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Development of versatile isotopic labeling reagents for profiling the amine submetabolome by liquid chromatography–mass spectrometry

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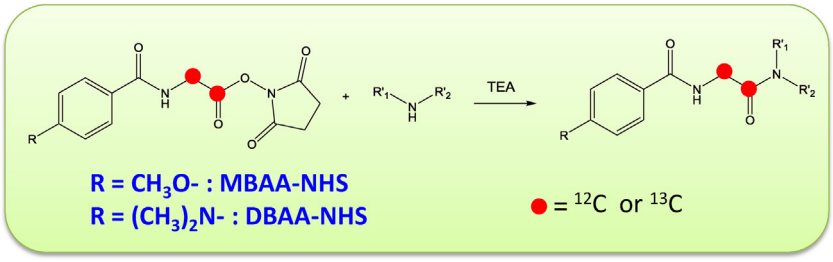
HIGHLIGHTS

- Two new reagents were developed for chemical isotope labeling mass spectrometry (MS).
- They could be used to label amine-containing metabolites in a metabolomic sample.
- The labeled metabolites could be detected with much improved sensitivity in MS.
- One of the reagents could also help generate useful MS/MS spectra for structural analysis.
- These reagents should be useful for quantitative metabolomics.

GRAPHICAL ABSTRACT



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ABSTRACT

Metabolomic profiling involves relative quantification of metabolites in comparative samples and identification of the significant metabolites that differentiate different groups (e.g., diseased vs. controls). Chemical isotope labeling (CIL) liquid chromatography–mass spectrometry (LC–MS) is an enabling technique that can provide improved metabolome coverage and metabolite quantification. However, chemical identification of labeled metabolites can still be a challenge. In this work, a new set of isotopic labeling reagents offering versatile properties to enhance both detection and identification are described. They were prepared by a glycine molecule (or its isotopic counterpart) and an aromatic acid with varying structures through a simple three-step synthesis route. In addition to relatively low costs of synthesizing the reagents, this reaction route allows adjusting reagent property in accordance with the desired application objective. To date, two isotopic reagents, 4-dimethylaminobenzoylamido acetic acid *N*-hydroxylsuccinimide ester (DBAA-NHS) and 4-methoxybenzoylamido acetic acid *N*-hydroxylsuccinimide ester (MBAA-NHS), for labeling the amine-containing metabolites (i.e., amine submetabolome) have been synthesized. The labeling conditions and the related LC–MS method have been optimized. We demonstrate that DBAA labeling can increase the metabolite detectability because of the presence of an electrospray ionization (ESI)-active dimethylaminobenzoyl group. On the other hand, MBAA labeled metabolites can be fragmented in MS/MS and pseudo MS^3 experiments to provide structural information on metabolites of interest. Thus, these reagents can be tailored to quantitative profiling of the amine submetabolome as well as metabolite identification in metabolomics applications.

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1. Introduction

Differential chemical isotope labeling (CIL) is a method that introduces a chemical tag (e.g., ^{12}C-reagent) and its isotopic analog (e.g., ^{13}C-reagent) into targeted analytes in a sample and a comparative control, respectively. After mixing the labeled sample and control, the mixture is analyzed by liquid chromatography–mass spectrometry (LC–MS). A differentially labeled metabolite is detected in a mass spectrum as a pair of peaks with mass difference defined by the isotope mass difference of the tag(s). The intensity ratio of the peak pair can be determined to provide relative quantification of the metabolite in the sample vs. the control. Absolute quantification is also possible if the concentration of the metabolite in the control is known, i.e., using a metabolite standard. CIL LC–MS has been shown to provide high accuracy and precision for metabolome profiling [1–4], which can be attributed to simultaneous measurement of the co-eluted analytes and their isotope counterparts to overcome ion suppression, instrument drift and other technical problems. Furthermore, chemical derivatization provides a useful means of improving the separation of polar or ionic metabolites in reversed phase (RP) LC as well as enhancing the ionization efficiency in electrospray ionization (ESI) by introducing a relatively hydrophobic and easily chargeable ESI-active tag [1,5,6].

To date, several CIL reagents have been developed to quantify organic amines, carboxylic acids, carbonyl compounds and some other types of metabolites with varying degrees of performance [1,4,5,7–24]. These research activities are mainly driven by the need of dealing with different classes of metabolites; a universally applicable derivatization reagent for LC–MS is not available at the present. On the other hand, chemical labeling can be used as a way of dividing the entire metabolome into several submetabolomes according to their functional groups (e.g., amine submetabolome, acid submetabolome, etc.), thereby reducing the complexity of a metabolomic sample [5,25]. This divide-and-conquer approach requires the use of robust chemical labeling reagents for profiling submetabolomes with different intended purposes (e.g., untargeted quantification, targeted quantification, chemical identification, etc.).

Development of a proper chemical labeling reagent is not an easy task, because multiple factors including reagent synthesis, labeling efficiency, analytical performance, applicability and cost need to be carefully considered. For example, Guo and Li developed a CIL method based on $^{12}C_2$- and $^{13}C_2$-dansyl chloride (DnsCl) [1]. Although the method allows for rapid and accurate quantification of amine-containing metabolites [26–29], metabolite identification, particularly for unknown metabolites, is a challenge due to the lack of structural information in the MS/MS spectra of dansyl labeled metabolites. Tsukamoto et al. developed H_6-/D_6-7-(*N*,*N*-dimethylaminosulfonyl)-4-(aminoethyl)-piperazino-2,1,3-benzoxadiazole (H_6-/D_6-DBD-PZ-NH_2) to profile fatty acids in rat plasma samples [8] and Shimbo et al. reported the use of *p*-*N*,*N*,*N*-trimethylammonioanilyl *N*′-hydroxysuccinimidyl carbamate iodide (TAHS) and D_3-TAHS for amino acid quantification [30]. Because deuterium was used in the labeling reagents, isotopic effect on retention time in RPLC was observed (i.e., H- and D-labeled same metabolite eluted out at different time, thereby subjecting to different matrix effect and ion suppression). Abello et al. described multiplex reagents for analyzing amine-containing metabolites in human cells based on pentafluorophenyl-activated ester of ^{13}C-containing poly(ethylene glycol) chains (PEG-OPFP) [10]. The family of these CIL reagents could be used to quantify three samples in parallel, but the reagents did not provide much signal enhancement in LC–MS. Yang et al. developed H_3-/D_3-*N*-hydroxysuccinimide ester of *N*-alkylnicotinic acid (H_3/D_3-C_n-NA-NHS) to measure concentrations of amino acids in rat urine by which the sensitivity of labeled amino acids was enhanced by up to 1000-fold [11]. While this reagent is useful for targeted analysis of amino acids, it may not be suitable for untargeted analysis, because of the deuterium isotopic effect and the use of an ionizable tag in solution which would limit efficient separation of labeled metabolites by RPLC, requiring the use of different columns for separating different classes of labeled metabolites.

