Anal. Chem. **2007,** *79,* 8631−8638

Stable-Isotope Dimethylation Labeling Combined with LC−ESI MS for Quantification of Amine-Containing Metabolites in Biological Samples

Kevin Guo, Chengjie Ji, and Liang Li*

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2

One of the challenges associated with metabolome profiling in complex biological samples is to generate quantitative information on the metabolites of interest. In this work, a targeted metabolome analysis strategy is presented for the quantification of amine-containing metabolites. A dimethylation reaction is used to introduce a stable isotopic tag onto amine-containing metabolites followed by LC−ESI MS analysis. This labeling reaction employs a common reagent, formaldehyde, to label globally the amine groups through reductive amination. The performance of this strategy was investigated in the analysis of 20 amino acids and 15 amines by LC−ESI MS. It is shown that the labeling chemistry is simple, fast (<10-min reaction time), specific, and provides high yields under mild reaction conditions. The issue of isotopic effects of the labeled amines on reversed-phase (RP) and hydrophilic interaction (HILIC) LC separations was examined. It was found that deuterium labeling causes an isotope effect on the elution of labeled amines on RPLC but has no effect on HILIC LC. However, ^{13}C-dimethylation does not show any isotope effect on either RPLC or HILIC LC, indicating that ^{13}C-labeling is a preferred approach for relative quantification of amine-containing metabolites in different samples. The isotopically labeled 35 amine-containing analogues were found to be stable and proved to be effective in overcoming matrix effects in both relative and absolute quantification of these analytes present in a complicated sample, human urine. Finally, the characteristic mass difference provides additional structural information that reveals the existence of primary or secondary amine functional groups in amine-containing metabolites. As an example, for a human urine sample, a total of 438 pairs of different amine-containing metabolites were detected, at signal-to-noise ratios of greater than 10, by using the labeling strategy in conjunction with RP LC-ESI Fourier-transform ion cyclotron resonance MS.

LC−MS has become an important tool for metabolome analysis. However, generation of accurate quantitative information by LC−MS is not straightforward.[1] One of the most reliable quantitative methods is to use stable-isotope-labeled (SIL) analogues as internal standards for quantifying the metabolites of interest. SIL internal standards are chemically and structurally similar to the analytes with specific atoms in the analytes replaced by their corresponding isotopes, such as deuterium for hydrogen, ^{13}C for ^{12}C, ^{15}N for ^{14}N, or ^{18}O for ^{16}O.[1,2] The use of SIL internal standards normalizes the MS intensity of analytes to their isotopic analogues and, therefore, effectively compensates for the matrix effect, ion suppression from other coeluting analytes, and variations caused by sample preparation, injection, and instrument parameters.[1−6] Unfortunately, only a limited number of SIL internal standards are commercially available. In the absence of SIL standards, structural analogues are used as the second best choice; but the use of structural analogues may result in poor quantification performance, particularly in analyzing metabolites present in a complex matrix.[7] Errors can be introduced during sample processing (e.g., different recovery rates in the extraction of a metabolite and its structural analogue) and the LC−MS analysis step. Structural analogues are often not coeluted with the analyte of interest, and therefore, they would experience levels of matrix effect different from that of analyte. As a result, the relative signal intensities of a metabolite and its analogue may not reflect their concentration ratio in the sample.

Another practical strategy for quantification in LC−MS is chemical labeling, which has been widely applied to obtain relative quantitative information of proteomes.[8,9] For metabolome analysis, there are a limited number of reports of using chemical derivatization to introduce a stable isotopic tag to metabolites to facilitate their quantitative analysis by LC−electrospray ionization (ESI) MS.[10−12] Development of facile derivatization methods for stable-isotope dilution-based quantification of metabolomes is needed.

* To whom correspondence should be addressed. E-mail: Liang.Li@ualberta.ca.

(1) Stokvis, E.; Rosing, H.; Beijnan, J. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 401−407.
(2) Liang, H.; Foltz, R.; Meng, M.; Bennet, P. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2815−2821.
(3) Annesley, T. M. *Clin. Chem.* **2003**, *49*, 1041−1044.
(4) Magni, F.; Pereira, S.; Leoni, M.; Grisenti, G.; Kienle, G. *J. Mass Spectrom.* **2001**, *36*, 670−676.
(5) Jacob, P.; Wilson, M.; Yu, L.; Mendelson, J.; Jones, R. T. *Anal. Chem.* **2002**, *74*, 5290−5296.
(6) Kuklenyik, Z.; Ashley, D. L.; Calafat, A. M. *Anal. Chem.* **2002**, *74*, 2058−2063.
(7) Stokvis, E.; Rosing, H.; Lopez-Lazaro, L.; Schellens, J.; Beijnan, J. *Biomed. Chromatogr.* **2004**, *18*, 400−402.
(8) Gygi, S. P.; Rist, B.; Gerber, S. A.; Turecek, F.; Gelb, M. H.; Aebersold, R. *Nat. Biotechnol.* **1999**, *17*, 994−999.
(9) Aebersold, R.; Mann, M. *Nature* **2003**, *422*, 198−207.

10.1021/ac0704356 CCC: $37.00 © 2007 American Chemical Society
Published on Web 10/10/2007

For an ideal derivatization protocol, the derivatization reagent should be specific for the target functional group, and the analytes of interest need to be rapidly and quantitatively derivatized with minimum byproducts (i.e., high yield). The reaction should be performed under mild conditions with minimum manipulation. For quantitative applications, the resulting products need to be stable.

Differential isotopic dimethyl labeling of N-terminal peptides with d(0) and d(2) or d(0), ^{12}C and d(2), ^{13}C-formaldehyde combined with LC-ESI or LC-MALDI have been successfully used for relative proteome quantification.[13−17] The labeling is carried out by using reductive amination chemistry.[18] In this work, we report our studies of using reductive amination to introduce isotopic tags to amine-containing metabolites and applying this strategy to the quantification by LC−ESI MS of both primary and secondary amine metabolites in human urine. Amine-containing metabolites play essential roles in biological functions. For example, amino acids and their derivatives are common biomarkers for human physiological process.[19] Their identification and quantification in human fluids provide significant insights related to human health. The polycationic polyamines are essential for eukaryotic cellular growth and viability; rapid tumor growth was associated with polyamine biosynthesis and accumulation.[20] Many studies indicate that significantly higher levels of polyamines and their metabolites were present in the biological fluids and the affected tissues of cancer patients and other hyperproliferative diseases.[20−22] Some therapeutic polyamine analogues are showing exciting potentials to treat cancer and other hyperproliferative disorders.[20] Thus, quantitative profiling of amine-containing metabolites could potentially be applied for the discovery of new disease biomarkers as well as for the monitoring of tumor growth and regression in cancer study.

