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　Le

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(54) **METABOLIC PROFILING BY REVERSE-PHASE/ION-EXCHANGE MASS SPECTROMETRY**

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(51) **Int. Cl.**
G01N 33/50 (2006.01)
G01N 30/72 (2006.01)
(Continued)

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CPC ***G01N 33/5038*** (2013.01); ***G01N 30/06*** (2013.01); ***G01N 30/72*** (2013.01); *G01N 2030/027* (2013.01)

(58) **Field of Classification Search**
CPC G01N 33/5038; G01N 30/06; G01N 30/72; G01N 2030/027; G01N 30/461;
(Continued)

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(57) **ABSTRACT**

Disclosed herein are methods of analyzing a biological sample comprising: separating components of the biological sample via reversed-phase (RP) chromatography to obtain an elute; subjecting the elute to separation via ion-exchange (IEX) chromatography or mixed-mode IEX chromatography; and detecting the separated compounds to determine the components of the biological sample. Also disclosed are devices comprising a reversed-phase (RP) chromatography column in communication with an ion-exchange (IEX) chromatography column or mixed-mode IEX chromatography column, wherein there is no switching valve between the columns.

11 Claims, 9 Drawing Sheets

Retention Time, min
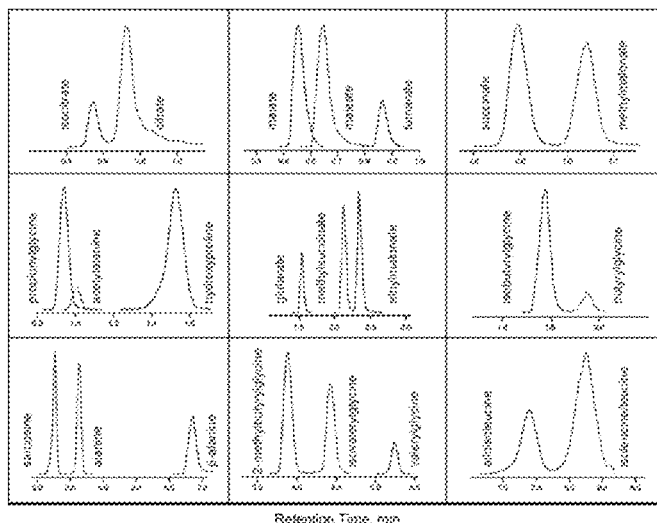
(51) **Int. Cl.**
G01N 30/06   (2006.01)
G01N 30/02   (2006.01)

(58) **Field of Classification Search**
CPC G01N 33/6806; G01N 33/92; G01N
2560/00; G01N 2800/04; B01D 15/325;
B01D 15/361
See application file for complete search history.

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FIGURE 1
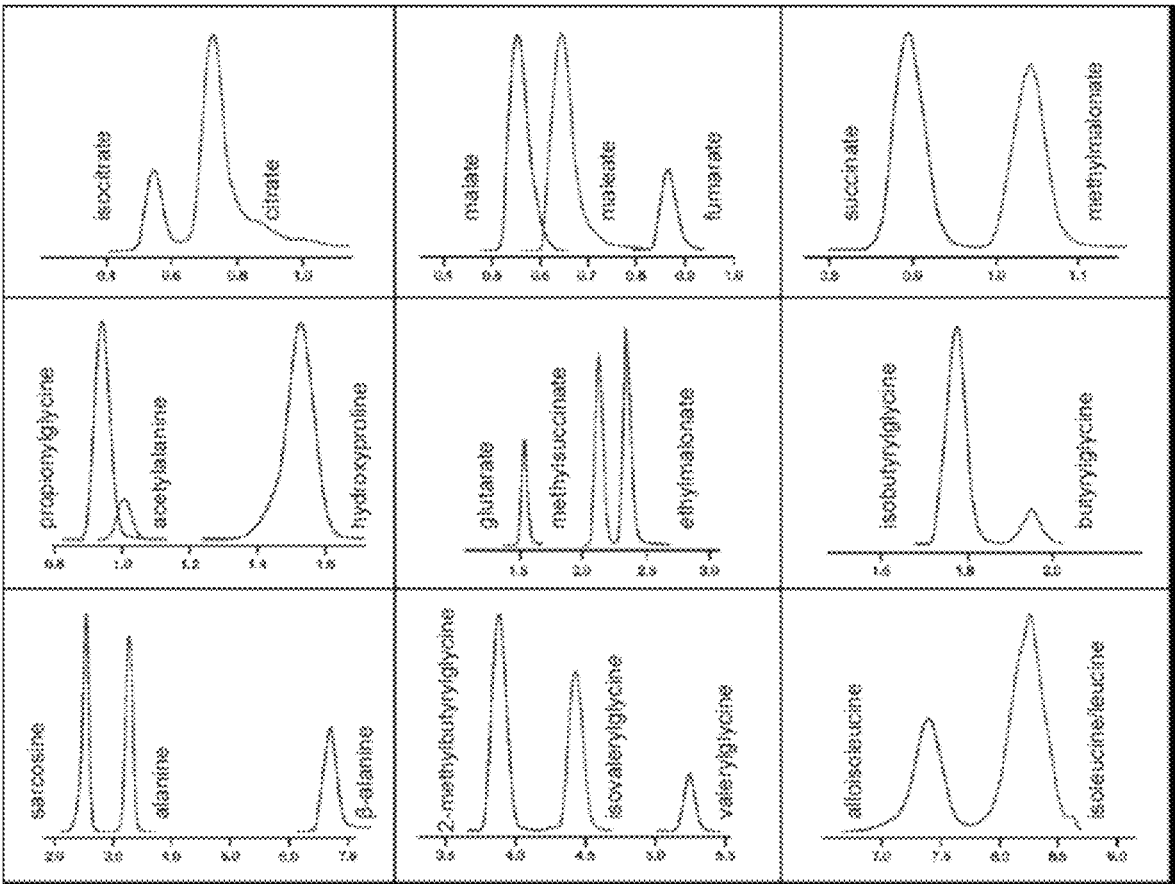
Retention Time, min

