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High-throughput quantitation of amino acids in rat and mouse biological matrices using stable isotope labeling and UPLC–MS/MS analysis☆

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ABSTRACT

Quantifying amino acids in biological matrices is typically performed using liquid chromatography (LC) coupled with fluorescent detection (FLD), requiring both derivatization and complete baseline separation of all amino acids. Due to its high specificity and sensitivity, the use of UPLC–MS/MS eliminates the derivatization step and allows for overlapping amino acid retention times thereby shortening the analysis time. Furthermore, combining UPLC–MS/MS with stable isotope labeling (e.g., isobaric tag for relative and absolute quantitation, i.e., iTRAQ) of amino acids enables quantitation while maintaining sensitivity, selectivity and speed of analysis. In this study, we report combining UPLC–MS/MS analysis with iTRAQ labeling of amino acids resulting in the elution and quantitation of 44 amino acids within 5 min demonstrating the speed and convenience of this assay over established approaches. This chromatographic analysis time represented a 5-fold improvement over the conventional HPLC–MS/MS method developed in our laboratory. In addition, the UPLC–MS/MS method demonstrated improvements in both specificity and sensitivity without loss of precision. In comparing UPLC–MS/MS and HPLC–MS/MS results of 32 detected amino acids, only 2 amino acids exhibited imprecision (RSD) >15% using UPLC–MS/MS, while 9 amino acids exhibited RSD >15% using HPLC–MS/MS. Evaluating intra- and inter-assay precision over 3 days, the quantitation range for 32 detected amino acids in rat plasma was 0.90–497 μM, with overall mean intra-day precision of less than 15% and mean inter-day precision of 12%. This UPLC–MS/MS assay was successfully implemented for the quantitative analysis of amino acids in rat and mouse plasma, along with mouse urine and tissue samples, resulting in the following concentration ranges: 0.98–431 μM in mouse plasma for 32 detected amino acids; 0.62–443 μM in rat plasma for 32 detected amino acids; 0.44–8590 μM in mouse liver for 33 detected amino acids; 0.61–1241 μM in mouse kidney for 37 detected amino acids; and 1.39–1681 μM in rat urine for 34 detected amino acids. The utility of the assay was further demonstrated by measuring and comparing plasma amino acid levels between pre-diabetic Zucker diabetic fatty rats (ZDF/Gmi fa/fa) and their lean littermates (ZDF/Gmi fa/?). Significant differences ($P < 0.001$) in 9 amino acid concentrations were observed, with the majority ranging from a 2- to 5-fold increase in pre-diabetic ZDF rats on comparison with ZDF lean rats, consistent with previous literature reports.

1. Introduction

Amino acids are the key building blocks of proteins and other essential biomolecules and hence represent a major group of endogenous compounds of interest in metabolite profiling in both preclinical and clinical settings [1,2]. Detection and accurate quantitation of amino acids play an important role in disease diagnostics, including diabetes [3,4] and diabetic ketoacidosis [5,6], kidney disease [7,8], inflammatory disorders [9] and cancer [10,11]. The traditional approach for amino acid analysis employs ion-exchange chromatography followed by post-column derivatization using ninhydrin and UV detection [12]. Other methods reported include use of pre-column fluorescent derivatization to enhance detection sensitivity followed by reversed-phase HPLC [13,14]. While these methods have been well established for routine amino acid analysis, they are limited in their abilities to offer

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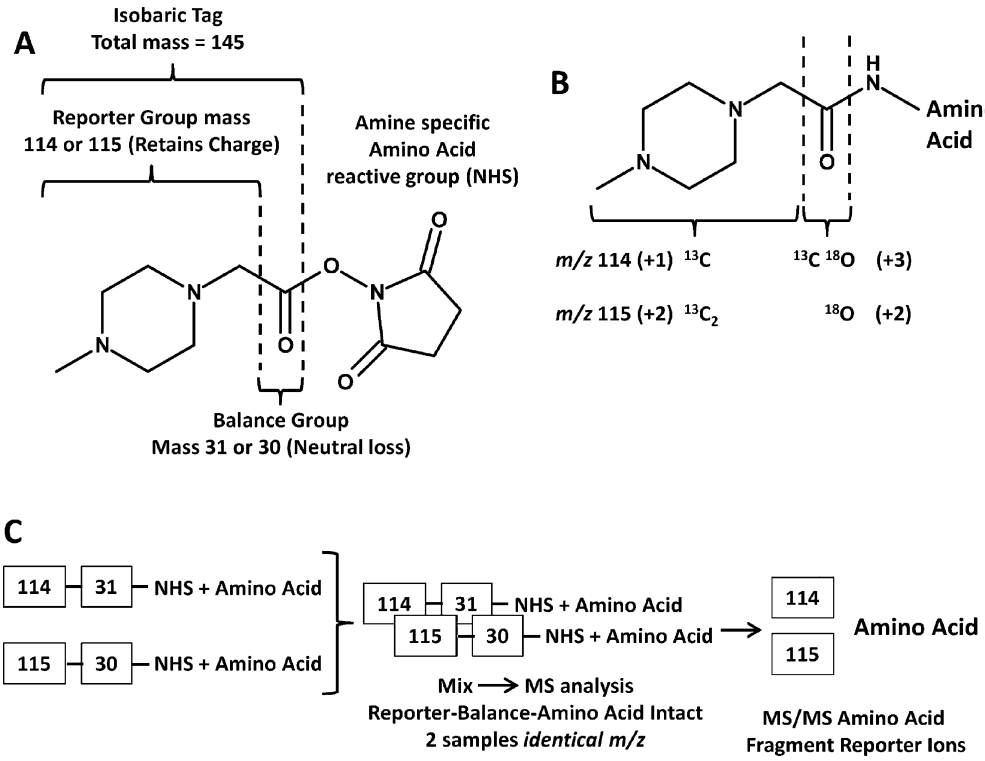


Scheme 1. (A) Description of the isobaric amino acid labeling reagent and tagging chemistry, consisting of a reporter group (based on *N*-methylpiperazine), a mass balance group (carbonyl), and an amine-reactive group (*N*-hydroxysuccinimide (NHS) ester). The overall mass of reporter and balance groups are kept constant using differential isotopic enrichment with ^{13}C and ^{18}O atoms. (B) Labeling reagents are linked to the amino group of amino acids. The reporter groups include *m/z* 114 (label for amino acid reference standards provided in the kit) and *m/z* 115 (label reacted with free amino acids in the samples), while the balance group ranges in mass from 31 and 30 Da, respectively, keeping the combined total mass consistent (145 Da) for each of the two labeling reagents. (C) Amino acid reference standards labeled with *m/z* 114 and samples labeled with *m/z* 115 are mixed prior to MS/MS analysis, resulting in detection of isobaric tag reporter ions at *m/z* 114 and 115 with resulting peak area ratios used for quantitative analysis. Information and diagrams have been previously published [19].

sufficient selectivity and sensitivity for samples in complex biological matrices. To address many of the challenges experienced when using the previously mentioned types of derivatization agents, while at the same time simplifying sample preparation and increasing throughput, recent publications have investigated the application of stable-isotope labeling coupled with LC–MS/MS [15,16]. One approach utilized iTRAQ (i.e., isobaric tag for relative and absolute quantitation) isotope labeling of physiological amino acids followed by LC–MS/MS quantitation [17]. This study was the first to compare iTRAQ stable-isotope labeling coupled with LC–MS/MS with the established approaches using GC–MS [18] and cation-exchange chromatography followed by post-column derivatization and UV detection [12]. Results from this study were shown to be similar, or superior to those from traditional methods.