EXPERIMENTAL SECTION

Chemicals and Reagents. Twenty amino acids, L-alanine, L-arginine, L-aspartic acid, L-asparagine, L-cysteine, L-glutamic acid, L-glutamine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine, L-tryptophan, and L-valine; and 15 amines, 1-ephedrine, 1,4-diaminobutane, (−)-epinephrine, 2-methylbenzylamine, 3-methyl-L-histidine, aniline, benzylamine, cysteamine, dopamine, histamine, L-4-hydroxyproline, *p*-aminohippuric acid, pyridoxamine, γ-aminobutyric acid, and tyramine, were purchased from Sigma Aldrich (Oakville, ON, Canada). Formaldehyde (37 wt % solution in water), sodium cyanoborohydride (95%), ammonium acetate, sodium acetate, LC−MS grade formic acid, and acetic acid were also obtained from Sigma-Aldrich. LC−MS grade of water, methanol, and acetonitrile were purchased from Fisher Scientific Canada (Edmonton, AB, Canada). Formaldehyde-^{13}C (20 wt % solution in water, >99% isotope purity) solution, and d(2)-formaldehyde (20 wt % solution in deuterated water, >98% isotope purity) were the products of Cambridge Isotope Laboratories, Inc. (Andover, MA).

Dimethylation Labeling Reaction. The freshly collected human urine was centrifuged for 10 min at 12 000 rpm. A total of 500 μL of urine supernatant, 20 amino acid solutions, or 15 amine standard solutions were mixed with an equal volume of ammonium acetate buffer (0.2 M, pH 5.3) in a reaction vial. The solutions were vortexed, centrifuged, and mixed with 125 μL of freshly prepared sodium cyanoborohydride (1.0 M). After further mixing, centrifugation, and the addition of 100 μL of 4% formaldehyde, or formaldehyde-^{13}C, or d(2)-formaldehyde solution, the mixtures were vortexed and centrifuged again, and the reaction was allowed to proceed for 10 min at 37 °C and 200 rpm in an Innova 4000 benchtop incubator shaker. The pH of mixtures was adjusted to pH 2−3 by adding ~25 μL of formic acid. The solutions were then centrifuged or filtered before being injected onto an LC column. The samples for hydrophilic interaction liquid chromatography (HILIC) were diluted with acetonitrile to obtain optimal separation efficiency. Because sodium cyanoborohydride is a highly toxic chemical that will produce hydrogen cyanide gas when exposed to acid, and formaldehyde is a known carcinogen on inhalation exposure, the dimethylation labeling reaction was carried out in a fume hood.

LC−ESI MS. The HPLC system used in conjunction with the mass spectrometer was an Agilent 1100 series binary system, and it was modified to reduce extra column system volume according to an Agilent protocol (Agilent Publication Number, 5988-2682EN). A reversed-phase (RP) Agilent Zorbax XDB C_{18} column (1.0 × 150 mm, 3.5-μm particle size, 80-Å pore size) and a Zorbax XDB C_{18} rapid resolution high-throughput cartridge column (2.1 × 15 mm, 1.8 μm, 80 Å) were purchased from Agilent Technologies, Inc. (Palo Alto, CA). For RP chromatography, solvent A was 0.1% formic acid, 5% methanol in water, and solvent B was 0.1% formic acid in methanol. All the formic acid, methanol, and water used were LC−MS grade. The 64-min binary gradient elution profile was as follows: t = 0, 0% B; t = 6 min, 0% B; t = 21 min, 30% B; t = 54 min, 90% B; t = 64 min, 90% B. The flow rate was 50 μL/min, and sample injection volumes were 10 μL.

A TSKgel Amide-80 HILIC column (1.0 × 250 mm, 5 μm) was obtained from Tosoh Bioscience LLC (Montgomeryville, PA). For HILIC, solvent A was 10% 15 mM ammonium acetate (pH 5.5) in LC−MS grade acetonitrile, and solvent B was 40% 15 mM ammonium acetate (pH 5.5) in LC−MS grade acetonitrile. The 45-min binary gradient elution profile was as follows: t = 0, 10% B; t = 30 min, 30% B; t = 37 min, 45% B; t = 42 min, 70% B; t = 45 min, 70% B. The flow rate was 55 μL/min, and sample injection volumes were 3 μL. The flow from RP or HILIC columns was directed to the ESI source of a Bruker Esquire-LC ion trap LC−MS system or a Bruker 9.4-T Fourier transform (FT) ion cyclotron resonance (ICR) mass spectrometer. All MS spectra were obtained in the positive ion mode. Negative ion detection was found to be not as sensitive as the positive ion detection for the labeled amines tested in these instruments.

(10) Fukusaki, E.; Harada, K.; Bamba, T.; Kobayashi, A. *J. Biosci. Bioeng.* **2005**, *99*, 75−77.
(11) Yang, W. C.; Mirzaei, H.; Liu, X.; Regnier, F. E. *Anal. Chem.* **2006**, *78*, 4702−4708.
(12) Shortreed, M. R.; Lamos, S. M.; Frey, B. L.; Phillips, M. F.; Patel, M.; Belshaw, P. J.; Smith, L. M. *Anal. Chem.* **2006**, *78*, 6398−6403.
(13) Hsu, J. L.; Huang, S. Y.; Chow, N. H.; Chen, S. H. *Anal. Chem.* **2003**, *75*, 6843−6852.
(14) Ji, C.; Li, L. *J. Proteome Res.* **2005**, *4*, 734−742.
(15) Ji, C.; Li, L. J.; Gebre, M.; Pasdar, M.; Li, L. *J. Proteome Res.* **2005**, *4*, 1419−1426.
(16) Ji, C.; Guo, N.; Li, L. *J. Proteome Res.* **2005**, *4*, 2099−2108.
(17) Ji, C.; Lo, A.; Marcus, S.; Li, L. *J. Proteome Res.* **2006**, *5*, 2567−2576.
(18) Means, G. E.; Feeney, R. E. *Biochemistry* **1968**, *7*, 2192−2201.
(19) Husek, P.; Simek, P. *LC-GC* **2001**, *19*, 986−999.
(20) Qureshi, G.; Khuhawar, M. *J. Chromatogr., B* **2001**, *764*, 385−407.
(21) Marton, L.; Casero, R., Jr. *Nat. Rev.* **2007**, *6*, 373−390.
(22) Wallace, H.; Saunder, F. *Biochem. Soc. Trans.* **2007**, *35*, 364−368.

