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Automated GC–MS analysis of free amino acids in biological fluids

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ABSTRACT

A gas chromatography–mass spectrometry (GC–MS) method was developed for the quantitative analysis of free amino acids as their propyl chloroformate derivatives in biological fluids. Derivatization with propyl chloroformate is carried out directly in the biological samples without prior protein precipitation or solid-phase extraction of the amino acids, thereby allowing automation of the entire procedure, including addition of reagents, extraction and injection into the GC–MS. The total analysis time was 30 min and 30 amino acids could be reliably quantified using 19 stable isotope-labeled amino acids as internal standards. Limits of detection (LOD) and lower limits of quantification (LLOQ) were in the range of 0.03–12 μM and 0.3–30 μM, respectively. The method was validated using a certified amino acid standard and reference plasma, and its applicability to different biological fluids was shown. Intra-day precision for the analysis of human urine, blood plasma, and cell culture medium was 2.0–8.8%, 0.9–8.3%, and 2.0–14.3%, respectively, while the inter-day precision for human urine was 1.5–14.1%.

1. Introduction

Metabolomics aims at the quantitative analysis of all metabolites in a given biological system [1,2]. In the absence of a single analytical technique that can cover the entire metabolome, analysis is typically limited to the quantitative profiling of selected pathways or building blocks of the metabolome [3]. Important targets for metabolic profiling are amino acids. Besides being the basic structural units of proteins, amino acids have several non-protein functions. They are a source of energy either through formation of keto acids from the ketogenic amino acids or through gluconeogenesis from glucogenic amino acids. Glutamate and γ-aminobutyric acid are neurotransmitters [4], while tryptophan and tyrosine are precursors of serotonin and catecholamines, respectively [5]. Glycine is a precursor of porphyrins, whereas ornithine is a precursor of polyamines [6] and arginine can be metabolized to form nitric oxide [7]. Elevated amino acid levels in blood plasma and urine are well-known markers for inborn errors of metabolism, such as phenylalanine in phenylketonuria (PKU) or branched-chain amino acids in maple syrup urine disease (MSUD) [8,9]. Amino acids also serve as markers for nutritional influences, e.g., urinary taurine levels are an indicator for fish intake [10], while the 1-methylhistidine level in urine correlates with meat protein intake [11].

Due to the important biological functions of amino acids, fast and reproducible analytical methods are needed for their quantitative analysis. There are several chromatographic methods available to quantify amino acids in biological samples. The most commonly used method is cation-exchange chromatography followed by post-column derivatization with ninhydrin and UV detection [12–14]. Derivatization with *o*-phthalaldehyde (OPA) has been used both post-column after cation-exchange chromatography and pre-column coupled with reversed phase high-performance liquid chromatography (RP-HPLC) [15,16]. Reaction with phenylisothiocyanate (PITC) produces phenylthiocarbamyl derivatives, which are separated by RP-HPLC and detected at 254 nm [17,18]. All these methods require manual sample preparation steps, including protein precipitation, and analysis may last up to 2.5 h per sample. Another drawback is the UV absorbance detection: compared to mass spectrometry it lacks substance specificity and, therefore, co-eluting matrix components can cause over-quantification.

GC–MS analysis of silylated amino acids is feasible [19], but not all derivatives are stable; arginine, for example, decomposes to ornithine [20], and glutamate rearranges to form pyro-glutamate. Another drawback is the sensitivity of the reagents and derivatives to moisture. Other derivatization procedures for GC analysis include reaction of the amino acids with pentafluoropropyl anhydrid/isopropanol [21,22] or trifluoroacetic anhydrid/isopropanol [23]. However, these methods involve reagent removal and solvent exchange, which renders their automation difficult.

Amino acids can be derivatized directly in aqueous solution using alkyl chloroformates [24–26]. The amino acids react very quickly, for instance, with propyl chloroformate and the derivates

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can be extracted with an organic solvent. From the organic phase an aliquot can be injected directly into the GC–MS. Applying this approach, a fast and fully automated quantitative method for the analysis of amino acids in physiological fluids by GC–MS was developed. The analysis was performed using a modified protocol based on the EZ:faast kit from Phenomenex (Phenomenex Inc., Torrence, CA, USA), whereby the cation-exchange cleanup step was omitted and the amino acids were derivatized directly in the aqueous biological sample. This simplified protocol allowed for the full automation of the procedure with an MPS-2 sample robot from Gerstel (Gerstel, Muehlheim, Germany), with reliable quantification of amino acids in various biological matrices having been accomplished over a wide dynamic range using stable-isotope labeled internal standards.

2. Experimental

2.1. Chemicals

A standard solution of 17 amino acids at 1 mM each in 0.1 M HCl, phenol, isooctane, methyl chloroformate, iso-propanol and thiodiglycol were purchased from Sigma (Sigma–Aldrich, Taufkirchen, Germany). The certified amino acid solution was purchased from NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA). Methanol (LC–MS grade) and chloroform (HPLC grade) were from Fisher (Fisher Scientific GmbH, Ulm, Germany). The [U-^{13}C, U-^{15}N] cell free amino acid mix was from Euriso-Top (Saint-Aubin Cedex, France) and α-aminoadipic acid [2,5, 5-$^{2}H_3$] was purchased from C/D/N Isotopes Inc. (Quebec, Canada). *N*-methyl-*N*-trifluoroacetamide (MSTFA) was obtained from Macherey-Nagel (Dueren, Germany), and the Phenomenex EZ:faast GC kit (Phenomenex Inc., Torrence, CA, USA) was used for the derivatization of amino acids with propyl chloroformate.

2.2. Biological samples

Human urine was collected from healthy volunteers. Mice urine was obtained from collaborators at the University of Regensburg, while urine and serum samples from patients with inborn errors of amino acid metabolism were provided by the Zentrum für Stoffwechseldiagnostik Reutlingen GmbH. The lyophilized human plasma control was purchased from Recipe (Munich, Germany) and reconstituted in HPLC water. The cell culture medium was RPMI 1640 (PAA Laboratories GmbH, Cölbe, Germany) with phenolred, to 500 mL of which penicillin (30 mg/L) and streptomycin (10.4 g/L) (Invitrogen, Karlsruhe, Germany) had been added, as well as 25 mL of fetal calf serum (PAA Laboratories GmbH), 153 mg L-glutamine and 115 mg sodium pyruvate (Sigma–Aldrich). To stabilize the amino acids in the biological sample, 20 μL of an aqueous solution containing 10% iso-propanol, 0.1% phenol and 2% thiodiglycol, were added to 20–50 μL of biological sample.

2.3. GC–MS analysis

An Agilent model 6890 GC (Agilent, Palo Alto, USA) equipped with a MSD model 5975 Inert XL, PTV injector (Gerstel, Muehlheim, Germany) and a MPS-2 Prepstation sample robot was used. The robot has two autosamplers equipped with one syringe each of different volume. A 10-μL syringe is used for addition of the internal standards and for sample injection, while a 250-μL syringe is used for adding reagents. Between the adding steps, the syringes were washed at least 3 times with chloroform and/or propanol. The syringes were washed with propanol after adding aqueous solutions and with chloroform and propanol after adding organic solutions. Biological samples were kept in a cooled tray (5 °C).

The GC-column was a ZB-AAA (Phenomenex Inc.), 15 m × 0.25 mm I.D., 0.1 μm film thickness. In addition, a RTX-35 Amine column and a RXI-5 MS column from Restek (GmbH, Bad Homburg, Germany) were tested. The oven temperature was initially held at 70 °C for 1 min, raised at 30 °C/min to 300 °C, and held for 3 min. The column flow was 1.1 mL He/min. The injection volume was 2.5 μL and the split ratio was 1:15. The temperature of the PTV Injector was set at 50 °C for 0.5 min and ramped at 12 °C/s to 320 °C (5 min).

