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Comparison of orthogonal liquid and gas chromatography–mass spectrometry platforms for the determination of amino acid concentrations in human plasma☆

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ABSTRACT

Concentrations of amino acids in a human plasma pool were determined using four independent quantification methods. Orthogonal separation schemes (LC, GC, or GC×GC) and detection systems (triple quadrupole or time-of-flight mass spectrometry) are shown to demonstrate excellent consistency among platforms for quantifying 18 amino acids in NIST Standard Reference Material (SRM) 1950 Metabolites in Human Plasma using a well-characterized isotope dilution (ID) quantification method. Measured levels were consistent with reference values in plasma from the literature. Individual amino acid concentrations in plasma varied by over an order of magnitude ranging from 1.83 μg/g to 28.0 μg/g (7.78 μmol/L to 321 μmol/L). Average variability (coefficient of variation) between experimental amino acid concentrations (excluding cysteine) among all methods was 6.3%. Certified mass fraction values for amino acids in NIST SRM 1950 will be established from statistically weighted means of all experimental results.

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1. Introduction

The human plasma metabolome consists of a complex matrix of small molecules – some of which are poorly characterized and unquantified. Free amino acids represent a significant fraction of the metabolome and can provide useful diagnostics for newborn screening of metabolic disorders [1,2] and physiological health [3–6]. Amino acids have been well characterized using LC- and GC-based mass spectrometry techniques for both plasma quantification and metabolomics studies [7–10]. Currently, National Institute of Standards and Technology (NIST) is collaborating with National Institutes of Health (NIH) to develop and characterize a human plasma-based Standard Reference Material (SRM 1950 Metabolites in Human Plasma) through qualitative and quantitative identification of biomolecules, including the 18 amino acids described here. SRM 1950 was produced with the intent of providing the scientific community with a uniform material for comparing results and analytical platforms over time as well as to demonstrate traceability to a higher-order secondary reference standard for plasma-based measurements. Here, we demonstrate uniformity in results obtained from distinct measurement platforms from which amino acid concentrations in plasma were determined with excellent accuracy and precision.

Comparisons between liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) platforms for amino acid quantification have been reported infrequently in the literature. Notably, one report details results from orthogonal isotope dilution GC–MS and LC–MS measurements of a single amino acid derivative, homocysteine, in plasma [11]. Another report compares GC–MS and LC–MS using propyl chloroformate and isobaric tag (iTRAQ) derivatization in the relative and absolute quantification, respectively, of urinary amino acid concentrations [12]. Further, there are several reports describing direct comparisons of LC–MS and GC–MS quantification platforms towards other classes of molecules: human serum creatinine [13], urinary free cortisol [14], serum testosterone [15], brominated flame retardants [16], steroid residues in bovine hair [17], and steroidal estrogens [18], as well as bacterial metabolites in various biofluids [19]. In general, these reports suggest that the use of orthogonal techniques is advantageous, if not necessary, for rigorous method validation, and cite that the selectivity and sensitivity of LC–MS/MS and the enhanced signal to noise (S/N) and limits of detection of GC–time-of-flight MS (TOF-MS) make quantification by these approaches highly comparable. At NIST the use of orthogonal techniques is desirable in value assignments for SRMs as assurance that sources of bias have been minimized.

SRM 1950 consists of a human plasma pool acquired from an equal mix of healthy male and female donors. The highly complex plasma matrix was minimally processed in all analyses, and only simplified using traditional precipitation protocols to remove

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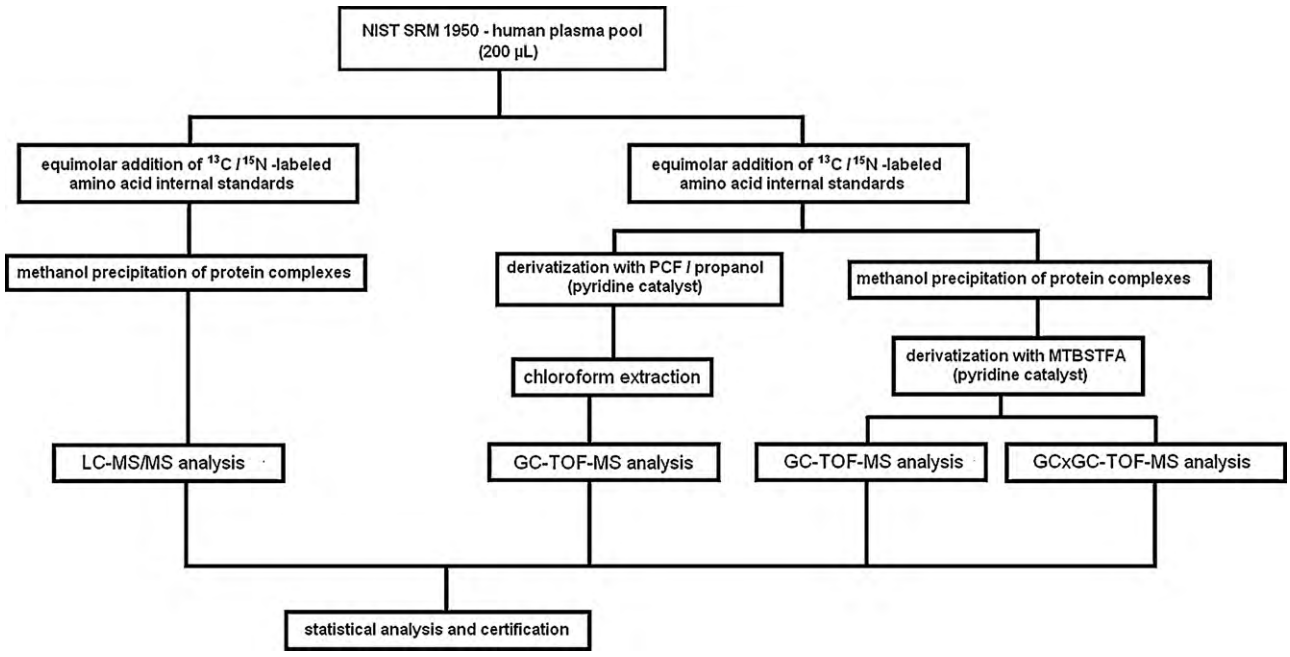

Fig. 1. Flow chart of parallel sample preparation for the four analytical methods used.

large complexes. GC approaches required additional derivatization prior to quantitative measurement. Isotope dilution (ID) methodology was applied to three distinct analytical platforms (LC–MS/MS, GC–TOF-MS, and GC×GC–TOF-MS) including the use of two distinct derivatizing agents in GC–TOF-MS analyses (*N*-methyl-*N*-[*tert*-butyldimethyl-silyl]trifluoroacetimide, MTBSTFA and propyl chloroformate, PCF). Isotope dilution is widely considered the gold-standard for absolute quantification of biomolecules using mass spectrometry [20] and, here, involves the use of stable $^{13}C/^{15}N$-labeled amino acids as internal standards. Moreover, a unique, isotopic analog was used for each biomolecule of interest. Sample concentrations in plasma were reported by interpolation through matrix-matched calibration curves.

To our knowledge, this is the first report to compare concentrations of multiple plasma-based amino acids using orthogonal LC and GC platforms. The comparisons are used to show similarities in the end results as well as to highlight differences between platforms. Highly accurate quantification is desired to best be able to compare measurement techniques. Not all amino acids were quantified in this study due to the poor stability and high reactivity of those measurands (*e.g.*, asparagine, glutamine, and tryptophan). For the 18 selected amino acids, all results will be statistically combined to obtain certified or reference mass fraction values which will be incorporated into a Certificate of Analysis for NIST SRM 1950 Metabolites in Human Plasma, due for release in 2010. This is part of an ongoing effort to characterize SRM 1950 – the first NIST serum or plasma-based SRM with certified values for amino acids.

