Amino acid analysis in biological fluids by GC-MS

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1 Table of Contents

2 Abbreviations and Acronyms

AAA	Amino acid analysis
AED	Atomic emission detector
AQC	6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate
BSTFA	N,O-bis-(trimethylsilyl)-trifluoroacetamide
CE	Collision energy
CE	Capillary electrophoresis
CoA	Coenzyme A
CUR	Curtain gas
CXP	Collision cell exit potential
đ	Mean difference
DABS-Cl	Dimethylamino-azobenzenesulfonyl chloride
DC	Direct current
DP	Declustering potential
FID	Flame ionization detector
ECD	Electron capture detector
EI	Electron impact ionization
EIC	Extracted ion chromatogram
ELCD	Electrolytic hall conductivity detector
EOF	Electroosmotic flow
EP	Entrance potential
ESI	Electrospray ionization
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FPD	Flame photometric detector
FMOC-Cl	9-fluorenylmethylchloroformate
GC	Gas chromatography
HFB	2,2,3,3,4,4,4-heptafluorobutanol
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High-performance liquid chromatography

HSQC	Heteronuclear single-quantum correlation
INTERMAP	INTERnational collaborative of Macronutrients and blood Pressure
IP	Ion pair
IS	Internal standard
IT	Ion trap
LC	Liquid chromatography
LLOQ	Lower limit of quantification
LOD	Limit of detection
LOQ	Limit of quantification
MCF	Methyl chloroformate
MRM	Multiple reaction monitoring
MS	Mass spectrometry / mass spectrometer
MS/MS	Tandem mass spectrometry
MPS	Multipurpose Sampler
MSTFA	*N*-methyl-trimethylsilyltrifluoroacetamide
MSUD	Maple syrup urine disease
MT	Migration time
NEFA	Non-esterified fatty acid
NMR	Nuclear magnetic resonance
NPD	Nitrogen phosphorus detector
NPD-F	7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole
OPA	*o*-phthalaldehyde
PCF	Propyl chloroformate
PID	Photoionisation detector
PITC	Phenylisothiocyanate
PKU	Phenylketonuria
PTV	Programmed-temperature vaporization
QC	Quality control
QTRAP	Triple quadrupole – linear ion trap hybrid mass spectrometer
R	Correlation coefficient

RF	Radio-frequency
RP	Reversed phase
RSD	Relative standard deviation
RSQ	Square of the correlation coefficient R
RT	Retention time
SD	Standard deviation
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Single reaction monitoring
TCD	Thermal conductivity detector
TE	Technical error
TEM	Auxilary gas temperature
TLC	Thin layer chromatography
TOF	Time-of-flight
TQ	Triple quadrupole
ULOQ	Upper limit of quantification
UPLC	Ultra-performance liquid chromatography
UV	Ultraviolet

The abbreviation for the amino acids are listed in chapter11, Table 11.

3 Motivation

Amino acids are important targets for metabolic profiling and their quantitative analysis is essential in many areas including clinical diagnostics of inborn errors of metabolism, biomedical research, bio-engineering and food sciences. [1, 2] There is an increasing need for fast and robust methods for the quantitative analysis of amino acids in large clinical and epidemiological studies.[3] The prevailing method for amino acid analysis has been cation exchange chromatography followed by post-column derivatization with ninhydrin and UV detection. But due to the low throughput and the low specificity of detection it is not suitable for the analysis of large sample batches of complex biological fluids such as urine and blood serum. There are several other methodologies available to analyze amino acids, which are based on chromatography, capillary electrophoresis, direct infusion coupled to different mass analyzers, as well as nuclear magnetic resonance (NMR). Protein precipitation is required for all LC and CE methods independent of the detection method used, which renders complete automation difficult. Shortcomings of NMR are relatively high limits of detection and large sample volumes required. Therefore there is still need for a method that allows the completely automated analysis of amino acids in biological fluids that can meet the demand for high sample throughput in large metabolomic studies.

Aim #1: Development of a fully automated method for the direct quantitative analysis of amino acids in various biological matrices

The aim was to develop a robust, accurate, fast and precise method for the analysis of urinary amino acids and its application to urine specimens from the INTERMAP study that examines the correlation between diet and ethnogeographic patterns of blood pressure, where urinary amino acids serve as surrogate markers of dietary protein sources. GC-MS was chosen because of its high separation efficiency and wide dynamic range. In order to obtain volatile analytes usually derivatization of metabolites is performed for GC analysis. GC-

MS based metabolomics studies commonly use silylation, which however causes degradation of some amino acids. The GC-MS method of choice builds on the direct derivatization of amino acids in diluted urine with propyl chloroformate, GC separation and mass spectrometric quantitation of derivatives using stable isotope labeled standards. Since derivatization with propyl chloroformate can be carried out directly in the aqueous biological sample without prior protein precipitation or solid-phase extraction of the amino acids, the entire analytical process, starting from the addition of reagents, over extraction, derivatization to injection into the GC-MS can be automated. Method parameters such as limit of detection (LOD), lower limit of quantification (LLOQ), linear range, reproducibilities and evaluation of matrixe spikes were to be determined to show to the method`s applicability to analyze amino acids in several biological samples. Propyl chlorofromate can react with all compounds containing amino and/or a carboxy function therefore there is space to include other metabolites e.g. fatty acids. The integration of fatty acids was to be determined, additionally.

Specific Aim #2: Urinary Amino Acid Analysis: A Comparison of iTRAQ®-LC-MS/MS, GC-MS and Amino Acid Analyzer

Another goal was the comparison of the performance of classical ion-exchange chromatography with postcolumn ninhydrin detection and the GC-MS method developed under aim #1 and a novel LC- MS/MS method based on the derivatization of amino acids with iTRAQ®. In this process, the performance of the iTRAQ® -LC-MS/MS method was to be evaluated.

Using two blinded sets of urine samples containing replicates and a certified amino acid standard, the precision and accuracy of the GC-MS method could be tested and the results compared with iTRAQ® derivatization LC-MS/MS and postcolumn ninhydrin detection of amino acids. The performance of the three methods was to be compared using various statistics, including technical error of mearuement, regression analysis and Bland-Altman plotting.

Specific Aim #3: Quantitative analysis of amino acids and related compounds by LC-MS/MS

Some important amino acids are thermally instable and cannot be quantified by GC-MS, such as arginine, citruline as well as 1- and 3- methyl histidines. Amino acids are highly polar analytes and, therefore, not suitable for conventional reversed-phase high-performance liquid chromatography (RP-HPLC). Thus, a derivatization is needed. The potential of derivatization with propyl chloroformates, follow by LC-MS/MS analysis for amino acid determination was to be tested and expanded to tryptophan metabolites and polyamines that are of great interest in several biological projects. Due to their amino function they can be derivatized with propyl chloroformate and analyzed by LC-MS/MS. For quantification aims it is important to use internal standards. However, isotope-labeled standards are not available commercially for all metabolites of interest. Instead of synthesizing individual standards for each metabolite, we wanted to exploite the derivatization of amino and carboxy functions with propyl chloroformate employing d_3-labeled propanol as a mean of generating an internal standard for each analyte.

4 Background

An abbreviated version of this chapter was published in Analytical Bioanalytical Chemistry.[4]

4.1 Metabolomics

The complete set of small molecules in an organism is termed metabolome. [5]

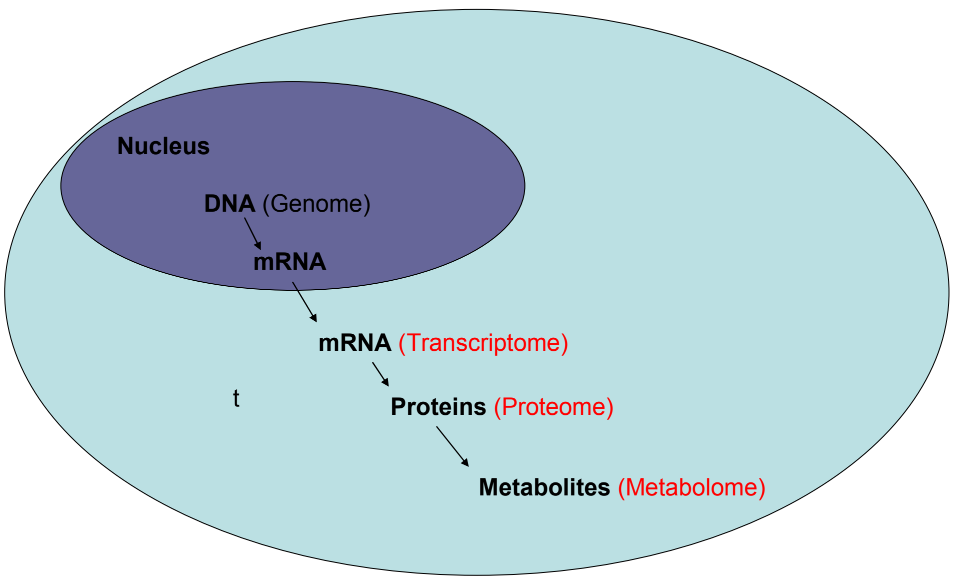


Figure 1: Information flow in a cell.

Metabolomics is the last step in the “omics” cascade (Figure 1). Metabolites are the end products of cellular processes. Therefore, their concentration can be regarded as the response of biological systems to genetic and/or environmental changes. Metabolomics aims at the quantitative analysis of all metabolites in a given biological system.[6] 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. [7]

There are different approaches in the field of metabolomics:

Metabolic profiling is the quantitative analysis of sets of metabolites in a selected biochemical pathway or a specific class of compounds. Important targets for metabolic profiling are e.g. amino acids, intermediates of the central carbon metabolism, nucleotides and polyamines, just to name a few. For this approach, it is necessary to develop accurate and robust methods to quantify those compounds.

Target analysis is more focused than metabolic profiling and only very few analytes are measured. They are often directly related to a genetic perturbation, such as substrates or products of enzymatic reactions, or they serve as biomarkers for a certain disease. [7]

Metabolic fingerprinting aims at the detection of as many analytes as possible. Metabolic fingerprinting is a global screening approach to classify samples based on metabolite patterns or “fingerprints.

Metabolic footprinting uses the same methods as fingerprinting but is limited to the analysis of metabolites in cell culture media. The reasoning is that compounds excreted by a cell or taken up from the medium will also give valuable insights into a cell’s phenotype and physiological state. [8]

4.2 Amino acids

Twenty standard amino acids are used by organisms in protein biosynthesis. The structures of the proteinogenic amino acids are shown in Figure 2.

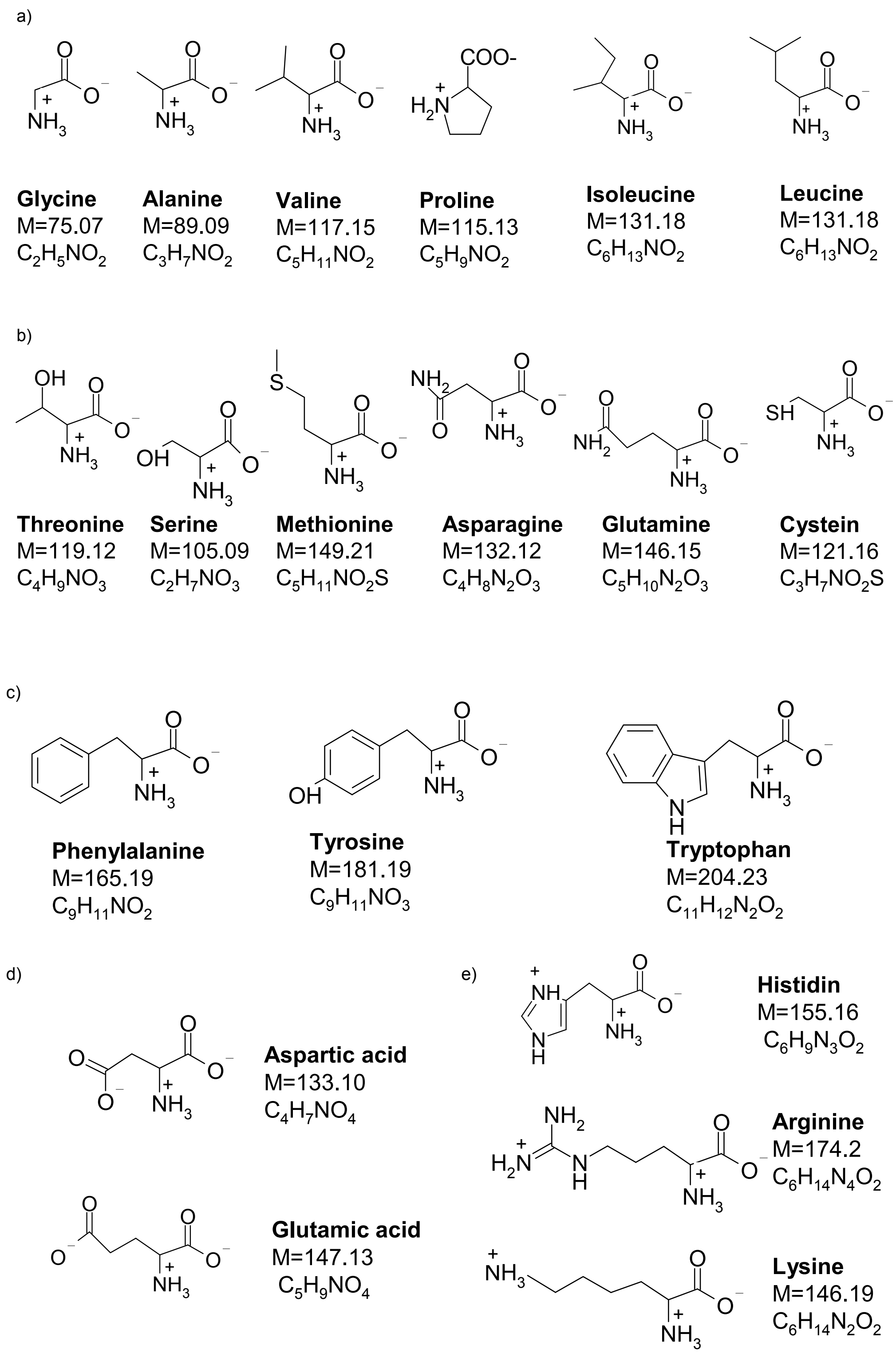


Figure 2: Molecular structure, formula weight and empirical formula for all 20 proteinogenic amino acids. Molecular structures are illustrated as they are at pH of 7; depending on their side chain, they are divided in a) unpolar side chain, b) polar uncharched side chain, c) aromatic side chain, d) negative charged side chain, e) positive charged side chain.

At pH 7 the α-amino group is protonated and the α-carboxy group is deprotonated. The positive and negative charges are equal, resulting in a neutral charge; therefore, they are called zwitterions. Depending on the chemical behavior of the side chains at pH 7 amino acids can be grouped as follows. Neutral amino acids have a non-charged (Figure 2a-c), acidic amino acids a negatively (Figure 2d) and basic amino acids a positively charged side chain (Figure 2e). Neutral amino acids can be subdived into neutral amino acids with unpolar side chain (Figure 2a), polar side chain (Figure 2b) and aromatic side chain (Figure 2c). Mammals including humans, can synthesize only 11 of the proteinogenic amino acids: tyrosine, aspartic acid, asparagine, alanine, serine, glycine, cysteine, glutamic acid, glutamine, proline and arginine. They are known as the non-essential amino acids. Tyrosine for example can be synthesized out of phenylalanine catalyzed by the enzyme phenylalanine monooxygenase (Figure 3)

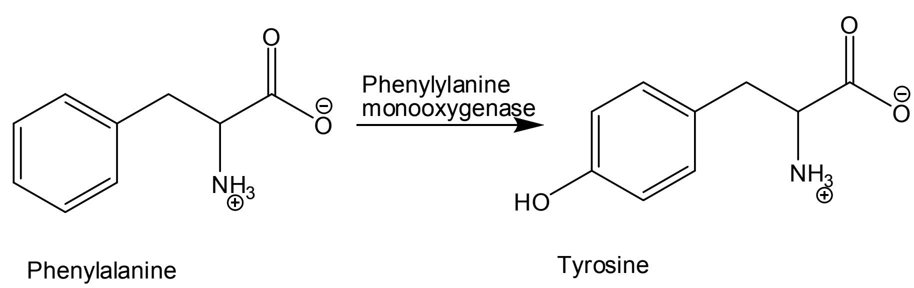


Figure 3: Biosynthesis of the non-essential amino acid tyrosine. PKU patients have a deficiency in the enzyme phenylalanine hydroxylase (PAH), also named Phenylalanine monooxygenase.

If the enzyme or its cofactors are defect, phenylylalanine is accumulating.[9] Phenylalanine accumulates and is converted into phenylketones, which can be detected in the urine and cause problems with brain development, leading to progressive mental retardation and seizures. This disease is called Phenylketonuria (PKU).[2, 9] Aminotransferase enzymes can catalyze the reaction from α-keto acids to the corresponding amino acid. Transamination of pyruvate, oxaloacetate, and α-ketoglutarate, yields alanine, aspartic acid, and glutamic acid, respectively. From glutamic acid the amino acids glutamine, proline and arginine can be formed and asparagine can be synthesized out of aspartic acid. Serine, glycine and cysteine are made from the intermediate 3-phosphoglyceric acid, formed by glycosis.[10] The other nine amino acids-phenylalanine, threonine, methionine, lysine, tryptophan, leucine, isoleucine, valine and histidine cannot be synthesized in mammals and must be provided in the diet. They are called essential amino acids.

4.3 Gas chromatography (GC)

4.3.1 Principles of GC

Gas chromatography is a separation technique that employs a gas as mobile phase and either a solid (gas solid chromatography) or a liquid (gas liquid chromatography) as stationary phase. Nowadays, most GC applications use capillary columns, with the stationary phase coated on the inner wall of the capillary. In case of a solid stationary phase these are called PLOT (porous layer open tubular) columns and if a liquid stationary phase is used they are called WCOT (wall coated open tubular) columns. This type of separation is suited for compounds, which can be vaporized wihout decomposition. The retention time of the analytes depends on the type of analyte and the interaction with the stationary phase. This is expressed by the partioning coefficient K, which is temperature dependend ($\ln K \sim 1/T$) and, therefore, the retention time can be controlled by column temperature. The temperature is either kept constant (isothermal) for analytes in a narrow boiling point range or is ramped for analytes in a wide boiling point range. The carrier gas that transports the sample through the column. Typical carrier gases are helium, argon, nitrogen or hydrogen.

For the quantitative analysis it is very important to have baseline resolved peaks. Chromatographic resolution is calculated as follows:

$$R_S = \frac{t_{R2} - t_{R1}}{(w_{b1} + w_{b2})/2} \quad \textbf{(1)}$$

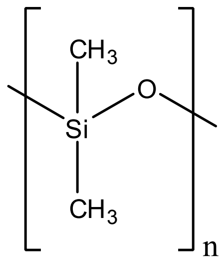
Where R_S is the resolution, t_{R1} and t_{R2} are the respective retention times of peak 1 and 2, and w_{b1} and w_{b2} are the respective base peak witdths of peak 1 and 2. For quantitative analysis the value for R_S should be higher then 1.5.[11]

4.3.2 Injector types

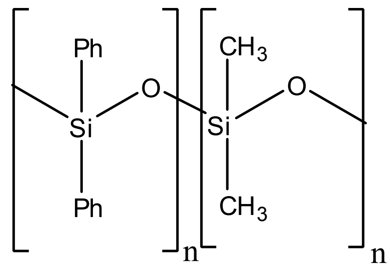
The sample is transfered onto the column by means of the injector. Commonly employed injectors are hot split/splitless and programmed-temperature vaporization (PTV) injection. Split and splitless injection are both performed using the same inlet, which is often termed a split/splitless inlet. For both applications the sample is introduced into a heated small chamber via a syringe through a septum. Split injection is used for concentrated samples, where only a small portion of the sample is transfered on the column and the major part is emerged through the split outlet. The amount of sample is controlled by the splt ratio. The whole sample amount is introduced onto the column using splitless injection. A programmed-temperature vaporization (PTV) inlet is a hybrid of the techniques described above. It is a split/splitless inlet that has been modified to allow cold injection and rapid temperature programming. This is a rather gentle injection technique, which is favorable for thermally labile compounds. A critical component of the injector is the liner. It is the chamber into which the sample is injected. The sample is vaporized and throughly mixed with the carrier gas. The liner shape must ensure complete sample vaporization, provide sufficient volume to accommodate the resulting vapor and must be inert to avoid analyte adsorption. Glass liners are used commonly and exist in wide range, differing in volume, special form or design, fillings (e.g. quarz or glas wool packed) or treatment for deactivation of the surface.

4.3.3 Gas chromatographic columns and stationary phases

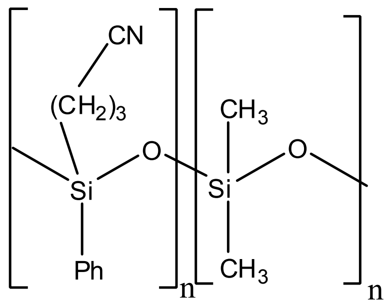
There are two main groups of columns, namely packed columns and capillary open tubular columns. For most applications capillary columns are used. Capillary columns are made of fused-silica with a polyimide outer coating and the stationary phase coated onto the inner surface. Presently, fused-silica capillary columns having a length of 10–100 m and an inner diameter of 0.10–0.53 mm are in widespread use. The most common stationary phases in gas-chromatography columns are polysiloxanes, which contain various substituent groups to change the polarity of the phase. The commercial nonpolar end of the spectrum is polydimethyl siloxane, which can be made more polar by increasing the percentage of phenyl- and/or cyanopropyl groups on the polymer. Wide spread stationary phases in metabolomics are 100% polydimethyl siloxane, 5% polydiphenyl- 95%- polydimethyl siloxane or with 14% polycyanopropylphenyl-86%- polydimethyl siloxane. For very polar analytes, polyethylene glycol (carbowax) is commonly used as stationary phase. The chemical structures of the four mentioned stationary phases are shown in Figure 4.



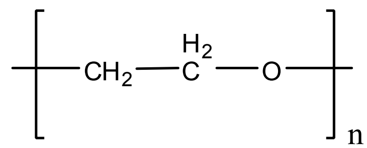
100% Polydimethyl siloxane



5% Polydiphenyl - 95% Dimethyl polysiloxane



6% Polycyanopropylphenyl - 94% polydimethyl siloxane



Polyethylene glycol

Figure 4: Chemical structur of popular stationary phases in GC.

The stationary phase can varry in the film thickness from 0.1 to 5 µm. The thickness of the film has an effect on the retentive character of the column. Thick films are used for the analysis of highly volatile analytes and thin films are used for the analysis of less volatile compounds.[11]

4.3.4 Detectors

A large number of GC detectors are available. The most popular detector is the flame ionizations detector (FID). A hydrogen/air flame is used to decompose the carbon containing analytes from the GC into ions by burning them and the changes in the current are measured afterwards. The FID detects most organic compounds when they are ionized and cause a voltage drop across the collector electrodes. The measured change is proportional to mass, and therefore number of carbon atoms, of the organic compound. One important benefit of a FID is that it is insensitive to H_2O, CO_2, CS_2, SO_2, CO, NO_x, and noble gases because they are not able to be oxidized/ionized by the flame. There are still a lot of applications using flame ionization detector (FID) as detector. However it is a non-specific detector and coeluting compounds cannot be separated.

Some gas chromatographs are connected to a mass spectrometer (MS) which acts as the detector. Suitable mass analyzers for GC are quadrupole/triple quadrupole, ion trap (IT), time of flight (TOF). The most common type of MS coupled to a GC is the quadrupole mass spectrometer. A quadrupole ion filter consists in four parallel rods. The rods have fixed DC (direct current) and alternating RF (radio-frequency) voltages applied to them. Depending on the electric field, only ions of a particular m/z will be allowed to pass, all the other ions will be deflected into the rods. Quadrupole ion filters are used in routine analysis due to their good reproducibility and excellent stability.

In conventional GC-MS electron impact (EI) ionization technique is employed. EI is an ionization method whereby energetic electrons interact with gas phase atoms or molecules to produce ions. This is a hard ionization technique and therefore the molecular ions break up into smaller fragments. The resulting mass spectrum is complex and provides important information about the structure of the molecule. Another possibilty to ionize compounds is the chemical ionization that begins with the ionization of methane, creating radicals which in turn impact the samples molecules rendering them postively charged as $[MH]^+$ molecular ions.

Other detectors for GC include nitrogen phosphorus detector (NPD), electron capture detector (ECD), photoionisation detector (PID), flame photometric detector (FPD), thermal conductivity detector (TCD), and atomic emission detector (AED).

4.3.5 Sample preparation

Sample preparation in biomedical analysis is mainly performed by liquid-liquid extraction and solid-phase extraction (SPE). In liquid-liquid extraction, dissolved components are transferred form one liquid phase to another. The most common application is the transfer of analytes from aqueous solution to an organic solvent that is more suitable for GC. [11] With the SPE technique the analytes are trapped on solid sorbent for concentration or cleanup. Another technique is the headspace technique used for the determination of cancerogen or toxic substances in urine or blood.[12, 13] It is most suited for the analysis of the highly volatile analytes in samples that can be efficiently partitioned into the headspace gas volume from the liquid or solid matrix sample. Higher boiling compounds and semi-volatiles are not detectable with this technique due to their low partition in the gas headspace volume. However, many biological analytes including amino acids have to be derivatized prior to GC to render them volatile. Several derivatization methods are available to obtain volatile derivatives suitable for GC. Derivatisations for GC analysis will be discussed in chapter 4.4.6.