The iTRAQ reagents are a set of isobaric reagents which are amine-specific, allowing for the identification and quantitation of multiple amine-containing components in the sample simultaneously, leading to their wide use in proteomics studies [19]. A schematic depicting the iTRAQ labeling reagents and their reactions with amino acids for detection is shown in Scheme 1. Two amine-specific isobaric reagents which possess two different reporter mass "tags" generated as MS/MS fragment ions are used for amino acid analysis. One reagent labels the free amino acids in the study sample extract (mass tag: 115). The second reagent consists of 44 different amino acids (mass tag: 114) of known concentrations, which are employed as internal reference standards. In LC–MS/MS analysis, both 115-labeled free amino acids and the 114-labeled known concentration internal standards appear at the same mass-to-charge ratio (*m/z*) and at the same retention time but possess different product ions which are quantitated in the multiple reaction monitoring (MRM) mode.

The application of ultra-performance liquid chromatography (UPLC) separation has demonstrated significant improvements compared with conventional HPLC, especially when coupled with mass spectrometry analysis. Utilizing sub-2 μm column particles and mobile phases at high linear velocities, and instrumentation that operates at higher pressures than those used in HPLC, drastic increases in resolution, sensitivity and speed of analysis can be achieved with UPLC. For example, reported applications of UPLC–MS/MS in metabolic profiling have demonstrated 10-fold increase in speed, a doubling of peak capacity, and a 3- to 5-fold increase in sensitivity compared with conventional reversed-phase HPLC [20–22].

Coupled with UPLC–photodiode array (PDA) and MS/MS analysis of amino acids, a more recent study reported the use of an alternative type of derivatization agent, i.e., AccQ·Tag (Waters Corporation, Milford, MA, USA), which was able to identify and quantify 16 amino acids in *Plasmodium falciparum* and 25 amino acids in human red blood cells [23]. The use of UPLC in this study provided a rapid LC separation time of less than 10 min, but required the use of both internally and externally generated calibration curves along with deuterated amino acids which served as internal standards. The goal of the present study was to develop a more rapid and selective UPLC–MS/MS method, when compared to the use of HPLC–MS/MS, coupled with iTRAQ-labeled amino acid analysis for the quantitation of amino acids in complex biological matrices. A modified

Table 1
Amino acids analyzed by UPLC–MS/MS and HPLC–MS/MS, together with their abbreviations, retention times, precision (RSD, %) of the retention time ($n = 6$), and MRM mass transitions applied.

Amino acid name	Amino acid abbreviation	Retention time (min)		RSD (%)		MRM (m/z)	
		UPLC	HPLC	UPLC	HPLC	Q1	Q3
1-Methyl-L-histidine	1MeHis	1.51	6.92	0.00	0.17	314.2	114.1
3-Methyl-L-histidine	3MeHis	1.67	7.16	0.00	0.14	314.2	114.1
L-α-Aminoadipic acid	Aad	1.87	7.58	0.00	0.15	306.2	114.1
L-α-Amino-*n*-butyric acid	Abu	1.99	8.29	0.00	0.12	248.2	114.1
L-Alanine	Ala	1.69	6.97	0.32	0.13	234.2	114.1
L-Anserine	Ans	1.87	7.51	0.00	0.16	385.2	114.1
L-Arginine	Arg	1.92	7.88	0.00	0.17	319.2	114.1
Argininosuccinic acid	Asa	1.69	7.03	0.24	0.21	435.2	114.1
L-Asparagine	Asn	0.81	4.78	0.13	0.13	277.2	114.1
L-Aspartic acid	Asp	1.14	5.61	0.36	0.23	278.1	114.1
D,L-β-Aminoisobutyric acid	bAib	1.99	7.98	0.00	0.12	248.2	114.1
β-Alanine	bAla	1.56	6.67	0.00	0.15	234.2	114.1
L-Carnosine	Car	1.85	7.50	0.00	0.20	371.2	114.1
L-Citrulline	Cit	1.48	6.21	0.00	0.17	320.2	114.1
Cystathionine	Cth	2.10	8.28	0.00	0.16	511.3	114.1
L-Cystine	Cys	2.11	8.32	0.19	0.09	529.2	114.1
Ethanolamine	EtN	1.12	5.50	0.00	0.28	206.2	114.1
γ-Amino-*n*-butyric acid	GABA	1.83	7.36	0.28	0.12	248.2	114.1
L-Glutamine	Gln	1.11	5.36	0.37	0.11	291.2	114.1
L-Glutamic acid	Glu	1.56	6.63	0.00	0.09	292.2	114.1
Glycine	Gly	1.01	5.33	0.00	0.17	220.1	114.1
Homocitrulline	Hcit	1.83	7.28	0.00	0.12	334.2	114.1
L-Homocysteine	Hcy	2.64	9.89	0.15	0.11	557.3	114.1
L-Histidine	His	1.46	6.91	0.00	0.50	300.2	114.1
D-Hydroxylysine	Hyl	2.04	8.03	0.27	0.15	451.3	114.1
Hydroxy-L-proline	Hyp	1.00	5.25	0.56	0.20	276.1	114.1
L-Isoleucine	Ile	2.86	10.80	0.14	0.10	276.2	114.1
L-Leucine	Leu	2.94	11.10	0.00	0.51	276.2	114.1
L-Lysine	Lys	2.24	8.66	0.00	0.11	435.3	114.1
L-Methionine	Met	2.44	9.58	0.00	0.09	294.2	114.1
L-Norleucine	Nle	3.00	11.30	0.14	0.00	276.2	114.1
L-Norvaline	Nva	2.53	9.77	0.00	0.09	262.2	114.1
L-Ornithine	Orn	2.10	8.23	0.00	0.16	421.3	114.1
O-Phosphoethanolamine	PEtN	0.45	2.73	0.23	0.20	286.1	114.1
L-Phenylalanine	Phe	2.98	11.30	0.14	0.00	310.2	114.1
L-Proline	Pro	2.01	8.01	0.27	0.12	260.2	114.1
O-Phospho-L-serine	Pser	0.40	2.56	0.34	0.22	330.1	114.1
Sarcosine	Sar	1.47	6.35	0.00	0.30	234.2	114.1
L-Serine	Ser	0.87	4.92	0.26	0.29	250.2	114.1
Taurine	Tau	0.47	2.77	0.32	0.29	270.1	114.1
L-Threonine	Thr	1.53	6.39	0.00	0.15	264.2	114.1
L-Tryptophan	Trp	3.21	12.10	0.00	0.00	349.2	114.1
L-Tyrosine	Tyr	2.44	9.68	0.21	0.07	326.2	114.1
L-Valine	Val	2.44	9.47	0.00	0.10	262.2	114.1

extraction procedure requiring a 4-fold less sample volume was also compared with the established extraction protocol. By this UPLC–MS/MS method, amino acid profiling was conducted in a range of biological matrices including plasma, urine and selected organ tissues. In an application of the assay, amino acid plasma concentrations were evaluated in prediabetic Zucker diabetic fatty rats (ZDF/Gmi fa/fa) and in ZDF lean (ZDF/Gmi fa/?). The results generated from this study were compared with literature data, where traditional HPLC-FLD methods were employed.

2. Experimental

2.1. Chemicals and reagents

All chemical reagents and solvents were purchased from the established commercial vendors and were HPLC grade except otherwise specified. All reagents used for amino acid extraction and iTRAQ stable isotope labeling were included in the Amino Acid Analysis for Physiological Samples Kit (AB-Sciex, Framingham, MA, USA; http://www.absciex.com/Documents/Downloads/Literature/Amino-Acid-Analysis-Physiological-Samples-iTRAQ-Reagents-Application-Kit-Use-LC-MS-MS-Systems-Protocol.pdf) unless otherwise specified.

