol injection. Thus, nLC-MS detected about 13% more metabolites than mLC-MS, likely due to improved dynamic range of detecting peak pairs. This result is consistent with what others have observed in bottom-up proteomics; more peptides or proteins could be detected with nLC-MS.[34] At the optimal injection amount for nLC-MS of 26.076 nmol, mLC-MS detected only 67% of the metabolites (i.e., 3034±161). This means that the optimal injection amount for nLC-MS was 2 times lower than using mLC-MS. The improved sensitivity of nLC-MS was more apparent at lower sample loading amounts; below 0.522 nmol loading, nLC-MS detected at least 8 times more metabolites than mLC-MS. It is clear that if sample amount is not limited, mLC-MS can still be used for metabolomic profiling without incurring too large drop in the number of metabolites detected. However, as Figure 5A shows, sample dilution has a much greater effect on mLC-MS than nLC-MS. For example, injecting 2.6 nmol detected less than 1/3 of the peak pairs found in the 26 nmol injection by mLC-MS, while injecting 0.26 nmol in nLC-MS detected more than half of the peak pairs found in the 2.6 nmol injection. Thus, nLC-MS would have a clear advantage in handling samples of limited amounts or diluted samples.

Sweat Submetabolome Profiling. The advantage of nLC-MS for analyzing a limited amount of sample can be demonstrated in profiling the human sweat metabolome. Typically, only several microliters of sweat can be collected from a subject without needing a prolonged

collection and using a very large collection area. For this study, about 10 µL of human sweat was collected after exercise from an arm of a healthy individual. The total concentration of the dansyl labeled metabolites in the sweat was determined to be 8.4 mM using LC-UV, which was 6 times lower than the total concentration of metabolites in urine. For sweat analysis, the lower total concentration and the lower volume require the extra sensitivity offered by the nLC-MS. Figure 5B shows that at the maximum injection amount of 5 nmol of dansylated sweat, 3908±62 peak pairs were detected for nLC-MS and 1064±6 peak pairs were detected for mLC-MS, or a 4-fold increase in the number of metabolites detected. Due to the limited amount of sample, it was not possible to inject an optimal amount for mLC-MS like with the urine sample. The higher sensitivity was again observed at lower sample loading amounts of 1 nmol where nLC-MS has 11-fold higher peak pair values of 3098±16 compared to 275±78 from mLC-MS. We envisage the use of nLC-MS for analyzing many types of metabolomic samples where the sample amount is limited, such as a microliter of sweat collected naturally, a droplet of blood from a finger prick, etc.

Robustness. For routine metabolomic analysis, an analytical tool needs to be robust in dealing with a large number of samples. Due to the small inner diameter of fused-silica capillaries, columns, and nESI emitters used in nLC-MS, the entire system is more finicky to maintain, compared to mLC-MS. Firstly, all of the fused-silica components are much more fragile than the polymer and stainless steel components used in mLC-MS and must be handled with care. The small internal diameter also means that the capillaries are more prone to clogging from particles in the samples and silica particles from poorly cut and ragged tubing edges. The small nESI emitters are more prone to clogging from sample matrix precipitation and silica particles from the fused-silica, and backpressure must be regularly monitored for clogging.

There are a few precautions that can be taken to significantly reduce the frequency of catastrophic clogging of nLC-MS. In our laboratory, a typical capillary internal diameter of 20 μm and outer diameter of 360 μm was found to be the optimal balance between robustness and chromatographic performance by reducing dead volume. For cutting fused-silica capillaries to the necessary lengths, a rotating diamond cutter is expensive but highly recommended for its ability to reproducibly give clean cuts that are free of capillary clogging particles. Following cutting, new fused-silica columns and capillaries must be flushed and their ends washed with clean solvent to remove any particles. Although the nLC-MS platform can be less robust than mLC-MS platform, by following these precautions and being careful, the system can be operated for months with little downtime. Recent advancements in nLC technology, such as the use of an integrated microfluidic column or capillary cartridge that can be conveniently connected to an MS interface,[35] are expected to make nLC-MS more robust for routine metabolomic analysis.