4.4 Amino acid analysis for metabolomics

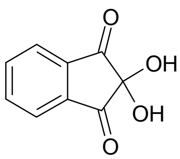
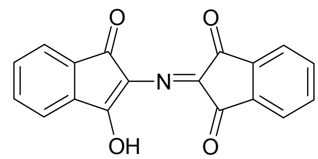
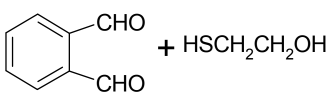
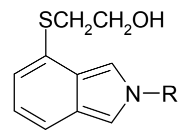
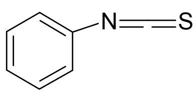
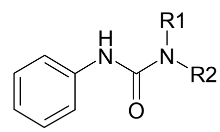
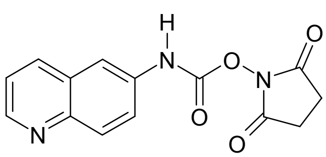
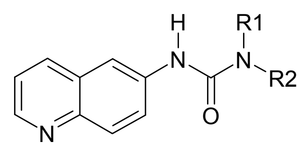
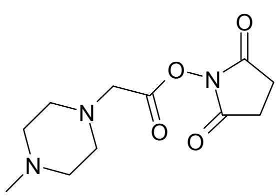
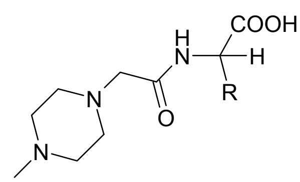
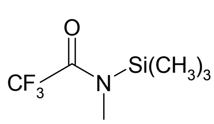
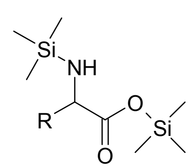
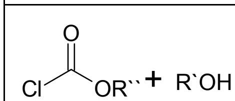
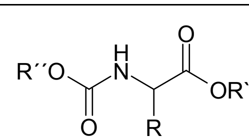
Amino acids are important targets for metabolic profiling. Besides being the basic structural units of proteins, amino acids have several non-protein functions. They are a source of energy either through formation of keto acids from the ketogenic amino acids or through gluconeogenesis from glucogenic amino acids. Glutamic acid and γ-aminobutyric acid are neurotransmitters,[14] while tryptophan and tyrosine are precursors of serotonin and catecholamines, respectively.[15] Glycine is a precursor of porphyrins, whereas ornithine is a precursor of polyamines[16] and arginine can be metabolized to form nitric oxide.[17] Elevated amino acid levels in blood plasma and urine are well-known markers for inborn errors of metabolism, such as phenylalanine in phenylketonuria or maple syrup urine disease.[1, 2] Amino acids also serve as markers for nutritional influences, e.g., urinary taurine levels serve as an indicator for fish intake,[18] while the 1-methylhistidine level in urine correlates with meat protein intake.[19]

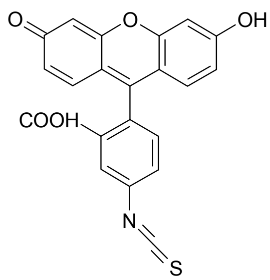
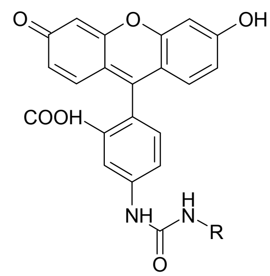
Due to the important biological functions of amino acids, their quantitative analysis is required in several fields, including clinical diagnostics of inborn errors of metabolism, biomedical research, bio-engineering and food sciences. Consequently, different analytical methods have been developed and commercialized over the past few decades for amino acid analysis. Nevertheless, efforts to improve existing methodology with regards to speed of analysis, robustness, reproducibility and sensitivity are ongoing and have been driven by a shift in application away from the analysis of protein hydrolysates to the analysis of free amino acids in various biological matrices. To that end, mass spectrometry coupled to chromatography has become a major means of amino acid analysis because of its selectivity and sensitivity.

4.4.1 Sample preparation

Analysis of free amino acids in biological samples often requires protein precipitation prior to analysis. Methods described for deproteinization include precipitation with acid or organic solvent, and ultrafiltration. The most common method to remove proteins is precipitation with sulphosalicylic acid.[20] Amino acids are highly polar analytes and, therefore, not suitable for conventional reversed-phase high-performance liquid chromatographic (RP-HPLC)[21] or gas chromatographic (GC) analysis. Capillary electrophoresis (CE) does not require derivatization, but sensitivity for CE-UV analysis can be increased by introduction of a UV active label. Therefore, a derivatization step is often employed. Most reagents used react with the amino group. Some derivatizing reagents react only with primary amines, but ideally secondary amines, such as proline and hydroxyproline, are also covered. Another option is to derivatize the carboxy function of the amino acids. The most common derivatization reagents are listed in Table 1 and their use will be discussed in the following chapters.

Table 1: Chemical structures of derivatization reagents and derivates or detected compounds.

Structure of the reagent	Structure of derivate or detected compound
 Ninhydrin	 Ruhemann`s Purple
 OPA (o-phthalaldehyde)	
 PITC=Phenyliso-thiocyanate; Waters: Pico•Tag	
 AQC (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; Waters: AccQ•Tag™)	
 iTRAQ™ (2,5-dioxopyrrolidin-1-yl-2-(4-methylpiperazin-1-yl)acetate)	
 MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoroacetamide)	
 Alkyl chloroformate	

 FITC (fluorescein isothiocyanate)	

4.4.2 Liquid chromatographic methods coupled with optical detection

There are several LC methods coupled with UV absorbance detection available for the quantification of amino acids. The two general approaches are either ion-exchange chromatography followed by post-column derivatization or pre-column derivatization preceding Reversed-phase (RP) HPLC. The gold standard method is cation-exchange chromatography using a lithium buffer system followed by post-column derivatization with ninhydrin and UV detection. The separation of the amino acids is achieved through changes in the pH and cationic strength of the mobile phase. Through the reaction of ninhydrin with amino acids containing a primary amine Ruhemann's purple (Figure 5) is generated, which is UV active (λ_{max} 570 nm). Secondary amines, such as proline, produce a yellow product (λ_{max} 440 nm).

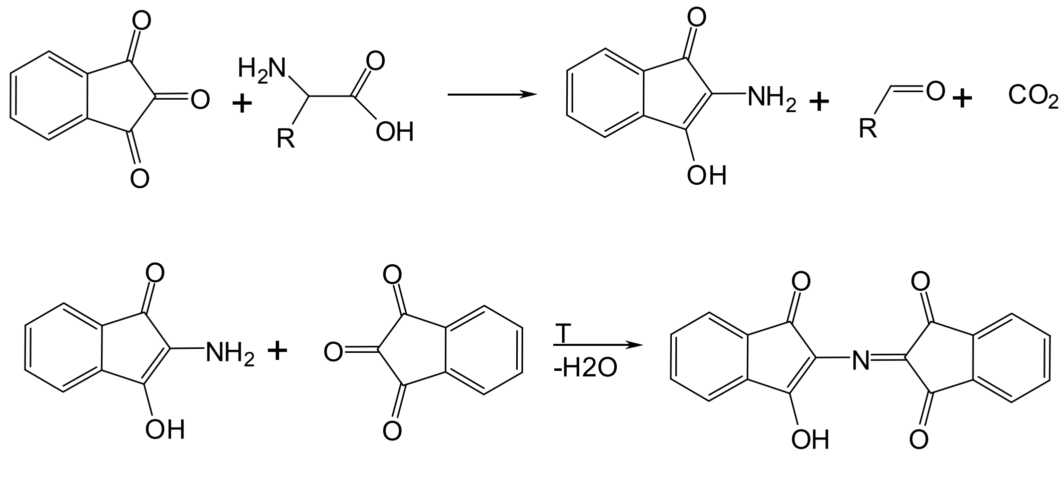


Figure 5: Reaction of amino acids with ninhydrin to Ruhemann`s Purple.

The eluate is monitored at 440 and 570 nm, respectively. Linearity ranges typically from 5 - 2500 µmol/L. Routinely, 38 amino acids are separated with a conventional amino acid analyzer in 115 min, but the method can be expanded to more than 140 min to resolve more analytes. A typical elution profile of urinary amino acids monitored at both 440 nm and 570 nm is shown in Figure 6.

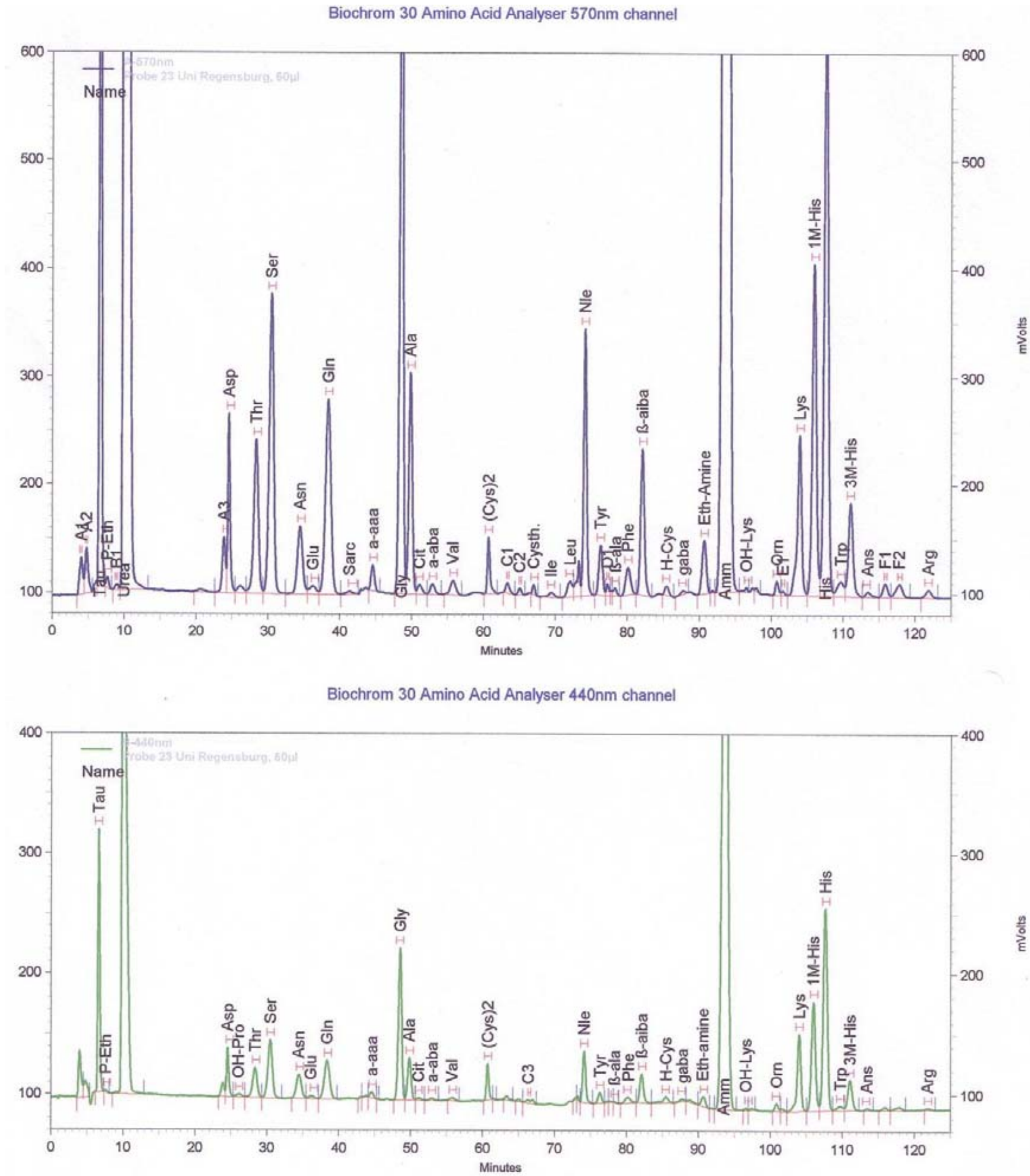


Figure 6: Typical elution profiles of urinary amino acids obtained on a Biochrom 30 amino acid analyzer with continuous UV absorbance monitoring at 440 and 570 nm, respectively.

Shortcomings of the method are the long runtime, the instability of ninhydrin, the necessity of protein precipitation, which impedes complete automation, and crosstalk by analytes other than amino acids and related compounds that may react with ninhydrin in complex biological samples and prevent accurate quantitation.[22] For example, methionine (Met) and homocitrulline (Hcit), phenylalanine (Phe) and aminoglycoside antibiotics, as well as histidine (His) and the anticonvulsant gabapentin, commonly have overlapping retention times.

Derivatization with o-phthalaldehyde[23] (OPA) has been used both post-column after cation-exchange chromatography and pre-column coupled with RP-HPLC. OPA reacts with amino compounds in the presence of a thiol such as mercaptoethanol to form a fluorescent derivative. RP-HPLC provides good selectivity for separating the OPA derivatives. The OPA derivatives of amino acids can be detected by UV absorbance at 340 nm, fluorimetry at excitation and emission wavelengths of 340 nm and 450 nm, respectively, amperometry for those OPA-derivatives that show little or no fluorescent activity, or a combination of the aforementioned detection methods. Alternative reagents for precolumn derivatization of free amino groups are phenylisothiocyanate (PITC), dimethylamino-azobenzenesulfonyl chloride (DABS-Cl), 9-fluorenylmethylchloro-formate (FMOC-Cl) and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F).[24] The derivatization time can vary between 1 min for OPA and 20 min for PITC. Depending on the number of the analytes, chromatographic run time varies between 13 min for 23 compounds and 95 min for 38 compounds.[24]

Based on the coupling reaction of the well-known Edman degradation, the reaction of phenylisothiocyanate (PITC) with both primary and secondary amino acids produces phenylthiocarbamyl derivatives, which are also separated by RP-HPLC and detected at 254 nm. This reaction served as the basis for the PICO•Tag method commercialized by Waters Inc. (Milford, MA, USA). More recently, Waters Inc. introduced a new kit (AccQ•Tag) based on the precolumn derivation of amino acids with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). AQC converts both primary and secondary amino acids into exceptionally stable, fluorescent derivatives that are amenable to UV-absorbance, fluorescence, electrochemical, and MS detection.[25]

To achieve faster analysis and improved resolution, the AccQ•Tag Ultra UPLC method has been introduced that employs columns packed with uniform 1.7-µm particles. The UPLC method is 3-5 times faster than conventional HPLC analysis, and baseline separation of all proteinogenic amino acids is achieved in less than 10 min. Boogers et al.[26] published a comparison between Pico•Tag HPLC and AccQ•Tag ultra UPLC analysis. They separated 16 amino acids in 23 min and 8 min, respectively, using the Pico•Tag HPLC and the AccQ•Tagultra UPLC method. For the Pico•Tag HPLC method lower limits of quantification (LLOQ) were in the range of 4.3-8.4 μM and for the AccQ•Tag ultra UPLC method in the range of 1.3-5.3 μM.

A drawback of the aforementioned methods is the lack of analyte specificity of optical detection. Therefore, uncertainties arise in the analysis of complex biological samples that may contain other non-protein amino acids or compounds with an amino function that display similar or identical retention behavior. This may be avoided by the use of mass spectrometry that allows the identification of co-eluting compounds unless they are isobaric and/or display identical fragmentation patterns. Optical detection systems are also not suited to distinguish between isotopes and, therefore, cannot be used for flux analysis in organisms fed with stable isotope labeled substrates. The major advantages of LC coupled to optical detection are good reproducibility, the comparatively inexpensive equipment and the high sensitivity in the low pmol range.

4.4.3 Ion pair reversed-phase liquid chromatography – tandem mass spectrometry (IP-LC-MS/MS)

It is feasible to analyze underivatized amino acids by ion pair IP-LC-MS/MS. Elimination of derivatization reduces sample preparation and minimizes the errors introduced by reagent and derivative instability, side reactions, and reagent interferences. Usually, charged hydrophobic species are used as IP reagents in combination with RP-C18-HPLC columns. There are two mechanisms discussed as basis for IP separation. The IP-reagent can be adsorbed at the interface between the stationary and mobile phase, creating a charged surface with the inorganic counterions forming a corresponding diffuse layer. Hence, the IP-reagent creates an electrostatic surface potential, and the magnitude of this potential is primarily determined by the surface concentration of the IP-reagent. Another hypothesis is the generation of uncharged complexes between the IP-reagent and the analyte that are less polar and will thus be retained on a C18 column.[27] The use of volatile IP reagents, such as perfluorocarboxylic acids, allows the hyphenation of LC to electrospray ionization mass spectrometry (ESI-MS). Piraud et al.[28] utilized HPLC separation on a C18 column with tridecafluoroheptanoic acid (TDFHA) as IP reagent coupled to tandem mass spectrometry for amino acid analysis. To quantify the amino acids, multiple reaction monitoring (MRM) was used. A total of 76 amino acids were quantified in less than 20 min and the quantification of 16 amino acids was validated using their stable isotope-labeled analogs as internal standards.

De Person et al.[29] studied the effect of five perfluorinated carboxylic acids (C3-TFA, C4-HFBA, C5-NFPA, C7-TDFHA and C8-PDFOA) on MS response. Signal intensity depended on type and concentration of IP reagent, as well as MS interface geometry. Limits of detection ranged 0.0003 – 9 µM depending on amino acid, type of mass spectrometer and IP reagent. Armstrong et al. [21] coupled IP-RP-HPLC using TDFHA as IP reagent to time-of-flight mass spectrometry. Twenty-five amino acids were quantified in human plasma and the calibration curves were linear over a range of 1.56 to 400 µM.

4.4.4 HILIC (Hydrophilic Interaction LIquid Chromatography)

Another approach to separate polar compounds is hydrophilic interaction liquid chromatography. Separation is achieved using a polar stationary phase, such as bare silica, amide-, hydroxyl-, cyano-, amino-, and ion-exchange columns, in combination with RP-type solvent systems. Gradient elution is started with a high percentage of organic solvent, typically acetonitrile, and the retained compounds are eluted by increasing the water-content in the mobile phase. Langrock *et al.*[30] demonstrated the separation of 16 proteinogenic amino acids in 25 min using an amide-column coupled to ESI-MS/MS. Detection was carried out using a neutral loss scan of formic acid. In a neutral-loss scan, all precursors that undergo loss of a specified common neutral, formic acd in this case, are monitored. Further, separation of all hydroxyproline isomers (trans-4-Hyp, trans-3-Hyp, and cis-4-Hyp) present in collagen hydrolysates was achieved. Detection limits were below 50 pmol for the Hyp-isomers

4.4.5 Capillary electrophoresis mass spectrometry (CE-MS)

Amino acids are chargeable analytes and, therefore, amenable to capillary electrophoresis (CE) separation without prior derivatization. However, if optical detection is employed, derivatization is needed to improve sensitivity. Labeling can be carried out with FMOC, NDA, OPA, or FITC.[31] Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) was used to analyze free amino acids in cerebrospinal fluid.[32] The amino acids were derivatized with FITC prior to analysis and the separation was completed within 22 min. Detection limits were in the low nanomolar range. Light-emitting diodes (LED) are replacing conventional gas lasers for CE-LIF. LEDs are very stable and provide high intensity at low cost. [33] Soga *et al.*[34] analyzed urinary amino acids without derivatization by bare fused-silica capillary electrophoresis-electrospray ionization-triple-quadrupole mass spectrometry. The method was validated for 32 amino acids with LODs between 0.1 and 14 µM and a linear dynamic range of approximately 10 – 200 µM. The relatively high LODs are due to the low injection volumes applied in CE.

4.4.6 Gas chromatography for amino acid analysis

The derivatization procedure most commonly employed in GC-MS is silylation, which replaces acetic hydrogen in functional groups by an alkylsilyl group, primarily trimethylsilyl, using reagents such as *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) or *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA). A reaction scheme for the derivatization with MSTFA is shown in Figure 7. GC analysis of silylated amino acids is feasible, but not all derivatives are stable; for example, arginine decomposes to ornithine, and glutamic acid rearranges to form pyro-glutamic acid. Another drawback is the sensitivity of the reagents and derivatives to moisture.[35]

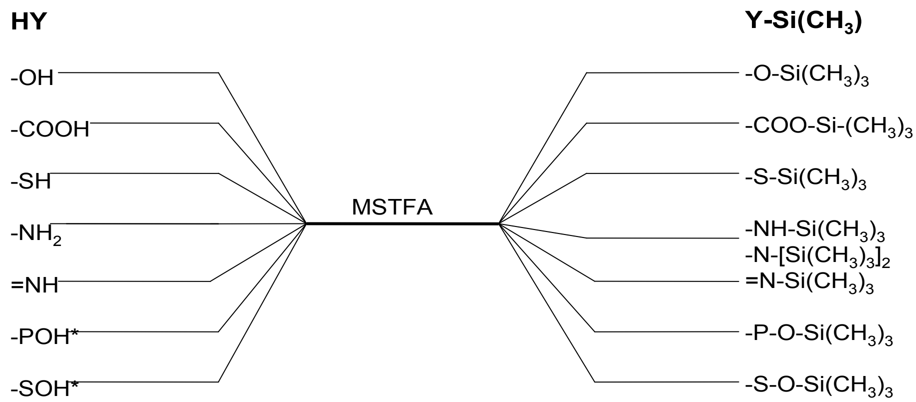


Figure 7: Silylation of functional groups with MSTFA.

Other derivatization procedures for GC analysis include acylation/esterification using various anhydride/alcohol combinations, such as pentafluorpropyl anhydride / isopropanol or trifluoroacetic anhydride / isopropanol.[36] An alternative is the derivatization of amino acids with alkyl chloroformates and alcohol. Carboxylic groups are converted directly to esters and amino groups to carbamates. This reaction can be catalyzed by pyridine or picoline. Using the alkyl chloroformate reaction, amino acids can be derivatized directly in aqueous solution without prior removal of proteins. The amino acids react very quickly, for instance, with propyl chloroformate and the derivates can be extracted with an organic solvent. From the organic phase an aliquot can be injected directly into the GC-MS.[37, 38] Fluorinated alcohols yield even more volatile compounds and have been applied to the separation of amino acid enantiomers.[39] Recently, fluoroalkyl chloroformates were used for the analysis of amino acids on 5% phenylmethylsilicone phase by GC with MS or FID.[40] Linearity was observed in the range of 0.1 - 100 nmol and LODs, defined as amount on column, ranged from 0.03 pmol for proline to 19.38 pmol for glutamic acid. More than 30 amino acids were separated in less than 10 min, including 1- and 3-methylhistidines, which were previously not described as amendable to GC analysis using alkyl chloroformate derivatization.

4.4.7 iTRAQ®-LC-MS/MS

In 2007, Applied Biosystems (Foster City, CA, USA) introduced a kit for the quantification of 42 physiological amino acids and related compounds based on the iTRAQ® chemistry originally developed for the quantification of peptides[41] by LC-MS/MS. Each reagent consists of a reporter group (with the masses m/z 114, 115, 116 and 117), a neutral balance linker (masses 24-32) and an amino reactive group (N-hydroxy-succinimide) (Figure 8).

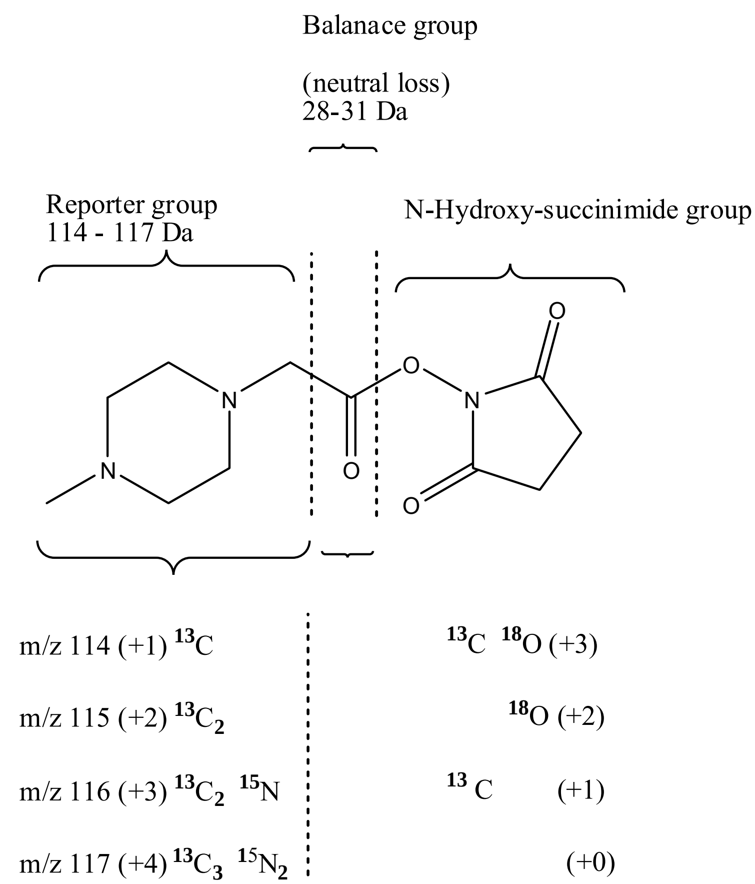


Figure 8: Structure and isotope patterns of iTRAQ® reagents.

The amino acids are derivatized with a reactive ester to introduce an isobaric tag. The N-hydroxy succinimide ester reacts with the amino group to give an amide (Figure 9)

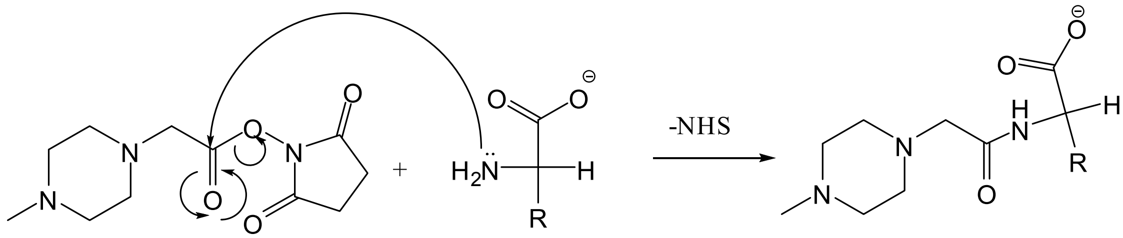


Figure 9: Reaction of iTRAQ® labeling reagent with amino acids.