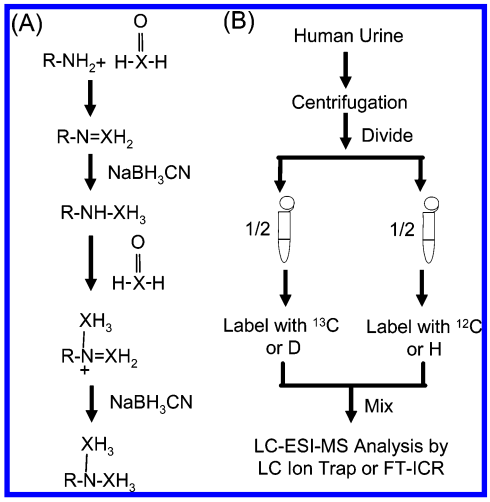


Figure 1. Overview of dimethylation labeling strategy for quantitative analysis of amine-containing metabolites. (A) Scheme of reductive amination labeling. X = ^{13}C or ^{12}C; in the case of D(2) labeling, two deuterium atoms replace two hydrogen atoms in formaldehyde. (B) Workflow for demonstrating the feasibility and performance of relative quantitative analysis of primary and secondary amine metabolites in urine samples by LC−ESI MS.

RESULTS AND DISCUSSION

Dimethyl Isotope Labeling. When amine-containing molecules are treated with low concentrations of simple aliphatic aldehydes and a small amount of sodium borohydride, amine groups can be converted in high yield into their corresponding mono- or dialkylamine derivatives.[18] We investigated the reaction of 20 amino acids and 15 amines with formaldehyde and observed the formation of predominantly dimethylated derivatives (28-Da difference for each labeled site) from primary amines, and monomethylated derivatives (14-Da difference) from secondary amines, such as proline. Judging from the LC−MS results of the labeled products, the conversion yield is better than 97%. This observation is similar to those found in other reported studies.[23,24] The use of dimethylation labeling is attractive for several reasons. The formaldehyde used as the labeling reagent is inexpensive and ^{13}C- or deuterium-labeled formaldehyde is commercially available. The experimental conditions for reductive amination are extremely mild, and the reaction is easily completed without any special reagents or reaction equipment. As Figure 1A shows, an intermediate Schiff base is formed in reductive amination. The high yield of methylated products observed in our experiment suggests that the Schiff base intermediate was reduced at a reaction rate much greater than that of formaldehyde. Consequently, the reactive intermediate readily reacts with formaldehyde and is reduced to dimethylated products. The dimethylation labeling reaction appears highly specific for amine groups for the 20 amino acids and the 15 amines we studied.[18]

We also tested the dimethylation reaction for each amino acid and amine, one by one, to examine any possible byproducts from the reaction. In general, no significant amount of side reaction products were observed, at least for the 20 amino acids and 15 amines tested. For Lys, there were two dimethylated tags introduced to the molecule. The number of tags introduced to an analyte corresponds to the number of primary or secondary amino groups present. This is another benefit of using this derivatization chemistry; i.e., one can deduce information about the number of amino groups present in an analyte, which may be useful for unknown metabolite identification or structural analysis. We also observed some ESI signal enhancement (~1−10-fold depending on the compound structures) for the products, compared to the unlabeled analytes. This can be attributed to the fact that dimethylation converts primary or secondary amine into a more easily protonated tertiary amine. In addition, in the case of amino acids, the labeled ones are slightly more hydrophobic than the unlabeled ones (e.g., they elute out at a longer retention time in RP LC). The increased hydrophobicity for some of the labeled polar amino acids may enhance the ESI signals.

It should be noted that the dimethylation labeling reaction is pH dependent. Ammonium acetate buffer (pH 5.3) was found to provide the optimal condition resulting in the highest yield possible for all the analytes. The pH of reaction mixtures was carefully controlled and checked when necessary. The labeling reaction is fast. No or very little byproducts were observed from a 10-min incubation at 37 °C. Some byproducts could be observed if the reaction time was too long or the incubation temperature was too high. The stability of the labeled products was also studied. LC−MS chromatograms show that the products were stable at room temperature over a period of at least two weeks. The labeled products can be stored at −20 °C for long-term storage.

In summary, reductive amination provides a simple means of labeling amine-containing compounds, such as amino acids and various amines. The labeling chemistry produces a high yield, requires a short reaction time, and is very specific under mild conditions. Due to its long-term stability, an isotope-labeled analogue can serve as the internal standard for absolute quantification, and differential labeling of comparative samples can be used for relative quantification of amine-containing compounds (see below).

Evaluation of Isotopic Effects. For quantitative analysis using the isotope dilution method, analytes and their isotopic analogues should coelute on LC; i.e., there should be no isotopic effect on analyte retention. In this way, a pair of isotope labeled analytes would experience the same degree of ion suppression or matrix effect in MS analysis. Thus, the overall analytical efficiencies for the pair would be the same, which is important for accurate relative quantification. Figure 2 shows the results of the isotopic effect studies. In Figure 2A, the heavy deuterium dimethylated isoleucine (Ile) and leucine (Leu) eluted noticeably faster than light hydrogen dimethylated Ile and Leu on RP chromatography. This result indicates that there was less interaction between the stationary phase and the deuterium-labeled compounds than there was between the stationary phase and the hydrogen-labeled counterpart. The origin of the deuterium isotopic effect has been attributed primarily to the differences in the lengths of the C−D and C−H bonds;[25−27] the smaller amplitude of vibrations, and therefore lower average volumes and polarizabilities for C−D bonds. Therefore, van der Waals interactions between the hydro-

(23) Bowman, R. E.; Stroud, H. H. *J. Chem. Soc.* **1950**, 1342−1345.
(24) Ingram, V. M. *J. Biol. Chem.* **1953**, *202*, 193−201.
(25) Turowski, M.; Yamakawa, N.; Meller, J.; Kimata, K.; Ikegami, T.; Hosoya, K.; Tanaka, N.; Thornton, E. R. *J. Am. Chem. Soc.* **2003**, *125*, 13836−13849.
(26) Wade, D. *Chem.-Biol. Interact.* **1999**, *117*, 191−217.
(27) Thornton, E. R. *Annu. Rev. Phys. Chem.* **1966**, *17*, 349−372.