2. Experimental

Fig. 1 illustrates the four parallel approaches used in this report for quantifying amino acids in plasma. In each approach plasma, standards, and calibrants were all obtained from identical sources. Isotopically-labeled amino acid internal standards were spiked into plasma prior to sample processing. An identical methanol precipitation protocol was followed in three of four analyses, with the exception of PCF-derivatized sample preparations where chloroform extraction was required.

2.1. Materials

A human plasma pool (NIST SRM 1950) was obtained from 50 male and 50 female volunteers between the ages of 40 and 50 consisting of a racial distribution equivalent to that of the U.S. population. Donors were required to fast overnight in advance of the blood draw, refrain from medication for >72 h prior, and have no overt diseases, extremes in body-mass index, exercise programs, or diets. The plasma was minimally processed, kept cold, and collected in lithium heparin anticoagulant tubes. The plasma was then dispensed into 1 mL vials and stored at −80 °C.

All reagents used in this analysis were obtained from commercial sources. High purity LC–MS grade CHROMASOLV water and acetonitrile were purchased from Riedel-de Haën (Hannover, Germany) through Sigma–Aldrich (Milwaukee, WI). HPLC grade methanol, HPLC grade hexanes (95% n-hexane), and pyridine PHOTREX reagent grade were purchased from JT Baker (Phillipsburg, NJ). Trifluoroacetic acid (TFA) was purchased from Fluka (Milwaukee, WI). Constant boiling hydrochloric acid (HCl) solution, high purity CHROMASOLV for HPLC 1-propanol, and propyl chloroformate, 98.9% were purchased from Sigma–Aldrich. Sodium hydroxide was ACS reagent grade purchased from Mallinckrodt (Phillipsburg, NJ). Chloroform OmniSolv grade was obtained from EM Scientific (Gibbstown, NJ). *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL). Labeled amino acids were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA) with a minimum isotopic enrichment of 3 Da. All labeled amino acids were analyzed for chemical purity at CIL and determined at >98%. The purity of the internal standard is trivial (at high purity) as it is added to both the samples and calibrants, thus canceled out in later calculations. Amino acids were purchased with the following isotopic labels and enrichment: L-alanine (U-$^{13}C_3$ 98%, ^{15}N 98%), L-arginine (U-$^{13}C_6$ 98%), L-cysteine (U-$^{13}C_3$ 97–99%, ^{15}N 97–99%), L-cystine (U-$^{13}C_6$ 98%, $^{15}N_2$ 98%), L-glutamic acid (U-$^{13}C_5$ 98%), glycine (U-$^{13}C_2$ 97–99%, ^{15}N 97–99%), L-histidine (U-$^{13}C_6$ 98%, <5%D), L-isoleucine (U-$^{13}C_6$ 98%), L-leucine (U-$^{13}C_6$ 98%), L-lysine (U-$^{13}C_6$ 98%), L-methionine (U-$^{13}C_5$ 97–99%, ^{15}N 97–99%), L-ornithine (U-$^{13}C_5$ 98%), L-phenylalanine (U-$^{13}C_9$ 97–99%, ^{15}N 97–99%), L-proline

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(U-$^{13}C_5$ 98%, ^{15}N 98%), L-serine (U-$^{13}C_3$ 98%, ^{15}N 98%), L-threonine (U-$^{13}C_4$ 97–99%), L-tyrosine (U-$^{13}C_9$ 98%, ^{15}N 98%), L-valine (U-$^{13}C_5$ 98%). Unlabeled amino acids were obtained from commercial vendors (Fluka, Sigma–Aldrich). Chemical purity was analyzed by the manufacturer prior to in-house analysis (see moisture and purity section). All amino acids were found to be 100.0% pure by either titration, LC, or thin layer chromatography, or a combination of these methods as per the manufacturer certificates with exceptions of L-phenylalanine and L-serine which were determined as >99% pure.

Masses were determined to an accuracy of 0.00001 g on a Mettler Toledo (Columbus, OH) XP205 or AX26 balance. Mass fractions were converted to concentration by approximating the density of H_2O at 20 °C = 0.9982 g/cm^3 and the density of SRM 1950 at ambient temperature = 1.02064 g/mL.

2.2. Methanol precipitation and sample preparation

Nine or ten sample vials (as noted below) were selected from a stratified random sample of the SRM 1950 production lot for analysis. Protein precipitation was accomplished using a methanol precipitation technique. Briefly, frozen plasma was thawed for 20 min at room temperature and vortexed for 30 s. One hundred μL of plasma for LC–MS or 200 μL of plasma for GC–TOF-MS and an equal molar addition of isotopically-labeled amino acid internal standard relative to the plasma content was added gravimetrically, vortexed for 30 s, and equilibrated for 30 min at 4 °C. A 4:1 dilution was made with methanol; samples were vortexed for 30 s and stored at −20 °C for 30 min. After thawing for 1 min, samples were again vortexed for 30 s and then centrifuged for 15 min at 16,000 × *g* at 4 °C in an Eppendorf (Hamburg, Germany) centrifuge 5403. For LC–MS analysis, the supernatant was decanted to a separate tube and lyophilized to dryness overnight without heating in a SPD1010 SpeedVac System (ThermoSavant). The following morning, samples were resolubilized in ≈100 μL of 1.0 mL/L formic acid, vortexed for 2 h at room temperature and equilibrated overnight at 4 °C. For GC–TOF-MS with MTBSTFA, 200 μL of supernatant was decanted and evaporated to dryness under N_2 and resolubilized during the derivatization step.

2.3. Internal standard and calibrant preparation

All isotopically labeled, and unlabeled, amino acid stock solutions were prepared gravimetrically in 0.1 mol/L HCl. A working internal standard solution was prepared gravimetrically from labeled stocks in an equal molar ratio to native plasma levels. Plasma concentrations were initially estimated using a calibration curve with a provisional internal standard solution of 50 μmol/L/amino acid and assuming the calibration line passes through the origin.

Two unique sets of four calibrant solutions (eight unique calibrants) were prepared gravimetrically for LC–MS/MS analyses; for each GC–TOF-MS analysis, six unique calibrant solutions were prepared by addition of internal standard solution to unlabeled stocks. The identical internal standard solution was used in corresponding sample preparations for each technique. Calibrants were prepared at targeted analyte-to-internal standard molar ratios of 0.7, 0.8, 1.3, and 1.4 for LC–MS/MS analyses, and approximately 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 for GC–TOF-MS analyses. The linearity of mass spectrometry response over a broad concentration range was assessed prior to sample analysis for each measurand.

2.4. MTBSTFA derivatization

Following methanol precipitation 50 μL of pyridine and 50 μL of MTBSTFA were added to the dried supernatant. The samples were vortexed for three min then allowed to sit at room temperature for one h. Samples were run on the GC–TOF-MS within 12 h of derivatization or they were stored at −20 °C until they could be run within 36 h of derivatization.