All derivates of one amino acid are isobaric and cannot be separated by RP-HPLC. The tag contains a cleavable reporter ion, which can be detected upon collision-induced dissociation in MS/MS mode (Figure 10). These reporter ions differ by one mass unit and can be used to quantify multiplexed biological samples. For the analysis of free amino acids, the biological sample is labeled with the tag containing the reporter ion *m/z* 115. Before analysis, the sample is mixed with an amino acid standard solution labeled with the reagent containing the reporter ion m/z 114. Because the two derivatives of one amino acid have the same mass, they elute at the same retention time and experience the same matrix effects during ESI. Consequently, each amino acid is quantified based on the ratio of the *m/z* 115-ion over the *m/z* 114-reporter ion. The main advantage of iTRAQ®-LC-MS/MS is the availability of 42 internal standards for all physiological amino acids and related compounds, such as taurine, ethanolamine or phosphoethanolamine. Disadvantages are the insufficient recovery of amino acids with sulfur containing groups, such methionine and cysteine, and the somewhat imprecise quantification due to the large number of transitions and the resultant insufficient acquisition of data points per peak in a single LC-MS/MS run. The latter may be alleviated by the use of time scheduled multiple reaction monitoring (sMRM).

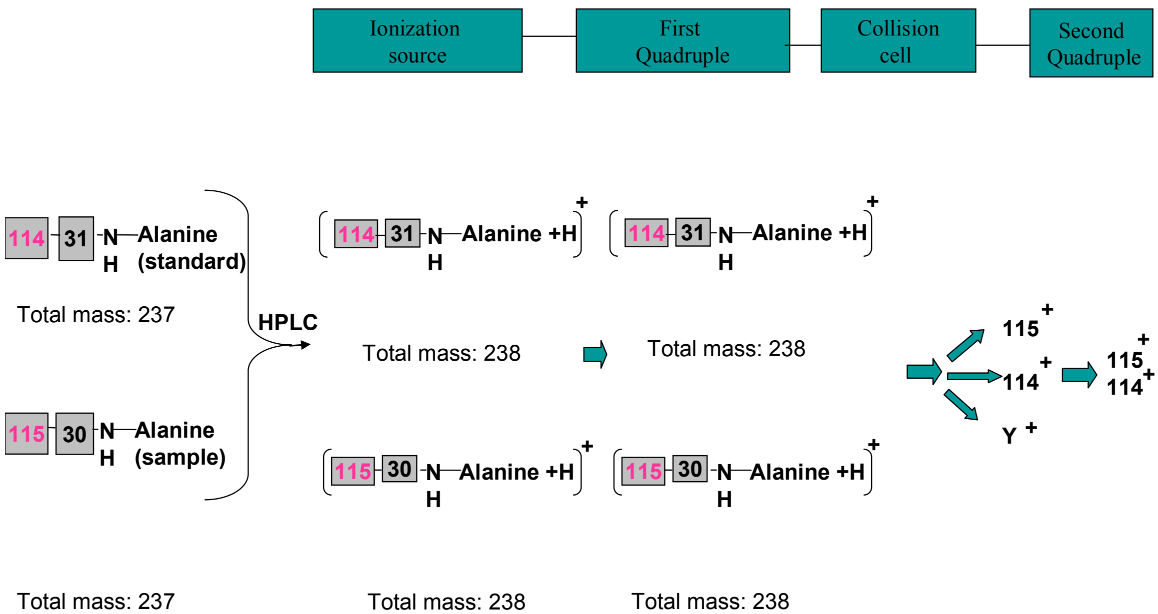


Figure 10: Amino acid analysis by iTRAQ®-LC-MS/MS: Separation of derivatives by HPLC and detection by MS/MS in multiple reaction monitoring (MRM), Each amino acid has its own internal standard correcting for matrix effects.

4.4.8 Direct infusion tandem mass spectrometry

Analysis of blood and urinary amino acids are used routinely in newborn screens for inherited metabolic disorders, such as phenylketonuria and maple syrup urine disease. Blood and urine samples are typically collected on filter paper, from which disks of defined size are punched out. Amino acids are then extracted with methanol containing stable isotope labeled amino acids. Extracted amino acids are converted into the corresponding butyl esters using hydrochloric acid in *n*-

butanol.[2] The screening for inborn errors of metabolism is performed using direct infusion MS/MS, which allows the very fast analysis of large number of samples. Additionally fatty acid and organic acid disorders can be detected in one brief analysis. However, isobaric amino acids, such as leucine, isoleucine and allo-isoleucine or alanine and sarcosine cannot be distinguished. For direct infusion, mass analyzers that provide high mass resolution, such as electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) and fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) are employed. This allows the identification of metabolites using accurate mass measurement. Dunn *et al.* showed the identification of amino acids and other metabolites in fruit extracts matching experimental accurate masses to the theoretical masses, for example glutamine and lysine are isobaric but can be distinguished by their accurate mass.[42]

4.4.9 Nuclear magnetic resonance (NMR)

The main advantage of NMR is its ability to detect all proton-containing metabolites in a sample simultaneously. Its sensitivity does not depend on chemical properties of the analytes such as pKa or hydrophobicity. Physiological fluids such as urine can be directly analyzed with only limited preparation. NMR is a very reproducible method and signals scale linearly with metabolite concentrations, which allows for reliable quantification. The main drawback of the method is its limited sensitivity compared to mass spectrometry. However, with the use of the newly developed cryo-probes limits of detection in the low µM range are obtained. Due to the high number of metabolites typically present in biological samples, however, significant overlap of amino acid signals with other signals is commonly observed in 1D ^{1}H NMR spectra as seen in Figure 11A. A mathematical solution to this problem is to fit overlapped signals with modelled peaks.[43] Alternatively, multidimensional NMR such as 2D ^{1}H-^{13}C heteronuclear single-quantum correlation (HSQC) spectra may be used to separate the overlapping metabolite signals in a second heteronuclear dimension.[44] A typical example obtained for human urine can be seen in Figure 11B

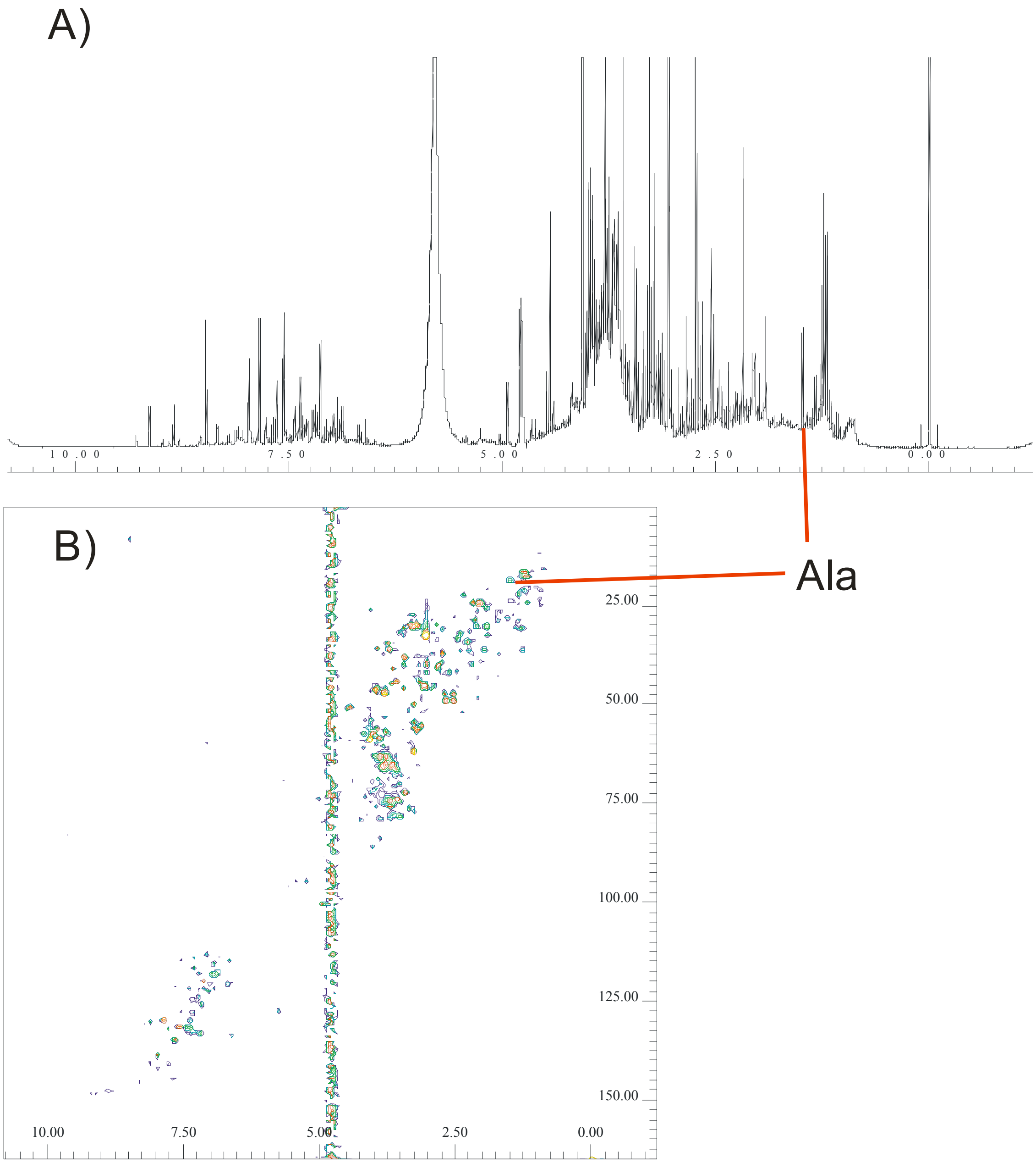


Figure 11: A) 1D ^{1}H spectrum of human urine measured at 600 MHz on a Bruker Avance III spectrometer equipped with a cryo-probe. B) The corresponding ^{1}H-^{13}C HSQC spectrum measured at natural abundance. As an example for amino acid metabolites in both spectra the signals corresponding to the alanine methyl groups are marked.

The availability of the newly developed cryo-probes allows partial compensation for the low natural abundance (≈1.1%) and low gyromagnetic ratio of the ^{13}C nuclei. In many instances it is advantageous to combine the results obtained by different methods such as NMR and mass spectrometry. As mentioned above some intensity loss is observed by going from 1D ^{1}H spectra to 2D ^{1}H-^{13}C HSQC spectra. One way of regaining this intensity loss due to the low natural abundance of ^{13}C is to chemically *N*-acetylate the amino-acid metabolites with ^{13}C-labeled acetic anhydride.[45] Using this approach, it is possible to obtain, on the one hand, highly sensitive ^{1}H-^{13}C HSQC spectra for amino acids and, on the other hand, background related to metabolites not modified by the derivatization procedure is drastically reduced, thus enabling lower limits of detection in the upper nanomolar range.

4.4.10 Comparison of methods for amino acid analysis

A comparison of the methods available for the analysis of amino acids is given in Table 2. The major advantage of NMR is that physiological fluids may be analyzed directly, albeit at the expense of sensitivity. Gains in sensitivity are feasible, but require N-acetylation of the amino acids with ^{13}C-labeled acetic anhydride. Another disadvantage is the large sample volume required, albeit due to the non-destructive nature of NMR, samples may be retrieved and subjected to further testing. The need for the acquisition of 2D-spectra limits throughput, but this is balanced by the ability of NMR to detect proton and carbon containing metabolites other than amino acids. Protein precipitation is required for all LC and CE methods independent of the detection method used, which renders complete automation difficult. Liquid chromatographic methods coupled with optical detection are well established and highly reproducible. However, classical pre- and post-column derivatization protocols employing OPA or ninhydrin suffer from long chromatographic runtimes, which render them poorly suited for large clinical and epidemiological studies. Another drawback shared by all methods based on optical detection is their lack of analyte specificity compared to mass spectrometry. The latter, however, is subjected to matrix and ion suppression effects that impair quantitative accuracy and necessitate the use of stable-isotope labeled internal standards. Nevertheless, MS based methods will prevail in the future. HILIC-MS and CE-MS allow the direct analysis of amino acids without prior derivatization, but they suffer from low throughput and comparatively poor reliability. Ion-pair LC-MS has been applied to the analysis of both native and iTRAQ®-labeled amino acids. The most important benefit of iTRAQ®-LC-MS/MS compared to other MS-based methods is the availability of internal standards not only for the 20 proteinogenic amino acids, but also for non-protein amino acids. But iTRAQ®-LC-MS/MS has a number of disadvantages including somewhat poor reproducibility due to the large number of transitions that have to be acquired, which may be alleviated in the future by scheduled multiple reaction monitoring (sMRM), the inability to accurately measure sulfur-containing amino acids, the difficulty of automating sample preparation, and the higher reagent costs.

GC-MS is a very robust method with excellent reproducibility of retention times. Especially with alkyl chloroformate derivatization excellent reproducibility of quantitative data has been observed and the method can be automated easily, thus, allowing high sample throughput. However, thermo-labile derivatives cannot be measured.

Finally, direct flow injection analysis with ESI-MS/MS offers high throughput and is now widely used in newborn screening for inborn errors of metabolism. The one major limitation is the inability to resolve isobaric amino acids. To date various methods exist for the quantification of amino acids in protein hydrolysates and physiological fluids. The great importance of amino acid analysis is reflected in a number of commercialized solutions ranging from kits to dedicated instruments. The development of new methods or the improvement of existing methods is still ongoing. Expansion of the analyte spectrum covered, reduction of sample preparation and analysis time, improved sensitivity, good robustness and reproducibility are the focus of research. An important aspect is method automation and high sample throughput, which is essential in studies with large sample numbers. There is room for new or improved methodology for amino acid analysis, including expansion of the analyte spectrum covered, reduction of sample preparation and analysis time, improved sensitivity, good robustness and reproducibility. Due to high selectivity and sensitivity, MS is expected to play a key role provided that stable isotope labelled standards, which are a prerequisite for robust quantification, become readily and cheaply available. Reduced sample pre-treatment is another important aspect for facilitating automation and improving robustness and sample throughput, which are essential in epidemiological studies with large sample numbers.

Table 2: Comparison of selected approaches for the metabolic analysis of amino acids

Method	Advantages	Disadvantages	LOD	Ref.
LC-methods coupled with optical detection	▸ Highly reproducible ▸ Inexpensive equipment ▸ Good linearity over a broad range	▸ Protein precipitation and derivatization necessary ▸ Lack of analyte specificity ▸ Co-eluting substances cannot be distinguished ▸ Not applicable to flux analysis	UV: 5 µM (LOQ)	22-25
UPLC-MS	▸ Fast separation ▸ Good resolution	▸ Protein precipitation necessary ▸ High pressure requires special equipment ▸ Limited number of amino acids covered ▸ Ion suppression	1.3 - 5.3 µM (LOQ)	26
IP-LC-MS/MS	▸ Derivatization not necessary ▸ High number of analytes covered ▸ Good resolution for polar amino acids	▸ Protein precipitation necessary ▸ Ion suppression ▸ Contamination of analytical system with IP reagent	0.0003 - 9 µM (LOD)	21, 28, 29
HILIC	▸ Derivatization not necessary ▸ Compatible with MS ▸ Well-suited for polar compounds	▸ Protein precipitation necessary ▸ Poor reproducibility ▸ Ion suppression in case of MS detection	5 µM (LOD) 10 µM (LOQ)	30
CE-MS	▸ Derivatization not necessary ▸ Low sample consumption	▸ Protein precipitation necessary ▸ Only low injection volume possible	0.1 - 14 µM (LOD)	34
GC-MS	▸ Robust method ▸ Highly reproducible ▸ Good resolution ▸ Fast separation	▸ Derivatization necessary ▸ Not suited for thermolabile amino acid derivatives	0.03 - 19.98 pmol on column (LOD)	40
iTRAQ®	▸ Fast separation ▸ Availability of internal standards for each analyte	▸ Protein precipitation necessary ▸ Insufficient recovery of sulfur containing amino acids	2-10 µM (LOQ)	Unpub-lished

		◗ Difficult to automate		own data
Direct infusion MS/MS, TOF	◗ No separation needed ◗ High throughput	◗ Extraction and derivatization required ◗ Isobaric amino acids cannot be resolved		
NMR	◗ No separation and derivatization needed ◗ Robust quantification ◗ Minimal sample preparation	◗ Insufficient sensitivity, albeit LOD can be lowered by derivatization ◗ Long analysis time	2D: 20 – 312 µM (LOD)	46

5 High-throughput analysis of free amino acids in biological fluids by GC-MS

5.1 Introduction

Our aim was to develop a robust, accurate, fast and precise method for urinary amino acid analysis. Amino acids can be derivatized directly in aqueous solution using alkyl chloroformate. The amino acids react very quickly, for instance, with propyl chloroformate and the derivates can be extracted with an organic solvent. From the organic phase an aliquot can be injected directly into the GC-MS.[37, 38] 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 standards. A shortened version of this chapter was published in the Journal of Chromatography B. [47]

5.2 Materials and methods

5.2.1 Chemicals

A standard solution of 17 amino acids at 1mM each in 0.1 M HCl, phenol, isooctane, methyl chloroformate, *n*-propanol, hippuric acid 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-13C, U-15N] cell free amino acid mix was from Euriso-top (Saint-Aubin Cedex, France) and *α*-aminoadipic acid [2, 5, 5-$^{2}H_{3}$] and [2,3,4,5,6-$^{2}H_{5}$] hippuric acid were 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.

5.2.2 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 phenol red, 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 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% n-propanol, 0.1% phenol and 2% thiodiglycol, were added to 20-50 µL biological sample.

5.2.3 Instrumentation

An Agilent model 6890 GC (Agilent, Palo Alto, USA) equipped with a MSD model 5975 Inert XL, PTV injector) and a MPS-2 Prepstation sample robot was used (Gerstel, Muehlheim, Germany. 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 MPS-2 Prepstation is shown in Figure 12.

Figure 12: GC-MS with MPS-2 Prepstation

The GC-column was a ZB-AAA (Phenomenex Inc.), 15 m x 0.25 mm ID, 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 here 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/sec 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 3.

Table 3: Ion traces selected for the SIM analysis of 33 physiological amino acids, dipeptides and norvaline. Amino acids printed in bold were quantified via stable isotope dilution using the internal standard quantification trace of the corresponding stable-isotope labeled amino acid.

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
ß-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
Hippuric acid	134	105	139
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	

5.2.4 Derivatization

In contrast to the original Phenomenex protocol, the cation exchange clean-up step was omitted. Amino acids were directly derivatized in the aqueous biological sample, 20-50 µL of which were transferred manually together with 20 µL of the stabilization reagent, described in chapter 5.2.2, 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 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 and [2,3,4,5,6-$^{2}H_5$] hippuric 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. 50 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 again 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. All steps were automated and done by the MPS-2 Prepstation from Gerstel. The different features of the MPS-2 Prepstation are shown in Figure 13.

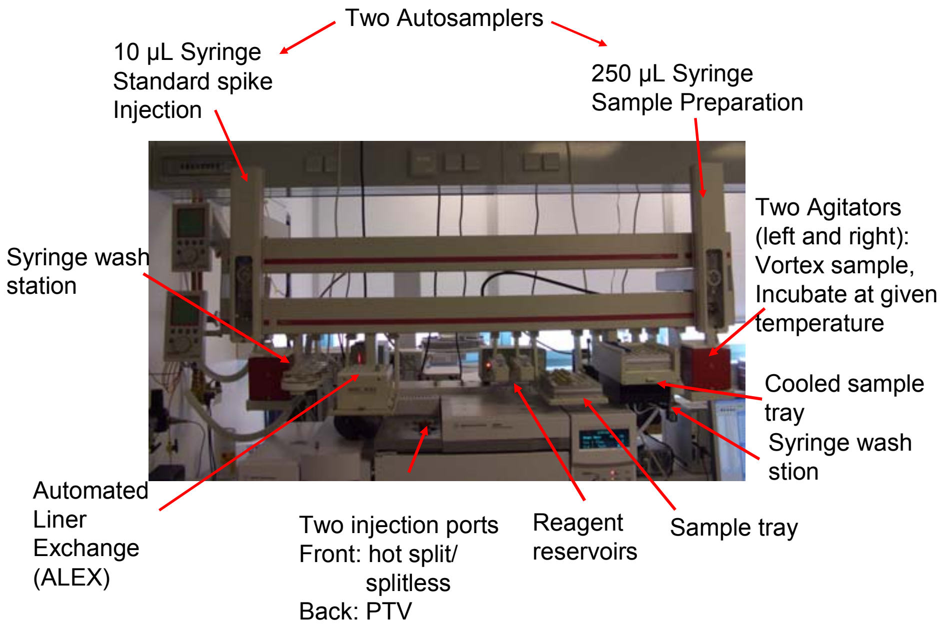


Figure 13: MPS Prepstation features.

5.2.5 Quantification

Absolute quantification of 33 compounds (alanine, sarcosine, glycine, *α*-aminobutyric acid, valine, *ß*-aminoisobutyric acid, leucine, allo-isoleucine, isoleucine, threonine, serine, proline, asparagine, thiaproline, aspartic acid, methionine, hippuric acid, 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 acids 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 and hippuric acid was added separately to yield a final concentration of 60 µM for each compound. The mix was further diluted to final concentrations of 6 µM 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 0.3-2,000 µM or normalized by the area of the closest eluting internal standard compound.

5.2.6 NMR

For NMR structural analysis, the propylformate derivative of asparagine was dissolved in 99.99% $CDCl_3$ that was also used as internal standard at 7.26 and 77.00 ppm for ^1H 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 ^1H, 2D ^1H-^{13}C HSQC and 2D ^1H-^{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 Figure 24, chapter 5.3.9).

5.3 Results and Discussion

5.3.1 Derivatization and column selection

Both the amino and the carboxyl group of amino acids react readily with alkyl chloroformates as shown in Figure 14 to yield volatile derivatives for GC-analysis.[37]

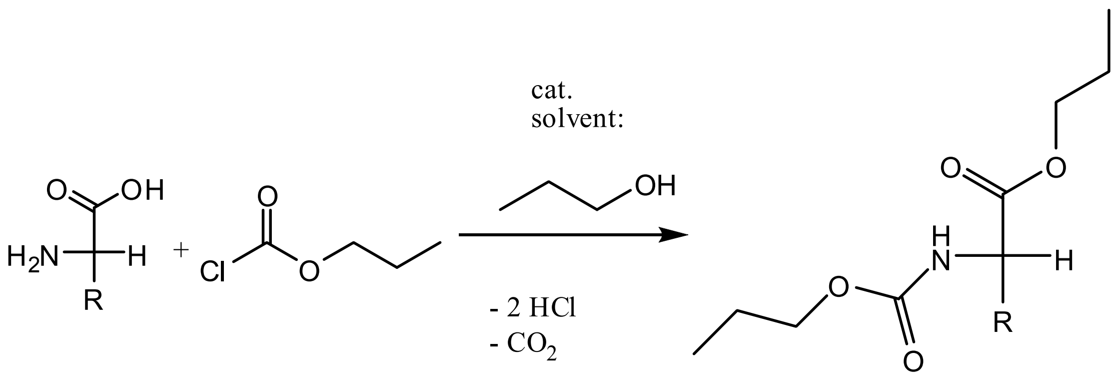


Figure 14: Reaction scheme for the derivatization of amino acids with propyl chloroformate.

Hydroxyl groups as found in serine and threonine have a very low reactivity and amide groups are not derivatized. Zampolli et al.[39] 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 x 0.25 mm ID, which was provided with the Phenomenex EZ:faast GC kit. The separation of the analytes was completed in less then 7 minutes (Figure 15).

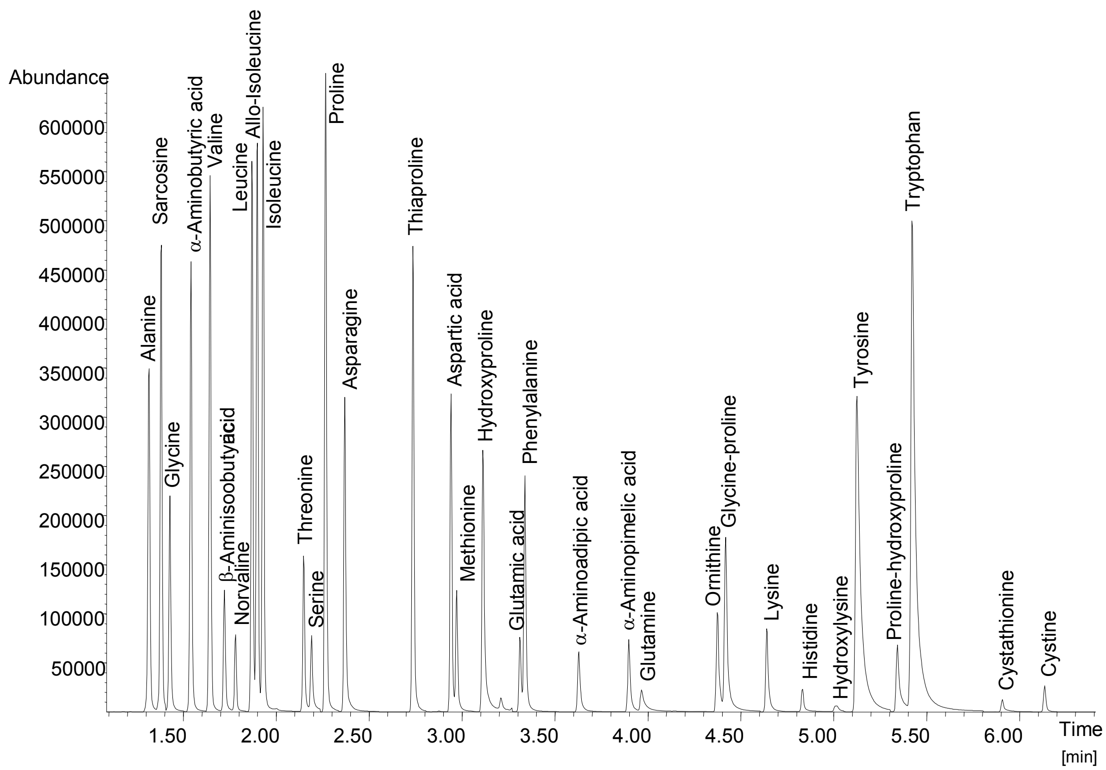


Figure 15: Typical GC-MS chromatogram for the analysis of an amino acid standard on a 10 m x 0.25 mm ID ZB-AAA column after derivatization with propyl chloroformate.