The following liners from Gerstel were tested: deactivated baffled glass liner, glass wool packed liner, quartz wool packed liner and the chemically inert SILTEC liner. The transfer line to the mass spectrometer was kept at 310 °C. The MS was operated in scan (50–420 *m*/*z*) and SIM (selected ion monitoring) mode. For SIM, appropriate ion sets were selected and two characteristic mass fragments of the derivatized amino acids were used for almost all amino acids, except for the labeled amino acids. The ion traces are listed in Table 1.

2.4. Derivatization

In contrast to the original Phenomenex protocol, the cation-exchange cleanup step was omitted. Amino acids were directly derivatized in the aqueous biological sample, 20–50 μL of which were transferred together with 20 μL of the stabilization reagent, described in Section 2.2, to a 2-mL autosampler vial (Gerstel). The vial was closed with a magnetic crimp cap to allow automated handling by the robot. The first step performed by the robot is the dilution of the sample with water up to 225 μL, followed by addition of 10 μL of a norvaline solution (200 μM) and 10 μL

Table 1
Ion traces selected for the SIM analysis of 32 physiological amino acids, norvaline and dipeptides

Amino acid	Quantification trace	Secondary ion trace	Internal standard quantification trace
Alanine	130	88	133
Sarcosine	130	217	
Glycine	102		105
α-Aminobutyric acid	144	102	
Valine	158	116	163
β-Aminoisobutyric acid	116		
Norvaline	158	72	
Leucine	172	130	178
allo-Isoleucine	172	130	
Isoleucine	172		178
Threonine	101	203	104
Serine	146	203	149
Proline	156		161
Asparagine	155	69	160
Thiaproline	174	147	
Aspartic acid	216	130	220
Methionine	203	277	206
Hydroxyproline	172	86	
Glutamic acid	230		235
Phenylalanine	190	206	199
α-Aminoadipic acid	244		247
α-Aminopimelic acid	258	84	
Glutamine	84	187	89
Ornithine	156	70	
Glycyl-proline	70	156	
Lysine	170	128	176
Histidine	282	168	290
Hydroxylysine	129	169	
Tyrosine	107	206	114
Proline-hydroxyproline	156		
Tryptophan	130		140
Cystathionine	203	272	
Cystine	248	216	

Amino acids printed in bold were quantified using the internal standard quantification trace of the corresponding stable-isotope labeled amino acid.

internal standard mix. A mixture of uniformly ^{13}C, ^{15}N-labeled alanine, glycine, valine, leucine, isoleucine, threonine, serine, proline, asparagine, aspartate, methionine, glutamate, phenylalanine, glutamine, lysine, histidine, tyrosine, and tryptophan, as well as [2,5,5-$^{2}H_3$] α-aminoadipic acid, were used as internal standards with a concentration range from 0.0438 to 1.4175 mM. To increase the pH of the solution, 120 μL of 0.33 M sodium hydroxide solution were added, followed by 50 μL of picoline in propanol, which acts as a catalyst for the derivatization reaction (solution provided by Phenomenex). The vial was moved to an agitator and the solution was mixed at 750 rpm for 0.2 min at 35 °C. Fifty μL of propyl chloroformate in chloroform were added to the sample, the solution was mixed for 0.2 min (750 rpm, 35 °C), equilibrated for 1 min and again mixed for 0.2 min. To extract the derivatives, 250 μL of isooctane were added and the vial was vortexed for 0.2 min (750 rpm, 35 °C). For analysis, an aliquot (2.5 μL) was taken from the upper organic phase and injected directly into the PTV.

2.5. Quantification

Absolute quantification of 32 compounds (alanine, sarcosine, glycine, α-aminobutyric acid, valine, β-aminoisobutyric acid, leucine, allo-isoleucine, isoleucine, threonine, serine, proline, asparagine, thiaproline, aspartic acid, methionine, hydroxyproline, glutamic acid, phenylalanine, α-aminoadipic acid, α-aminopimelic acid, glutamine, ornithine, glycyl-proline, lysine, histidine, hydroxylysine, tyrosine, proline-hydroxyproline, tryptophan, cystathionine and cystine) was performed by analyzing standard solutions containing equimolar amounts of all amino acids. The Phenomenex kit contains 3 different standard amino acid mixtures at 200 μM each. The first mixture consists of 23 amino acids. The second mixture contains amino acids not stable in acidic solution (asparagine, glutamine and tryptophan), while the third mixture includes complementary amino acids and dipeptides occurring in urine (α-aminoadipic acid, cystathionine, glycyl-proline, hydroxylysine, proline-hydroxyproline and thiaproline). For calibration, the three different mixtures were mixed in equal amounts to yield a final concentration of 60 μM for each compound. The mix was further diluted to final concentrations of 6 and 0.6 μM, respectively. For calibration, increasing volumes of the diluted and non-diluted standards were pipetted automatically by the autosampler into empty vials and then derivatized as described above. A 1 mM amino acid standard solution from Sigma was used to extend the calibration curve to higher concentrations. The amino acids were normalized by the area of the labeled amino acid for the generation of calibration curves in the range of 0.3–2000 μM or normalized by the area of the closest eluting internal standard compound.

2.6. NMR analysis

For NMR structural analysis, the propylformate derivative of asparagine was dissolved in 99.99% pure $CDCl_3$ that was also used as internal standard at 7.26 and 77.00 ppm for ^{1}H and ^{13}C, respectively.

NMR experiments were recorded at 300 K on a Bruker Avance III spectrometer equipped with two channels and a cryo-cooled pulse field gradient triple resonance probe with z-gradients. The conformation of the molecule was confirmed by 1D ^{1}H, 2D ^{1}H-^{13}C HSQC and 2D ^{1}H-^{13}C HMBC experiments.

NMR assignments: C2 155.6 ppm; C4 67.2 ppm; H4A/H4B 3.98 ppm; C5 22.0 ppm; H5A/H5B 1.59 ppm; C6 10.0 ppm; H6A/H6B/H6C 0.89 ppm; C8 50.5 ppm; H8 4.47 ppm; C9 21.6 ppm; H9A 2.96 ppm; H9B 2.87 ppm; C10 115.8 ppm; C12 168.5 ppm; C15 68.2 ppm; H15A/H15B 4.12 ppm; C16 21.6 ppm; H16A/H16B 1.65 ppm; C17 10.0 ppm; H17A/H17B/H17C 0.89 ppm (numbering is shown in Fig. 1).

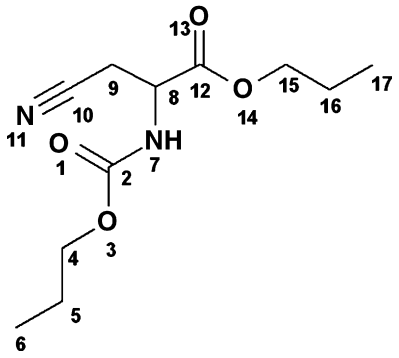

Fig. 1. Propyl chloroformate derivate of asparagine after the loss of water.

3. Results and discussion

3.1. Derivatization and column selection

Both the amino and the carboxyl group of amino acids react readily with alkyl chloroformates as shown in Fig. 2, to yield volatile derivatives for GC-analysis [24]. Hydroxyl groups as found in serine and threonine have a very low reactivity and amide groups are not derivatized. Zampolli et al. [40] showed that methyl chloroformate (MCF) and 2,2,3,3,4,4,4 – heptafluorobutanol (HFB) produce mono – and bis-acylated derivatives for serine, while no acylation of the hydroxyl group in threonine was observed. For amino acids without any additional functional groups two equivalents of alkyl chloroformate are needed. The acid function is converted to the ester, under loss of CO_2, and the amino group reacts to the corresponding amide. Using U-^{13}C, U-^{15}N-labeled amino acids it was shown that the CO_2 loss originated from the derivatization reagent (data not shown).

For derivatization of the amino acids with propyl chloroformate prior to GC–MS analysis the Phenomenex EZ:faast GC kit was employed. To allow for complete automation of sample pretreatment and injection, we explored whether the cation-exchange solid-phase extraction step recommended by Phenomenex prior to derivatization could be omitted given the high selectivity of a quadrupole mass spectrometer operated in SIM mode. Indeed, no significant differences in retention times and number of amino acids detected were observed between urine and plasma samples subjected to either solid-phase extraction or derivatized directly (data not shown).