Conclusions

We report a nanoflow LC-MS system combined with chemical isotope labeling of metabolites for metabolomic profiling with high coverage. A reversed phase trap column is used to capture the labeled metabolites at a flow rate of 7 μL/min, followed by separation on a capillary RPLC column at a flow rate of 350 nL/min. The sample injection volume is typically at 5 μL, allowing the analysis of a diluted sample solution. Dansylation CIL was demonstrated for sensitive profiling of the amine/phenol submetabolome in human urine and sweat; however, the technique should be applicable to other labeling chemistries where labeled metabolites can be retained on RPLC. Because the configuration of the nLC-MS system described herein is similar

to those widely used for shotgun proteome analysis, this metabolomic profiling platform should be readily adapted.

Acknowledgements

This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Canada Research Chairs program, Genome Canada and Alberta Innovates.

Supporting Information Available

The Supporting Information is available free of charge on the ACS Publications website at DOI: (to be added).

Note N1 for experimental section, Figures S1-S3, and Tables T1-T2.

References

(1) Yin, P. Y.; Xu, G. W. *J. Chromatogr. A* **2015**, *1374*, 1-13.
(2) Rainville, P. D.; Theodoridis, G.; Plumb, R. S.; Wilson, I. D. *Trac-Trends in Analytical Chemistry* **2015**, *61*, 181-191.
(3) Zhou, R.; Huan, T.; Li, L. *Anal. Chim. Acta* **2015**, *881*, 107-116.
(4) Guo, K.; Li, L. *Anal. Chem.* **2009**, *81*, 3919-3932.
(5) Guo, K.; Li, L. *Anal. Chem.* **2010**, *82*, 8789-8793.
(6) Liu, P.; Huang, Y. Q.; Cai, W. J.; Yuan, B. F.; Feng, Y. Q. *Anal. Chem.* **2014**, *86*, 9765-9773.
(7) Tayyari, F.; Gowda, G. A. N.; Gu, H. W.; Raftery, D. *Anal. Chem.* **2013**, *85*, 8715-8721.
(8) Dai, W. D.; Huang, Q.; Yin, P. Y.; Li, J.; Zhou, J.; Kong, H. W.; Zhao, C. X.; Lu, X.; Xu, G. W. *Anal. Chem.* **2012**, *84*, 10245-10251.
(9) Yuan, W.; Anderson, K. W.; Li, S. W.; Edwards, J. L. *Anal. Chem.* **2012**, *84*, 2892-2899.
(10) Song, P.; Mabrouk, O. S.; Hershey, N. D.; Kennedy, R. T. *Anal. Chem.* **2012**, *84*, 412-419.
(11) Zhou, R.; Tseng, C. L.; Huan, T.; Li, L. *Anal. Chem.* **2014**, *86*, 4675-4679.
(12) Huan, T.; Li, L. *Anal. Chem.* **2015**, *87*, 7011-7016.
(13) Guo, K.; Peng, J.; Zhou, R. K.; Li, L. *J. Chromatogr. A* **2011**, *1218*, 3689-3694.
(14) Mirnaghi, F. S.; Caudy, A. A. *Bioanalysis* **2014**, *6*, 3393-3416.
(15) Vuckovic, D. *Anal. Bioanal. Chem.* **2012**, *403*, 1523-1548.
(16) Nilsson, T.; Mann, M.; Aebersold, R.; Yates, J. R.; Bairoch, A.; Bergeron, J. J. M. *Nature methods* **2010**, *7*, 681-685.
(17) Jones, D. R.; Wu, Z.; Chauhan, D.; Anderson, K. C.; Peng, J. *Anal. Chem.* **2014**, *86*, 3667-3675.
(18) Chetwynd, A. J.; Abdul-Sada, A.; Hill, E. M. *Anal. Chem.* **2015**, *87*, 1158-1165.
(19) David, A.; Abdul-Sada, A.; Lange, A.; Tyler, C. R.; Hill, E. M. *J. Chromatogr. A* **2014**, *1365*, 72-85.
(20) Uehara, T.; Yokoi, A.; Aoshima, K.; Tanaka, S.; Kadowaki, T.; Tanaka, M.; Oda, Y. *Anal. Chem.* **2009**, *81*, 3836-3842.
(21) Hao, L.; Zhong, X. F.; Greer, T.; Ye, H.; Li, L. J. *Analyst* **2015**, *140*, 467-475.
(22) Yuan, W.; Zhang, J. X.; Li, S. W.; Edwards, J. L. *J. Proteome Res.* **2011**, *10*, 5242-5250.
(23) Ivanisevic, J.; Zhu, Z. J.; Plate, L.; Tautenhahn, R.; Chen, S.; O'Brien, P. J.; Johnson, C. H.; Marletta, M. A.; Patti, G. J.; Siuzdak, G. *Anal. Chem.* **2013**, *85*, 6876-6884.
(24) Contrepois, K.; Jiang, L. H.; Snyder, M. *Mol. Cell. Proteomics* **2015**, *14*, 1684-1695.
(25) Vorkas, P. A.; Isaac, G.; Anwar, M. A.; Davies, A. H.; Want, E. J.; Nicholson, J. K.; Holmes, E. *Anal. Chem.* **2015**, *87*, 4184-4193.
(26) Tulipani, S.; Mora-Cubillos, X.; Jauregui, O.; Llorach, R.; Garcia-Fuentes, E.; Tinahones, F. J.; Andres-Lacueva, C. *Anal. Chem.* **2015**, *87*, 2639-2647.
(27) Mahieu, N. G.; Huang, X. J.; Chen, Y. J.; Patti, G. J. *Anal. Chem.* **2014**, *86*, 9583-9589.
(28) Wu, Y. M.; Li, L. *Anal. Chem.* **2013**, *85*, 5755-5763.
(29) Percy, A. J.; Chambers, A. G.; Yang, J. C.; Domanski, D.; Borchers, C. H. *Anal. Bioanal. Chem.* **2012**, *404*, 1089-1101.
(30) Smith, R.; Ventura, D.; Prince, J. T. *Brief. Bioinform.* **2015**, *16*, 104-117.
(31) Wilm, M.; Mann, M. *Anal. Chem.* **1996**, *68*, 1-8.
(32) Schmidt, A.; Karas, M.; Dulcks, T. *J. Am. Soc. Mass Spectrom.* **2003**, *14*, 492-500.