FIGURE 2
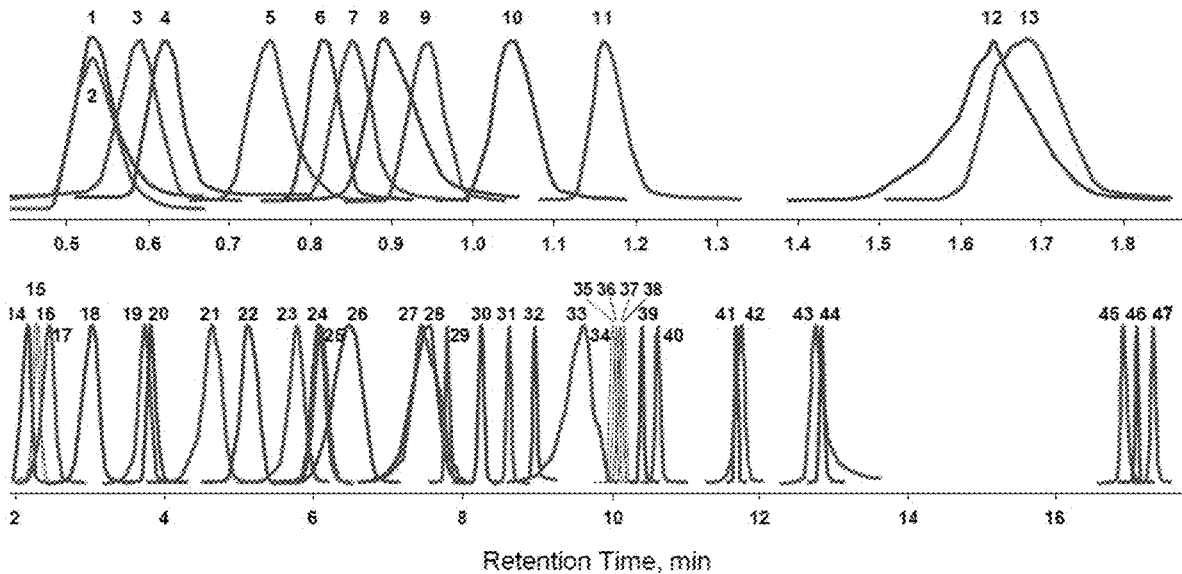
FIGURE 3

FIGURE 4

FIGURE 5
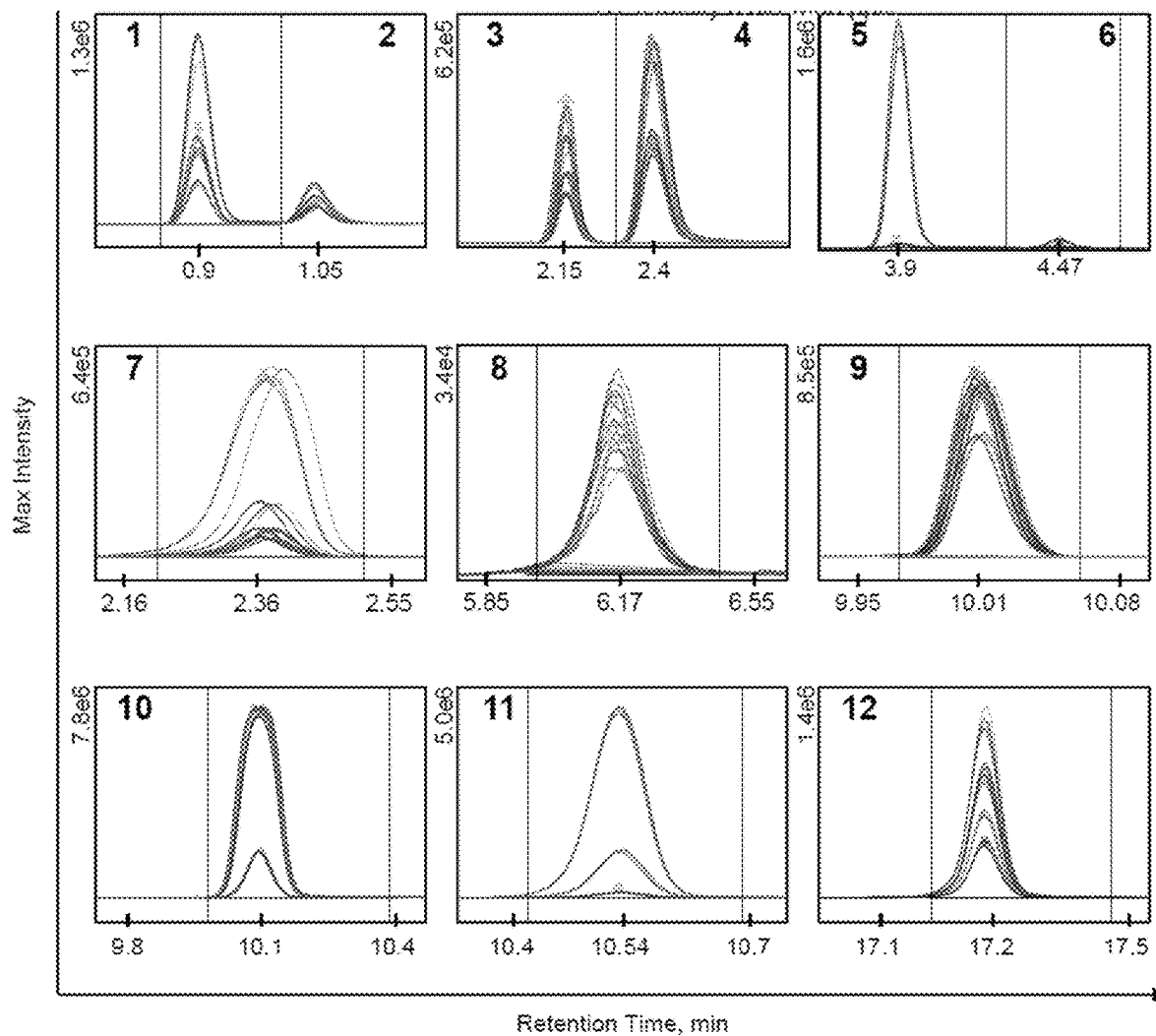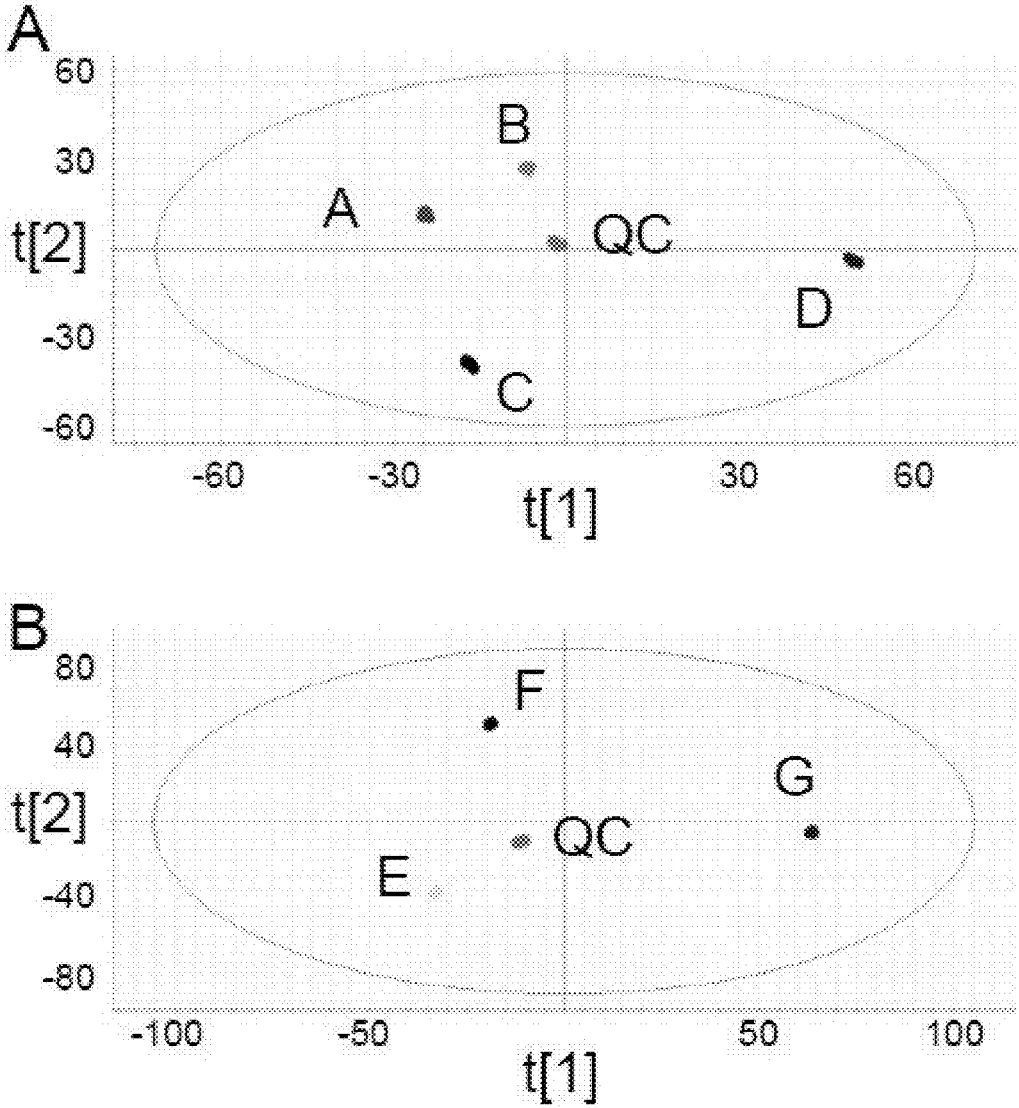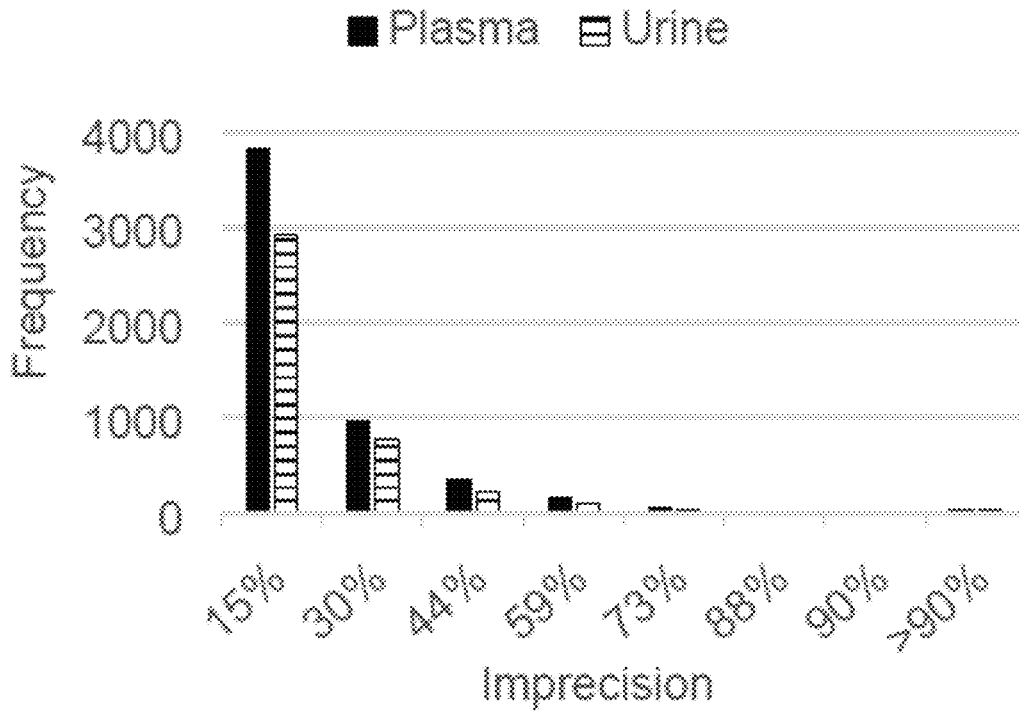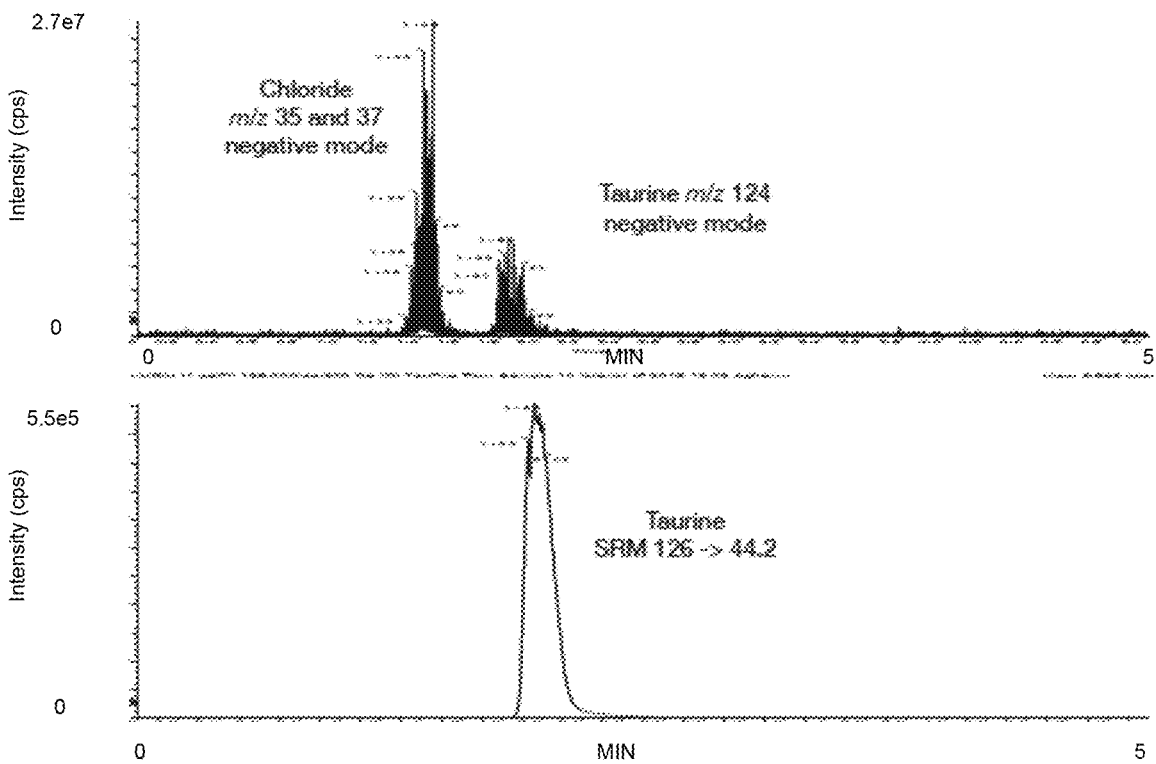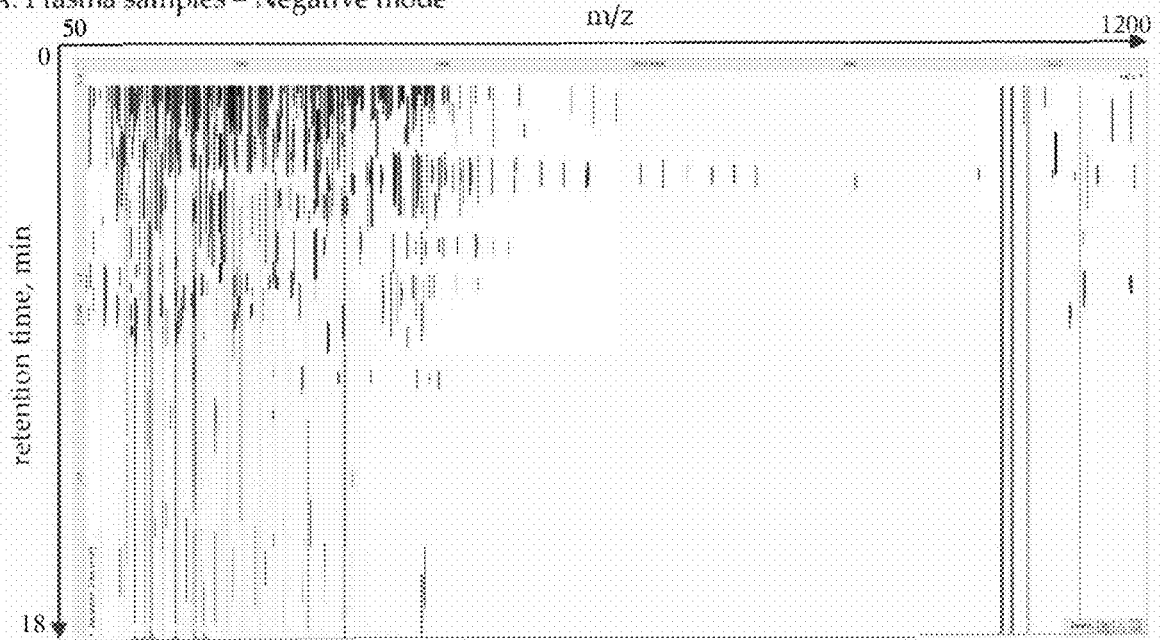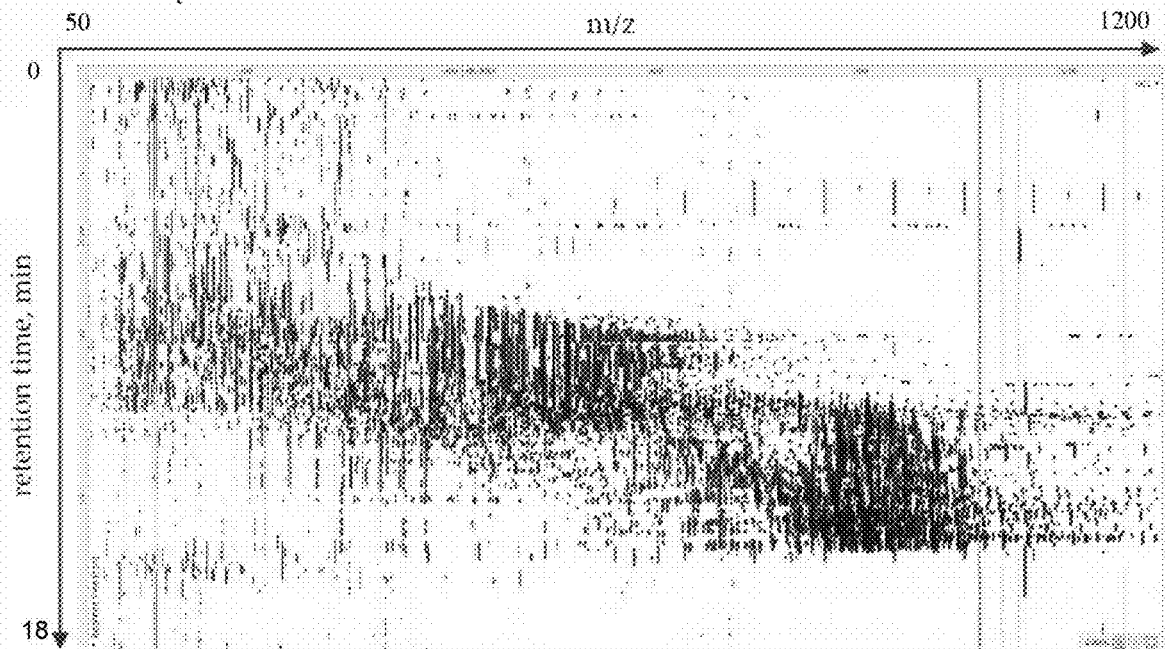

