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Partially ^{13}C-labeled mouse tissue as reference for LC-MS based untargeted metabolomics

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ABSTRACT

The inclusion of stable isotope-labeled reference standards in the sample is an established method for the detection and relative quantification of metabolic features in untargeted metabolomics. In order to quantify as many metabolites as possible, the reference should ideally include the same metabolites in their stable isotope-labeled form as the sample under investigation. We present here an attempt to use partially ^{13}C-labeled mouse material as internal standard for relative metabolite quantification of mouse and human samples in untargeted metabolomics.

We fed mice for 14 days with a^{13}C-labeled *Ralstonia eutropha* based diet. Tissue and blood amino acids from these mice showed ^{13}C enrichment levels that ranged from 6% to 75%. We used MetExtract II software to automatically detect native and labeled peak pairs in an untargeted manner. In a dilution series and with the implementation of a correction factor, partially ^{13}C-labeled mouse plasma resulted in accurate relative quantification of human plasma amino acids using liquid chromatography coupled to mass spectrometry, The coefficient of variation for the relative quantification is reduced from 27% without internal standard to 10% with inclusion of partially ^{13}C-labeled internal standard.

We anticipate the method to be of general use for the relative metabolite quantification of human specimens.

Introduction

The great physico-chemical variability of low molecular weight metabolites combined with their wide dynamic range of concentrations complicate their analyses. The identification of 'true' metabolite features among thousands of signals remains challenging with untargeted LC-ESI-MS metabolomics approaches. Metabolic features are often derived from background and chemical noise [1–3], and frequently also represent ion species from the same metabolite including isotopologues, in-source fragments and adducts of various kind. Relative metabolite quantification presents another challenge, especially when using soft ionization techniques where matrix effects [4] can cause ion suppression or enhancement.

For targeted metabolomics samples can be spiked with an internal standard (IS) such as a stable isotope-labeled compound that has the same structure and physico-chemical properties as the targeted compound, which can help to overcome the above problems [5]. The IS coelutes with the target metabolite and has the same ion suppression/enhancement allowing accurate relative quantification.

Typically, stable isotope-labeled reference standards are chemically synthesized enabling the accurate quantification of a limited number of compounds. In order to overcome this limitation another strategy is to derivatize a sample aliquot with a stable isotope-labeled reagent [6–9] to create a sample specific IS. This approach works well for specific compound classes but not for all compounds present in the sample.

If the goal is to detect and relatively quantify a large number of metabolites in an untargeted manner, lower organisms or mammalian cells can be labeled in culture by growing them in media where ^{12}C is

Abbreviations: AA, amino acids; ACN, acetonitrile; EIC, extracted ion chromatogram; ESI, electrospray ionization; GMD, Golm Metabolome Database; HILIC, hydrophilic interaction chromatography; HPLC, high performance liquid chromatography; IS, internal standard; LC, liquid chromatography; MS, mass spectrometry; MSTFA, N-Methyl-N-(trimethylsilyl) trifluoroacetamide; PLM, partially ^{13}C-labeled mouse material; TMS, trimethylsilyl; TOF, time of flight

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replaced with the ^{13}C isotope for generating an isotope-labeled reference that can be used as IS. This approach has been successfully established for microorganisms [10,11], and was subsequently extended to plants [12] and small animals [13].

We report on an attempt to enrich mice with the ^{13}C isotope with the aim of producing isotope-labeled material as reference for untargeted metabolomics studies. Due to the high costs involved in generating ^{13}C stable isotope labeled mice, we explored whether partially ^{13}C-labeled mouse material (PLM) can be used for this purpose. Specifically, we wanted to investigate if such PLM can be used as IS for relative quantification in untargeted metabolomics experiments with human specimens. We use an established mouse feed where only the protein content is labeled with a stable isotope. In addition, we explored whether the PLM can aid the automatic detection of "true" metabolic features.

Material and methods

Chemicals

HPLC-grade methanol (MeOH), acetonitrile (ACN) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Merck (Merck, Darmstadt, Germany). Chloroform ($CHCl_3$) was purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA). Ammonium acetate was purchased from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA, USA). Ultrapure water was obtained from ELGA Purelab ultra Labwater System (Bucks, UK).

Animals

Eight-week-old male C57BL/6 mice (Charles River Laboratories, Maastricht, The Netherlands) were housed under standard conditions (12-h light/dark cycle, lights on at 0600 h, room temperature 23 ± 2 °C, humidity 60%, tap water and food *ad libitum*) and fed with standard rodent diet (Harlan Laboratories, Inc., Indianapolis, IN, USA) for one week. For adaptation prior to labeling the animals were first fed an unlabeled *Ralstonia eutropha* bacterial protein-based rodent diet (Silantes GmbH, Munich, Germany) for 4 days. The food supply was then switched to ^{13}C-labeled *Ralstonia eutropha* bacterial diet (Silantes GmbH) for 14 days. The diet is made up of standard rodent protein free feed (Harlan Sprague Dawley Inc., Indianapolis, IN, USA) supplemented with either unlabeled or ^{13}C-labeled *Ralstonia eutropha* hydrolysate as protein source. Following labeling the animals were sacrificed and organs and blood isolated. The partially ^{13}C-labeled animals did not show any discernible health effects compared to animals fed with a standard diet, and had similar weight gains as animals fed with standard food (data not shown).

Metabolite plasma sample extraction for LC-MS analysis

For metabolite analysis samples were extracted with liquid/-liquid partitioning as described previously [14]. Briefly, 50 µl plasma samples were incubated with 165 µl MeOH for 1 h at 30 °C, upon which 115 µl $CHCl_3$ and 200 µl H_2O were added to induce a phase separation. Samples were centrifuged and aqueous supernatant dried by vacuum concentration and stored dry under nitrogen gas at −20 °C until further processing.