To increase the versatility of CIL LC–MS for metabolomics, we have developed a general strategy based on the use of $^{12}C_2$-glycine and $^{13}C_2$-glycine to construct a family of CIL reagents that can be tailored to different applications. In this work, we report two CIL reagents, $^{12}C_2$-/$^{13}C_2$-4-dimethylamino-benzoylamido acetic acid *N*-hydroxysuccinimide ester ($^{12}C_2$-/$^{13}C_2$-DBAA-NHS) and $^{12}C_2$-/$^{13}C_2$-4-methoxybenzoylamido acetic acid *N*-hydroxysuccinimide ester ($^{12}C_2$-/$^{13}C_2$-MBAA-NHS), that can be used to improve detection and identification of amine-containing metabolites while maintaining efficient retention and separation on RPLC. The synthesis of these reagents and their analytical performance in profiling the amine submetabolome of biofluids such as human urine are described.

2. Experimental

2.1. Chemicals and urine samples

All chemicals and reagents were purchased from Sigma–Aldrich (Oakville, ON) unless otherwise noted. $^{13}C_2$-glycine was purchased from Cambridge Isotopes Laboratories (Andover, MA). Stock solutions of twenty amino acid standards (20 mM each) were prepared in H_2O:ACN (50:50 (v/v)) and stored at 4 °C. To aid the dissolution of amino acids, 6 M HCl could be added drop-by-drop (less than a few microliter) to a solution to completely dissolve an amino acid. A pooled amino acid (1 mM each) was prepared by mixing aliquots of twenty stock solutions. The 300 mM triethylamine (TEA) solution was prepared by dilution of 500 μL of LC-grade TEA in 28.5 mL acetonitrile. The 300 mM formic acid (FA) solution was prepared by adding 1.13 mL of formic acid into 100 mL of H_2O. Both solutions were stored at 4 °C.

The study of human urine samples was approved by the Ethics Approval Board of the University of Alberta. Human urine sample collection and processing were performed according to a previous reported protocol [6].

2.2. CIL reagent synthesis

Fig. 1(A) shows the three-step synthesis pathway to prepare the reagents and their isotopic analogs. *N*,*N*-Dicyclohexylcarbodiimide (DCC) (1.25 g) and *N*-hydroxyl succinimide (NHS) (0.67 g) were added into a solution of 4-methoxybenzoic acid (0.92 g) for preparing MBAA-NHS or 4-dimethylaminobenzoic acid (0.99 g) for preparing DBAA-NHS in 40 mL DMF at 25 °C. The mixture was stirred for 24 h and then filtered to give a clear solution. Glycine (0.75 g) or its isotopic counterpart ($^{13}C_2$-glycine) was dissolved in a mixture of H_2O (20 mL) and triethylamine (2.7 mL). The solution was quickly added into the filtrate from the first reaction. After 30 min, the mixture was neutralized by formic acid to give a white solid, 4-methoxybenzoylamido acetic acid or 4-dimethylbenzoylamido acetic acid, which was filtered out and washed by cold acetone (3 × 10 mL). The product could be directly used in the next step without purification. The third step was exactly the same as the first reaction. 4-Methoxybenzoylamido acetic acid (0.90 g) or 4-dimethylbenzoylamido acetic acid (0.98 g) was dissolved in 40 mL DMF. NHS (0.50 g) and DCC (0.89 g) were added into the solution which was stirred for 24 h at 25 °C. The final product was purified by recrystallization with Hexane:*i*-PrOH (3:1 v/v). The

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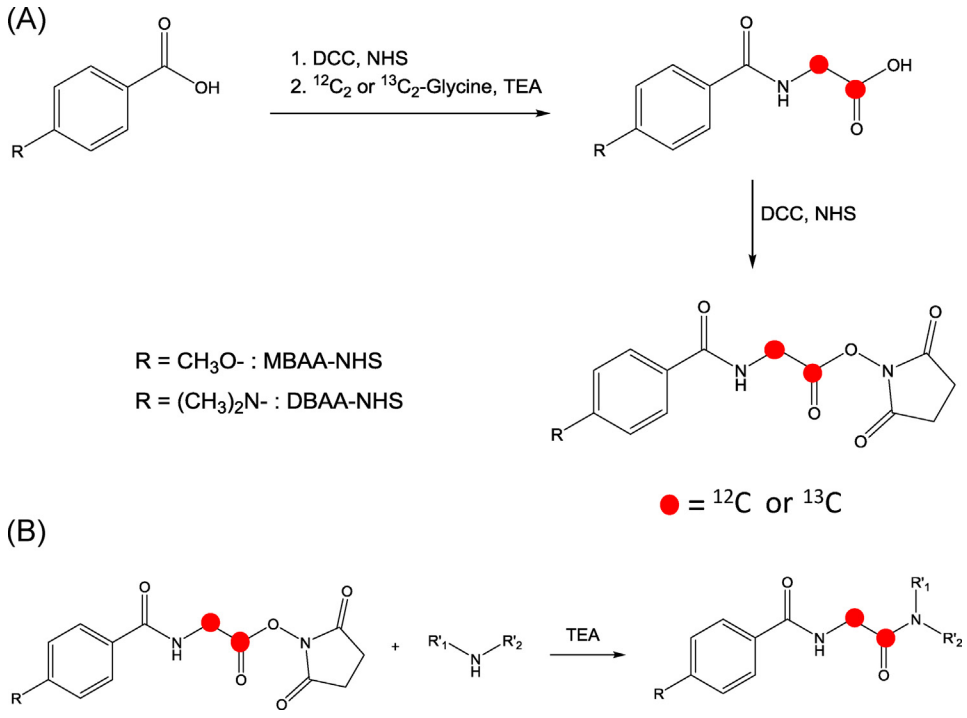

Fig. 1. (A) Structures and synthesis of $^{12}C_2$-/$^{13}C_2$-MBAA-NHS and $^{12}C_2$-/$^{13}C_2$-DBAA-NHS. (B) Labeling reaction scheme of MBAA-NHS and DBAA-NHS.

total yield from the three steps of synthesis was 68% and the product was stored at −20 °C.