2.5. Propyl chloroformate (PCF) derivatization

Samples were prepared as described above except no methanol was added to these samples. Following addition of internal standard, derivatization was carried out directly in plasma. Reagents used were pyridine in propanol (≈10% volume fraction) and propyl chloroformate in chloroform (≈32% volume fraction). Propyl chloroformate and propanol are reactants while pyridine acts as a catalyst. All reagent concentrations were calculated to be in ≈100× molar excess to the expected amount of amino acid present. First 50 μL of pyridine in propanol was added, and samples were vortexed for 15 s. Next 50 μL of propyl chloroformate in chloroform was added; the samples were vortexed for 1 min then allowed to rest 1 min, and then vortexed again 1 min. After vortexing, the sample tubes were vented to release carbon dioxide produced from the derivatization reaction. Samples were allowed to react with PCF for 10 min before extraction. Chloroform (250 μL) was added and samples were vortexed for 15 min to extract the derivatized portion into the organic layer. The chloroform layer was then removed and directly injected for GC–TOF-MS analysis. This procedure was modified from one previously published by Kaspar et al. [7].

2.6. Experimental design

Amino acids were quantified by LC–MS/MS in three distinct sets (a) alanine, arginine, glycine, histidine, lysine, methionine, proline, serine, threonine, tyrosine; (b) glutamic acid, ornithine, phenylalanine; (c) cysteine, cystine, isoleucine, leucine, valine. Each amino acid was quantified from nine unique ampoules distributed from all segments of the SRM 1950 lot, and were analyzed with duplicate sample preparations by LC–MS/MS. The injection order of samples was systematically arranged into two groups positioned between discrete sets of calibrants with both sample and calibrant injection orders randomized among replicates. For GC–TOF-MS analyses, all amino acids were quantified within one chromatographic experiment. Calibrants were injected in triplicate at the beginning, middle, and end of the injection order. Samples of SRM 1950 were injected once in two groups, with calibrants run before and after each group. For both LC–MS a solvent blank was used and for GC–TOF-MS analyses a procedural blank was used, blanks were run between sample and calibrant injections.

2.7. LC–MS/MS analysis

Liquid chromatographic separation was achieved by 5 μL injections onto a SIELC (Prospect Heights, IL) Primesep 100 mixed-mode (ion-exclusion and reverse phase) analytical column (2.1 × 250 mm, 5 μm particles, 10 nm pores) with a Primesep 100 guard column (2.1 × 10 mm, 5 μm particle size, 100 å pore size) at a flow rate of 200 μL/min. An Agilent 1200 LC system (Santa Clara, CA) was coupled in-line with an Applied Biosystems API 5000 triple quadrupole mass spectrometer (Foster City, CA) equipped with a standard micro-flow source. Ions were detected using multiple-reaction monitoring (MRM) in the positive ion mode. For sample sets (a) and (b), chromatographic separation was accomplished using an increasing linear gradient of organic/aqueous solvent (ACN/H_2O) in tandem with a decreasing pH gradient (increasing TFA concentration) followed by a column wash and re-equilibration. Mobile phases A and B consisted of 0.5 mL/L and 4.5 mL/L TFA, respectively in 0.3 L/L aqueous ACN. Sample set (c), requiring separation of Ile/Leu isomers, was separated under iso-

Table 1
Multiple-reaction monitoring (MRM) settings for LC–MS/MS analyses.

	Unlabeled MW (g/mol)	MRM transition	$^{13}C/^{15}N$-isotope MW (g/mol)	MRM transition	Declustering potential (V)	Collision energy (V)	Cell exit potential (V)	Entrance potential (V)
L-Alanine	89.1	90.1 → 44.0	93.1	94.1 → 47.0	30	22	20	9
L-Arginine	174.2	175.2 → 70.0	180.2	181.2 → 74.0	55	43	24	9
		175.2 → 60.0		181.2 → 61.0	55	23	11	9
L-Cysteine	121.2	122.2 → 58.9	125.2	126.2 → 60.9	48	31.5	9	9
		122.2 → 76.0		126.2 → 79.0	48	19	12	9
L-Cystine	240.3	241.3 → 74.0	248.3	249.3 → 77.0	40	37	14	9
		241.3 → 152.0		249.3 → 156.0	40	18.5	14	9
L-Glutamic acid	147.1	148.1 → 84.0	152.1	153.1 → 88.0	45	24	14	9
		148.1 → 56.0		153.1 → 59.0	45	36	10	9
Glycine	75.1	76.1 → 30.1	78.1	79.1 → 32.1	30	23	14	9
L-Histidine	155.2	156.2 → 110.0	161.2	162.2 → 115.0	50	31	20	9
		156.2 → 93.0		162.2 → 98.0	50	31	20	9
L-Isoleucine	131.2	132.2 → 86.0	137.2	138.2 → 91.0	42	16	15	9
		132.2 → 69.0		138.2 → 74.0	42	25	15	9
L-Leucine	131.2	132.2 → 86.0	137.2	138.2 → 91.0	42	16	15	9
L-Lysine	146.2	147.2 → 84.1	152.2	153.2 → 89.1	60	24	15	7
		147.2 → 130.1		153.2 → 136.1	60	14	15	7
L-Methionine	149.2	150.2 → 61.1	155.2	156.2 → 63.0	45	30	11	9
		150.2 → 56.1		156.2 → 60.0	45	24	9.5	9
L-Ornithine	132.2	133.2 → 70.0	137.2	138.2 → 74.0	33	25	15	9
		133.2 → 116.0		138.2 → 121.0	33	14	19	9
L-Phenylalalnine	165.2	166.2 → 120.1	175.2	176.2 → 129.1	50	21	17	9
		166.2 → 103.1		176.2 → 111.1	50	38	14	9
L-Proline	115.1	116.1 → 70.0	121.1	122.1 → 75.0	45	39	21	7
		116.1 → 43.0		122.1 → 46.0	45	39	21	7
L-Serine	105.1	106.1 → 60.0	109.1	110.1 → 63.0	40	16	18	10
		106.1 → 42.1		110.1 → 45.0	40	28	18	10
L-Threonine	119.1	120.1 → 56.0	123.1	124.1 → 59.0	36	22	22	9
		120.1 → 74.0		124.1 → 77.0	36	16	16	9
L-Tyrosine	181.2	182.2 → 136.2	191.2	192.2 → 145.1	52	19.5	19.5	9
		182.2 → 123.1		192.2 → 130.0	52	23	23	9
L-Valine	117.2	118.2 → 72.1	122.2	123.2 → 76.1	50	19	19	9
		118.2 → 55.1		123.2 → 59.1	50	29	29	9

cratic conditions (30% solvent B) where mobile phase A consisted of 0.5 mL/L TFA in water only. Column temperature was maintained at 30 °C for all experiments; autosampler plate temperature control was set at 10 °C.

Data acquisition was performed using Analyst (Applied Biosystems) scheduled-MRM. Optimized source and fragmentation parameters (declustering potential, entrance potential, collision energy, and collision cell exit potential) were selected independently for all transitions by monitoring MS-response over a broad range and noting maximum signal intensity. The labeled analogs for each amino acid were subsequently infused to validate the optimization results and to verify negligible isotope effect. Parameters were optimized through direct infusion of ≈100 μmol/L solutions at 10 μL/min coupled in a "T" setup with a 50/50 mix of solvents (200 μL/min) from the LC pump. During data acquisition, these parameters were set identically for unlabeled-labeled pairs: collision gas = 3.4×10^4 Pa (5 psi), unit resolution in Q1 and Q3, curtain gas (CUR) = 4.1×10^5 Pa (60 psi), intensity threshold = 0, ion source gas 1 (GS1) = 2.8×10^5 Pa (40 psi), settling time = 5 ms, ion source gas 2 (GS2) = 2.8×10^5 Pa (40 psi), pause between mass ranges = 5 ms, ion spray voltage (IS) = 5000 V, *x*-axis spray position (vert.) = 0 mm, capillary temperature (TEM) = 500 °C, *y*-axis spray position (horiz.) = 7 mm, target scan time = 1.3 s, interface heater = ON, and MRM detection window = 180 s.