Plasma protein hydrolysis

After metabolite extraction protein containing pellets were washed twice with MeOH and hydrolyzed with 6M HCl for 24 h at 110 °C in a temperature controlled sand bath [15]. Hydrolysates were transferred to fresh safe lock tubes and dried by vacuum concentration and stored dry under inert gas at −20 °C until further processing.

GC-MS measurement

GC-MS based profiling of amino acid pool sizes from heart tissue and plasma specimens

Gas chromatography mass spectrometry (GC-MS) was used for an initial assessment of isotope enrichment. This was an important test to find out if the PLM is suited for untargeted metabolite detection and IS based relative quantification [16]. Polar small molecules were extracted from mouse heart (approximately 180 mg per replicate) and 50 µl plasma aliquots. The metabolites were extracted from each aliquot in 1 ml of a homogenous mixture of −20 °C methanol: methyl-*tert*-butyl-ether: water (1: 3: 1), with shaking for 30 min at 4 °C (Thermo Stat Plus; Eppendorf, Germany), followed by another 10 min incubation in an ice cold ultrasonication bath. After adding 650 µl UPLC-grade methanol:water (1: 3), the homogenate was vortexed and centrifuged for 5 min at 4 °C in a table-top centrifuge (Eppendorf). After phase separation the upper organic phase containing lipophilic metabolites and the lower aqueous phase containing polar and semipolar metabolites were isolated.

The polar phase was dried by vacuum concentration. After drying a 10 µl aliquot was dissolved at 20 mg/mL in pure pyridine (Merck, Darmstadt, Germany) and methoxiaminated with 10 µl methoxyamine hydrochloride (Sigma, Munich, Germany) for 90 min at 40 °C. Subsequently the sample was derivatized by trimethylsilylation with 17.5 µl N-methyl-N-(trimethylsilyl)trifluoroaceta-mide (MSTFA; Macherey and Nagel, Düren, Germany) and subjected with 2.5 µl retention time standard for 45 min at 40 °C in a second step. Both steps were done "online" prior to injection according to Erban et al. [17]. Analysis was performed using an Agilent 7890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) on an MDN-35 or equivalent column with fatty acid methylesters (FAMEs) as retention time standards, which was coupled to a Pegasus HT mass spectrometer (LECO, St. Joseph, MI, USA).

GC-MS based profiling of amino acids from protein hydrolysates

Metabolite profiling was performed as described previously [14,18,19] by GC-MS using an Agilent 6890N24 gas chromatograph (Agilent Technologies) with a FactorFour VF-5ms column (Varian-Agilent Technologies), which was connected to a Pegasus III time-of-flight mass spectrometer (LECO).

Dried hydrolysates were methoxyaminated and trimethylsilylated manually prior to GC-MS analysis [17–21]. Retention indices were calibrated by the addition of a C10, C12, C15, C18, C19, C22, C28, C32, C36 n-alkane mixture to each sample [22].

GC-MS data processing

GC-MS chromatograms were visually inspected, baseline-corrected and exported in NetCDF file format using ChromaTOF software (Version 4.22; LECO). After processing into a standardized numerical data matrix, compound identification was performed using the Tagfinder software [23,24]. Compounds were identified by mass spectral and retention time index matching with the reference collection of the Golm Metabolome Database (GMD, http://gmd.mpimpgolm.mpg.de/) [25–27] and NIST08 database (http://www.nist.gov/srd/mslist.htm). Criteria for manual supervised metabolite identification were the presence of at least 3 specific mass fragments per compound and a retention index deviation < 1.0% [22].

Amino acid (AA) features of selected TMS products were extracted from the Tagfinder generated data and manually corrected when necessary. These data were subjected to the CORRECTOR software tool and ^{13}C enrichment values were calculated as described previously [28,29].

LC-MS measurement

LC-MS based profiling of metabolite pool sizes

Plasma extracts were analyzed using an 1100 HPLC system (Agilent Technologies) connected to an Impact II TOF MS system controlled by Hystar 3.2 software (Bruker Corporation, Billerica, MA, USA). HPLC eluent A: 5% ACN, 10 mM ammonium acetate pH 6.8, eluent B: 95% ACN, 10 mM ammonium acetate pH 6.8, flow rate: 100 µl/min^{-1}, SeQuant ZIC®-HILIC 2.1 × 100 mm column (3.5 µm particle size, 100 Å pore size, SeQuant ZIC®-HILIC; Merck KGaA, Darmstadt, Germany) with guard column, both heated to 40 °C. Samples were kept at 6 °C in the sample rack and 2 µl were injected. The HPLC was run isocratic for 2 min with 95% of eluent B followed by a 4 min gradient to 85% eluent B, followed by another 6.5 min gradient to 21% eluent B. The column was then washed for 5.5 min with 95% B and re-equilibrated for 14 min.

The sample flow was introduced splitless into the ESI source. Compounds were ionized with an end plate offset of −500 V, a capillary voltage of −4500 V in positive mode and 4000 V in negative mode. The nebulizer gas N_2 flow was 8 l/min^{-1} and a pressure of 1 bar heated to 220 °C into the source. Profile data were acquired with a spectra rate of 1 Hz and a mass range of 50–1300 *m/z* (Bruker Compass 1.9) in full scan MS1. The mass accuracy was adjusted by internal calibration using sodium format clusters in ESI+ mode (Bruker DataAnalysis 4.4 software). After calibration chromatograms were converted to mzXML files by MSConvert (Proteowizard http://proteowizard.sourceforge.net, version 3.0.9987).