(33) Zhou, R.; Li, L. *J Proteomics* **2015**, *118*, 130-139.
(34) Ficarro, S. B.; Zhang, Y.; Lu, Y.; Moghimi, A. R.; Askenazi, M.; Hyatt, E.; Smith, E. D.; Boyer, L.; Schlaeger, T. M.; Luckey, C. J.; Marto, J. A. *Anal. Chem.* **2009**, *81*, 3440-3447.
(35) Rainville, P. D.; Langridge, J. I.; Wrona, M. D.; Wilson, I. D.; Plumb, R. S. *Bioanalysis* **2015**, *7*, 1397-1411.

Figure Captions

Figure 1. nLC-MS chromatograms of a mixture of 18 dansylated amino acids obtained (A) without using a trap column and (B) with the use of a trap column. The peak at 23.63 min was from dansyl-OH, a product of dansyl reagent after quenching with NaOH. This product did not retain on the RP trap column and thus did not show up in (B).

Figure 2. Chromatographic peak areas of dansylated amino acids obtained by injecting the same sample amount (120 fmol) while changing the injection volumes for different concentrations of solutions.

Figure 3. (A) Chromatographic peak area of dansyl alanine as a function of injected sample solution concentration for mLC-MS and nLC-MS. Error bar represents one standard deviation (n=3). (B) Molecular ion region of the mass spectrum obtained from 1:2 mixture of ^{12}C-dansyl alanine and ^{13}C-dansyl alanine at 5 nM with an injection of 5 µL solution (i.e., 25 fmol) in nLC-MS. The extra peak next to the ^{12}C-dansyl alanine was from a background species.