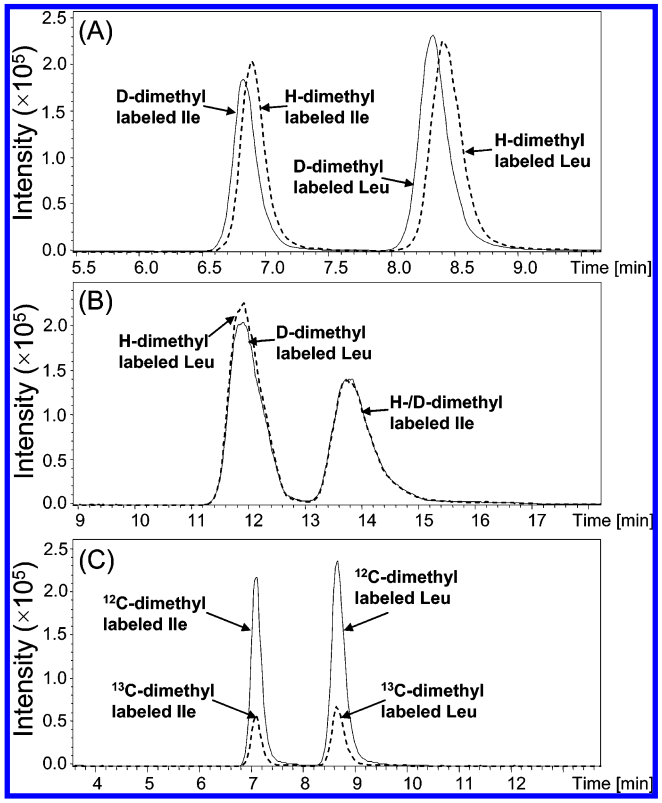


Figure 2. Evaluation of isotopic effects. (A) Deuterium dimethyl-labeled isoleucine and leucine eluted earlier than those of hydrogen counterparts in RP chromatography. (B) Deuterium/hydrogen dimethyl-labeled isoleucine and leucine coeluted perfectly in HILIC separation. (C) 1:4 ratio of ^{13}C-/^{12}C-dimethyl-labeled isoleucine and leucine coeluted perfectly in RP separation.

phobic stationary phase and deuterium-labeled species or dispersion forces that result in attraction binding forces of deuterium analogues within the hydrophobic stationary phase are less than that of hydrogen-labeled counterparts. Thus, the deuterium-labeled internal standard is not the best choice for stable-isotope dilution quantitative analysis due to the isotopic effect in RP LC.

In contrast, as shown in Figure 2B, the deuterium dimethylated Ile and Leu perfectly coeluted with their hydrogen counterparts in HILIC mode separation. There appears to be no difference in the magnitude of interactions with the stationary phase of the deuterium-labeled species and that of their hydrogen counterparts. In HILIC LC, the combination of the hydrophilic interaction and ion-exchange mechanisms results in the enhanced polar retention, and the hydrophilic interaction is dominated when the mobile phase is above 70% organic solvent. The hydrophilic interaction, the partitioning of polar analytes into and out of the adsorbed water layer for deuterium and the corresponding hydrogen species, are approximately the same. Thus, no isotopic effect was observed in HILIC LC. Thornton et al. reported that much less isotopic effect was observed when a less hydrophobic stationary phase (compared to C_{18}) was used, even in reversed-phase LC.[25] Our results are consistent with the notion that increasing hydrophilic interactions decreases the isotopic effect of deuterium-labeled compounds.

As expected, the heavy ^{13}C-dimethylated Ile and Leu eluted with exactly the same retention time as the light ^{12}C-dimethylated Ile and Leu in RP LC (see Figure 2C). Not surprisingly, ^{13}C-dimethylated standards did not show any isotopic effect in HILIC separation either (data not shown). Thus, ^{13}C-dimethylation is a preferred labeling method for stable-isotope quantitative analysis. This reaction produces at least a 2-Da mass difference between the light- and heavy-labeled analytes except for a secondary amine where the mass difference would be 1 Da. However, the more expensive ^{13}C-labeled analogues may not be needed if a HILIC separation is used for LC−ESI MS. In this case, deuterium labeling is sufficient and provides at least a 4-Da mass difference, which can be advantageous in avoiding the overlaps of isotope envelopes, particularly for high-mass analytes.

Quantitative Response. To study the feasibility of our labeling strategy for quantitative analysis, ^{13}C- and ^{12}C-dimethylated amino acid and amine standards were mixed in ratios of 1:8, 1:4, 1:1, 4:1, and 8:1 in aqueous solution. The standard mixtures were then injected onto a RP column followed by ESI-MS. The resulting extracted ion chromatograms were obtained from the corresponding mass for heavy ^{13}C- and light ^{12}C-dimethylated products. The ratios of chromatographic peak areas were calculated for each pair of 20 amino acids and 15 amines from their corresponding extracted ion chromatograms. The linear regression plots of tryptophan, phenylalanine, and methionine, as examples, are shown in Figure 3A. An R^2 value of above 0.99 was obtained for all the retained species, indicating good correlation of the experimental data with the theoretical ratios. Thus, the amine-containing compounds were quantitatively and reproducibly derivatized by this protocol. The D- and H-dimethylated standards in water with the same mixing ratios were also examined using HILIC LC−ESI MS. Similar R^2 values for all the polar species were obtained (data not shown).

To demonstrate the feasibility of our labeling strategy to overcome the matrix effect in complex biological samples, ^{13}C- and ^{12}C-dimethylated standards were mixed in ratios of 1:8, 1:4, 1:1, 4:1, and 8:1 in water containing 20% unlabeled human urine to mimic a complex matrix. The unlabeled urine would not contribute to the signals of the labeled ion pairs of interest, but would introduce chemical noise and coeluting components to the analysis. With the same procedure described above, linear regression plots were performed and three examples are shown in Figure 3B. Similarly, the D- and H-dimethylated standards in 20% unlabeled urine with the same mixing ratios were also examined for polar species in HILIC separation. In general, the R^2 values for those in 20% unlabeled urine were over 0.99 (Figure 3C and D), about the same as those in pure water. These results of good linearity indicate that the use of dimethylated analogues as internal standards can effectively overcome the matrix effect.