FIGURE 6

FIGURE 7A

A. Plasma samples – Negative mode

FIGURE 7B

B. Plasma Samples – Positive Mode

C. Urine Samples – Negative Mode   **FIGURE 7C**
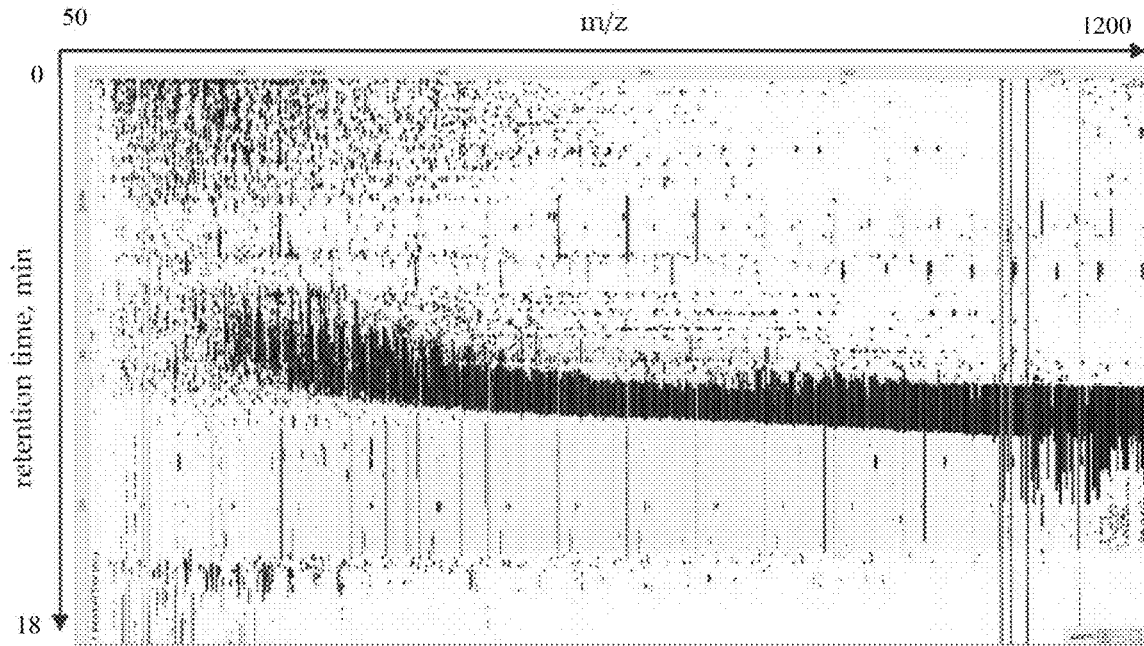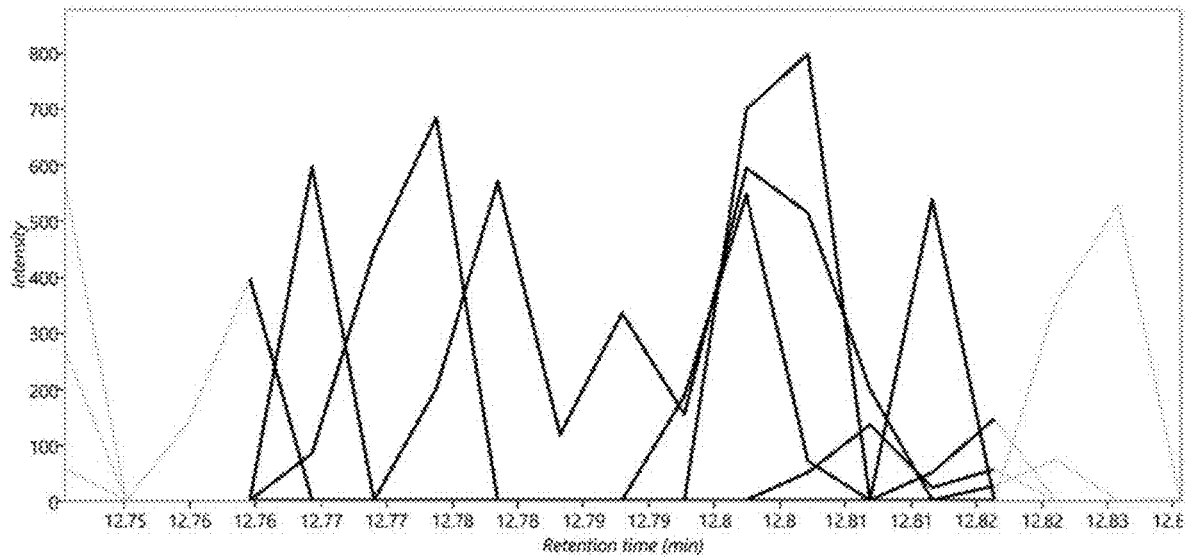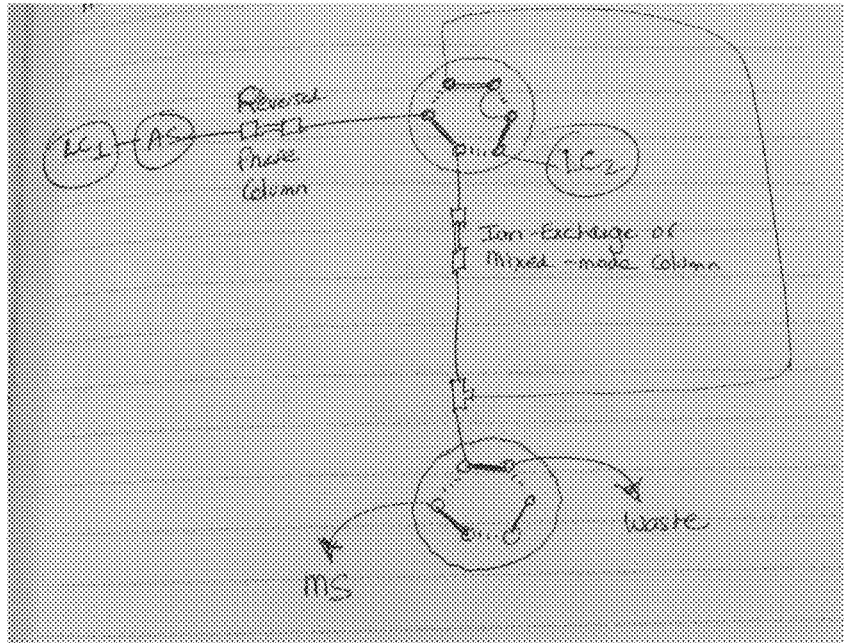

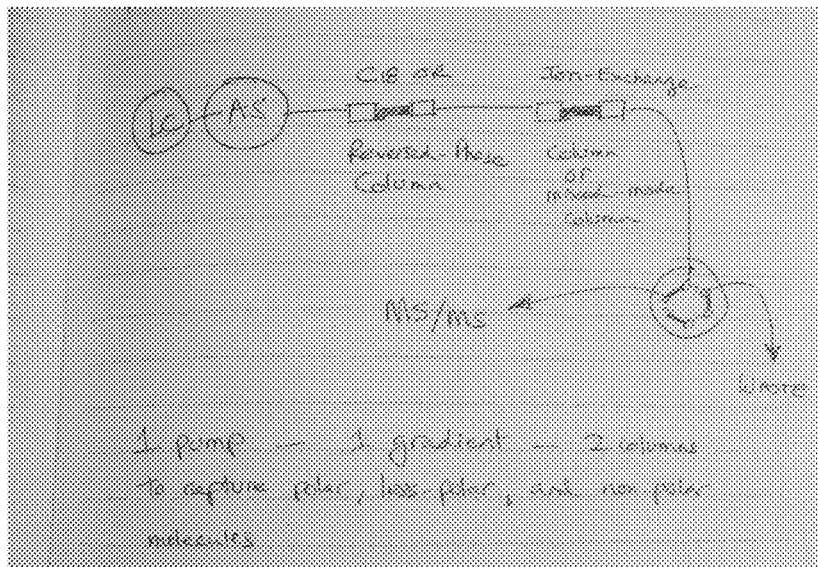**FIGURE 8**

FIGURE 9

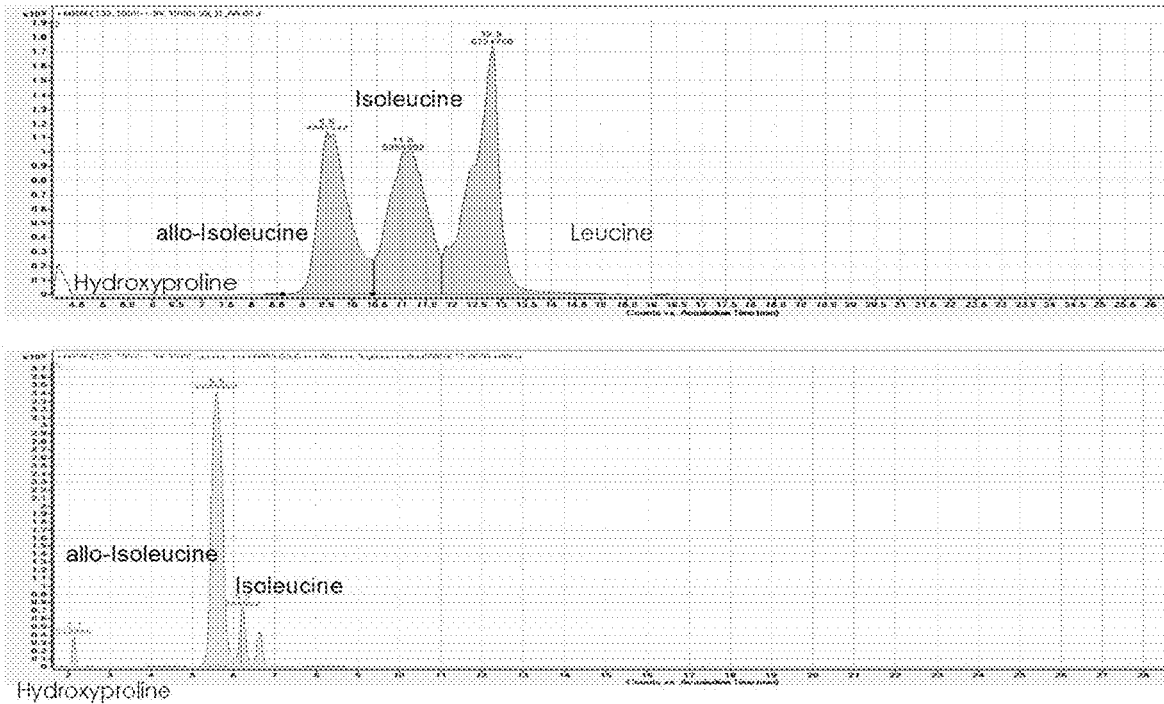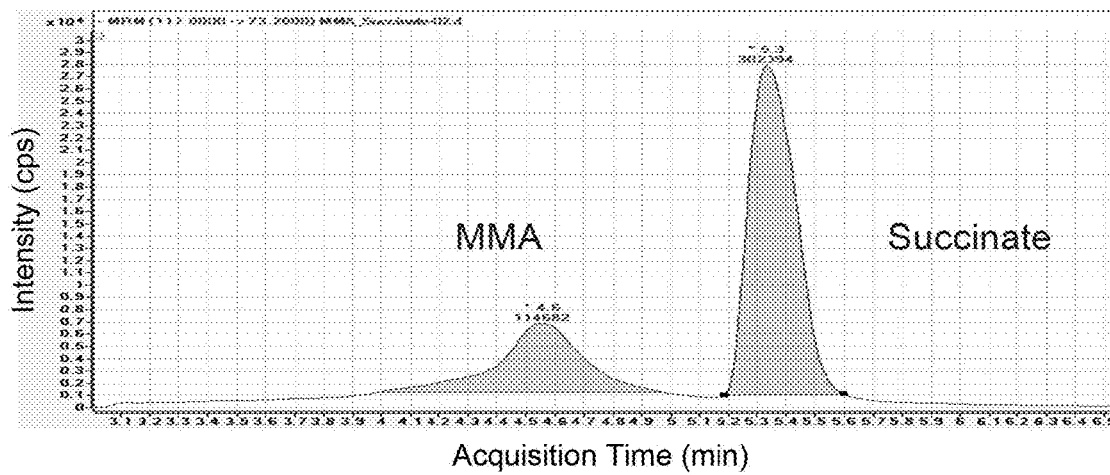**FIGURE 10**

FIGURE 11

FIGURE 12

METABOLIC PROFILING BY REVERSE-PHASE/ION-EXCHANGE MASS SPECTROMETRY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of and priority to U.S. Patent Application No. 62/992,383, filed on Mar. 20, 2020, the contents of which are incorporated herein in their entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with Government support under contract HD081355 awarded by the National Institutes of Health. The Government has certain rights in the invention.

BACKGROUND

The human metabolome encompasses lipids, carbohydrates, and metabolic intermediates (e.g., organic acids, amino acids, and acylcarnitines). Detection of these diverse compound classes using liquid chromatography-mass spectrometry (LC-MS) currently requires multiple chromatographic techniques. Commonly, lipidomic methods use reversed-phase (RP) chromatography, hydrophilic interaction chromatography (HILIC), or direct infusion; and glycomic methods use HILIC. Because methods based on either RP or HILIC alone can miss key metabolites, results from the independent use of these two approaches are often combined to capture and detect the full range of compounds by full-scan MS. However, this approach requires separate sample preparations for each chromatographic technique, and leads to overlapping datasets (i.e., the same metabolite being detected on both techniques) that must be meticulously curated to achieve a single, unique result set.

Various chromatographic strategies have been investigated to address limitations of the independent use of RP and HILIC. These include RP methods using mobile phase modifiers, such as ion-pairing reagents or ammonium fluoride, and columns with increased polar retention, such as C18-pentafluorophenyl (PFP) and porous graphitic carbon (Hypercarb), as well as combined RP-HILIC or HILIC-RP arrangements. While these strategies expand metabolome coverage, they are unable to resolve key pathognomonic metabolites (e.g., alloisoleucine, seen in maple syrup urine disease) without sacrificing negative mode ionization, or they require at least two LC systems to overcome mobile phase incompatibility. Ion-exchange (IEX) chromatography and mixed-mode IEX have also been investigated to widen metabolite coverage, especially to retain highly charged metabolites, but, under the conditions studied, were associated with prolonged retention of hydrophobic or highly charged compounds, or the lack of hydrophobic retention.

Alternatively, an in-line dual-column IEX-RP configuration using a single LC system has been used to increase peak capacity in proteomic applications. and the RP column would separate the remaining, less polar metabolites. By pairing RP with IEX, it was predicted that both polar and non-polar metabolites should bind to and elute from their appropriate columns, resulting in expanded metabolite coverage with one LC system.

