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Comparative Metabolite Profiling of Carboxylic Acids in Rat Urine by CE-ESI MS/MS through Positively Pre-charged and ^{2}H-coded Derivatization

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Abstract

A new approach for the selective comparative metabolite profiling of carboxylic acids in rat urine was established using capillary electrophoresis-mass spectrometry (CE-MS) and a method for positively pre-charged and ^{2}H-coded derivatization. Novel derivatizing reagents, *N*-alkyl-4-aminomethylpyridinum iodide (alkyl=butyl, butyl-d9 or hexyl), containing quaternary amine and stable isotope atoms (deuterium), were introduced for the derivatization of carboxylic acids. CE separation in positive polarity showed high reproducibility (0.99 –1.32% RSD of migration time) and eliminated problems with capillary coating known in CE-MS anion analyses. Essentially complete ionization and increased hydrophobicity after the derivatization also enhanced MS detection sensitivity (e.g. formic acid was detected at 0.5 pg). Simultaneous derivatization of one sample using two structurally similar reagents, *N*-butyl-4-aminomethylpyridinum iodide (BAMP) and *N*-hexyl-4-aminomethylpyridinum iodide (HAMP), provided additional information for recognizing a carboxylic acid in an unknown sample. Moreover, characteristic fragmentation acquired by online CE-MS/MS allowed for identification and categorization of carboxylic acids. Applying this method on rat urine, we found 59 ions matching the characteristic patterns of carboxylic acids. From these 59, 32 ions were positively identified and confirmed with standards. For comparative analysis, 24 standard carboxylic acids were derivatized by chemically identical but isotopically distinct BAMP and BAMP-d9, and their derivatization limits and linearity ranges were determined. Comparative analysis was also performed on two individual urine samples derivatized with BAMP and BAMP-d9. The metabolite profiling variation between these two samples was clearly visualized.

Keywords

Metabolomics; Carboxylic acid derivatives; CE; Group Specific Internal Standard Technology (GSIST)

1 Introduction

Metabolomics is the comprehensive study of metabolites under a given set of conditions [1,2]. The most popular techniques employed for metabolomic studies include NMR spectroscopy and mass spectrometry [3-7]. Although considerable advances have been made in the development of metabolomic tools, it is commonly believed that a fully

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comprehensive analysis of metabolite's is unfeasible [8-10]. This pessimism arises from the complexity (as many as 200,000 metabolites arise in plants) [11], chemical diversity (molecular weight, polarity, solubility) and physical properties of the average metabolome. A further complication is the broad concentration range of metabolites [12].

Metabolite profiling is an alternative to NMR and MS for metabolomics focusing on sub-metabolome studies that involve identification and quantification of a pre-defined set of metabolites, generally related to a specific metabolic pathway(s) [12]. Profiling a selected group of metabolites is an important indicator of normal and pathological phenotypes [13,14], and may lead to biomarker discovery. Urinary carboxylic acids represent such a selected group of metabolites. Profiling urinary carboxylic acids provides information on the pathophysiological status of various diseases, such as organic acidurias, diabetes mellitus, or kidney and liver disorders. Carboxylic acids are usually analyzed using GC [15]. Most HPLC-related reports on this group of compounds target specific carboxylic acids [16,17]. Comprehensive carboxylic acid analysis in bio-fluids is rarely seen. Recently, Lafaye reported metabolite profiling in rat urine using LC-MS that included characterization of some carboxylic acids, such as hippuric acid, ferulic acid and sebacic acid [17]. One limitation of LC-MS methods based on reversed phase chromatography is poor resolution, probably due to the strong polarity of carboxylic acids. The second limitation is lack of sensitive quantification due to iosnization suppression. It has been demonstrated that common mobile phase modifier, such as formic acid, in reverse phase HPLC suppresses the ionization efficiency of carboxylic acids in ion source when MS is operated in negative mode, hence, reduces the detection sensitivity [18]. Similarly, variable ionization efficiencies of analytes also means that MS is unable to provide absolute quantification. Although it is routine to quantify targeted molecules using stable-isotope internal standards or their structural analogs, this approach is neither routine nor simple in metabolite profiling. A widely use strategy to circumvent this problem is comparative quantification using *in vivo* isotope labeling with ^{2}H, ^{13}C, or ^{15}N labeled substrates. Although this strategy is well recognized in model systems such as *E. coli*, yeast, algae, fungi, and plants [19-21], it is difficult to apply in animal and human studies.

To address the quantification issue, we developed a new MS-based strategy for comparative metabolomics called Group Specific Internal Standard Technology (GSIST) [18, 22, 23]. Quantification in GSIST is accomplished by derivatization of metabolites of interest with isotopically coded reagents. These reagents usually have permanent positive charges and length-adjustable alkyl chains that enhance their ionization efficiency and increase their hydrophobicity [23-26]. This method also allows for comparative quantification which is accomplished by comparing individually derivatized samples with chemically identical, but isotopically distinct, labeling reagents. Absolute quantification of target metabolites can also be achieved by mixing an isotope-coded experimental sample with a known amount of standard derivatives [22]. In this report, we expand this methodology to carboxylic acid profiling using CE-MS and novel derivatizing reagents, *N*-alkyl-4-aminomethylpyridinum iodide.

CE-MS is an analytical tool characterized by high separation efficiency, increased detection sensitivity, low sample and solvent consumption, and short analysis time [27-29]. Interestingly, only a few studies evaluating carboxylic acids with CE-MS have been reported [29-33]. This is most likely because small carboxylic acids have a strong negative electrophoretic mobility that makes CE-MS analysis difficult. In CE, ionic species are separated based on their charge and size (mobility in the electric field). Carboxylic acids migrate from cathode to anode, opposite to the direction of electroosmotic flow (EOF), and at a higher migration rate than the EOF rate under ordinary conditions. Therefore, they are usually separated in a co-EOF mode using a cation-coated capillary (dynamic or covalent

coating) and reversed polarity. However due to problems with dynamic coatings, this strategy is a challenge in CE-MS. Coatings formed by dynamical adsorption of a cationic surfactant, such as by using cetyltrimethylammonium bromide (CTAB) in the background electrolyte (BGE), are unstable in the CE-MS system. This is because the CE-MS system does not possess an outlet vial with BGE that can provide a continuous source of CTAB. As a consequence, CTAB desorbs from the wall and migrates to the inlet (cathode). This leaves unsequestered silanol groups (SiO^-) in the column that generate normal EOF toward the cathode (away from the MS). Beyond changing analyte migration rates this can create a liquid void at the capillary exit that interrupt the separation process[34]. To overcome this issue, a permanently coated capillary is required. Disadvantages are that this type of capillary is more expensive and the stability of most coatings strongly depends on the running pH, even though some coatings are indeed pH indsependent [29].

An alternative solution described in this paper that changes the chemical and physical properties of carboxylic acids using a positively charged derivatizing agent. After derivatization, these positively charged derivatives always migrate to the cathode, which allows CE-MS to operate in the normal CE mode with the cathode at the column outlet. Capillary modification is unnecessary, which makes the analytical process simpler and more robust. The permanent positive charge also eliminates the effects of pH and BGE on the charge status of analytes. Operating pH of the BGE can then be optimized over a wide range. Particularly important is that formic acid, a commonly used BGE, can be used without any limitations. For anion analysis by CE-MS, ammonium acetate at pH >8.5 has frequently been used as a BGE [29,35]. However, the detection sensitivity for anions in CE-MS using ammonium acetate is several-fold lower than that of cations [29]. We think this is a result of ion suppression by acetate. A second advantage to our approach is that positively charged carboxylic acid derivatives will migrate faster than the underivatized neutral or negative species (in front of the sample zone) and suppress ionization efficiency of positive ions, resulting in a highly selective analysis in a shorter time. In addition, enhanced MS detection by extending alkyl chain length and comparative quantification using deuterium coding are included in this approach.

2 Materials and methods

2.1 Chemicals

All standard carboxylic acids, methanol, 4-aminomethylpyridine, alkyl iodide, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), HBr (68%, v/v) and phthalic anhydride were purchased from Sigma-Aldrich (St. Louis, MO). 1-iodobutane-*d*9 was purchased from Cambridge Isotope Laboratories (Andover, MA).

The procedure for synthesis of *N*-butyl-4-aminomethylpyridinium iodide (BAMP) and *N*-hexyl-4-aminomethylpyridinium iodide (HAMP) was adapted from Bardsley [36]. Briefly, 15 g of phthalic anhydride (100 mmol) was mixed with 10 ml of 4-aminomethylpyridine (100 mmol) and incubated for 30 min at 170°C. The product, crude phthalimido compound, was recrystallized from methanol and 5 g (about 20 mmol) dissolved in 20 ml methanol with either iodobutane (88 mmol, BAMP) or iodohexane (67 mmol, HAMP) in a 1:1 ratio. To complete the synthesis, the reaction was refluxed for 48 (BAMP) or 72 hr (HAMP). The solution was cooled to room temperature, the solvent removed by evaporation under vacuum, and the solid phase recrystallized from acetone. The purified crystals were refluxed in 20 ml HBr (68%, v/v) for 6 hours followed by addition of 10 ml water. The solution was filtered to remove phthalic acid, the filtrate extracted with ether (2 × 10 ml) and the extract evaporated to dryness under vacuum. The products (BAMP or HAMP) were recrystallized from acetone. *N*-butyl-d9-4-aminomethylpyridinium iodide (BAMP-d9) was synthesized using the same procedure, with the substitution of iodobutane-d9 for iodobutane.

2.2 Rat urine sample collection

Sprague Dawley female rats, 7-8 weeks old (weight ~220 g), purchasesd from Harlan (Harlan,Indianapolis, IN) were fed ad libitum with a standard rodent diet (Harlan, Indianapolis, IN). Collected urine was stored at -80°C until analysis.

2.3 Derivatization procedure

Derivatizing reagents and EDC were prepared in water at a concentration of 0.1 M (derivatization solutions were stored up to one month at 4°C; EDC was prepared fresh daily). A pooled standard solution of 24 carboxylic acids (0.45 mM each) was prepared in water. Individual standards (Table 1) contained 10 mM of each carboxylic acid. To establish the derivatization method, 50 μl of the standard solution was added to 100 μl of a derivatizing reagent and 100 μl of EDC solution (~20-fold excess of each). Derivatization was performed by incubating the mixture for 2 hours at 50°C. For analysis of rat urine samples, urine was centrifuged for 10 min at 3000 × *g*, 500 μl of the supernatant was added to 200 μl of a derivatizing reagent and 15 mg solid EDC. After pH adjustment to a range of 4-6 by titration with either 2 M HCl or 2M NaOH, the samples were incubated for 2 hours at 50°C. For comparative analysis, the samples were derivatized under identical conditions with BAMP and BAMP-d9 (reaction shown in Figure 1), at various ratios as described below.

2.4 Solid phase extraction (SPE)

The derivatized compounds were purified using Oasis® WCX cartridges (Waters, Milford, MA). A cartridge (1 ml) was pre-conditioned by washing with 1 ml of methanol followed by 1 ml water. The samples were diluted with 300 μl of 5% ammonium hydroxide, loaded onto the cartridge, and washed with an additional 1 ml of 5% ammonium hydroxide and 1 ml of methanol. The derivatives were eluted with 2% formic acid in methanol (1 ml), dried under vacuum, and reconstituted to the original volume using CE running buffer.

2.5 CE-MS instrumentation and conditions

A Beckman-Coulter PA800 CE System (Beckman-Coulter, Fullerton, CA) was coupled to a QStar mass spectrometer (Applied Biosystems; Foster City, CA) through the CE-MS Upgrade Kit interface (Applied Biosystems/MDS SCIEX; Concord, ON, Canada). Bare fused silica capillaries (100 cm length, 50 μm ID × 365 μm OD) were used for CE separations (Polymicro Technologies, Phoenix, AZ). The separation background electrolyte (BGE) was formic acid (1 M). To gain high electroosmotic flow (EOF) for rapid separation, each capillary (before connecting CE and MS) was flushed overnight with 0.1 M NaOH, followed by water and BGE for 10 min. A potential of 30 V/cm was applied off-line for 30 min to precondition columns. Sampling was performed by pressure injection for 5 s at 2 psi. The separation was carried out at a potential of 30 V/cm, unless otherwise stated. Between each run, the capillary was flushed for 2 min with 0.1 M NaOH and BGE at 50 psi. Sheath flow liquid [50% (v/v) methanol with 0.1% (v/v) formic acid in water] was delivered to the CE-MS sheath flow interface at 1 μl/min using the MS syringe pump.

The MS was operated in the positive mode with scan range of 190 - 500 *m/z* for BAMP derivatives or 200 - 500 *m/z* for HAMP derivatives at 1 scan/s. Optimization of ion source parameters, sheath flow rate, maximum ESI sensitivity, and stability were achieved using electrokinetic pumping at a potential of 300 V/cm and 0.2 psi assisting pressure to the sample vial containing standards in BGE buffer. Typical settings were as follows: ion spray voltage (IS), 4800 V; curtain gas (CUR), 30; ion source gas 1 (GS 1), 40; ion source gas 2 (GS2), 0; declustering potential (DP), 50 V; focusing potential (FP), 220 V; declustering potential (DP2), 10 V. Data acquisition was manually started immediately after the

separation voltage reached the set point. For CE-ESI MS/MS experiments, Information Dependent Acquisition (IDA) was performed with collision-induced dissociation (CID) The collision energy and collision gas set at 40 and 5, respectively. Data were acquired and processed using Analyst software (Applied Biosystems/MDS SCIEX; Concord, ON, Canada).

3 Results and discussion

3.1 Optimization of derivatization reaction and SPE

For derivatization of carboxyl groups, a variety of reagents have been investigated and found to be valuable under certain conditions [37]. Besides general considerations for derivatization reactions such as quantitative yield, mild conditions, stable and identifiable product, additional requirements may be necessary for our specific situations. Reagents should have permanent positive charges, and should be easily coded with stable isotopes. Because both the carboxylic acids and derivatizing reagents are small water-soluble molecules, performing the reaction in an aqueous solution is ideal. To meet such requirements, the HAMP, BAMP and BAMP-d9 derivatizing reagents were developed. The labeling procedure involves initial activation of the carboxyl group by a water-soluble EDC, and subsequent reaction in aqueous solutions [38].

EDC is a well-known condensing reagent for carboxyl groups. When used in organic solvents, a catalyst 4-(dimethylamino) pyridine (DMAP) is often used [39,40]. Our experimental results showed that in aqueous solutions alkaline conditions have little or no effect on the derivatization reaction, and the optimum pH for derivatization is between 3 and 6, with no need for DMAP. Since the pH of EDC-HCl solution is approximately 4.6 and the solution has inherent buffering capability, it was not necessary to adjust the reaction pH for carboxylic acid standards. Derivatization of urine samples, on the other hand, required pH adjustment to the above-mentioned range. These observations are consistent with previous reports [38,41]. The optimum yield of the derivatization reaction was reached with approximately a 20-fold molar excess of both derivatizing reagents and EDC.

Our preliminary CE analyses of these derivatives showed a large broad peak ahead of the analyte zone in the electrophogram. This front peak diminished separation in that all dicarboxylic acid derivatives migrated in one peak. The interfering peak was effectively removed by adding an SPE cleanup step (Figure 2). SPE was carried out with an Oasis® WCX cartridge packed with bifunctional sorbent (ion exchange and reversed-phase) that provides superior sample enrichment of strong bases and quaternary amines [42].

3.2 Optimization of CE-MS conditions

The choice of the separation BGE is crucial for CE separation as this has great impact on the mobility of analytes, EOF, peak shape, and therefore on the overall separation efficiency. In the case of CE-MS, the composition of BGE also affects MS detection. Since the derivatives studied here are permanently charged, BGE pH is not an issue in separations. The major concern is for compatibility with MS analysis. For this reason, formic acid (a common volatile acid additive for ESI-MS) was used. The impact of formic acid concentration on separation and MS sensitivity was evaluated in the range of 0.1 M to 2.0 M. With increasing concentrations of formic acid, up to 1 M, significant improvement in peak shape and MS sensitivity was observed. Considerable peak broadening was found at formic acid concentrations less than 0.5 M and was likely due to the ionic interaction between positively charged analytes and SiO^- of the inner capillary wall. Even with a pH as low as 2, silanol groups on the fused-silica capillary may still exist in anionic form [43]. On the other hand, concentrations of formic acid greater than 1 M increased the separation current and Joule

heating, leading to deterioration of the separation. The optimum concentration of formic acid in BGE was determined to be 1M.

Optimization of the composition and the flow rate of the sheath liquid are also important for separation and detection. The matrix of sheath liquid must be volatile, and the mixture of methanol and water is commonly used. On the hand, the choice of electrolytes in sheath liquid, which provides sheath liquid conductivity, should be carefully made and its concentration should optimized. Differences in the composition of sheath liquid and BGE of actual CE separation may create a moving ionic boundary effect, and lead to discontinuous separation and irreproducible results [44]. This is especially likely under zero-EOF conditions that would be created with a neutral polymer coated capillary or at a high concentration of formic acid. To minimize this effect, 50% (v/v) methanol in water containing 0.1% (v/v) formic acid was used as the sheath liquid.

The effect of sheath flow rate was investigated over the range of 0.5-10 μl/min. As expected, higher flow rates decreased sensitivity due to dilution in the sample zone. At a flow rate of 0.5 μl/min, on the other hand, separation current was unstable and occasionally interrupted. The optimal combination of stable separation current and sensitivity was achieved at a sheath flow rate of 1.0 μl/min.

3.3 Evaluation of CE-MS analysis with HAMP and BAMP carboxylic acid standard derivatives

Figure 2 shows the base peak chromatograms of 24 BAMP and HAMP derivatized carboxylic acid standards including short-chain fatty acids, di- and tri-carboxylic acids, hydroxyl carboxylic acids, α-keto carboxylic acids, cyclic carboxylic acids and aromatic carboxylic acids. Di-carboxylic acids were exclusively doubly derivatized. Two types of derivatives, both double derivatized and dehydrated double derivatized, were found for citric and isocitric acids. The mechanism for dehydration is not clear. Perhaps lactone is formed that then dehydrates. Doubly derivatized acids possess two positive charges. This causes them to migrate faster and appeared at the front of the electropherogram, followed by mono-derivatized carboxylic acids. Since the HAMP derivatives are larger than BAMP derivatives, all HAMP derivatives exhibited longer migration times than the corresponding BAMP derivatives. Although CE was not able to resolve all the carboxylic acids, differentiation between acids in unresolved peaks was easily achieved in the MS. In addition, CE resolution of some stereo isomers (fumaric and maleic acids) as well as positional isomers (citric and isocitric acids) was achieved. Presumably, it could be attributed to the fact that drivatization make the difference in structure more significant, resulting in better separation. Unfortunately, the method failed to distinguish butyric and isobutyric acids. These isomers have exactly the same mass and co-eluted.

The sensitivity and reproducibility of this method were examined by analyzing 24 carboxylic acid standards (Table 1). Based on a signal-to-noise ratio of 3, HAMP derivatives showed approximately 4- to 5-fold higher sensitivity than BAMP derivatives. This is consistent with previous observations for amino acid derivatives [23]. I Ionization efficiency increases with analyte hydrophobicity or with the length of the alkyl chain in the derivative. Even though the concentration detection limits of CE-MS are mediocre, it was found that the mass detection limits for HAMP derivatives are superior to HPLC fluorescence based detection [16]. It was initially observed that migration time gradually decreased during a series of runs, but satisfactory reproducibility was obtained by replacing the BGE after each run. Since the BGE is not a buffered solution, a significant pH changes of the BGE due to electrolysis are expected over time [45]. This will cause a gradual decrease in EOF.

3.4 Determination of carboxylic acids in rat urine

Pooled rat urine was used to examine the capability of the developed CE-MS method in a real biological system (base peak chromatograms are shown in Figure 3). Aliquots of sample were derivatized with BAMP and HAMP, derivatives enriched with an SPE cartridge, and analyzed as described in the experimental section. After visual examination of individual extracted ion chromatograms, we found that most of the high intensity peaks were potentially derivatized carboxylic acids. Both CE-MS and CE-MS/MS methods were used to verify these results. In the CE-MS mode, we knew that two ions with a mass difference of **n** × 28 amu must be found in two CE-MS electropherograms corresponding to BAMP and HAMP derivatives (**n** represents the number of the derivatizing tags per labeled molecule and 28 amu is molecular weight difference between BAMP and HAMP - two CH_2 units). In the CE-MS/MS mode, verification was carried out by analyzing MS/MS spectra for characteristic fragments found in the standards. MS/MS data for all carboxylic acid standards were acquired in information dependent acquisition (IDA) mode and the fragmentation pattern examined. Although MS/MS spectra of these standards did not provide enough information for precise structural analysis, characteristic fragments were used to indicate the presence of carboxylic acids and suggest a structural class of carboxylic acid. For example, all small fatty acids were represented by 107.0, 109.0, 135.0, 177.1, and 219.1 m/z fragments. Mono-carboxylic acids showed fragments from the loss of a C_6H_9 group (M-84.0) in the derivatizing reagent tag. These fragments were not seen with di- or tri-carboxylic acids. Phenyl or hetero ring-substituted formic acids showed a fragment at m/z = 105.0 and loss of the C_6H_9 group (M-84.0). Figure 4 illustrates the MS/MS fragmentation pattern of HAMP-derivatized citric acid. Using this approach 59 potential carboxylic acids were found (Table 2) distributed in 32 peaks (Figure 3).

Carboxylic acids were identified in the following way, it was assumed that ions with a charge of +2 or +3 correspond to double and triple derivatized molecules, respectively. Molecular mass of the original (underivatized) compound was calculated from the molecular mass of the ion found in the MS scan by subtracting the molecular mass of the derivatizing tag. These calculated molecular masses were then used to search the LIGAND database (http://www.genome.ad.jp/kegg/ligand.html; released on June 12, 2006 by Kyoto University, Japan) [46]. Non-carboxyl group-containing compounds were excluded from the list obtained. The number of potential candidates was further reduced by stipulating that the number of carboxyl groups should match the charge status of the ion. .

Of 59 candidate ions, 32 were positively identified using standards (labeled **S** in Table 2). Seven more were not confirmed with standards but had been previously reported in human urine (labeled **R** in Table 2) [15]. Another 7 candidate ions were tentatively identified as carboxylic acids (labeled **L** in Table 2) and 13 more ions were not identified (labeled **U** in Table 2).

The above results illustrate the strengths of this new method for carboxylic acid analysis. The permanent positive charge of quaternary amines in the derivatives simplifies the SPE enrichment step, increases CE selectivity, and enhances the MS signal due to their high ionization efficiency, presumably by suppression of other non-derivatived molecules in the sample [23]. The high selectivity and sensitivity obtained through derivatization greatly facilitate the profiling of carboxylic acids in real biological samples.

3.5 Comparative quantification using isotopically encoded BAMP and BAMP-d9 labeling reagents

To evaluate the quantification ability of the newly developed method, 24 standard carboxylic acids were derivatized by chemically identical, but isotopically distinct, reagents.

Two aliquots of individual carboxylic acid standards were derivatized with BAMP and BAMP-d9, and mixed at specific ratios for CE-MS analysis. Comparative quantification was performed by analyzing the extracted ion chromatograms corresponding to the light (BAMP) and heavy (BAMP-d9) forms of the standard derivatives and their corresponding MS spectra (Figure 5). Interestingly, no isotope effects were seen in the CE separation as opposed to HPLC [23]. Both the light and heavy derivatives co-elute and can be differentiated by MS only (Figure 5). This suggests the superiority of CE over HPLC for deuterium-coded GSIST derivatives. The range of linearity and derivatization limits of the standards are summarized in Table 3. Derivatization limit is the lowest concentration of an analyte that can be derivatized and detected at a signal-to-noise ratio of 3. Unlike determination of detection limit by diluting an analyte prepared at much higher concentration, derivatization limits are determined by derivatization of substances at the concentration they will be analyzed. Thus, the derivatization limit reflects the actual analytical capability of a method.

The linear ranges varied from 1:1 to 1:8 with correlation coefficients of 0.978 to 0.999. Derivatization limits were found at 25 μM or less for 20 of 23 standards. The relatively high derivatization limit (50 μM) of oxaloacetic and oxalic acids may be attributed to their well-known thermal instability. We have no explanation for the high derivatization limit of quinic acids (100 μM).

Efficacy of the GSIST comparative profiling strategy using CE-MS was also evaluated in a biological fluid. Urine samples from two individual rats were derivatized by BAMP and BAMP-d9 under identical conditions, and mixed in a 1:1 ratio. Following CE-MS analysis, quantitative profiles of these two samples were obtained from the peak intensities corresponding to the light and heavy forms (doublet) of individual carboxylic acid derivatives (Figure 6). If there is no significant biological variation in carboxylic acid production, the peak intensities of the light and heavy forms of individual metabolites should be approximately equal between rats. Seventeen of the 31 identified metabolites showed little or no variation. The other 14 carboxylic acids had a much larger variation in concentration between rats (e.g. ~7 fold for suberic acid and ~10 fold for adipic acid). Since the comparison was done under identical conditions and the experimental variation was minimized, these differences likely reflect individual variations in metabolism.

4 Concluding remarks

Based on the results presented above it is concluded that a pre-charged deuterium-labeled coding agent for carboxylic acid metabolites enhances both detection sensitivity and quantification in CE-MS and CE-MS/MS. The described method enables comprehensive, sensitive, and comparative profiling of carboxylic acids in biological fluids such as urine. Fifty-nine potential carboxylic acids were found in pooled urine with 32 positively identified using standards and 14 tentatively assigned. Comparative analysis of two samples from individual rats illustrated the capability of comparative metabolite profiling. Since no sample preparation is required before derivatization, it is concluded the method can be applied to a wide range of biological samples for profiling, biomarker discovery, analysis of drug metabolism, and pharmacokinetics of carboxylic acid-related compounds.

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Abbreviations

EOF	electroosmotic flow
BGE	background electrolyte
HAMP	*N*-hexyl-4-aminomethylpyridinum iodide
BAMP	*N*-butyl-4-aminomethylpyridinum iodide
BAMP-d9	*N*-butyl-d9 -4-aminomethyl-pyridinium iodide

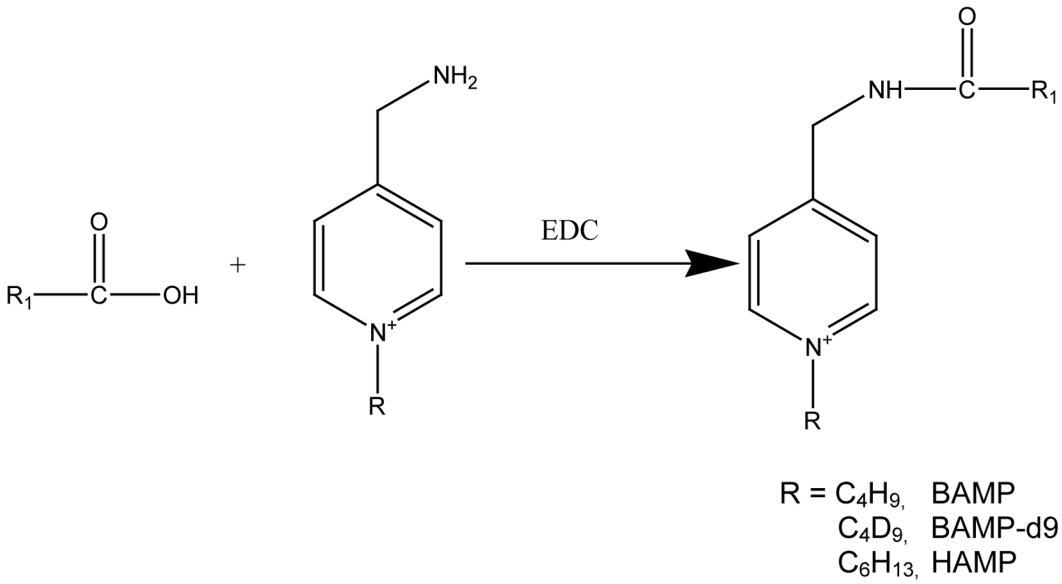

Figure 1.
Derivatization scheme for carboxylic acids.

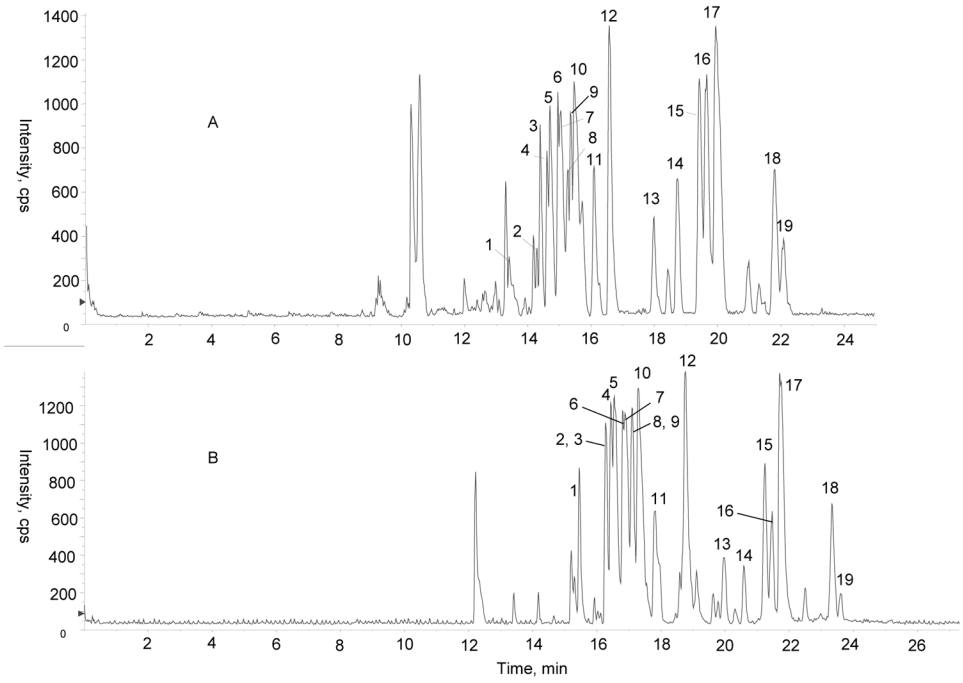

Figure 2.

CE-ESI-MS base peak electropherogram of BAMP and HAMP derivatives of standard carboxylic acids. Experimental conditions: carboxylic acid concentration, 100 M each; capillary, bare fused silica 50 m i.d. × 100 cm; electrolyte, 1M formic acid; separation, 30kV + 0.2psi forward pressure; injection, 2 s at 5 psi; sheath flow, 1 l/min of 50% (v/v) methanol with 0.1% (v/v) formic acid in water. Trace A: BAMP derivatives. Trace B: HAMP derivatives. Peak identifications are provided in Table 1.

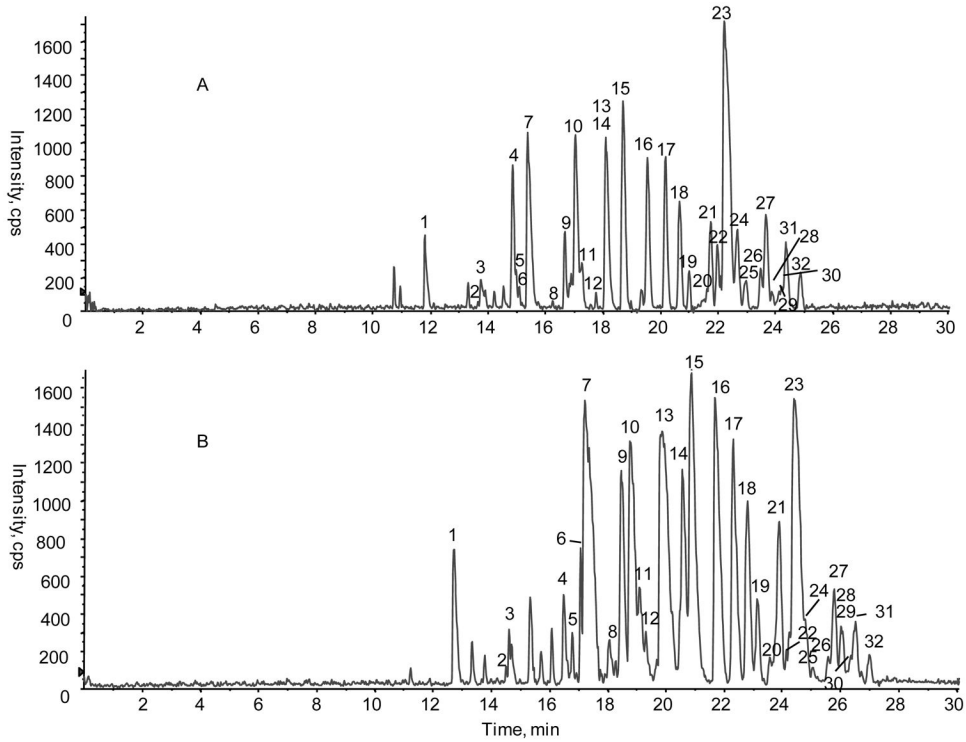

Figure 3.
CE-ESI-MS base peak chromatogram of BAMP and HAMP derivatives of the pooled rat urine. Trace A: BAMP derivatives. Trace B: HAMP derivatives. Peak identifications are provided in Table 2. Experimental conditions are the same as in Figure 2.

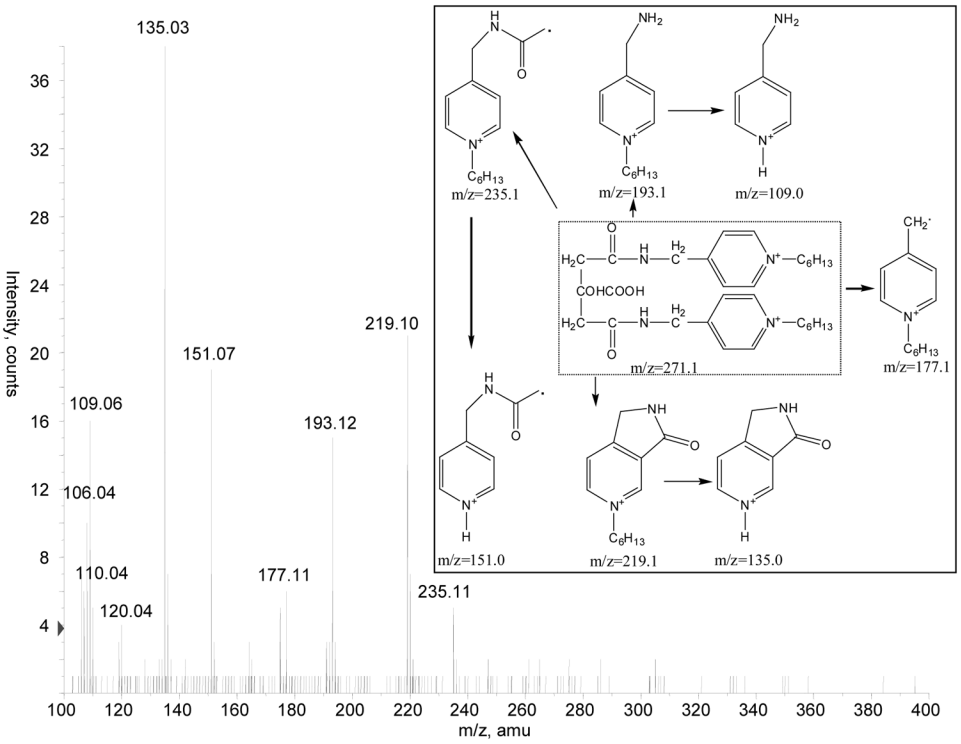

Figure 4.

Tandem mass spectrum and fragmentation of HAMP derivative of citric acid. Experimental conditions are the same as in Figure 2.

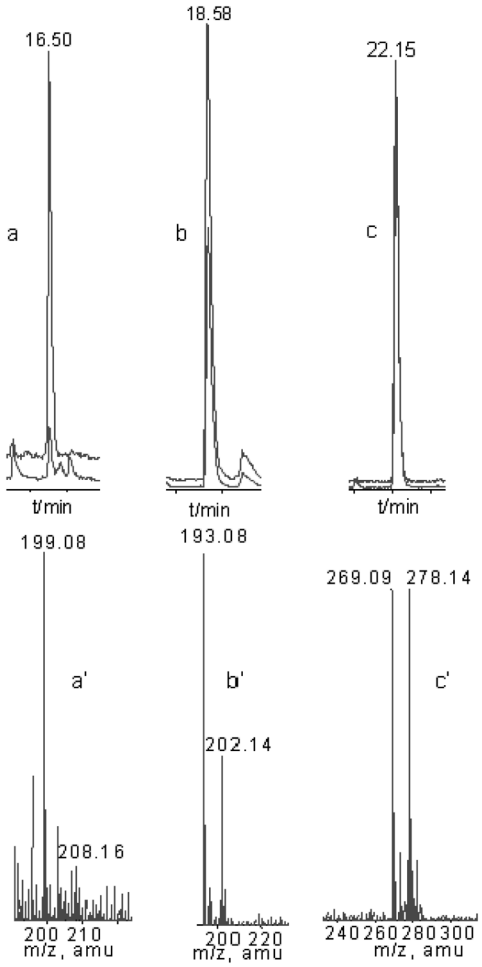

Figure 5.

Extracted ion chromatograms of representative carboxylic acids at specific light/heavy concentration ratios. Upper traces (a, b, c) are light-form derivatives; lower traces are heavy-form derivatives. Corresponding MS spectrum (a', b', c') of the pairs of light- and heavy-form derivatives are also shown. a, a': Malonic acid at ratio 8:1 (light/heavy); b, b': formic acid at ratio 2:1; c, c': benzoic acid at the ratio 1:1. Other experimental conditions are the same as in Figure 2.

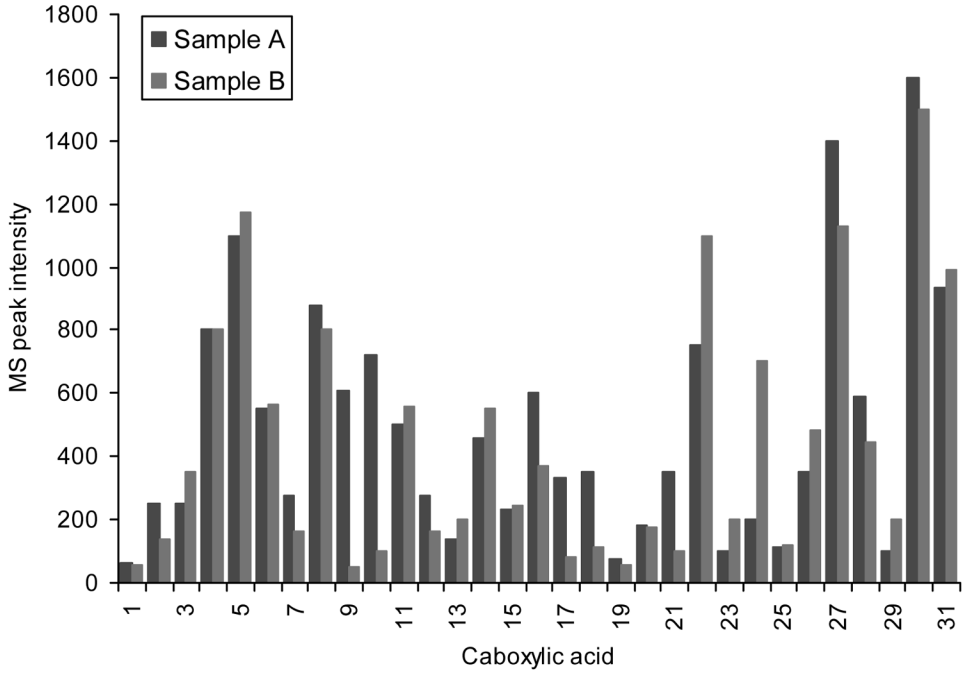

Figure 6.

Distribution of carboxylic acids in urine from two normal rats. Samples A and B were derivatized with BAMP and BAMP-d9, respectively, and mixed at the ratio of 1:1. The results provided are the averages from 5 analyses of each sample. The experimental conditions are the same as in Figure 2. The numbers on the x-axis correspond to the following carboxylic acids: 1. nicotinic acid; 2. oxaloacetic acid; 3. oxalic acid; 4. succinic acid; 5. citric acid; 6. malic acid; 7. ketoglutaric acid; 8. methylcitric acid; 9. adipic acid; 10. suberic acid; 11. isocitric acid; 12. 3-methylgluteric acid; 13. formic acid;14. carbonic acid; 15. glyoxylic acid; 16. acetic acid; 17. propionic acid; 18. pyruvic acid; 19. oxamic acid; 20. lactic acid; 21. butyric acid, isobutyric acid; 22. benzonic acid; 23. 5-methylfuran-2-carboxylic acid; 24. 2-hydroxy-2-ethyl-succinic acid; 25. phenylacetic acid; 26. 3-indoleacetic acid; 27. 3-phenyl-propionic acid; 28. α-hydroxyphenylacetic acid; 29. suberic acid; 30. hippuric acid; 31. nalidixic acid.

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Table 1

m/z, detection limit and migration reproducibility of BAMP and HAMP derivatives of carboxylic acids.

		BAMP			HAMP			
			Detection limit[a]			Detection limit		
Peak	Carboxylic acid	m/z	Conc. (μM)	Mass (pg)	m/z	Conc. (μM)	Mass (pg)	Migration time RSD (%, n=5)[b]
1	Oxaloacetic acid, di-	213.37 (+2)	20.0	26.0	241.38 (+2)	5.0	6.0	1.20
2	Oxalic acid, di-	192.35 (+2)	20.0	18.0	220.40 (+2)	5.0	4.0	1.11
3	Malonic acid, di-	199.33 (+2)	10.0	10.0	227.36 (+2)	2.0	2.0	0.92
4	Fumaric acid, di-	205.37 (+2)	5.0	5.0	233.35 (+2)	1.0	1.0	1.16
5	Succinic acid di-	206.36 (+2)	2.0	2.0	234.37 (+2)	0.5	0.6	0.99
6	Maleic acid, di-	205.37 (+2)	5.0	5.0	233.35 (+2)	1.0	1.0	1.29
7	Malic acid, di-	214.37 (+2)	2.0	2.0	242.39 (+2)	0.5	0.6	1.18
7	Citric acid, di-, -H_2O	234.33 (+2)	5.0	9.0	262.36 (+2)	1.0	2.0	1.18
7	ketoglutaric acid, di-	220.38 (+2)	10.0	14.0	248.38 (+2)	2.0	3.0	1.18
8	isocitric acid, di-, -H_2O	234.33 (+2)	10.0	19.0	262.36 (+2)	2.0	4.0	1.23
9	Tartaric acid, di-	222.36 (+2)	5.0	7.0	250.37 (+2)	1.0	1.0	1.26
10	Adipic acid, di-	220.57 (+2)	2.0	2.0	248.38 (+2)	0.5	0.7	1.32
11	Citric acid, di-	243.33 (+2)	5.0	9.0	271.33 (+2)	1.0	2.0	1.01
12	isocitric acid, di-	243.33 (+2)	5.0	9.0	271.03 (+2)	1.0	2.0	1.00
12	Formic acid	193.33 (+1)	5.0	2.0	221.32 (+1)	1.0	0.5	1.00
13	Acetic acid	207.34 (+1)	10.0	6.0	235.33 (+1)	5.0	3.0	1.06
14	Propionic acid	221.37 (+1)	2.0	1.0	249.14 (+1)	1.0	0.7	1.08
15	Pyruvic acid	235.37 (+1)	10.0	8.0	263.37 (+1)	2.0	1.0	1.14
15	Lactic acid	237.34 (+1)	10.0	9.0	265.37 (+1)	2.0	2.0	1.14
16	Butyric + isobutyric acid [c]	235.32 (+1)	2.0	1.0	263.37 (+1)	1.0	0.8	1.20
17	Benzonic acid	269.33 (+1)	2.0	2.0	297.32 (+1)	0.5	0.6	1.28
18	Shikimic acid	321.36 (+1)	10.0	17.0	349.34 (+1)	2.0	3.0	1.24
19	Quinic acid	339.35 (+1)	100.0	192.0	367.36 (+1)	20.0	38.0	1.27

[a] Mean of three injections of five different levels in the concentration range of 100-1mM, injection volume 10 nl.

[b] Consecutive injections (n = 5) of standard mixture at the concentration of 100mM.

[c] Not separated by CE/MS.

Table 2

Carboxylic acid peaks found in pooled rat urine and identification results.

Peak	BAMP			HAMP			Identification[b]	
	Tr (min)	m/z	Int.[a] (%)	Tr (min)	m/z	Int.[a] (%)	Mr	Compound
1	11.81	235.19(+1)	1.11	12.7	264.21(+1)	0.78	88.21	U
1	11.81	320.46(+1)	0.71	12.7	348.47(+1)	0.5	173.17	n-acetylleucine (R)
2	13.67	299.37(+1)	0.49	14.52	327.42(+1)	0.35	152.12	U
3	13.76	313.35(+1)	0.88	14.72	341.39(+1)	0.63	166.09	U
4	14.99	204.78(+3)	0.88	16.5	232.79(+3)	0.63	172.47	U
4	14.99	254.35(+1)	0.88	16.5	282.36(+1)	0.63	107.06	U
4	14.99	303.30(+1)	1.28	16.5	331.33(+1)	0.91	156.03	furan-2,5-dicarboxylic acid, mono- (R)
4	14.99	278.40(+2)	3.99	16.5	306.87(+2)	2.83	263.14	U
5	15.08	270.33(+1)	0.88	16.8	298.33(+1)	0.63	123.03	Nicotinic acid (S)
6	15.2	213.37(+2)	0.8	17	241.38(+2)	0.56	132.16	oxaloacetic acid, di- (S)
6	15.22	211.35(+1)	0.26	17.08	239.37(+1)	0.18	64.07	U
7	15.38	191.31(+1)	3.98	17.22	219.35(+1)	2.82	44.05	U
7	15.38	299.37(+1)	4.86	17.23	327.32(+1)	3.45	152.02	U
7	15.45	274.32(+1)	1.46	17.4	302.40(+1)	1.03	127.1	δ-Piperideine-6-L-carboxylate δ-Piperideine-2-carboxylate (L)
8	16.25	192.36(+2)	0.32	18.08	220.40(+2)	0.66	90.2	oxalic acid (S)
8	16.27	203.84(+2)	0.48	18.08	231.87(+2)	0.34	113.14	U
9	16.68	206.36(+2)	2.37	18.48	234.37(+2)	1.68	118.14	succinic aicd,di- (S)
10	17.05	234.34(+2)	4.87	18.82	262.34(+2)	3.45	174.08	citric acid, di-,-H2O (S)
10	17.04	214.37(+2	0.38	18.92	242.39(+2)	0.27	134.18	malic acid, di- (S)
11	17.27	220.38(+2)	0.27	19.09	248.38(+2)	0.19	146.16	α-ketoglutaric acid, di- (S)
11	17.27	234.34(+2)	1.6	19.14	262.36(+2)	1.01	174.12	iso-citric acid, di-,-H2O (S)
11	17.27	225.37(+2)	1.33	19.17	253.35(+2)	0.94	156.1	furan-2,5-dicarboxylic acid, di- (R)
11	17.3	255.25(+2)	1.16	19.34	283.25(+2)	0.82	215.9	5-Oxopent-3-ene-1,2,5-tricarboxylate 5-Carboxymethyl-2-hydroxymuconate (L)
12	17.75	269.85(+2)	0.76	19.45	297.86(+2)	0.54	245.12	U
13	18.05	250.84(+2)	1.59	19.76	278.88(+2)	1.12	207.16	methylcitric acid, di- (S)

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Peak	BAMP			HAMP			Identification[b]	
	Tr (min)	m/z	Int.[a] (%)	Tr (min)	m/z	Int.[a] (%)	Mr	Compound
13	18.07	220.57(+2)	0.44	19.8	248.38(+2)	0.31	146.16	adipic acid, di-, (S)
13	18.1	207.38(+1)	1.11	19.84	235.31(+1)	0.78	60.01	U
13	18.1	243.33(+2)	5.13	19.92	271.32(+2)	3.64	192.08	citric acid, di-, (S)
14	18.28	234.33(+2)	0.8	20.23	262.36(+2)	0.57	174.12	suberic acid, di-, (S)
14	18.3	257.39(+2)	0.35	20.52	285.40(+2)	0.25	220.02	3-hydroxy-3-(carboxymethyl)-adipic acid, di-, (R)
14	18.32	243.35(+2)	0.71	20.58	271.34(+2)	0.5	192.08	isocitric acid, di-, (S)
14	18.37	220.39(+2)	0.42	20.59	248.37(+2)	0.29	146.16	3-methylglutaric acid, di-, (S)
15	18.7	193.31(+1)	5.77	20.88	221.32(+1)	4.09	46.02	formic acid, (S)
16	19.55	208.31(+1)	4.32	21.72	236.32(+1)	3.06	61.02	carbonic acid, (S)
17	20.27	221.37(+1)	0.27	22.3	249.37(+1)	0.19	74.07	glyoxylic acid, (S)
17	20.18	207.31(+1)	4.43	22.33	235.33(+1)	3.14	60.03	acetic acid, (S)
18	20.45	247.32(+1)	3.07	22.83	275.33(+1)	2.17	artifact [c]	
19	21	221.37(+1)	1.26	23.15	249.38(+1)	0.89	74.08	propionic acid, (S)
20	21.5	261.33(+1)	0.46	23.6	289.36(+1)	0.32	114.06	2-Hydroxy-2,4-pentadienoate
								cis-2-Hydroxypenta-2,4-dienoate
								cis-Acetylacrylate, (L)
20	21.53	235.37(+1)	0.57	23.64	263.37(+1)	0.4	88.07	Pyruvic acid, (S)
21	21.65	236.36(+1)	0.46	23.81	264.38(+1)	0.33	89.08	Oxamic acid, (S)
21	21.75	235.37(+1)	1.77	23.9	263.37(+1)	1.25	88.07	butyric acid, isobutyric acid, (S)
21	21.75	237.34(+1)	2.78	23.92	265.35(+1)	1.97	90.05	lactic acid, (S)
22	21.98	295.30(+1)	2.18	24.17	323.34(+1)	1.54	148.04	o-methylmalic acid, mono-, (R)
22	22.1	264.33(+1)	1.22	24.26	292.36(+1)	0.86	117.06	3-Nitroacrylate, (L)
23	22.23	269.34(+1)	7.97	24.4	297.32(+1)	5.65	122.02	benzoic acid, (S)
23	22.55	273.36(+1)	1.14	24.47	301.36(+1)	0.81	126.06	5-methylfuran-2-carboxylic acid, (R)
23	22.58	287.36(+1)	1.06	24.79	315.34(+1)	0.75	140.04	2-hydroxy-2-ethylsuccinic acid, mono, (R)
24	22.68	283.31(+1)	2.37	24.82	311.39(+1)	1.68	136.09	phenylacetic acid, (S)
25	22.98	263.39(+1)	0.9	25.07	291.41(+1)	0.63	116.01	3-Methyl-2-oxobutanoic acid
								Hexylic acid
								3-Oxopentanoic acid

Peak	BAMP			HAMP			Identification[b]	
	Tr (min)	m/z	Int.[a] (%)	Tr (min)	m/z	Int.[a] (%)	Mr	Compound
								2-Oxopentanoic acid, (L)
25	22.98	297.36(+1)	1.01	25.08	325.41(+1)	0.72	150.11	3-Phenyl-propionic acid, (S)
26	23.48	322.35(+1)	1.34	25.6	350.39(+1)	0.95	175.09	3-indoleacetic acid, (S)
26	23.57	299.35(+1)	0.6	25.68	327.40(+1)	0.43	152.1	α-hydroxyphenylacetic acid, (S)
27	23.67	313.35(+1)	2.89	25.78	341.38(+1)	2.04	166.08	4-hydroxyphenylhydracrylic acid, (R)
28	23.88	323.36(+1)	0.73	26.1	351.38(+1)	0.52	176.08	5,6,7,8-Tetrahydro-2-naphthoic acid, (L)
29	24.05	321.32(+1)	0.44	26.2	349.34(+1)	0.31	174.04	suberic acid, mono-, (S)
30	24.19	348.36(+1)	0.53	26.31	376.38(+1)	0.37	201.08	U
30	24.22	326.37(+1)	0.84	26.38	354.37(+1)	0.59	179.07	hippuric acid, (S)
31	24.37	340.37(+1)	2.17	26.53	368.37(+1)	1.54	193.07	5,6-Dihydroxyindole-2-carboxylate
								Phenylacetylglycine
								D-Dopachrome, (L)
32	24.87	379.36(+1)	1.23	27	407.38(+1)	0.87	232.08	Nalidixic acid, (S)

[a] Individual peak intensity was normalized to highest peak intensity of all peaks shown in the table.

[b] Molecular weight was calculated from the *m/z* of a derivative and the mass of the tag. **U** - unidentified carboxylic acid; **L** - identification based on molecular mass matching from the LIGAND database; **R** - compound was also found in the literature; **S** - identification was confirmed by the standards.

[c] This ion was also found in the standard chromatogram and was not represent it any carboxylic acid standards. It was treated as artifact.

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Table 3

Quantitative aspects of isotope-labeling derivatization[a]

	m/z			Linearity	
Carboxylic acid	**Light form**	**Heavy form**	**Derivatization limit (μM)**	**Correlation**[b]	**Dynamic range**[c]
Oxaloacetic acid, di-	213.36	222.32	50	y=0.892x, r2=0.997	1:1~1:5
Oxalic acid, di-	192.25	201.26	50	y=0.925x, r2=0.995	1:1~1:5
Malonic acid, di-	199.33	208.34	25	y=1.124x, r2=0.999	1:1~1:8
Fumaric acid, di-	205.37	214.38	10	y=0.935x, r2=0.995	1:1~1:10
Succinic acid di-	206.36	215.36	5	y=1.085x, r2=0.999	1:1~1:50
Maleic acid, di-	205.38	214.38	10	y=0.943x, r2=0.997	1:1~1:10
Malic acid, di-	214.37	223.37	5	y=0.914x, r2=0.992	1:1~1:50
Citric acid, di-, -H2O	234.33	243.34	10	y=0.967x, r2=0.993	1:1~1:10
a-ketoglutaric acid, di-	220.38	229.33	25	y=0.907x, r2=0.993	1:1~1:10
isocitric acid, di-, -H2O	234.33	243.34	25	y=0.975x, r2=0.990	1:1~1:10
Tartaric acid, di-	222.36	231.35	10	y=0.843x, r2=0.996	1:1~1:10
Adipic acid, di-	220.57	229.56	5	y=1.142x, r2=0.999	1:1~1:50
Citric acid, di-	243.33	252.34	10	y=1.135x, r2=0.999	1:1~1:10
isocitric acid, di-	243.33	252.34	10	y=1.112x, r2=0.994	1:1~1:10
Formic acid	193.32	202.34	10	y=1.201x, r2=0.978	1:1~1:10
Acetic acid	207.33	216.34	25	y=0.956x, r2=0.997	1:1~1:10
Propionic acid	221.37	230.33	5	y=1.230x, r2=0.999	1:1~1:50
Pyruvic acid	235.37	244.34	25	y=1.160x, r2=0.992	1:1~1:8
Lactic acid	237.34	246.36	25	y=1.109x, r2=0.993	1:1~1:8
Butyric + isobutyric acid	235.33	244.34	5	y=0.871x, r2=0.996	1:1~1:50
Benzonic acid	269.33	278.33	5	y=1.063x, r2=0.999	1:1~1:50
Shikimic acid	321.36	330.38	25	y=1.231x, r2=0.996	1:1~1:8
Quinic acid	339.35	348.37	100	nd	nd

[a] Analytes were derivatized with BAMP and BAMP-d9.

[b] y= experimental mass spectrometric peak intensity ratio of light form to heavy form; x= theoretic ratio.

[c] Determined by keeping the light form concentration constant (100mM) and reducing the heavy form concentration.

[d] Not detected.