2.3. Derivatization and mixing

Fig. 1(B) shows the derivatization reaction scheme. 50 μL of the pooled amino acid standards or human urine was mixed with 25 μL of 0.3 M TEA, and then added to freshly prepared 100 μL of 20 mM $^{12}C_2$-MBAA solution in ACN:DMSO (1:1 v/v) for light labeling, or an equal volume of $^{13}C_2$-MBAA for heavy labeling. The solutions were incubated at 25 °C for 10 min, and then acidified by adding 25 μL of 0.3 M FA solution. After centrifugation at 2000 × *g* for 5 min, equal aliquots of $^{12}C_2$-/$^{13}C_2$-MBAA-NHS labeled samples were taken and then mixed to give the final sample for LC–MS analysis.

2.4. Absolute quantification of amino acids

Individual stock solutions of twenty amino acid standards were derivatized by $^{13}C_2$-MBAA-NHS. Appropriate volumes of stock solutions were mixed based on their individual concentrations commonly found in human urine [6]. The mixture was spiked into a pooled human urine labeled by $^{12}C_2$-MBAA-NHS prior to LC–MS analysis. Absolute concentrations of 20 amino acids were calculated by the intensity ratios of peak pairs between the labeled amino acid isotopomers found in mass spectra.

2.5. LC–MS

LC–MS analysis was performed using an Agilent 1100 series binary LC system (Agilent, Palo Alto, CA) hyphenated to a Bruker Impact HD quadrupole time-of-flight (Q-TOF) MS (Bruker, Bremen, GE). All data were acquired in the positive ion mode. Chromatographic separations were carried out on an Agilent Zorbax Eclipse Plus C18 column (2.1 mm × 100 mm, 1.8 μm, New Castle, DE). The mobile phase A was 0.1% formic acid in ACN/H_2O (5/95, v/v) and the mobile phase B was 0.1% formic acid in ACN. A 20-min gradient: 0 min (10% B), 0–0.5 min (10% B), 0.5–7 min (10–25% B), 7–18 min (25–99% B), and 18–20 min (99% B). The column was re-equilibrated with the initial mobile phase conditions for 15 min prior to the next sample run. The flow rate was 180 μL min^{-1} and the sample injection volume was 2.0 μL.

2.6. Flow injection MS/MS and pseudo MS^3

The Bruker Q-TOF-MS was used to perform MS/MS and pseudo MS^3 analysis. A labeled sample solution was infused directly to the mass spectrometer by a syringe pump at a flow rate of 3 μL min^{-1}. The MS instrument was operated under the following conditions: nebulizer gas 1.8 bar, dry gas 8.0 L min^{-1}, dry gas heater 220 °C, capillary voltage 4500 V, end plate offset −500 V, the mass range was set at *m*/*z* 50–500. A mass scan was first performed to find the protonated molecular ion. In the MS/MS scan, the precursor ion of a labeled amino acid was isolated within ±1 Da mass window. After collision induced dissociation (CID) in the CID cell, the product ions were detected by the TOF analyzer. In the pseudo MS^3 scan, the first generation fragment ions were generated in the skimmer region by raising the in-source CID voltage. The protonated unlabeled amino acid ion was isolated by the first quadrupole within ±1 Da of mass window and then subjected to CID in the CID cell. Finally, the second generation product ions were analyzed in the TOF. All of the MS scan, MS/MS scan and pseudo MS^3 experiments were performed in the positive ion mode.

2.7. Data processing

The MS data were internally calibrated and processed using a peak-pair picking software, IsoMS. The level 1 peak pairs were aligned from multiple runs by retention time within 30 s and accurate mass within 5 ppm. Only the common peak-pairs were retained for statistical analysis. Principle component analysis (PCA) and volcano plot analysis were performed by Metaboanalyst (www.metaboanalyst.ca) [31]. The data were mean-centered and auto-scaled (unit variance) prior to analysis.

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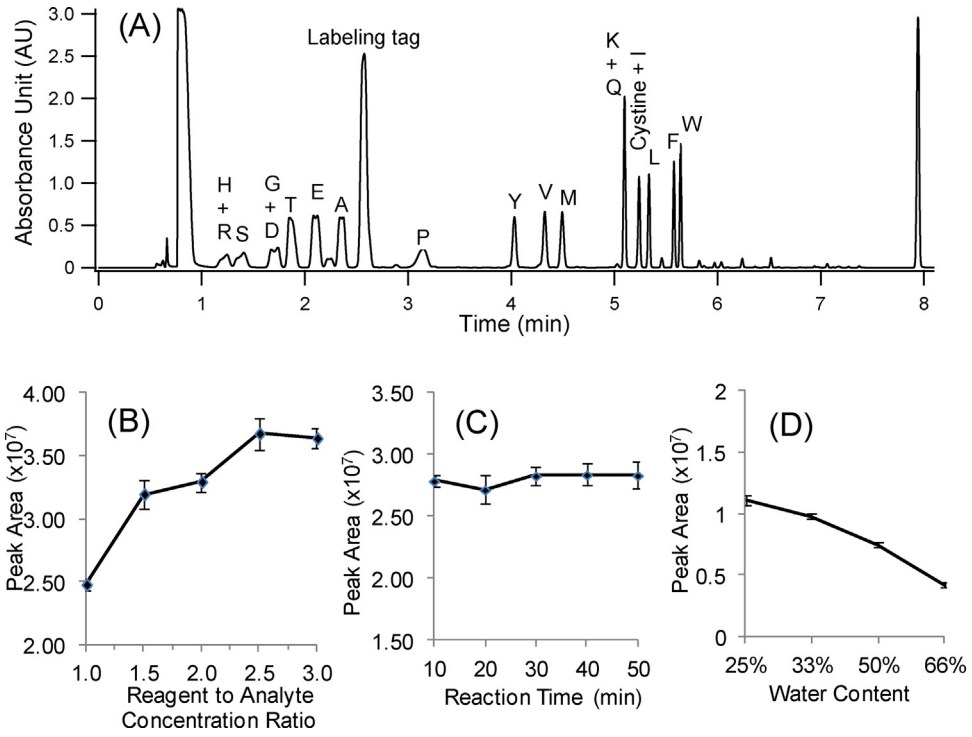

Fig. 2. (A) UV chromatogram of MBAA labeled amino acid standards obtained by LC-UV with absorbance measurement at 254 nm. Total peak area of the LC-UV chromatographic peaks is plotted as a function of (B) concentration ratio of reagent and analyte, (C) labeling reaction time, and (D) water content in the labeling solution.