Figure 4. Effect of detection saturation on the calculated peak pair ratio in mLC-MS and nLC-MS. Derivation from the expected 1:2 ratio is plotted as a function of the solution concentration of 1:2 mixture of ^{12}C-dansyl amino acid and ^{13}C-dansyl amino acid.

Figure 5. Number of peak pairs detected as a function of the sample injection amount from mLC-MS and nLC-MS analysis of (A) ^{12}C-/^{13}C-labeled human urine sample and (B) ^{12}C-/^{13}C-labeled human sweat sample.

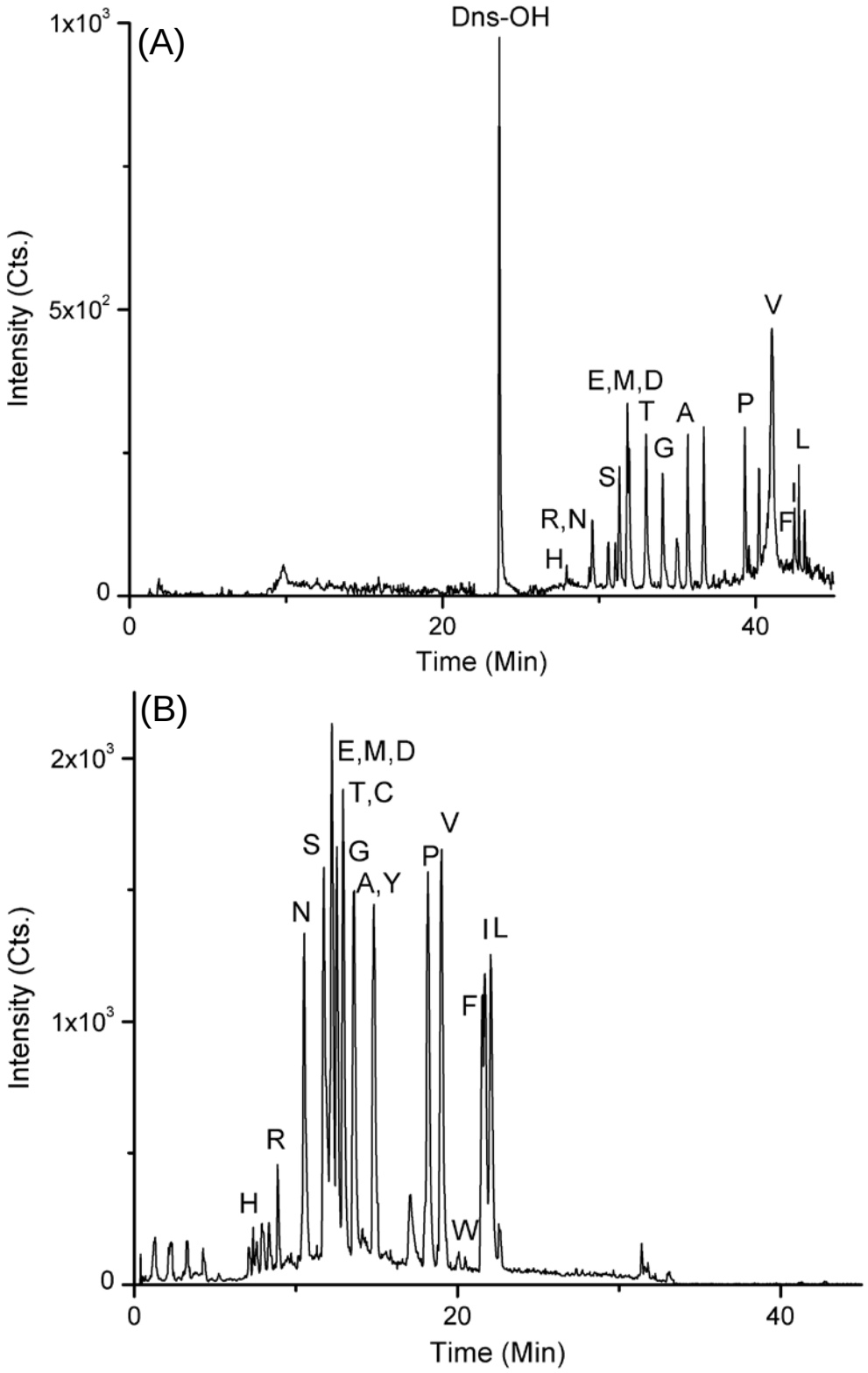
(A)
Dns-OH
1x10^3
5x10^2
0
Intensity (Cts.)
0
20
40
Time (Min)
E,M,D
T
A
P
V
G
S
L
I
F
R,N
H
(B)
2x10^3
1x10^3
0
Intensity (Cts.)
E,M,D
T,C
S
G
V
P
A,Y
N
IL
F
R
H
W
0
20
40
Time (Min)