In general, the performance of stable-isotope dilution quantification largely depends on the control of the two labeling reactions, i.e., heavy and light dimethylation, and also the quality of the mass spectra obtained that display the isotope peak pairs. These spectra can be affected by the ionization processes of the analytes. Reductive amination occurs with a minimum change in hydrophobicity of amino acids and amines. For some of the less polar species, which can be retained on C_{18} reversed-phase columns, good-quality mass spectra of the dimethylated products with high signal-to-noise ratios can be obtained. However, many of the amino acids and amines studied in this work are very polar. They are poorly retained on the hydrophobic C_{18} stationary phase

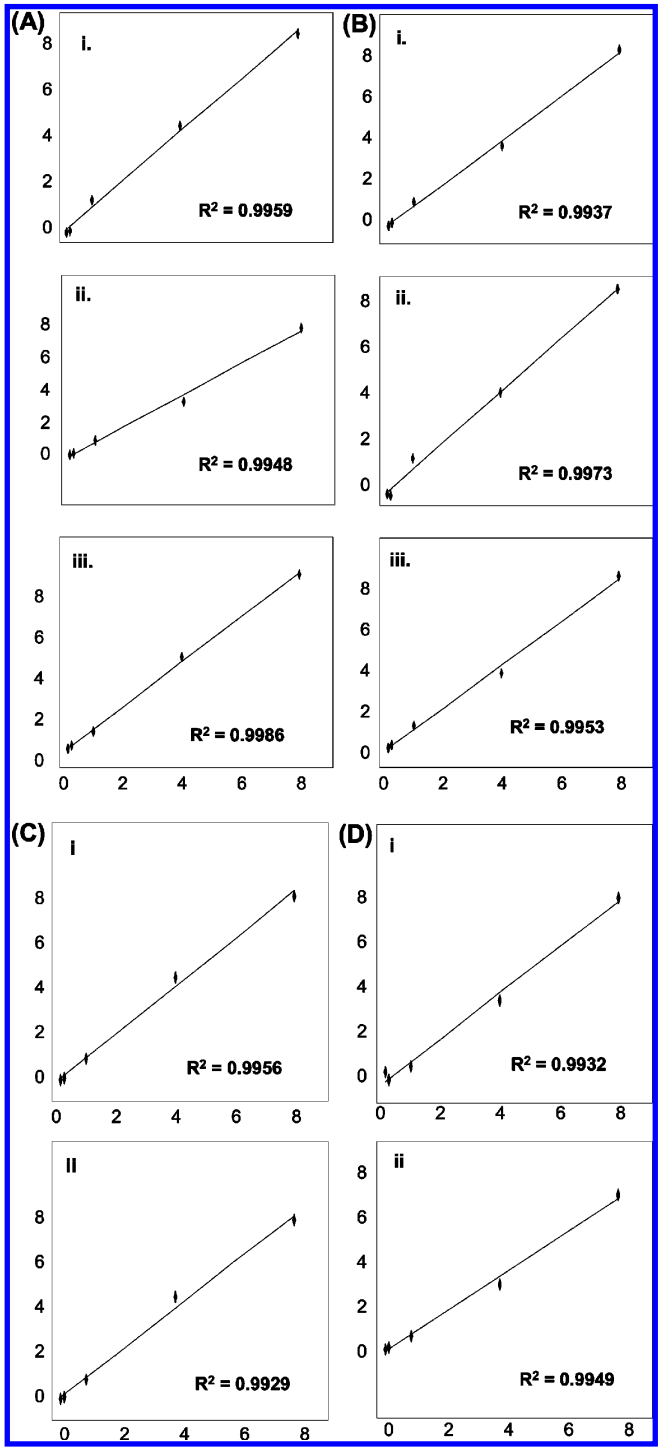


Figure 3. Linear regression plot of (A) RP chromatography of (i) ^{13}C-/^{12}C-dimethyl-labeled tryptophan in water, (ii) ^{13}C-/^{12}C-dimethyl-labeled phenylalanine in water, and (iii) ^{13}C-/^{12}C-dimethyl-labeled methionine in water. Linear regression plot of (B) HILIC separation of (i) ^{13}C-/^{12}C-dimethyl-labeled leucine in water, (ii) ^{13}C-/^{12}C-dimethyl-labeled tyrosine in water, and (iii) ^{13}C-/^{12}C-dimethyl-labeled asparagine in water. Linear regression plot of (C) RP chromatography of (i) ^{13}C-/^{12}C-dimethyl-labeled tryptophan in 20% urine and (ii) ^{13}C-/^{12}C-dimethyl-labeled phenylalanine in 20% urine. Linear regression plot of (D) HILIC separation of (i) ^{13}C-/^{12}C-dimethyl-labeled tyrosine in 20% urine and (ii) ^{13}C-/^{12}C-dimethyl-labeled asparagine in 20% urine.

and elute in the initial void volume, resulting in low sensitivity of ESI-MS detection due to analyte ion suppression and high background levels. For those highly polar species, HILIC separation that utilizes high organic mobile phases (>70%) provides excellent complementary selectivity to that of RP chromatography. A polar species generally elutes after a less polar one, and at a high percentage of organic solvent. As a consequence, much higher sensitivity can be obtained from HILIC LC−ESI MS for the polar compounds. This is important for achieving quantitative results from the stable-isotope dilution method. Thus, in this work, all the data for the polar species were obtained from the HILIC mode, and that of less polar species were from RP LC. Dimethylated products of some less polar species, such as leucine and isoleucine, were retained in both modes and could be used for comparison studies. As illustrated above, the linear regression R^2 values of both modes were similar, indicating that both separation modes can be used for quantitative analysis with the dimethylation labeling strategy.