Manual extraction of isotopologue intensities from LC-MS data

For the analysis of PLM-spiked samples with untargeted LC-ESI-MS the MetExtract II software [30] was used. To test if the software was able to automatically detect the peaks from partially ^{13}C-labeled metabolic features in a reliable and accurate manner the spectra were manually inspected. Isotopologue intensity information was extracted with a target list (Bruker PathwayScreener 1.0). AA were identified in plasma samples by $[M+H]^+$ adduct matching to exact mass (3 ppm) and verification with MS/MS fragmentation pattern matching (Bruker HMDB library) when possible. Retention time (RT) and exact mass were used to find the target AA. For the target list a .csv file was generated that had the exact AA mass, isotopologue and RT information as input for PathwayScreener 1.0. Peak quality was checked by mass difference (< 1 mDa = good; < 3 mDa = acceptable) and RT (< 0.1 min = good and < 0.2 min = acceptable). Only closest matching RT peaks were picked and their intensity and area exported to a .csv file. For calculations exported .csv files were further processed with Microsoft Excel (Microsoft, Redmond, WA, USA). The labeled IS ion signal is then used for normalization of the sample signal.

Automatic detection and normalization of native and labeled feature pairs

Raw, centroid LC-MS data files in the mzXML format were processed with the AllExtract module of the MetExtract II software [30] with the aim to search for pairs of native and co-eluting ^{13}C-labeled metabolite ions (^{13}C-isotopic enrichment: 97.5%, min. signal intensity: 1000 counts, Δm/z between ^{13}C and ^{12}C: 1.00335, carbon atoms searched for: 3–60, max. m/z difference between native and ^{13}C-labeled ion signals: 5 ppm (of the monoisotopic, native isotopologue), maximum extracted ion chromatogram (EIC) *m/z* deviation: 10 ppm, chromatographic peak width: 6–30 scans, min. Pearson correlation for chromatographic co-elution: 0.95). Subsequently, the software automatically convoluted detected metabolite ion pairs into groups, each representing a unique metabolite (minimum Pearson correlation: 0.85). Finally, metabolite ions and groups detected in the different samples were bracketed into a two-dimensional data matrix, which contained the monoisotopic, native and ^{13}C-labeled metabolite ion intensities for all detected metabolite ions and processed samples (maximum allowed RT-shift: 0.15 min, maximum allowed *m/z* deviation: 15 ppm).

Relative quantification of metabolite features

Since not all carbon atoms in the diet were ^{13}C, mouse metabolite carbons were only partially labeled with the heavy isotope. Use of PLM therefore required the refinement of existing approaches for internal standardization by implementing a correction factor. When the PLM is spiked into an unlabeled sample the ^{12}C part adds on to the ^{12}C signal of the unlabeled sample. To take this into account we calculate a correction factor from the ^{13}C signal of the PLM for each metabolic feature.

For relative quantification equal amounts of PLM were spiked into all samples of the study. Signal intensities of each metabolite M were normalized to its internal, stable isotope-labeled reference (Mx_{13}). In case of partially ^{13}C-labeled metabolites the native signal and the signals of the spiked, labeled metabolite may overlap. To account for such interferences a correction value (a) was calculated from the LC-HRMS data obtained after measurement of the PLM (r, ^{13}C control samples) without native sample material.

This correction value (a), which is the intensity ratio of the native signal Mr_{12} to the labeled, principle isotopologue signal Mr_{13} ($Mr_{12}/Mr_{13} = a$) of the respective metabolite (M), was calculated independently of the spiked samples using the ^{13}C PLM r (average ratio of the two technical replicates).

$$IS\ normalized\ value\ Mx_{IS} = \frac{Mx_{12}}{Mx_{13}} - a \quad (1)$$

Final stable isotope assisted normalization (Mx_{IS}) was carried out by calculating Mx_{IS} values according to equation (1), where Mx_{12} represents the intensity of the native isotopologue signal of the metabolite (M) and Mx_{13} is the labeled, principle isotopologue signal of the metabolite (M) in the spiked sample (x).

Results

Generation of ^{13}C stable isotope-labeled mouse material

^{13}C-labeled specimens for relative metabolite quantification are frequently used to compensate for ion suppression/enhancement effects and to increase accuracy. This approach has been successfully applied to lower organisms [10,11] but not yet to mammals. We present here an attempt to use ^{13}C-labeled mouse material as a stable isotope-labeled IS for the relative quantification of metabolites in human and mouse specimens. For this purpose a mouse diet was generated (www.silantes.com) that includes ^{13}C-labeled *Ralstonia euthropha* bacteria hydrolysates as sole protein source with an overall ^{13}C enrichment > 95%. This type of feed had previously been established for labeling mice with ^{15}N [31]. Eight week old male DBA/2Ola mice were fed for 14 days with the ^{13}C *Ralstonia euthropha* diet and blood and organs were collected.

Analysis of amino acids in ^{13}C-labeled mice

For characterization of the ^{13}C mouse material we first used GC-MS (see 2.5.1), a well-established method to detect and identify amino acids (AA). As proteins were the only source of ^{13}C in the feed, we expected to find the main share of ^{13}C signal in AA. We assessed the degree of isotope enrichment to verify that the partially ^{13}C-labeled mouse material was suitable as PLM for untargeted detection, annotation and quantification purposes.