2.2. Amino acid extraction and stable-isotope labeling

Plasma, tissue and urine from male Sprague-Dawley rats and male C57BL6 mice were obtained from Bioreclamation, Inc. (Hicksville, NY, USA). Plasma from male ZDF rats (ZDF/Gmi fa/fa) and male lean ZDF littermates (ZDF/Gmi fa/?), age 28 days, was obtained from Charles River Laboratories (Stone Ridge, NY, USA). Mouse plasma was pooled from a single lot and processed in 6 duplicates for comparison of the modified extraction procedure. Rat plasma was pooled from a single lot and processed as 6 replicates for UPLC and HPLC method comparisons and inter- and intra-day variability determination. Tissue homogenates were prepared at a 1:4 (w/v) ratio in water using the Covaris E210 Focused-ultrasonicator (Covaris, Inc., Woburn, MA, USA). Tissue and urine samples were run in triplicate for amino acid concentration comparisons. Plasma samples from ZDF rats (ZDF/Gmi fa/fa) and lean littermates (ZDF/Gmi fa/?) were run in triplicate for comparison

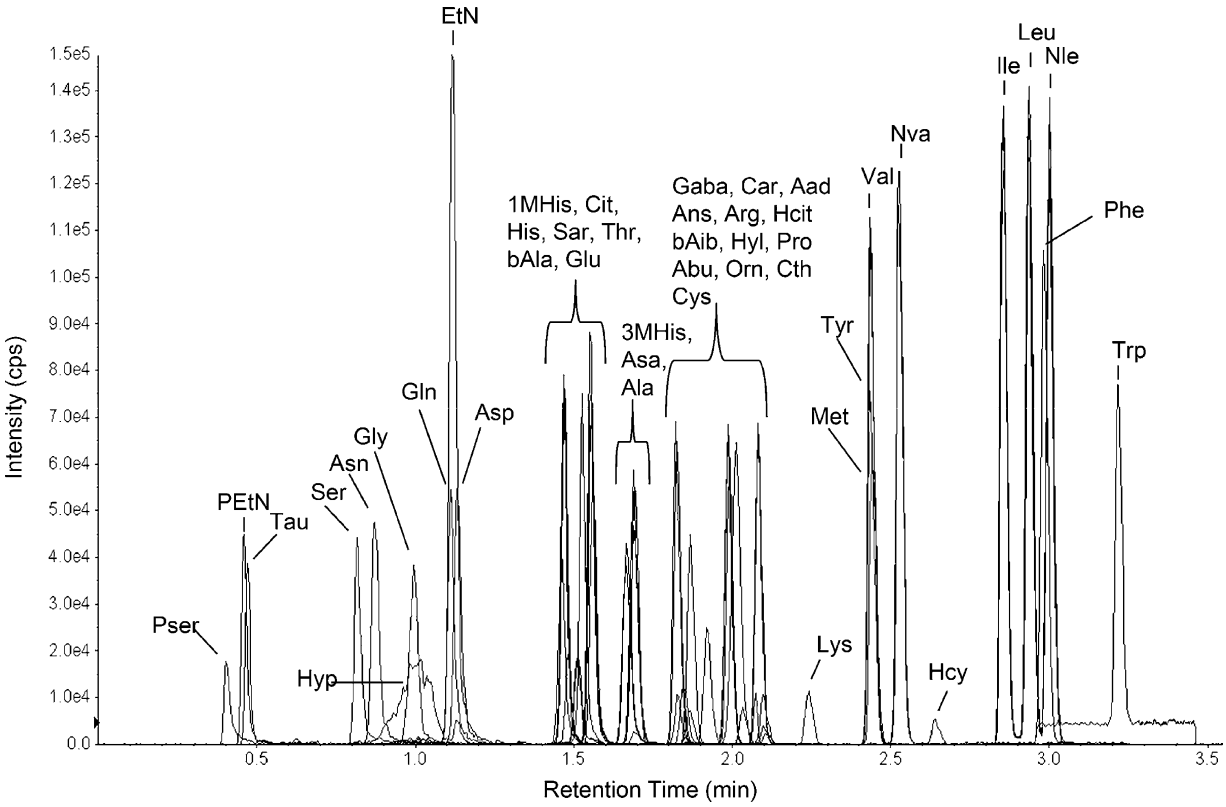
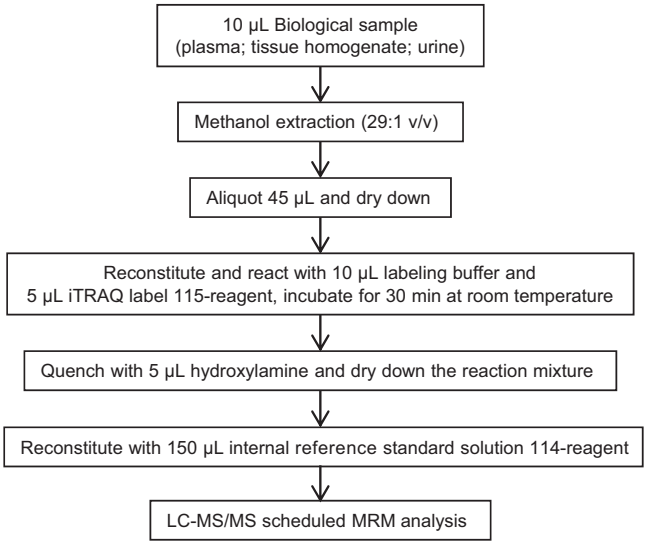


Fig. 1. Overlaid extracted ion chromatograms for individual amino acid scheduled MRM signals for the iTRAQ reagent 114-labeled internal reference standard mix, 100 μM of each amino acid except for L-cystine present at 50 μM, using the UPLC–MS/MS method.

of amino acid concentrations. All replicates were subjected to the entire sample preparation process prior to analysis.

Free amino acids were extracted from the plasma, tissue and urine samples using methanol at a 1:29 (v/v) ratio. The extracted amino acids were then directly reacted with iTRAQ reagents, using supplied reagents and a modified protocol from the Amino Acid Analysis for Physiological Samples Kit (AB-Sciex, Framingham, MA, USA). A summary of the sample preparation workflow is shown in Scheme 2. In brief, 10-μL aliquots of plasma, tissue homogenate, or urine were added to 290-μL aliquots of methanol. For comparison, the manufacturer's extraction protocol was also performed using a 40-μL aliquot of plasma and 10-μL of acid precipitation solution. Samples were vortexed and centrifuged (10 min, 12,800 × *g*, room temperature) and 45-μL aliquots of the supernatant were decanted and evaporated to dryness under nitrogen using the TurboVap 96 Evaporation System (Caliper Life Sciences, Hopkinton, MA, USA) for approximately 30 min at 30 °C. To each dried sample, 10 μL of the labeling buffer (i.e., 0.45 M borate buffer, pH 8.5, containing 20 pmol/μL norvaline for determination of derivatization efficiency) and 5 μL of iTRAQ 115-labeled reagent solution were added, followed by short vortexing and centrifugation (5 s, 1000 × *g*, room temperature). Samples were then incubated for 30 min at room temperature. The reaction was terminated by addition of 5 μL of the hydroxylamine kit reagent, and samples were vortexed and then centrifuged (5 s, 1000 × *g*, room temperature). Samples were evaporated to dryness under nitrogen for approximately 30 min at 30 °C using the TurboVap 96 Evaporation System. The dried samples were reconstituted in 150-μL aliquots of the iTRAQ reagent 114-labeled standard mix (100 pmol of each amino acid/μL except for L-cystine which was present at 50 pmol/μL), used as the internal reference standards for quantitation. The samples were then diluted with 750 μL of 0.5% formic acid (Suprapur, EMD, Gibbstown, NJ, USA) in water prior to HPLC–MS/MS or UPLC–MS/MS analysis.



Scheme 2. Workflow summary of the amino acid extraction, iTRAQ labeling process, and sample analysis.