Table 1. LC-Ion Trap-MS Results of Four Replicate Measurements of 1:1 Mixtures of 20 Isotope-Labeled Amino Acids Using Dimethylation

metabolite	measured ratio of the isotope-labeled pair	RSD %
glycine	1.03	8.0
alanine	0.96	4.3
proline	1.02	6.6
serine	1.08	7.7
valine	0.97	4.5
threonine	1.07	8.1
cysteine	1.10	8.9
leucine	1.01	4.5
isoleucine	1.02	5.6
asparagine	1.09	3.9
aspartic acid	1.10	4.4
glutamine	1.05	2.1
glutamic acid	1.09	9.3
methionine	1.00	3.1
histidine	1.07	6.2
phenylalanine	1.00	2.7
arginine	1.08	7.0
lysine	1.05	8.5
tyrosine	1.07	5.2
tryptophan	1.02	2.3

Relative Quantification of Amine-Containing Metabolites in Human Urine. Relative quantification of different metabolome samples is important for many biological studies, including the search for potential biomarkers of diseases. Our labeling strategy combined with LC−ESI MS can potentially be used to determine relative quantities of all the primary and secondary amines in two biological samples. The reproducibility of our method was evaluated by running four replicate experiments of mixtures containing 1:1 ^{12}C- and ^{13}C-dimethylated 20 amino acids in aqueous solution. Relative standard deviation (RSD) of the measured intensity ratio for tryptophan, phenylalanine, leucine, isoleucine, tyrosine, arginine, methionine, valine, and lysine was calculated from extracted ion chromatograms of RP LC−MS runs. RSD of other dimethylated amino acids were calculated from extracted ion chromatograms of HILIC LC−MS runs. The results are summarized in Table 1. As Table 1 shows, the measured relative intensity ratio of an amino acid pair varies from 0.96 to 1.10 with RSD ranging from 2.1 to 9.3 (average RSD = 5.6%).

To demonstrate the feasibility of comparative quantification by this labeling strategy, we divided a biological sample into two equal

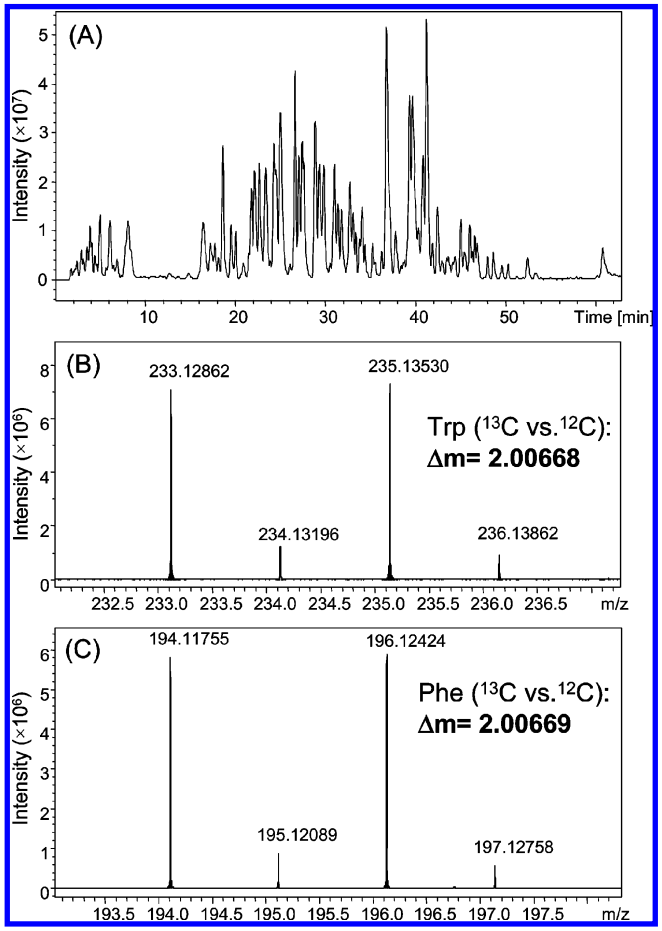


Figure 4. Feasibility of relative quantification of metabolites in human urine. (A) Reversed-phase base peak ion chromatogram of equivalent mixing of ^{13}C- and ^{12}C-dimethyl-labeled human urine obtained by using RPLC FT-ICR-MS; (B) mass spectrum of ^{13}C-/^{12}C-dimethyl-labeled tryptophan in human urine at the retention time of 22.35 min; (C) mass spectrum of ^{13}C-/^{12}C-dimethyl-labeled phenylalanine in human urine at the retention time of 16.52 min.

fractions (see Figure 1B), followed by labeling with heavy and light dimethyl isotopes, and then examined whether the ratio of heavy to light labeled pair was close to 1:1. For this demonstration, a human urine sample was split into four fractions which were ^{13}C- or ^{12}C- and D(2)- or H-dimethyl labeled under the same reaction conditions. Then the fractions from the ^{13}C- and ^{12}C-labeled samples or D(2)- and H-labeled samples, respectively, were well mixed by vortexing. The aliquots of mixtures were injected onto RP or HILIC columns, followed by ESI MS detection. Note that, for the treatment of the urine sample, only centrifugation was applied to remove possible particles in urine. Molecular weight cutoff filters were initially used to remove proteins, but found to be not necessary. The amount of sample injected was very small, and the protein concentration in urine should be very low. We have not observed any adverse effect on the lifetime of a column or the chromatographic performance of the urine metabolites from injecting the urine samples without removal of the proteins. As an example of the urine analysis, Figure 4A shows the resulting base peak ion chromatogram from RP LC on the Bruker 9.4-T FT-ICR mass spectrometer. The ratio of integrated peak areas or peak heights for the ^{13}C- and ^{12}C-dimethylated tryptophan pair (m/z 233.128 62 and 235.135 30, see Figure 4B) were 1.02 and 1.04, respectively. The ratio of the phenylalanine pair (m/z 194.117 55 and 196.124 24, see Figure 4C) were 1.00 and 1.02. For the 20 amino acids, the measured ratio of an amino acid pair ranges from 0.96 to 1.10 with an average ratio of 1.04 and a coefficients of variation (CV) of 4.1%. These LC−MS experimental values are in excellent agreement with the expected ratios of 1.00.

Note that, as pointed out earlier, the characteristic mass differences between heavy and light dimethylated compounds in mass spectra indicate the existence of primary or secondary amine functional group in a metabolite, and this could potentially be used for unknown amine identification, in addition to the information gained from retention time, MS/MS, accurate mass, and isotopic pattern by high-resolution MS. In our FT-ICR-MS, the mass measurement accuracy for the metabolites is typically less than 2 ppm. This high mass accuracy facilitates the identification of ion pairs of amine-containing metabolites in the urine sample. Supporting Information Table S1 lists 33 amine-containing metabolites detected by either RP or HILIC LC-ESI FT-ICR-MS with each pair having signal-to-noise ratios of greater than 10. The 20 amino acids plus 13 amines were positively identified based on their accurate mass and retention time in comparison to those of labeled standards. Among them, 16 were found in both RP and HILIC LC−MS runs, 2 was found in RP LC−MS alone, and 15 were found in HILIC LC−MS alone.