Figure 1

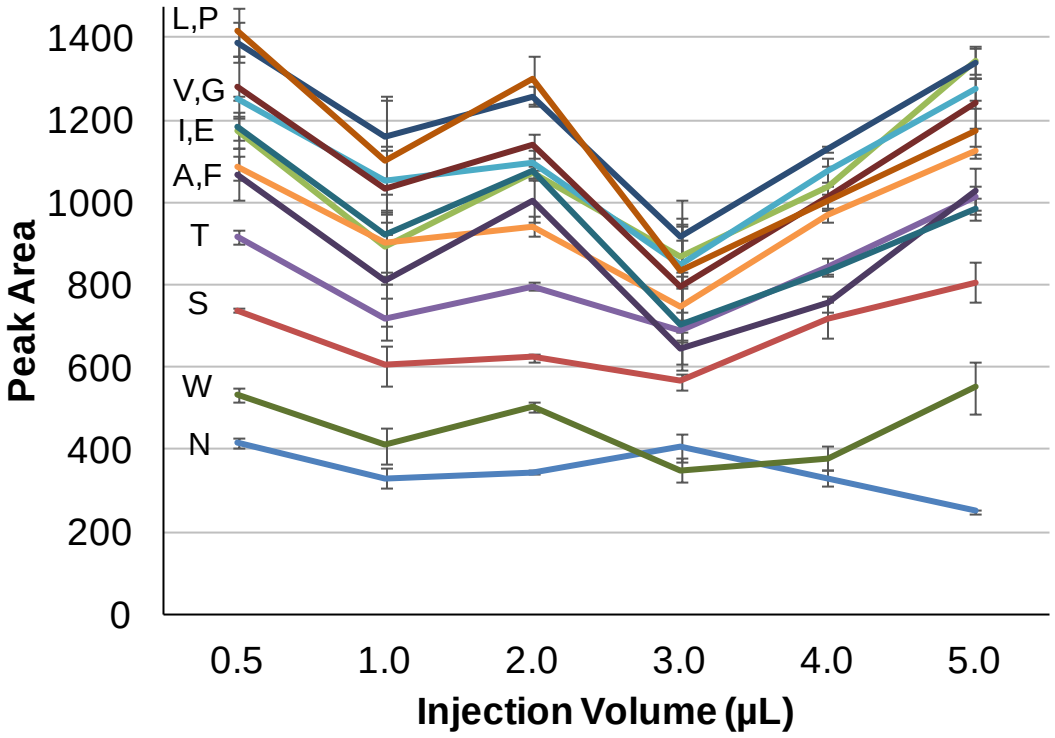
L,P
V,G
I,E
A,F
T
S
W
N
1400
1200
1000
800
600
400
200
0
Peak Area
0.5
1.0
2.0
3.0
4.0
5.0
Injection Volume (µL)