2.3. LC–MS/MS instrumentation and conditions

LC–MS/MS analyses were performed on an Applied Biosystems Sciex API-4000 triple quadruple mass spectrometer equipped with an electrospray probe (AB-Sciex, Framingham, MA, USA) interfaced to an Acquity UPLC system (Waters, Milford, MA, USA). The analytical columns employed were an AccQ·Tag, 1.7 μm, 2.1 mm i.d. × 100 mm (Waters, Milford, MA, USA) for UPLC and

an AAA C_{18}, 5 μm, 4.6 mm i.d. × 150 mm (AB-Sciex, Framingham, MA, USA) for HPLC. Mobile phase A was composed of 0.1 vol.% formic acid and 0.01 vol.% hexafluorobutyric acid in water. Mobile phase B was composed of 0.1 vol.% formic acid and 0.01 vol.% hexafluorobutyric acid in acetonitrile. For UPLC, the mobile phase was held at 0%B for 0.5 min followed by linear gradients from 0%B to 22%B over 3 min and from 22%B to 80%B over 0.37 min and then maintained at 80%B for additional 0.37 min; the flow rate was 0.7 mL/min. For HPLC, the mobile phase was a linear gradient from 2%B to 22%B over 10 min, from 22%B to 80%B over 10 min and then maintained at 80%B for additional 6 min; the flow rate was 0.8 mL/min. Autosampler and column temperatures were maintained at 10 °C and 50 °C, respectively. The injection volume was 1 μL for UPLC and 5 μL for HPLC.

Amino acids were ionized in positive ion mode using an electrospray probe. Ion spray voltage was set at 5000 V. Auxiliary gas temperature (TEM) was set at 600 °C. Curtain gas (CUR), nebulizer gas (GS1), auxiliary gas (GS2), and collision gas were set at 30, 55, 65 and 6 arbitrary units, respectively; entrance potential (EP), declustering potential (DP), collision energy (CE) collision cell exit potential (CXP) were set at 10 V, 30 V, 30 V and 15 V, respectively. Quantitation was performed using scheduled multiple reaction monitoring (MRM) specifying a 30-s detection window and 0.3-s target scan time. A total of 88 MRM transitions were acquired in a single run; each 44 MRM transitions were used for the 114-labeled internal reference standards for individual amino acids and the 115-labeled endogenous amino acids. Peak area integration was performed using Analyst version 1.5 IntelliQuan quantitation software (Applied Biosystems, Foster City, CA, USA).

2.4. Data analysis

Formula (F1) was used to calculate amino acid concentration (i.e., [AA]):

$$[\mathrm{AA}]\ (\mu\mathrm{M}) = \frac{\text{Analyte area} \times [\mathrm{IS}]\ (\mu\mathrm{M})}{\text{IS area} - (\text{Analyte area} \times \text{Contribution factor})} \times \text{Conversion factor} \quad \text{(F1)}$$

The internal standard (IS) concentration [IS] was 100 μM for all amino acids and 50 μM for L-cysteine. Amino acids are quantified separately based on the corresponding 115-labeled analyte peak area (analyte area) and 114-labeled internal reference standard peak area (IS area). The contribution factor is the ratio of the 114-labeled peak area to the 115-labeled peak area when only the iTRAQ Reagent 115-labeled reference standard was injected. This contribution factor was calculated separately for each individual amino acid and accounts for the small contribution from the analyte labeling reagent (115-labeled amino acids) signal into the internal reference standard (114-labeled amino acids) signal. The conversion factor is calculated based on the ratio of 114-labeled internal standard concentration in comparison with the manufactures protocol (50–100 pmol/μL) divided by the final volume of plasma, urine or tissue homogenate injected onto the column after the extraction and labeling protocol (0.15 μL), resulting in a value of 0.667 for the conversion factor used in the modified extraction protocol. The labeling process internal standard norvaline (Nva) was used to normalize the final concentrations using the known spiked concentration of Nva as a reference. Tissue amino acid concentrations were calculated using a 5-fold dilution factor, based on a 1:4 (w/v) tissue homogenate and an assumption of 1 g/mL tissue density, and reported as micromolar.

Amino acid concentrations in ZDF rat plasma were reported as mean ± standard deviation (SD). Means were compared using Student's unpaired *t* test function in Excel (Microsoft office 2003).

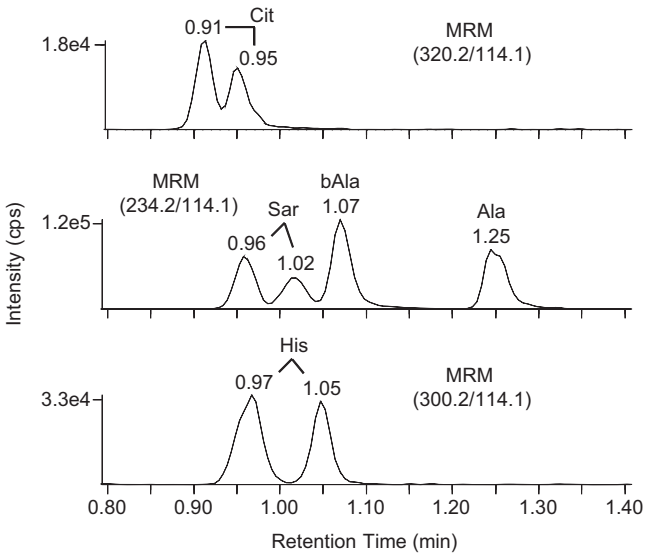


Fig. 2. Peak splitting for Cit, Sar and His caused by the labeling buffer present in the extracted sample during early UPLC method development. Extracted ion chromatogram MRM mass transition signals shown only for the 114-labeled internal reference standards.

3. Results and discussion

The amino acids analyzed in the present study, along with their corresponding retention times, precision of the retention time, and MRM transitions used, are presented in Table 1. Shown in Fig. 1 is an extracted ion chromatogram overlay using UPLC for the 114-labeled amino acid standards. All 44 amino acids were eluted in less than 3.5 min with the majority eluting between 1.4 and 2.2 min. There were a number of overlapping MRM extracted ion chromatogram (XIC) peaks in the 0.8-min time window. These closely-spaced peaks, narrow peak widths (i.e., averaging 3 s), and

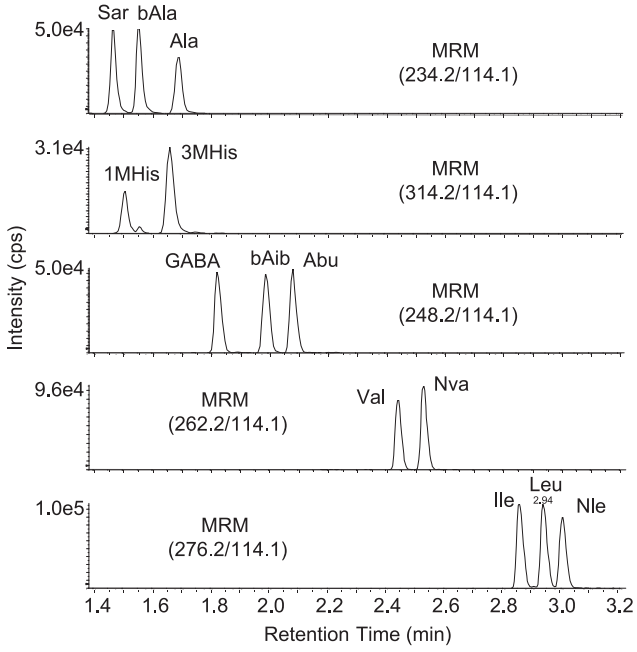


Fig. 3. Separation of isobaric amino acids: Sar, bAla, and Ala; 1MeHis and 3MeHis; GABA, bAib, and Abu; Val and Nva; Ile, Leu, and Nle, and their extracted ion chromatograms using the UPLC–MS/MS method. Extracted ion chromatogram MRM mass transition signals shown only for the 114-labeled internal reference standards.