It is against this background that a need arose to develop the embodiments described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an embodiment of the resolution of isomers. Extracted ion chromatograms of isomers commonly found

in metabolic profiling. Authentic standards were used and are listed in the figure. Malic acid undergoes in-source fragmentation and can interfere with the analysis of fumaric acid or maleic acid [38]

FIG. 2 shows an embodiment of the separation of polar and non-polar metabolites. Overlaid extracted ion chromatograms of standards demonstrate the wide-polarity range of compounds that can be separated by this RP-IEX method. (1) uracil, (2) orotic acid, (3) 2-ketoglutaric acid, (4) acetylglycine, (5) uric acid, (6) pyroglutamic acid, (7) uracil 5-carboxylic acid, (8) adenosine monophosphate, (9) propionylglycine, (10) xanthine, (11) 2-hydroxybutyric acid, (12) aspartic acid, (13) cyclic adenosine monophosphate, (14) asparagine, (15) glutamic acid, (16) threonine, (17) glutamine, (18) proline, (19) guanosine, (20) adipic acid, (21) citrulline, (22) methionine, (23) cysteine, (24) tyrosine, (25) phenylpyruvic acid, (26) homocitrulline, (27) guanidinoacetic acid, (28) creatine, (29) biotin, (30) hexanoylglycine, (31) phenylpropionylglycine, (32) suberic acid, (33) adenosine, (34) carnitine, (35) glutarylcarnitine, (36) deoxycytidine, (37) methylthioadenosine, (38) tryptophan, (39) propionylcarnitine, (40) cystine, (41) cholic acid, serotonin, (43) argininosuccinic acid, (44) stearoylcarnitine, (45) histidine, (46) lysine, and (47) arginine. Preliminary annotations of plasma samples show that lipids might elute between 13 to 16 min (Table 5).

FIG. 3 shows an embodiment of Retention Time Stability. Retention time stability was evaluated by injecting each sample five times. For eachanalyte, all injections were overlaid (plasma: n=25; urine: n=20). (1) succinic acid, (2) methylmalonic acid, (3) methylsuccinic acid, (4) ethylmalonic acid, (5) 2-methylbutyrylglycine, and (6) isovalerylglycine were from urine samples, and (7) glutamic acid, (8) tyrosine, (9) alloisoleucine, (10) isoleucine/leucine, (11) phenylalanine, and (12) propionylcarnitine were from plasma samples.

FIG. 4 shows an embodiment of the assessment of data quality by principal component analysis. Data quality was evaluated by unsupervised principal component analysis. (A) Five plasma and (B) four urine samples were each injected five times. Principal components one (t[1]) and two (t[2]) are shown. Groups A-G each represent individual metabolic diseases.

FIG. 5 shows an embodiment of the frequency of peak area precision. Histogram of peak area CVs calculated from five replicate injections of the plasma (black) or urine (stripe) QC sample. The distributions were calculated from 5,445 and 4,111 features for plasma and urine, respectively.

FIG. 6 shows an embodiment of the separation of chloride and taurine.

FIG. 7 shows an embodiment of ion intensity maps from Progenesis for the plasma samples, acquired with one injection beginning with (a) negative mode (480 ions) and then switching to (b) positive mode (7092 ions), and for the (c) urine samples acquired in negative mode (7371 ions) are shown. The detected ion features are indicated in blue.

FIG. 8 shows an embodiment of the method precision evaluated using the plasma QC samples. The chromatogram shows peak integrations (black traces; all five injections are shown) of an ion feature that were discarded from the precision study in FIG. 5. Of the five replicate injections, this feature had a maximum abundance of 83.

FIG. 9 shows an embodiment of a device used for the present methods with a switching valve between the two columns. In some embodiments this type of device is suitable for universal metabolomic analysis with two in-

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series columns with different functionalities by LC-MS/MS. As shown in FIG. **9**, an autosampler (AS) is upstream of the two in-series columns.

FIG. **10** shows an embodiment of a device used for the present methods without a switching valve between the two columns. In some embodiments this type of device is suitable for universal metabolomic analysis with two in-series columns with different functionalities by LC-MS/MS. As shown in FIG. **10**, an autosampler (AS) is upstream of the two in-series columns.

FIG. **11** shows the separation of isomers of leucine using embodiments of this disclosure.

FIG. **12** shows the separation of MMA and succinate using embodiments of this disclosure.

DETAILED DESCRIPTION

The present disclosure includes embodiments directed to methods of analyzing a biological sample comprising: separating components of the biological sample via reversed-phase (RP) chromatography to obtain an elute; subjecting the elute to separation via ion-exchange (IEX) chromatography or mixed-mode IEX chromatography; and detecting the separated compounds to determine the components of the biological sample. In some embodiments, the biological sample is obtained from a subject having or suspected of having a metabolic disruption. In some embodiments, the biological sample is a plasma sample or a urine sample. In some embodiments, the biological sample comprises lipids, carbohydrates, and metabolic intermediates. In some embodiments, the biological sample comprises polar and non-polar metabolites. In some embodiments, the detecting step is performed using mass spectrometry. In some embodiments, the detecting step includes qualitative analysis. In some embodiments, the biological sample is separated in the RP chromatography and IEX chromatography with one solvent gradient. In some embodiments, there is no switching valve between the RP chromatography and IEX chromatography. In some embodiments, isomers of metabolites in the biological sample are separated. Representative metabolites are described in the following sections and tables.

Other embodiments include a device comprising a reversed-phase (RP) chromatography column in communication with an ion-exchange (IEX) chromatography column or mixed-mode IEX chromatography column, wherein there is no switching valve between the columns. In some embodiments, the device is configured to inject a biological sample into the reversed-phase (RP) chromatography column and the elute from the RP chromatography column is transported directly to the IEX chromatography column or mixed-mode IEX chromatography column. In some embodiments, the device is in communication with a mass spectrometer configured to measure the mass of components exiting the IEX chromatography column or mixed-mode IEX chromatography column. In some embodiments, the mass spectrometer is a triple quadrupole mass spectrometer. Representative aspects of the device are also described in the following sections and tables and figures.

The following examples are given for the purpose of illustrating various embodiments of the disclosure and are not meant to limit the present disclosure in any fashion. One skilled in the art will appreciate readily that the present disclosure is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. The present examples, along with the methods described herein are presently

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representative of embodiments and are exemplary, and are not intended as limitations on the scope of the disclosure. Changes therein and other uses which are encompassed within the spirit of the disclosure as defined by the scope of the claims will occur to those skilled in the art.

Examples

Materials and Methods

Materials

LC-MS grade methanol and formic acid were purchased from Fisher Scientific (Chino, CA). LC-MS grade water, HPLC grade acetonitrile, and HPLC grade isopropanol were purchased from VWR (Visalia, CA). LC-MS grade ammonium formate salt, high-purity ammonium hydroxide (25% v/v), and Pall Omega 3-kDa filters were purchased from Sigma-Aldrich (St. Louis, MO). The Mass Spectrometry Metabolite Library of Standards was purchased from IROA Technologies (Bolton, MA). All other standards were purchased from Sigma-Aldrich. MS calibration and reference mass solutions were purchased from Agilent Technologies (Santa Clara, CA).

Liquid Chromatography

LC separations were performed on an Agilent 1290 Quaternary LC system (Agilent Technologies, CA). The RP column, a 2.1×50 mm 1.8-micron HSS T3 (Waters, MA), was placed before the IEX column, a 2.0×30 mm 3-micron Intrada Amino Acid (Imtakt, OR). The RP column was protected with an EXP2 filter and both columns were joined with EXP2 fittings (Optimize Technologies, OR).

Chromatographic Gradient Optimization

A screening approach was used for optimization of the IEX column. The gradient table used for optimization is detailed in Table 1. The flow rate was 0.5 mL/min. The column compartment temperature was set to 45° C. The injection volume was 5 μL. The total run time was 20 minutes, inject-to-inject.

The optimized mobile phases were (A) 150 mg of ammonium formate per liter of water with 0.4% formic acid (v/v), (B) 1.2 g of ammonium formate per liter of methanol with 0.2% formic acid (v/v), and (C) water with 1% each of formic acid and ammonium hydroxide (v/v). The optimized gradient table started at 2.5% B and 0% C, then ramped to 4% B and 0% C at 3 min, 25% B and 0% C at 7 min, 95% B and 0% C at 10.8 min, 97% B and 0% C at 15.1 min, 10% B and 90% C at 15.4 min, which was held for 1.2 min, and ended with re-equilibration at the initial conditions from 16.65 to 19.5 min.

Quadrupole Time of Flight (QTOF) MS

Mass spectrometry was performed on an Agilent 6545 QTOF (Agilent Technologies, CA) equipped with dual Agilent JetStream electrospray ionization (ESI). The 6545 was operated in sensitivity-mode, with extended dynamic range enabled, and was set to acquire profile-mode data from 60-1100 m/z at a cycle time of 250 ms. The mass resolution was 11,000 at 120 m/z. Reference mass calibration solution was infused by the on-board calibrant delivery system. The source temperature was 150° C., the gas flow was 8 L/min, the nebulizer pressure was 30 psig, the sheath gas temperature was 400° C., and the sheath gas flow was 8 L/min. The capillary voltages were set to +3000 and −2500 for positive and negative polarity ionization, respectively. The LC stream was directed into the QTOF at 0.4 min. While many clinically relevant metabolites evaluated in plasma can be detected in positive polarity ionization, certain polar acids require negative polarity ionization. Therefore, to analyze plasma samples with just one injection, the MS was set to

negative polarity from 0.4 to 1.85 min to detect methylmalonic acid, and then set to positive polarity for the remainder of the acquisition. Because many clinically relevant organic acids evaluated in urine ionize better or solely in negative polarity, the urine samples were acquired in negative polarity.

Samples

A test mixture consisting of five organic acids (succinic acid, methylmalonic acid, methylsuccinic acid, ethylmalonic acid, sebacic acid; all 10 µM), eight amino acids (glutamine, sarcosine, alanine, methionine, alloisoleucine, isoleucine, leucine, arginine; all 50 µM), and stearoylcarnitine (3.8 µM) was made for chromatographic method optimization and diluted, like processing a plasma sample, before analysis.

Four plasma and three urine samples from patients with metabolic disruptions were obtained as residual, de-identified specimens following routine clinical testing in the Stanford Clinical Biochemical Genetics Laboratory.

Metabolite Extraction and Analysis

To process plasma samples, 50 µL of sample was vortex mixed with 150 µL of precipitation reagent (methanol: isopropanol:formic acid 2:1:0.1% v/v/v) and centrifuged at 3° C. for 30 minutes at 17,000 xg. The supernatant was diluted 1:5 with water and analyzed. For urine, 50 µL of sample was deproteinized by ultrafiltration (3-kDa) at 3° C. for 10 minutes at 17,000 xg. The filtrate was diluted 1:2 with 1% formic acid in water (v/v) and analyzed. Two quality control (QC) samples, one plasma and one urine, were created by pooling the four plasma and the three urine samples described above. Analytical precision was assessed by injecting each sample, including the individual samples with metabolic disruptions, five times in a non-repeating sequence.

Data Analysis

Targeted qualitative data review was performed using MassHunter Qualitative 10 by searching the metabolite library of authentic standards (Agilent Technologies, CA). Run alignment, peak picking (automatic, level 4), adduct deconvolution, and feature identification using the library of authentic standards (10 ppm mass error and 0.5 min retention time error) were performed using Progenesis (Waters, MA). In both MassHunter and Progenesis, the adducts used for positive polarity analysis were [M+H], [M+NH4], and [M+Na], and the adduct used for negative polarity analysis was [M−H]. Preliminary annotation of ion features not in the library was performed using the HMDB [Nucleic Acids Res., 46 (2018) D608-D617] and KEGG [Nucleic Acids Res., 28 (2000) 27-30] plug-ins in Progenesis with search settings set to 10 ppm mass error. Unsupervised principle component analysis (PCA), with automatic log transformation and pareto scaling selected, was performed using the EZ Info Progenesis module (Umetrics, SE). For plasma samples, the negative and positive mode datasets were merged using Excel (Microsoft, WA) and then the combined dataset was imported into EZ Info for data analysis. The peak area coefficients of variation (CVs) were calculated using Excel.

Chromatographic Method Development

Two details were realized at the inception of this study. First, analytical performance would benefit from de-coupling the mixed-mode RP-IEX design. Placement of a robust RP column rated to 18,000 PSI before the IEX column, which are typically rated to 6,000 PSI, would enable faster run-times since the RP column would absorb much of the LC pressure. Furthermore, this allowed us to dictate each column's length, and thus better balance chromatographic

resolution with analytical speed. Longer columns delayed both the elution of non-polar compounds captured by the RP column and highly basic metabolites captured by the IEX column (Data not shown). Second, a quaternary LC system would be necessary in some embodiments. Along with the our experience, one publication using a mixed mode C18-IEX column for small molecule separations suggested that a binary solvent system would be insufficient to control the two orthogonal stationary phases [Anal. Chem., 83 (2011) 2152-2161]. An organic gradient would be needed to control elution on the RP column and a volatile salt gradient would simultaneously be needed to control the IEX column.

Method development was focused on understanding IEX separations for small molecules. During initial screening, it was confirmed that the IEX column captured amino acids using an acidic, low salt aqueous mobile phase. Importantly, it was learned that the most basic amino acids did not elute with high salt in methanol, but did elute with high salt in water.

Fifteen combinations of ammonium formate (0, 50, 100, 150, 300 mg/L) and formic acid (0.1%, 0.2%, 0.4%) for mobile phase A were evaluated to determine their influence on the retention times and peak areas of compounds in the test mixture (Table 2). For consistency, the methanol gradient, mobile phases B and C, and the 20-minute program time were unchanged. Results demonstrate that increasing both ionic strength and formic acid did not impact the retention times of compounds strongly retained by the RP column, such as sebacic acid and stearoylcarnitine, but did decrease their sensitivities. Concurrently, increasing formic acid increased retention of polar organic acids and amino acids, but decreased the sensitivity of the polar amino acids. For both polar and neutral amino acids, increasing ionic strength decreased their retention, but did not greatly affect their sensitivity until the ionic strength increased to 300 mg of ammonium formate per liter of water. Unexpectedly, increasing ionic strength nearly baseline resolved the leucine isomers (Condition 13: 300 mg of ammonium formate per liter of water and 0.1% formic acid). However, Condition 13 could not be used due to unacceptable retention and sensitivity for other polar compounds. In sum, only Condition 12 (150 mg of ammonium formate per liter of water and 0.4% formic acid) provided resolution of succinic acid from methylmalonic acid, methylsuccinic acid from ethylmalonic acid, and alloisoleucine from isoleucine and leucine with sufficient sensitivity. For final adjustments, the proportion of methanol was decreased to 25% at 7 min for additional RP resolution and the ionic strength of mobile phase B was slightly increased for sharper amino acid peaks.

Resolution of isomers is crucial for metabolic profiling. For example, 2-methylbutyrylglycine and isovalerylglycine, both 5-carbon glycine conjugates, require separation because they represent distinct pathways in branched-chain amino acid metabolism. In another example, alloisoleucine requires separation because it is pathognomonic for maple syrup urine disease. FIG. 1 shows the separation of select isomers relevant to human metabolism. The separation of additional isomers is provided in the library (Table 3).

The limited commercial selection of similar IEX columns compatible with organic solvents severely limited exploration of the RP-IEX dynamic. Although the Imtakt Intrada Amino Acid column chemistry is proprietary, it is possible that it predominately consists of negatively charged functional groups that can capture the positively charged amino acids. Attempts to separate amino acids using the HP-SCX column from Sepax Technologies (Newark, DE) resulted in excessive retention of many amino acids. Therefore, the

synthesis of a more optimal IEX stationary phase represents a clear approach to increasing chromatographic performance.

Metabolite Library

A library with 397 metabolites was generated using authentic standards (Table 3). This method captures a wide polarity-range of analytes, including acylcarnitines, amino acids, bile acids, nucleosides, organic acids, steroid hormones, and vitamin cofactors (FIG. 2). Because it captures compounds based on both hydrophobicity and charge, two main chemical properties governing retention, this chromatographic arrangement likely can retain many additional compounds. Nonetheless, sugars and compounds with multiple phosphate groups, which are negatively affected by trace metals in the LC system [J. Sep. Sci., 28 (2005) 1823-1830], were not retained by this method. Studies are underway to address this limitation.

Metabolite Detection with Human Samples

Clinical metabolic profiling is typically performed using plasma and urine samples, both containing metabolites that require both positive and negative mode ionization. Since certain embodiments of the method does not use ion-pairing reagents, both ionization polarities can be used. Overall ion feature detection at clinically relevant concentrations was evaluated by analyzing plasma and urine samples with specific metabolic disruptions, as well as corresponding plasma or urine QC pools. Samples were selected based on the presence of clinically relevant polar and non-polar metabolites, ranging from methylmalonic acid to alloisoleucine to stearoylcarnitine. In total, Progenesis detected 7,572 ion features for the plasma samples and 7,371 ion features for the urine samples (FIG. 7). However, manual peak review showed that ion features with median peak areas below 100 (2,127 features for plasma and 3,260 features for urine) were essentially noise (FIG. 8). In total, 5,445 and 4,111 ion features for the plasma and urine groups respectively were not marked as noise.

Metabolite identification in the plasma and urine QC samples, which contained pathologic compounds at diluted concentrations and endogenous compounds at physiologic concentrations, using a library of authentic standards revealed 88 and 82 metabolites, respectively (Table 4). This method detected metabolites such as methylmalonic acid, glutamine, glycine, citrulline, methionine, alloisoleucine, arginine, creatine, carnitine, propionylcarnitine, stearoylcarnitine, 2-methylcitric acid, propionylglycine, 2-methylbutyrylglycine, 3-methylcrotonylglycine, adipic acid, suberic acid, and glutaric acid. Notably, plasma samples were evaluated at a 1:20 final dilution to reflect simple and rapid sample processing desired by clinical laboratories. Despite the dilution factors, the peak areas for most metabolites still ranged from 104 to 106. Should higher sensitivity be necessary, an evaporation step could be incorporated.

While in some embodiments the method was not intended for lipidomic applications, many semi-lipophilic metabolites were detected in some embodiments. Stearoylcarnitine is one the most non-polar metabolic intermediates in the library and has a retention time of 12.8 min. Therefore, to elute additional semi-lipophilic metabolites, RP regeneration was extended to 15.1 min. An identification search of m/z features in plasma (FIG. 7b) eluting between minutes 13 and 16 mainly resulted in preliminary annotations for lipids. For example, six lipids were listed as potential annotations for a feature eluting at 14.47 min with a m/z of 844.5093 (Table 5). After five non-sequential replicate injections, this feature had a peak area CV of 4.7% (average peak area=29, 235), indicating consistent elution. Based on these observations, using isopropanol in the fourth solvent channel may expand lipid coverage.

Data Quality of the LC-QTOF Method

Consistent retention times and compound ionization can be critical for peak alignment and downstream reporting quality [Metabolomics, 14 (2018) 1-17]. Within-run analytical precision was evaluated by injecting each sample five times, totaling 45 injections. FIG. 3 shows overlaid extracted ion chromatograms of select compounds for all injections, demonstrating consistent retention times and peak areas throughout the acquisition. Retention times remained within a 0.5-minute retention time window at least one month after generation of the in-house library, indicating good retention time stability.

Next, overall within-run analytical data quality was assessed by unsupervised PCA, including features marked as noise. FIG. 4 shows that the replicate injections overlap, indicating excellent analytical quality. Precision was assessed by calculating the peak area CVs of the QC samples, excluding median peak areas below 100 (FIG. 5). Of the remaining 5,445 features in the plasma QC group, 88% had CVs<30% and 79% had CVs<20%. Of the remaining 4,111 features in the urine QC group, 91% had CVs<30% and 81% had CVs<20%. This precision meets current QC guidelines and is comparable to current RP-HRMS methods, where 70-90% of the features detected in the QC group have CVs<30% [Bioanalysis, 4 (2012) 2249-2264.].

While the two columns contain distinct stationary phases, the possibility of disturbed metabolite elution due to the in-line column configuration was investigated by analyzing the peak area precision of select acylcarnitines, which are first retained by the RP column and then go through the IEX column, and amino acids, which pass through the RP column and are retained by the IEX column (Table 6). The plasma QC data showed CVs less than 4% for propionylcarnitine, palmitoylcarnitine, and stearoylcarnitine, suggesting that the IEX column is unlikely to interfere with the elution of compounds first retained by the RP column. For amino acids, the peak area CVs ranged from 1.7% to 6.9% for glutamine, alanine, citrulline, methionine, alloisoleucine, leucine, and tryptophan, demonstrating that the RP column does not interfere with the movement of amino acids onto the IEX column. This data and the high-quality peaks presented in FIGS. 1 and 2 suggest that the in-line column configuration is unlikely to interfere with metabolite elution.

CONCLUSION

The chromatographic design outlined here underscores the critical role of chromatography in metabolic profiling. In-line RP-IEX chromatography enables the separation of a wide polarity range of clinically relevant metabolic intermediates, including isomers, in 20-minutes with one LC-MS system. Notably, the simple RP-IEX setup retains and separates amino acids, acylcarnitines, and organic acids, which currently require multiple LC systems or independent RP and HILIC analyses. Each column can be independently controlled and behaves synergistically to increase the number of detected metabolites. The method showed high analytical quality and provided suitable sensitivity for human plasma and urine samples. Because ion-pairing reagents are not used, the MS system can be used in both negative and positive polarity. For targeted applications, the LC method can be coupled to a triple quadrupole mass spectrometer, which can provide enhanced sensitivity, precision, dynamic range, and rapid polarity switching.

Gradient Program Used for LC Optimization

Mobile phase A was optimized as indicated in Table 2. Mobile phase B contained 1 g of ammoniumformate per liter of methanol and 0.2% formic acid (v/v) and mobile phase C contained water with 1% each of formic acid and ammonium hydroxide (v/v).

TABLE 1

Gradient program used for LC optimization

Time, min	% A	% B	% C	% D
0	97.5	2.5	0	0
3	96	4	0	0
8	65	35	0	0
10.9	5	95	0	0
15.1	3	97	0	0
15.5	0	10	90	0
16.6	0	10	90	0
16.65	97.5	2.5	0	0
19.5	97.5	2.5	0	0

LC Optimization Results

The levels of ionic strength and formic acid were optimized for mobile phase A by tracking retention time (minutes) and peak area (in millions). LC conditions used for the optimization are detailed in Table 1.

For peaks without baseline resolution, the retention times are reported together and the peak areas are summed. AF, ammonium formate salt; formic acid, FA; SA, succinic acid; MMA, methylmalonic acid; MS, methylsuccinic acid; EMA, ethylmalonic acid; Seb, sebacic acid; Gln, glutamine; Sarc, sarcosine; Ala, alanine; Met, methionine; Aile, alloisoleucine; Ile, isoleucine; Leu, leucine; Arg, arginine; C18, stearoylcarnitine.

TABLE 2

LC Optimization results
Mobile Phase A Retention time, min

Condition	AF (mg/L)	FA % (v/v)	SA	MMA	MS	EMA	Seb	Gln	Sarc	Ala	Met	Aile	Ile	Leu	Arg	C18
1	0	0.1	0.89	0.96	2.2		10.6	2.8	2.9	4.2	7.3		11.4		17.4	13.1
2	0	0.2	0.90	1.00	2.3	2.6	10.6	3.1	3.4	4.4	8.0		11.8		17.4	13.1
3	0	0.4	0.88	1.10	2.5	2.8	10.6	3.3	3.3	4.4	7.8		11.9		17.4	13.1
4	50	0.1	0.90		2.1		10.6	2.4	2.4	3.5	6.2	9.7	10.8		17.4	13.1
5	50	0.2	0.90	1.00	2.2	2.5	10.6	2.7	2.8	4.0	6.8	10.2	11.1		17.4	13.1
6	50	0.4	0.88	1.00	2.4	2.7	10.6	3.0	3.0	4.1	6.8	10.0	11.4		17.4	13.1
7	100	0.1	0.90		1.8		10.6	2.1	2.1	3.1	5.3	7.8	9.1		17.4	13.1
8	100	0.2	0.90		2.2		10.6	2.7	2.7	3.7	5.8	8.6	9.9		17.4	13.1
9	100	0.4	0.88	1.00	2.4		10.6	2.9	2.9	3.8	5.6	8.8	9.9		17.4	13.1
10	150	0.1	0.89		1.9	2.2	10.6	1.9	1.9	2.7	4.6	6.5	7.4		17.4	13.1
11	150	0.2	0.89	0.96	2.1		10.6	2.2	2.2	3.1	5.1	7.4	8.4		17.4	13.1
12	150	0.4	0.88	1.00	2.1	2.3	10.6	2.5	2.6	3.4	5.1	7.7	8.5		17.4	13.1
13	300	0.1	0.83		1.7	2.0	10.6	1.3	1.3	1.7	2.7	3.9	4.2	4.5	17.4	13.1
14	300	0.2	0.92		2.0		10.6	1.6	1.6	2.1	3.9		5.1	5.7	17.4	13.1
15	300	0.4	0.87	0.97	2.0	2.2	10.6	1.9	1.9	2.5	4.1	5.7	6.0	6.6	17.4	13.1
1	0	0.1	0.17	0.99	7.40		0.46	4.00	2.94	1.66	1.14		0.22		0.65	5.21
2	0	0.2	0.18	0.99	3.51	0.48	0.35	0.90	1.35	0.33	0.41		0.11		1.70	4.30
3	0	0.4	0.19	0.87	1.40	0.19	0.34	0.34	0.84	0.14	0.20		0.62		1.80	3.70
4	50	0.1	1.30		3.64		0.48	3.34	2.45	1.09	1.00	0.61	0.95		2.00	3.34
5	50	0.2	0.26	1.08	5.54	0.59	0.07	1.07	1.41	0.34	0.44	0.26	0.11		1.89	3.05
6	50	0.4	0.24	0.85	0.92	0.27	0.32	0.35	0.87	0.15	0.25	0.60	0.50		1.90	3.20
7	100	0.1	1.46		4.55		0.04	3.50	2.64	1.17	1.08	0.75	1.22		0.90	1.60
8	100	0.2	0.28	1.19	2.50		0.05	0.88	1.44	0.32	0.43	0.21	1.02		2.10	1.43
9	100	0.4	0.23	0.87	1.62		0.30	0.33	0.90	0.14	0.31	0.83	0.91		2.10	1.41
10	150	0.1	1.14		6.75	3.47	0.05	2.98	2.14	0.85	0.69	0.54	1.04		1.88	1.09
11	150	0.2	0.24	1	7.37		0.04	1.00	1.31	0.28	0.37	0.31	0.85		1.79	1.25
12	150	0.4	0.24	0.8	0.20	0.34	0.04	0.30	0.79	0.12	0.25	0.62	0.83		1.90	1.05
13	300	0.1	1.09		0.94	0.21	0.09	1.71	1.56	0.39	0.68	0.57	0.48	0.80	1.87	0.87
14	300	0.2	1.2		0.43		0.09	0.48	0.92	0.13	0.28		0.80	0.29	0.80	0.48
15	300	0.4	0.24	0.45	0.15		0.08	0.18	0.60	0.09	0.26	0.42	0.52	0.90	1.30	0.51

TABLE 3

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
cysteic acid	C3H7NO5S	169.004	0.42
gluconolactone	C6H10O6	178.048	0.42
monoethylmalonic acid	C5H8O4	132.042	0.42
n-acetylneuraminic acid	C11H19NO9	309.106	0.42
phosphoserine	C3H8NO6P	185.009	0.42
rhamnose	C6H12O5	164.068	0.42
saccharic acid	C6H10O8	210.038	0.42
sulfocysteine	C3H7NO5S2	200.976	0.42
taurine	C2H7NO3S	125.014	0.42
glucuronic acid	C6H10O7	194.043	0.43
mesoxalate	C3H2O5	117.990	0.43
uridine diphosphate glucose	C15H24N2O17P2	566.055	0.43
gluconic acid	C6H12O7	196.058	0.44
galactaric acid	C6H10O8	210.038	0.45

TABLE 3-continued

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
uridine diphosphate-n-acetylgalactosamine	C17H27N3O17P2	607.082	0.45
uridine diphosphate-n-acetylglucosamine	C17H27N3O17P2	607.082	0.45
orotic acid	C5H4N2O4	156.017	0.47
meso-tartaric acid	C4H6O6	150.016	0.49
n-acetylmannosamine	C8H15NO6	221.090	0.49
adp-glucose	C16H25N5O15P2	589.082	0.50
dihydroorotic acid	C5H6N2O4	158.033	0.50
n-acetylgalactosamine	C8H15NO6	221.090	0.50
n-formylglycine	C3H5NO3	103.027	0.50
phosphoethanolamine	C2H8NO4P	141.019	0.50
quinic acid	C7H12O6	192.063	0.50
uracil	C4H4N2O2	112.027	0.50
uridine monophosphate	C9H13N2O9P	324.036	0.50
malonic acid	C3H4O4	104.011	0.51
isocitric acid	C6H8O7	192.027	0.55
malic acid	C4H6O5	134.022	0.55
n-acetylasparagine	C6H10N2O4	174.064	0.55
2-ketoglutaric acid	C5H6O5	146.022	0.55
shikimic acid	C7H10O5	174.053	0.57
acetylglycine	C4H7NO3	117.043	0.60
glucosamine 6-phosphate	C6H14NO8P	259.046	0.60
pyruvic acid	C3H4O3	88.016	0.60
inosine monophosphate	C10H13N4O8P	348.047	0.62
ureidopropionic acid	C4H8N2O3	132.053	0.63
2-propenoic acid	C3H4O2	72.021	0.64
lactic acid	C3H6O3	90.032	0.64
maleic acid	C4H4O4	116.011	0.64
phosphorylcholine	C5H14NO4P	183.066	0.64
xanthosine-monophosphate	C10H13N4O9P	364.042	0.64
3-dehydroshikimic acid	C7H8O5	172.037	0.65
maleamic acid	C4H5NO3	115.027	0.65
nicotinamide mononucleotide	C11H15N2O8P	334.057	0.65
n-acetylaspartic acid	C6H9NO5	175.048	0.67
5-aminoimidazole-4-carboxamide ribonucleotide	C9H15N4O8P	338.063	0.68
dtdp-d-glucose	C16H26N2O16P2	564.076	0.69
guanosine monophosphate	C10H14N5O8P	363.058	0.70
3,4 dihydroxymandelate	C8H8O5	184.037	0.73
uric acid	C5H4N4O3	168.028	0.73
citric acid	C6H8O7	192.027	0.75
quinolinic acid	C7H5NO4	167.022	0.75
2-oxoadipic acid	C6H8O5	160.037	0.77
3,4-dihydroxyphenylglycol	C8H10O4	170.058	0.82
aniline-2-sulfonate	C6H7NO3S	173.015	0.83
deoxycytidine monophosphate	C9H14N3O7P	307.057	0.83
pyroglutamic acid	C5H7NO3	129.043	0.83
2-methylmaleic acid	C5H6O4	130.027	0.84
succinic acid	C4H6O4	118.027	0.85
uracil 5-carboxylic acid	C5H4N2O4	156.017	0.85
fumaric acid	C4H4O4	116.011	0.86
mevalolactone	C6H10O3	130.063	0.87
mevalonic acid	C6H12O4	148.074	0.88
monomethylglutaric acid	C6H10O4	146.058	0.88
n-acetylglutamic acid	C7H11NO5	189.064	0.88
ketoleucine	C6H10O3	130.063	0.89
3-methoxy-4-hydroxymandelic acid (VMA)	C9H10O5	198.053	0.90
4-hydroxy-3-methoxyphenylglycol	C9H12O4	184.074	0.90
adenosine-monophosphate	C10H14N5O7P	347.063	0.90
homogentisic acid	C8H8O4	168.042	0.90
uridine	C9H12N2O6	244.070	0.90
citramalic acid	C5H8O5	148.037	0.94
propionylglycine	C5H9NO3	131.058	0.95
deoxyguanosine-monophosphate	C10H14N5O7P	347.063	1.00
n-acetylalanine	C5H9NO3	131.058	1.00
thymidine-monophosphate	C10H15N2O8P	322.057	1.00
methylmalonic acid	C4H6O4	118.027	1.05
xanthine	C5H4N4O2	152.033	1.05
aconitic acid	C6H6O6	174.016	1.07
pyridoxal-phosphate	C8H10NO6P	247.025	1.07
itaconic acid	C5H6O4	130.027	1.10
3-hydroxymethylglutaric acid	C6H10O5	162.053	1.13
succinate semialdehyde	C4H6O3	102.032	1.15
2-hydroxybutyric acid	C4H8O3	104.047	1.18
deoxyuridine	C9H12N2O5	228.075	1.23

TABLE 3-continued

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
deoxyadenosine monophosphate	C10H14N5O6P	331.068	1.25
thymine	C5H6N2O2	126.043	1.25
2,4-dihydroxpteridine	C6H4N4O2	164.033	1.27
n-acetylcysteine	C5H9NO3S	163.030	1.30
4-acetamidobutanoic acid	C6H11NO3	145.074	1.32
n-methylaspartic acid	C5H9NO4	147.053	1.32
mevalonolactone	C6H10O3	130.063	1.37
2-methylcitric acid	C7H10O7	206.043	1.41
6-hydroxynicotinic acid	C6H5NO3	139.027	1.43
2,6-dihydroxypyridine	C5H5NO2	111.032	1.45
citicoline	C14H26N4O11P2	488.107	1.47
acetoin	C4H8O2	88.052	1.56
glutaric acid	C5H8O4	132.042	1.58
hydroxyproline	C5H9NO3	131.058	1.60
cyclic guanosine monophosphate	C10H12N5O7P	345.047	1.62
aspartic acid	C4H7NO4	133.038	1.66
s-carboxymethylcysteine	C5H9NO4S	179.025	1.67
cyclic adenosine monophosphate	C10H12N5O6P	329.053	1.69
inosine	C10H12N4O5	268.081	1.70
n-methylglutamic acid	C6H11NO4	161.069	1.72
4-pyridoxic acid	C8H9NO4	183.053	1.75
isobutyrylglycine	C6H11NO3	145.074	1.79
2-hydroxypyridine	C5H5NO	95.037	1.86
butyrylglycine	C6H11NO3	145.074	1.97
dimethylglycine	C4H9NO2	103.063	2.10
methylsuccinic acid	C5H8O4	132.042	2.15
asparagine	C4H8N2O3	132.053	2.20
sarcosine	C3H7NO2	89.048	2.20
serine	C3H7NO3	105.043	2.20
threonine	C4H9NO3	119.058	2.20
allothreonine	C4H9NO3	119.058	2.24
hypoxanthine	C5H4N4O	136.039	2.35
ethylmalonic acid	C5H8O4	132.042	2.40
glutamic acid	C5H9NO4	147.053	2.40
xanthosine	C10H12N4O6	284.076	2.41
glutamine	C5H10N2O3	146.069	2.50
3,4-dihydroxybenzoic acid	C7H6O4	154.027	2.57
pyrocatechol	C6H6O2	110.037	2.57
succinylacetone	C7H10O4	158.058	2.60
homoserine	C4H9NO3	119.058	2.65
thymidine	C10H14N2O5	242.090	2.82
ophthalmic acid	C11H19N3O6	289.127	2.85
2-hydroxy-3-methylbutyric acid	C5H10O3	118.063	2.90
2-aminoadipic acid	C6H11NO4	161.069	2.95
glycine	C2H5NO2	75.032	2.95
betaine	C5H11NO2	117.079	3.00
indoxyl sulfate	C8H7NO4S	213.010	3.00
2-hydroxy-4-(methylthio)butanoic acid	C5H10O3S	150.035	3.03
proline	C5H9NO2	115.063	3.10
o-succinyl-homoserine	C8H13NO6	219.074	3.14
n-formyl-l-methionine	C6H11NO3S	177.046	3.34
n-acetylproline	C7H11NO3	157.074	3.36
alanine	C3H7NO2	89.048	3.40
4-quinolinecarboxylic acid	C10H7NO2	173.048	3.46
cyanocobalamin	C63H89CoN14O14P	1355.575	3.47
glycerol 3-phosphate	C3H9O6P	172.014	3.47
pantothenic acid	C9H17NO5	219.111	3.53
3-hydroxybenzyl alcohol	C7H8O2	124.052	3.68
guaiacol	C7H8O2	124.052	3.68
histidinol	C6H11N3O	141.090	3.70
3,4-dihydroxyphenylacetic acid	C8H8O4	168.042	3.73
guanosine	C10H13N5O5	283.092	3.76
methylglutaric acid	C6H10O4	146.058	3.83
adipic acid	C6H10O4	146.058	3.86
2-aminobutyric acid	C4H9NO2	103.063	3.89
2-methylbutyrylglycine	C7H13NO3	159.090	3.91
mandelic acid	C8H8O3	152.047	4.00
2-aminoisobutyric acid	C4H9NO2	103.063	4.10
tiglylglycine	C7H11NO3	157.074	4.15
3-methyl-2-oxovaleric acid	C6H10O3	130.063	4.36
heptanoic acid	C7H14O2	130.099	4.36
hydroquinone	C6H6O2	110.037	4.40
4-hydroxybenzoic acid	C7H6O3	138.032	4.45
nicotinic acid	C6H5NO2	123.032	4.45

TABLE 3-continued

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
isovalerylglycine	C7H13NO3	159.090	4.46
2,5-dihydroxybenzoic acid	C7H6O4	154.027	4.48
3-methylcrotonylglycine	C7H11NO3	157.074	4.52
4-hydroxyphenylacetic acid	C8H8O3	152.047	4.59
citrulline	C6H13N3O3	175.096	4.60
l-dopa	C9H11NO4	197.069	4.65
1-aminocyclopropanecarboxylic acid	C4H7NO2	101.048	4.70
n-acetylmethionine	C7H13NO3S	191.062	4.76
theobromine	C7H8N4O2	180.065	4.78
valine	C5H11NO2	117.080	4.79
guanidinosuccinic acid	C5H9N3O4	175.059	4.80
glucosamine	C6H13NO5	179.079	5.05
d-mannosamine	C6H13NO5	179.079	5.10
valerylglycine	C7H13NO3	159.090	5.20
galactosamine	C6H13NO5	179.079	5.20
methionine	C5H11NO2S	149.051	5.22
2-acetamido-2-deoxy-beta-d-glucosylamine	C8H16N2O5	220.106	5.36
trigonelline	C7H7NO2	137.048	5.69
cysteine	C3H7NO2S	121.020	5.78
hippuric acid	C9H9NO3	179.058	5.80
4-hydroxybenzaldehyde	C7H6O2	122.037	5.84
2,3-dihydroxybenzoic acid	C7H6O4	154.027	5.90
3-hydroxybenzoic acid	C7H6O3	138.032	5.94
phenylpyruvic acid	C9H8O3	164.047	6.00
theophylline	C7H8N4O2	180.065	6.00
paraxanthine	C7H8N4O2	180.065	6.07
pipecolic acid	C6H11NO2	129.079	6.10
tyrosine	C9H11NO3	181.074	6.25
salicylamide	C7H7NO2	137.048	6.40
beta-alanine	C3H7NO2	89.048	6.58
xanthurenic acid	C10H7NO4	205.038	6.58
n-acetylserotonin	C12H14N2O2	218.106	6.60
homocitrulline	C7H15N3O3	189.111	6.62
caffeic acid	C9H8O4	180.042	6.75
n-acetylornithine	C7H14N2O3	174.100	6.79
6-carboxyhexanoic acid	C7H12O4	160.074	6.99
indole-3-acetamide	C10H10N2O	174.079	7.02
homovanillic acid	C9H10O4	182.058	7.20
4-aminobenzoic acid	C7H7NO2	137.048	7.24
kynurenic acid	C10H7NO3	189.043	7.27
3-hydroxyanthranilic acid	C7H7NO3	153.043	7.29
5-aminolevulinic acid	C5H9NO3	131.058	7.30
suberylglycine	C10H17NO5	231.111	7.30
2-quinolinecarboxylic acid	C10H7NO2	173.048	7.34
creatine	C4H9N3O2	131.069	7.50
guanidinoacetic acid	C3H7N3O2	117.054	7.50
caffeine	C8H10N4O2	194.080	7.53
pterin	C6H5N5O	163.049	7.53
dihydrobiopterin	C9H13N5O3	239.102	7.62
alloisoleucine	C6H13NO2	131.095	7.60
4-aminobutyric acid	C4H9NO2	103.063	7.75
biotin	C10H16N2O3S	244.088	7.77
3-methoxytyrosine	C10H13NO4	211.084	7.84
3-aminoisobutyric acid	C4H9NO2	103.063	8.05
4-coumaric acid	C9H8O3	164.047	8.20
thiopurine s-methylether	C6H6N4S	166.031	8.23
n-alpha-acetyllysine	C8H16N2O3	188.116	8.25
hexanoylglycine	C8H15NO3	173.105	8.30
n-acetylphenylalanine	C11H13NO3	207.090	8.58
isoleucine	C6H13NO2	131.095	8.50
leucine	C6H13NO2	131.095	8.50
phenylpropionylglycine	C11H13NO3	207.090	8.64
2',4'-dihydroxyacetophenone	C8H8O3	152.047	8.69
4-imidazoleacetic acid	C5H6N2O2	126.043	8.70
3-(2-hydroxyphenyl)propanoic acid	C9H10O3	166.063	8.73
salicylic acid	C7H6O3	138.032	8.78
riboflavin	C17H20N4O6	376.138	8.80
n-acetyltryptophan	C13H14N2O3	246.100	8.90
suberic acid	C8H14O4	174.089	8.98
dethiobiotin	C10H18N2O3	214.132	9.00
indoleacetaldehyde	C10H9NO	159.068	9.00
indole-3-ethanol	C10H11NO	161.084	9.05
melatonin	C13H16N2O2	232.121	9.26
3-methyl-2-oxindole	C9H9NO	147.068	9.37