Figure 2

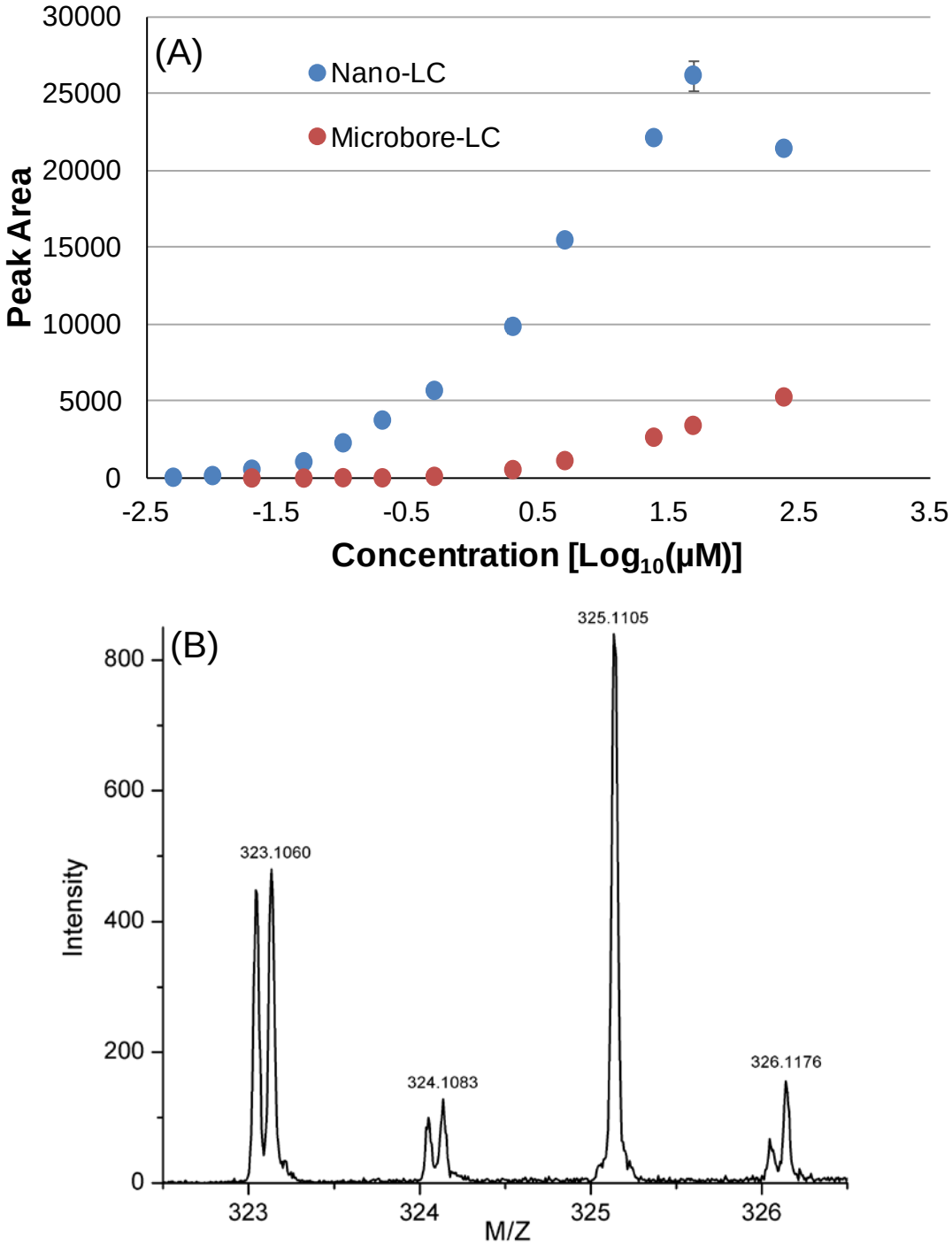
(A)
Nano-LC
Microbore-LC
Peak Area
30000
25000
20000
15000
10000
5000
0
-2.5
-1.5
-0.5
0.5
1.5
2.5
3.5
Concentration [Log10(μM)]
(B)
325.1105
323.1060
324.1083
326.1176
Intensity
800
600
400
200
0
323
324
325
326
M/Z