Free plasma and heart AA from ^{13}C-labeled mice were extracted and subjected to GC-MS analysis (see 2.5.1). AA in proteins were measured (see 2.5.2) from hydrolysates of metabolite extracted plasma protein pellets (see 2.4). Data were extracted from the GC-MS runs using Tagfinder software (see 2.5.3) [23] and subjected to the CORRECTOR [28,29] software to correct for natural occurring isotopes. The results were mapped on a simplified pathway (Fig. 1). Glutamine and pyroglutamic acid cannot be differentiated in this analysis and are

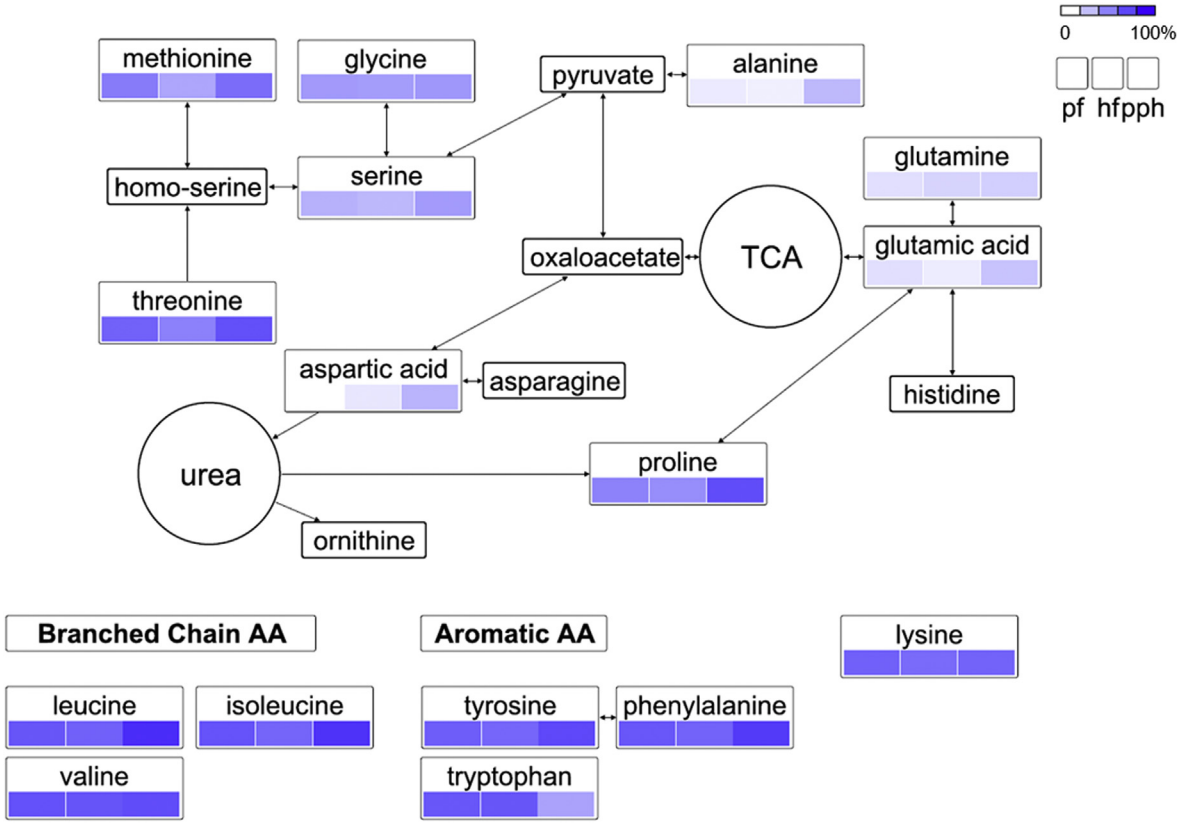

Fig. 1. ^{13}C enrichment of free amino acids in mouse plasma (pf) (see 2.5.1) and heart tissue (hf) (see 2.5.1) and in plasma protein hydrolysates (pph) (see 2.5.2) mapped on a simplified metabolic pathway. Amino acids were measured using GC-MS based metabolomics methods. Isotopologue intensities were extracted and corrected for the natural occurring isotopes with the CORRECTOR software tool. Color code represents average ^{13}C enrichment of 3 biological replicates (pf; hf) and 3 technical replicates (pph). The overall ^{13}C enrichment was approximately 65%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

combined under glutamine.

For the essential AA, branched chain AA and aromatic AA ^{13}C enrichment was very high in all samples (Fig. 1). In contrast, the ^{13}C enrichment was low for alanine, glutamic acid, glutamine and aspartic acid which are all in proximity to the glycolysis pathway (Fig. 1, Supplemental Table 2). This indicates the presence of two independent AA sources. Whereas one batch of AA is synthesized via glycolysis and associated pathways resulting in lower ^{13}C incorporation, all other AA are derived from diet protein degradation and hence have high ^{13}C incorporation levels.

Overall the ^{13}C enrichment is higher in plasma protein hydrolysate AA (pph) compared to free AA, especially for alanine, aspartic acid and glutamic acid. This indicates that AA in plasma proteins are probably derived to a larger extent from diet protein degradation.

The AA in the PLM exhibited a wide range of ^{13}C incorporation levels and thus resulted in complex AA isotopologue peak patterns that complicated relative quantification efforts.

Relative AA quantification with manual and automatic peak detection

For stable isotope assisted relative quantification equal amounts of a stable isotope labeled IS are spiked into each sample. The labeled IS ion signal is then used for normalization of the sample signal. For targeted analysis with a known mix of labeled compounds this is straightforward as the *m/z* values are known. However, for an untargeted approach with labeled biological material high resolution MS instruments and sophisticated software tools like MetExtract II [30] are required in order to detect true peak pairs. To test whether the MetExtract II software can reliably and accurately detect the native and labeled AA peak pairs of a sample spiked with PLM, we compared the obtained results with manual detection of AA signal pairs. Human plasma samples were spiked with PLM and measured with an LC-MS based platform with a high resolution qTOF instrument (see 2.6.1). Manual peak detection was performed with Bruker Daltonics Pathway Screener 1.0 (see 2.6.2) and a target list that contained information for the $[M+H]^+$ ion and all AA isotopologue signals. Detected peak areas were reintegrated if necessary. Figs. 2 and 3 show data for AA that were detected manually (see 2.6.2) and automatically (see 2.6.3) with MetExtract II software. For manual peak detection the absence or presence of an isotopologue signal assisted in determining the correct compound. In order to see if there were discrepancies between automatic and manual peak detection results we calculated the Pearson correlation coefficient (Fig. 2A, Supplemental Fig. 5). The correlation is based on the peak areas of all replicates for the native $[M+H]^+$ peak in the labeled samples. The correlation values were 0.992 and ~1 for asparagine and citrulline, respectively. This very high correlation is supported by a linear regression fit with a high R^2 value when plotting automatically versus manually picked AA peaks (Fig. 2B), indicating that both peak detection methods give very similar results for determining the peak area.

We next determined the ratio of expected and measured relative AA concentrations and determined the Coefficient of Variance (CV) for each AA based on the peak area of both replicates and all points of the dilution series. Only 4 manually detected AA and 3 automatically detected AA showed a CV greater than 30% (Fig. 2C). In most cases the CV for automatic peak detection was as good as or slightly better than the CV for manual peak detection with the exception of asparagine that had very low peak intensity and noisy isotopologue peak shapes resulting in poor peak integration with the software (Fig. 2C, Supplemental Fig. 5).

We conclude that the MetExtract II software tool is able to detect the correct AA peak pairs. Furthermore, peak area and intensity calculation

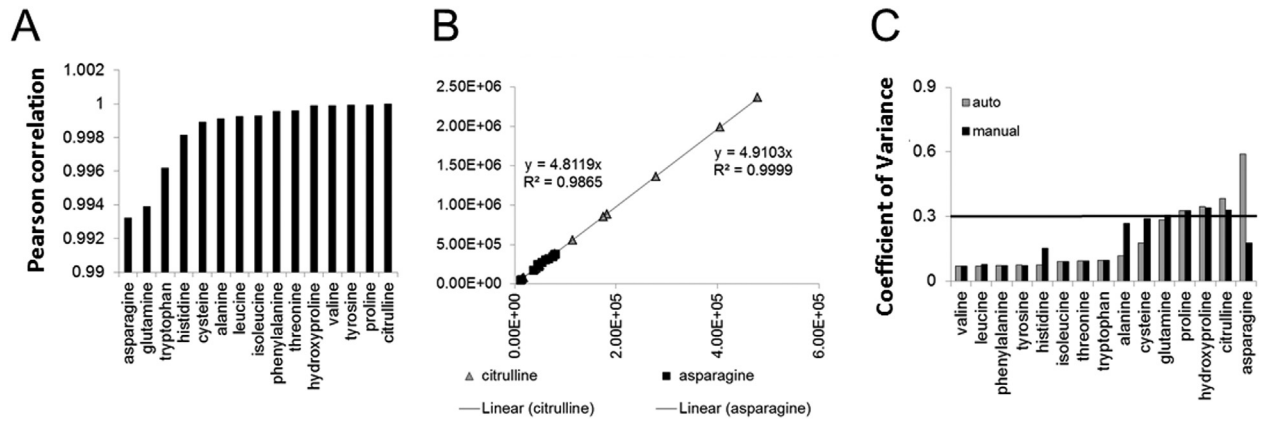

Fig. 2. Correlation between automatically picked peaks with MetExtract II software and manually picked peaks. Samples were measured with an LC-MS based metabolomics method (2.6). Raw data were processed twice, first with MetExtract II software (automatic) the then with PathwayScreener and a target list that contained *m/z* and RT information for each isotopologue (manual) **(A)** Pearson correlation between all detected amino acids based on peak area of the $[M+H]^+$ adduct. **(B)** Scatter plot with linear regression model of worst (asparagine) and best (citrulline) performing correlations. **(C)** Comparison of relative SD of measured/expected relative concentration ratios between automatically (auto) and manually (manual) picked data.

are as good as with manual calculation.

Mouse and human plasma amino acid quantification using partially labeled mouse reference material and automated data processing with MetExtract II

To test whether the generated PLM can be used as IS for the accurate relative quantification in an untargeted LC-MS based metabolomics approach (see 2.6.1) we spiked equal amounts of PLM into human plasma samples as part of a dilution series. MS1 spectra were extracted (see 2.6.3) with MetExtract II [30] and the software parameters adjusted to find features that expose the specific isotopologue patterns of the individual ^{13}C-labeled AA. By manually optimizing the data processing parameters with known metabolites we were able to use the software for an untargeted analysis.

The software detected 72 feature pairs in 26 peak groups in human plasma spiked with ^{13}C mouse reference plasma (Supplemental Table 1). We were able to annotate several metabolic features to adducts and neutral losses (Supplemental Table 1). Some of the fragments were of unknown identity but could be grouped by peak shape correlation (> 0.9) and the number of ^{13}C atoms. Taking into account adducts, groups and number of carbon atoms per ion, allowed the assignment of 43 metabolic features to 20 known metabolites (Supplemental Table 1). Assignment was done with a list of common adducts in ESI Source. Metabolite annotation was based on the exact mass (± 5 ppm) of the $[M+H]^+$ adduct from the Human Metabolite Database (HMDB) [32–34].

In order to test the accuracy of known AA relative quantification we compared the dilution series without and with spiked PLM (see 2.7). For this purpose we maximum normalized the data to half of the average highest concentration for each AA (Fig. 3). As is evident from the plot, relative quantification without the use of the PLM (no IS) resulted in a much higher standard deviation compared to relative quantification (see 2.7) with PLM (^{13}C IS) (Fig. 3A). A linear regression model supports this finding with $R^2 = 0.64$ for no IS and $R^2 = 0.99$ for ^{13}C IS.

In order to find out how the PLM performs for the individual AA, we calculated the ratio between the experimental and expected relative concentrations. Except for glutamine and asparagine all AA ratios were between one and two (Fig. 3B). Glutamine and asparagine coelute with the EDTA peak and suffer from ion suppression (Supplemental figure 2).

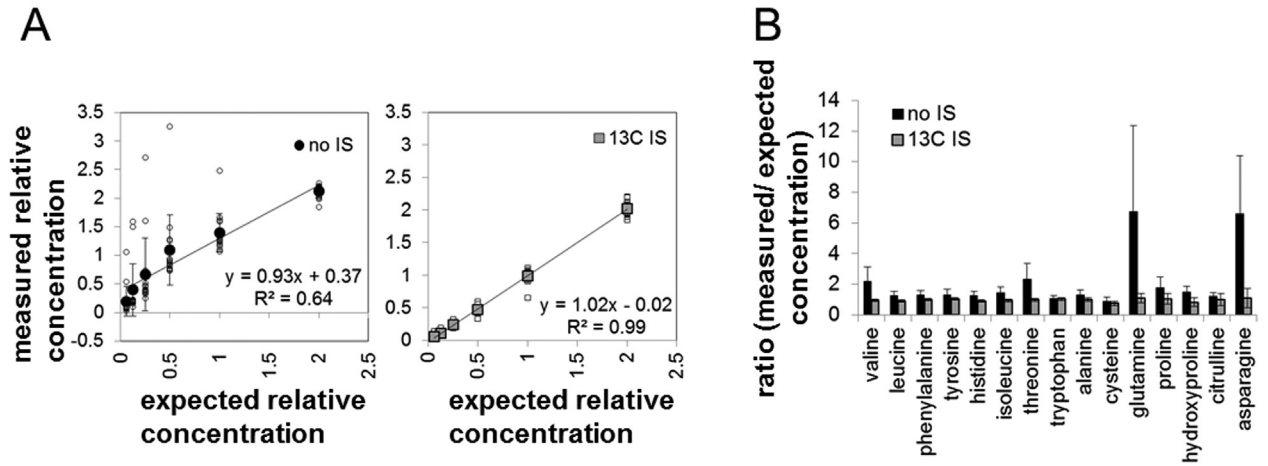

Fig. 3. Relative amino acid quantification in human plasma spiked with partially ^{13}C-labeled mouse plasma. Two independent dilution series were analyzed in two technical replicates using an LC-MS based method (2.6). One series was measured without partially ^{13}C-labeled spiked mouse plasma (no IS), the other series was spiked with equal amounts of partially ^{13}C-labeled mouse plasma (norm to IS). **(A)** Expected vs. measured concentrations normalized to half average highest concentration with (^{13}C IS) and without mouse reference plasma (no IS). Average individual amino acid values are displayed for each expected relative concentration, error bars represent SD (n = 15). When no error bar is displayed the size of the symbol exceeds SD. **(B)** Ratio of measured and expected relative concentrations. All values were normalized to the highest concentration and then divided by the relative concentration. Average values of all expected relative concentrations for each AA are shown, error bars represent SD (n = 6).

For the majority of the tested metabolites, the ratios for the samples with PLM were closer to 1 and had smaller SD, with CV values (median CV = 10%) in most cases much lower than samples without PLM with the respective correction factor adjusted ^{13}C isotopologue peaks (median CV = 27%; Supplemental Table 2).

Our results demonstrate that AA concentrations can be determined with higher accuracy in samples spiked with PLM as IS than in samples without.

Discussion

In the present study we have explored the use of ^{13}C-labeled mouse tissues and body fluids for untargeted metabolomics. We have tested partially ^{13}C-labeled mouse plasma as reference for relative quantitation of human metabolites by mass spectrometry analyses. In the PLM plasma essential AA had the highest ^{13}C incorporation levels. Since essential AA cannot be produced by the organism the labeled feed protein is their major source. AA can be recycled when proteins are degraded, which is why complete ^{13}C labeling of essential AA is not expected. Non-essential AA are synthesized by the organism and receive their carbon from the feed, in particular from carbohydrates. According to their synthesis rate and the carbon source ^{13}C enrichment of those AA tends to be lower and reflects the ^{13}C dilution by direct synthesis in the organism. As a consequence the PLM has a high metabolite ^{13}C enrichment variability (Fig. 1).

All metabolites in the PLM have a^{12}C signal, corresponding to the native ^{12}C isotope which makes the calculation of a correction value for the quantification of each metabolite necessary. This value is used to subtract the contribution from the PLM to the ^{12}C isotope signal from the sample. When applying the correction value, relative metabolite quantification of a plasma dilution series resulted in accurate results for most AA. Especially in the case of glutamine and asparagine the relative concentrations could be determined with higher accuracy when including the IS than without. Only proline, hydroxyproline, citrulline and asparagine show a CV greater than 30% (Fig. 2C). For proline we found one outlier where the peak area of the native peak was much higher than expected.

For hydroxyproline, citrulline and asparagine our relative quantification method did not perform well. Small signal variations can have a large effect for low compound concentrations. For the correction of the partially ^{13}C-labeled IS we need to estimate the ^{12}C peak area of the IS in the spiked sample. For low compound concentrations the IS input signal is relatively high (Supplemental figure 3). In this case, estimating the IS input error has a large effect on the calculated sample signal and results in high CVs. We conclude that metabolites with a low intensity and low ^{13}C enrichment cannot be used as IS for relative quantification.

We have successfully developed an untargeted metabolomics method for the relative quantification of metabolites in human plasma samples using partially ^{13}C-labeled reference from mouse. Since mice share many metabolic pathways with humans it is conceivable that our approach can be further extended to other metabolites and used for human metabolomics research, when all carbon sources can be exchanged to ^{13}C. In addition, PLM blood and tissue material can serve as valuable reference for metabolomics studies of mouse models. We submit that such PLM can be used to verify biomarker candidates and aid in their identification.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2018.06.023.