TABLE 3-continued

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
norleucine	C6H13NO2	131.095	9.50
trans-cinnamaldehyde	C9H8O	132.058	9.58
cytidine	C9H13N3O5	243.086	9.60
rosmarinic acid	C18H16O8	360.085	9.61
lipoamide	C8H15NOS2	205.060	9.66
trans-cinnamic acid	C9H8O2	148.052	9.70
heptanoylglycine	C9H17NO3	187.121	9.73
azelaic acid	C9H16O4	188.105	9.77
adenosine	C10H13N5O4	267.097	9.80
folate	C19H19N7O6	441.140	9.80
3-nitro-l-tyrosine	C9H10N2O5	226.059	10.00
carnitine	C7H15NO3	161.105	10.00
deoxyadenosine	C10H13N5O3	251.102	10.00
guanine	C5H5N5O	151.049	10.00
indole-3-methyl acetic acid	C11H11NO2	189.079	10.00
nicotinamide	C6H6N2O	122.048	10.00
phenylalanine	C9H11NO2	165.079	10.00
5-hydroxytryptophan	C11H12N2O3	220.085	10.01
succinylcarnitine	C11H19NO6	261.121	10.01
glutarylcarnitine	C12H21NO6	275.137	10.09
1-methyladenosine	C11H15N5O4	281.112	10.10
octanoylglycine	C10H19NO3	201.136	10.17
deoxycytidine	C9H13N3O4	227.091	10.17
methylthioadenosine	C11H15N5O3S	297.090	10.17
tryptophan	C11H12N2O2	204.090	10.20
3-hydroxyisovalerylcarnitine	C12H23NO5	261.158	10.23
6-hydroxydopamine	C8H11NO3	169.074	10.24
noradrenaline	C8H11NO3	169.074	10.24
trans-cyclohexanediol	C6H12O2	116.084	10.24
urocanate	C6H6N2O2	138.043	10.24
3,5-diiodo-l-tyrosine	C9H9I2NO3	432.867	10.25
butyric acid	C4H8O2	88.052	10.25
diethanolamine	C4H11NO2	105.079	10.25
isobutyric acid	C4H8O2	88.052	10.25
sebacic acid	C10H18O4	202.121	10.25
cortisone	C21H28O5	360.194	10.29
acetylcarnitine	C9H17NO4	203.116	10.30
kynurenine	C10H12N2O3	208.085	10.32
2-methylpropanal	C4H8O	72.058	10.33
10-hydroxydecanoic acid	C10H20O3	188.141	10.37
pyridoxal	C8H9NO3	167.058	10.39
epinephrine	C9H13NO3	183.090	10.40
adenine	C5H5N5	135.054	10.42
cortisol	C21H30O5	362.209	10.42
thyrotropin releasing hormone	C16H22N6O4	362.170	10.48
methyl 4-aminobutyric acid	C5H11NO2	117.079	10.51
propionylcarnitine	C10H19NO4	217.131	10.54
cytosine	C4H5N3O	111.043	10.59
3,5-diiodo-l-thyronine	C15H13I2NO4	524.893	10.60
cystine	C6H12N2O4S2	240.024	10.60
n-acetylputrescine	C6H14N2O	130.111	10.60
normetanephrine	C9H13NO3	183.090	10.60
deoxycarnitine	C7H15NO2	145.110	10.66
pyridoxine	C8H11NO3	169.074	10.66
4-guanidinobutanoic acid	C5H11N3O2	145.085	10.70
corticosterone	C21H30O4	346.214	10.70
2-aminophenol	C6H7NO	109.053	10.75
1-hydroxy-2-naphthoic acid	C11H8O3	188.047	10.77
butyrylcarnitine	C11H21NO4	231.147	10.77
cortisol 21-acetic acid	C23H32O6	404.220	10.78
cortexolone	C21H30O4	346.214	10.80
dopamine	C8H11NO2	153.079	10.80
n-acetylleucine	C8H15NO3	173.105	10.83
creatinine	C4H7N3O	113.059	10.85
isovalerylcarnitine	C12H23NO4	254.219	10.90
homocysteine thiolactone	C4H7NOS	117.025	10.90
5-methylcytosine	C5H7N3O	125.059	10.95
methionine sulfoximine	C5H12N2O3S	180.057	10.95
liothyronine	C15H12I3NO4	650.790	10.96
saccharopine	C11H20N2O6	276.132	11.03
omega-hydroxydodecanoic acid	C12H24O3	216.173	11.05
tyramine	C8H11NO	137.084	11.08
3-hydroxyphenylacetic acid	C8H8O3	152.047	11.13
methylguanidine	C2H7N3	73.064	11.16

TABLE 3-continued

Metabolite library
Authentic standards were injected to generate a retention time library for this method.

Name	Formula	Mass	Retention Time, min
thyroxine	C15H11I4NO4	776.687	11.18
pregnenolone sulfate	C21H32O5S	396.197	11.25
salsolinol	C10H13NO2	179.095	11.25
3-methoxytyramine	C9H13NO2	167.095	11.30
gamma,gamma-dimethylallyl pyrophosphate	C5H12O7P2	246.006	11.33
cystathionine	C7H14N2O4S	222.067	11.35
glycocholic acid	C26H43NO6	465.309	11.37
phenylacetaldehyde	C8H8O	120.058	11.40
deoxycorticosterone acetic acid	C23H32O4	372.230	11.42
phenylethanolamine	C8H11NO	137.084	11.42
octanoylcarnitine	C15H29NO4	287.210	11.47
biliverdin	C33H34N4O6	582.248	11.50
lumichrome	C12H10N4O2	242.080	11.58
glycochenodeoxycholic acid	C26H43NO5	449.314	11.63
lithocholyltaurine	C26H45NO5S	483.302	11.65
lauric acid	C12H24O2	200.178	11.66
pyruvic aldehyde	C3H4O2	72.021	11.66
cholic acid	C24H40O5	408.288	11.70
s-adenosylhomocysteine	C14H20N6O5S	384.122	11.74
decanoylcarnitine	C17H33NO4	315.241	11.77
serotonin	C10H12N2O	176.095	11.80
ethyl 3-indoleacetic acid	C12H13NO2	203.095	11.82
phenethylamine	C8H11N	121.089	11.82
gamma-linolenic acid	C18H30O2	278.225	11.86
chenodeoxycholic acid	C24H40O4	392.293	11.94
linoleic acid	C18H32O2	280.240	11.95
l-tryptophanamide	C11H13N3O	203.106	11.98
deoxycholic acid	C24H40O4	392.293	11.99
homocystine	C8H16N2O4S2	268.055	12.00
lithocholic acid	C24H40O3	376.298	12.00
quinoline	C9H7N	129.058	12.00
ursodeoxycholic acid	C24H40O4	392.293	12.00
dodecanoylcarnitine	C19H37NO4	343.272	12.10
1-methylnicotinamide	C7H9N2O	137.071	12.11
alpha-tocopherol	C29H50O2	430.381	12.11
glycerol-myristic acid	C17H34O4	302.246	12.14
beta-carotene	C40H56	536.438	12.17
palmitic acid	C16H32O2	256.240	12.20
n-methyltryptamine	C11H14N2	174.116	12.22
3-methyladenine	C6H7N5	149.070	12.25
heptadecanoic acid	C17H34O2	270.256	12.26
2-undecanone	C11H22O	170.167	12.29
dodecanoylglycine	C14H27NO3	257.199	12.29
5-valerolactone	C5H8O2	100.052	12.30
tryptamine	C10H12N2	160.100	12.30
deoxyguanosine	C10H13N5O4	267.097	12.31
pentanoic acid	C5H10O2	102.068	12.32
retinoic acid	C20H28O2	300.209	12.35
diaminopimelic acid	C7H14N2O4	190.095	12.38
3-hydroxypalmitoylcarnitine	C23H45NO5	415.330	12.40
didecanoyl-glycerophosphocholine	C28H56NO8P	565.374	12.41
porphobilinogen	C10H14N2O4	226.095	12.44
n,n-dimethyl-1,4-phenylenediamine	C8H12N2	136.100	12.45
25-hydroxyvitamin d (calcidiol)	C27H44O2	400.334	12.47
retinol	C20H30O	286.230	12.50
oleoyl-glycerol	C21H40O4	356.293	12.52
docosahexaenoic acid	C22H32O2	328.240	12.55
25-hydroxycholesterol	C27H46O2	402.350	12.56
desmosterol	C27H44O	384.339	12.56
nervonic acid	C24H46O2	366.350	12.56
elaidic acid	C18H34O2	282.256	12.60
palmitoylcarnitine	C23H45NO4	399.335	12.61
lanosterol	C30H50O	426.386	12.64
7-dehydrocholesterol	C27H44O	384.339	12.65
arachidic acid	C20H40O2	312.303	12.72
octadecanoylcarnitine	C25H49NO4	427.366	12.83
erucic acid	C22H42O2	338.318	12.83
argininosuccinic acid	C10H18N4O6	290.123	12.84
5,6 dimethylbenzimidazole	C9H10N2	146.084	12.89
sphinganine	C18H39NO2	301.298	12.90
bis(2-ethylhexyl)phthalate	C24H38O4	390.277	12.93
1-phenylethanol	C8H10O	122.073	12.95
protoporphyrin	C34H34N4O4	562.258	13.40
nicotine	C10H14N2	162.116	14.50

TABLE 3-continued

Metabolite library Authentic standards were injected to generate a retention time library for this method.			
Name	Formula	Mass	Retention Time, min
retinyl palmitic acid	C36H60O2	524.459	15.40
histidine	C6H9N3O2	155.069	16.80
1-methylhistidine	C7H11N3O2	169.085	16.90
3-methylhistidine	C7H11N3O2	169.085	16.90
n,n-dimethyl-arginine	C8H18N4O2	202.143	17.00
5-hydroxylysine	C6H14N2O3	162.100	17.05
lysine	C6H14N2O2	146.106	17.10
ornithine	C5H12N2O2	132.090	17.10
n,n,n-trimethyllysine	C9H20N2O2	188.152	17.20
arginine	C6H14N4O2	174.112	17.30

Ion feature identifications from one representative (a) plasma or (b) urine QC sample were made by searching the library of authentic standards (Table 3) using MassHunter Qualitative. The peak height filter was set to 2,000, the allowable retention time error was set to ±0.3 minutes, and the allowable mass error was set to ±10 ppm.

TABLE 4

Metabolites detected in human samples		
ID - Plasma	RT, min	Area
Identifications of the plasma QC sample.		
pyruvic acid	0.5	2.45E+05
isocitric acid	0.5	6.59E+05
malic acid	0.5	5.60E+05
maleic acid	0.6	6.25E+04
2-ketoglutaric acid	0.6	3.98E+05
lactic acid	0.7	2.58E+06
n-acetylaspartic acid	0.7	3.31E+04
citric acid	0.7	1.20E+06
2-methylmaleic acid	0.7	8.51E+04
uric acid	0.7	1.05E+06
pyroglutamic acid	0.8	2.16E+05
succinic acid	0.9	1.15E+05
uridine	0.9	3.33E+04
propionylglycine	1.0	1.93E+04
methylmalonic acid	1.0	4.44E+05
xanthine	1.0	7.13E+04
itaconic acid	1.1	2.77E+04
2-hydroxybutyric acid	1.1	2.21E+05
hydroxyproline	1.6	5.17E+04
aspartic acid	1.7	4.49E+04
butyrylglycine	2.1	3.98E+05
asparagine	2.2	8.25E+05
serine	2.2	2.53E+05
dimethylglycine	2.3	1.49E+06
hypoxanthine	2.3	1.92E+06
threonine	2.4	1.19E+06
glutamic acid	2.4	8.87E+05
glutamine	2.5	8.07E+06
glycine	3.0	1.73E+04
betaine	3.0	3.97E+07
proline	3.1	2.34E+07
pantothenic acid	3.3	1.48E+05
alanine	3.3	1.40E+06
2-aminobutyric acid	3.9	9.76E+04
citrulline	4.5	4.23E+05
theobromine	4.5	7.27E+04
methionine	5.2	7.43E+04
phenylpyruvic acid	6.2	1.06E+05
tyrosine	6.2	1.51E+05
alloisoleucine	7.6	1.20E+06
creatine	7.6	6.99E+06
isoleucine/leucine	8.4	4.92E+06
suberic acid	9.0	1.25E+05
trans-cinnamaldehyde	9.6	8.25E+04