Initially, propyl chloroformate derivates were analyzed on a Phenomenex ZB-AAA column, 10 m × 0.25 mm I.D., which was provided with the Phenomenex EZ:faast GC kit. However, for some amino acids either peak tailing (e.g., tryptophan and tyrosine) or non-linear calibration curves (e.g., glutamine and tryptophan) were observed. Further, not all amino acids, including the isobaric leucine, were baseline separated. Therefore, other stationary phases were evaluated.

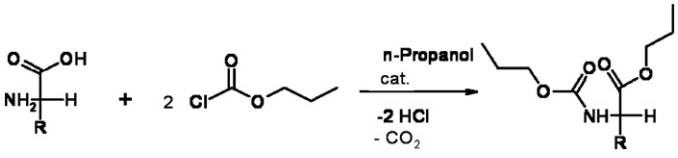

Fig. 2. Reaction scheme for the derivatization of amino acids with propyl chloroformate.

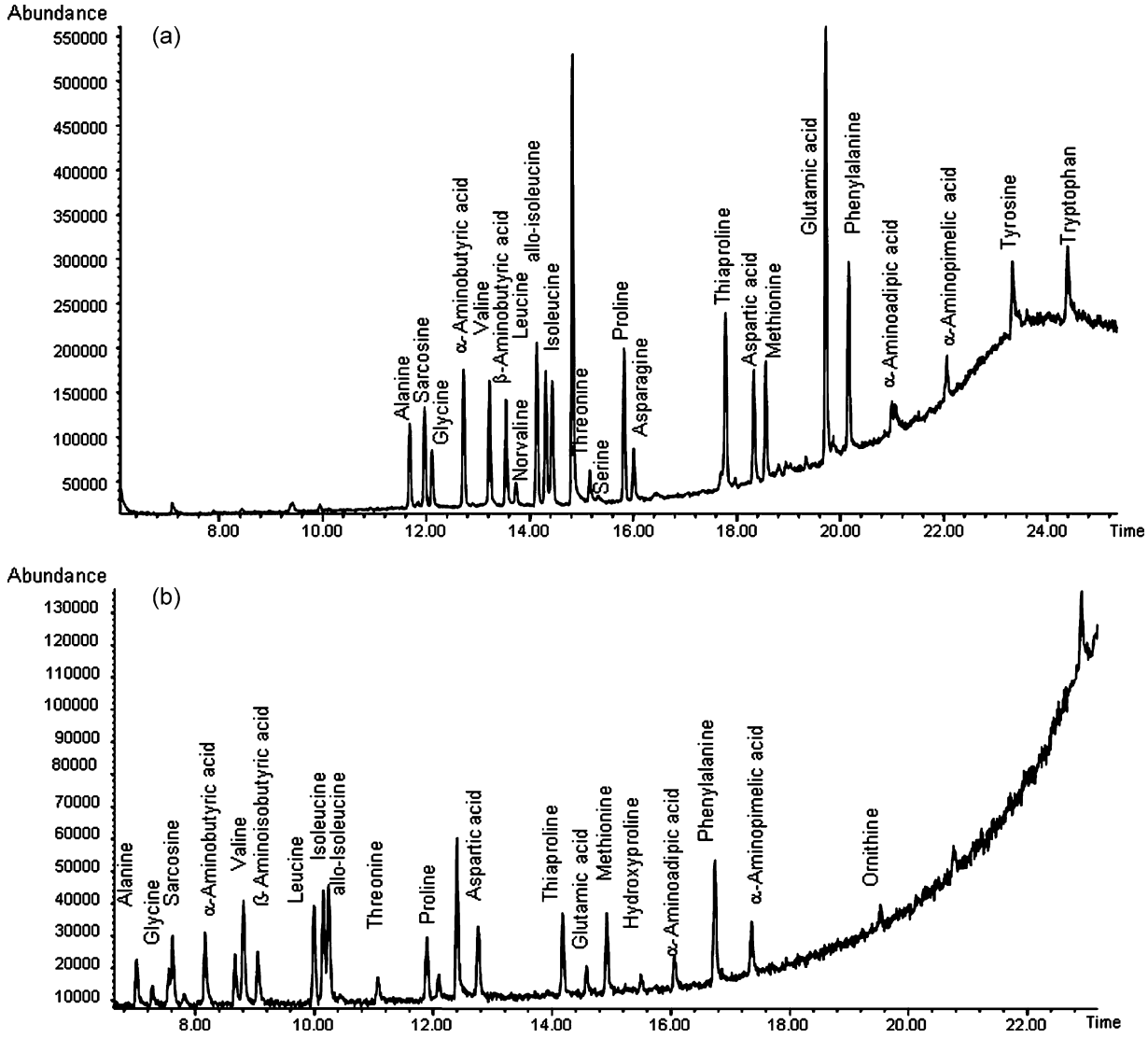

Fig. 3. GC–MS chromatograms of an amino acid standard separated on a 30-m RTX-35 Amine column after derivatization with (a) propyl and (b) methyl chloroformate, respectively.

The first column tested was a RTX-35 Amine column (30 m × 0.25 mm I.D., 0.5 μm film thickness), which is specifically designed for the separation of amines. Peak tailing was significantly reduced (Fig. 3a). However ornithine, histidine, glutamine, glycyl-proline, lysine, hydroxylysine, proline-hydrxyproline, cystathionine and cystine were not detected due to either the significant column bleeding occurring at high temperatures, which might mask late eluting analytes, or the fairly high film thickness (0.5 μm) of the RTX-35 Amine column, which might retain amino acid derivates indefinitely. The column is not commercially available with a thinner film. To obtain more volatile derivates the reaction with methyl chloroformate was tested [25]. But even then, many amino acids, including asparagine, serine, glutamine, glycyl-proline, lysine, histidine, hydroxylysine, tyrosine, proline-hydroxyproline, tryptophan, cystathionine and cystine, were not detected on the RTX-35 Amine column (Fig. 3b). In addition to the polar column, a low bleeding non-polar RXI-5 MS column was tested (30 m × 0.25 mm I.D., 0.25 μm film thickness). Using the propyl chloroformate reaction, five amino acids were not detected (threonine, serine, glutamine, cystathionine and cystine), while with the methyl chloroformate reaction aspargine, serine, threonine, ornithine, hydroxyproline, proline-hydroxyproline, cystathionine and cystine could not be detected.

We also compared the separation of the propyl chloroformate derivatives on a 15-m ZB-AAA column versus the original 10-m column. Employing the same temperature program, better resolution was obtained on the longer column for asparagine and methionine as well as glutamic acid and phenylalanine, which facilitates a more robust selection of SIM windows. For both amino acid pairs the resolution was 1.7 with the 10-m column and it improved to >2.5 using the 15-m column. Fig. 4 presents a typical chromatogram of the 33 compounds including norvaline, which is a non-endogenous compound used as an internal standard. Less than 10 min were required to resolve all compounds.

3.2. Injection and liner selection

Sample injection was done using programmed-temperature vaporization. The sample was introduced into the cold insert (50 °C), which was then rapidly heated to vaporize and transfer the analytes into the GC column. This is a rather gentle injection technique, which is favorable for thermally labile compounds. Since

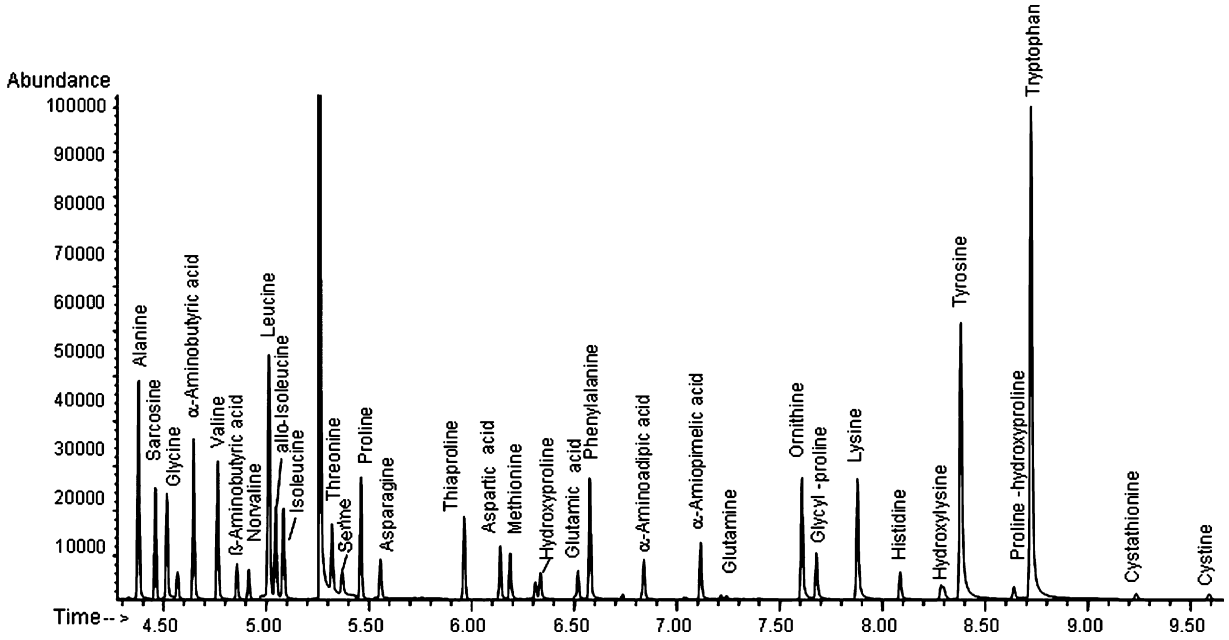

Fig. 4. Typical GC–MS chromatogram for the analysis of an amino acid standard on a 15 m × 0.25 mm I.D. ZB-AAA column after derivatization with propyl chloroformate.

the amino acid derivatives are still rather polar analytes, adsorption to the insert surface can occur, reducing the reproducibility of the analysis. Proper selection of the insert type is important. Therefore, different liners were tested with regard to the reproducibility of urine analysis: deactivated baffled glass liner, glass wool packed liner, quartz wool packed liner and the chemically inert SILTEC liner. Using the glass or quartz wool packed liner increases the liner surface to retain the liquid sample injected, which can then evaporate from the glass or quartz wool surface. However, there is the risk of increased analyte adsorption at the active sites on the surface. A urine sample was analyzed 5 times with each liner and the relative standard deviation (R.S.D.) was calculated (Fig. 5). The glass wool packed liner showed the worst reproducibility, in particular for amino acids with polar functional groups such as aspartic acid, glutamic acid and asparagine. Additionally, it was not possible to detect glutamine, cystathionine and cystine. Reproducibility was better for the quartz wool packed liner, but still inferior to the SILTEC liner. With the baffled liner, there were more amino acids with an R.S.D. > 10%, and for thiaproline the R.S.D. exceeded 20%. Only with the SILTEC liner, the R.S.D.s were <10%, except for sarcosine with 10.6%, and all amino acids were detected successfully. Therefore, the SILTEC liner was used for all further analyses following its prior conditioning by the subsequent injection of the silylation reagent MSTFA, a 1 mM amino acid standard solution, and blanks to deactivate any active sites on the glass surface.

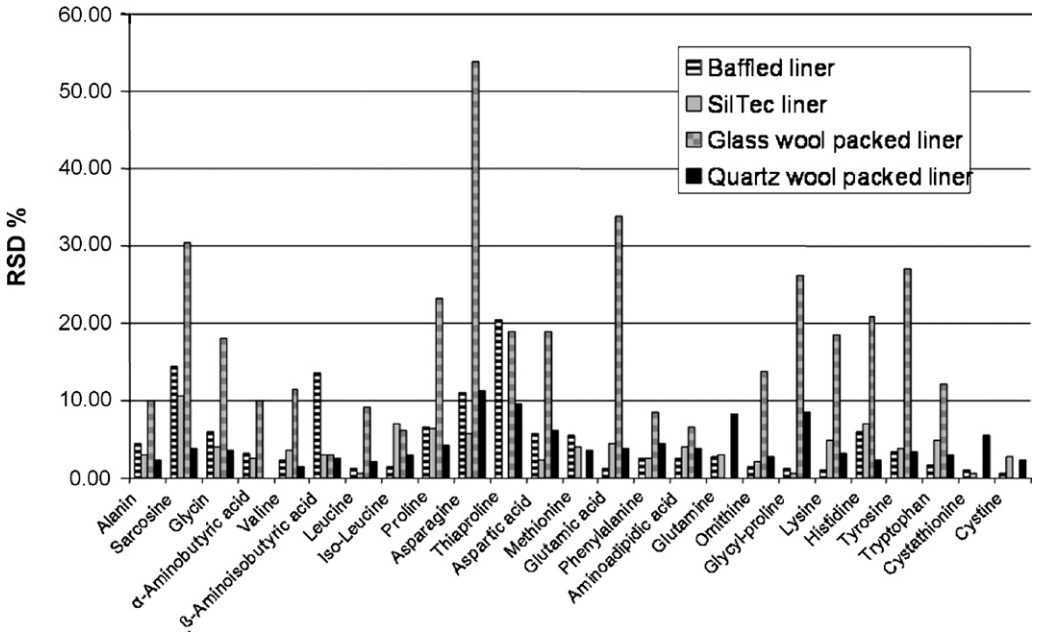

Fig. 5. Comparison of the relative standard deviation values obtained for the repeated analysis ($n = 5$) of urinary amino acids using different injector inserts.

Table 2
Comparison of the *R*-square values and R.S.D. values obtained for the calibration curves of selected amino acids using either norvaline (Nval) or stable-isotope labeled amino acids as internal standards

Amino acid[a]	R^2 Nval	R^2 Stable isotopes	R.S.D. (%) Nval	R.S.D. (%) Stable isotopes
Alanine	0.9732	0.9997	7.04	1.5
Sarcosine	0.9974	0.9969	10.91	5.7
Glycine	0.9893	0.9998	9.06	2.2
α-Aminobutyric acid	0.9984	0.9984	4.07	4.2
Valine	0.8904	0.9996	1.96	2.1
β-Aminoisobutyric acid	0.9977	0.9970	4.92	5.6
Leucine	0.9988	0.9992	2.64	2.8
allo-Isoleucine	0.9953	0.9985	2.86	2.5
Isoleucine	0.9061	0.9996	2.96	2.7
Threonine	0.9191	0.9988	n.d.	n.d.
Serine	0.8637	0.9975	n.d.	n.d.
Proline	0.9955	0.9960	6.90	3
Asparagine	0.9754	0.9986	13.70	2.2
Thiaproline	0.9858	0.9900	n.d.	n.d.
Aspartic acid	0.9939	0.9997	15.49	14.1
Methionine	0.9915	0.9958	7.36	11.8
Hydroxyproline	0.9725	0.9758	n.d.	n.d.
Glutamic acid	0.9993	0.9999	8.13	3.3
Phenylalanine	0.9972	0.9997	5.34	3.4
α-Aminoadipic acid	0.9908	0.9982	6.81	2.5
α-Aminopimelic acid	0.9956	0.9925	n.d.	n.d.
Glutamine	0.9523	0.994	15.66	4.2
Ornithine	0.9909	0.9971	9.07	4.8
Glycyl-proline	0.9659	0.984	n.d.	n.d.
Lysine	0.975	0.996	7.79	3.9
Histidine	0.8937	0.9987	12.28	2.2
Hydroxylysine	0.985	0.9976	n.d	n.d.
Tyrosine	0.9688	0.9984	5.99	2.5
Proline-hydroxyproline	0.9807	0.9906	n.d.	n.d.
Tryptophan	0.9802	0.9987	4.02	2.8
Cystathionine	0.9959	0.993	5.42	2.5
Cystine	0.9861	0.995	9.56	11.3

The R.S.D. values represent the inter-day reproducibility of urinary amino acid levels for 11 repeated injections using either quantification method.
n.d.: not detected above the LLOQ.
[a] Amino acids printed in bold were quantified with a corresponding stable isotope.

3.3. Internal standard selection

For the generation of reliable quantitative data, the use of an internal standard is required to correct for chemical and analytical losses during derivatization and analysis. We observed for amino acids of similar structure and retention to norvaline, such as leucine or glycine, that norvaline corrected quite well for such losses. But for amino acids with a more complex structure and more functional groups, e.g., glutamine, histidine and tyrosine, the linearity was lost over a wider concentration range, as shown in Table 2. Additionally, the reproducibility decreased. This led to the conclusion that more internal standards structurally similar to as many analytes as possible were needed. This is best realized by stable-isotope labeled amino acids. A standard mix of 18 uniformly ^{13}C and ^{15}N-labeled amino acids was chosen. The amino acids are extracted from algae. Consequently, their individual concentrations, as analyzed by HPLC, differ and range from 0.043 to 1.417 mM. Additionally [2,5,5-$^{2}H_3$] α-aminoadipic acid was used as internal standard. To compare the difference with and without using the internal standard mixture, the *R* square (R^2)-values of the calibrations of all amino acids are shown in Table 2. The *R* square-values are at least 0.99 using the labeled amino acids as internal standards except for hydroxyproline and glycyl-proline. In comparison, the *R* square-values of the calibration curves using norvaline as the only internal standard were mostly <0.99. In summary, the *R* square-values improved for all amino acids except sarcosine, α-aminobutyric acid, α-aminopimelic acid and cystathionine, for which no stable-isotope labeled amino acids were available.

In addition, we compared the inter-day reproducibility of 11 biological replicates of a urine sample. This biological sample was measured 11 times during a batch of 351 biological samples. The R.S.D.s using norvaline as the sole internal standard ranged from 1.98% to 18.6%. But they improved significantly (1.5–5.7%) for most amino acids, except for aspartic acid, methionine and cystathionine, when stable-isotope labeled amino acid standards were employed. For the last exception no stable isotope labeled standard had been available. Stable-isotope labeled amino acids were used as internal standards for all further analyses.

3.4. Method characterization

For absolute quantification, calibration curves were generated. Calibration curve parameters, retention time, range of quantification, *R* square-values and limits of detection are presented in Table 3. The quantification range is determined by the lower (LLOQ) and the upper limit of quantification (ULOQ), which are defined as the lowest, respectively, highest point of the calibration curve with an accuracy between 80% and 120%, in agreement with the FDA Guide for Bioanalytical Method Validation [27]. The *R* square-value or coefficient of determination was calculated as the square of the correlation coefficient *r* of the regression analysis over the quantification range. The limit of detection (LOD) is defined as the concentration producing a signal to noise (S/N) ratio of at least 3:1. Concentrations reported in Table 3 were calculated from the analysis of 50-μL aliquots. The lowest LOD was 0.03 μM, corresponding to an absolute injection amount of 15 fmol.

The LOD of 0.03 μM was determined for alanine, glycine and tryptophan. The LODs for most other amino acids were below 1 μM except for serine, asparagines, histidine, hydroxylysine, cystathionine and cystine, which yielded an LOD of 3 μM. The highest LODs with 12 μM were obtained for proline-hydroxyproline and glutamine. For glutamine, this was due to partial decomposition of the propylformate derivative through elimination of water, as evidenced by two peaks in the chromatogram. For asparagine, elimination of water was complete. Nevertheless, both glutamine and asparagine could be determined by derivatization with propyl chloroformate, thereby not confirming the observation by Casal et al. [41] that glutamine and asparagine are converted to aspartate and glutamate during derivatization with ethyl chloroformate and 2,2,3,3,4,4,4-heptafluoro-1-butanol. The LOD for all amino acids can be improved by using less organic solvent for extraction or injecting more sample using large volume technique. The calibration ranges ranged from 0.3 to 2000 μM for most amino acids. Satisfactory linearity was obtained for the calibration curves with a *R* square-value ≥0.99 for all amino acids except hydroxyproline (0.9758) and glycyl-proline (0.984). However, for these amino acids no corresponding stable-isotope had been available.

3.5. Method validation

A certified amino acid standard from NIST was analyzed to check the accuracy of the method. This Standard Reference Material (SRM) is an aqueous mixture of 17 amino acids in 0.1 M hydrochloric acid. We were able to quantify 16/17 amino acids. Arginine could not be determined because of the thermal instability of its propylformate derivative that carries a free guanidine group. The certified concentrations and estimated uncertainties for the 16 amino acids are given in Table 4. These values are based on in-house analysis at NIST and a round robin study that was conducted in cooperation

Table 3
Calibration curve parameters

Amino acid	R^2[a]	LOD[b] (μM)	Range of quantification [μM][c]	Regression line	Retention time (min)
Alanine	0.9997	0.03	0.3–2000	$1.507 \times x + 0.011940$	4.35
Sarcosine	0.9969	0.3	0.9–270	$1.611 \times x + -0.008277$	4.43
Glycine	0.9998	0.03	3–2000	$1.238 \times x + 0.068277$	4.49
α-Aminobutyric acid	0.9984	0.12	0.3–270	$2.521 \times x + 0.001067$	4.62
Valine	0.9996	0.18	0.3–2000	$1.174 \times x + 0.001163$	4.73
β-Aminoisobutyric acid	0.9970	0.27	0.9–270	$0.613 \times x + -0.003078$	4.83
Leucine	0.9992	0.06	0.3–2000	$1.102 \times x + 0.005131$	4.98
allo-Isoleucine	0.9985	0.3	0.9–270	$1.252 \times x + -0.005865$	5.02
Isoleucine	0.9996	0.12	0.9–2000	$1.122 \times x + -0.002333$	5.05
Threonine	0.9988	0.18	0.3–2000	$1.03 \times x + -0.001590$	5.29
Serine	0.9975	3.0	12–2000	$1.22 \times x + 0.443377$	5.34
Proline	0.9960	0.27	0.3–2000	$0.623 \times x + -0.010681$	5.43
Asparagine	0.9986	3.0	12–270	$1.125 \times x + -0.036928$	5.53
Thiaproline	0.9900	0.3	0.9–270	$4.349 \times x + -0.006387$	5.93
Aspartic acid	0.9997	0.3	3–2000	$1.251 \times x + -0.018064$	6.11
Methionine	0.9958	0.9	3–1000	$1.177 \times x + -0.028463$	6.16
Hydroxyproline	0.9758	0.9	3–270	$0.334 \times x + -0.004975$	6.30
Glutamic acid	0.9999	0.9	3–2000	$1.249 \times x + -0.023798$	6.49
Phenylalanine	0.9997	0.3	0.9–2000	$1.191 \times x + -0.005860$	6.55
α-Aminoadipic acid	0.9982	0.9	3–270	$1.089 \times x + -0.033340$	6.81
α-Aminopimelic acid	0.9925	0.9	3–270	$2.351 \times x + -0.131996$	7.09
Glutamine	0.9940	12.0	30–270	$1.197 \times x + -0.012232$	7.58
Ornithine	0.9971	0.3	0.9–270	$1.526 \times x + -0.018075$	7.18
Glycyl-proline	0.9840	0.9	3–270	$0.161 \times x + -0.006280$	7.65
Lysine	0.9960	0.3	0.9–2000	$1.122 \times x + -0.024828$	7.85
Histidine	0.9987	3.0	12–2000	$0.372 \times x + -0.043117$	8.06
Hydroxylysine	0.9976	3.0	12–270	$0.208 \times x + 0.006536$	8.26
Tyrosine	0.9984	0.3	0.9–2000	$2.449 \times x + -0.001631$	8.35
Proline-hydroxyproline	0.9906	12.0	12–270	$0.047 \times x + -0.002474$	8.61
Tryptophan	0.9987	0.03	0.3–270	$1.44 \times x + -0.003679$	8.69
Cystathionine	0.9930	3.0	12–270	$0.133 \times x + 0.001518$	9.20
Cystine	0.9950	3.0	12–1000	$0.186 \times x + 0.012430$	9.55

Ranges of quantification were defined by the lower and upper limits of quantification. Amino acids printed in bold were quantified using the corresponding stable isotope-labeled amino acid.

[a] Coefficient of determination (square of the correlation coefficient r of the regression analysis).

[b] Limit of detection ($S/N \geq 3$).

[c] LOD and LOQ were calculated for a sample volume of 50 μL.

with the Association of Biomolecular Research Facilities. The certified value is the equally weighted mean of the NIST average and the round robin average. Additionally gravimetric values given by NIST are shown in the table. The gravimetric value is based on the weighed amount of each amino acid used to prepare the solution. For all amino acids, there is an excellent correspondence between the results obtained by GC–MS and the certified values obtained on conventional amino acid analyzers. In addition, a recovery based on the gravimetric values was calculated. It ranges from 94.6% for methionine up to 105.3% for lysine. Only the recovery for histidine is high (123.7%). But for this amino acid, the certified concentration measured by NIST is also higher than the gravimetric value.

The applicability of the method to biological samples was demonstrated by analyzing amino acids in a certified biological matrix. We chose Clinchek plasma controls from RECIPE, which are used for internal quality assurance in clinical-chemical laboratories. The mean values and confidence intervals have been established by independent reference laboratories using conventional amino acid analyzers. To quantify the amino acid concentration in plasma, plasma was measured 10 times by GC–MS.

Table 4
Arithmetic means and standard deviations of the concentrations (mM) of amino acids in a certified standard compared to the reference values given by NIST and compared to the gravimetric values in terms of recovery

Amino acid	GC–MS ($n = 6$)	NIST	Gravimetric value	Recovery (%) of the GC–MS data based on gravimetric values
Alanine	2.506 ± 0.027	2.51 ± 0.09	2.5	100.2
Glycine	2.604 ± 0.026	2.45 ± 0.08	2.51	103.7
Valine	2.623 ± 0.020	2.44 ± 0.08	2.55	102.9
Leucine	2.562 ± 0.018	2.48 ± 0.09	2.6	98.5
Isoleucine	2.650 ± 0.013	2.39 ± 0.07	2.54	104.3
Threonine	2.549 ± 0.069	2.39 ± 0.08	2.44	104.5
Serine	2.584 ± 0.082	2.43 ± 0.09	2.47	104.6
Proline	2.592 ± 0.035	2.44 ± 0.09	2.5	103.7
Aspartic acid	2.576 ± 0.020	2.5 ± 0.09	2.55	101.0
Methionine	2.386 ± 0.144	2.43 ± 0.09	2.53	94.3
Glutamic acid	2.513 ± 0.055	2.27 ± 0.10	2.44	103.0
Phenylalanine	2.566 ± 0.025	2.44 ± 0.08	2.58	99.5
Lysine	2.642 ± 0.032	2.47 ± 0.10	2.51	105.3
Histidine	3.080 ± 0.052	2.83 ± 0.11	2.49	123.7
Tyrosine	2.609 ± 0.047	2.47 ± 0.09	2.49	104.8
Cystine	1.157 ± 0.071	1.16 ± 0.06	1.2	96.4

Table 5
Amino acid concentrations in a plasma reference as determined by GC–MS in comparison to the reported control range (data given by the manufacturer)

Amino acid	Concentration by GC–MS (μM) Mean ± S.D. (n = 10)	Control range (μM)
Alanine	349.9 ± 3.51	327–491
Glycine	168.7 ± 2.84	148–222
α-Aminobutyric acid	9.8 ± 0.55	10.8–16.2
Valine	195.7 ± 1.60	178–266
Leucine	192.3 ± 1.82	157–235
Isoleucine	68.7 ± 0.86	56.7–85.1
Proline	191.7 ± 1.66	159–239
Asparagine	29.7 ± 0.88	17.3–25.9
Aspartic acid	19.0 ± 0.39	16.9–25.3
Methionine	38.8 ± 1.03	30.3–45.5
Glutamic acid	243.3 ± 2.84	236–354
Phenylalanine	80.6 ± 1.67	65.8–98.8
Ornithine	125.7 ± 3.34	112–168
Glutamine	205.1 ± 9.01	199–299
Lysine	154.6 ± 1.57	128–192
Histidine	71.4 ± 5.61	60.6–91
Tyrosine	64.1 ± 1.47	47.6–71.4
Tryptophan	50.7 ± 0.78	37.8–56.6

We were able to determine 18 amino acids in the plasma. All measured values were well inside the control range given by RECIPE (Table 5). The sole exception was asparagine, for which the GC–MS value was slightly too high. The control range for asparagine was 17.3–25.9 μM and the concentration measured by GC–MS was 29.7 μM.

3.6. Precision of GC–MS analysis of amino acids in different biological matrices

The method's precision in the determination of amino acid concentrations in different biological matrices was evaluated by analyzing human urine, mice urine, control plasma and cell culture medium. Ten or more replicates were analyzed for each sample and the R.S.D.s obtained for different amino acids are listed in Table 6. For human urine, we determined not only the intra-day but also the inter-day precision. The reproducibility in all biological samples for all amino acids was excellent, with R.S.D.s typically <5%. Generally, the R.S.D.s are higher in urine than in cell culture medium or plasma, but consistently <9% in the intra-day experiments. For most amino acids, the precision for intra-day and inter-day measurements is comparable, except for aspartic acid, methionine and cystine. For the latter amino acids, the R.S.D.s increased above 10% in the inter-day measurements with a maximum value of 14.1% for aspartic acid.

3.7. Quantification in biological matrices

Matrix spike experiments were performed in human urine to evaluate the impact of the biological matrix on the quantification. Amino acid standards in three different absolute amounts (1.5, 6.0 and 10.5 nmol) were added to three different urine samples and measured in triplicate. Linear regression analysis was performed for the standard addition and the calculated slopes were compared with those obtained from the calibration with the aqueous standards. The correlation between the slopes for the amino acids found in human urine is shown in Fig. 6. A slope of 1.08 and a correlation coefficient (RSQ) of 0.95 indicate the absence of matrix effects for most amino acids and justify the use of aqueous standards for calibration. Only glycine, sarcosine, α-aminobutyric acid and tyrosine are slightly over- or underestimated. The average recovery for all amino acids calculated over all spike levels and all replicates was 93.6%, ranging from 70.9% for glutamine to 120% for glycine. However, glutamine and glycine have high levels in urine and the spike levels used are too low to evaluate these amino acids correctly.

3.8. Inborn errors of amino acid metabolism

Analysis of blood and urinary amino acids are used routinely in the diagnosis and treatment of inherited metabolic disorders, such

Table 6
Reproducibility of GC–MS analysis of amino acids in different biological matrices using aliquots of 20 μL of sample, except for 50 μL of human urine

Amino acid[a]	Relative standard deviation (%)				
	Human urine (n = 10) Intra-day	Human urine (n = 11) Inter-day	Mice urine (n = 10) Intra-day	Plasma (n = 11) Intra-day	Media (n = 10) Intra-day
Alanine	2.0	1.5	1.3	1.0	2.6
Sarcosine	5.6	5.7	4.6	n.d.	n.d.
Glycine	3.1	2.2	2.1	1.7	2.0
α-Aminobutyric acid	7.9	4.2	3.9	5.6	3.5
Valine	2.4	2.1	1.3	0.8	3.4
β-Aminoisobutyric acid	5.7	5.6	8.9	n.d.	n.d.
Leucine	3.0	2.8	2.4	0.9	3.0
allo-Isoleucine	3.6	2.5	1.6	1.6	n.d.
Isoleucine	2.5	2.7	2.3	1.3	2.6
Proline	3.2	3.0	3.1	0.9	3.0
Asparagine	3.5	2.2	5.4	3.0	4.0
Aspartic acid	8.1	14.1	6.9	2.0	3.8
Methionine	7.1	11.8	4.8	2.7	4.3
Glutamic acid	3.1	3.3	2.5	1.2	3.0
Phenylalanine	2.5	3.4	3.6	8.3	n.d.
α-Aminoadipic acid	4.3	2.5	4.3	3.6	n.d.
Ornithine	3.7	4.8	3.2	2.7	8.2
Glutamine	8.7	4.2	9.1	4.4	3.9
Lysine	2.4	3.9	2.0	1.0	3.6
Histidine	4.9	2.2	7.5	7.8	5.4
Tyrosine	4.0	2.5	5.8	2.3	3.7
Tryptophan	2.9	2.8	3.3	1.5	3.9
Cystathionine	8.8	2.5	n.d.	n.d.	n.d.
Cystine	7.1	11.3	8.0	n.d.	14.3

n.d.: not detected above the LLOQ.
[a] Amino acids printed in bold were quantified with a corresponding stable isotope.

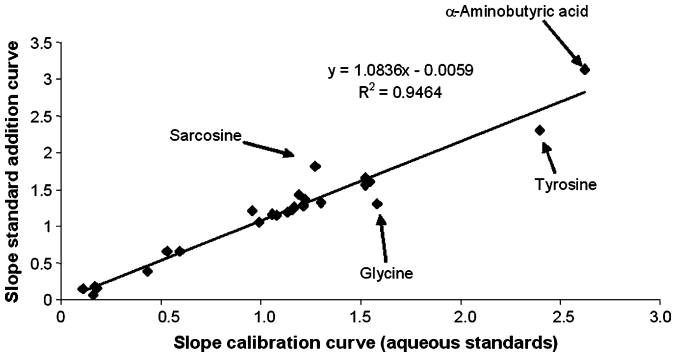

Fig. 6. Evaluation of matrix effects by comparison of the slopes of the calibration curve (*x*-axis) with the slope of the standard addition curve in human urine (*y*-axis).

as phenylketonuria and maple syrup urine disease. The screening for inborn errors of metabolism is widely done using direct infusion LC-MS/MS methods [8,28,29], which allows the very fast analysis of large number of samples. However, isobaric amino acids, such as leucine, isoleucine and allo-isoleucine or alanine and sarcosine cannot be distinguished. In contrast, the GC–MS method takes longer, but separation of those isobars is achieved.

To demonstrate the applicability of the GC–MS method to the determination of abnormal amino acid levels in inherited disorders of amino acid metabolism, serum and urine samples were ascertained from patients with various inborn errors of metabolism. Four different serum samples and 4 different urine samples were analyzed. The serum samples originated from patients with maple syrup urine disease, phenylketonuria, propionic acidemia and tyrosinemia I, whereas the urine samples were from patients with argininosuccinic aciduria, propionic acidemia, maple syrup urine disease and aminoaciduria. All samples were measured in triplicate. The amino acid concentrations observed in these patients are listed in Tables 7 and 8.

Phenylketonuria (PKU) is caused by a deficiency of the enzyme phenylalanine hydroxylase or its cofactors [9], leading to the accumulation of phenylalanine [30]. PKU can be diagnosed by an increased ratio of phenylalanine to tyrosine in serum [31]. Indeed, a high concentration of phenylalanine (296.8 μM) was detected in the PKU serum sample compared to the other samples analyzed that yielded an average phenylalanine concentration of 39.1 μM. Patients with maple syrup urine disease have a defect in branched-chain α-keto acid decarboxylase, resulting in increased serum concentrations of keto acids and their corresponding amino acids. The amino acid that accumulates the most is leucine. Further, increased concentrations of valine and isoleucine are often observed [8]. MSUD can be diagnosed by an increased ratio of leucine and isoleucine to phenylalanine [31]. As shown in Table 7, leucine is the most abundant amino acid with serum concentration of 394 μM, while the average concentration was only 58 μM in the three MSUD-negative serum samples. The concentrations of valine and isoleucine in the MSUD serum sample were also higher than in the other serum samples. In addition, allo-isoleucine was detected in the serum sample with a concentration of 32.1 μM. There were also pronounced differences in the urinary amino acid profiles between MSUD-positive and MSUD-negative samples. In comparison to argininosuccinic aciduria and propionic acidemia, the urinary concentrations for valine, leucine and isoleucine were increased 8-, 15- and 17-fold, respectively. Even allo-isoleucine could be detected and quantified with a concentration of 56 μM. In addition, high urinary concentrations of threonine, serine, α-aminoadipic acid, lysine, histidine and proline-hydroxyproline were detected.

Tyrosinemia I and II are characterized by an accumulation of tyrosine. The tyrosinemia type I is caused by a deficiency of fumarylacetoacetase [8]. The tyrosinemia-positive urine sample has a ten times higher concentration of tyrosine compared to the other urine samples analyzed. Propionic acidemia is categorized as a deficiency of propionyl-CoA-carboxylase. Methylcitrate and propionic acid are the key indicators for that disorder [32–34]. Additionally, high concentrations of glycine can occur in urine and serum [35]. Accordingly, high glycine concentrations were detected in the propionic acidemia positive serum and urine samples. Argininosuccinic aciduria is an inborn error with a urea cycle defect that causes

Table 7
Serum amino acid concentrations (μM) for patients with inborn errors of metabolism

Amino acid	Maple syrup urine disease	Phenylketonuria	Propionicacidemia	Tyrosinemia I
Alanine	69.56 ± 1.46	174.81 ± 0.95	86.51 ± 0.48	187.75 ± 1.29
Glycine	81.67 ± 2.05	151.57 ± 4.93	489.14 ± 2.93	187.45 ± 6.03
α-Aminobutyric acid	3.81 ± 0.17	3.07 ± 0.12	2.97 ± 0.02	3.46 ± 0.08
Valine	245.49 ± 5.16	126.69 ± 1.11	80.67 ± 0.31	70.55 ± 0.39
β-Aminoisobutyric acid	n.d.	n.d.	1.2 ± 0.07	1.06 ± 0.04
Leucine	394.3 ± 7.95	55.15 ± 0.47	69.44 ± 0.68	49.7 ± 0.68
allo-Isoleucine	32.11 ± 1.06	n.d.	n.d.	n.d.
Isoleucine	123.37 ± 2.44	31.65 ± 0.21	29.11 ± 0.1	21.71 ± 0.35
Threonine	39.91 ± 1.49	50.39 ± 0.40	39.02 ± 1.08	50.74 ± 0.84
Serine	56.1 ± 0.55	79.86 ± 0.31	60.49 ± 1.89	77.21 ± 1.46
Proline	47.4 ± 1.21	95.59 ± 1.76	65.21 ± 0.41	80.75 ± 0.54
Asparagine	13.47 ± 0.29	n.d.	16.43 ± 0.62	17.33 ± 1.22
Aspartic acid	14.17 ± 0.45	13.62 ± 0.3	8.27 ± 0.27	13.95 ± 0.26
Methionine	8.58 ± 0.17	7.06 ± 0.43	8.12 ± 0.21	7.52 ± 0.45
Hydroxyproline	n.d.	n.d.	n.d.	9.26 ± 1.29
Glutamic acid	36.35 ± 0.62	50.65 ± 0.22	21.3 ± 0.3	47.39 ± 0.46
Phenylalanine	45.54 ± 0.65	296.75 ± 1.81	33.43 ± 0.65	38.4 ± 0.42
α-Aminoadipidic acid	n.d.	n.d.	1.05 ± 0.05	n.d.
Glutamine	120.63 ± 1.76	151.81 ± 3.58	103.23 ± 3.87	173.81 ± 3.27
Ornithine	18.87 ± 1.25	51.45 ± 2.86	11.67 ± 0.15	32.83 ± 2.25
Lysine	50.85 ± 1.07	67.61 ± 1.14	118.23 ± 1.08	70.65 ± 0.77
Histidine	31.43 ± 1.15	32.05 ± 1.95	27.67 ± 0.58	39.8 ± 0.43
Tyrosine	25.02 ± 0.56	34.97 ± 0.30	19.46 ± 0.25	277.05 ± 1.83
Proline-hydroxyproline	66.07 ± 5.68	53.23 ± 21.84	53.99 ± 21.27	50.26 ± 17.09
Tryptophan	13.92 ± 0.23	23.55 ± 0.09	15.21 ± 0.02	18.06 ± 0.07

Each sample was measured in triplicate.
n.d.: not detected above the LLOQ.

Table 8
Urinary amino acid concentrations (μM) for patients with inborn errors of metabolism

Amino acid	Argininosuccinic aciduria	Propionic acidemia	Maple syrup urine disease	Aminoaciduria
Alanine	123.87 ± 1.64	180.57 ± 1.33	129.34 ± 1.70	2424.03 ± 38.64
Sarcosine	n.d.	1.06 ± 0.18	2.06 ± 0.07	40.02 ± 1.51
Glycine	489.81 ± 1.67	5524.1 ± 188.5	2034.0 ± 18.3	27090 ± 1259.7
α-Aminobutyric acid	6.58 ± 0.18	4.73 ± 0.18	4.35 ± 0.14	5.3 ± 0.11
Valine	32.27 ± 0.69	16.64 ± 0.21	194.71 ± 1.17	129.38 ± 1.92
β-Aminoisobutyric acid	n.d.	25.35 ± 1.04	3.9 ± 0.02	262.69 ± 6.43
Leucine	19.55 ± 0.15	23.03 ± 0.06	305.33 ± 2.26	80.88 ± 1.16
allo-Isoleucine	n.d.	n.d.	56.12 ± 0.37	n.d.
Isoleucine	6.31 ± 0.04	9.89 ± 0.16	134.8 ± 0.61	8.67 ± 0.22
Threonine	68.13 ± 3.27	57.41 ± 4.43	178.41 ± 10.72	2398.44 ± 20.06
Serine	202.89 ± 4.52	294.62 ± 13.09	903.29 ± 7.70	1885.23 ± 40.18
Proline	13.38 ± 0.18	11.09 ± 0.83	23.63 ± 0.08	4909.14 ± 96.19
Asparagine	51.77 ± 0.74	86.95 ± 0.75	82.71 ± 1.54	1236.85 ± 13.46
Thiaproline	1.43 ± 0.21	2.38 ± 0.18	1.46 ± 0.1	4.13 ± 0.2
Aspartic acid	19.13 ± 0.32	11.77 ± 0.73	12.94 ± 0.55	12.87 ± 0.14
Methionine	54.57 ± 1.04	11.31 ± 0.22	21.68 ± 0.71	33.87 ± 1.14
Hydroxyproline	n.d.	n.d.	41.29 ± 1.82	1083.45 ± 52.37
Glutamic acid	11.99 ± 0.34	18.39 ± 1.31	60.49 ± 0.36	175.23 ± 8.95
Phenylalanine	46.44 ± 0.71	48.93 ± 0.66	87.4 ± 1.87	596.53 ± 10.76
α-Aminoadipic acid	26.68 ± 1.08	29.71 ± 0.45	136.79 ± 1.22	6.65 ± 0.2
Glutamine	556.96 ± 19.8	220.95 ± 6.94	447.29 ± 25.19	2899.77 ± 73.38
Ornithine	11.1 ± 0.72	8.75 ± 0.20	15.43 ± 0.51	338.05 ± 8.88
Glycyl-proline	24.93 ± 1.86	8.24 ± 1.49	119.95 ± 5.08	n.d.
Lysine	46.78 ± 0.31	49.85 ± 0.87	173.27 ± 1.53	3565.4 ± 60.90
Histidine	172.27 ± 0.99	182.45 ± 1.26	1416.05 ± 65.85	1806.71 ± 15.21
Tyrosine	30.99 ± 1.09	141.63 ± 5.22	125.92 ± 3.95	754.26 ± 21.63
Proline-hydroxyproline	135.29 ± 9.95	167.34 ± 36.54	742.37 ± 70.66	87.01 ± 8.72
Tryptophan	22.63 ± 0.53	42.79 ± 0.41	72.45 ± 0.35	86.45 ± 1.25
Cystathionine	34.6 ± 1.85	n.d.	5.25 ± 0.18	18.33 ± 1.40
Cystine	44.73 ± 6.26	51.18 ± 0.93	84.28 ± 3.59	286.35 ± 22.9

Each sample was measured in triplicate.
n.d.: not detected above the LLOQ.

ammonia to accumulate in the blood. It is caused by a deficiency of argininosuccinate lyase [36,37]. There were no characteristic concentration changes for any of the amino acids quantified by GC–MS in the argininosuccinic aciduria-positive urine. Aminoaciduria is a condition that can occur in several disorders, like Hartnup disease, Dent's disease and Fanconi syndrome. The aminoaciduria is generally characterized by high urinary amino acid excretion [38]. Levels of almost all amino acids were increased except for α-aminobutyric acid, isoleucine, aspartic acid, and methionine. Interestingly, the concentration for α-aminoadipic acid decreased by a factor of 4 in comparison to the levels detected in the urine of patients with argininosuccinic aciduria or propionic acidemia.

3.9. Method limitations

Arginine is an important amino acid that cannot be analyzed by GC–MS following alkyl chloroformate derivatization [25]. This is due to the thermal instability of the derivative that carries a free guanidine group. We could not confirm the report by Namera et al. [39] that threonine, serine, asparagine and glutamine cannot be derivatized and analyzed by GC–MS successfully. However, we did observe the complete, respectively, partial elimination of water during the derivatization of asparagine and glutamine. As a result, the corresponding derivatives contain a nitrile function instead of the amide group as confirmed by NMR (see Section 2.6).

The quantitation of asparagine and glutamine proved robust in our hands, while this was not consistently the case for serine and threonine. Standards of serine and threonine could be detected readily after proper conditioning of the SILTEC liner as described above. For human adult urine samples, however, a rapid deterioration of the liner performance was observed, which resulted in increasingly broader peaks over very few injections. Reconditioning of the liner did not alleviate this problem. Interestingly, we were able to detect the 3-fold derivatized serine and threonine with the hydroxyl group being also acylated. In both cases the 3-fold derivatized product is the minor product. In comparison to the major derivative the 3-fold derivative was observed in the urine samples in low quantity. This observation led to the assumption that the free hydroxyl group and the biological matrix can interact with the liner.

4. Conclusions

A robust and accurate GC–MS method was developed for the automated quantitative analysis of amino acids as their propyl chloroformate derivatives in various biological matrices. At present, 30 amino acids and dipeptides can be reliably quantified by using 19 stable-isotope labeled amino acids as internal standards.

The advantage of the method in comparison to the commercial kits is the complete automation and a more robust quantification. By omitting manual sample preparation steps, the sample throughput is increased, which is of high importance in metabolomics studies. Moreover, analyte losses are minimized and the amount of sample required per analysis is reduced. The introduction of stable-isotope labeled amino acids as internal standards immensely improved the method reproducibility over using only norvaline as internal standard, which allows the accurate and robust quantification of amino acids in large sample batches.

Limitations of the method are firstly that serine and threonine, depending on the biological matrix, may not always be measured reliably and, secondly, that certain amino acids such as arginine, cysteine, citrulline, taurine, and the 1- and 3-methylhistidines are not amenable to GC–MS analysis due to their thermal instability. The latter amino acids may be readily measured by LC–MS. However, for LC–MS the organic phase containing the propylformate derivatives needs to be evaporated prior to analysis, thereby imped-

ing complete automation of the method as demonstrated here for GC–MS.

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