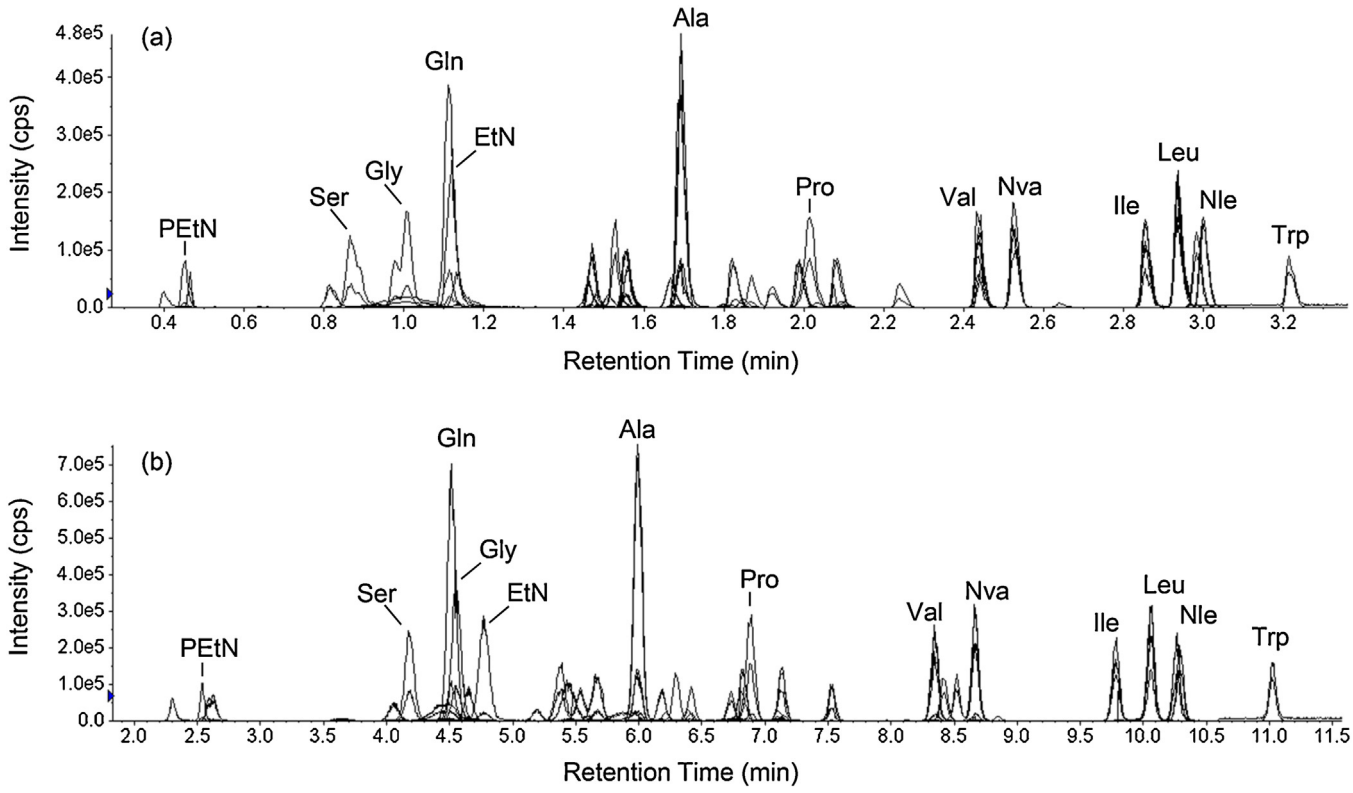


Fig. 4. Comparison of extracted ion chromatograms from rat plasma for both 115-labeled amino acids and 114-labeled internal reference standards using (a) UPLC–MS/MS and (b) HPLC–MS/MS.

Table 2
Amino acids and their concentrations (μM) measured in mouse plasma by UPLC–MS/MS after protein precipitation by acid or methanol extraction.[a]

Amino acid	Acid precipitation[a]	RSD (%)	Methanol extraction[a]	RSD (%)	% Difference[b]
3MeHis	6.37	10.4	5.67	13.4	−10.9
Aad	3.68	11.0	3.19	9.73	−13.4
Abu	14.7	4.22	13.6	3.09	−7.62
Ala	428	8.00	374	7.49	−12.7
Arg	137	3.49	104	2.90	−24.5
Asn	56.0	5.68	49.5	6.43	−11.7
Asp	8.55	3.63	7.44	4.17	−12.9
bAla	23.8	26.0	26.3	20.6	10.4
Cit	108	3.35	93.1	3.88	−13.8
EtN	15.8	6.89	12.6	8.67	−20.5
Gln	485	2.51	430	3.26	−11.5
Glu	30.9	4.23	26.6	4.91	−13.9
Gly	357	2.08	315	2.36	−11.7
His	66.7	7.12	61.7	7.68	−7.38
Hyp	42.3	3.09	35.1	3.73	−17.1
Ile	71.8	2.42	65.5	2.65	−8.78
Leu	130	2.93	108	3.53	−17.0
Lys	331	3.51	301	5.01	−8.89
Met	117	4.63	111	5.38	−5.03
Orn	56.7	4.51	43.5	5.88	−23.2
PEtN	9.02	8.17	7.91	9.32	−12.4
Phe	63.7	1.86	56.1	2.11	−12.0
Pro	140	4.29	122	4.92	−12.8
Sar	5.46	6.19	4.42	7.65	−19.1
Ser	179	5.75	161	7.46	−10.4
Tau	185	2.57	154	2.14	−16.7
Thr	137	5.17	115	6.97	−16.4
Trp	59.6	1.88	57.7	2.44	−3.28
Tyr	77.0	2.83	68.6	4.69	−10.9
Val	200	11.4	167	14.4	−16.8

[a] Mouse plasma was extracted using a commercially available kit either by acid precipitation (40 μL, $n = 6$) or by methanol extraction (10 μL, $n = 6$).
[b] % Difference = (concentration measured with methanol extraction − concentration measured with acid precipitation)/concentration measured with acid precipitation * 100%.