Table 1 describes details of the MRM functions used during tandem mass spectrometry analysis for each amino acid. Two transitions were monitored for each amino acid (and its isotopic analog) and were required to yield quantitative agreement (exceptions for alanine, glycine, and leucine due to limited fragmentation). Applied Biosystems Analyst software (v1.5) was used for data analysis. Peaks were identified manually, and were automatically selected by the Analyst Quantitation Wizard; peak areas were integrated by Analyst using a bunching factor = 3, number of smooths = 1, and all other parameters set to default values. All peak integrations were visually inspected, and in some cases, manual integration was necessary. Peak area ratios were exported into Microsoft Excel for data management.

2.8. GC–TOF-MS and GC×GC–TOF-MS analysis

Samples were analyzed by gas chromatography with mass spectrometry (time-of-flight) detection (Leco PegasusIV, St. Joseph, MI). Approximately 1 μL of sample was injected using an autosampler (Agilent 7683B) into a 270 °C split/splitless injector in the splitless mode (60 s purge time).

Separation in 1D mode (GC–TOF-MS) for the MSTBSTFA samples was completed still using two columns in series, however, no cryotrapping was performed between the two columns resulting in a 1D separation. The two columns were an Rtx-5 (5% diphenyl/95% dimethyl polysiloxane) (Restek, Bellefonte, PA), 38 m in length, 180 μm internal diameter, and 0.2 μm film thickness as the first column and a Rxi-17 (50% diphenyl/50% dimethyl polysiloxane) (Restek), 1 m in length, 100 μm internal diameter, and 0.1 μm film thickness as the second column, joined by a Siltek lined fitting (Siltek MXT Connector for 0.25 mm, Restek). A flow rate of 1 mL/min of He was used. The temperature program was 100 °C initial hold for 4 min, followed by a ramp of 5 °C/min to 300 °C, hold 10 min at 300 °C. The secondary oven, which houses the second column, tracked the main oven at +5 °C. The modulator was +20 °C compared to the main oven. Mass spectrometer transfer line was maintained at 300 °C. The mass spectrometer program had a 600 s solvent delay and then tracked masses (*m*/*z*) 40–800 at a rate of 20 spectra/s for the duration of the run. Separation in 2D mode (GC×GC–TOF-MS) used the same conditions described above except the second dimension separation time was set to 3 s and the mass spectrometer collected spectra at a rate of 200 spectra/s.

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For PCF derivatization in 1D mode (GC–TOF-MS) again two columns were used in series without cryotrapping, an Rtx-17 (50% diphenyl/50% dimethyl polysiloxane) (Restek), 10 m in length, 180 μm internal diameter, and 0.18 μm film thickness as the first column and a Rxi-17 (Restek), 1 m in length, 100 μm internal diameter, and 0.1 μm film thickness as the second column, joined by a Siltek lined fitting (Siltek MXT Connector for 0.25 mm, Restek) was used. The temperature program was 50 °C initial hold for 1 min, followed by a ramp of 30 °C/min to 295 °C, hold 2 min at 295 °C. The secondary oven tracked the main oven at +5 °C. A solvent delay of 120 s was used for the mass spectrometer. All other conditions were the same as described above for GC–TOF-MS MTBSTFA analyses.

Masses used for quantification are shown in Table 2. The most abundant fragment was typically chosen and it was preferred if the fragment contained the R group unique to each amino acid. Peak areas were obtained using the LECO ChromaTOF software. These peak areas were then exported into Microsoft Excel for data processing.

2.9. Purity and moisture analysis

Impurities in the unlabeled amino acid powders were assessed in-house by liquid chromatography with ultraviolet detection (LC-UV) and MS detection (data not shown). Elemental analysis was performed, and moisture content was measured; both analyses were performed at Galbraith Labs (Knoxville, TN), and selected amino acids were validated by Karl Fischer titration in-house. Purity values were determined by quantifying residual moisture of the material and any known impurities, such as salts, metals, degradation products, and contaminants [21], these values can be found in the supplementary table. The purity values were factored into calculations of calibration curves to get the most accurate concentration determination in the plasma. Further, error associated with the purity of the standards was accounted for by factoring a 1–3% uncertainty in the final statistical analysis.

3. Results

Representative MRM chromatograms are displayed in Fig. 2(A–C) as total ion currents (TIC) from the LC–MS/MS analysis of plasma samples (SRM 1950). Representative TIC chromatograms for GC–TOF-MS are shown in Fig. 3. As a rule, integrated total peak areas were measured for all amino acids. Chromatography was reproducible to within ±10 s for peak maxima for LC, ±1 s for GC (MTBSTFA), and ±0.2 s for GC (PCF) analyses. Partially resolved or co-eluting amino acids within the same set could be quantified over the same time period in LC–MS/MS experiments because peak widths were sufficiently wide to allow alternating scans of multiple transitions while still acquiring sufficient data points over the curve for accurate quantification.

3.1. Amino acid concentrations

Measurement results for each amino acid are provided with an associated uncertainty estimate in Table 3 and are displayed graphically in Fig. 4. For LC–MS/MS analyses, each amino acid was measured by two discrete fragmentation transitions from nine unique vials of SRM 1950, and measured in duplicate – accounting for 36 data points comprising the statistical means (alanine, glycine, and leucine are noted exceptions). For GC–TOF-MS and GC×GC–TOF-MS analyses, each amino acid was measured in nine (MTBSTFA) or ten (PCF) unique vials of SRM 1950. Purity of the amino acid standards used to generate concentration curves were taken into account in calculations of concentrations in SRM 1950. Amino acid concentrations vary from ≈2 μg/g

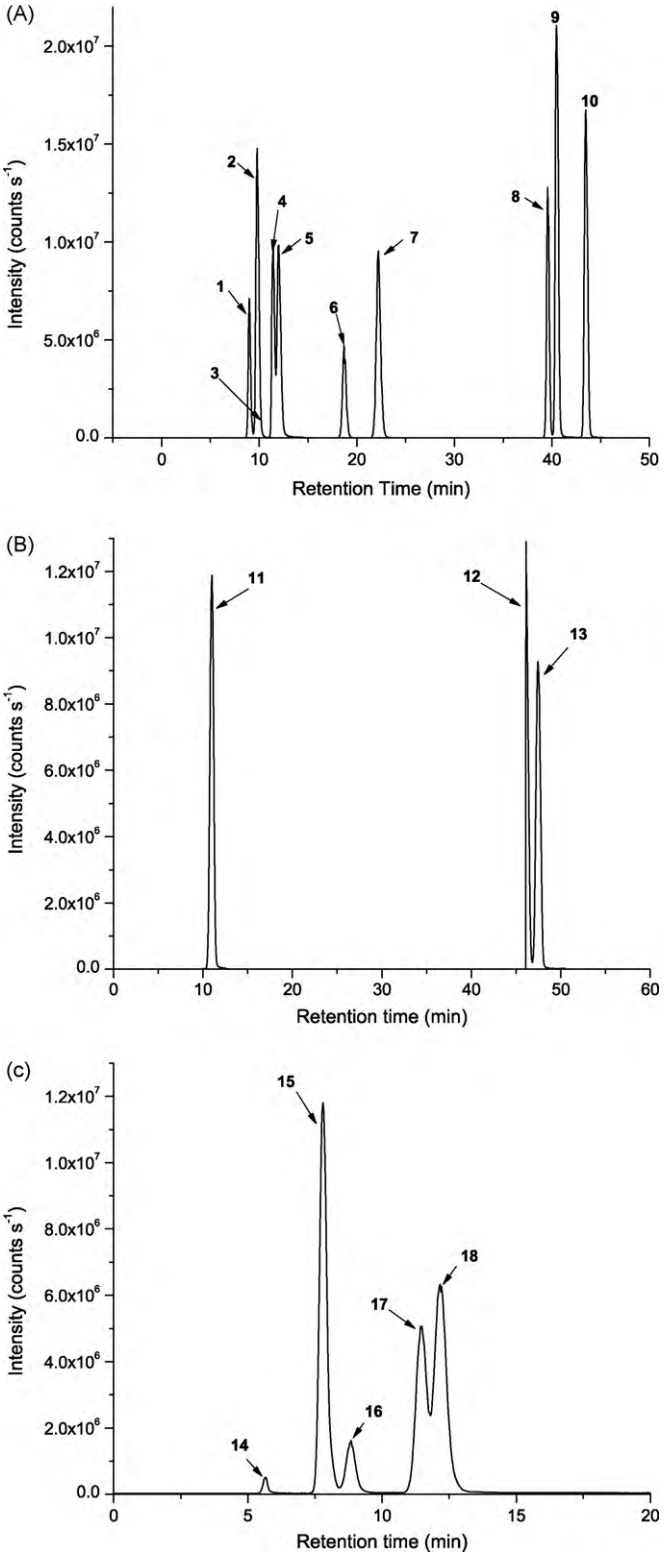

Fig. 2. LC–MS/MS total ion chromatograms of SRM 1950. Amino acid sets 1 (A), 2 (B) and 3 (C). Peak identifications are: L-serine (1), L-threonine (2), glycine (3), L-alanine (4), L-proline (5), L-methionine (6), L-tyrosine (7), L-histidine (8), L-lysine (9), L-arginine (10), L-glutamic acid (11), L-phenylalanine (12), L-ornithine (13), L-cysteine (14), L-valine (15), L-cystine (16), L-isoleucine (17), and L-leucine (18).

(cystine) and 3 μg/g (methionine) up to 24 μg/g to 28 μg/g (alanine) corresponding to molar concentrations in the range of approximately 8 μmol/L to 320 μmol/L. These concentrations compare well to literature averages (obtained from Human Metabolome Database,http://www.hmdb.ca [22]) of matrix-

Table 2
Quantification masses for GC[×GC]-TOF-MS.

	MTBSTFA		PCF	
	Derivatized mass (M)	Quantification ion	Derivatized mass (M)	Quantification ion
L-Alanine	317	158 (M-159)[a]	217	130 (M-87)[b]
L-Cysteine	463	406 (M-57)[a]	249	162 (M-87)[b]
L-Glutamic acid	489	432 (M-57)[a]	–	–
Glycine	303	246 (M-57)[a]	203	116 (M-87)[b]
L-Histidine	383	196	–	–
L-Isoleucine	359	200 (M-159)[a]	259	172 (M-87)[b]
L-Leucine	359	200 (M-159)[a]	259	172 (M-87)[b]
L-Lysine	488	300	274	170
L-Methionine	377	218 (M-159)[a]	277	143
L-Phenylalanine	393	234 (M-159)[a]	293	148
L-Proline	343	258	243	156 (M-87)[b]
L-Serine	447	390 (M-57)[a]	–	–
L-Threonine	461	303	247	143
L-Tyrosine	523	302	309	164
L-Valine	345	186 (M-159)[a]	245	116

"–" indicates that the amino acid was not measured by this method.
[a] Common fragments of derivatization products of MTBSTFA are M-57 and M-159.
[b] Common fragment of derivatization products of PCF M-87.

matched values (blood draws from healthy, male and female adult patients).

3.2. Measurement precision

Coefficients of variation (CV) of the measurement repeatability for LC–MS/MS analyses ranged from 0.73% (glutamic acid) to 11.5% (cysteine) with a mean CV (18 measurands) of 2.6%, denoting excellent overall measurement precision. CVs for GC methods ranged from 4.8% (glycine) to 13% (leucine) for GC–TOF-MS (MTBSTFA), 5.2% (isoleucine) to 14% (leucine) for GC×GC–TOF-MS (MTBSTFA), and 7.4% (valine) to 17% (methionine) for GC–TOF-MS (PCF). Cysteine had much higher CVs (31% GC×GC–TOF-MS (MTBSTFA) and 24% GC–TOF-MS (PCF)), however the signal was significantly suppressed in the plasma and the S/N for these peaks was 9 (MTBSTFA) and 40 (PCF) – a factor of 10 to 100 smaller than other amino acids of the same concentration. For the purpose of the discussion of measurement precision, these results were excluded as outliers. Average CVs (9 to10 measurands) were 7.8% for GC–TOF-MS (MTBSTFA), 8.5% for GC×GC–TOF-MS (MTBSTFA), and 11% for GC–TOF-MS (PCF). While the average CV is higher than that of the LC–MS/MS analysis, the additional derivatization step in the GC analyses contributed to the error, although the resulting CVs still demonstrate acceptable precision.

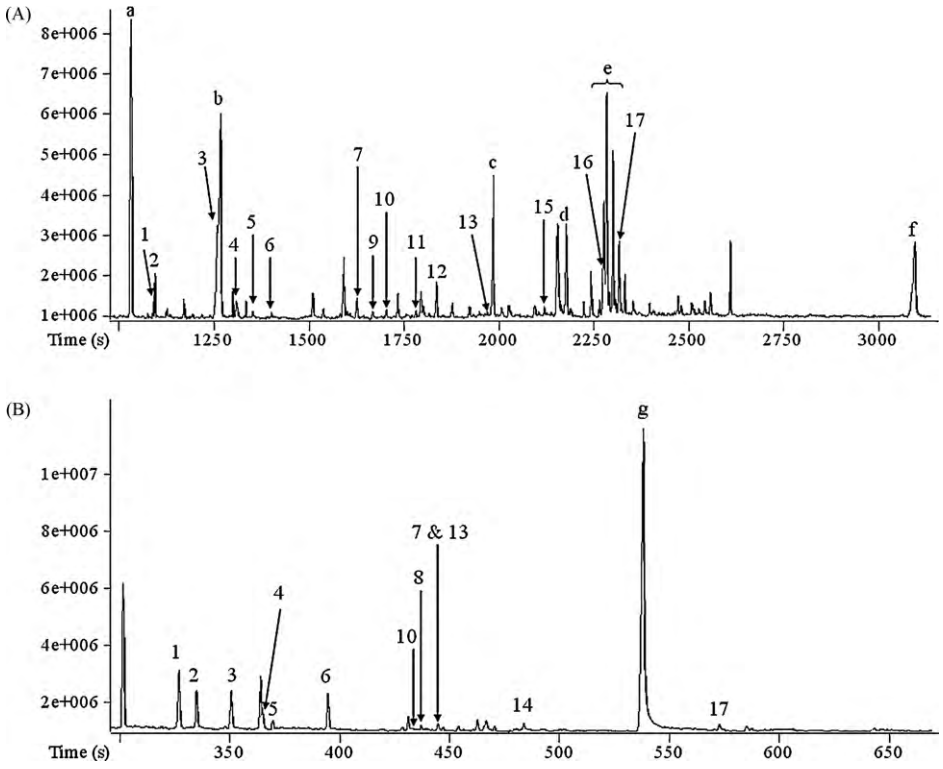

Fig. 3. GC–TOF-MS total ion chromatograms of SRM 1950 with MTBSTFA (A) and PCF (B). Peak identifications are: L-alanine (1), glycine (2), L-valine (3), L-leucine (4), L-isoleucine (5), L-proline (6), L-pyroglutamic acid (7), L-methionine (8) L-serine (9), L-threonine (10), L-phenylalanine (11), L-aspartic acid (12), L-glutamic acid (13), L-cysteine (14), L-glutamine (15), L-histidine (16), L-tyrosine (17), lactic acid (a), urea (b), hexadecanoic acid (c), octadecanoic and oleic acid (d), sugars (e), and cholesterol (f), and PCF derivatizing agent (g).

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Table 3
Comparison of results from orthogonal platforms for human plasma (SRM 1950) and literature averages of normal plasma.

	LC–MS/MS		GC–TOF-MS (MTBSTFA)		GC×GC–TOF-MS (MTBSTFA)		GC–TOF-MS (PCF)		Literature averages[a]	
	Avg. MF[b] (μg/g)	SD[c]	Avg. MF[b] (μg/g)	SD[c]	Avg. MF[b] (μg/g)	SD[c]	Avg. MF[b] (μg/g)	SD[c]	Avg. MF[b] (μg/g)	SD[c,d]
L-Alanine	24.4	0.3	26.6	1.6	25.6	1.8	28.0	2.1	29.0	6.5 (1)
L-Arginine	13.9	0.2	–	–	–	–	–	–	14.3	9.5 (5)
L-Cysteine	5.63	0.64	–	–	11.9	3.7	4.9	1.2	2.6	3.2 (3)
L-Cystine	1.83	0.05	–	–	–	–	–	–	24.9	–
L-Glutamic acid	8.46	0.06	10.9	1.1	11.0	0.8	–	–	7.6	3.0 (2)
Glycine	18.6	0.4	15.7	0.8	18.0	1.7	18.6	1.3	17.3	8.1 (4)
L-Histidine	10.8	0.2	9.02	0.49	11.3	0.7	–	–	12.9	3.1 (3)
L-Isoleucine	6.92	0.13	7.13	0.48	7.61	0.40	7.10	0.57	9.1	4.0 (4)
L-Leucine	12.6	0.2	13.6	1.8	13.0	1.8	12.9	1.6	18.9	6.1 (3)
L-Lysine	19.0	0.3	20.6	1.7	20.7	1.1	20.5	2.7	27.2	8.0 (3)
L-Methionine	3.07	0.03	3.40	0.32	3.24	0.19	3.40	0.58	4.4	1.5 (4)
L-Ornithine	6.74	0.21	–	–	–	–	–	–	6.7	4.3 (4)
L-Phenylalanine	8.13	0.39	6.31	0.72	8.70	0.66	9.03	0.79	9.2	2.5 (4)
L-Proline	19.7	0.3	19.7	1.4	20.7	1.2	21.9	1.7	21.6	11.8 (3)
L-Serine	9.80	0.57	10.3	0.9	9.60	0.59	–	–	12.1	7.0 (7)
L-Threonine	13.9	0.2	14.7	1.0	14.2	0.8	13.5	1.8	17.1	6.0 (3)
L-Tyrosine	9.99	0.23	10.4	0.7	10.5	0.6	10.1	1.0	11.4	4.1 (3)
L-Valine	20.2	0.2	21.6	1.2	21.3	1.2	21.1	1.6	26.5	7.5 (3)

"–" indicates that the amino acid was not measured by this method.
[a] Literature averages were taken directly from http://www.hmdb.ca/ (Human Metabolome Database) as a resource overview of many published values. Only matrix-matched values were considered (blood draws from healthy, adult patients).
[b] MF is mass fraction.
[c] SD is standard deviation.
[d] (*n*) denotes the number of distinct literature values averaged for the calculation of standard deviation.

3.3. Comparison of methods

Relative statistical deviations between each measurement platform were calculated as percent differences (% Diff = $[(x_1 - x_2)/((x_1 + x_2)/2)] \times 100$) of measurement means, as shown in Table 4. Percent differences are in both the positive and negative directions and, with exception of the amino acid cysteine, range from <1% to 35%. On average, LC measurements vary by 8.9% from the mean of all GC methods. Overall, the mean percent difference among all four methods was 10.7%, with 43 of 52 inter-method comparisons varying by less than 10%. The best agreement (7.6%) was demonstrated between GC–TOF-MS and GC×GC–TOF-MS platforms using the same derivatizing agent (MTBSTFA), while the use of different derivatizing agents (GC vs. GC×GC) yielded the largest mean percent deviation (10.8%) among the four platforms.

Of the 15 amino acids that were measured by multiple methods, 11 were measured with means within one standard deviation of each other, between any measurement platform (glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine). Further, all 11 amino acids were quantified to within one standard deviation of the mean literature value for a similar plasma sample. Of the remaining four amino acid measurands (alanine, cysteine, glutamic acid, histidine), results from at least two of the three platforms (LC, GC, and GC×GC) show no statistical difference; however, each amino acid is a unique case.

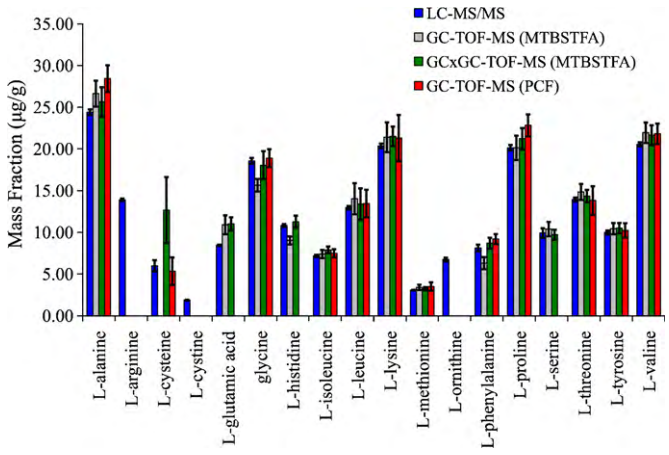

Fig. 4. Comparison of amino acid concentration results for orthogonal methods. Concentrations are means of nine or ten sample vials of SRM 1950. Error bars represent standard deviations.

Quantification of alanine exhibits statistical agreement between LC and GC×GC, as well as between GC and GC×GC. However, LC quantification yielded slightly lower values when compared to one-dimensional GC (both MTBSTFA and PCF derivatization) emphasizing that in certain cases where chromatographic matrix interferences are high, a GC×GC separation scheme is advantageous. Cysteine shows statistical agreement between LC and GC (PCF); however, LC and GC×GC averages differ by two-fold. Cysteine is an extreme case in that plasma concentrations approach instrumental limits of quantification (LOQs) *and* both LC and GC efforts to quantify this amino acid were hindered by significant signal suppression, likely leading to relatively high measurement error. Quantification of glutamic acid exhibits consistency between all GC platforms, yet LC measurements are found to be slightly lower. There may be concern that glutamic acid measurements are affected by stability issues in plasma, however, this possibility has not yet been confirmed. Finally, histidine shows statistical similarity between LC and GC×GC measurements, with the outlier being GC (MTBSTFA) quantification. However, chromatograms for the MTBSTFA-derivatized amino acids show that histidine co-elutes with a large glucose peak which interferes with the quantification in 1D, see Fig. 3. The second dimension separation of the GC×GC allows complete resolution of the histidine peak thus reducing interference and improving the confidence in this measurement, see Fig. 5.