3. Results and discussion

Rational design of a labeling reagent is important for metabolome profiling. An ideal reagent would provide simultaneous improvement in separation, detection and identification of labeled metabolites. However, the extent of improvement in each process may need to be balanced to achieve the optimal overall performance. For example, to enhance the detection sensitivity of ESI, it is preferable to add a readily chargeable group (e.g., dimethylamino group) or even a charged group (e.g., trimethylamino group) to a metabolite. However, a charged group can greatly affect the retention properties of the labeled metabolites, resulting in non-optimal LC separation. This is particularly true in cases where RPLC, an ideal mode of separation for online MS detection, is used for high efficiency metabolite separation. A readily chargeable group during ESI can enhance detection sensitivity while not compromising RPLC separation. However, the protonated labeled-metabolite with its charge locked at the labeling tag may not produce any useful fragment ions from the metabolite moiety itself in MS/MS. Thus, some compromises are needed to balance the extent of sensitivity enhancement and the degree of fragmentation required for structural analysis. Based on this principle, we developed a reaction scheme to synthesize the DBAA-NHS and MBAA-NHS reagents that are versatile for balancing the detectability and fragmentation ability.

DBAA-NHS was designed to provide more sensitive detection of labeled metabolites because of a basic amino group on the aromatic ring that can be more readily protonated in ESI [1]. MBAA-NHS does not have the basic amino group and thus protonation at the metabolite moiety becomes more likely. This increases the possibility of producing fragment ions from the metabolite itself. The hydrophobic aromatic ring in both reagents is important for increasing the overall hydrophobicity of labeled metabolites so that very hydrophilic or even ionic metabolites can be retained on RPLC after derivatization. This ring is also important for enhancing ESI by stabilizing a charge after protonation of nitrogen or oxygen that is connected to the ring, and by increasing the propensity of moving the analyte to the surface of an ESI droplet due to an increase in hydrophobicity. After the synthesis of the two reagents, the protocol for labeling amines was optimized using amino acid standards. The performance of MBAA-NHS and DBAA-NHS for labeling a real world sample for CIL LC–MS was studied using human urine samples. Finally, the MS/MS experiments of MBAA-NHS labeled amino acids were performed to show that the fragment ions of MBAA-NHS derivatives can provide useful structure information for metabolite identification.

3.1. MBAA-NHS and DBAA-NHS

A high-performance isotope labeling reagent can be divided into three parts according to their functions: (1) reaction group, (2) isotope group, and (3) physiochemical property tuning group. The reaction group is responsible for the derivatization reaction between the labeling reagent and the targeted analytes. The isotope group containing differential isotope atoms (e.g., ^{12}C vs. ^{13}C) provides the mass difference in a peak pair found in a mass spectrum. The tuning group affects the separation, ionization and fragmentation. Selection of an appropriate isotope group is important, because it determines how isotope atoms are encoded to the molecule and the cost of making the reagent. In this work, glycine was chosen as an isotope group. In addition to its low cost, glycine has a desirable structure for doing the following tasks. First of all, glycine has an amino group which can react with various compounds including carbonyl chlorides or alkyl bromide. Thus, the isotope group can be easily linked to many tuning compounds. Secondly, the amide bond is sufficiently strong to resist other reaction conditions, an important consideration for multi-step synthesis. Thirdly, the carboxylic acid of glycine can be readily converted into active *N*-hydroxyl succinimide (NHS) ester that is well known for labeling amines under mild conditions [16]. Since glycine is a polar compound, an aromatic compound is a good candidate of the tuning group to increase the hydrophobicity of the labeling reagents. Contrary to other reagents

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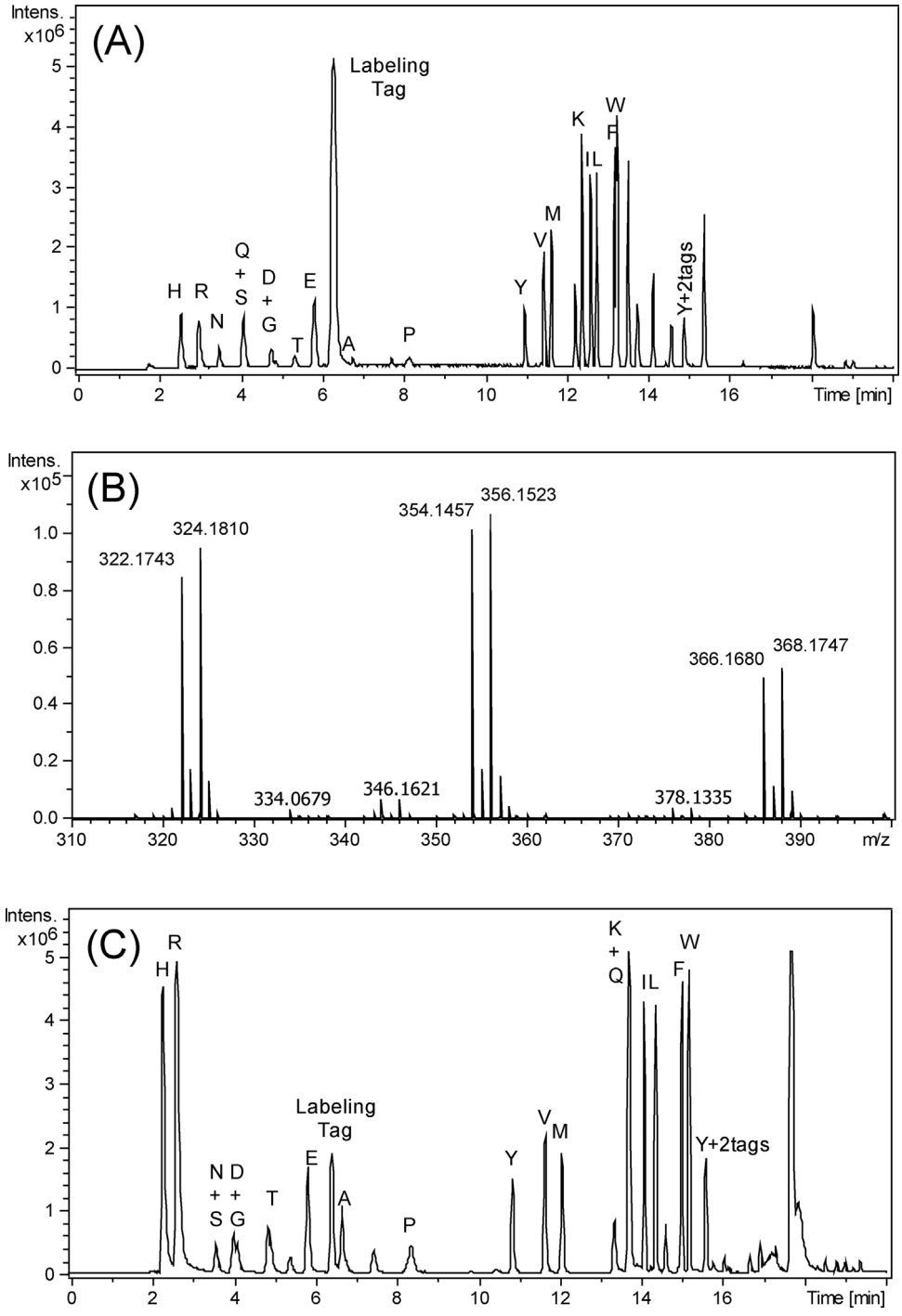