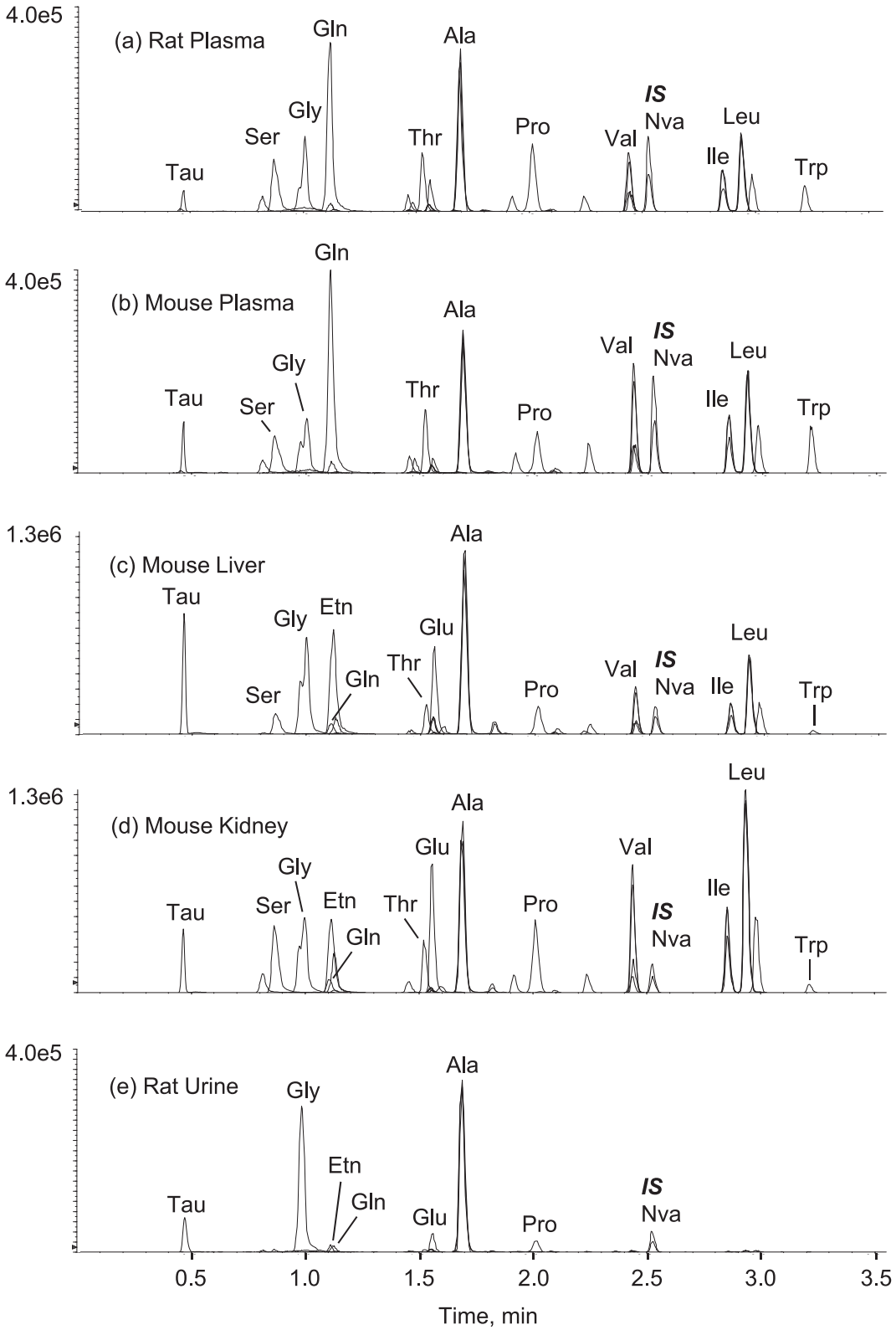


Fig. 5. Comparison of extracted ion chromatograms (the 115-labeled amino acid signals only) for extracted amino acids from 5 different matrices: (a) rat plasma, (b) mouse plasma, (c) mouse liver, (d) mouse kidney, and (e) rat urine. The internal standard Nva is labeled as "IS" on each chromatogram.

overall short chromatographic run time, made it difficult to define separate MRM periods for mass spectrometry analysis, as has been reported previously using HPLC methods [17]. Due to the narrow peak widths observed in UPLC and the use of a typical MRM dwell time of less than 100 ms, it is important to adjust the dwell time and chromatography run time to maximize the number of data points or scans per peak for accurate quantitation and run-to-run reproducibility. Previous studies [17,23] have reported the use of longer chromatographic run time along with dividing the total MRM scans into functions or periods. In our study, the scheduled

Table 3
Intra- and inter-assay imprecision (RSD, %, $n = 6$) for rat plasma amino acid concentrations (μM) determined using UPLC–MS/MS.

Amino acid	Intra-day precision						Inter-day precision	
	Day 1		Day 2		Day 3		Mean	RSD (%)
	Mean	RSD (%)	Mean	RSD (%)	Mean	RSD (%)		
1MeHis	3.55	26.1	3.90	10.0	3.44	19.6	3.63	6.55
3MeHis	3.09	19.4	3.25	19.0	3.64	21.6	3.32	8.46
Aad	0.79	30.9	1.05	34.1	0.87	36.5	0.90	15.0
Abu	1.62	8.32	2.03	8.87	2.48	10.0	2.04	21.1
Ala	361	9.59	403	15.5	435	4.86	400	9.31
Ans	ND	ND	ND	ND	ND	ND	ND	ND
Arg	81.0	10.6	88.4	10.6	101	8.64	90.3	11.4
Asa	ND	ND	ND	ND	ND	ND	ND	ND
Asn	52.3	4.98	60.3	9.50	63.0	4.61	58.5	9.55
Asp	5.56	8.74	6.17	13.7	6.38	9.32	6.04	7.09
bAib	ND	ND	ND	ND	ND	ND	ND	ND
bAla	12.9	18.5	14.3	15.7	14.5	2.63	13.9	6.47
Car	ND	ND	ND	ND	ND	ND	ND	ND
Cit	59.4	6.46	64.6	15.8	69.6	6.25	64.5	7.92
Cth	ND	ND	ND	ND	ND	ND	ND	ND
Cys	ND	ND	ND	ND	ND	ND	ND	ND
EtN	5.23	10.9	6.22	15.6	7.47	15.1	6.31	17.8
GABA	ND	ND	ND	ND	ND	ND	ND	ND
Gln	431	6.50	508	5.74	553	5.13	497	12.4
Glu	67.1	4.07	80.3	8.58	85.1	13.3	77.5	12.0
Gly	238	7.83	309	6.07	305	7.70	284	14.1
Hcit	1.93	33.9	1.97	27.5	3.04	37.8	2.31	27.0
Hcy	ND	ND	ND	ND	ND	ND	ND	ND
His	48.9	5.88	54.8	11.8	60.8	5.52	54.8	10.9
Hyl	ND	ND	ND	ND	ND	ND	ND	ND
Hyp	30.1	4.38	35.8	7.92	38.8	6.95	34.9	12.7
Ile	26.0	6.22	30.4	7.23	31.5	4.45	29.3	9.88
Leu	83.4	3.59	97.1	10.3	104	8.53	94.7	10.9
Lys	174	8.54	209	12.4	234	8.33	206	14.7
Met	37.2	3.36	43.0	10.5	47.0	13.2	42.4	11.6
Orn	30.3	15.2	32.4	13.0	42.3	4.72	35.0	18.3
PEtN	5.99	8.02	6.96	9.81	7.28	5.28	6.75	9.94
Phe	43.9	2.63	49.6	7.82	53.5	6.31	49.0	9.91
Pro	123	3.67	144	9.78	151	3.97	139	10.6
Pser	ND	ND	ND	ND	ND	ND	ND	ND
Sar	2.82	5.68	3.19	10.5	3.19	15.8	3.06	6.90
Ser	185	8.34	221	7.45	238	10.4	215	12.8
Tau	87.9	4.36	101	10.3	113	12.9	101	12.4
Thr	107	5.08	126	9.27	145	8.76	126	14.9
Trp	46.9	7.63	54.4	8.42	57.3	5.18	52.9	10.2
Tyr	45.8	4.64	53.3	9.21	56.4	5.69	51.8	10.5
Val	67.8	4.52	78.2	10.0	84.6	4.33	76.9	11.0

ND: not detected.