Figure 3

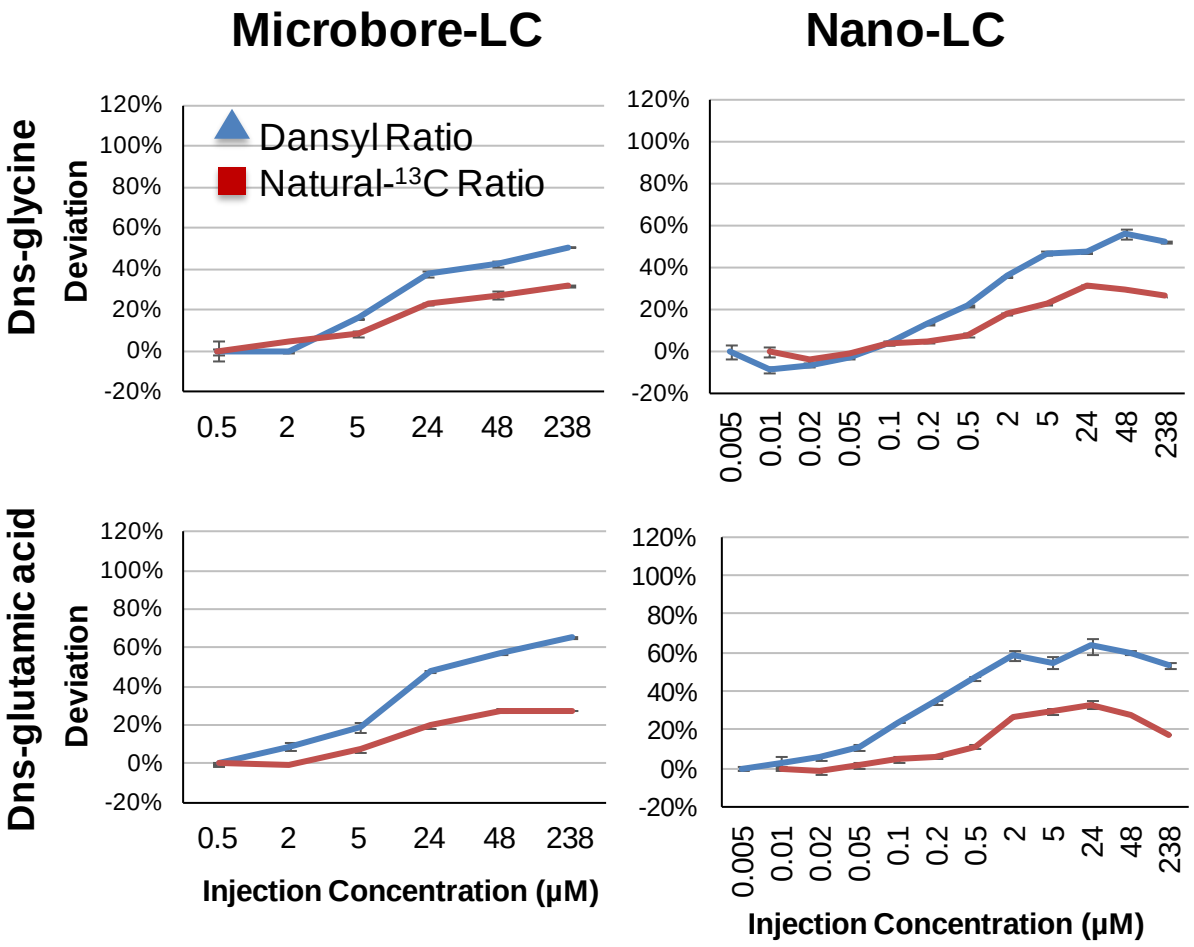
Microbore-LC
Nano-LC
Dansyl Ratio
Natural-13C Ratio
Dns-glycine Deviation
Dns-glutamic acid Deviation
120%
100%
80%
60%
40%
20%
0%
-20%
0.5
2
5
24
48
238
0.005
0.01
0.02
0.05
0.1
0.2
0.5
2
5
24
48
238
Injection Concentration (μM)
Injection Concentration (μM)