In the RP LC-ESI FT-ICR-MS analysis of the labeled urine sample, besides the 18 metabolites identified and listed in Table S1, we actually detected additional 420 ion pairs (see Supplorting Information Table S2). All these pairs displayed a signal-to-noise ratio of greater than 10. Over 100 ion pairs showed signal-to-noise ratios of greater than 80, indicating they are high-abundance metabolites. Based on accurate mass and retention time information, we conclude that these ion pairs belong to different metabolites. However, these metabolites remain to be identified. Accurate mass measurement alone does not lead to unambiguous metabolite identification, considering there are many metabolites potentially present in body fluids. Nevertheless, this work demonstrates that, using the labeling strategy combined with RP LC-ESI FT-ICR-MS, we can potentially profile over 438 amine-containing metabolites in human urine. One would expect that multiple dimensional separation of the labeled metabolites followed by LC−MS analysis should further expand the metabolome coverage.

Absolute Quantification of 20 Amino Acids and 15 Amines in Human Urine. Absolute quantification of analytes of interest can provide direct information on the expression of a given metabolite in relation to other metabolites present in a sample. Absolute quantification by stable-isotope dilution LC−MS can be done by using this dimethylation labeling strategy, as long as the targeted amines are commercially available. The availability of the ^{13}C- or deuterium isotope-labeled analogue of an amine of interest is no longer an issue, because stable isotopic tags will be introduced onto the parent compounds using the dimethylation labeling reaction to produce the isotope analogue, while the same reaction will be done on the sample to be analyzed.

As an example of absolute quantification, aliquots of human urine were heavy dimethyl labeled with ^{13}C- or D(2)-formaldehyde. The 20 amino acid and 15 amine standard solutions of known concentration were light dimethyl labeled with normal formalde-

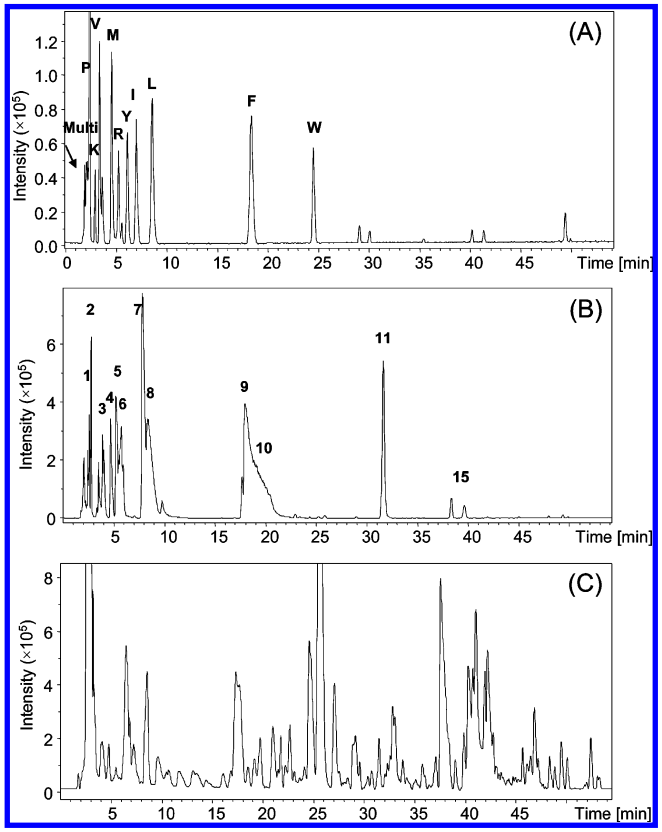


Figure 5. Base peak ion chromatograms of RP chromatography. (A) ^{13}C-/^{12}C-dimethyl-labeled 20 amino acids; the components that eluted in the void volume are A, C, E, Q, G, S, and T, and (B) ^{13}C-/^{12}C-dimethyl-labeled 15 amines. Histamine, diaminobutane, 4-hydroxproline, 3-methylhistidine, and γ-aminobutyric acid were eluted in the column void volume. 1, pyridoxamine and cysteamine; 2, (−)-epinephrine; 3, dopamine; 4 and 5, unknown; 6, tyramine; 7, benzylamine; 8, aniline; 9, 2-methylbenzylamine; 10, 1-ephedrine; 11, *p*-aminohippuric acid; (C) ^{13}C-dimethyl-labeled human urine mixed with ^{12}C-dimethyl-labeled standards.