TABLE 4-continued

Metabolites detected in human samples		
ID - Plasma	RT, min	Area
trans-cinnamic acid	9.7	8.30E+04
azelaic acid	9.8	9.34E+05
phenylalanine	10.0	1.19E+06
nicotinamide	10.0	1.38E+05
carnitine	10.1	3.83E+07
deoxyadenosine	10.2	2.73E+05
3-hydroxyisovalerylcarnitine	10.2	4.79E+04
tryptophan	10.2	2.32E+05
sebacic acid	10.3	1.55E+05
1-methyladenosine	10.3	9.27E+04
isobutyric acid	10.3	6.77E+05
diethanolamine	10.3	7.41E+05
acetylcarnitine	10.3	2.04E+07
propionylcarnitine	10.5	5.23E+06
n-acetylputrescine	10.6	8.15E+04
deoxycarnitine	10.7	3.98E+06
butyrylcarnitine	10.8	4.37E+05
creatinine	10.8	1.38E+07
glycocholic acid	11.4	1.74E+05
phenylacetaldehyde	11.4	5.15E+05
phenylethanolamine	11.4	3.82E+05
octanoylcarnitine	11.5	8.27E+05
biliverdin	11.5	2.61E+04
glycochenodeoxycholic acid	11.6	1.24E+05
decanoylcarnitine	11.8	2.41E+06
linoleic acid	12.0	4.34E+05
lithocholic acid	12.0	6.43E+04
dodecanoylcarnitine	12.1	3.20E+05
glycerol-myristic acid	12.1	1.57E+05
palmitic acid	12.2	2.23E+05
heptadecanoic acid	12.3	3.79E+05
docosahexaenoic acid	12.5	2.23E+04
oleoyl-glycerol	12.5	8.02E+05
palmitoylcarnitine	12.6	1.27E+06
arachidic acid	12.7	7.53E+04
erucic acid	12.8	7.63E+04
octadecanoylcarnitine	12.8	3.91E+05
1-phenylethanol	13.0	8.32E+05
histidine	16.9	8.16E+05
n,n-dimethyl-arginine	17.0	1.95E+05
ornithine	17.1	7.05E+05
lysine	17.1	8.96E+05
n,n,n-trimethyllysine	17.3	1.64E+05
arginine	17.3	2.90E+05
Identifications of the urine QC sample		
2-propenoic acid	0.6	6.85E+04
maleic acid	0.6	5.86E+05
lactic acid	0.7	2.04E+05
n-acetylaspartic acid	0.7	1.20E+06
2-oxoadipic acid	0.7	4.35E+04
citric acid	0.7	8.64E+06
pyruvic acid	0.7	1.44E+05
2-methylmaleic acid	0.8	4.17E+06

TABLE 4-continued

Metabolites detected in human samples

ID - Plasma	RT, min	Area
uric acid	0.8	1.26E+05
propionylglycine	0.9	4.21E+04
n-acetylglutamic acid	0.9	2.07E+05
succinic acid	0.9	2.38E+06
citramalic acid	1.0	2.83E+06
fumaric acid	1.0	7.47E+03
homogentisic acid	1.0	1.44E+04
methylmalonic acid	1.1	3.69E+05
xanthine	1.1	5.14E+04
aconitic acid	1.1	2.85E+05
itaconic acid	1.1	4.57E+05
3-hydroxymethylglutaric acid	1.1	1.95E+06
succinate semialdehyde	1.1	4.16E+04
2-hydroxybutyric acid	1.2	3.12E+05
n-acetylcysteine	1.3	1.35E+04
4-acetamidobutanoic acid	1.4	6.80E+05
2-methylcitric acid	1.4	1.65E+06
acetoin	1.6	1.14E+04
glutaric acid	1.6	8.04E+04
2-hydroxypyridine	1.6	2.89E+04
6-hydroxynicotinic acid	1.6	6.97E+05
cyclic adenosine monophosphate	1.7	7.58E+03
4-pyridoxic acid	1.8	7.48E+06
isobutyrylglycine	1.8	8.92E+04
methylsuccinic acid	2.2	9.70E+05
serine	2.2	4.90E+04
threonine	2.3	2.89E+04
hypoxanthine	2.3	8.00E+04
glutamic acid	2.3	1.19E+05
ethylmalonic acid	2.4	2.67E+06
xanthosine	2.4	4.99E+04
glutamine	2.5	8.13E+05
2-aminoadipic acid	2.9	1.43E+05
betaine	2.9	2.59E+04
2-hydroxy-3-methylbutyric acid	2.9	8.80E+04
indoxyl sulfuric acid	2.9	3.55E+06
alanine	3.5	2.39E+04
pantothenic acid	3.5	1.54E+06
3-hydroxybenzyl alcohol	3.8	5.43E+04
adipic acid	3.9	4.61E+05
2-methylbutyrylglycine	3.9	1.52E+05

TABLE 4-continued

Metabolites detected in human samples

ID - Plasma	RT, min	Area
mandelic acid	4.0	3.91E+04
tiglylglycine	4.2	1.48E+05
citrulline	4.4	5.52E+04
isovalerylglycine	4.5	3.13E+05
3-methylcrotonylglycine	4.5	2.28E+06
guanidinosuccinic acid	4.6	5.52E+04
4-hydroxyphenyllactic acid	4.6	4.63E+06
2,5-dihydroxybenzoic acid	4.7	5.65E+05
hippuric acid	5.8	9.32E+06
4-hydroxybenzaldehyde	5.9	2.80E+05
kynurenic acid	7.3	1.02E+04
creatine	7.6	3.60E+05
guanidinoacetic acid	7.7	4.54E+04
hexanoylglycine	8.3	4.55E+05
n-alpha-acetyllysine	8.4	1.40E+05
salicylic acid	8.8	2.40E+04
n-acetyltryptophan	8.9	2.56E+04
suberic acid	9.0	3.17E+05
azelaic acid	9.8	5.10E+05
1-methyladenosine	10.2	2.46E+04
urocanic acid	10.2	2.20E+05
octanoylglycine	10.3	5.67E+04
pyridoxal	10.4	1.51E+04
adenine	10.5	6.43E+04
cystine	10.7	1.35E+05
4-guanidinobutanoic acid	10.7	2.57E+04
creatinine	10.8	1.42E+05
saccharopine	11.1	2.44E+04
histidine	16.8	1.78E+06
1-methylhistidine	16.9	1.52E+05
n,n-dimethyl-arginine	17.0	2.60E+04
anserine	17.4	1.03E+05
carnosine	17.4	5.22E+04

Among all plasma samples, compounds eluting between minutes 13 to 16 were searched for potential annotations using the HMDB and KEGG modules in Progenesis. Six potential results within 3 ppm mass error were found for a selected feature eluting at 14.47 min with a m/z of 844.5093.

TABLE 5

Search results of a non-polar compound in plasma

ChemSpider ID	Description	Adducts	Formula	Mass error (ppm)	Isotope similarity
CSID35032656	(19R,25S)-22,25,28,28-Tetrahydroxy-22,28-dioxido-16-oxo-17,21,23,27-tetraoxa-22lambda~5~,28lambda~5~-diphosphaoctacosan-19-yl (9Z,12Z)-9,12-octadecadienoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73
CSID35032669	(7Z,19R,25S)-22,25,28,28-Tetrahydroxy-22,28-dioxido-16-oxo-17,21,23,27-tetraoxa-22lambda~5~,28lambda~5~-diphosphaoctacos-7-en-19-yl (11Z)-11-octadecenoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73
CSID35032670	(7Z,19R,25S)-22,25,28,28-Tetrahydroxy-22,28-dioxido-16-oxo-17,21,23,27-tetraoxa-22lambda~5~,28lambda~5~-diphosphaoctacos-7-en-19-yl (9Z)-9-octadecenoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73
CSID35032696	(2R,8S)-2-[(9Z)-9-Hexadecenoyloxy]-5,8,11,11-tetrahydroxy-5,11-dioxido-4,6,10-trioxa-5lambda~5~,11lambda~5~-diphosphaundec-1-yl (11Z)-11-octadecenoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73
CSID35032711	(2R,8S)-2-[(9Z)-9-Hexadecenoyloxy]-5,8,11,11-tetrahydroxy-5,11-dioxido-4,6,10-trioxa-5lambda~5~,11lambda~5~-diphosphaundec-1-yl (9Z)-9-octadecenoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73

TABLE 5-continued

Search results of a non-polar compound in plasma

ChemSpider ID	Description	Adducts	Formula	Mass error (ppm)	Isotope similarity
CSID35032724	(2R,8S)-5,8,11,11-Tetrahydroxy-5,11-dioxido-2-(palmitoyloxy)-4,6,10-trioxa-5lambda~5~,11lambda~5~-diphosphaundec-1-yl (9Z,12Z)-9,12-octadecadienoate	M + NH4	$C_{40}H_{76}O_{13}P_2$	−0.85	88.73

The CVs of select amino acids and acylcarnitines were calculated from the peak areas of 5 replicate injections of the plasma QC sample.

TABLE 6

Peak area precision of select plasma amino acids and acylcarnitines

Compound	Mean Peak Area	CV, %
Glutamine	8.1E+06	3.9
Alanine	1.5E+06	4.6
Citrulline	4.3E+05	1.7
Methionine	6.9E+04	6.9
Alloisoleucine	9.8E+05	6.8
Isoleucine/Leucine	4.8E+06	5.1
Tryptophan	2.3E+05	3.9
Propionylcarnitine	5.2E+06	3.3
Palmitoylcarnitine	1.2E+06	3.5
Stearoylcarnitine	3.7E+05	3.6

TABLE 7

A metabolomics analysis example of polar molecules, followed by less polar molecules, and then non-polar molecules.

Compound Name	Retention Time (min)
Succinate	2
Methylmalonate (MMA)	2.9
Lactate	3.9
Glycine	4.4
Glutamate	4.5
allo-Isoleucine (Allo-Ile)	5.6
Ethyl Malonate	6
Isoleucine (Ile)	6.2
Leucine (Leu)	6.6
Glutarate	8.5
Tyrosine	10.8
Adipic Acid	13.2
25-Hydroxy Vitamin D2	14.5
Phenylalanine	15.2
Sebacic Acid	17.9
25-Hydroxy Vitamin D3	21.8
C4-Acylcarnitine	22.4

As used herein, the singular terms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to an object can include multiple objects unless the context clearly dictates otherwise.

As used herein, the terms "substantially," "substantial," "approximately," and "about" are used to describe and account for small variations. When used in conjunction with an event or circumstance, the terms can refer to instances in which the event or circumstance occurs precisely as well as instances in which the event or circumstance occurs to a close approximation. When used in conjunction with a numerical value, the terms can refer to a range of variation of less than or equal to ±10% of that numerical value, such as less than or equal to ±5%, less than or equal to ±4%, less than or equal to ±3%, less than or equal to ±2%, less than or equal to ±1%, less than or equal to ±0.5%, less than or equal to ±0.1%, or less than or equal to ±0.05%. For example, a first numerical value can be deemed to be "substantially" the same or equal to a second numerical value if the first numerical value is within a range of variation of less than or equal to ±10% of the second numerical value, such as less than or equal to ±5%, less than or equal to ±4%, less than or equal to ±3%, less than or equal to ±2%, less than or equal to ±1%, less than or equal to ±0.5%, less than or equal to ±0.1%, or less than or equal to ±0.05%.

Additionally, amounts, ratios, and other numerical values are sometimes presented herein in a range format. It is to be understood that such range format is used for convenience and brevity and should be understood flexibly to include numerical values explicitly specified as limits of a range, but also to include all individual numerical values or sub-ranges encompassed within that range as if each numerical value and sub-range is explicitly specified. For example, a ratio in the range of about 1 to about 200 should be understood to include the explicitly recited limits of about 1 and about 200, but also to include individual ratios such as about 2, about 3, and about 4, and sub-ranges such as about 10 to about 50, about 20 to about 100, and so forth.

While the disclosure has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the disclosure as defined by the appended claim(s). In addition, many modifications may be made to adapt a particular situation, material, composition of matter, method, operation or operations, to the objective, spirit and scope of the disclosure. All such modifications are intended to be within the scope of the claim(s) appended hereto. In particular, while certain methods may have been described with reference to particular operations performed in a particular order, it will be understood that these operations may be combined, sub-divided, or re-ordered to form an equivalent method without departing from the teachings of the disclosure. Accordingly, unless specifically indicated herein, the order and grouping of the operations are not a limitation of the disclosure.

What is claimed is:

1. A method of analyzing a biological sample comprising:
separating components of the biological sample via reversed-phase (RP) chromatography to obtain an elute;
subsequently, subjecting the elute to separation via ion-exchange (IEX) chromatography or mixed-mode IEX chromatography; and
detecting separated compounds to determine the components of the biological sample;

wherein:

the separating and the subjecting are conducted with one aqueous to organic solvent gradient; and

there is no switching valve between the RP chromatography and IEX chromatography.

2. The method of claim 1, wherein the biological sample is from a subject having or suspected of having a metabolic disruption.

3. The method of claim 1, wherein the biological sample is a plasma sample or a urine sample.

4. The method of claim 1, wherein the biological sample comprises lipids, carbohydrates, and metabolic intermediates.

5. The method of claim 1, wherein the biological sample comprises polar and non-polar metabolites.

6. The method of claim 1, wherein the detecting step is performed using mass spectrometry.

7. The method of claim 1, wherein the detecting step includes qualitative analysis.

8. The method of claim 1, wherein isomers of metabolites in the biological sample are separated.

9. The method of claim 1, wherein the biological sample comprises methylmalonic acid, glutamine, glycine, citrulline, methionine, alloisoleucine, arginine, creatine, carnitine, propionylcarnitine, stearoylcarnitine, 2-methylcitric acid, propionylglycine, 2-methylbutyrylglycine, 3-methylcrotonylglycine, adipic acid, suberic acid, and glutaric acid and wherein the detecting separated compounds comprises detecting the methylmalonic acid, glutamine, glycine, citrulline, methionine, alloisoleucine, arginine, creatine, carnitine, propionylcarnitine, stearoylcarnitine, 2-methylcitric acid, propionylglycine, 2-methylbutyrylglycine, 3-methylcrotonylglycine, adipic acid, suberic acid, and glutaric acid.

10. The method of claim 1, wherein the biological sample comprises plasma; and wherein the step of detecting comprises detecting 88 metabolites; and wherein the method is conducted in 20 minutes or less.

11. The method of claim 1, wherein the biological sample comprises urine, and wherein the step of detecting comprises detecting 82 metabolites; and wherein the method is conducted in 20 minutes or less.

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