Figure 4

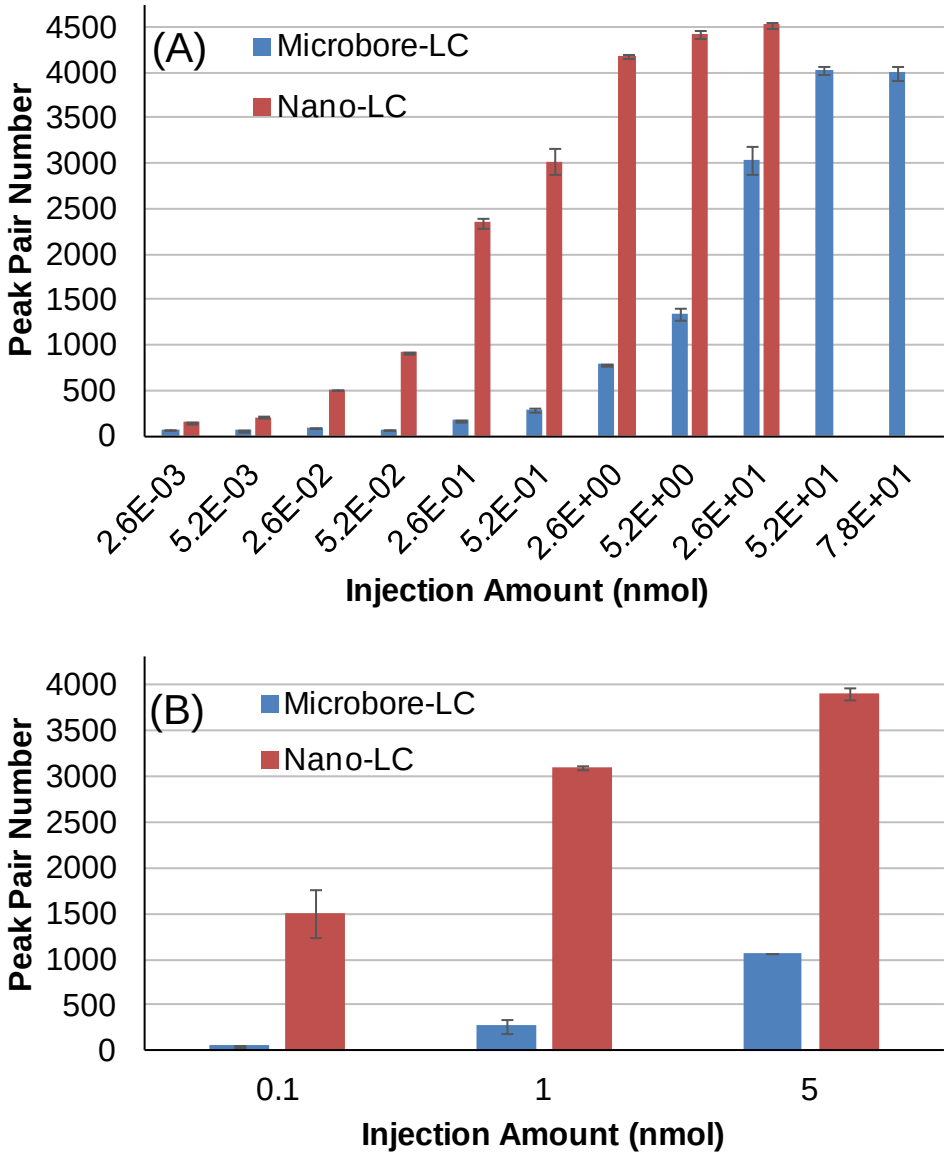
(A)
Microbore-LC
Nano-LC
Peak Pair Number
4500
4000
3500
3000
2500
2000
1500
1000
500
0
2.6E-03
5.2E-03
2.6E-02
5.2E-02
2.6E-01
5.2E-01
2.6E+00
5.2E+00
2.6E+01
5.2E+01
7.8E+01
Injection Amount (nmol)
(B)
Microbore-LC
Nano-LC
Peak Pair Number
4000
3500
3000
2500
2000
1500
1000
500
0
0.1
1
5
Injection Amount (nmol)

Figure 5

For Table of Contents: