# Differential Metabolomics Using Stable Isotope Labeling and Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometry

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# Differential Metabolomics Using Stable Isotope Labeling and Two-Dimensional Gas Chromatography with Time-of-Flight Mass Spectrometry

**Xiaodong Huang and Fred E. Regnier***

*Department of Chemistry, Purdue University, West Lafayette, Indiana 47907*

**This work describes an approach to differential metabolomics that involves stable isotope labeling for relative quantification as part of sample analysis by two-dimensional gas chromatography/mass spectrometry (GC×GC/MS). The polar metabolome in control and experimental samples was extracted and differentially derivatized using isotopically light and heavy ($D_6$) forms of the silylation reagent *N*-methyl-*N*-*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA). MTBSTFA derivatives are of much greater hydrolytic stability than the more common trimethylsilyl derivatives, thus diminishing the possibility of isotopomer scrambling during GC analysis. Subsequent to derivatization with MTBSTFA, differentially labeled samples were mixed and analyzed by GC×GC/MS. Metabolites were identified, and the isotope ratio of isotopomers was quantified. The method was tested using three classes of metabolites; amino acids, fatty acids, and organic acids. The relative concentration of isotopically labeled metabolites was determined by isotope ratio analysis. The accuracy and precision, respectively, in quantification of standard mixtures was 9.5 and 4.77% for the 16 amino acids, 9.7 and 2.83% for the mixture of 19 fatty acids, and 14 and 4.53% for the 20 organic acids. Suitability of the method for the examination of complex samples was demonstrated in analyses of the spiked blood serum samples. This differential isotope coding method proved to be an effective means to compare the concentration of metabolites between two samples simultaneously.**

Metabolomics focuses on the analysis of low molecular weight, endogenous metabolites in tissues and biofluids. The objective in metabolomics is generally to provide a nontargeted, qualitative, and quantitative description of the metabolic milieu in an organism. Along with genomics, transcriptomics, and proteomics, metabolomics is playing an important role in defining complex biological systems during the course of growth, development, and response to environmental stimuli[1,2] along with serving as a powerful new tool in drug discovery,[3,4] toxicology,[5,6] phytochemistry,[7,8] clinical chemistry,[9,10] and disease diagnosis,[11,12] where it is used to examine genotype−phenotype, genotype−envirotype, and disease−metabolite relationships.[13] When coupled with principal component analysis,[14,15] hierarchical cluster analysis,[16] partial least-squares discriminant analysis,[17] and parallel factor analysis,[14] a broad range of new biomarkers and biological relationships can often be recognized.[18]

Fourier transform infrared spectroscopy,[19] mass spectrometry,[19,20] nuclear magnetic resonance spectrometry,[20−22] liquid chromatography (LC), capillary electrophoresis, gas chromatography (GC), and hybrid combinations of these instruments[19,23−25] have all been found to be of utility in metabolomics. Among the

* To whom correspondence should be addressed. Phone: (765) 494-9390. Fax: (765) 494-0239. E-mail: fregnier@purdue.edu.

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new hybrid methods, comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry (GC×GC/TOF-MS) is gaining acceptance as one of the most rapid, high-resolution systems available.[26,27] Compared with conventional one-dimensional GC, two-dimensional gas chromatography with time-of-flight mass spectrometry offers an order of magnitude increase in separation capacity, a significant increase of signal-to-noise ratio and dynamic range, and faster acquisition of mass spectral data.[28−30] But there is still the issue of quantification. This is the focus of the work described here. It is frequently the goal in metabolomics to identify qualitative and quantitative differences in the metabolome of hundreds of individuals as part of studying the impact of disease and environmental stimuli.

Stable isotope coding has been widely used in vitro for quantification of large numbers of proteins in comparative proteomics. This technique is based on the fact that (1) families of analytes often contain a common functional group that can be derivatized in vitro and (2) these analytes can be differentially coded according to sample origin by derivatization with an isotopically distinct coding reagent. This is the case in proteomics where amino groups in peptide mixtures of 1 million or more components are derivatized by stable isotope coded acylating agents. Analytes in a control sample are derivatized with one isotopomer of the acylating agent while those from an experimental sample are derivatized with a second, isotopically unique form of the derivatizing agents. The two samples are then mixed and analyzed by LC/MS, where the relative concentration of coded analytes in the two samples is determined by isotope ratio analysis of the isotopomers.[31] The difference between this approach and other well-established stable isotope based internal standard methods is that large numbers of analytes are being globally coded and compared between two samples simultaneously.[32−34] Similar methods have recently been described in metabolomics for the quantification of specific functional group containing metabolites by LC/MS/MS.[35−40]

This paper explores the extension of global isotopic coding to GC×GC/TOF-MS. Prior to GC, metabolites are often derivatized by trimethylsilylation to increase their volatility. This provides the ideal opportunity for isotope coding in the manner described above by substituting $^{13}C$ or $^{2}H$ for $^{12}C$ and $^{1}H$ in the silylating agent. The advantage of the powerful trimethylsilylating agents currently in use is that they derivatize a broad variety of functional groups ranging from hydroxyl, thiol, amino, and imino groups to carboxyl and phosphate groups.[41] Over 1000 metabolites can be seen in a cell extract or blood sample in a single analysis through silylation and GC×GC/TOF-MS analysis. A concern with trimethylsilyl coding is that trimethylsilyl groups are easily hydrolyzed and scrambling of the labeling might occur between differentially coded analytes during gas chromatography. This led to the use of (*N*-methyl-*N*-*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) as the silylating agent in this work. An alcohol derivatized with the more sterically hindered *tert*-butyldimethylsilyl group is at least 10 000-fold less likely to hydrolyze than a trimethylsilyl-derivatized alcohol.[42−45] Three classes of standard metabolites were examined by MTBSTFA derivatization, amino acids, fatty acids, and organic acids. Following method development, blood samples were analyzed to determine the utility of the method with complex natural samples.

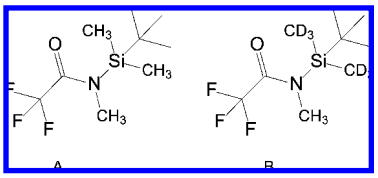


**Figure 1.** Structure of silylation reagents **A**, *N*-methyl-*N*-*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA); **B**, $D_6$-MTBSTFA.

## EXPERIMENTAL SECTION

**Materials and Reagents.** The amino acid standard mixture, fatty acid standards, organic acid standards, anhydrous pyridine, anhydrous acetonitrile, and human serum were obtained from Sigma-Aldrich (St. Louis, MO). Individual amino acids were at a concentration of 2.5 $\mu$mol/mL in 0.1 N HCl except L-cystine at 1.25 $\mu$mol/mL. The fatty acid and organic acid mixtures contained 0.5 mg/mL of the individual acids in pyridine. Anhydrous methanol and chloroform were purchased from Mallinckrodt Baker (Phillipsburg, NJ). Ethoxyamine hydrochloride and hydroxylamine hydrochloride were obtained from Sigma-Aldrich and used to prepare 50 mg/mL solutions in pyridine, respectively. The MTBSTFA and deuterio-$D_6$-MTBSTFA derivatization reagents were obtained from Regis Technologies (Morton Grove, IL). Their structures are showed in Figure 1.

**Derivatization.** A 100-$\mu$L amino acid mixture was dried with a flow of nitrogen and redissolved in 50 $\mu$L of pyridine before derivatization. Individual 50-$\mu$L aliquots of the amino acid mixture, fatty acid mixture, and organic acid mixture were derivatized with 50 $\mu$L of MTBSTFA or the $D_6$-isotopomer for 30 min at 60 °C, respectively. After derivatization, 20 $\mu$L of the sample derivatized with MTBSTFA was mixed with 20 $\mu$L of the corresponding $D_6$-MTBSTFA derivatized sample, respectively.


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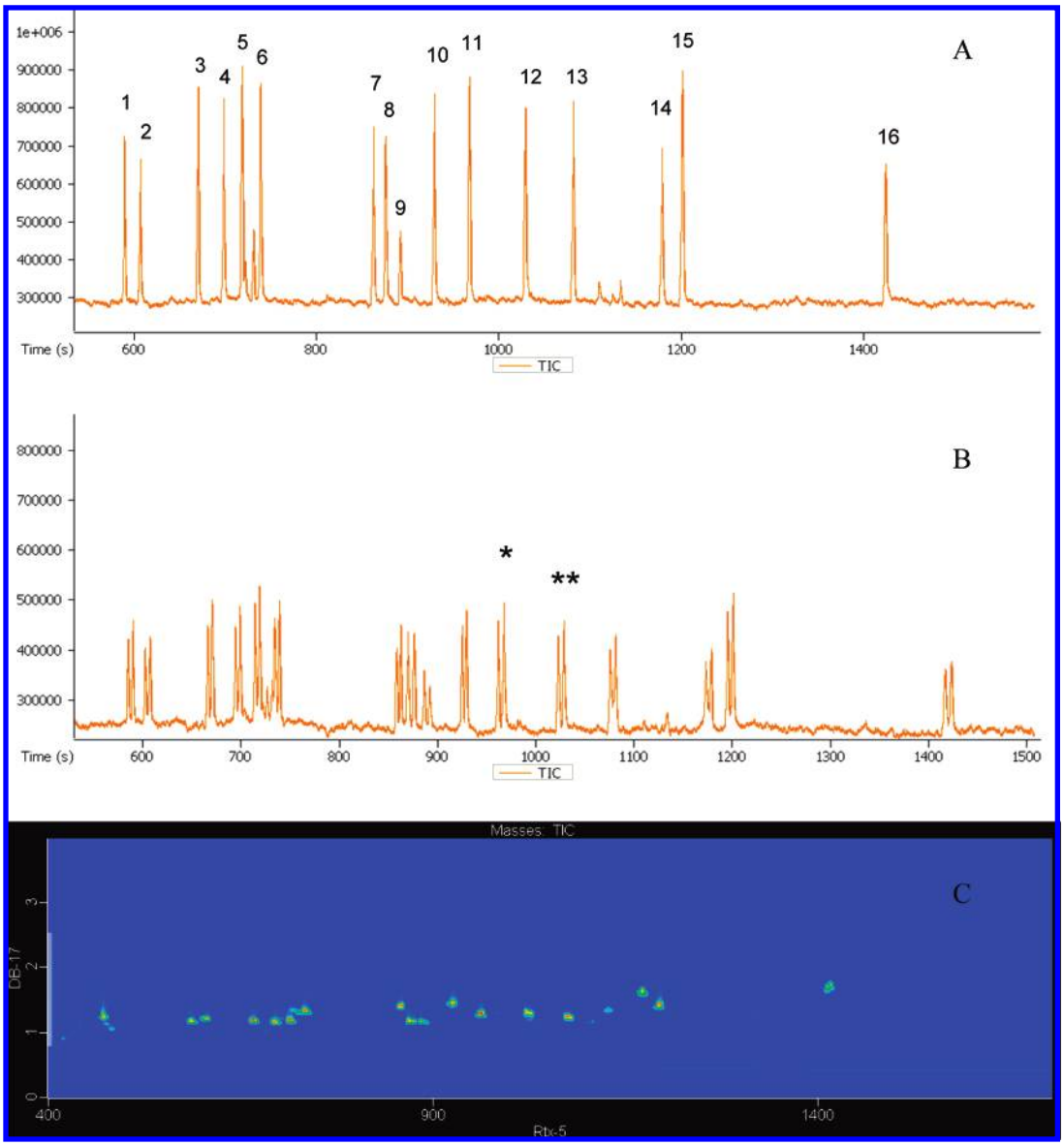


**Figure 2.** Total ion current (TIC) chromatograms of 16 amino acids using 1D GC/TOF-MS (A). The mixture of MTBSTFA derivatives and $D_6$-MTBSTFA derivatives (1:1) of 16 amino acids using 1D GC/TOF-MS is shown in (B). and the 2D GC/TOF-MS separation is in (C). 1, L-alanine; 2, glycine; 3, L-valine; 4, L-leucine; 5, L-isoleucine; 6, L-proline; 7, L-methionine; 8, L-serine; 9, L-threonine; 10, L-phenylalanine; 11, L-aspartic acid*; 12, L-glutamic acid**; 13, L-lysine; 14, L-histidine; 15, L-tyrosine; 16, L-cystine.

**Extraction and Derivatization of Serum Sample.** Two 200-$\mu$L aliquots of a human serum sample, one of which had 10 $\mu$g each of L-valine, L-isoleucine, L-threonine, succinic acid, fumaric acid, adipic acid, oxalic acid, pentadecanoic acid, heptadecanoic acid, and octadecanoic acid added, were mixed with 400 $\mu$L of methanol. The methanol both precipitated proteins and extracted metabolites from samples. Alternatively acetonitrile, pyridine, and chloroform/methanol/water (2:5:2) were used in place of methanol. After sitting at room temperature for 1 h and being sonicated for 10 min, protein were removed from mixtures with centrifugation 15000*g*. Supernatants from the two mixtures were collected and evaporated to dryness with a SpeedVac and then redissolved in 50 $\mu$L of pyridine. The 20-$\mu$L aliquots from the two extracts were treated with 10 $\mu$L of 50 mg/mL ethoxyamine hydrochloride solution for 30 min at 60 °C, respectively. Subsequently, the spiked extracts were differentially derivatized with 20 $\mu$L of an MTBSTFA isotopomer for 1 h at 60 °C. After derivatization, 10 $\mu$L of the differentially isotope coded samples was mixed and subjected to GC×GC/TOF-MS.

**GC×GC/TOF-MS Analysis.** The analyses of derivatized standard mixtures and the extracted serum sample were performed using a Leco Pegasus 4D GC×GC/TOF-MS instrument (Leco Corp., St. Joseph, MI) equipped with a cryogenic modulator. The GC part of the instrument is an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA), and the injector is a CTC Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland). The first-dimension chromatographic column was a 10-m DB-5 capillary column with an internal diameter of 180 $\mu$m and a stationary-phase film thickness of 0.18 $\mu$m, and the second-dimension chromatographic column was a 1-m DB-17 capillary column with an internal diameter of 100 $\mu$m and a film thickness of 0.1 $\mu$m. High-purity helium was used as the carrier gas at a flow rate of 1.0 mL/min. The first-dimension column oven ramp began at 50 °C with a 0.2-min hold after which the temperature

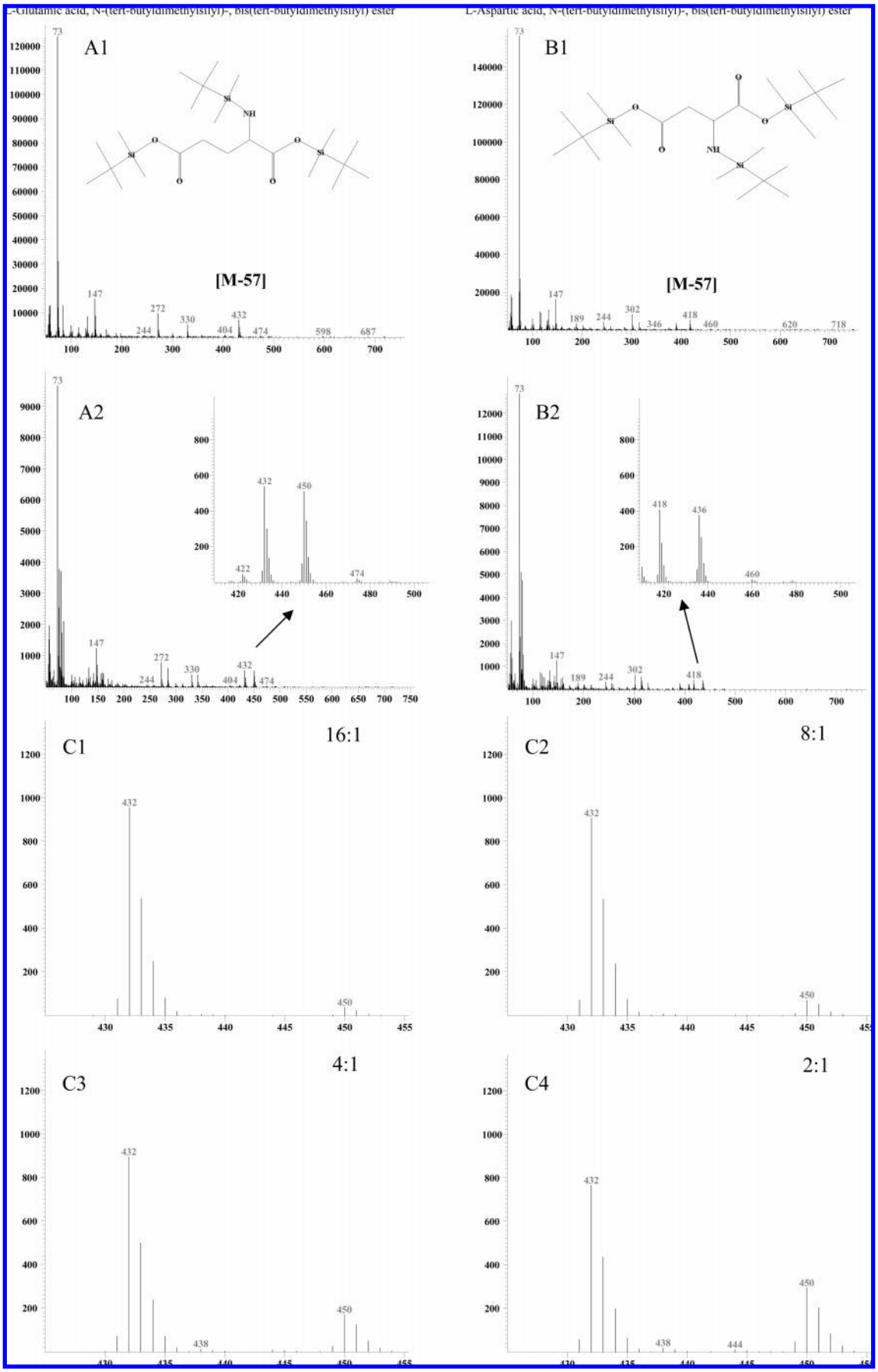


**Figure 3.** Unique mass of L-glutamic acid MTBSTFA derivative (A1) and L-aspartic acid MTBSTFA derivative (B1), respectively. Doublet mass cluster of undeuterated and deuterated derivatives of L-glutamic acid (A2) and L-aspartic acid (B2), respectively; Determination and illustration of isotope ratios for the mixtures of MTBSTFA and $D_6$-MTBSTFA derivatives (16:1) (C1), (8:1) (C2), (4:1) (C3), and (2:1) (C4), respectively.

was programmed to 300 °C at a rate of 10 °C/min and then held at this temperature for 5 min. The second-dimension column temperature was maintained 20 °C higher than the corresponding first-dimension column. The programming rate and hold times were the same for the two columns. A 4-s separation time was used in the second dimension. A 240-s solvent delay was used with the amino acid mixture whereas a 300-s delay was used with the fatty acid mixture and a 110-s delay with the organic acid mixture. The combined mixture of amino acids, fatty acids, and organic acids along with the serum sample was examined using a 120-s delay. The 2 $\mu$L of sample solution was injected in the split mode of injection at a ratio of 100:1 in all cases except the serum sample where a 20:1 split ratio was used. The inlet temperature and transfer line were set at 280 °C. The ion source was held at 200 °C. The detector voltage was set at 1600 V, and the filament bias was at −70 V. Mass spectra were collected from 50 to 900 $m/z$ at 50 spectra/s. The Leco ChromaTOF software version 2.32 equipped with NIST MS database (NIST MS Search 2.0, NIST/EPA/NIH Mass Spectral Library; NIST 2002) was used for data processing and peak matching.

## RESULTS AND DISCUSSION

**Derivatization.** Increasing the volatility of analytes is widely achieved in GC by trimethylsilylation. But because it was an objective of this work to reduce the potential for interanalyte scrambling of label among stable isotope coded analytes during GC analysis, derivatization was carried out with MTBSTFA. The *tert*-butyldimethylsilyl group is at least 10 000 times more hydrolytically stable than that of a trimethylsilyl group.[42−45] Nonlabeled MTBSTFA and a heavy $D_6$-coded isotopomer (Figure 1) were selected to differentially code samples. Control samples were dried with a stream of nitrogen and after addition of pyridine derivatized with MTBSTFA for 30 min at 60 °C. Deuterio $D_6$-MTBSTFA derivatization of experimental samples was achieved in the same way. Equal aliquots of the isotopically coded samples were then mixed and analyzed. Analytes with a single reactive functional group coded in this manner differed by 6 atomic mass units (amu) in molecular weight. Each additional reactive functional group in an analyte added another 6 amu in mass difference between differentially coded isoforms of analytes. When deuterated and nondeuterated analytes were examined together, *tert*-butyldimethylsilyl (*t*-BDMS)-derivatized fragment ions of an analyte appeared as doublet clusters separated by 6 amu while nonsilylated fragment ions from isotopomeric parents were of identical mass. Amino acids, fatty acids, and organic acid standards were used to test the efficacy of the method.

**Comparative Quantification by Isotope Ratio Analysis.** Standard sample mixtures were prepared by splitting the mixtures of amino acid standards, fatty acid standards, and organic acid standards into equal size aliquots, respectively, and differentially coding them with *t*-BDMS and $D_6$-*t*-BDMS groups, and then mixing them before GC/MS analysis. These sample mixtures were analyzed by one-dimensional (1D) GC/MS and 2D GC×GC/MS, respectively. In the case of amino acids (Figure 2), the isotopomers differed by at least 12 amu because both an amino and a carboxyl group were derivatized. The presence of additional reactive functional groups in amino acids such as an alcohol, sulfhydryl, carboxyl, or amino group resulted in the addition of a third silyl group.

**Table 1. Quantitative Analysis of Differentially Labeled Amino Acids**

| amino acid | unique mass | observed ratio | accuracy (% error) | precision CV (%) ($n = 5$) |
|---|---|---|---|---|
| L-alanine | 260:272 | 1.20 | 20 | 2.50 |
| glycine | 246:258 | 1.11 | 11 | 3.67 |
| L-valine | 288:300 | 1.17 | 17 | 3.68 |
| L-leucine | 302:314 | 1.13 | 13 | 2.56 |
| L-isoleucine | 302:314 | 1.08 | 8 | 6.72 |
| L-proline | 286:298 | 1.15 | 15 | 2.69 |
| L-methionine | 320:332 | 1.08 | 8 | 4.88 |
| L-serine | 390:408 | 1.01 | 1 | 8.56 |
| L-threonine | 404:422 | 0.93 | 7 | 3.37 |
| L-phenylalanine | 336:348 | 1.12 | 12 | 3.59 |
| L-aspartic acid | 418:436 | 1.07 | 7 | 6.08 |
| L-glutamic acid | 432:450 | 1.06 | 6 | 3.00 |
| L-lysine | 300:312[a] | 1.03 | 3 | 9.30 |
| L-histidine | 440:458 | 1.14 | 14 | 3.75 |
| L-tyrosine | 466:484 | 1.00 | 0 | 7.17 |
| L-cystine | 348:360[a] | 1.00 | 0 | 9.59 |
| average | | 1.08 | 9.5 | 4.77 |

[a] The unique mass is not from the ion of $[M - 57]^+$: $m/z$ 300 comes from [M − 131 − 57] ion; $m/z$ 348 is from the cleavage of S−S bond of cystine.

At least partial resolution of isotopomers differing in deuterium content was noted. This chromatographic isotope effect has been previously observed in high-resolution chromatography systems.[46,47] The degree to which resolution occurs depends on both the structural position and the number of deuterium atoms in analytes. Whether coded isotopomers coelute influences how isotope ratio analysis can be achieved. When isotopomers exactly coelute, their isotope ratio can be determined from mass spectra taken at any point during their elution from the column. When they do not, each analyte must be quantified independently based on peak area measurements and the isotope ratio calculated after the isotopomers have eluted from the column. Isotope ratio analysis by this second method is easier with the electron impact mode of ionization used in this work because there is no matrix suppression of ionization as with electrospray ionization in LC. Matrix suppression can cause isotopomers eluting at different times to experience wide differences in ionization efficiency and produce large quantification errors in isotope ratios.[48]

The magnitude of chromatographic isotope effects on GC separations was most easily seen with carboxylic acids. Because fatty acids have a single derivatizable function group, the heavy and light *t*-BDMS-coded isotopomers differ by six deuterium atoms and are partially resolved. Greater resolution was seen with di- and tricarboxylic acids where the heavy isoforms contain 12 and 18 deuterium atoms, respectively. The fact that heavy and light isoforms were resolved to some degree in most cases necessitated that isotope ratio quantification be based on the comparison of peak areas from selection ion chromatograms.

Glutamic and aspartic acid were selected for illustration of isotope ratio analysis (Figure 3). With most of the MTBSTFA

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**Table 2. Quantitative Analysis of Differentially Labeled Fatty Acids**

| fatty acid | unique mass | observed ratio | accuracy (%) | precision (CV %) ($n = 5$) |
|---|---|---|---|---|
| hexanoic acid | 173:179 | 1.03 | 3 | 7.71 |
| heptanoic acid | 187:193 | 0.66 | 34 | 9.42 |
| octanoic acid | 201:207 | 1.02 | 2 | 1.38 |
| nonanoic acid | 215:221 | 1.06 | 6 | 3.74 |
| decanoic acid | 229:235 | 1.16 | 16 | 0.48 |
| undecanoic acid | 243:249 | 1.08 | 8 | 0.78 |
| dodecanoic acid | 257:263 | 1.19 | 19 | 2.48 |
| tridecanoic acid | 271:277 | 1.10 | 10 | 0.80 |
| tetradecanoic acid | 285:291 | 1.15 | 15 | 1.54 |
| pentadecanoic acid | 299:305 | 1.11 | 11 | 1.71 |
| hexadecanoic acid | 313:319 | 1.06 | 6 | 2.80 |
| heptadecanoic acid | 327:333 | 1.05 | 5 | 1.77 |
| octadecanoic acid | 341:347 | 1.03 | 3 | 2.02 |
| nonadecanoic acid | 355:361 | 1.04 | 4 | 0.71 |
| icosanoic acid | 369:375 | 1.14 | 14 | 3.60 |
| heneicosanoic acid | 383:389 | 1.05 | 5 | 2.39 |
| docosanoic acid | 397:403 | 1.07 | 7 | 4.84 |
| tricosanoic acid | 411:417 | 1.09 | 9 | 2.84 |
| tetracosanoicc acid | 425:431 | 1.08 | 8 | 2.69 |
| average | | 1.06 | 9.7 | 2.83 |
| | | 1.08[a] | 8.4[a] | 2.46[a] |

[a] The value to the exclusion of heptanoic acid.