Table 4 provides a clear way to visualize outliers among analytes or among measurement platforms. For example, both lysine (2.5%) and valine (2.5%) were quantified to within a very small mean difference among measurement techniques suggesting good agreement of the methods. The least abundant plasma-based amino

Table 4
Percent difference in amino acid concentration in human plasma (SRM 1950) between orthogonal methods.

	GC (MTBSTFA) and GC×GC (MTBSTFA)	GC (PCF) and GC×GC (MTBSTFA)	GC (PCF) and GC (MTBSTFA)	GC (average) and LC–MS/MS	Mean % difference[a]
L-Alanine	3.8%	−8.7%	4.9%	9.0%	6.6%
L-Arginine	–	–	–	–	–
L-Cysteine	–	83.6%	–	39.6%	61.6%
L-Cystine	–	–	–	–	–
L-Glutamic acid	−1.1%	–	–	25.8%	13.4%
Glycine	−14.2%	−3.2%	17.4%	−6.3%	10.3%
L-Histidine	−22.1%	–	–	−6.4%	14.2%
L-Isoleucine	−6.5%	6.9%	−0.4%	5.1%	4.7%
L-Leucine	4.5%	0.8%	−5.3%	4.8%	3.8%
L-Lysine	−0.5%	1.0%	−0.5%	8.1%	2.5%
L-Methionine	5.1%	−5.0%	−0.1%	8.5%	4.7%
L-Ornithine	–	–	–	–	–
L-Phenylalanine	−31.8%	−3.7%	35.4%	−1.4%	18.1%
L-Proline	−5.0%	−5.8%	10.7%	5.5%	6.7%
L-Serine	6.7%	–	–	1.4%	4.1%
L-Threonine	3.2%	5.4%	−8.6%	2.1%	4.8%
L-Tyrosine	−0.6%	4.4%	−2.0%	33%	3.0%
L-Valine	1.5%	0.8%	−2.3%	5.4%	2.5%
Mean % difference[a] between methods	7.6%	10.8%	8.1%	8.9%	10.7%

"–" indicates that the amino acid was not measured by one or both of these methods.
[a] Mean % differences are calculated with the absolute value of percent differences.

acid, methionine, was quantified by all four methods with mean agreement to within 4.7%. Cysteine measurements (average relative deviation of 61.6%) were clearly the poorest. Individual cysteine measurements provided in Table 3 imply that the GC×GC approach does not match the other two measurement techniques. The error and S/N were demonstrated to be very high relative to other amino acid measurements suggesting the possibility of matrix suppression using GC×GC. Further, there is precedence in the literature [23–25] describing the interconversion of cysteine to cystine. Large mean differences between measurement techniques for other amino acids suggest method bias. For example, as noted above for histidine, glucose interferences not found in PCF derivatizations cause one-dimensional GC (MTBSTFA) measurements to yield lower mass fractions than all of the other methods leading to a high mean difference for this amino acid. Phenylalanine similarly shows a higher mean difference among methods as a result of an outlier in GC (MTBSTFA) measurements. The cause of this outlier is not obvious, however, and it is noted that for all amino acid measurements that are not in agreement among methods, NIST is providing a reference value and not a certified value. The mean relative difference between all measurements was found to be 10.8% (and only 7.4% when ignoring cysteine). On average, the percent difference between all measurand means for GC and GC×GC (both MTBSTFA-derivatized) was 7.6%; for GC×GC (MTBSTFA) and GC (PCF) that mean was 11.1% (4.7% without cysteine); for GC (MTBSTFA) and PCF the difference was 8.1%. Finally the mean percent difference between LC–MS/MS means and the average of all GC measurements was calculated to be 8.9% (6.8% ignoring cysteine).

Calibration curves were generated from eight (LC–MS/MS) or six (GC[×GC]-TOF-MS) independent calibration points for each amino acid by plotting mass ratios vs. peak area ratios. Regression model analysis demonstrates linear response within the targeted concentration range in all calibration curves; associated correlation coefficients (R^2) were ≥0.99 signifying a low fraction of variance and good predictability of sample concentration. The slope of the calibration curve is the ratio of masses divided by the ratio of the peak areas for an amino acid and its isotopic analog. For the GC analyses, the slope of the calibration curves varied between 0.7 and 2.9 (with the exception of phenylalanine for MTBSTFA which was around 11). For LC–MS/MS analyses, slopes of the calibration curves ranged from 0.69 to 1.7. Notably, distinct fragmentation transitions of the same precursor analyte do not necessarily give identical calibration curves.

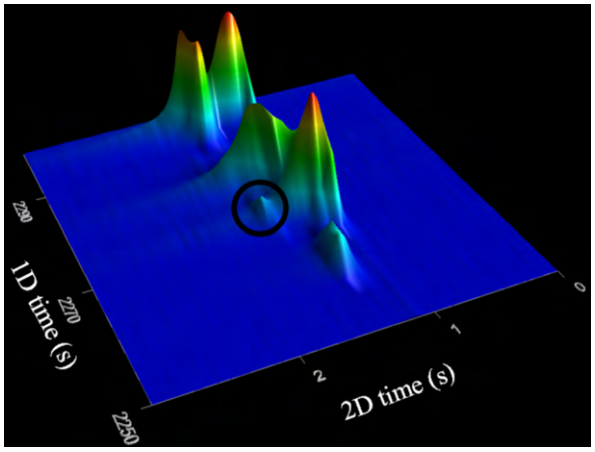

Fig. 5. Total ion chromatogram of GC×GC–TOF-MS (MTBSTFA). The 2D-chromatogram displays the retention of L-histidine (circled) in both time dimensions resolved from interfering peaks.

3.4. Statistical analysis

Statistical analysis of the data sets for certification of values followed standard conventions as suggested by NIST [26] with uncertainty values conforming to International Organization for Standardization (ISO) guidelines [27]. Statistical significance was evaluated, but not found, between samples, replicates, and transitions. Type A uncertainty measurements from the experimental data were determined by pooling amino acid concentration values as determined by a single platform (*i.e.*, LC–MS/MS or GC–TOF-MS). Uncertainties at the 95% confidence level were calculated based on the (Type B) assumptions of a 0.5% uncertainty in the purity of the reference compounds, a 0.5% uncertainty due to mass spectral interferences, and a 0.1% uncertainty due to gravimetric measurement. The values from the four distinct methods will be combined to generate a NIST certified value and associated uncertainty that will appear on the Certificate of Analysis for SRM 1950. The cer-

Table 5
Certified and reference mass fraction values and associated uncertainties for amino acids in NIST SRM 1950 Metabolites in Human Plasma. Official Certificate of Analysis is available at www.nist.gov.

	Certified mass fraction (μg/g)	Certified concentration (μmol/L)
L-Alanine	26.2 ± 2.2	300 ± 26
Glycine	18.0 ± 1.2	245 ± 16
L-Histidine	11.0 ± 0.6	72.6 ± 3.6
L-Isoleucine	7.13 ± 0.42	55.5 ± 3.4
L-Leucine	12.9 ± 0.8	100 ± 6
L-Lysine	20.0 ± 1.9	140 ± 14
L-Methionine	3.26 ± 0.26	22.3 ± 1.8
L-Proline	19.9 ± 1.1	177 ± 9
L-Serine	9.87 ± 0.44	95.9 ± 4.3
L-Threonine	13.9 ± 0.7	119 ± 6
L-Tyrosine	10.2 ± 0.5	57.3 ± 3.0
L-Valine	20.9 ± 1.2	182 ± 10
	Reference mass fraction (μg/g)	**Reference concentration (μmol/L)**
L-Arginine	13.9 ± 0.4	81.4 ± 2.3
L-Cysteine	5.26 ± 0.81	44.3 ± 6.9
L-Cystine	1.83 ± 0.08	7.78 ± 0.36
L-Glutamic acid	9.71 ± 2.53	67.4 ± 18
L-Ornithine	6.74 ± 0.37	52.1 ± 2.8
L-Phenylalanine	8.22 ± 1.13	50.8 ± 7.0

tified mass fraction value will be a weighted mean of the method means. The uncertainty will include contributions from both the within-method precisions and the between-method variability; it will be expressed as an approximate 95% expanded uncertainty interval on the certified value. Dodder et al. can provide further discussion on calculations of certified values and associated uncertainties [13]. NIST certified and reference values for the amino acids discussed in the paper are provided in Table 5. Certified values are assigned to NIST measurements of the highest confidence, such as those validated by orthogonal techniques. Measurements with a lower degree of confidence, such as those with a higher level of uncertainty or those not determined by multiple techniques, are typically assigned reference values.

4. Discussion

Most of the amino acids could be measured using both LC and GC techniques yielding comparable results: notable exceptions were arginine, cystine, and ornithine which were quantified by LC–MS/MS methods and not by any GC–TOF-MS methods. Cystine is significantly suppressed in the plasma using the GC techniques. A calibration curve was generated, but due to signal suppression the levels of cystine in plasma were below the detection limit of the GC techniques and could not be quantified by these methods. Arginine cannot be quantified by the specific GC methods used here because these derivatizing agents do not stabilize the guanidino group in the side chain making the molecule thermally unstable, thus converting arginine to ornithine [10,28]. The conversion takes place in the GC injector port prior to separation so it is impossible to distinguish the portion of the signal due to arginine and the portion due to ornithine. If it is known that ornithine is present in the absence of arginine it can be accurately quantified by GC, however the presence of arginine will give an artificially high value for ornithine. This is not an issue in LC–MS/MS because chromatographic separation is performed at low temperature.

Thiol groups within cysteine have been shown under various conditions to oxidize in plasma forming cystine dimers or to form other disulfide bonds such as those of glutathione [23–25]. No specific reducing agents were added to prevent thiol group reactions in this study, however, the experimental approach was optimized to minimize potential thiol reactions by keeping plasma samples at or below 4 °C at all times during LC analyses, or until the derivatization step was reached during GC–MS analyses. Derivatization using MTBSTFA would effectively block any thiol reactions. Due to the measurement variation for cysteine among methods, along with the potential for thiol reactions to different extent among sample preparations, NIST is assigning cysteine mass fractions in SRM 1950 as a reference value rather than a certified value, reflecting a lower certainty in measurement accuracy.

Each of the analytical platforms has advantages and disadvantages. LC–MS/MS demonstrated the highest measurement precision among the techniques and a better overall sensitivity when compared to other approaches. The specificity and selectivity of multiple-reaction monitoring is highly advantageous when working with complex matrices. Further, the ease of sample preparation (no chemical derivatization) means less random error and shorter analysis times. LC–MS/MS also makes it possible to validate quantitative measurements by monitoring multiple precursor → product ion transitions for each measurand. One disadvantage to using LC–MS/MS tends to be finding chromatographic conditions suitable to resolution of many analytes in a single run.

Major advantages to using GC approaches relative to LC is improved peak resolution, better separation of isomers, and the availability of extensive mass spectral data libraries for identifying compounds. For the GC techniques, the biggest disadvantage was that arginine, ornithine, and cystine could not be quantified in the plasma. The two derivatizing agents (MTBSTFA, PCF) give similar concentrations for the amino acids. MTBSTFA is more broadly applicable to metabolomics because the total ion chromatogram (Fig. 3A) contains peaks for many other physiologically relevant compounds. However, because it is more complex there is more chance of peak overlap that could cause bias in the concentration, such as with histidine. The two dimensional analysis (GC×GC) was able to separate histidine from the sugar peaks and allow better quantification. However, in general the integration for the two dimensional separation is much more complex and time consuming and the added dimension did not improve precision (except in the case where there is an interference). The PCF derivatization is targeted to amino acids, so this minimizes the extra peaks in the total ion chromatogram (Fig. 3B). Both the derivatization and run time are very short compared to MTBSTFA allowing the entire analysis to be completed in under an hour. However, some of the amino acids could not be quantified with PCF. Glutamic acid and pyroglutamic acid co-elute and have the same major mass fragments, and serine and histidine did not show any distinguishing peaks when injected at high concentration.

4.1. Certification

This measurement procedure was thoroughly assessed for sources of bias and uncertainty. Mean coefficients of variation for all amino acid measurements were calculated as less than 2.6% for LC–MS/MS, and 11% or 8.5% for GC– and GC×GC–TOF-MS, respectively. The majority of the total uncertainty in the measurements was contributed from Type B components, suggesting the precision of the measurement procedure was of high quality. Further, the accuracy of the measurements is confirmed by four orthogonal measurement techniques and comparison to literature references, and is therefore also considered to be of high quality.

The isotope dilution measurement procedure described here refers to the technique of adding isotopically-labeled internal standards to both the samples and calibrants. In this work, we used a distinct internal standard to quantify each measurand. Stable isotopes [theoretically] act identically during sample preparation, chromatographic retention, ionization, fragmentation, and detection. These mass spectrometry methods are characterized by

high selectivity, the ability to limit interferences, accuracy over a large dynamic range, and the ease of adding stable isotope internal standards contingent on sample preparation. For LC–MS/MS, using MRM selective detection of ions in a triple quadrupole MS results in negligible susceptibility to interferences. Quantitative agreement was established using two fragmentation transitions from an amino acid. For GC analyses, selective detection was achieved through adherence to stringent chromatographic retention indices, accurate mass in a TOF system, and validation of electron impact (EI) ionization fragmentation patterns with known standards. Quantitative agreement was demonstrated using two unique derivatizing agents, as well as two unique chromatographic separation approaches. Both approaches satisfy all aspects of a well-characterized measurement procedure.

5. Conclusion

SRM 1950 is the first SRM produced to support metabolomics measurements, and the first serum or plasma-based amino acid SRM produced from NIST. Eighteen amino acids were accurately and precisely quantified from a healthy, human plasma pool using up to four orthogonal isotope-dilution mass spectrometry techniques. Only amino acids that were observed as stable, and were within quantifiable ranges in plasma, and have commercially available stable isotopic analogs were included as measurands. Results compare well between orthogonal platforms and also with published literature values, establishing high confidence in measurement accuracy and demonstrating that very different analytical approaches can, and should, provide like results. Statistical evaluation of all sets of data has provided certified mass fraction values for the pending release of NIST SRM 1950 Metabolites in Human Plasma. Development of a certified reference material for amino acid in plasma provides the scientific community essential metrological traceability to routine analyses, establishing a common, stable, and accurate baseline for instrument calibration and thus linking exploratory and clinical analyses to an established higher-order standard.

Disclaimer

Certain commercial equipment, instruments, or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.07.025.

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