Fig. 3. (A) Base-peak ion chromatogram of $^{12}C_2$-/$^{13}C_2$-DBAA labeled amino acid standards obtained by LC–MS. (B) Expanded mass spectrum showing the molecular ion regions of three labeled amino acids. (C) Base-peak ion chromatogram of $^{12}C_2$-/$^{13}C_2$-MBAA labeled amino acid standards.

such as dansyl chloride, glycine serves as both the isotope group and the coupling group, while the aromatic group is only used to change the performance of the final product. This feature allows us to optimize the performance of these CIL reagents by simply changing the structure of the aromatic compound.

The synthesis of the reagents is quite straightforward. The three-step reactions are performed under very mild conditions and the final products are purified by a simple recrystallization. As shown in Fig. 1(B), the amino group of glycine (or $^{13}C_2$-glycine) reacts with a carboxylic acid NHS ester to form an amide intermediate. Then the carboxylic acid of the glycine moiety in the intermediate is converted into an active NHS ester which can selectively derivatize primary and secondary amines in an aqueous solution. Two kinds of CIL reagents, DBAA-NHS and MBAA-NHS, have been synthesized based on the common reaction scheme with a difference in the aromatic side-chain structure. Although we have not tried other types of tuning groups, we expect that many groups should be workable using the same synthesis route.

3.2. Optimization of labeling efficiency

A reproducible and efficient chemical reaction is essential for any isotope labeling method. In addition, the reaction should be carried out under mild conditions to avoid any undesirable side-reactions.

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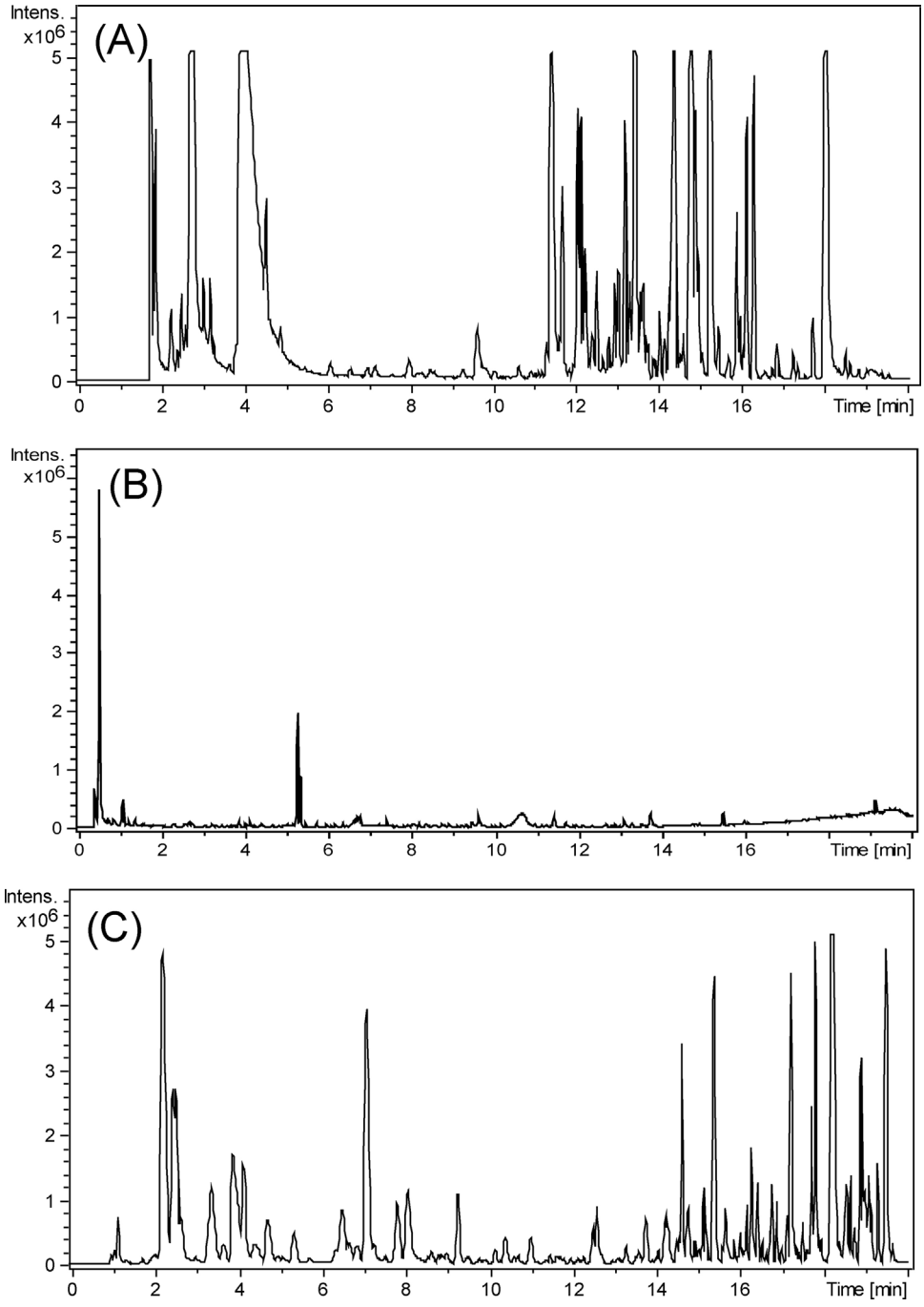

Fig. 4. Base-peak ion chromatograms of (A) $^{12}C_2$-/$^{13}C_2$-DBAA labeled human urine, (B) plain urine and (C) $^{12}C_2$-/$^{13}C_2$-MBAA labeled human urine.

Because of the similarity between MBAA-NHS and DBAA-NHS, the optimal labeling efficiency was similar. Only the optimization results for MBAA-NHS are described below. In this case, a mixture of 20 amino acid standards was used to react with MBAA-NHS and LC-UV was used to determine the labeling efficiency. These 20 amino acids have a wide range of hydrophobicity with their values covering those of most of small molecules. Hydrophobicity greatly influences separation in RPLC and ionization in the positive ESI source. Thus, the results from analyzing 20 amino acids are representative of a great number of small molecules.

Fig. 2(A) shows a LC-UV chromatogram of the labeled amino acids. The amino acids elutes within about 5 min and separation is sufficient for determining the peak areas of labeled amino acids. To optimize the derivatization conditions, the effects of MABB-NHS concentration, reaction time and solvent composition were investigated at room temperature. Fig. 2(B) shows the total UV peak area of the amino acids as a function of the reagent concentration ranging from 20 mM to 60 mM while the concentration of the standards was fixed at 20 mM. The area increases as the concentration of MBAA-NHS increases and reaches a plateau when MBAA-NHS is 2.5-fold more than that of the standards. Fig. 2(C) shows the effect of reaction time (10–50 min) on labeling efficiency. As Fig. 2(C) shows, the UV peak area is independent of the reaction time, which means that the labeling reaction reaches the maximal yield within 10 min. This observation is consistent with a previous report of similar reactions [32].

The solvent composition has a large influence on the labeling efficiency. The presence of a small amount of water in a metabolic

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sample (e.g., up to 25%) is often needed in order to dissolve metabolites. The effect of water content on labeling efficiency is shown in Fig. 2(D) where the UV peak area is inversely proportional to the water content in the solvent. Water is not a favorable solvent for nucleophilic substitution reaction, as it can form hydrogen bonds with the amino group and thus the molecular interaction around the amino group is reduced. Furthermore, water also causes hydrolysis of the labeling reagent. However, from Fig. 2(D), it is clear that a small amount of water in a sample solution is still fine for labeling. From the above studies, we concluded that an optimal labeling reaction can be performed by using 50 mM MABB-NHS to label 20 mM amino acid standards or undiluted urine samples in 25% aqueous solution with 10 min reaction at room temperature.

3.3. Analytical performance of DBAA-NHS and MBAA-NHS labeling

A set of experiments were carried out to gauge the analytical performance of DBAA-NHS and MBAA-NHS labeling for metabolite analysis. Fig. 3(A) shows the base-peak ion chromatogram of a 1:1 mixture of amino acid standards differentially labeled by $^{13}C_2$- and $^{12}C_2$-DBAA. Most of the amino acids are well separated. Some background peaks from the labeled sample solution (unlabeled peaks in Fig. 3(A)) are detected and likely from impurities in the sample. Fig. 3(B) shows the expanded mass spectrum of $^{13}C_2$-/$^{12}C_2$-DBAA labeled Val, Met and Tyr. Besides the protonated peak pair found for each labeled amino acid, some low intensity peaks from sodium adduct ions are also observed. These adduct peaks are filtered out during the data processing using IsoMS to produce the final peak pair list.

Fig. 3(C)shows the base-peak ion chromatogram of amino acid standards labeled by $^{13}C_2$-/$^{12}C_2$-MBAA. In this case, the amount of sample injected was 4 times more than that used for analyzing the DBAA labeled sample. The intensities of chromatographic peaks obtained in Fig. 3(A) and (C) are by and large very similar. Thus, the detection sensitivity of MBAA labeled amino acids is about 4 times lower than that of DBAA labeled amino acids except histidine (H) and arginine (R). These two amino acids themselves are more readily charged and thus have higher ESI ionization efficiency. The retention times of the labeled amino acids by the two reagents are different, which is not surprising considering that the two tags have different chargeability in the separation solutions used.

Fig. 4(A) shows the base-peak ion chromatogram of a $^{13}C_2$-/$^{12}C_2$-DBAA labeled human urine sample. Two microliter of the labeled sample, equivalent to 0.5 μL of the original urine sample, was injected for generating this chromatogram. There are many peaks detected from this labeled sample, compared to a very few peaks detectable using 0.5 μL of unlabeled urine (see Fig. 4(B)). This indicates that DBAA labeling can improve the detectability of urine metabolites significantly. Fig. 4(C) shows the chromatogram obtained from the $^{13}C_2$-/$^{12}C_2$-MBAA labeled human urine sample. The amount of the labeled urine sample injected was 4 times of that used for the DBAA labeled sample. The two chromatograms look different, which is not surprising considering the differences in retention time and detection sensitivity of the metabolites labeled by the two different tags.

To compare the metabolomic results obtained by the two labeling methods, we used IsoMS to process the LC–MS data to determine the number of peak pairs detected from each method. Triplicate experiments were done. In the MBAA labeled samples, an average of 1100 ± 55 ($n = 3$) peak pairs could be detected per sample for a combined total of 1217 peak pairs, compared to a average of 1027 ± 15 and a total of 1104 peak pairs found from the DBAA labeled sample. Based on the accurate mass information (<10 ppm) of each peak pair, we searched the Human Metabolome Database (HMDB) [33] and the Evidence-based Metabolome Library (EML) [34] using MyCompoundID [34] to generate a list of putative metabolites (see Supplemental Tables T1–T4 for HMDB and EML matches). Among the 1217 peak pairs detected from the MBAA labeled samples, 242 matched the metabolites in HMDB and 715 matched the predicted metabolites in EML with one metabolic reaction. Thus, 78.6% of the peak pairs could be matched. For the DBAA labeled samples, 817 out of the 1104 peak pairs (74.0%) could be matched (376 matched to HMDB and 441 matched to EML with one reaction). There were 663 putative metabolites commonly detected from the MBAA and DBAA labeled samples. 294 peak pairs were uniquely found in MBAA samples, while 151 pairs were uniquely found in DBAA samples. Thus, many common metabolites were found in the two labeled methods. The unique peak pairs found in each method may be attributed to the differences in ionization efficiency and ion suppression effect from co-eluting analytes. Out of the 663 commonly detected metabolites, we randomly checked 50 of them and found that there were 43 amine-containing compounds (86%), 7 phenol-containing compounds (14%), 5 hydroxyl-containing compounds (10%). Only one

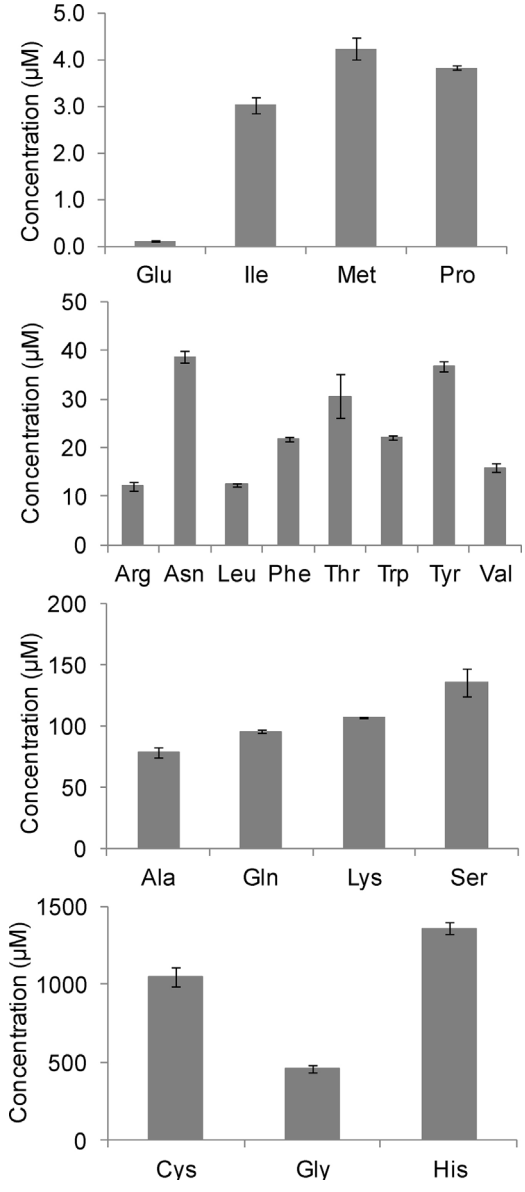

Fig. 5. Absolute concentration measurement of amino acids in a human urine sample.

metabolite did not contain any possible functional groups for labeling (2%), which might be a false-match.

The above results also indicate that although MBAA labeling may be about 4 times less sensitive than DBAA labeling, by increasing the injection amount by 4-fold, the metabolome coverage generated by MBAA labeling was similar to that obtained by DBAA labeling (1100 ± 55 vs. 1027 ± 15 per run). However, as it will be shown in Section 3.5, MBAA labeling offers the opportunity of generating useful fragment ion spectra for structure analysis or metabolite identification using MS/MS spectral search.

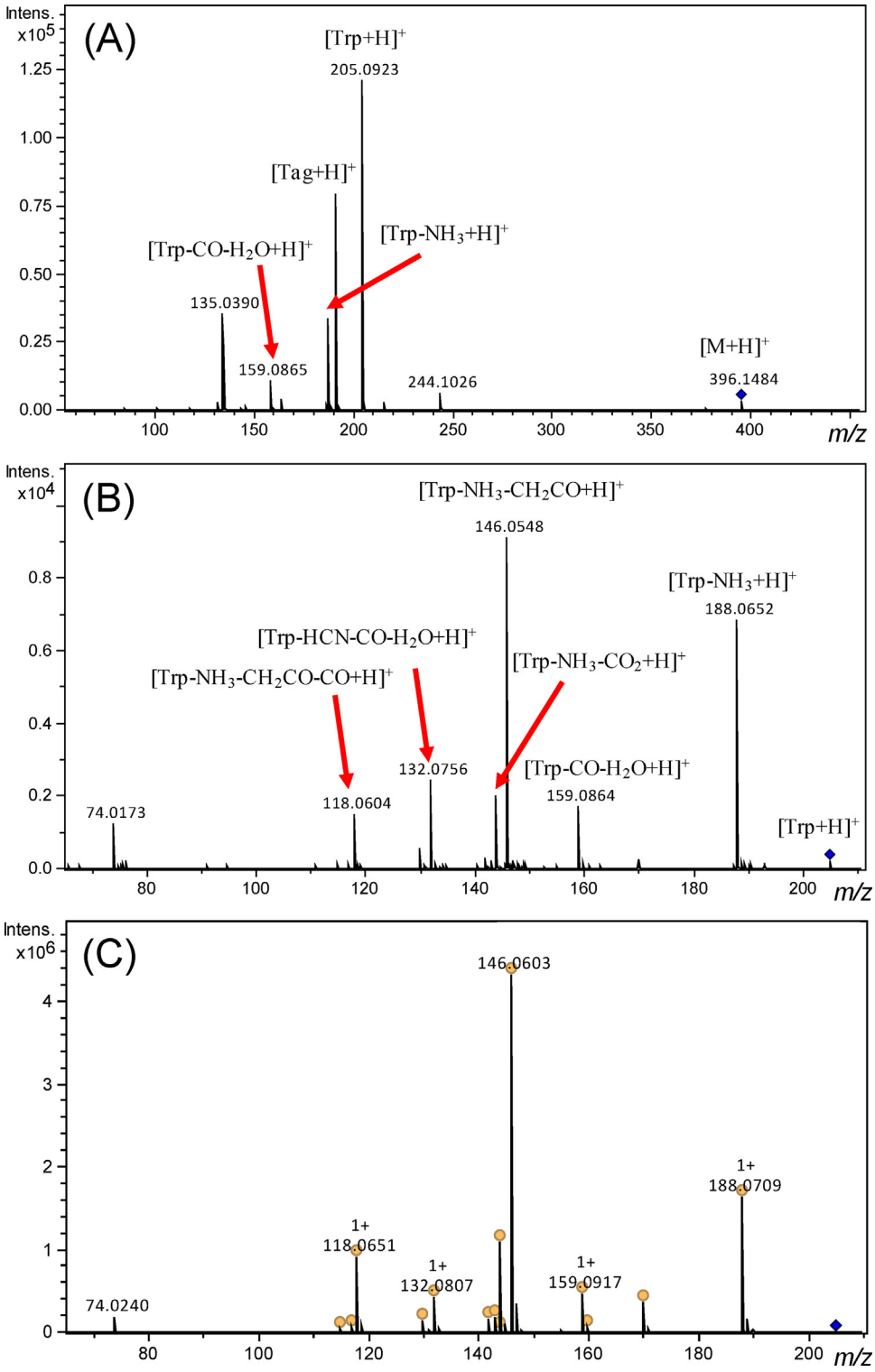

Fig. 6. (A) MS/MS spectrum of MBAA labeled tryptophan. (B) Psedo MS^3 spectrum of the protonated tryptophan generated from the skimmer region fragmentation of protonated MBAA labeled tryptophan. (C) MS/MS spectrum of tryptophan standard obtained using 10 μM tryptophan in 1:1 H_2O:acetonitrile 0.1% formic acid infused by syringe pump at 3 μL min^{-1}. The collision energy for MS/MS was set to 20 eV.

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3.4. Absolute quantification of amino acids in human urine

Guo and Li have developed a strategy to quantify the absolute concentration of metabolites in a biological sample by using a pooled sample as an internal standard [1]. The absolute concentration of a metabolite in the pooled sample can be determined by spiking the $^{13}C_2$-labeled metabolite standard into the $^{12}C_2$-labeled pooled sample. The absolute concentration of this metabolite in individual samples is determined by comparison of the $^{12}C_2$-labeled individual sample with the $^{13}C_2$-labeled pooled sample. In this work, the absolute concentrations of 20 amino acids in a pooled urine sample were determined by MBAA labeling in the same manner as reported using dansylation labeling. Based on our previous results [6], a pooled amino acid solution was prepared by mixing appropriate volumes of 20 amino acid standards. The mixture was labeled by $^{12}C_2$-MBAA and then spiked into the $^{13}C_2$-labeled pooled urine prior to LC–MS analysis. Fig. 5 shows the results of absolute quantification of amino acids in the pooled urine sample. Aspartic acid could not be quantified, likely due to very low concentration of this amino acid in the sample. The standard deviation value shown in Fig. 5 for each amino acid ($n = 3$) also indicates that good precision can be obtained by the method. These results indicate that MBAA labeling can be used for absolute quantification of metabolites should there be a need to do so in a metabolomics application.

It should be noted that because of only 2 Da mass difference between the $^{13}C_2$-dansyl labeled peak and the $^{12}C_2$-dansyl labeled peak within a peak pair, there is a small overlap of the $^{13}C_2$ natural isotope peak of the $^{12}C_2$-dansyl labeled peak with the $^{13}C_2$-dansyl labeled peak. However, for most metabolites with $m/z < 800$, the peak intensity contribution of the natural abundance $^{13}C_2$ peak of the $^{12}C_2$-labeled metabolite to that of the $^{13}C_2$-labeled metabolite peak is usually less than 10%. If accurate measurement of the absolute concentration of a metabolite is needed, we can calculate this intensity contribution based on the intensity of the $^{12}C_2$-labeled peak and subtract this contribution from the intensity of the $^{13}C_2$-labeled peak for the peak ratio calculation. In this way, the natural abundance peak contribution can be corrected. In this work, the accuracy of absolute quantification of amino acids using MBAA labeling was not investigated, as this was not the major focus of this work; for metabolomics applications, relative quantification is more important.

3.5. Fragmentation analysis of amino acids labeled by MBAA-NHS

One major objective of developing the MBAA-NHS reagent was to perform MS/MS analysis of labeled metabolites while still improving separation and detection. We have examined the fragmentation behaviors of labeled amino acids and found that only a few neutral loss ions (i.e., $-H_2O$, $-NH_3$ or $-CO_2$) were observed for some amino acids, such as labeled tryptophan (see Fig. 6(A)). These ions are not very informative for structure analysis. In order to provide adequate information for structural determination of an unknown or for metabolite identification based on the use of a fragment ion spectral library, it is important to generate as many fragment ions as possible from the metabolite moiety itself. Fortunately, fragmentation of a protonated MBAA labeled amino acid can produce a fragment ion with its structure corresponding to the protonated unlabeled amino acid (e.g., see the peak at m/z 205 in Fig. 6(A) from the protonated tryptophan). This fragment ion can be further dissociated to produce a MS^3 spectrum. Although Q-TOF-MS does not have the capability of performing a conventional MS^3 experiment, it offers a function known as in-source CID for generating the first generation fragments in the skimmer region [35]. Thus, the protonated unlabeled amino acid generated in the skimmer region can be isolated in the first quadrupole, then subjected to the second CID in the second quadrupole to produce the second generation fragment ions that are subsequently detected in TOF-MS.

An example of the pseudo MS^3 spectrum is shown in Fig. 6(B), acquired from the precursor ion with m/z 205 that was generated in the skimmer region of Q-TOF-MS. Compared to Fig. 6(A), more fragment ions are produced by pseudo MS^3 from the backbone structure fragmentation. Fig. 6(B) is then compared to the MS/MS spectrum of unlabeled tryptophan (see Fig. 6(C)). The two spectra display the same types of fragment ions. Thus, the pseudo MS^3 spectrum could be used for structure analysis or metabolite identification based on spectral match. Among the 20 amino acid standards, except glycine, all could produce similar fragmentation patterns in pseudo MS^3 to those of the MS/MS spectra from the unlabeled standards.

The sensitivity of the pseudo MS^3 strategy was investigated. In the direct infusion experiment, library-searchable MS/MS spectra could be readily generated from a labeled amino acid at a concentration of 5 μM. The applications of MBAA labeling for metabolome profiling and metabolite identification in real world metabolomics will be reported in the future.

4. Conclusions

We have developed a strategy to prepare differential isotopic labeling reagents for profiling amine-containing metabolites by which a CIL reagent is divided into three components: isotope group, coupling reaction group and physiochemical property tuning group. We used isotopologues of glycine to serve as an isotope core as well as a coupling group in CIL reagents so that we can easily modify the properties of CIL reagents by simply changing the tuning group. In addition, the cost of the reagents is low due to the use of inexpensive $^{13}C_2$-glycine. We have prepared two CIL reagents using the strategy, $^{12}C_2$-/$^{13}C_2$-MBAA-NHS and $^{12}C_2$-/$^{13}C_2$-DBAA-NHS. MBAA-NHS is particularly useful for metabolic identification. We have investigated the fragmentation behaviors of 20 MBAA labeled amino acids. Their pseudo MS^3 spectra were then compared to the MS/MS library spectra of unlabeled standards. The results indicate that the pseudo MS^3 spectra of all labeled amino acids except glycine can provide almost identical fragmentation patterns as their corresponding unlabeled amino acids, illustrating the possibility of using the MBAA NHS method for metabolic identification based on MS/MS spectral search. We have shown that an average of 1100 ± 55 ($n = 3$) putative metabolites per run could be detected from human urine using MBAA labeling LC–MS. The application of this approach for metabolomics will be reported in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.04.021.

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