MRM software function was used to optimize peak detection for each amino acid allowing a significantly faster UPLC elution time of 3.5 min. This function designated separate retention times for individual amino acid MRM transitions, automatically optimizing MRM dwell time depending on the number of peaks within a given time window, resulting in improved peak shape and sensitivity. The only notable non-symmetrical, broader peak shape observed was for hydroxyproline (Hyp), however this phenomenon was expected due to the *cis* and *trans* positional isomers of this amino acid. During the initial UPLC method development, peak splitting was observed during the retention time window between 0.8 and 1.2 min for the amino acids Cit, Sar and His as illustrated in Fig. 2. The UPLC conditions were modified to increase the retention of these amino acids. As a result, only a slight shoulder was observed on the leading edge of the peak for a different amino acid, glycine, which did not appear to interfere with peak detection and integration. The size of the injection volume was found to be important for achieving optimal chromatography; an injection volume greater than 1 μL resulted in pronounced peak splitting for glycine, anserine and serine. Chromatographic resolution was also improved during UPLC method optimization, decreasing the peak width from 6 s (Fig. 2) to 3 s as displayed in Figs. 1, 3–5. The borate labeling buffer was ultimately identified as the cause of this peak splitting since no splitting was observed when analyzing the internal reference standard solution or plasma spiked with internal reference standard solution in the absence of the labeling buffer. Another important factor during the UPLC method development was to ensure complete baseline separation of the isobaric amino acids as these amino acids have identical MRM mass transitions (Table 1). In this analysis there were five sets of isobaric amino acids, namely Sar, bAla, and Ala; 1MeHis and 3MeHis; GABA, bAib, and Abu; Val and Nva; Ile, Leu, and Nle. To achieve the selectivity required for quantitation, chromatographic conditions were optimized to ensure baseline separation and examples of the chromatographic separation for each set of isobaric amino acids are shown in Fig. 3. Baseline separation of these amino acids using HPLC [17] and UPLC [23] have been demonstrated with elution times of 10 min or greater, while in contrast the present study was able to still provide baseline separation required for accurate quantitation within 3.5 min.

Prior to employing the UPLC–MS/MS method and standard iTRAQ labeling procedures, a modified amino acid extraction procedure was developed. The commercially available kit specified an acid precipitation requiring 40 μL of plasma. Due to plasma volume

Table 4
Amino acid concentrations (μM) measured using UPLC–MS/MS and HPLC–MS/MS for rat plasma ($n = 6$) and UPLC–MS/MS for mouse plasma, mouse liver, mouse kidney and rat urine ($n = 3$ per matrix).

Amino acid	UPLC	HPLC	UPLC			
	Rat plasma		Mouse plasma	Mouse liver	Mouse kidney	Rat urine
1MeHis	3.93[a,b]	2.36[a,b]	1.13[a]	ND	1.14[a]	5.52
3MeHis	3.13[a]	3.19	2.41[a]	0.49[a]	5.88[a]	10.1
Aad	0.62	0.66[a]	1.55[a]	6.45[a]	4.44	1.77[a]
Abu	1.61[b]	3.77[b]	2.53	5.67	0.78	3.08
Ala	379	361	239	1016	1120	1490
Ans	ND	ND	ND	ND	ND	ND
Arg	84.0	85.3	75.3	ND	273	14.5
Asa	ND	ND	ND	ND	12.2[a]	ND
Asn	53.9	53.8	38.8	10.1	233	17.7
Asp	5.87	4.92	2.59	111	310	54.4
bAib	ND	ND	ND	ND	ND	2.89[a]
bAla	12.2	14.7	11.1	52.3	14.3	19.1
Car	ND	ND	ND	ND	ND	ND
Cit	60.0	56.1[a]	64.0[a]	3.74[a]	5.71[a]	20.6
Cth	ND	ND	ND	6.02[a]	ND	ND
Cys	ND	ND	ND	34.8	37.2	ND
EtN	5.46	5.32[a]	5.06	269	196	17.8
GABA	ND	ND	ND	40.3	26.2	6.29
Gln	443	471	431	74.6	116	4.48
Glu	66.5	73.7	25.2	544	852	157
Gly	248	260[a]	180	905	887	1681
Hcit	1.53	1.79[a]	0.98	ND	1.00	ND
Hcy	ND	ND	ND	ND	ND	ND
His	49.6	60.3[a]	41.9	33.1	112	10.6
Hyl	ND	ND	ND	ND	55.8	18.3
Hyp	30.4	33.0[a]	17.9	4.08[a]	8.46[a]	47.6
Ile	26.8[b]	77.4[b]	29.8	49.7	156	1.40[a]
Leu	85.0	70.4	81.1	192	578	5.82
Lys	179	183	219	295	504	23.9
Met	37.5	40.0	38.4	54.4	84.7	3.11
Orn	33.3	27.5	41.2	160	69.5	16.8
PEtN	6.15	5.86	3.73	1.53[a]	0.61[a]	2.64
Phe	44.1	45.0	45.9	93.8[a]	292	6.41
Pro	125	127	59.0	133	384	77.3
Pser	ND	ND	ND	0.44[a]	0.76[a]	ND
Sar	2.77[b]	1.85[a,b]	1.93	0.84[a]	1.30[a]	4.09
Ser	189	193	107	184	623	21.1
Tau	87.9	81.6	185	8590[a]	1241	616
Thr	108	115	93.7	138[a]	302	15.4
Trp	48.6	49.1	61.8	15.7	43.9	1.39
Tyr	46.5	48.3	49.3	72.5	193	5.69[a]
Val	68.3[b]	98.8[b]	89.8	131	381	7.47

ND: not detected.
[a] RSD, % >15% for replicate samples.
[b] RSD, % >15% concentration difference between UPLC and HPLC methods.

limitations commonly encountered in preclinical studies, particularly in mouse studies, it was desirable to use a lower volume of plasma. The modified procedure used only 10 μL of plasma with 290 μL of methanol for extraction. Results (Table 2) demonstrated that the values for amino acid concentration and precision using the modified sample extraction procedure, based on a comparison of observed 30 detected amino acid concentrations in mouse plasma, were comparable (difference less than 20%) to those using the kit protocol provided by the manufacturer. Controlling for variability in extraction efficiency using the modified procedure could be further improved by the addition of an internal standard in the extraction step.

Chromatograms from rat plasma extracts obtained from UPLC and HPLC analyses are shown in Fig. 4. All amino acids were eluted within 3.5 min using UPLC compared to 11.5 min using HPLC separation. The total run time including column re-equilibration was 5 and 25 min for the UPLC and HPLC separation, respectively. Improvements in resolution and peak shape were also obtained with UPLC resulting in average peak widths of 3 s versus 12 s for HPLC. Similar intensity profiles were observed for both separation approaches despite the injection volume for UPLC being 5-fold smaller than the volume used for HPLC. Variability in retention time was originally a concern for the narrow UPLC peaks compared with the broader HPLC peaks. However, retention time reproducibility (<0.6%) was similar for the two methods (Table 1) and comparable to previously reported UPLC retention time reproducibility of 0.08–1.08% [23].

Intra- and inter-day assay precision for amino acid concentrations in rat plasma samples using UPLC–MS/MS are shown in Table 3. Five amino acids (1MeHis, 3MeHis, Aad, bAla, Hcit) out of 32 amino acids, with concentrations ranging from 0.90 to 497 μM, displayed an intra-day assay imprecision greater than 15%; among them 1MeHis, 3MeHis, Aad, and Hcit had concentrations lower than 5 μM. Four amino acids (Abu, EtN, Hcit, Orn) displayed an inter-day assay imprecision greater than 15%; among them Abu and Hcit had concentrations lower than 5 μM. The other two amino acids (EtN, Orn) had intra-day imprecision less than 15% and displayed inter-day assay variability greater than 15%. The total mean inter-day imprecision was 12% over a 3-day time period. These intra and inter-day reproducibility values are in agreement with those reported previously [23,26], with an increase in variability of approximately 15% for amino acids with concentrations in

Table 5
Plasma amino acid concentrations (μM) measured by UPLC–MS/MS in three ZDF/Gmi fa/? lean and three ZDF/Gmi fa/fa fatty rats.