Authors contribution

FD, HH and CT conceived the study; FD performed LC-MS measurements, sample preparation, GC-MS data analysis; CB and RS analyzed LC-MS data; CT and HH developed ^{13}C mouse feed; FD, RS and CT integrated the data; all authors wrote the manuscript.

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Conflicts of interest

HH is the CEO of Silantes GmbH.

References

[1] T.R. Covey, B.A. Thomson, B.B. Schneider, Atmospheric pressure ion sources, Mass Spectrometry Reviews 28 (2009) 870–897.
[2] B.O. Keller, J. Sui, A.B. Young, R.M. Whittal, Interferences and contaminants encountered in modern mass spectrometry, Anal. Chim. Acta 627 (2008) 71–81.
[3] M. Trotzmuller, X. Guo, A. Fauland, H. Kofeler, E. Lankmayr, Characteristics and origins of common chemical noise ions in negative ESI LC-MS, J. Mass Spectrom. 46 (2011) 553–560.
[4] L.T.a.R. Seraglia, Matrix effect, signal suppression and enhancement in LC–ESI–MS, in: A. Cappiello (Ed.), Advances in LC–MS Instrumentation, Elsevier Science, 2007, pp. 193–210.
[5] S. Klein, E. Heinzle, Isotope Labeling Experiments in Metabolomics and Fluxomics, Wiley Interdisciplinary Reviews: Systems Biology and Medicine vol. 4, (2012), pp. 261–272.
[6] K. Guo, C. Ji, L. Li, Stable-isotope dimethylation labeling combined with LC-ESI MS for quantification of amine-containing metabolites in biological samples, Anal. Chem. 79 (2007) 8631–8638.
[7] K. Guo, L. Li, Differential 12C-/13C-isotope dansylation labeling and fast liquid chromatography/mass spectrometry for absolute and relative quantification of the metabolome, Anal. Chem. 81 (2009) 3919–3932.
[8] K. Guo, L. Li, High-performance isotope labeling for profiling carboxylic acid-containing metabolites in biofluids by mass spectrometry, Anal. Chem. 82 (2010) 8789–8793.
[9] W. Yuan, K.W. Anderson, S. Li, J.L. Edwards, Subsecond absolute quantitation of amine metabolites using isobaric tags for discovery of pathway activation in mammalian cells, Anal. Chem. 84 (2012) 2892–2899.
[10] L. Wu, M.R. Mashego, J.C. van Dam, A.M. Proell, J.L. Vinke, C. Ras, W.A. van Winden, W.M. van Gulik, J.J. Heijnen, Quantitative analysis of the microbial metabolome by isotope dilution mass spectrometry using uniformly 13C-labeled cell extracts as internal standards, Anal. Biochem. 336 (2005) 164–171.
[11] C. Bueschl, B. Kluger, M. Lemmens, G. Adam, G. Wiesenberger, V. Maschietto, A. Marocco, J. Strauss, S. Bodi, G.G. Thallinger, R. Krska, R. Schuhmacher, A novel stable isotope labelling assisted workflow for improved untargeted LC-HRMS based metabolomics research, Metabolomics 10 (2014) 754–769.
[12] P. Giavalisco, J. Hummel, J. Lisec, A.C. Inostroza, G. Catchpole, L. Willmitzer, High-resolution direct infusion-based mass spectrometry in combination with whole 13C metabolome isotope labeling allows unambiguous assignment of chemical sum formulas, Anal. Chem. 80 (2008) 9417–9425.
[13] A.S. Edison, C.S. Clendinen, R. Ajredini, C. Beecher, F.V. Ponce, G.S. Stupp, Metabolomics and natural-products strategies to study chemical ecology in nematodes, Integr. Comp. Biol. 55 (2015) 478–485.
[14] F. Dethloff, A. Erban, I. Orf, J. Alpers, I. Fehrle, O. Beine-Golovchuk, S. Schmidt, J. Schwachtje, J. Kopka, Profiling methods to identify cold-regulated primary metabolites using gas chromatography coupled to mass spectrometry, Meth. Mol. Biol. 1166 (2014) 171–197.
[15] A. Nanchen, T. Fuhrer, U. Sauer, Determination of metabolic flux ratios from 13C-experiments and gas chromatography-mass spectrometry data: protocol and principles, Meth. Mol. Biol. 358 (2007) 177–197.
[16] P. Giavalisco, Y. Li, A. Matthes, A. Eckhardt, H.-M. Hubberten, H. Hesse, S. Segu, J. Hummel, K. Köhl, L. Willmitzer, Elemental formula annotation of polar and lipophilic metabolites using 13C, 15N and 34S isotope labelling, in combination with high-resolution mass spectrometry, Plant J. 68 (2011) 364–376.
[17] A. Erban, N. Schauer, A.R. Fernie, J. Kopka, Nonsupervised construction and application of mass spectral and retention time index libraries from time-of-flight gas chromatography-mass spectrometry metabolite profiles, Meth. Mol. Biol. 358