**Table 3. Quantitative Analysis of Differentially Labeled Organic Acids**

| organic acid | unique mass | observed ratio | accuracy (%) | precision (CV %) ($n = 5$) |
|---|---|---|---|---|
| acetic acid | 103:109 | 0.90 | 10 | 2.67 |
| formic acid | 117:123 | 0.94 | 6 | 9.72 |
| propionic acid | 131:137 | 1.20 | 20 | 3.01 |
| isobutyric acid | 145:151 | 0.88 | 12 | 5.83 |
| butyric acid | 145:151 | 0.90 | 10 | 2.62 |
| L-(+)lactic acid | 261:273 | 1.06 | 6 | 3.16 |
| benzoic acid | 179:185 | 1.01 | 1 | 2.92 |
| oxalic acid | 261:273 | 1.30 | 30 | 7.08 |
| malonic acid | 275:287 | 1.11 | 11 | 2.34 |
| maleic acid | 287:299 | 1.04 | 4 | 2.07 |
| succinic acid | 289:301 | 1.13 | 13 | 0.92 |
| fumaric acid | 287:299 | 1.28 | 28 | 2.11 |
| adipic acid | 317:329 | 1.19 | 19 | 1.14 |
| D-malic acid | 419:437 | 1.30 | 30 | 3.41 |
| D-tartaric acid | 549:573 | 1.25 | 25 | 5.07 |
| (-)quinic acid | 477:495 | 1.08 | 8 | 6.55 |
| shikimic acid | 459:477 | 1.23 | 23 | 7.88 |
| citric acid | 591:615 | 1.08 | 8 | 8.84 |
| DL-isocitric acid | 591:615 | 1.08 | 8 | 8.84 |
| L-ascorbic acid | 461:479 | 1.13 | 13 | 4.47 |
| average | | 1.10 | 14 | 4.53 |

derivatives, the ion at $[M - 57]^+$ (loss of the *tert*-butyl group from the derivative) is the base peak and much larger than the parent ion. This mass was used for quantification in all cases. In the cases of glutamic acid and aspartic acid, there are three functional groups derivatized with either *t*-BDMS or $D_6$-*t*-BDMS. Major peaks at $[M - 57]^+$ for these two amino acids are *m/z* 432 and 418. The corresponding peaks for the heavy isotopomer at $[M - 57 + 3 \times 6]^+$ are *m/z* 450 and 436. Isotope ratios of a 1:1 mixture of the isotopomers of glutamic and aspartic acid were found to be 1.06 and 1.07 (Table 1), respectively. Use of this stable isotope coding method to compare the relative concentration of components between samples is illustrated in Figure 3 (C1−C4).

Tables 1−3 list the experimentally determined isotope ratios with amino acids, fatty acids, and organic acids, respectively,

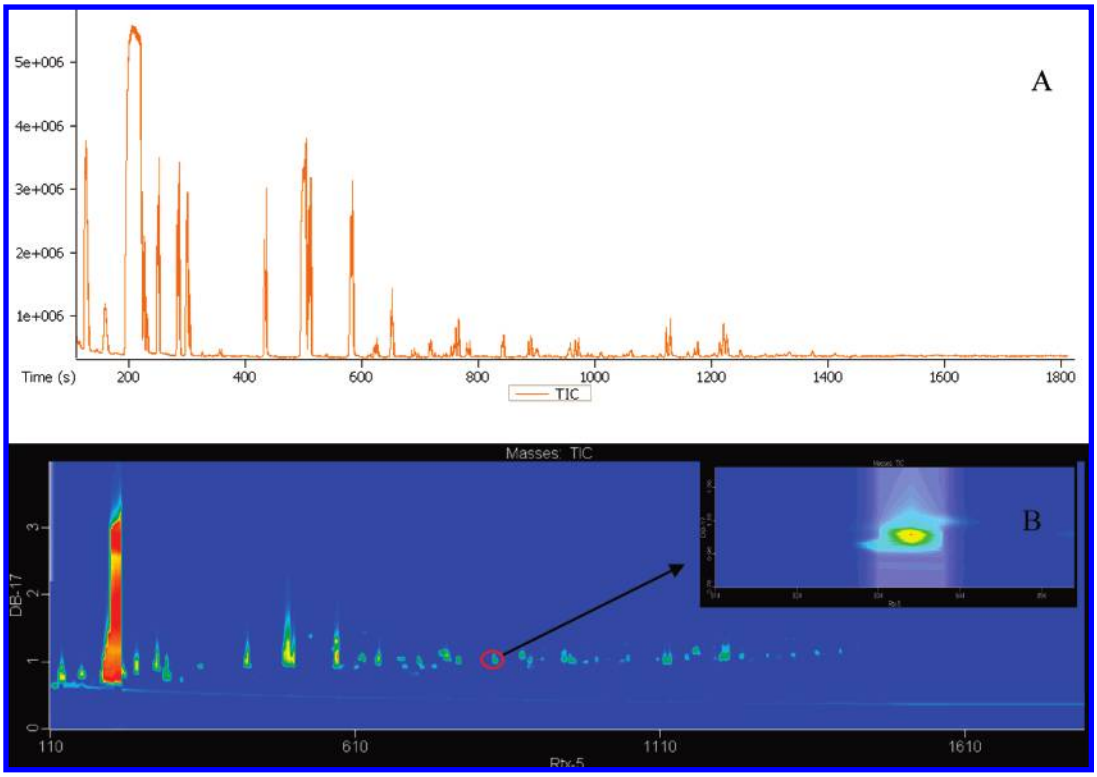


**Figure 4.** TIC of a mixture of MTBSTFA derivatives and $D_6$-MTBSTFA derivatives (1:1) of 16 amino acids, 19 fatty acids, and 19 organic acids using 1D GC/TOF-MS (A) and 2D GC/TOF-MS (B), respectively. An identified peak, dodecanoic acid was shown in small window of (B).