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 leucines, were baseline separated. Therefore, other stationary phases were evaluated. The first column tested was a RTX-35 Amine column (30 m x 0.25 mm ID, 0.5 μm film thickness), which is specifically designed for the separation of amines. (Figure 16a)

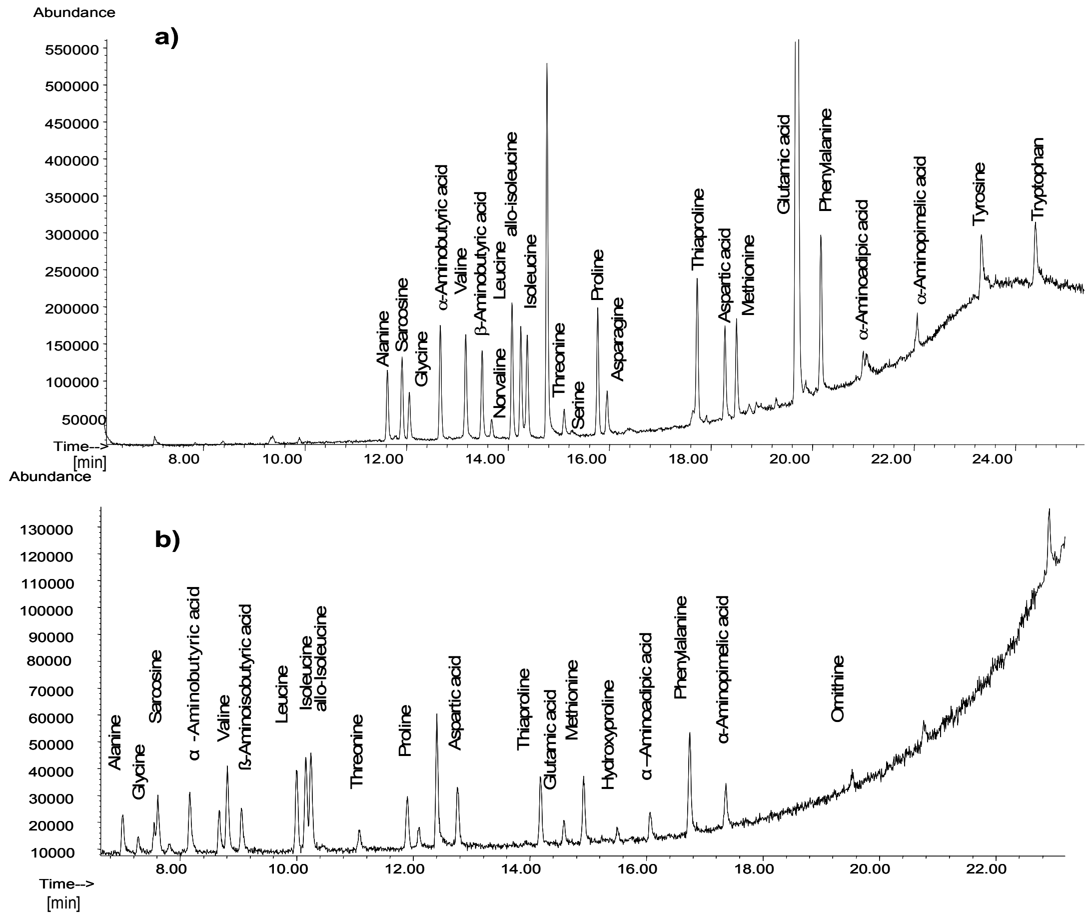


Figure 16: GC-MS chromatograms of an amino acid standard separated on a 30-m RTX-35 column after derivatization with (a) propyl and (b) methyl chloroformate, respectively.

Peak tailing was significantly reduced. 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.[38] 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 (Figure 16b). In addition to the polar column, a low bleeding non-polar RXI-5 MS column was tested (30 m x 0.25 mm ID, 0.25 µm film thickness). Using the propyl chloroformate reaction, five amino acids were not detected (threonine, serine, glutamine, cystathionine and cystine) (Figure 17a), while with the methyl chloroformate reaction aspargine, serine, threonine, ornithine, hydroxyproline, proline-hydroxyproline, cystathionine and cystine could not be detected (Figure 17b).

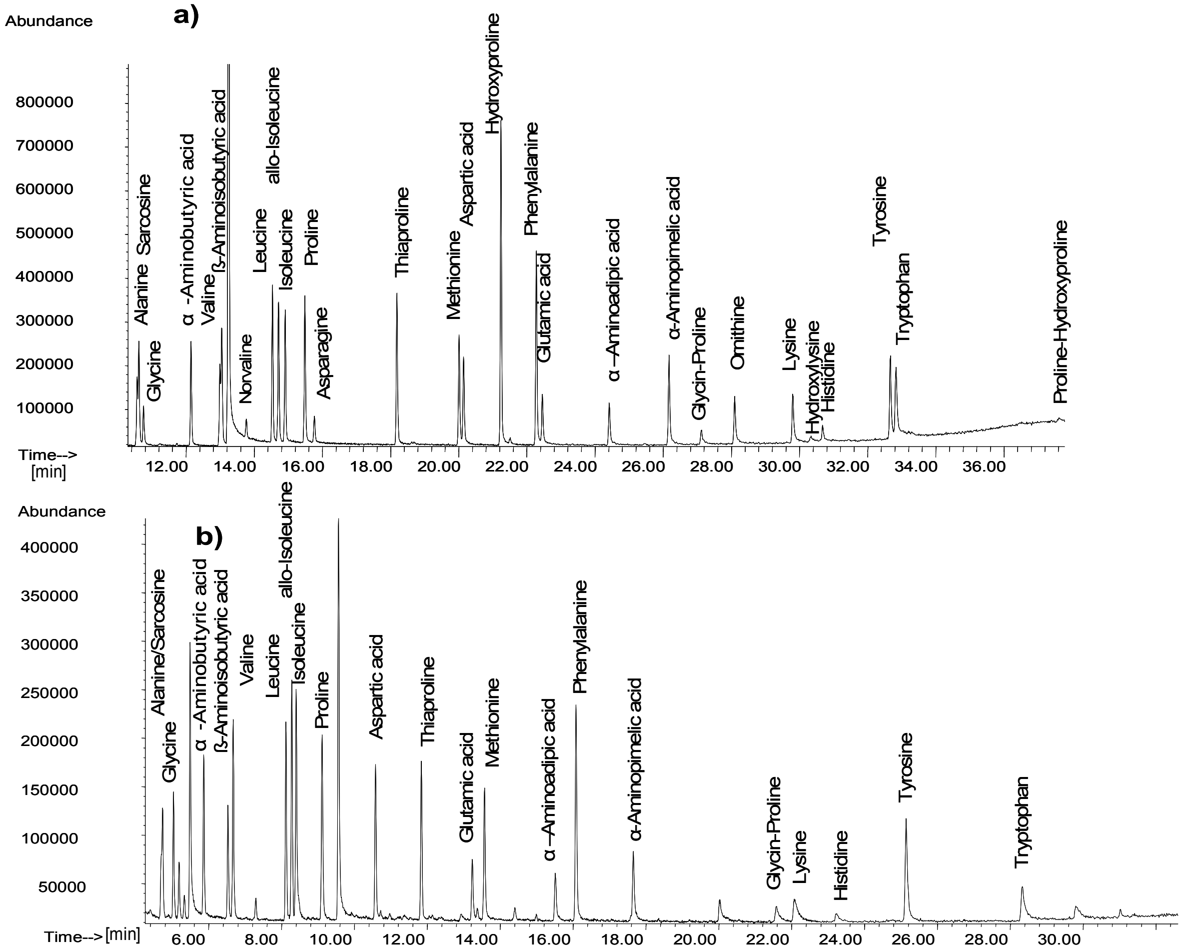


Figure 17: GC-MS chromatograms of an amino acid standard separated on a 30-m RXI-5MS column after derivatization with (a) propyl and (b) methyl chloroformate, respectively.

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 (defined in chapter 4.3.1) was 1.7 with the 10-m column and it improved to > 2.5 using the 15-m column. Figure 18 represents a typical chromatogram of the 34 compounds including norvaline, which is a non-endogenous compound and used as an internal standard. Less than ten minutes were required to resolve all compounds.

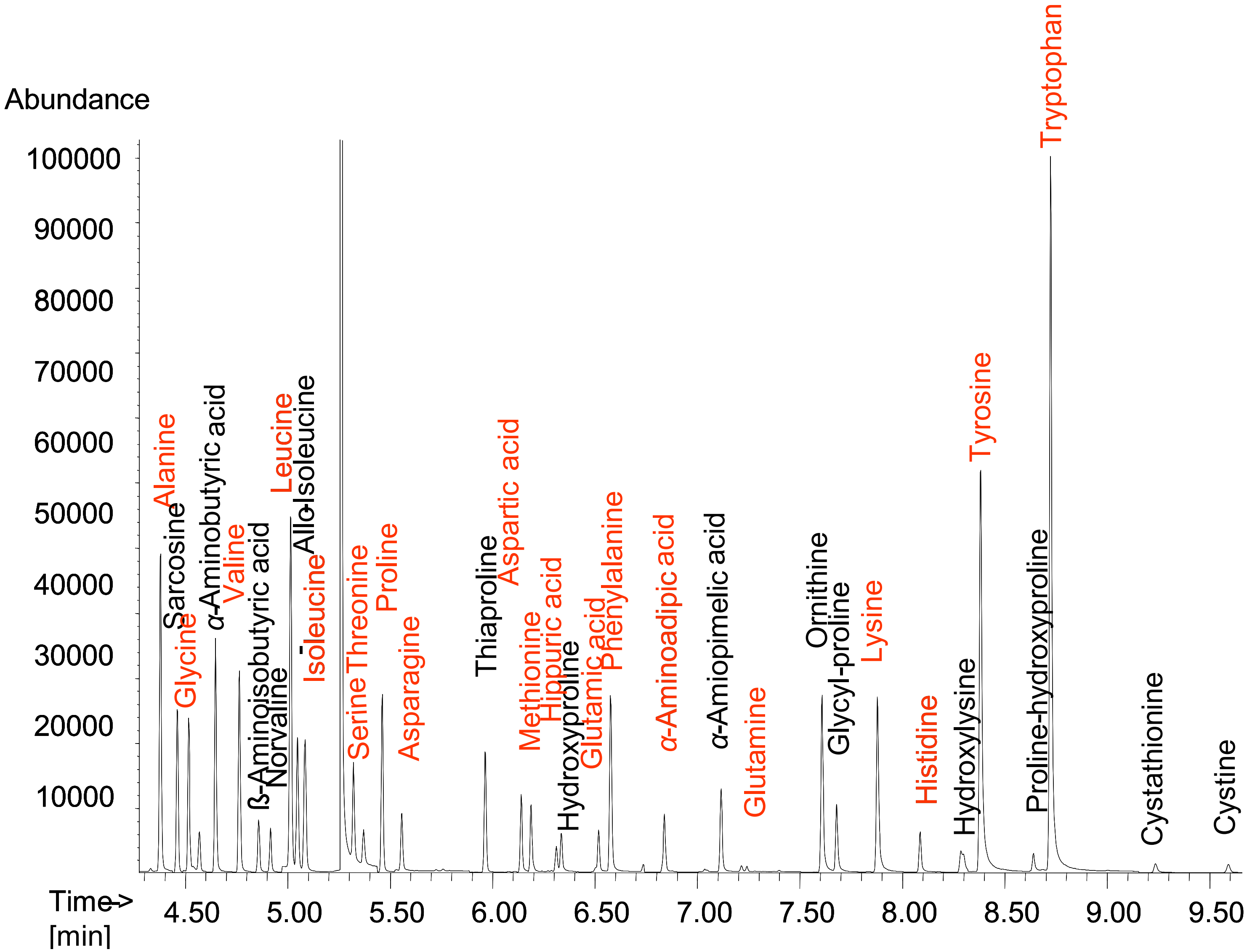


Figure 18: Typical GC-MS chromatogram for the analysis of an amino acid standard on a 15 m x 0.25 mm ID ZB-AAA column after derivatization with propyl chloroformate. Amino acids printed in red were quantified using the corresponding stable-isotope-labeled amino acid as internal standards for quantification.

5.3.2 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 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 (Figure 19.). 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 to the active sites on the surface.

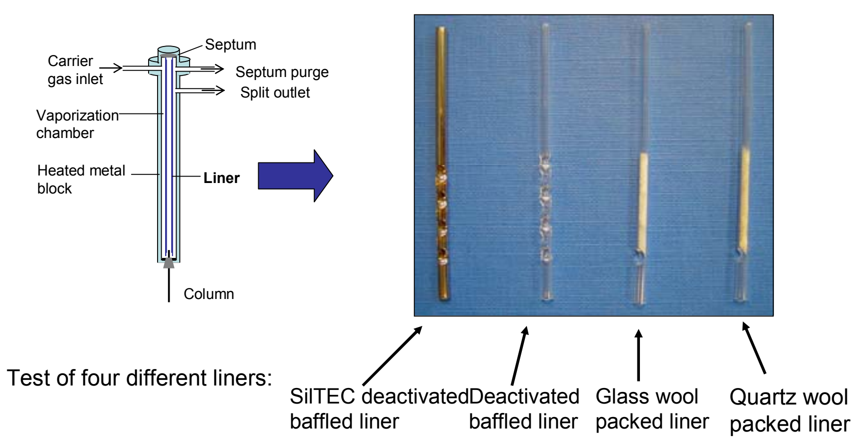


Figure 19: Injector scheme and four different liners tested for reproducibility

A urine sample was analyzed five times using each liner and the relative standard deviation (RSD) was calculated (Figure 20).

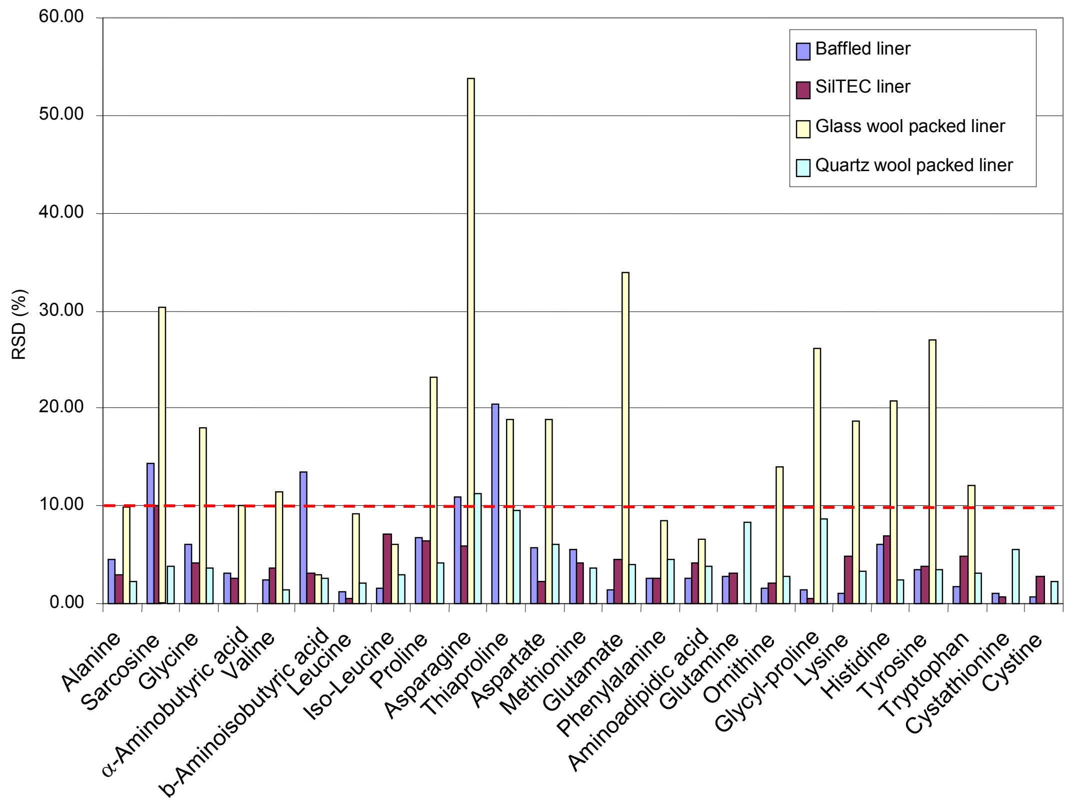


Figure 20: Comparison of the relative standard deviation values obtained for the repeated analysis (n=5) of urinary amino acids using different injector inserts.

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 RSD > 10%, and for thiaproline the RSD exceeded 20 %.

Only with the SILTEC liner, the RSDs 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 consecutive injection of the silylation reagent MSTFA, a 1 mM amino acid standard solution, and blanks to deactivate any active sites on the glass surface.

5.3.3 Internal standard selection

For the generation of reliable quantitative data, internal standards are required to correct for chemical and analytical losses during derivatization and analysis. We observed that norvaline corrected quite well for such losses for amino acids similar structure and retention to norvaline, e.g. leucine and glycine. 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 4. 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. It is important that the mass difference between analyte and internal standard is more than one unit to avoid the overlap with the content of the natural isotope ^{13}C. A standard mix of 18 uniformly ^{13}C and ^{15}N labeled amino acids was chosen. The labeled amino acids are extracted from algae. Consequently, their individual concentrations, as analyzed by HPLC, differ and range from 0.043 - 1.417 mM. Additionally [2,5,5-$^{2}H_3$] *α*-aminoadipic acid and [2,3,4,5,6-$^{2}H_5$] hippuric acid were used as internal standard. To compare the difference with and without using the internal standard mixture, the R square-values of the calibrations of all amino acids are shown in Table 4. 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.

Table 4: Comparison of the R-square values obtained for the calibration curves of selected amino acids using either norvaline (Nval) or stable-isotope labeled amino acids as internal standards. The RSD values represent the inter-day reproducibility of urinary amino acid levels for 11 repeated injections using either quantification method.

Amino acid[a]	**R^2**	**R^2**	**RSD (%)**	**RSD (%)**
	Nval	Stable isotopes	Nval	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
ß-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
Hippuric acid	0.9921	0.99	18.60	16.7
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

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

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 RSDs using norvaline as the sole internal standard ranged from 1.98% to 18.6%. But they improved significantly (1.5% to 5.7%) for most amino acids, except for aspartic acid, methionine,hippuric acid and cystathionine, when stable-isotope labeled amino acid standards were employed. For the latter no stable isotope labeled standards had been available.

5.3.4 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 5. 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 80-120%, in agreement with the FDA Guide for Bioanalytical Method Validation.[48] 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 5 were calculated from the analysis of 50-µL aliquots of human urine. The lowest LOD was 0.03 µM, corresponding to an absolute injected 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, asparagine, 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.*[49], 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 might be improved by using less organic solvent for extraction or injecting more sample using large volume technique.

Table 5: Calibration curve parameters. Limits of detection and 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.

Amino acid	R-square[a]	LOD[b] (µM)	Range of quantification [µM] [c]	Regression line	Retention time (min)
Alanine	0.9997	0.03	0.3-2000	1.507 * x + 0.011940	4.35
Sarcosine	0.9969	0.3	0.9-270	1.611 * x + -0.008277	4.43
Glycine	0.9998	0.03	3-2000	1.238 * x + 0.068277	4.49
α-Aminobutyric acid	0.9984	0.12	0.3-270	2.521 * x + 0.001067	4.62
Valine	0.9996	0.18	0.3-2000	1.174 * x + 0.001163	4.73
ß-Aminoisobutyric acid	0.9970	0.27	0.9-270	0.613 * x + -0.003078	4.83
Leucine	0.9992	0.06	0.3-2000	1.102 * x + 0.005131	4.98
allo-Isoleucine	0.9985	0.3	0.9-270	1.252 * x + -0.005865	5.02
Isoleucine	0.9996	0.12	0.9-2000	1.122 * x + -0.002333	5.05
Threonine	0.9988	0.18	0.3-2000	1.03* x + -0.001590	5.29
Serine	0.9975	3.0	12-2000	1.22 * x + 0.443377	5.34
Proline	0.9960	0.27	0.3-2000	0.623 * x + -0.010681	5.43
Asparagine	0.9986	3.0	12-270	1.125 * x + -0.036928	5.53
Thiaproline	0.9900	0.3	0.9-270	4.349 * x + -0.006387	5.93
Aspartic acid	0.9997	0.3	3-2000	1.251 * x + -0.018064	6.11
Methionine	0.9958	0.9	3-1000	1.177 * x + -0.028463	6.16
Hippuric acid	0.99	3	12-2000	0.062 * x + -0.003033	6.28
Hydroxyproline	0.9758	0.9	3-270	0.334 * x + -0.004975	6.30
Glutamic acid	0.9999	0.9	3-2000	1.249 * x + -0.023798	6.49
Phenylalanine	0.9997	0.3	0.9-2000	1.191 * x + -0.005860	6.55
α-Aminoadipic acid	0.9982	0.9	3-270	1.089 * x + -0.033340	6.81
α-Aminopimelic acid	0.9925	0.9	3-270	2.351 * x + -0.131996	7.09
Glutamine	0.9940	12.0	30-270	1.197 * x + -0.012232	7.58
Ornithine	0.9971	0.3	0.9-270	1.526 * x + -0.018075	7.18
Glycyl-proline	0.9840	0.9	3-270	0.161 * x + -0.006280	7.65
Lysine	0.9960	0.3	0.9-2000	1.122 * x + -0.024828	7.85
Histidine	0.9987	3.0	12-2000	0.372 * x + -0.043117	8.06
Hydroxylysine	0.9976	3.0	12-270	0.208 * x + 0.006536	8.26
Tyrosine	0.9984	0.3	0.9-2000	2.449* x + -0.001631	8.35
Proline-Hydroxyproline	0.9906	12.0	12-270	0.047 * x + -0.002474	8.61
Tryptophan	0.9987	0.03	0.3-270	1.44 * x + -0.003679	8.69
Cystathionine	0.9930	3.0	12-270	0.133 * x + 0.001518	9.20
Cystine	0.9950	3.0	12-1000	0.186 * x + 0.012430	9.55

[a] Coefficient of determination (square of the correlation coefficient r of the regression analysis)
[b] Limit of Detection (S/N ≥ 3)
[c] LOD and LOQ were calculated for a sample volume of 50 µL

The calibration ranges ranged from 0.3 µM 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.

5.3.5 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 out of 17 amino acids. Arginine could not be determined because of the thermal instability of its propyl chloroformate derivative that carries a free guanidine group. The certified concentrations and estimated uncertainties for the 16 amino acids are given in Table 6. These values are based on in-house analysis at NIST and a round-robin study that was conducted in cooperation 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 6. 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 by means of conventional amino acid analyzers. In addition, a recovery based on the gravimetric values was calculated. It ranged from 94.3% up to 105.3% for methionine and lysine, respectively. 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.

Table 6: 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	'avimetric lue	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

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. We were able to determine 18 amino acids in the plasma. All measured values were well inside the control range given by RECIPE (Table 7). The sole exception was asparagine, for which the GC-MS value was slightly too high. The control range for asparagine was 17.3 to 25.9 µM and the concentration measured by GC-MS was 29.7 µM.

Table 7: 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 [µ*M*] Mean ± SD (n =10)	Control range [µ*M*]
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

5.3.6 Precision of GC-MS analysis of amino acids in different biological matrices

The method's precision low 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 RSDs obtained for different amino acids are listed in Table 8. 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 RSDs typically < 5%. Generally, the RSDs 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 are comparable, except for aspartic acid, methionine and cystine. For the latter amino acids, the RSDs increased above 10% in the inter-day measurements with a maximum value of 14.1% for aspartic acid.

Table 8: 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**.**

	Relative standard deviation [%]				
Amino acid[a]	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
ß-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
Hippuric acid	8.7	16.7	7.5	n.d	n.d
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

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

5.3.7 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 Figure 21. 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 were 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.

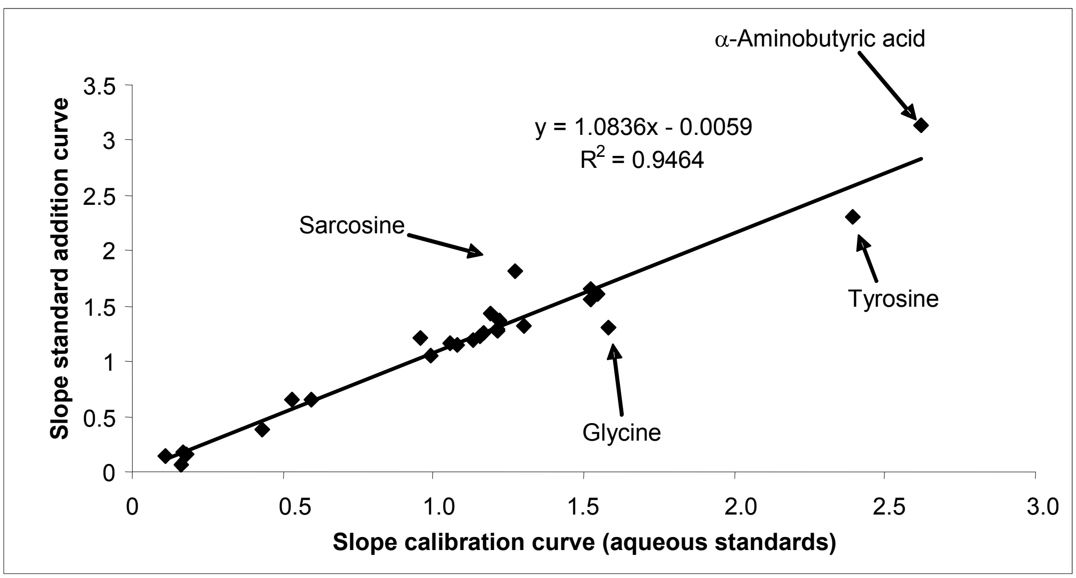


Figure 21: Evaluation of matrix effects by comparison of the slopes of the calibration curve (x-axis) with the slope of the standard addition curve in human urine (y-axis).

5.3.8 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 as phenylketonuria (PKU) and maple syrup urine disease (MSUD). The screening for inborn errors of metabolism is widely done using direct infusion LC-MS-MS methods,[2, 50, 51] 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 four 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 Table 9 and Table 10 in this chapter. Phenylketonuria (PKU) is caused by a deficiency of the enzyme phenylalanine hydroxylase or its cofactors,1 leading to the accumulation of phenylalanine (Figure 3, chapter 4.2).[45] PKU can be diagnosed by an increased ratio of phenylalanine to tyrosine in serum.[52] In the serum samples with this inborn error, there is a high concentration of phenylalanine, in comparison to the other samples. This is obvious from the dominant phenylalanine peak (q) in the GC-MS total ion current chromatograms shown in Figure 22a. Figure 22a and Figure 22b show chromatograms of the propyl chloroformate derivatives of amino acids from a PKU-positiv serum and MSUD-positv serum, respectively.

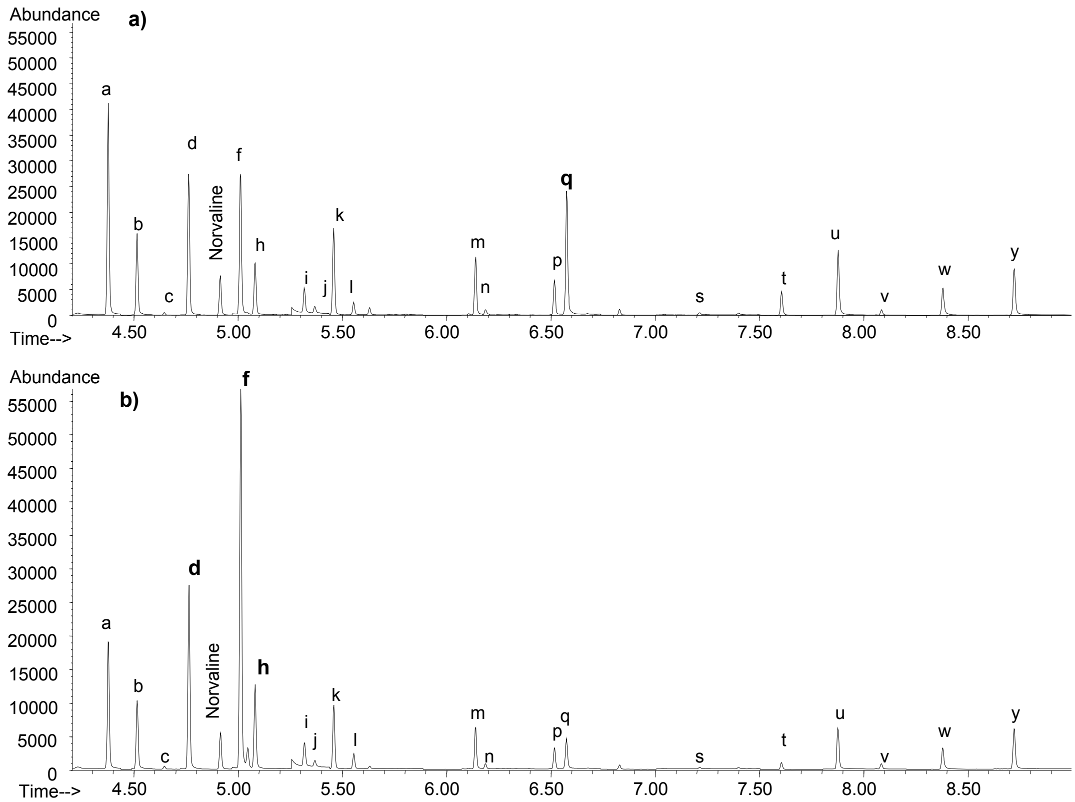


Figure 22: GC-MS total ion current chromatograms of propyl chloroformate derivatives of amino acids from a PKU-positive serum (a) and a MSUD-positive serum (b). Labeled peaks are the derivatives of a) Ala, b) Gly, d) Val, f) Leu, h) Ile, i) Thr, j) Ser, k) Pro, l) Asn, m) Asp, n) Met, p) Glu, q) Phe, s) Gln, t) Orn, u)Lys, v) His, w) Tyr, and y) Trp.

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 (MSUD) have a defect in branched-chain α-keto acid decarboxylase, resulting in increased serum concentrations of keto acids and their corresponding amino acids. The pathways of the degradation of the branched chain amino acids are shown in Figure 23. The amino acid that accumulates the most is leucine. Further, increased concentrations of valine and isoleucine are often observed.[2]

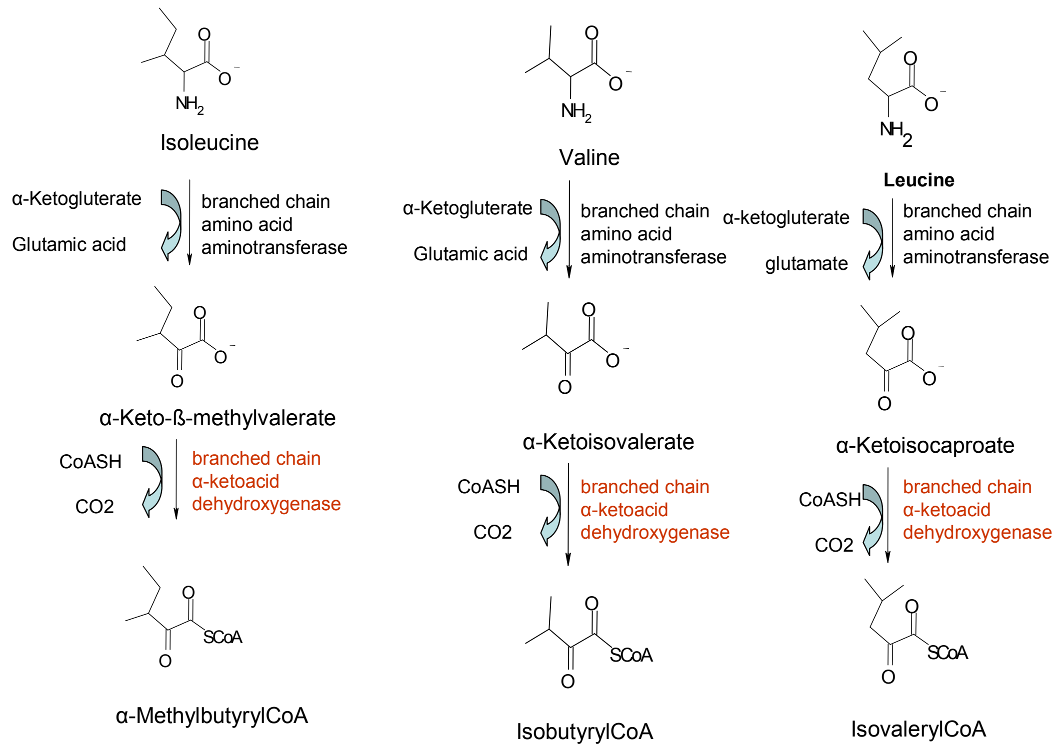


Figure 23: Branched chain amino acid metabolism. MSUD is caused by a deficiency of the metabolic enzyme branched chain α-keto acid dehydrogenase (BCKDH).

MSUD can be diagnosed by an increased ratio of leucine and isoleucine to phenylalanine.[52] As shown in Table 9, 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.[2] The tyrosinemia type I is caused by a deficiency of fumarylacetoacetase. 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.[53-55] Additionally, high concentrations of glycine can occur in urine and serum.[56] 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 ammonia to accumulate in the blood. It is caused by a deficiency of argininosuccinate lyase.[9, 57] 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.[58] 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 four in comparison to the levels detected in the urine of patients with argininosuccinic aciduria or propionic acidemia.

Table 9: Plasma amino acid concentrations [µM] for patients with inborn errors of metabolism. Each sample was measured in triplicate.

Amino acid	Maple syrup urine disease	Phenyl-ketonuria	Propionic acidemia	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
ß-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

n.d. - not detected above the LLOQ.

Table 10: Urinary amino acid concentrations [µM] for patients with inborn errors of metabolism. Each sample was measured in triplicate.

Amino acid	Argininosuccinic aciduria	Propionic acidemia	MaMaple syrup D urine disease	Amino-aciduria
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
ß-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

n.d. - not detected above the LLOQ

5.3.9 Method limitations

Arginine is an important amino acid that cannot be analyzed by GC-MS following alkyl chloroformate derivatization.[38] 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*.[59] 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 experimental section 5.6). The reaction is shown in Figure 24.

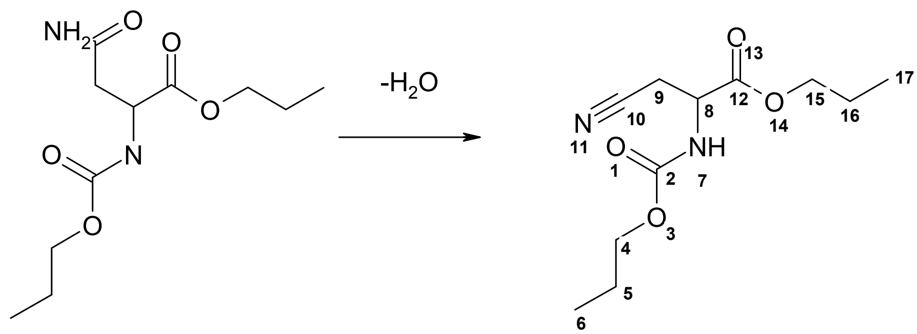


Figure 24: Reaction scheme for the loss of water in the propyl chloroformate derivate of asparagines.

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 threefold derivatized serine and threonine with the hydroxyl group being also acylated. In both cases the threefold derivatized product is the minor product. In comparison to the major derivative the threefold 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.

5.4 Applications to different biological projects

5.4.1 Metabolome analysis of *E. coli*

A capillary electrophoresis – mass spectrometry (CE-MS) method was developed and validated for the quantitative analysis of negatively charged metabolites, using a time-of-flight (TOF) mass by Timischl et al.[60] The method was used to elucidate metabolic changes in an Escherichia coli mutant, UdhA-PntAB, a double kock out for the nicotinamide nucleotide transhydrogenase.

To validate the CE-MS method and to get a more comprehensive coverage of the E. coli metabolome, the E. coli samples were also subjected to the amino acid analysis by GC-MS with propyl chlorofromate. For the amino acids glutamic acid and aspartic acid, which were detected by both methods, a very good correlation was observed. Many of the other amino acids showed significant differences between the wild type and the mutant strain. The results of the amino acid analysis are also puplished by Timischl *et al*. [60]

5.4.2 Cross-validation with 2D NMR

A two-dimensional (2D) nuclear magnetic resonance (NMR) spectroscopy method was developed by Gronwald et al. for the quantification of metabolites in biological fluids and tissue extracts. [46] The quantitative 2D NMR data of a selected set of the urinary metabolites were compared with data obtained by GC-MS amino acids analysis with and propyl chloroformates. To crossvalidate the NMR measurement with other analytical methods a blinded set of 50 human urine samples originating from the INTERMAP study were analyzed by NMR, GC-MS and LC-MS. The set contained 6 triplicates (18 samples) 5 duplicates (10 samples), 14 single samples, and 8 samples that were mixed at a ratio of 1:1 (Figure 25).

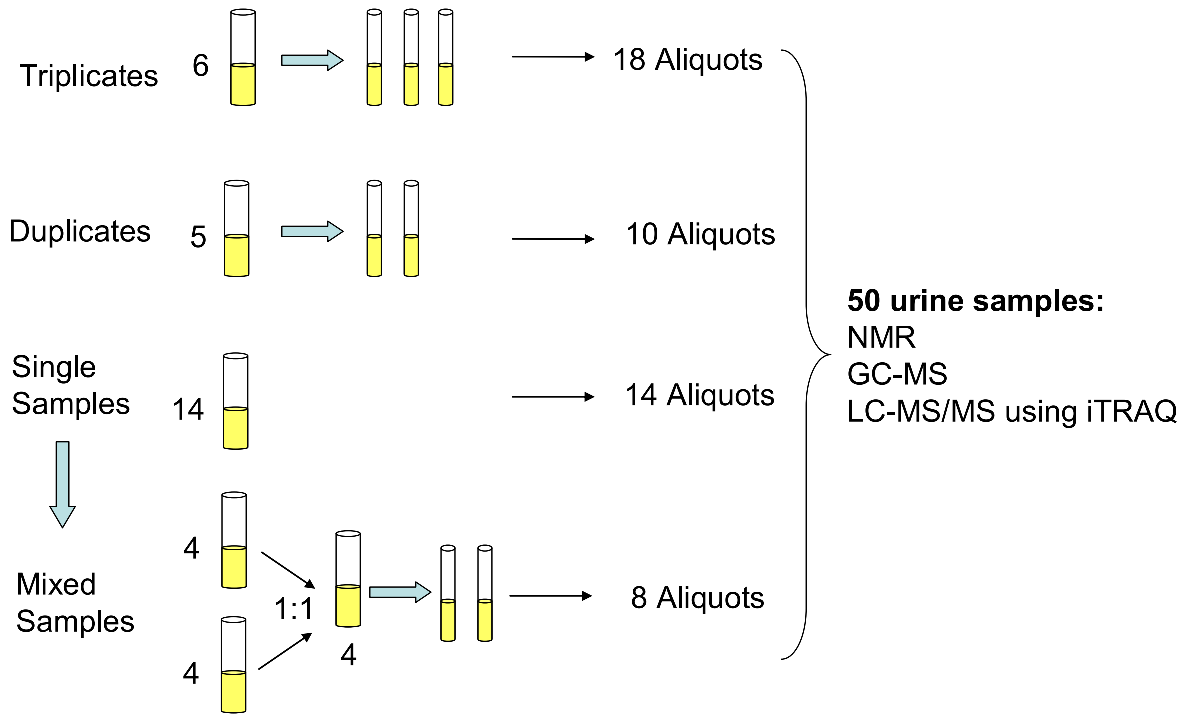


Figure 25: Study design of the blinded sample set.

The RSDs (%) for the GC-MS analysis are shown in Figure 26 for the triplicates, duplicates, mixed samples and for the average over all.

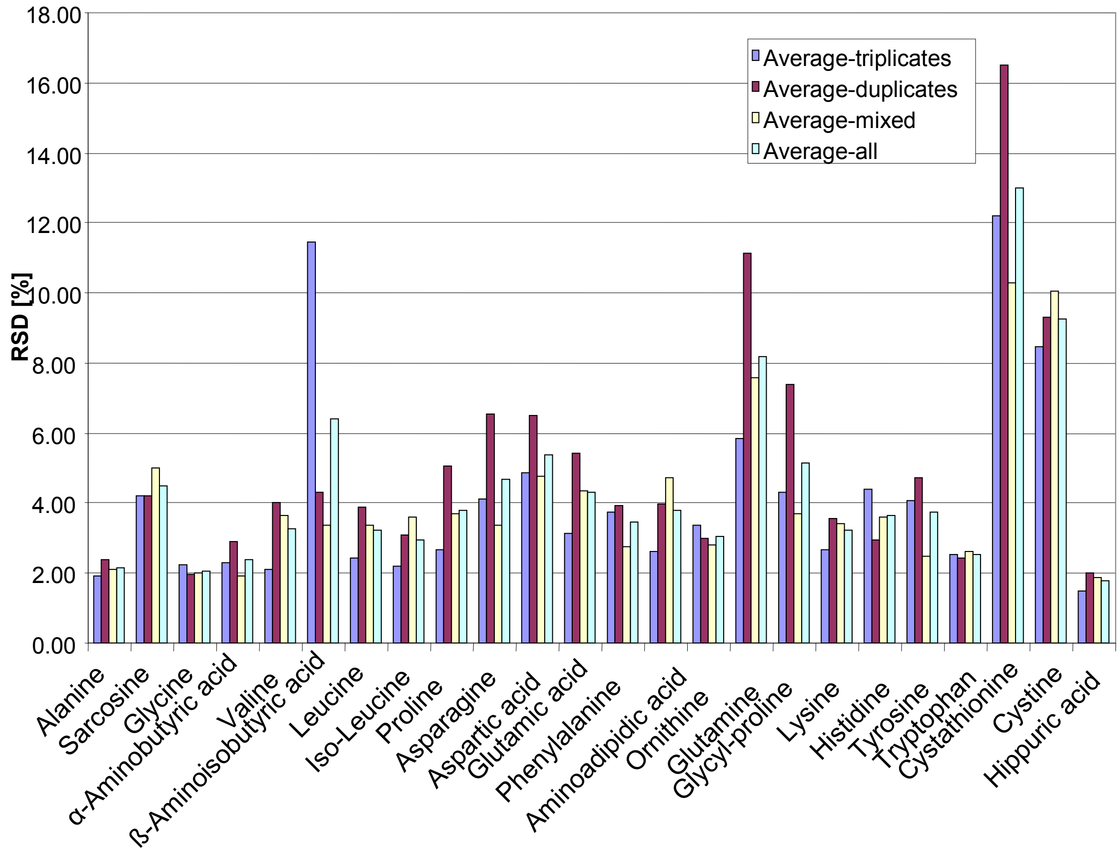


Figure 26: Reproducibility as relative standard deviation of urinary GC-MS amino acids analysis of the 50 samples using 50 µL sample aliquots.

The 2D NMR results of the 50 urine samples taken from the INTERMAP study for 7 compounds (alanine, glutamine, glycine, hippuric acid, histidine and lysine) were linearly regressed with the data obatianed by GC-MS and the R-square values were calculated. One important urinary metabolite amenable to both NMR and GC-MS is hippuric acid. Figure 27 shows the comparison of the corresponding NMR and GC-MS results for the 50 urine samples investigated. As indicated by the high R-square value of 0.99, both methods allowed the precise determination of hippuric acid and showed a linear correlation over the entire observed concentration range.[46]

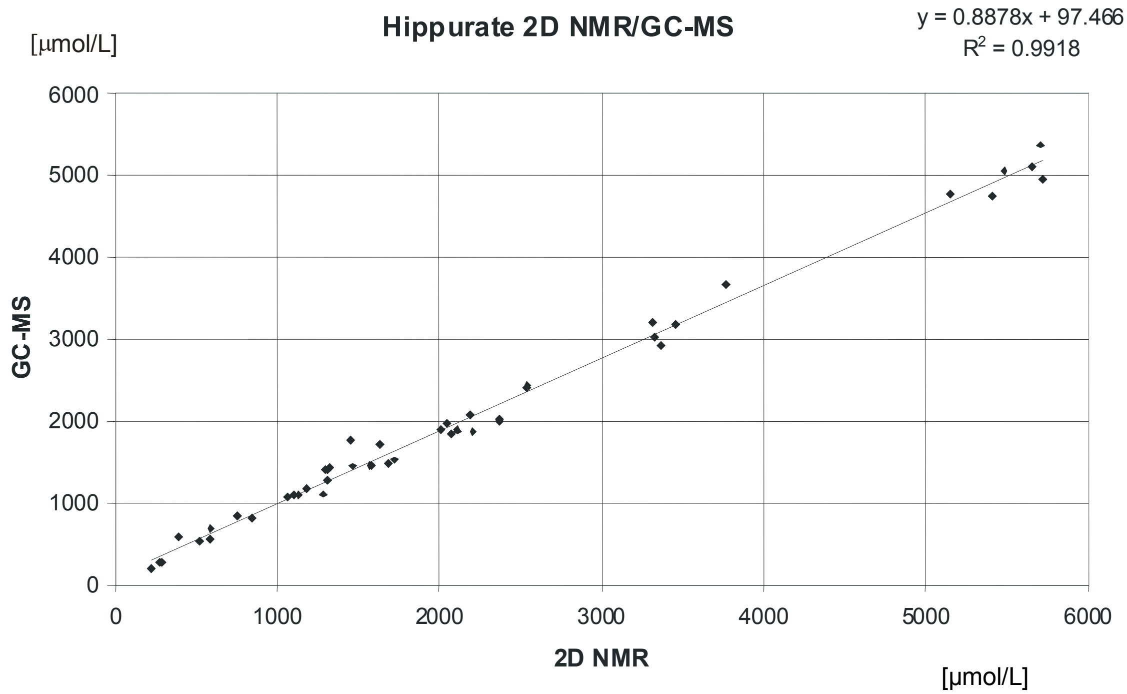


Figure 27: Correlation between 2D NMR analysis and GC-MS analysis for hippuric acid.

5.4.3 Other biological projects

Due to the broad applicability of the method a lot of different samples were measured. Samples ranging from sera and plasma to urine, cell media, cell extracts and milk. Mice urine samples were measured for a working group of the biology department, cell media was measured for our in house working group examining the intake of glutamine, human urine samples were measured for the correlation of GC-MS and NMR for hipuric acid.[61] Tryptophan analysis was performed for a project investigating mesenchymal stem cells (MSC) inhibiting the immune response in vitro.[62]

6 A Comparison of iTRAQ®-LC-MS/MS, GC-MS and Amino Acid Analyzer

6.1 Introduction

The goal of the present study was to compare sample preparation, amount of sample needed for analysis, runtime, number of analytes amenable to quantification, cost, lower limit of quantification (LLOQ), reproducibility, reliability, and validity of three methods for urinary amino acids analysis: A conventional amino acid analyzer, GC-MS of propyl chloroformate derivatives,[47] and iTRAQ®-LC-MS/MS.[41] Use of iTRAQ®-LC-MS/MS for this purpose is described here for the first time. The iTRAQ® method is based on differential derivatization of standard and sample amino acids with isobaric tags that show identical chromatographic retention, but can be distinguished by tandem mass spectrometry upon collision-induced dissociation of reporter ions that differ by one mass unit. The advantage of iTRAQ®-LC-MS/MS over other existing methods is the availability of 42 internal standards of physiological amino acids and related amines that enable absolute quantification by isotope ratio analysis.

For the comparison of the three methods blinded sets of 98 and 341 urine specimens, respectively, were analyzed. The urine specimens were aliquots from the timed 24-hour urine collections of the INTERMAP study (INTERnational collaborative of MAcronutrients and blood Pressure) on relation between diet and blood pressure among 4,680 men and women ages 40-59 years in Japan, Peoples Republic of China (PRC), UK and USA.[3, 63] This chapter will be published in the Journal of Chromatography B.[64]

6.2 *Materials and Methods*

6.2.1 Urine specimens

The urine specimens were aliquots taken from the timed 24-hour urine collection of the 17 population samples collected by the INTERMAP Study from 1996 to 1999 in 4 countries. Boric acid had been added as a preservative to the urine samples upon collection. Before preparation of aliquots for this study, specimens had been stored at -20°C. Aliquots were shipped from London to Regensburg and Framingham, respectively, on dry ice and, thereafter, stored at -20°C until analysis.

The first test set comprised 30 triplicates and 4 duplicates from 34 INTERMAP urine specimens randomly selected from five (of 17) population samples: Sapporo (Japan), Aito Town (Japan), Guangxi (PRC), Chicago (US), and Minneapolis (US), respectively. The second set comprised 341 aliquots from 144 INTERMAP urine specimens that were different from those in batch I, but selected from the same five population samples. Of the 144 different urine specimens, 91 were represented as duplicates and 53 as triplicates.

6.2.2 iTRAQ®-LC-MS/MS

The analysis by LC-MS/MS was carried out by Applied Biosystems. Derivatization of urinary amino acids with iTRAQ® was performed semi-automated using the Apricot Designs TPS-24 Total Pipetting Solution™ liquid handler with a 12-position pipetting head. Forty µL of urine were manually pipetted into 96-well plates. The plates were placed on the liquid handler and 10 µL of 10% sulfosalicylic acid containing 4 nmol of norleucine were added to each well to precipitate the proteins. Norleucine served as internal standard to calculate extraction efficiency. The plates were removed from the liquid handler, mixed for 30 s, and then centrifuged in an Eppendorf Centrifuge 5810R for 5 min at 2,000 RPM (700xg). The plates were returned to the liquid handler, 10 µL of supernatant were transferred to new wells and mixed with 40 µL labeling buffer (0.45 M borate buffer, pH 8.5, containing 20 pmol/µL norvaline as internal standard to calculate derivatization efficiency). Ten µL of the diluted supernatant were transferred to new wells and mixed with 5 µL of a diluted iTRAQ® reagent 115 solution (1 tube mixed with 70 µL of isopropanol) and incubated at room temperature for 30 min. Then 5 µL of 1.2% hydroxylamine solution were added to each well. The samples were allowed to evaporate overnight to dryness and were reconstituted the next day with 32 µL of iTRAQ® reagent 114-labeled standard mix (5 pmol of each amino acid/µL - with the exception of L-cystine, present at 2.5 pmole/µL - in 0.5% formic acid). Chromatographic separation of amino acids with an identical nominal mass was achieved at 50°C using an Agilent 1100 HPLC system. An Applied Biosystems C18-5 µm column (4.6 i.d. x 150 mm) was used. LC separation was carried out using a mobile phase consisting of 0.1% formic acid and 0.01% heptafluorobutyric acid in water (solvent A) and 0.1% formic acid and 0.01% heptafluorobutyric acid in acetonitrile (solvent B). The column was equilibrated in 98% A and the gradient was 98%-72% A over 10 min, 72%-0% A over 0.1 min, hold at 100% B for 5.9 min. A flow rate of 800 µL min^{-1} was used and the injected sample volume was 2 µL. Tandem mass spectrometry was performed on an API 3200 mass spectrometer (Applied Biosystems) with turbo ion spray in positive mode using the following parameters: Ion spray voltage (IS) 1500 V; auxiliary gas temperature (TEM) 700ºC; curtain gas (CUR), nebulizer gas (GS1), and auxiliary gas (GS2) 20, 70, and 70 arbitrary units, respectively; collision gas medium. Entrance potential (EP) was set at 10 V, declustering potential (DP) at 20 V, collision energy (CE) at 30 V, and collision cell exit potential (CXP) at 5 V. Quantitative determination was performed in multiple reaction-monitoring (MRM) mode using one transition for the analyte and one for the internal standard, according to the manufacturer's instructions. Processing of the chromatograms was performed using a beta version of the Cliquid® software (Appl. Biosys.) for automated tracking of mass traces and stable isotope ratio analysis. A chromatogram of a urinary sample, using MRM, is shown in Figure 28.

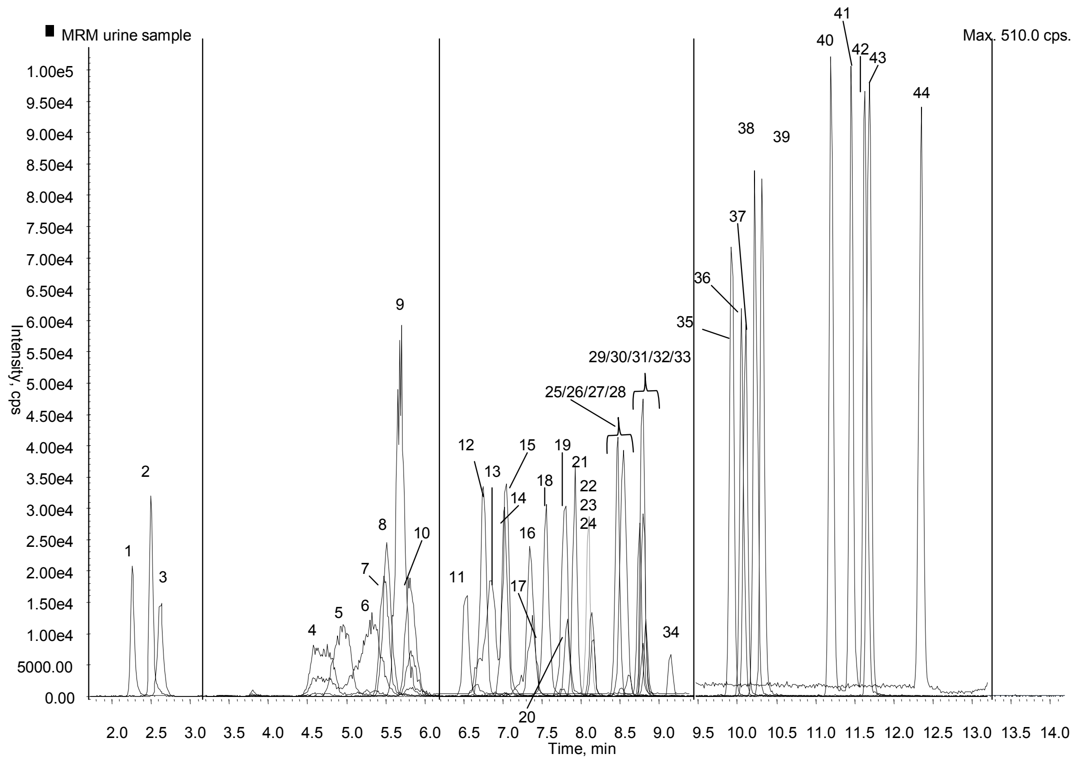


Figure 28: Separation of 44 amino acids in an urine sample on a C18 column with iTRAQ® (labeled with numbers) in MRM mode, divided into 4 different time windows. Labeled peaks are the derivatives of: 1) Pser, 2) PEtN, 3) Tau, 4) Asn, 5) Ser, 6) Hyp, 7) Gly, 8) Gln, 9) Asp, 10) EtN, 11) Cit, 12) Sar, 13) bAla, 14) Ala, 15) Thr, 16) Glu, 17) His, 18) M1His, 19) M3His, 20) Hcit, 21) GABA, 22) ßAib, 23) Abu, 24) Aad, 25) Ans, 26) Car, 27) Pro, 28) Arg, 29) Hly, 30) Orn, 31) Cth, 32) Cys-Cys, 33) Asa, 34) Lys, 35) Val, 36) Nva, 37) Met, 38) Tyr, 39) Hcys, 40) Ile, 41) Leu, 42) Nle, 43) Phe, 44) Trp.

6.2.3 Amino acid analyzer

The amino acid analyzer Biochrom 30 was used (Laborservice Onken, Gründau, Germany) for the analysis of the first batch of urine specimens. Sample preparation and analysis were performed using the manufacturer's standard protocols, including protein precipitation with sulfosalicylic acid before chromatographic separation. The amino acids were eluted with lithium citrate buffer from a cation-exchange column using a step gradient, followed by post-column ninhydrin derivatization and UV absorbance detection at 570 nm and 440 nm, respectively. Data on urinary levels of 21 selected amino acids for the second batch of specimens had been measured previously at the INTERMAP central laboratory in Leuven, Belgium, using a Biochrom 20 (Biochrom Ltd, Cambridge, UK) amino acid analyzer.

6.2.4 Statistics

Intra-specimen reliability of amino acid duplicate and triplicate measurements was tested separately on the 34 and 144 urine specimens of batches 1 and 2, respectively, by calculating the technical error (TE) of measurements, which is interpreted to be the typical magnitude of measurement error that one can expect to incur.[65] It was computed as the square root of the sum of variance between corresponding measurements divided by the number of urine specimens analyzed. For calculation of percent TE (%TE), TE was divided by the mean of all split sample values and multiplied by 100. Pearson's correlation analysis was implemented to calculate the association between the three techniques for each urinary amino acid. The Bland-Altman test[66] was employed to determine the 95% limits of agreement between the techniques. The urinary amino acid concentrations measured are reported as micromolar (µmol/L) ranges both uncorrected and corrected for the molar concentration of urinary creatinine, which had been measured previously at the INTERMAP central laboratory in Leuven by the Jaffe method.[63] The uncorrected values are given for direct comparison of urinary amino acid concentrations with the respective lower limits of quantitation (LLOQ) for GC-MS and iTRAQ®-LC-MS/MS. The LLOQ is defined as the lowest point of the calibration curve that can be determined with 80-120% accuracy, in agreement with the FDA Guide for Bioanalytical Method Validation.[48] We abstained from reporting means and standard deviations for urinary amino acid concentrations because of the small number of ethnogeographically diverse specimens. Data were analyzed using SAS/STAT 9.1 software (SAS Institute Inc., Cary, NC) and descriptive statistical functions implemented in Excel® 2004 for Mac (version 11.4.1, Microsoft Corp., Redmond, WA).

6.3 Results and Discussion

6.3.1 Reproducibility

First a blinded set of 98 urine samples was analyzed by GC-MS, iTRAQ®-LC-MS/MS and the amino acid analyzer. The set comprised 34 different urine specimens, from which 2 or 3 replicate aliquots had been prepared. All 34 urine specimens were analyzed by the amino acid analyzer, while only 33 and 31 specimens were subjected to GC-MS and iTRAQ®-LC-MS/MS, respectively, due to specimen volume limitations. Not all amino acids and their derivatives were amenable to analysis by all three methods and the same applied to the availability of stable isotope labeled internal standards for GC-MS analysis (Table 11).

Table 11: List of amino acids amenable to analysis by each of the three methods**.**

Amino Acid	Abbreviation	iTRAQ	GC-MS	Biochrom30
α-Aminoadipic Acid	Aad	X	X	X
α -Aminobutyric acid	Abu	X	X	X
Alanine	Ala	X	X	X
Anserine	Ans	X		X
Arginine	Arg	X		X
Argininosuccinic Acid	Asa	X		
Asparagine	Asn	X	X	X
Aspartic Acid	Asp	X	X	X
β-Alanine	β-Ala	X		
β-Aminoisobutyric acid	βAib	X	X	X
Carnosine	Car	X		X
Citrulline	Cit	X		X
Cystathione	Cth	X	X	X
Cystine	Cys-Cys	X	X	X
Ethanolamine	EtN	X		X
γ-Aminobutyric Acid	GABA	X		X
Glutamine	Gln	X	X	X
Glutamic acid	Glu	X	X	X
Glycine	Gly	X	X	X
Glycine-Proline	Gpr		X	
Homocitrulline	Hcit	X		
Homocystine	Hcys			
Hippuric acid	Hip		X	
Histidine	His	X	X	X

Hydroxylysine	Hyl	X	X	X
Hydroxyproline	Hyp	X	X	
allo-Isoleucine	allo-Ile		X	
Isoleucine	Ile	X	X	X
Leucine	Leu	X	X	X
Lysine	Lys	X	X	X
1-Methylhistidine	M1His	X		X
3-Methylhistidine	M3His	X		X
Methionine	Met	X	X	X
Ornithine	Orn	X	X	X
Phosphoethanolamine	PEtN	X		X
Phenylalanine	Phe	X	X	X
Proline	Pro	X	X	
Phosphoserine	Pser	X		X
Sarcosine	Sar	X	X	X
Serine	Ser	X		X
Taurine	Tau	X		X
Threonine	Thr	X		X
Tryptophan	Trp	X	X	X
Tyrosine	Tyr	X	X	X
Valine	Val	X	X	X

Number of analytes covered by the different methods ranged from 26 analytes for GC-MS to 40 and 42 for the amino acid analyzer and iTRAQ®-LC-MS/MS, respectively. As described previously,[47] urinary serine, threonine, hydroxylysine, and hydroxyproline, could not be measured reliably by the GC-MS method because of secondary interactions of their underivatized hydroxyl group with the liner in the injector, resulting in increasingly broader peaks within only a few injections. In addition, anserine, arginine, argininosuccinic acid, carnosine, citrulline, ethanolamine, γ-aminobutyric acid, homocitrulline, phosphoethanolamine, phosphoserine, taurine, and the methylhistidines were not amenable to GC-MS because of either their thermal instability (e.g., arginine) or low vapor pressure and high polarity (e.g., phosphoethanolamine). Quantification of ß-alanine by iTRAQ® was impeded by coeluting matrix components, hence it was excluded. Urinary levels of some amino acids, such as phosphoserine, cystathionine and proline, were low and, consequently, not all urine specimens analyzed yielded concentration values above the lower limits of quantitation, which are listed together with the ranges of urinary amino acid levels observed for both batches of urine specimens in Table 12.

Table 12: Range of urinary amino acid concentrations [μmol/L] uncorrected and corrected for urinary creatinine [μmol/mmol creatinine] in batches 1 and 2 (434 and 433 urine aliquots, respectively), and LLOQs [μmol/L] for GC-MS and iTRAQ®-LC-MS/MS.

Amino Acid	GC-MS (N=434) μmol/L	iTRAQ® (N=433) μmol/L	GC-MS μmol/mmol creatinine*	iTRAQ® μmol/mmol creatinine*	GC-MS LLOQ μmol/L	iTRAQ® LLOQ μmol/L
Aad	<3.00 – 99.28	3.58 – 153.12	0.05 – 0.83	0.07 – 1.22	3	0.5
Abu	0.38 – 35.80	<0.5 – 40.40	0.01 – 0.23	0.02 – 0.27	0.3	0.5
Ala	19.56 – 1072.70	22.19 – 1376.46	0.4 – 7.82	0.39 – 10.9	0.3	1
Ans	UD	<1.00 – 806.71	-	0.01 – 9.12	-	1
Arg	UD	<5.00 – 128.58	-	0.06 – 1.73	-	5
Asa	UD	<5 – 37.83	-	0.03 – 0.49		5
Asn	10.62 – 550.48	17.31 – 713.97	0.22 – 5.0	0.36 – 6.39	12	5
Asp	<3.00 – 65.44	0.66 – 49.67	0.02 – 0.48	0.01 – 0.35	3	0.5
βAib	6.50 – 2299.96	4.64 – 2523.59	0.09 – 27.3	0.06 – 23.05	0.9	0.5
Car	UD	1.43 – 260.80	-	0.02 – 5.87	-	1
Cit	UD	<0.50 – 30.80	-	0.09 – 0.4	-	0.5
Cys-Cys	<12.00 – 355.24	<10 – 1491.36	0.15 – 2.43	0.21 – 15.62	12	10
EtN	UD	60.45 – 803.76	-	0.67 – 10.53	-	0.5
GABA	UD	<1 – 23.96	-	0.01 – 0.49	-	1
Gln	32.06 – 1753.00	37.15 – 1867.69	0.66 – 21.51	0.59 – 31.49	30	0.5
Glu	1.60 – 38.76	2.18 – 36.19	0.06 – 0.72	0.05 – 0.65	3	0.5
Gly	70.60 – 5175.28	124.50 – 6524.52	1.44 – 69.44	1.89 – 121.8	3	0.5
Gpr	<3.00 – 35.36	UD	0.02 – 0.45	-	3	-
Hcit	UD	<5.00 – 163.69	-	0.07 – 1.13	-	5
Hip	42.08 – 5148.88	UD	0.34 – 111.3	-	30	-
His	54.58 – 2444.74	55.27 – 2865.53	1.16 – 19.25	0.95 – 34.4	12	0.5
Hyl	UD	<1.00 – 76.31	-	0.02 – 0.94	12	1
Hyp	UD	<0.5 – 65.15	-	0.003 – 0.41	3	0.5
Allo-Ile	<0.9 – 10.3	UD	0.004 – 0.06	-	0.9	-
Ile	1.44 – 40.72	1.47 – 51.24	0.03 – 0.25	0.03 – 0.32	0.9	0.5
Leu	3.42 – 96.56	3.63 – 103.33	0.07 – 0.63	0.07 – 2.45	0.3	0.5
Lys	7.06 – 1862.82	8.6 – 2206.81	0.14 – 25.0	0.15 – 29.61	0.9	0.5
Met	<3.00 – 18.90	<0.5 – 18.81	0.01 – 0.13	0.004 – 0.24	3	0.5
M1His	UD	7.98 – 5614.71	-	0.1 – 63.44	-	1
M3His	UD	10.15 – 2966.78	-	0.14 – 40.04	-	0.5
Orn	1.66 – 75.78	<5.00 – 110.66	0.03 – 1.02	0.04 – 1.49	0.9	5
PEtN	UD	2.40 – 106.43	-	0.05 – 0.9	-	0.5
Phe	6.62 – 192.74	6.50 – 220.00	0.12 – 1.14	0.12 – 1.2	0.9	0.5
Pro	0.94 – 24.60	<5 – 24.19	0.02 – 0.32	0.03 – 0.25	0.3	5
Sar	0.92 – 7.94	0.6 – 11.01	0.01 – 0.14	0.01 – 0.14	0.9	0.5
Ser	UD	48.99 – 1092.64	-	0.72 – 9.77	-	0.5
Tau	UD	11.88 – 5238.65	-	0.27 – 71.84	-	1
Thr	UD	10.33 – 498.27	-	0.2 – 6.11	-	1
Trp	5.88 – 242.08	7.17 – 269.72	0.12 – 1.3	0.1 – 1.45	0.3	0.5
Tyr	8.76 – 350.36	8.8 – 363.43	0.18 – 2.03	0.14 – 2.4	0.9	1
Val	4.98 – 136.10	4.21 – 146.67	0.1 - 0.82	0.1 – 0.92	0.3	1

*****Ranges are only given for amino acid concentrations above the LLOQ, UD, undeterminable.

For amino acids, for which not all urine specimens could be included in computation of %TE due to limits of quantitation, the actual number of specimens is given in brackets next to the %TE value in Table 13. Average percent technical error (%TE) over all sample replicates was calculated for each amino acid in Table 13.

Table 13: Percent technical errors computed from duplicate and triplicate measurements of urinary amino acids for batches #1 and #2 of urine specimens. Number of duplicates or triplicates used for computing percent technical error is given in brackets. Urine specimens with amino acid levels below the lower limit of quantitation were excluded**.**

Amino acid	First batch			Second batch		
	iTRAQ (N=31)	GC-MS (N=33)	Biochrom30 (N=34)	iTRAQ (N=143)	GC-MS (N=144)	Biochrom20 (N=101)
Aad	11.08	34.84 (30)	6.72	22.73	4.08	ND
Abu	22.15 (30)	56.54	5.26	20.37	6.63	ND
Ala	9.90	16.33	2.20	23.54	3.38	4.02
β-Ala	UD	ND	5.65 (10)	UD	ND	ND
Ans	46.81 (22)	UD	5.24 (18)	50.53 (132)	UD	ND
Arg	17.67 (28)	UD	7.45	22.25 (140)	UD	15.60 (84)
Asa	<LLOQ	UD	<LLOQ	43.15 (94)	UD	ND
Asn	13.40	16.21	5.00	18.86	4.16	5.86
Asp	21.43	12.80 (16)	12.00	25.55	15.02 (138)	ND
β-Aib	64.26	33.49	10.95 (30)	63.99	11.02	ND
Car	18.59	UD	9.36 (3)	29.32	UD	8.23 (100)
Cit	22.45	UD	6.60	30.01 (141)	UD	ND
Cth	8.72 (9)	13.18 (6)	17.62 (26)	25.81 (6)	9.98 (18)	ND
Cys-Cys	14.91	31.65	3.29	73.31* (142)	14.02 (139)	5.84
EtN	7.30	UD	5.27	13.88	UD	7.53
GABA	26.01 (22)	UD	25.42	26.57	UD	ND
Gln	25.11	22.70	3.98	22.27	13.95	3.84
Glu	11.99	19.92	19.03 (32)	22.03	3.93	ND
Gly	13.91* (30)	19.22	2.98	40.64	4.47	2.66
Gpr	UD	36.25 (17)	ND	UD	28.69 (121)	ND
Hcit	21.50* (26)	UD	ND	30.24 (138)	UD	ND
Hip	UD	ND	UD	UD	25.08	UD
His	18.26	10.14	2.13	27.15	4.39	3.30
Hyl	33.72 (28)	UD	11.72 (24)	43.01 (133)	UD	ND
Hyp	36.93 (31)	UD	<LLOQ	23.05 (37)	UD	ND
allo-Ile	UD	<LLOQ	ND	UD	5.23 (30)	ND
Ile	6.60	15.24	16.05 (28)	18.32	5.22	16.86 (60)
Leu	52.15	14.29	9.06 (30)	16.59	4.13	ND
Lys	18.96	20.73	6.27	50.60	4.53	5.72
Met	16.12 (27)	20.16 (8)	<LLOQ	115.64 (102)	10.30 (79)	19.01 (95)
M1His	14.89	UD	6.76	35.78	UD	3.30
M3His	17.01	UD	2.92	21.17	UD	4.80

Orn	15.40 (25)	23.11	4.00	33.76 (121)	9.13	ND
PEtN	6.90	UD	5.58* (33)	17.56	UD	ND
Phe	11.92	16.15	4.07	16.45	4.10	10.60 (99)
Pro	7.51 (7)	18.76	<LLOQ	18.21 (89)	5.65	ND
Pser	13.11 (2)	UD	<LLOQ	23.05 (37)	UD	ND
Sar	22.20	ND	11.40 (32)	23.74	7.49 (104)	ND
Ser	19.28	UD	2.39	15.38	UD	3.56 (100)
Tau	15.75	UD	4.53	20.84	UD	3.01
Thr	13.33	UD	2.56	23.75	UD	4.18
Trp	9.49	12.80	5.04	18.22	4.29	12.69 (82)
Tyr	9.51	22.10	2.57	16.30	4.37	6.63
Val	7.74	12.15	7.15	18.54	3.85	12.07 (98)

*One outlier exceeding 8 SDs of the mean excluded;
ND, not determined;
UD, undeterminable.

For 20 urinary amino acids, quantitative data were available from all three methods; the following comparison of reproducibility is limited to those analytes. Mean ± SD of %TE (range) for the 20 amino acids was 7.27±5.22 (2.13-19.03), 21.18±10.94 (10.14-56.54), and 18.34±14.67 (6.60-64.26), respectively, for amino acid analyzer, GC-MS, and iTRAQ®-LC-MS/MS. For α-aminoadipic acid (Aad), α-aminobutyric acid (Abu), ß-aminoisobutyric acid (β Aib), cystathionine (Cth), and cystine (Cys-Cys), no stable isotope labeled standards had been available for GC-MS analysis of the first batch of urine specimens. As a result, the concentrations of Aad, Abu, β-Aib, Cth, and Cys-Cys, had to be calculated using the nearest eluting stable isotope standard as a reference. However, this fails to account fully for any variation of ionization that may occur between analyses and, therefore, technical error is expected to be higher. This was confirmed by excluding Aad, Aba, βAib, and Cys-Cys, from computation of TE. For GC-MS, %TE improved from 21.60±11.07 (mean ± SD) to 16.93±4.15, range 10.14-23.11. For iTRAQ®-LC-MS/MS, the corresponding values also improved slightly from 18.85±14.89 to 16.38±11.19, range 6.60-52.15, due to omission of the high %TE associated with the measurement of βAib.

Limiting the above comparison to amino acid analyzer and iTRAQ®-LC-MS/MS and the 34 amino acids that could be measured by both methods, mean ± SD (range) of %TE was 7.43±5.43 (2.13-25.42) and 19.08±12.92 (6.60-64.26), respectively.

GC-MS and iTRAQ®-LC-MS/MS were further evaluated with a second batch of 341 split samples from 144 INTERMAP urine specimens randomly selected from the same five population samples used for the first batch. For 101 urine specimens from these population samples, urinary levels of 21 selected amino acids had been analyzed previously in duplicate at the INTERMAP central laboratory using a Biochrom 20 amino acid analyzer. For 13/21 amino acids with urinary levels determined successfully by amino acid analyzer, GC-MS, and iTRAQ®-LC-MS/MS, mean ± SD (range) of %TE was 8.39±5.35 (2.66-19.01), 6.23±3.84 (3.38-14.02), and 35.37±29.42 (16.30-115.64), respectively. Excluding methionine, whose urinary levels were the least reproducible for amino acid analyzer and iTRAQ®-LC-MS/MS with %TEs of 19.01 and 115.64, respectively, reduced the corresponding average %TEs to 7.51±4.48, 5.89±3.80, and 28.68±17.59. Expanding the comparison to all 21 amino acids amenable to both amino acid analyzer and iTRAQ®-LC-MS/MS yielded average %TEs of 7.59±4.96 and 30.90±23.88, respectively.

Overall, including the %TEs of all amino acids whose urinary levels could be determined (Table 13), the amino acid analyzer yielded the most consistent results with average %TEs of 7.43±5.43 and 7.59±4.96 (mean ± SD) for batches 1 and 2, respectively, despite the fact that the measurements had been done on different instruments at different locations. GC-MS matched the reliability of the amino acid analyzer for the second batch of urine specimens only with an average %TE of 8.28±6.64, while the average %TE for the first smaller batch of urine specimen had been 21.69±10.67. There is no obvious reason for the improvement in precision for the second batch other than the gain in experience over time by the operator of GC-MS. Reproducibility of GC-MS measurements depends to a significant extent on the availability of stable isotope labeled amino acid standards that allow to account for variation of electron impact ionization due to matrix effects. This is immediately obvious from comparing the average %TE of 5.87±3.59 for the 17 amino acids, for which stable isotope labeled internal standards were available, with the average %TE of 13.03±8.31 for the 8 amino acids, whose concentrations were determined using the nearest eluting stable isotope labeled standard as reference. Hence, further improvements of GC-MS performance will depend on the successful synthesis of additional stable isotope labeled amino acids. This will not benefit the most significant drawback of GC-MS, namely, the comparatively small number of only 26 urinary amino acids and related amines amenable to successful analysis versus 34 and 40 for amino acid analyzer and iTRAQ®-LC-MS/MS, respectively. The latter method also carries the advantage of having stable isotope labeled internal standards available for 44 amino acids, including norleucine and norvaline that are added to biological samples to account for extraction and derivatization efficiency, respectively. As a consequence, one would expect iTRAQ®-LC-MS/MS to be highly reproducible. But for reasons discussed below, iTRAQ®-LC-MS/MS yielded the highest average %TE of 30.38±19.16 for the second batch of urine specimens and surpassed only slightly GC-MS with an average %TE of 19.08±12.58 for the first batch.

6.3.2 Correlation between methods

The second batch of urine samples had been analyzed previously on a Biochrom 20 amino acid analyzer at the INTERMAP central laboratory in Leuven, Belgium. These data and those obtained by GC-MS and iTRAQ®-LC-MS/MS were correlated with each other; Pearson r-values are listed in Table 14. The Pearson's correlation coefficients for the 12 amino acids that could be measured by both GC-MS and the amino acid analyzer ranged from 0.800 (Trp) to 0.980 (Gly). GC-MS and iTRAQ®-LC-MS/MS had 19 amino acids in common and showed generally good correlation (cystathionine was excluded, because its urinary levels were with few exceptions below the LLOQs of GC-MS and iTRAQ®-LC-MS/MS). The single exception was the sulfur containing amino acid cystine (r=0.822). The correlation coefficients for the remaining 18 analytes ranged between 0.934 (Glu) and 0.988 (Tyr). Urinary levels of 20 amino acids were available for the comparison of iTRAQ®-LC-MS/MS with the amino acid analyzer. Correlation coefficients for arginine (0.561), carnosine (0.801), cystine (0.811), isoleucine (0.802), taurine (0.885) tryptophan (0.764), and tyrosine (0.780) were poor. The correlation coefficients for the remaining 13 amino acids varied from 0.899 (Phe, Val) to 0.951 (Lys).

Table 14: Pearson correlation coefficients (R) and slopes computed from the mean concentrations of duplicate and triplicate measurements of 144 urine specimens using the amino acid analyzer Biochrom 20 , GC-MS and iTRAQ® -LC-MS/MS.

Amino Acid	GC-MS *vs.* Biochrom 20		iTRAQ®-LC-MS/MS *vs.* GC-MS		iTRAQ®-LC-MS/MS *vs.* Biochrom 20	
	R	slope	R	slope	R	Slope
Aad	-	-	0.968	1.258	-	-
Abu	-	-	0.953	0.974	-	-
βAib	-	-	0.967	0.722	-	-
Ala	0.970	0.928	0.979	1.175	0.944	0.823
Arg	-	-	-	-	0.561	0.900
Asn	0.953	0.719	0.986	1.050	0.940	1.170
Asp	-	-	0.929	0.618	-	-
Car	-	-	-	-	0.801	1.462
Cys	0.944	0.684	0.822	1.49	0.811	0.616
EtN	-	-	-	-	0.917	0.873
Glu	-	-	0.934	0.752	-	-
Gln	0.956	1.111	0.958	0.628	0.938	1.231
Gly	0.980	0.968	0.937	1.198	0.921	0.730
His	0.969	1.056	0.965	1.042	0.940	0.799
Ile	0.812	0.812	0.976	1.059	0.802	0.737
Leu	-	-	0.984	0.997	-	-
Lys	0.969	0.966	0.977	0.963	0.951	0.968
M1His	-	-	-	-	0.934	0.799
M3His	-	-	-	-	0.906	0.753
Orn	-	-	0.963	1.310	-	-
Phe	0.909	0.778	0.986	1.018	0.899	1.015
Ser	-	-	-	-	0.939	0.856
Tau	-	-	-	-	0.885	0.694
Thr	-	-	-	-	0.946	1.071
Trp	0.800	0.782	0.981	0.907	0.760	0.841
Tyr	0.844	0.525	0.988	0.974	0.807	1.318
Val	0.912	0.995	0.983	0.952	0.899	0.851

6.3.3 Bland-Altman plots

Bland-Altman plots depict agreement between two different analytical methods: This graphical method plots the concentration difference between the two techniques for each specimen against the average of the two techniques. In addition, the mean difference *(đ)* and lower and upper limits of agreement are shown as horizontal lines. The limits of agreement are defined as the mean difference plus/minus 1.96 times the standard deviation (đ ± 1.96 SD). The mean difference, limits of agreement and the type of plot obtained are listed in Table 15.

Table 15: Mean differences ($\bar{d}$) and limits of agreement ($\bar{d}$ ± 1.96 SD) between methods in µM and types of Bland-Altman plots (TP*).

AA	Biochrom vs. GC-MS			GC-MS vs. iTRAQ			BIOCHROM vs. iTRAQ		
	đ	**±1.96 SD**	**TP**	**đ**	**±1.96 SD**	**TP**	**đ**	**±1.96 SD**	**TP**
Aad				-7.45	-24.95 – 10.04	**E**			
Abu				-0.89	-4.96 – 3.18	**A**			
bAib				98.96	-320.6 – 518.6	**D**			
Ala	23.2	-55.7 – 102.0	**A**	-11.2	-134.1 – 111.7	**F**	11.9	-135.9 – 159.8	**A**
Arg							-4.76	-42.1 – 32.5	**C**
Asn	31.57	-39.2 – 102.4	**D**	-7.96	-49.1 – 33.1	**F**	23.7	-54.0 – 101.4	**F**
Asp				4.54	-2.1 – 11.1	**D**			
Car							70.8	1.1 – 140.5	**D**
Cys	18.0	-14.8 – 50.8	**D**	-26.29	-139.31 – 86.72	**E**	-8.27	-117.83 – 101.28	**E**
EtN							-15.1	-127.6 – 97.5	**A**
Gln	- 59.3	-219.9 – 101.3	**C**	141.7	-83.0 – 366.3	**D**	82.4	-84.7 – 249.4	**D**
Glu				2.95	-3.3 – 9.2	**B**			
Gly	2.2	-292.1 – 296.5	**A**	-44.9	-927.0 – 837.2	**A**	-42.6	-954.8 – 869.6	**A**
His	- 44.0	-254.4 – 166.3	**E**	-2.53	-340.7 – 335.7	**F**	-46.6	-440.3 – 347.1	**F**
Ile	- 1.9	-6.2 – 2.4	**C**	-0.75	-4.5 – 3.0	**A**	-2.7	-8.5 – 3.1	**C**
Leu				-0.12	-8.2 – 8.0	**A**			
Lys	68.5	-67.3 – 204.3	**D**	1.8	-192.6 – 196.1	**F**	70.4	-158.0 – 298.8	**F**
M1His							28.9	-524.0 – 581.7	**A**

M3His							-8.3	-107.5 – 90.9	**A**
Orn				-2.8	-14.3 – 8.7	**E**			
Phe	6.4	-16.6 – 29.3	**B**	-2.7	-15.6 – 10.1	**A**	3.7	-22.0 – 29.3	**A**
Ser							-3.0	-128.6 – 122.7	**A**
Tau							-121.5	-993.1 – 750.2	**E**
Thr							23.2	-48.3 – 94.8	**B**
Trp	-9.03	-48.5 – 30.4	**C**	5.1	-11.0 – 21.3	**A**	-4.7	-33.9 – 24.4	**A**
Tyr	5.49	-82.2 – 93.2	**A**	2.7	-20.0 – 25.4	**A**	4.86	-38.5 – 48.2	**A**
Val	- 2.35	-16.3 – 11.6	**F**	1.4	-8.0 – 10.8	**F**	-0.94	-17.0 – 15.1	**F**

*A, methods are interchangeable; B, absolute mean difference between two methods has a positive value exceeding 15% of mean concentration for all measurements; C, absolute mean difference between two methods has a negative value exceeding 15% of mean concentration for all measurements; D, absolute mean difference becomes proportionatly more positive the higher the analyte concentration; E, absolute mean difference becomes proportionatly more negative the higher the analyte concentration; F, absolute mean difference increases with analyte concentration.

Since it is not possible to display all plots, each Bland-Altman plot was categorized according to its graphical appearance and six major plot types were defined.

Type A: Type A represents the ideal agreement between two methods. The mean difference is almost zero and the individual differences scatter randomly with no apparent systematic error. For type A plots, the mean of the difference is lower than 15 % of the mean concentration over all measurements obtained with two methods. A typical plot is shown in Figure 29 a for glycine (comparison of GC-MS to iTRAQ®-LC-MS/MS). Here the mean of the concentration over all measurements for both methods is 991.6 µmol/L and the mean of the difference is -44.9 µmol/L.

Type B: If the mean difference has a negative value and is higher than 15 % of the mean concentration over all measurements, the Bland-Altman plot is labeled as type B. In this case an absolute systematic error is detected, because the first analytical method underquantifys compared to the second method as is exemplified in Figure 29 b for the analysis of arginine by Biochrom and iTRAQ®-LC-MS/MS.

Type C: Type C equals type B, but the mean difference has a positive value indicating that the first method overquantifys relative to the second method. An example is shown in Figure 29 c for glutamic acid and the comparison of GC-MS with iTRAQ®-LC-MS/MS.

Type D: Type D plots represent a proportional error in the agreement between the methods. In this case the first method underquantitates the more the higher the concentration of the analyte. An example for type D is the comparison between Biochrom and GC-MS for lysine (Figure 29 d).

Type E: In case of type E plots the first method overquantitys the more the higher the concentration of the analyte. This is exemplified for the comparison between GC-MS and iTRAQ®-LC-MS/MS for cystine (Figure 29 e).

Type F: Type F indicates that variation of at least one method depends strongly on the magnitude of measurements as shown in Figure 29 f for valine (Biochrom vs. iTRAQ®-LC-MS/MS).

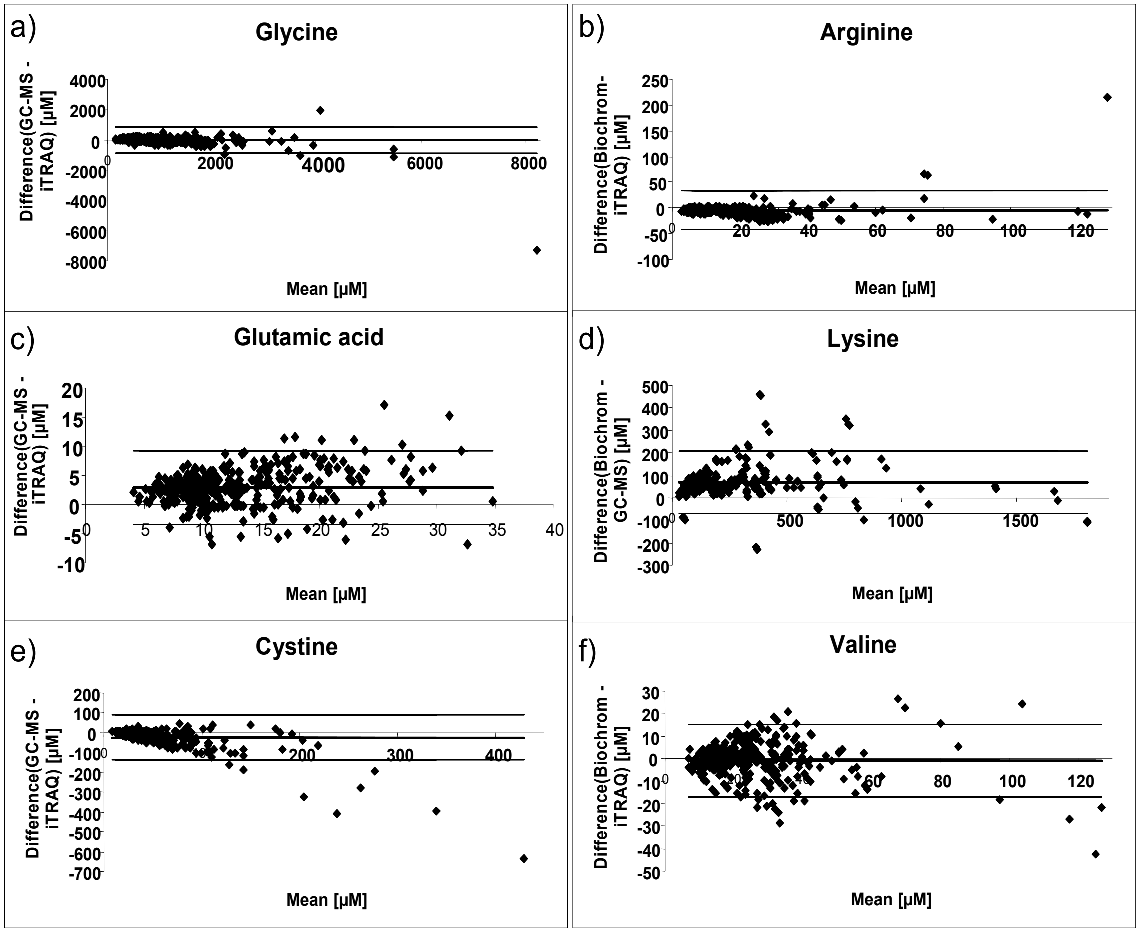


Figure 29: Different types of Bland-Altman plots: (a) type A with glycine shown as an example; (b) type B with arginine as an example; (c) type C with glutamic acid as an example; (d) type D with lysine as an example; (e) type E with cystine as and example; and (f) type F with valine as an example.

Overall, only 19 out of 51 (37.3%) Bland-Altman plots revealed an excellent type A agreement between any of two methods compared. Glycine and tyrosine were the only amino acids with quantitative data that agreed well across all three methods, i.e. for these amino acids the three methods are interchangeable. For phenylalanine and tryptophan, type A agreements were observed between GC-MS and iTRAQ®-LC-MS/MS as well as BIOCHROM and iTRAQ®-LC-MS/MS, while absolute systematic errors were found between BIOCHROM and GC-MS, with the former method either slightly under- (Trp) or overquantifying (Phe) in comparison to GC-MS. In case of isoleucine, BIOCHROM underquantitated relative to both GC-MS and iTRAQ®-LC-MS/MS, while the latter two methods showed type A agreement. Overall, absolute systematic errors were observed in 8 (15.7%) instances; proportional errors, i.e. mean difference rises (type D) or falls (type E) with increasing urinary amino acid concentrations, in 8 (15.7%) and 6 (11.8%) cases, respectively; in 10 (19.6%) cases, variation of at least one method depended strongly on magnitude of measurements (type F), i.e. error proportional to concentration of the quantity being measured.

Especially, since only 7 out of 19 (36.8%) comparisons between GC-MS and iTRAQ® showed excellent agreement over the urinary amino acid concentrations measured, and 5 other comparisons revealed a multiplicative error (type F), we validated the accuracy of these methods using a NIST certified amino acid standard.

6.3.4 Validation with a certified standard

The certified NIST standard, comprising a total of 17 amino acids, was analyzed to validate GC-MS and iTRAQ®-LC-MS/MS. We quantitated 16 amino acids with the GC-MS method. Arginine could not be determined due to the thermal instability of its propyl chloroformate derivative. An excellent correspondence with the NIST certified values was obtained for all amino acids measured by GC-MS and iTRAQ®-LC-MS/MS (Figure 30). The recoveries for GC-MS varied from 98-111% and for iTRAQ®-LC-MS/MS from 91-106%. Overall, GC-MS tended to overestimate the NIST certified values by 5.33±3.70% (mean ± standard deviation), whereas iTRAQ®-LC-MS/MS, on average, matched the certified values well with -0.04±4.18%. The reproducibility of the GC-MS data was excellent with relative standard deviations (RSDs) of about 1% (based on 6 replicate measurements) for most amino acids. The iTRAQ®-LC-MS/MS data showed RSDs of about 3-6% based on 40 replicate measurements.

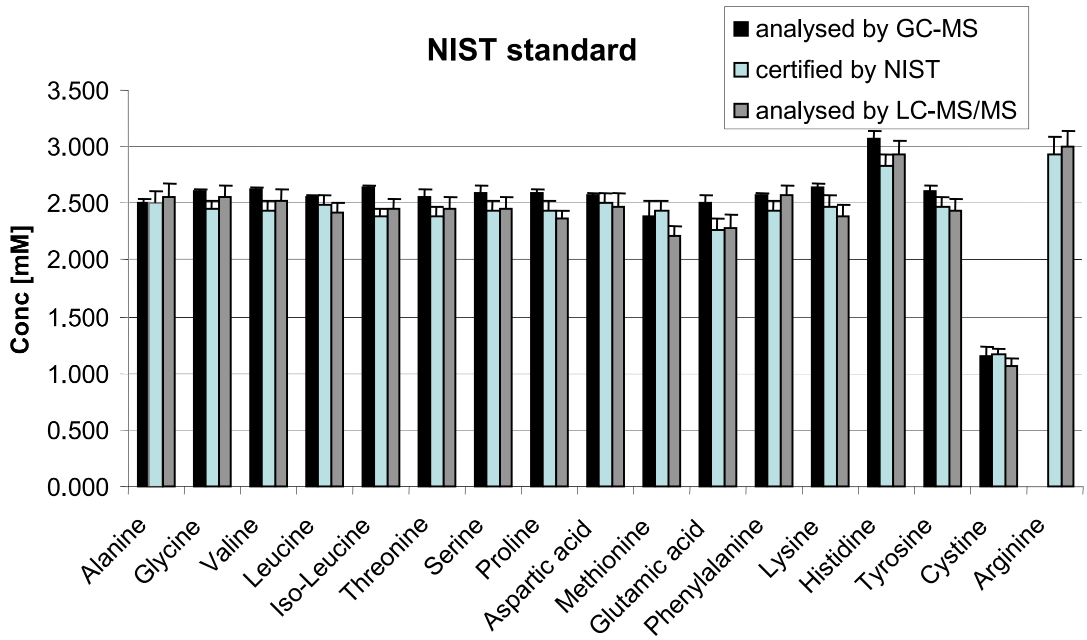


Figure 30: Arithmetic means and standard deviations of amino acid concentrations [mM] in a NIST-certified standard that was analyzed by GC-MS (n=6) and LC-MS/MS (n=40).

Both GC-MS and iTRAQ®-LC-MS/MS quantitated accurately the concentration of cystine in the acidified NIST standard, which does not contain any free cysteine. In urine, however, iTRAQ®-LC-MS/MS consistently overquantitated cystine with the difference from GC-MS and the amino acid analyzer becoming greater with higher urinary cystine levels (Table 14, Figure 29 e). Cysteine may oxidize under non-acidic conditions to cystine; the rapid disappearance of small amounts of urinary cysteine has been reported in non-acidified urine in contact with air.[67] Although the urine specimens were alkalized with borate buffer to pH 8.5 for the labeling of amino acids with the iTRAQ® reagent, followed by the addition of a 1.2% hydroxylamine solution after completion of the labeling reaction to reverse partial labeling of the phenolic hydroxyl group of tyrosine and to stabilize cysteine to prevent its oxidation to cystine, the excess in urinary cystine by iTRAQ®-LC-MS/MS far exceeded the reported levels of urinary cysteine, which is typically present at about 10% of cystine.[67] Therefore, reasons other than the potential oxidation of cysteine to cystine have to account for the apparent overquantitation of urinary cystine.

6.3.5 Comparison of methods

Both, amino acid analyzer and iTRAQ®-LC-MS/MS require protein precipitation. GC-MS allows the direct derivatization of amino acids with propyl chloroformate in native urine and, therefore, automation of the entire analytical procedure. The urine volumes needed for GC-MS and iTRAQ®-LC-MS/MS analysis are 40-50 µL, while 200 µL are required for the amino acid analyzer. Given that urine is typically available in large quantities, these differences in sample volume are negligible.

A drawback of the amino acid analyzer is the typical runtime of 130 min. In contrast, total runtimes for GC-MS and iTRAQ®-LC-MS/MS are 20 and 25 min, respectively. The LLOQs for the amino acid analyzer (2-3 µmol/L) are also on average higher than those for GC-MS (0.3-30 µmol/L) and iTRAQ®-LC-MS/MS (0.5-10 µmol/L).

A disadvantage of GC-MS is the smaller number of amino acids amenable to analysis. In principle, 33 urinary amino acids can be detected by GC-MS, but only 22 amino acids were measurable above the LLOQ in ≥ 80% of the 144 urine specimens of the second batch. In contrast, it was possible to quantify 34 analytes in at least 80% of the urine specimens by iTRAQ®-LC-MS/MS.

The higher TEs of iTRAQ®-LC-MS/MS appear to be mainly due to excess of multiple reaction-monitoring transitions acquired in the third of the four predefined time windows. In the first, second, and fourth period, 3 (PSer, PEtN, Tau), 7 (Asn, Ser, Hyp, Gly, Gln, EtN, Asp), and 10 (Val, Nva, Met, Tyr, Hcy, Ile, Leu, Nle, Phe, Trp) amino acids are monitored, respectively. In contrast, in the third period 24 amino acids (Cit, Sar, bAla, Ala, Thr, Glu, His, 3MHis, 1MHis, Hcit, Asa, GABA, bAib, Abu, Aad, Ans, Car, Pro, Arg, Hyl, Orn, Cth, Cys, Lys) are monitored, with only half as many data points recorded. This has a significant influence on the reproducibility of peak areas. For the second batch of urine specimens, mean ± SD of %TE (range) was 33.09±14.60 (18.21-73.31) for period 3, while it was (excluding methionine) 21.16±7.39 (13.88-40.64) for periods 1, 2, and 4. This shortcoming may be alleviated by recent implementation of scheduled sMRM that allow definition of as many overlapping periods as there are amino acids, with each amino acid monitored only for the time period of its expected elution from the column. For maximum precision, chromatographic resolution of amino acids will have to be improved to limit number of overlapping periods.

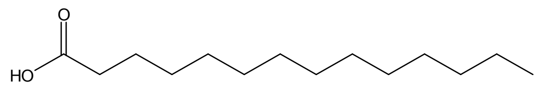
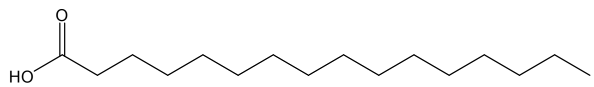
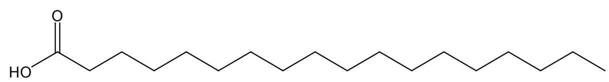
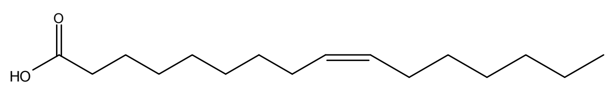
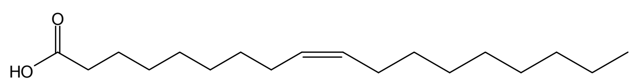
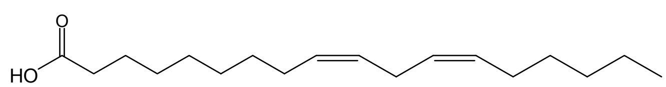
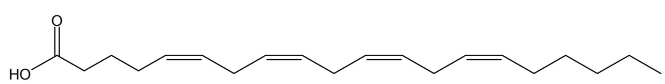
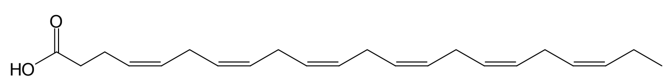
In conclusion, GC-MS and LC-MS/MS are attractive alternatives to the amino acid analyzer. The advantages of GC-MS are its complete automation, short runtime, and higher precision; its one limitation is the smaller number of amino acids amenable to analysis. In comparison, iTRAQ®-LC-MS/MS excels in greater number of amino acids amenable to analysis and current availability of 42 stable isotope labeled standards. Incorporation of scheduled MRM, improved chromatographic resolution, and an advanced integration algorithm may improve reproducibility of the iTRAQ® method.

7 Combined amino and fatty acid analysis by GC-MS

7.1 Introduction

An important sub-compartment of the metabolome are lipids and their comprehensive analysis is the subject of lipidomics.[68, 69] Lipids can be categorized into several classes, including for example non-esterified fatty acids (NEFAs), triglycerides and phospholipids. Essentially all NEFAs in serum are bound to albumin.[70] Fatty acids are compound with a carboxyl group and an alkyl chain which is either saturated or unsaturated and differ in length. Most of the natural fatty acids have an even number of carbon atoms, usually 14, 16, 18 or 20 because their biosynthesis involves acetyl-CoA, a coenzyme carrying a two-carbon-atom group. The double bond can occur in a cis or trans configuration. In general, the fatty acids in biological systems contain only cis double bonds. The most common fatty acids in mammals are shown in Table 1. Fatty acids, in particular stearic acid, oleic acid, linolenic acid and palmitic acid, are precursors for the synthesis of cholesterol and steroid hormones that regulate a wide range of functions, including blood pressure, blood clotting, blood lipid levels, immune and inflammatory responses to injury and infection.[71, 72] Several analytical methods have been developed for lipid analysis. Lipid extraction is commonly performed by Folch[73] or Bligh & Dyer extraction.[74] Another approach is the determination of the fatty acid composition by GC-FID or GC-MS after conversion into fatty acid methyl esters (FAMEs). Esterification is achieved using acidic methanol[75], iodomethane or BF_3/methanol. To avoid lengthy sample preparation steps Lepage & Roy developed a method for the direct transesterification of all classes of lipids in plasma using an acetyl chloride/methanol mixture.[76] This method was further simplified by Masood *et al.* by introducing a one-step stock solution method, without the requirement for subsequent neutralization or centrifugation to separate phases.[77]

Table16: Common natural fatty acids (saturated and unsaturated)

Common name of acid	Abbreviation	Chemical structur
Saturated fatty acids		
Myristic acid	C14:0	
Palmitic acid	C16:0	
Stearic acid	C18:0	
Unsaturated acid		
Palmitoleic acid	C16:1	
Oleic acid	C18:1	
Linoleic acid	C18:2	
Arachidonic acid	C20:4	
Docosahexanoic acid	C22:6	

Husek *et al.* reported the applicapility of alkyl chloroformate derivatization to fatty acids.[37] Mateo-Castro *et al.* used ethyl chloroformate derivatization in aqueous medium to analyze amino, fatty and bile acids by GC-FID in binders used in artistic paintings after the samples were hydrolyzed by HCl.[78] Our aim was to integrate fatty acids in the fully automated quantitative method by GC-MS using propyl chloroformate derivatization to analyze amino acids and fatty acids in the same analysis. The analysis of the fatty acids should allow either the analysis of free fatty acids, or the total fatty acids from all lipid classes. Due to the behavior of propyl chloroformate in base as a good esterifying reagent, the fatty acids that are attached to other molecules should react with the reagent immediately.

7.2 Materials and methods

7.2.1 Chemicals

The fatty acids C10:0, C12:0, C14:0, C16:1, C16:0, C18:1 cis, C18:0, C18:1 trans, C18: 2, C18:3, C20:0, C20:4, C22:1, C22:0, C22:6, C24:1, and C24:0 were purchased from Sigma. The [U-13C] fatty acid mix was from Medical Isotopes Inc. (Pelham, USA).

7.2.2 Biological samples

The applicability of the method was tested using human, mice and bovine serum. Human serum was collected from healthy volunteers. Bovine serum was provided by collaborators from the clinic for ruminants in the veterinary faculty of the Ludwig-Maximilians-University Munich (LMU). Mice serum was obtained from collaborators at the University of Regensburg. Due to low sample volume available from the latter, several samples were pooled to perform reproducibility studies. Twenty microliters of serum were always used.

7.2.3 GC-MS analysis

To that end, the method was adapted to allow the combined analysis of fatty acids and free amino acids in a single gas chromatographic run. The retention times and specific ion traces for the SIM analysis of the fatty acids C10:0, C12:0, C14:0, C16:1, C16:0, C18:1 cis, C18:0, C18:1 trans, C18:2, C18:3, C20:0, C20:4, C22:1, C22:0, C22:6, C24:1, and C24:0, as well as 25 endogenous amino acids were determined and listed in Table 17. A temperature program was used starting at a temperature of 70°C and a heating rate of 5°C/min to 300°C, and then held for 3 min. Column gas flow was set to 1.1 mL He/min and a sample volume of 2.5 µL was injected with a split ratio of 1:5. A stable isotope labeled fatty acid mix, containing uniformly ^{13}C labeled C14:0, C16:0, C16:1, C18:0, C18:1 cis, C18:2 and C18:3 was integrated as internal standard for the fatty acids. The column and GC-MS is identical as discribed in chapter 5.2.3

Table 17: Retention times and ion traces selected for the SIM analysis of endogenous amino acids plus norvaline and 17 fatty acids. Analytes printed in bold were quantified using the internal standard quantification trace of the corresponding stable-isotope labeled compound as reference.

Analyte	Retention time (min)	Quantification trace	Secondary ion trace	Internal standard trace
C10:0	10.78	173	214	
Alanine	12.45	130	88	133
Sarcosine	12.9	130	217	
Glycine	13.19	102		105
α-Aminobutyric acid	13.96	144	102	
Valine	14.64	158	116	163
C12:0	14.92	183	242	
ß-Aminoisobutyric acid	13.19	116		
Norvaline	15.52	158	72	
Leucine	16.1	172	130	178
allo-Isoleucine	16.24	172	130	
Isoleucine	16.46	172		178
Proline	18.37	156		161
C14:0	18.79	270	211	284
Asparagine	19.04	155	69	160
C16:1n7	22.39	296	237	312
C16:0	22.36	298	239	314
Aspartic acid	22.53	216	130	220
Methionine	22.61	203	277	206
Hippuric acid		134	105	139
Glutamic acid	24.74	230		235
Phenylalanine	24.73	190	206	199
C18:1n9cis	25.59	324	265	342
C18:0	25.68	326	267	344
C18:1n9trans	25.71	324	265	
C18:2n6cis	25.85	322	263	340
C18:3n3	26.28	320	261	338
α-Aminoadipic acid	26.6	244		247
Glutamine	28.41	84	187	89
C20:0	28.7	354	354	
C20:4n6	28.73	346	287	
Ornithine	30.95	156	70	
C22:1n9	31.52	321	380	
C22:0	31.54	323	382	
C22:6n3	32.07	91	105	
Lysine	32.52	170	128	176

Histidine	33.59	282	168	290
C24:1	34.2	349	408	
C24:0	34.19	410	351	
Tyrosine	35.28	107	206	114
Tryptophan	36.8	130		140
Cystathionine	37.51	203	272	
Cystine	41.3	248	216	252

7.2.4 Derivatization

Twenty microliters of serum were transferred together with 20 µL of a stabilization reagent containing 10% iso-propanol, 0.1% phenol and 2% thiodiglycol to a 2-mL autosampler vial. Then 10 µL of the stable isotope labeled fatty acid mix, containing uniformly ^{13}C labeled C14:0, C16:0, C16:1, C18:0, C18:1 cis, C18:2 and C18:3 in *n*-propanol was added to the vial, followed by the addition of 10 µL of stable isotope labeled amino acid mix (descriped in chapter 5.2.4). Addition of the stable isotope labeled fatty acid mix was performed manually because addition of the *n*-propanol solution by the autosampler proved to be not reproducible (data not shown). The vial was then 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 135 µL water, followed by addition of 50 µL of *n*-propanol. Addition of n-propanol was performed, because for calibration purposes 50 µL of fatty acid standard in *n*-propanol were used and the percentage of *n*-propanol in the reaction mixture should be kept constant. The next steps were identical to those described in chapter 5.2.4.