(2007) 19–38.
[18] O. Fiehn, J. Kopka, P. Dormann, T. Altmann, R.N. Trethewey, L. Willmitzer, Metabolite profiling for plant functional genomics, Nat. Biotechnol. 18 (2000) 1157–1161.
[19] J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A.R. Fernie, Gas chromatography mass spectrometry-based metabolite profiling in plants, Nat. Protoc. 1 (2006) 387–396.
[20] U. Roessner, C. Wagner, J. Kopka, R.N. Trethewey, L. Willmitzer, Simultaneous analysis of metabolites in potato tuber by gas chromatography–mass spectrometry, Plant J. 23 (2000) 131–142.
[21] C. Wagner, M. Sefkow, J. Kopka, Construction and application of a mass spectral and retention time index database generated from plant GC/EI-TOF-MS metabolite profiles, Phytochemistry 62 (2003) 887–900.
[22] N. Strehmel, J. Hummel, A. Erban, K. Strassburg, J. Kopka, Retention index thresholds for compound matching in GC-MS metabolite profiling, J. Chromatogr. B, Anal. Technol. Biomed. life sci. 871 (2008) 182–190.
[23] A. Luedemann, K. Strassburg, A. Erban, J. Kopka, TagFinder for the quantitative analysis of gas chromatography–mass spectrometry (GC-MS)-based metabolite profiling experiments, Bioinformatics 24 (2008) 732–737.
[24] J.W. Allwood, A. Erban, S. de Koning, W.B. Dunn, A. Luedemann, A. Lommen, L. Kay, R. Loscher, J. Kopka, R. Goodacre, Inter-laboratory reproducibility of fast gas chromatography-electron impact-time of flight mass spectrometry (GC-EI-TOF/MS) based plant metabolomics, Metabolomics 5 (2009) 479–496.
[25] J. Kopka, N. Schauer, S. Krueger, C. Birkemeyer, B. Usadel, E. Bergmuller, P. Dormann, W. Weckwerth, Y. Gibon, M. Stitt, L. Willmitzer, A.R. Fernie, D. Steinhauser, GMD@CSB.DB: the Golm metabolome database, Bioinformatics 21 (2005) 1635–1638.
[26] N. Schauer, D. Steinhauser, S. Strelkov, D. Schomburg, G. Allison, T. Moritz, K. Lundgren, U. Roessner-Tunali, M.G. Forbes, L. Willmitzer, A.R. Fernie, J. Kopka, GC-MS libraries for the rapid identification of metabolites in complex biological samples, FEBS Lett. 579 (2005) 1332–1337.
[27] J. Hummel, N. Strehmel, J. Selbig, D. Walther, J. Kopka, Decision tree supported substructure prediction of metabolites from GC-MS profiles, Metabolomics 6 (2010) 322–333.
[28] J. Huege, J. Goetze, D. Schwarz, H. Bauwe, M. Hagemann, J. Kopka, Modulation of the major paths of carbon in photorespiratory mutants of synechocystis, PLoS One 6 (2011) e16278.
[29] J. Huege, Jan Goetze, Frederik Dethloff, Bjoern Junker, J. Kopka, Quantification of Stable Isotope Label in Metabolites via Mass Spectrometry, Plant Chemical Genomics, Humana Press Inc, NJ, USA, 2012.
[30] C. Bueschl, B. Kluger, N.K.N. Neumann, M. Doppler, V. Maschietto, G.G. Thallinger, J. Meng-Reiterer, R. Krska, R. Schuhmacher, MetExtract II: a software suite for stable isotope-assisted untargeted metabolomics, Anal. Chem. 89 (2017) 9518–9526.
[31] E. Frank, M.S. Kessler, M.D. Filiou, Y. Zhang, G. Maccarrone, S. Reckow, M. Bunck, H. Heumann, C.W. Turck, R. Landgraf, B. Hambsch, Stable isotope metabolic labeling with a novel (15)N-enriched bacteria diet for improved proteomic analyses of mouse models for psychopathologies, PLoS One 4 (2009) e7821.
[32] D.S. Wishart, D. Tzur, C. Knox, R. Eisner, A.C. Guo, N. Young, D. Cheng, K. Jewell, D. Arndt, S. Sawhney, C. Fung, L. Nikolai, M. Lewis, M.A. Coutouly, I. Forsythe, P. Tang, S. Shrivastava, K. Jeroncic, P. Stothard, G. Amegbey, D. Block, D.D. Hau, J. Wagner, J. Miniaci, M. Clements, M. Gebremedhin, N. Guo, Y. Zhang, G.E. Duggan, G.D. Macinnis, A.M. Weljie, R. Dowlatabadi, F. Bamforth, D. Clive, R. Greiner, L. Li, T. Marrie, B.D. Sykes, H.J. Vogel, L. Querengesser, HMDB: the human metabolome database, Nucleic Acids Res. 35 (2007) D521–D526.
[33] D.S. Wishart, C. Knox, A.C. Guo, R. Eisner, N. Young, B. Gautam, D.D. Hau, N. Psychogios, E. Dong, S. Bouatra, R. Mandal, I. Sinelnikov, J. Xia, L. Jia, J.A. Cruz, E. Lim, C.A. Sobsey, S. Shrivastava, P. Huang, P. Liu, L. Fang, J. Peng, R. Fradette, D. Cheng, D. Tzur, M. Clements, A. Lewis, A. De Souza, A. Zuniga, M. Dawe, Y. Xiong, D. Clive, R. Greiner, A. Nazyrova, R. Shaykhutdinov, L. Li, H.J. Vogel, I. Forsythe, HMDB: a knowledgebase for the human metabolome, Nucleic Acids Res. 37 (2009) D603–D610.
[34] D.S. Wishart, T. Jewison, A.C. Guo, M. Wilson, C. Knox, Y. Liu, Y. Djoumbou, R. Mandal, F. Aziat, E. Dong, S. Bouatra, I. Sinelnikov, D. Arndt, J. Xia, P. Liu, F. Yallou, T. Bjorndahl, R. Perez-Pineiro, R. Eisner, F. Allen, V. Neveu, R. Greiner, A. Scalbert, HMDB 3.0–the human metabolome database in 2013, Nucleic Acids Res. 41 (2013) D801–D807.