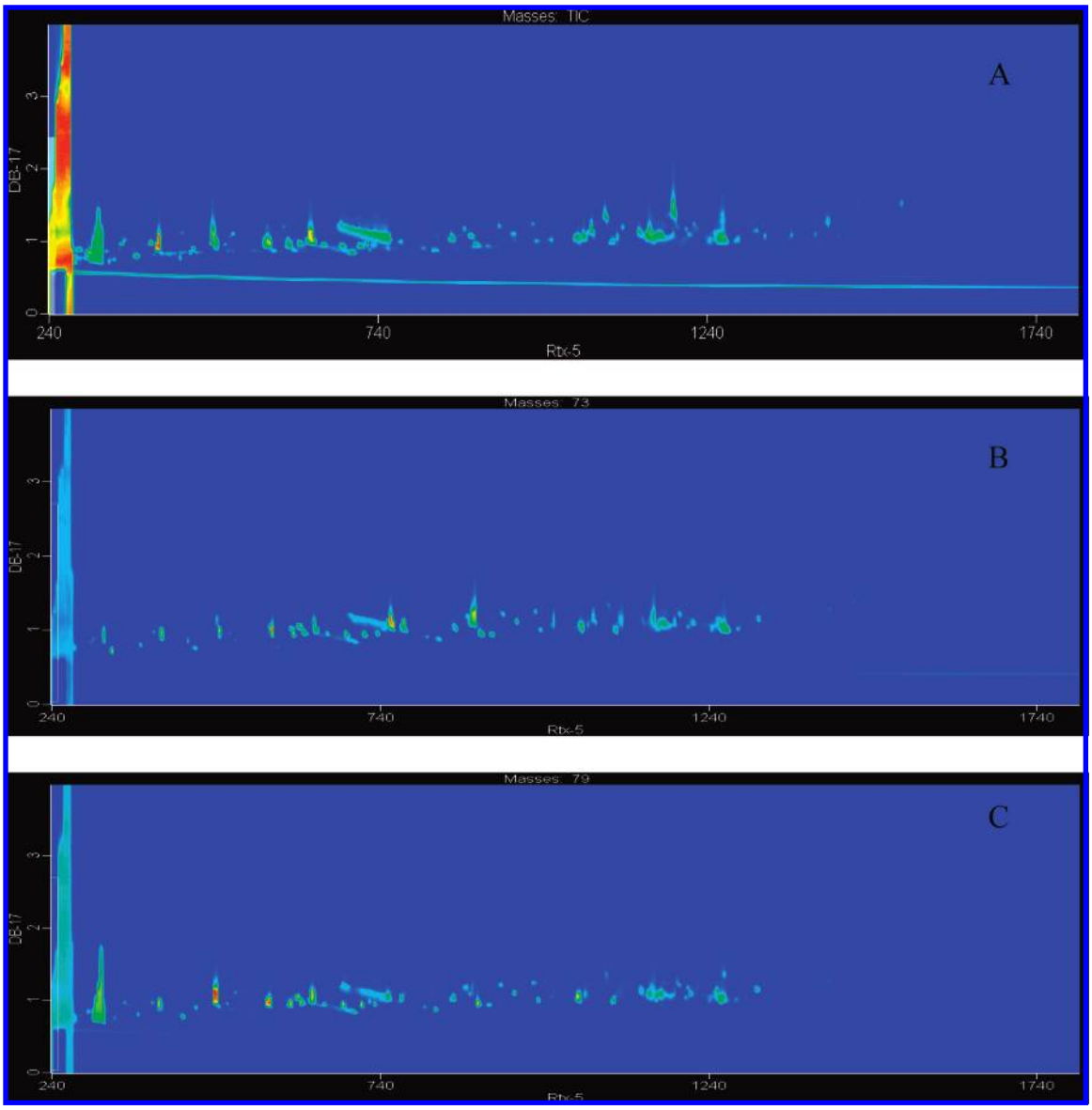


**Figure 5.** TIC (A), selective ion chromatogram at *m*/*z* 73 (B) and at *m*/*z* 79 (C) of the mixture of MTBSTFA derivatives of the spiked serum and $D_6$-MTBSTFA derivatives of the unspiked serum (1:1) using 2D GC/TOF-MS, respectively.

comparing samples of identical concentration. Values in these tables are based on five analyses. Detection was achieved by selected ion monitoring at $m/z = [M - 57]^+$ with the exception of L-lysine and L-cystine. Intensity of the $[M - 57]^+$ ion was very low with these two analytes. The average error of the method for all analytes was 9.5%, although individual deviations ranging from 0 to 20% error were noted in some cases. In the case of fatty acids, the average error was 9.7% with individual deviations ranging from a 2 to 19% error. Heptanoic acid was an exception in that a 34% error was noted in the isotope ratio. Why the error was so high with this simple fatty acid cannot be explained. Excluding heptanoic acid from the calculations, the average error for fatty acids was 8.4%. The average error in organic acid analysis was 14%. We also directly compared the concentration of analytes in samples without using stable isotope labeling. The accuracy and precision of amino acids, fatty acids, and organic acids was from 11 to 40% and 3 to 28%, respectively, as previously noted in metabolite analysis by GC/MS.[49]

The global coding approach was further tested using two aliquots of a mixture containing 54 components consisting of 16 amino acids, 19 fatty acids, and 19 organic acids. After differential isotope coding with MTBSTFA and $D_6$-MTBSTFA, the samples were mixed and analyzed by both one and two-dimensional GC/MS. As seen in Figure 4, the separation with two-dimensional GC is better than that with one-dimensional GC. The isotope ratios were then calculated. The differences between the observed ratios and expected ratios were ranged from 0 to 20%. The mean error for all analytes was 1% with a standard deviation of 0.10 excluding heptanoic acid (data not shown). This is roughly the same as when the amino acids, fatty acids, and organic acids were examined individually (Tables 1−3).