Amino acid	ZDF fa/?	SD	ZDF fa/fa	SD	% Change[a]
1MeHis	1.12	0.20	1.18	0.31	NS
3MeHis	1.66	0.20	1.51	0.46	NS
Aad	0.59	0.01	3.44[d]	0.36	485
Abu	1.60	0.02	1.96[b]	0.19	22
Ala	291	13.6	509[d]	56.6	75
Arg	80.7	3.11	14.8[d]	8.75	−82
Asn	65.2	2.84	64.7	3.87	NS
Asp	14.7	0.54	57.3[c]	14.4	289
bAib	0.11	0.05	0.19	0.10	NS
bAla	8.75	0.89	10.4	2.82	NS
Cit	55.8	0.39	64.4	7.47	NS
EtN	12.6	1.78	25.1[c]	3.41	99
GABA	0.49	0.04	0.99[c]	0.17	102
Gln	522	19.5	602[b]	41.6	15
Glu	77.2	3.96	235[c]	43.4	205
Gly	262	30.0	250	25.5	NS
Hcit	2.58	0.75	3.10	0.52	NS
His	49.0	2.00	62.7[b]	1.63	28
Hyp	35.0	1.54	24.9[c]	1.24	−29
Ile	40.8	2.00	76.5[d]	1.91	88
Leu	84.1	1.95	156[d]	8.82	85
Lys	157	5.85	167	19.4	NS
Met	35.0	5.00	40.5	11.8	NS
Orn	26.9	0.67	126[d]	8.19	367
PEtN	1.51	0.03	3.30[b]	0.94	NS
Phe	36.6	2.37	61.0[d]	3.32	67
Pro	128	4.25	149[b]	2.48	16
Sar	2.09	0.33	3.41[b]	0.56	63
Ser	132	5.00	152[b]	9.96	15
Tau	138	30.1	410[d]	50.8	197
Thr	125	3.53	146	12.7	NS
Trp	57.2	2.31	64.9[b]	2.95	13
Tyr	59.0	2.07	75.1[c]	5.04	27
Val	97.1	2.75	169[d]	4.01	74

Values are expressed as mean values ($n = 3$) for each group.
[a] % Change = (concentration ZDF (fa/fa) − concentration ZDF (fa/?))/concentration ZDF (fa/?) * 100.
[b] $P < 0.05$ when compared with lean controls (fa/?).
[c] $P < 0.01$ when compared with lean controls (fa/?).
[d] $P < 0.001$ when compared with lean controls (fa/?). NS: not significant based on P values.

the range of 5–10 μM [26]. One of these studies [23] used replicates consisting of three repeated sample reinjections to determine reported reproducibility. In contrast, in our study we used replicates including both the labeling process and the UPLC–MS/MS analysis.

The amino acid concentrations determined in rat plasma by UPLC–MS/MS and HPLC–MS/MS are shown in Table 4. In both methods, using 6 replicates, a total of 32 amino acids were detected with concentrations ranging from 0.62 to 443 μM in the UPLC–MS/MS analysis and 0.66 to 471 μM in the HPLC–MS/MS analysis. Two amino acids exhibited imprecision greater than 15% using UPLC–MS/MS compared to nine amino acids using HPLC–MS/MS. The difference in reproducibility and greater variability using HPLC–MS/MS are likely due to the lower chromatographic resolution and a larger number of overlapping peaks in the HPLC–MS/MS method (Fig. 4). Also, four out of the nine amino acids with higher imprecision were present at concentrations below 5 μM, indicating a greater challenge in detecting low abundance amino acids when using HPLC–MS/MS compared with UPLC–MS/MS. In contrast, using UPLC, the only two amino acids exhibiting imprecision greater than 15% were 1MeHis and 3MeHis; however the concentrations of these two amino acids were also relatively low in rat plasma. Variations in amino acid concentrations between HPLC–MS/MS and UPLC–MS/MS (Table 4) were observed for five amino acids which displayed differences greater than 15%. Among these five amino acids, 1MeHis, Abu, and Sar had concentrations below 5 μM. The low concentrations of these three amino acids may account for the larger percentage difference observed between HPLC–MS/MS and UPLC–MS/MS. The reason for the 15% difference in concentration observed for Ile and Val is unknown.

Also listed in Table 4 are amino acids measured in mouse plasma, liver, kidney, and rat urine using UPLC–MS/MS. Differences in amino acid profile and concentrations were observed among these biological samples (Fig. 5). The amino acid Nva, which was used at a fixed concentration as an internal standard to control for the iTRAQ labeling efficiency step, is labeled in each chromatogram shown in Fig. 5. Since Nva is present at a fixed concentration in these samples this amino acid can be used as a relative intensity reference in comparing amino acid abundances among the different biological matrices. The amino acid concentrations were similar in rat plasma (range, 0.62–443 μM) and mouse plasma (range, 0.98–431 μM), except for the lower concentrations for Pro and Ser and the higher concentration of Tau in mouse plasma. The amino acid concentrations in mouse liver and kidney differed markedly from those in plasma, with concentrations ranging from 0.44 to 8590 μM in mouse liver for 33 detected amino acids and from 0.61 to 1241 μM in mouse kidney for 37 detected amino acids. Higher concentrations were observed for Ala, Asp, EtN, Glu, Gly, Tau and Val, but lower concentrations for Gln, compared with plasma. Interestingly, 1MeHis, Arg, Hcit and Hyl were present in mouse kidney but were not observed in mouse liver tissue. The larger imprecision obtained for many amino acids in the mouse liver and kidney tissues may result from a greater variability in the tissue extraction step which could be further optimized. The amino acid profile for rat urine was notably different from that of the other matrices (i.e., plasma, liver, and kidney). Urine amino acid concentrations ranged from

1.39 to 1681 μM for 34 detected amino acids. For the majority of the amino acids relatively low concentrations were observed, suggesting that these amino acids are reabsorbed from the primary urine for recycled utilization in the body, except for Ala, Gly and Tau.

Amino acid metabolism in the male ZDF/Gmi fa/fa rats during the prediabetic insulin-resistant stage (5 weeks age) and the frank type 2 diabetic stage (11 weeks age) was found to exhibit significant differences in plasma amino acids when compared with those of the ZDF/Gmi fa/? lean littermates [24,25]. Significant increases of 2- to 5-fold for amino acid plasma levels (Table 5) were observed in the male ZDF/Gmi fa/fa rats during the prediabetic insulin-resistant stage including Aad, Phe, Tau, Trp and the 3 branched chain amino acids Ile, leu and Val, correlating well with previously reported observations [24]. The amino acids Asp, Gln, His, Ser previously shown to have minor decreases [24] in the prediabetic stage displayed increases in our study. But for the majority of these amino acids the percentage increase was small with the exception of Aad which exhibited an almost 5-fold increase. Among the amino acids which were previously reported [24] to show no change in the prediabetic stage, our study observed significant decreases in Arg and Hyp and a significant increase in Glu at the early prediabetic stage. This deviation may be due to improvements in specificity, sensitivity and precision in the methodology described in this study. However, it is important to note that differences seen in amino acid levels compared to previous studies may also result from differences in study animals (e.g., diet, age, and sampling). Among the amino acids which were not measured or reported in previous studies significant increases were observed for Abu, EtN, GABA, Orn, Sar, and PEtN comparing ZDF/Gmi fa/fa rats with ZDF/Gmi fa/? lean littermates, indicating their potential as disease biomarkers in these animal models.

4. Conclusion

A high-throughput method for quantitation of amino acids in biological matrices including plasma, urine, liver and kidney using stable-isotope labeling followed by UPLC–MS/MS analysis was developed. Employing the ease of iTRAQ isotope labeling coupled with the speed, selectivity and sensitivity of UPLC–MS/MS provides an effective alternative approach for amino acid analysis. The UPLC–MS/MS assay allowed analysis of 44 amino acids in less than 3.5 min resulting in a total run time of 5 min. Precision for amino acid concentrations in rat plasma samples was <15% for 30 out of 32 amino acids. The developed assay provides an efficient platform for amino acid biomarker studies as demonstrated in this application for the profiling of ZDF rat plasma.

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