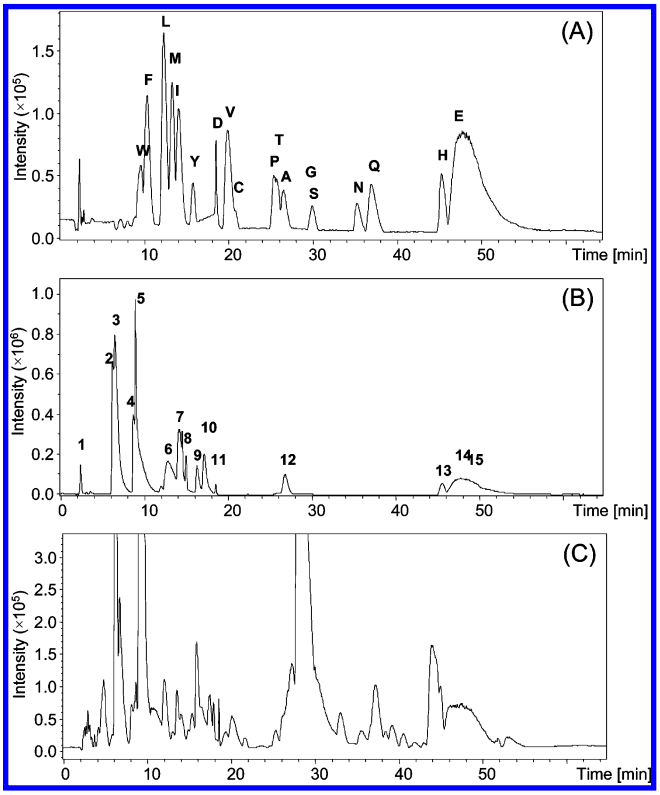


Figure 6. Base peak ion chromatograms of hydrophilic interaction chromatography of (A) ^{13}C-/^{12}C dimethyl-labeled 20 amino acids, (B) ^{13}C-/^{12}C-dimethyl-labeled 15 amines, and (C) ^{13}C-dimethyl-labeled human urine mixed with ^{12}C-dimethyl-labeled standards. The 15 amines in (B): 1, aniline; 2, 2-methylbenzylamine; 3, *p*-aminohippuric acid; 4, benzylamine; 5, 1-ephedrine; 6, pyridoxamine; 7, tyramine; 8, histamine; 9, dopamine; 10, cysteamine; 11, (−)-epinephrine; 12, 4-hydroxyproline; 13, diaminobutane; 14, γ-aminobutyric acid; 15, 3-methylhistidine.

hyde under exactly the same reaction conditions. Heavy-labeled urine was mixed with light-labeled standards and then injected onto RP and HILIC columns followed by ESI-MS analysis. Figures 5 and 6 show the base peak ion chromatograms of the ^{13}C- and ^{12}C-dimethyl-labeled amino acid standards, amine standards, and the heavy-labeled human urine sample mixed with light-labeled standards obtained by RP LC-ESI MS and HILIC LC-ESI MS, respectively. The calculated ratios of heavy- to light-labeled pairs of amines from their extracted ion chromatograms were used to perform absolute quantification, since the concentrations of the standards are known. The absolute concentrations of 20 amino acids and 15 amines in human urine were determined and are listed in Table 2. As Table 2 shows, the metabolites detected in the urine sample have concentrations ranging from 0.05 (arginine or tyramine) to 21.2 mg/L (glutamine) with a concentration dynamic range of at least 424-fold.

While the preliminary results shown in Table 2 illustrate the possibility of carrying out absolute metabolite quantification using the isotope dilution method with dimethylation. Additional work is required to validate this method. To this end, we are planning to study the analyte recovery issue during sample workup with spiked samples, compare this method with other widely used techniques such as LC-UV method, and investigate the run-to-run and day-to-day reproducibility of the method. We envisage that, once the method is validated with the amino acid standards, we should be able to extend this method for absolute quantification of many other amine-containing metabolites.

It should be noted that, in addition to allowing absolute quantification, spiking a complex sample with isotopically labeled

Table 2. Absolute Quantificiation of 20 Amino Acids and 15 Amines in Human Urine

metabolites	concn (mg/L)	metabolites	concn (mg/L)
glycine	10.1	tyrosine	3.00
alanine	0.97	tryptophan	4.80
proline	0.14	aniline	<DL
serine	1.40	2-methylbenzylamine	<DL
valine	0.51	benzylamine	0.09
threonine	2.20	1-ephedrine	1.90
cysteine	0.20	*p*-aminohippuric acid	1.40
leucine	0.37	tyramine	0.05
isoleucine	0.27	γ-aminobutyric acid	0.22
asparagine	0.96	histamine	0.26
aspartic acid	1.40	L,4-hydroxyproline	0.29
glutamine	21.2	cysteamine	0.12
glutamic acid	0.17	1,4-diaminobutane	0.14
methionine	0.22	(−)-epinephrine	0.06
histidine	16.2	pyridoxamine	0.25
phenylalanine	1.00	dopamine	0.38
arginine	0.05	3-methyl-L-histidine	0.08
lysine	1.60		

standards can facilitate the identification of targeted amines. This can be done by looking at the extracted ion chromatogram of the labeled standards: the analytes of interest in urine appear in the same mass spectrum with a characteristic mass difference.

CONCLUSIONS

In this work, we examined the use of reductive amination to provide isotope tags for amine-containing metabolites. The simple and rapid labeling reaction is carried out under very mild conditions with minimum sample and reagent manipulation. The targeted amine-containing metabolites could be quantitatively derivatized under controlled reaction conditions. The dimethylated products were stable at room temperature, and the ionization efficiency of the products was not compromised. This labeling strategy essentially produces inexpensive ^{13}C- or deuterium-labeled analogues of targeted amines for stable-isotope dilution analysis in RP and HILIC LC−ESI MS. This method was proved to be effective to overcome matrix effects. It was shown to be feasible to quantify amine-containing metabolites in the complex biofluid, human urine. Absolute quantification can be performed using this strategy as long as the parent standards are available for dimethylation labeling. Relative quantification can be easily performed on biological samples by differential isotope labeling. Another advantage of labeling is that the characteristic mass difference between the heavy and light dimethylamines provides additional information to facilitate peak and compound identification. Using FT-ICR-MS combined with one-dimensional RP LC separation, we detected a total of 438 ion pairs of amine-containing metabolites in a human urine sample. Although most of them have not been identified, they appear to be different metabolites based on the accurate mass and retention time information. Future work will involve expanding the list of 35 standards to many more biologically relevant amines and apply this isotope dilution strategy to generate quantitative profiles of amine-containing metabolites in different natures of complex samples, such as urine samples collected from disease and control objects and cells of different states. We note that, while the method can potentially be used for high-throughput quantitative profiling of amine-containing metabolome, the major challenge is for the identification of unknown metabolites. To this end, we are in the process of developing a human metabolome MS/MS database, which should facilitate the identification of unknown metabolites.[28]

(28) Wishart, D. S.; Tzur, D.; Knox, C.; et al. *Nucleic Acids Res.* **2007**, *35*, D521−D526.

ACKNOWLEDGMENT

This work was funded by Genome Canada through Genome Alberta's Human Metabolomics Toolbox project and the Canada Research Chairs Program. We thank the Canada Foundation for Innovation and the Alberta Science and Research Investments Program for providing the equipment fund for purchasing the FT-ICR-MS instrument.

SUPPORTING INFORMATION AVAILABLE

A list of 33 ion pairs detected and identified by RP and HILIC LC-ESI FT-ICR-MS from a mixture of 1:1 human urine samples labeled by ^{12}C- and ^{13}C-formaldehyde, respectively (Table S1), and a list of additional 420 ion pairs detected by RP LC-ESI FT-ICR-MS (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review March 3, 2007. Accepted August 16, 2007.

AC0704356