**Analysis of Serum Sample.** The complexity of human blood serum makes it an ideal sample for evaluation of the methods described above. Known amounts of some metabolites were added

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**Table 4. Quantitative Analysis of the Human Serum Sample**

| component | unique mass | observed ratio | ratio related to mean[b] |
|---|---|---|---|
| L-alanine | 260:272 | 1.10 | |
| glycine | 246:258 | 0.92 | |
| L-valine[a] | 288:300 | 1.47 | 1.41 |
| L-leucine | 302:314 | 1.07 | |
| L-isoleucine[a] | 302:314 | 1.44 | 1.38 |
| L-serine | 390:408 | 0.91 | |
| L-threonine[a] | 404:422 | 2.33 | 2.24 |
| L-phenylalanine | 336:348 | 1.13 | |
| L-glutamic acid | 432:450 | 0.93 | |
| L-lysine | 300:312 | 1.12 | |
| L-tyrosine | 466:484 | 1.10 | |
| nonanoic acid | 215:221 | 1.17 | |
| tetradecanoic acid | 285:291 | 0.87 | |
| pentadecanoic acid[a] | 299:305 | 9.75 | 9.38 |
| hexadecanoic acid | 313:319 | 0.87 | |
| heptadecanoic acid[a] | 327:333 | 6.00 | 5.77 |
| octadecanoic acid[a] | 341:347 | 2.05 | 1.97 |
| icosanoic acid | 369:375 | 1.12 | |
| L-(+)lactic acid | 261:273 | 1.01 | |
| benzoic acid | 179:185 | 1.00 | |
| oxalic acid[a] | 261:273 | 1.66 | 1.60 |
| succinic acid[a] | 289:301 | 39.25 | 37.7 |
| fumaric acid[a] | 287:299 | 4.74 | 4.56 |
| adipic acid[a] | 317:329 | 80.00 | 76.9 |
| shikimic acid | 459:477 | 1.17 | |
| citric acid | 591:615 | 1.14 | |
| mean ± SD[c] | | 1.04 ± 0.11 | |

[a] The concentration of these components was changed by spiking. [b] The isotope ratios of the spiked components related to the mean of the unspiked components. [c] The value of mean ± SD of the unspiked components.

to the serum and compared with the initial serum sample to determine the degree of change by the isotope labeling method. Two methods of extracting metabolites from serum were evaluated in this experiment. The popular methanol/chloroform/water (2.5:1:1) solvent mixture was examined first, but formation of the protein pellet between the upper methanol/water phase and the lower chloroform phase during centrifugation made it difficult to collect metabolites from the bottom chloroform layer without contamination. As previously reported, methanol alone was found to be superior to mixed solvent extraction, acetonitrile extraction, and the pyridine extraction methods.[49,50] The methanol extract was dried and redissolved in pyridine and then treated with hydroxylamine to derivatize carbonyl groups.[51] This step is widely used before silylation to minimize formation of geometric isomers that poduce multiple peaks during GC separations. Although both ethoxyamine hydrochloride and hydroxylamine hydrochloride have been used in oximation of sugars before silylation and GC analysis, ethoxyamine hydrochloride was used in this work because it was found to be less likely to produce multiple peaks with monosaccharides. After oximation, samples were silylated with with either MTBSTFA or $D_6$-MTBSTFA to achieve isotopic sample coding.

Samples differentially coded through isotopomer derivatization were mixed and analyzed by GC×GC/MS (Figure 5A). It was seen that many compounds in samples derivatized with MTBSTFA characteristically produced an ion at $m/z$ 73 while those treated with the deuterated reagent yielded an ion 6 amu higher at $m/z$ 79. Clearly, this 6 amu difference is due to the presence of the six deuterium atoms in the deuterated reagent. These ions are thought to occur by structural rearrangement during fragmentation and have the structures ($[Si(CH_3)_3]^+$) and ($[Si(CD_3)_2(CH_3)]^+$), respectively. Compounds having either of these two ions are thus easily identified as being silylated as seen in Figure 5B and C. A total of 639 components were recognized in serum. Among them were 11 amino acids, 7 fatty acids, and 8 organic acids (Tables 1−3). Furthermore, the concentration of three amino acids, three fatty acids, and four organic acids was elevated by the addition of standards, meaning they were in the samples initially. By this differential derivatization procedure, the degree of relative change was obtained by analyzing their isotope ratios (Table 4). The mean isotope ratio of unchanged components in serum samples was 1.04. Of the components that changed in concentration, the smallest was a 1.38-fold change and the largest one was 77-fold. It further turned out that the approach is applicable to complex sample matrixes.

## CONCLUSIONS

It is concluded from the data present in this paper that comparing the concentration of metabolites between two samples can be easily achieved in the GC/MS mode of analysis simultaneously by sample specifically coding the functional groups in large numbers of metabolites in each sample. Coding in this case was achieved by labeling reactive functional groups with sample-specific isotopomers of a silylating agent and mixing the samples before analysis. This approach should provide an effective and simple means for relative quantification of metabolites in complex biological samples and be of great value in differential metabolomics.

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