7.2.5 Quantification

Absolute quantification of 25 amino acids (alanine, sarcosine, glycine, α-aminobutyric acid, valine, ß-aminoisobutyric acid, leucine, allo-isoleucine, isoleucine, proline, asparagine, aspartic acid, methionine, hippuric acid, glutamic acid, phenylalanine, α-aminoadipic acid, glutamine, ornithine, lysine, histidine, tyrosine, tryptophan, cystathionine and cystine) and 17 fatty acids (C10:0, C12:0, C14:0, C16:1, C16:0, C18:1 cis, C18:0, C18:1 trans, C18:2, C18:3, C20:0, C20:4, C22:1, C22:0, C22:6, C24:1, and C24:0) was performed by analyzing standard solutions. Single stock solutions of the fatty acids were prepared in *n*-propanol at concentrations of 100 mM (C10:0, C12:0, C14:0, C16:0, and C18:1 trans, C18:3), 81mM (C16:1), 65.9mM (C22:0), 50mM (C18:2, C18:1 cis, C18:0, and C20:0), 39 mM (C24:0), 14 mM (C20:4), and 5 mM (C22:6, C22:1, and C24:1). Using the single stock solutions a master mix of all fatty acids was prepared in *n*-propanol at a concentration of 1 mM for each analyte. A serial dilution containing 13 points of the master mix in *n*-propanol was prepared resulting in a concentration range of 0.24 µM- 1000 µM, keeping the volume of propanol constant at 50 µL.

7.3 Results and discussion

7.3.1 Method development

In comparison to the original protocol described in chapter 5 for the analysis of amino acids the number of amino acids quantified in the present method was modified. Threonine and serine were excluded because the quantification of these analytes proved to be not reproducible as already described in chapter 5.3.9. Moreover, thiaproline, hydroxyproline, hydroxylysine, glycyl-proline, α-aminopimelic acid and proline-hydroxyproline were not quantified because these analytes were not detected in the biological samples, specifically serum, analyzed so far in our laboratory. Another modification made to the original protocol is a longer temperature program. Using a temperature program with a start temperature of 70°C and a heating rate of 5°C/min provided sufficient separation of oleic (C18:1 n9 cis) and elaidic acid (C18:1 n9 trans). These two monounsaturated C18-acids yielded the same fragments after ionization by electron impact (EI) making baseline chromatographic separation crucial for their analysis. Stearic acid (C18:0) and elaidic acid were not baseline separated, but could be distinguished based on characteristic mass fragments. The ion traces for C18:0 and 18:1 isomers are shown in Figure 31.

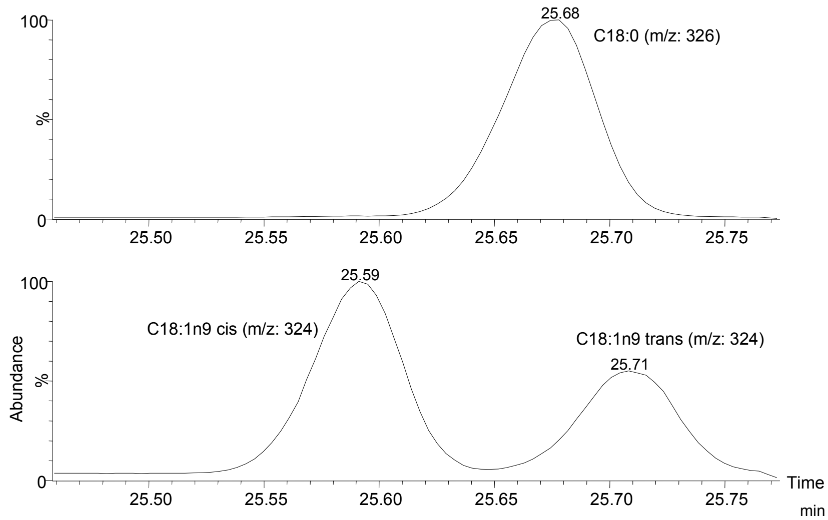


Figure 31: Separation of oleic acid (C18:1n9 cis), elaidic acid (C18:1 1n9 trans)and stearic acid (C18:0).

Therefore, the molecular ion, although not being very intense, is used for quantification because this allows the differentiation between fatty acids with the same carbon number but different degrees of unsaturation, such as C18:0 and C18:1 or C16:0 and 16:1. Figure 32 presents a typical chromatogram of the amino acids including norvaline and the 17 fatty acids. The analysis time was 50 min.

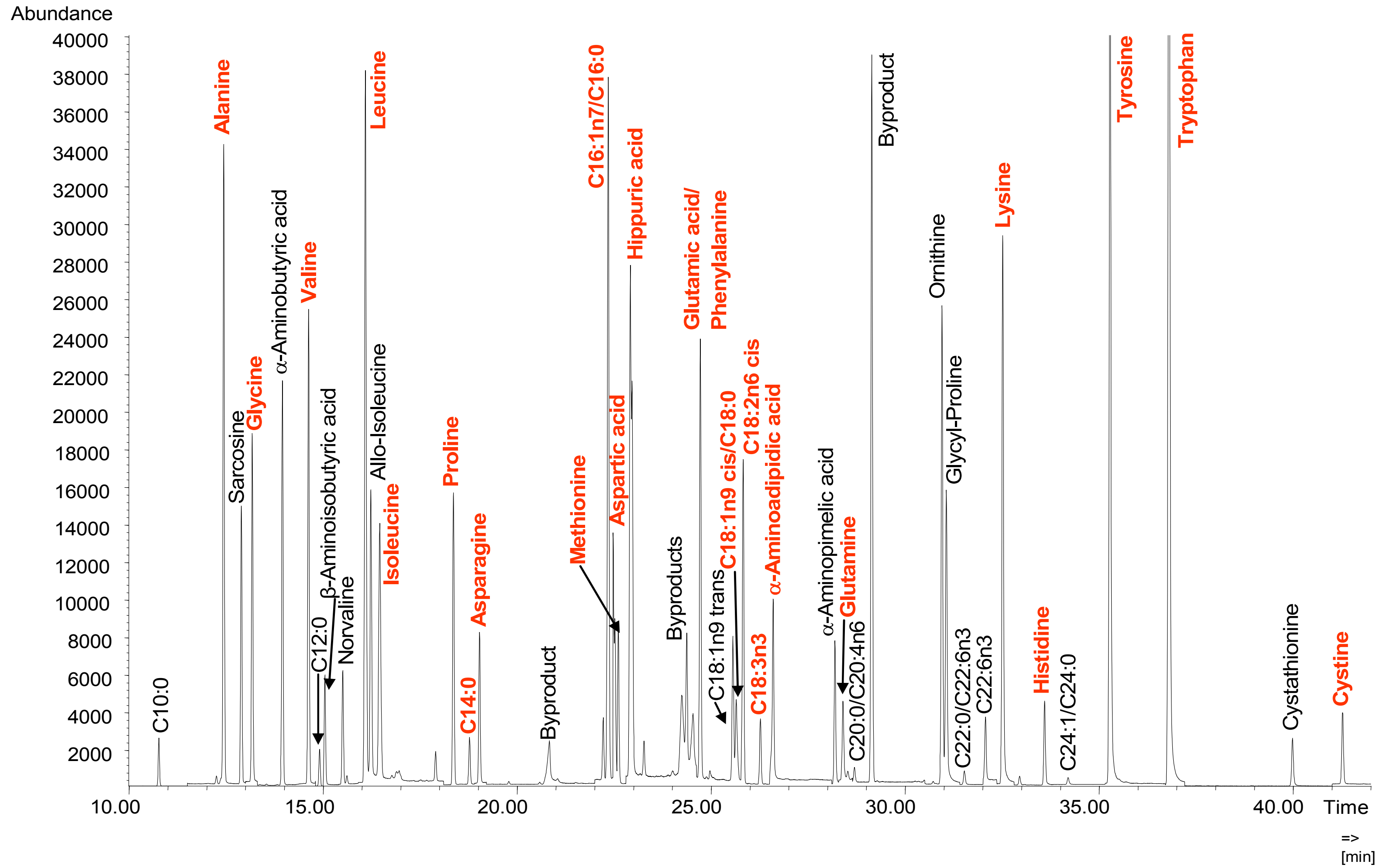


Figure 32: GC-MS analysis of fatty acids and amino acids standards after derivatization with propyl chloroformate.Analytes with their corresponding stable-isotope labeled are marked red.

7.3.2 Method characterization

A calibration was carried out using 13 calibration points. Figures of merit and calibration curve parameters are presented in Table 18. The R square-value or coefficient of determination was calculated as the square of the correlation coeffiient R of the regression analysis over the quantification range. 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 80-120%. The limit of detection (LOD) is defined as the concentration producing a signal to noise (S/N) ratio of at least 3:1. For some analytes it was observed that the stable isotope labeled internal standards contain minute amounts of the unlabeled analytes. In that case the LOD was defined as background analyte level plus three times the standard deviation of the background signal.

Table 18: Figures of merit and calibration curve parameters.

Analyte	**LOD[a,b] (µM)**	**LOQ[b] (µM)**	**ULOQ[b] (µM)**	**Regression line**	**R^{2c}**
C10:0	0.08	1.11	4375	45.687 * x + 1.302893	0.9994
Alanine	0.15	1.50	4375	1.370* x + 0.016749	0.9997
Sarcosine	0.30	1.50	525	1.207 * x + -0.008837	0.9996
Glycine	0.15	4.50	4375	1.455 * x + 0.461975	0.9993
α-Aminobutyric acid	0.15	4.50	4375	2.336 * x + -0.067501	0.9966
Valine	0.15	0.53	4375	1.306* x + 0.004960	0.9999
C12:0	0.31	2.44	2500	35.456 * x + 0.885587	0.9996
ß-Aminoisobutyric acid	1.50	4.50	525	0.767 * x + -0.027075	0.9976
Leucine	0.15	0.53	4375	1.257 * x + 0.005071	0.9998
allo-Isoleucine	0.30	0.53	4375	1.402 * x + 0.000658	0.9996
Isoleucine	0.15	0.53	4375	1.286 * x + 0.007381	0.9999
Proline	0.15	52.50	4375	0.817 * x + -0.337205	0.9972
C14:0	1.11	9.78	2500	6.481 * x + 0.305019	0.9996
Asparagine	1.50	4.50	525	1.325 * x + -0.013764	0.9980
C16:1n7	2.44	19.58	2500	14.855 * x + -1.077235	0.9990
C16:0	4.88	19.58	2500	0.425 * x + 0.306325	0.9999
Aspartic acid	1.50	7.50	4375	1.218 * x + -0.279939	0.9980
Methionine	0.30	30.00	4375	1.069 * x + 0.190182	0.9992
Hippuric acid	0.53	4.50	4375	0.093 * x + 0.073425	0.9999
Glutamic acid	4.50	30.00	1250	1.030 * x + 0.317407	0.9934
Phenylalanine	1.50	4.50	4375	1.369 * x + 0.011706	0.9993
C18:1n9cis	9.78	19.58	2500	0.681 * x + 0.240229	0.9999

C18:0	0.61	4.88	2500	9.410 * x + 1.560254	0.9986
C18:1n9trans	19.58	39.08	2500	0.401 * x + -0.084591	0.9969
C18:2n6cis	0.31	19.58	2500	2.951 * x + 1.154595	0.9992
C18:3n3	4.88	19.58	2500	5.302 * x + 2.658921	0.9995
α-Aminoadipic acid	0.53	30.00	525	5.426 * x + -1.15785	0.9934
Glutamine	7.50	30.00	525	1.564 * x + 0.073241	0.9995
C20:0	9.78	19.58	2500	6.720 * x + -1.453153	0.9966
C20:4n6	39.08	78.13	2500	0.704 * x + -0.416711	0.9986
Ornithine	0.30	1.50	525	1.219 * x + -0.013352	0.9992
C22:1n9	19.58	78.13	2500	0.214 * x + -0.157461	0.9981
C22:0	4.88	39.08	2500	4.983 * x + -2.349723	0.9962
C22:6n3	19.58	39.08	2500	143.757 * x + -89.363077	0.9967
Lysine	0.15	0.30	4375	1.348 * x + 0.006601	0.9997
Histidine	1.50	4.50	4375	1.764 * x + -0.017558	0.9996
C24:1	39.08	78.13	2500	0.132 * x + -0.123526	0.9976
C24:0	9.78	39.08	1250	4.097 * x + -1.994844	0.9945
Tyrosine	0.15	1.50	4375	1.207 * x + 0.009607	0.9998
Tryptophan	0.15	1.50	4375	1.327 * x + 0.002861	0.9999
Cystathionine	1.50	7.50	525	3.588 * x + -0.158190	0.9895
Cystine	0.53	7.50	1250	3.937 * x + 0.092151	0.9958

[a] Limit of detection (S/N≥3 or method blank plus 3 times standard deviation of method blank)
[b] LOD, LLOQ and ULOQ were calculated for a sample volume of 20 µL
[c] Coefficient of determination (square of the correlation coefficient r of the regression analysis)
Analytes printed in bold were quantified with a corresponding stable isotope labeled standard.

Concentrations reported in Table 18 were calculated for the analysis of 20-µL sample aliquots. LODs for the fatty acids ranged from 0.08 µM up to 39 µM. The lowest LOD (0.08 µM) was observed for C10:0, corresponding to an absolute injection amount of 16 fmol. However, C10:0 is also a fatty acid for which no stable isotope labeled internal standard was available and which is consequently not disturbed by a background signal. C12:0, C18:0 and C18:2n6 cis had also LODs below 1 µM, while for the remaining fatty acids higher values were determined. The highest LOD (39 µM) was found for C20:4, which is caused by the high degree of fragmentation observed during EI ionization. Therefore no intense fragment ion was available for quantification resulting in the high LOD. LODs for the amino acids ranged from 0.15 µM to 7.5 µM. The lowest LOD (0.15 µM) was observed for alanine, glycine, leucine, isoleucine, lysine, proline, tryptophan, tyrosine, valine and α-aminoadipic acid, while the highest value was determined for glutamine (7.5 µM). The range of LODs is similar to those described in chapter 5.3.4 Overall, lower detection limits were determined for the amino acids. Analysis of a standard solution (absolute amount in solution derivatized: fatty acids 6.25 nmol, amino acids 10.5 nmol) in six replicates yielded an accuracy ranging from 83.9 to 105.6% for the fatty acids and from 90.4 to 115.3% for the amino acids, respectively (datas not shown). Relative standard deviations (RSDs) were between 1.6 and 10.5% for the fatty acids with C24:0 showing the highest RSD. For the amino acids RSDs were below 4% with the exception of α-aminoadipic acid (12.1%). Inter-day reproducibility of replicate standard analyses was in the same range as observed for intra-day reproducibility (Table 19). In addition to a standard, the method precision was tested for the analysis of human serum, bovine serum and mice serum. Human and bovine sera were analyzed in six replicates while for mice serum only 4 replicates were measured due to the limited sample volume. The RSDs obtained for the fatty acids and amino acids analyzed in the different matrices are listed in Table 19. The reproducibility in all biological samples for all analytes was good, with RSDs ranging from 0.7 to 11%. The average reproducibility across all biological samples was excellent, but with 2.8% somewhat lower for the amino acids compared to the fatty acids with 5.5%. An influence of the serum type on the reproducibility was not observed. A number of analytes, such as C20:0, C22:1n9, C22:0, C24:1, C24:0, ß-aminoisobutyric acid, allo-Isoleucine, α-aminoadipic acid, and cystathionine were not detected above the LLOQ in the serum samples, while detection of some analytes above the LLOQ depended on the serum type, for example C10:0 and C12:0 were only detected in human serum and hippuric acid was only found bovine serum.

Table 19: Reproducibility of GC-MS analysis of fatty acids and amino acids solved in *n*-propanol and water, respectively and in different biological matrices using 20 µL sample aliquots. Reproducibility is given as relative standard deviation [%]. Concentration of standard in absolute amount: fatty acids 6.25 nmol, amino acids 10.5 nmol.

Analyte	**Human serum (n=6)**	**Bovine serum (n=6)**	**Mice serum (n=4)**	**Standard Intra-day (n=6)**	**Standard Inter-day (n=3)**
C10:0	9.29	n.d.	n.d.	4.21	5.36
Alanine	0.67	2.97	1.30	0.45	0.24
Sarcosine	n.d.	n.d.	4.62	3.20	5.05
Glycine	3.04	3.23	1.08	0.45	0.45
α-Aminobutyric acid	4.16	11.03	2.02	1.74	3.46
Valine	1.17	1.18	1.35	0.61	0.40
C12:0	6.54	n.d.	n.d.	3.49	6.03

ß-Aminoisobutyric acid	n.d.	n.d.	n.d.	2.55	2.17
Leucine	2.46	2.17	1.17	0.46	0.29
allo-Isoleucine	n.d.	n.d.	n.d.	1.09	0.73
Isoleucine	1.55	0.96	1.32	0.92	0.19
Proline	0.86	1.81	0.87	0.45	0.89
C14:0	8.75	10.30	n.d.	3.55	4.18
Asparagine	3.55	4.76	4.07	2.89	0.51
C16:1n7	5.46	6.58	10.72	8.90	2.88
C16:0	4.50	3.14	3.92	2.61	1.99
Aspartic acid	5.56	10.70	0.95	1.30	1.11
Methionine	n.d.	n.d.	2.19	1.80	1.82
Hippuric acid	n.d.	2.17	n.d.	0.41	0.49
Glutamic acid	5.33	n.d.	1.85	3.35	2.57
Phenylalanine	0.84	2.46	4.96	3.63	0.66
C18:1n9cis	3.73	2.75	6.12	2.27	3.71
C18:0	1.56	2.83	3.62	5.36	5.07
C18:1n9trans	8.69	n.d.	10.30	3.76	7.15
C18:2n6cis	2.74	3.24	4.99	1.86	1.15
C18:3n3	n.d.	2.83	n.d.	1.63	2.80
α-Aminoadipic acid	n.d.	n.d.	n.d.	12.06	12.52
Glutamine	4.12	2.35	3.78	3.24	2.79
C20:0	n.d.	n.d.	n.d.	6.80	0.30
C20:4n6	6.15	6.28	5.11	4.01	4.69
Ornithine	5.67	2.62	5.12	1.90	1.69
C22:1n9	n.d.	n.d.	n.d.	6.08	2.63
C22:0	n.d.	n.d.	n.d.	8.72	4.49
C22:6n3	3.87	n.d.	5.40	3.39	8.76
Lysine	1.56	1.95	1.82	0.40	0.05
Histidine	4.18	3.24	2.21	3.84	0.03
C24:1	n.d.	n.d.	n.d.	7.85	4.23
C24:0	n.d.	n.d.	n.d.	10.50	6.36
Tyrosine	1.68	1.81	1.57	0.64	0.93
Tryptophan	1.21	1.91	3.14	0.44	0.61
Cystathionine	n.d.	n.d.	n.d.	2.65	5.93
Cystine	0.87	n.d.	n.d.	1.03	4.84

n.d.: not detected above the LLOQ.
Analytes printed in bold were quantified with a corresponding stable isotope labeled standard.

Matrix spike experiments were performed using human and bovine serum to further validate the accuracy of the method. Twenty-µL aliquots of serum were spiked with 0, 1.56, 6.25 and 12.5 nmol absolute of the fatty acid standard and 0, 1.5, 6 and 9.3 nmol absolute of the amino acid standard. Each spike level was prepared and analyzed in triplicate. The zero spike level was used to subtract the endogenous analyte concentration in order to calculate a recovery for the three spike levels. The average recovery of the analytes at the three different levels in the human and bovine serum matrix in both matrices was 103.6%, ranging from

91% for C24:0 in bovine serum to 125.4% for C18:0 in bovine serum as well. The average recovery for the amino acids was 102.8%, ranging from 88.2% for α-aminobutyric acid up to 128.5% for glutamic acid, both in bovine serum. For some analytes in bovine serum, such as C18:0, glycine and proline, higher deviations from the spiked amount were observed at the first spike level. However, these analytes have higher endogenous levels in serum and the spike levels might be too low to evaluate these analytes correctly.

Table20: Recovery rates for amino and fatty acids in human and bovine serum at three different spike levels.

Analyte	**Spike level 1**		**Spike level 2**		**Spike level 3**	
	Human	**Bovine**	**Human**	**Bovine**	**Human**	**Bovine**
C10:0	106.8±5.1	108.4±9.2	107.2±6.1	106.3±7.8	106.5±3.5	108.7±4.5
Alanine	108.8±5.2	121.3±4.1	104.4±2.1	105±1	103±0.4	102.1±0.8
Sarcosine	106.9±8.4	111.7±10.2	115±2.7	112.8±10.2	114.4±7.9	103.8±8.9
Glycine	120.1±10.2	148.7±13.2	100.6±5.2	97.3±1.7	96.4±0.2	94.5±1.3
α-Aminobutyric acid	87.1±1.3	79.2±10.1	93.2±1.3	90.6±5.2	93.6±1.6	94.8±4.9
Valine	99.6±4.1	101.6±4.4	99.3±1.1	101.3±0.8	98.2±0.2	97.8±0.4
C12:0	101.4±2.2	101.9±6.9	103.9±5.3	104.4±3.1	104.6±3.6	106.7±5.9
ß-Aminoiso-butyric acid	92.3±2.4	87.1±5.8	95.7±1.1	93.3±2.3	93.6±0.3	95.2±1.8
Leucine	99.2±3.3	105.5±2.8	97.7±1	97.6±0.6	96.3±0.2	95.4±0.1
allo-Isoleucine	101.8±0.9	98.3±1.8	106.3±0.6	103.1±1	107.2±0.6	102.9±1
Isoleucine	102.8±1.1	103.5±2.7	102.5±0.9	103.5±0.4	101.9±0.2	100.9±0.2
Proline	116.9±6.1	182.9±0.5	85.4±1.1	86±2.1	85.1±0.2	84.3±0.2
C14:0	105.5±2.2	98.5±5.3	106.2±6.2	103.7±2.3	106.8±2.8	106.3±6
Asparagine	89.2±1.5	99.3±5.6	91.2±0.5	94.6±5.3	99.2±1.1	97.8±5.6
C16:1n7	97.3±2.6	84.7±2.1	103.6±6.3	89.6±4.8	105.2±2.5	100±6.2
C16:0	117.1±43.4	94.6±17	118.8±4.9	110.5±17.4	112.2±7.8	113±9.3
Aspartic acid	107.9±4.8	78.3±4.5	106.1±2.6	103.4±3.8	105.1±1.4	110.8±1.6
Methionine	110.5±1.9	107.7±5	110.5±1.2	102.7±2.2	110.7±1	105.6±4
Hippuric acid	99.4±2.2	106.3±0.7	98.7±0.7	98.5±1.6	97.8±0.2	96.3±0.7
Glutamic acid	121.6±6.9	118.7±6.7	120.5±0.9	140.2±3.5	114.7±6	126.7±3.7
Phenylalanine	101.4±1.5	101.7±0.9	99.6±0.8	99.4±0.3	98.5±0	97.8±0.7
C18:1n9cis	111.3±9	101.4±34.7	104.5±3.8	99.9+5.6	103.7±2.4	109.9±5.1
C18:0	115.1±29.1	141.5±32.2	111.1±14.5	115.3±4	108.7±6.2	119.3±2
C18:1n9trans	98.4±1.6	114.3±3.4	94.6±1.2	102.9±3.3	98.8±5.1	107.4±4.9
C18:2n6cis	118.5±14.8	111.6±28.5	118.1±4.2	112.8±2.6	108.3±2.8	115±7.6
C18:3n3	93.9±6.2	115.6±13.7	95.3±1.2	108.2±4.5	99.2±1.1	113.4±4.8

α-Aminoadipic acid	107.8±7.8	105.8±4.1	108.6±3.7	106.6±3.8	105.9±1.9	105.8±5.1
Glutamine	118.2±29.7	121.3±17.7	88.3±11	111.4±11.4	108.3±11.3	104.6±8.1
C20:0	99.5±3.1	103.7±10.2	98.7±4.5	97.4±1.5	101.2±3	100.7±4
C20:4n6	80.5±2	98.3±14.3	103.2±8.1	117.3±3.2	106.8±3.4	119.1±3.8
Ornithine	104.9±5.6	113±5.7	104.9±3.9	102.9±1	103.3±1.5	96±1.6
C22:1n9	117.9±6.3	101.7±3.1	94.2±3.1	89.4±5.7	99.6±1.2	97.7±6.3
C22:0	100.3±4.3	93.6±0.7	96.6±4.7	89±2.9	101.2±2.4	98.5±5.2
C22:6n3	96.8±2.8	105.7±6.7	91.1±6.6	93±1.7	99.2±1.5	102.9±7.5
Lysine	102.1±3.2	114.9±1.4	101±0.9	101.4±0.4	100.5±0.6	100.4±0.7
Histidine	98.3±1.3	95.8±2.4	97.4±1.2	99.1±2.9	97.2±1	97.7±0.2
C24:1	112±12.2	114.4±6.2	91.3±5.7	89.3±3.1	98.1±2.7	97.9±7.2
C24:0	88±3.5	81.5±2	96±6.3	88.8±3.8	106.1±2.3	102.7±6.6
Tyrosine	104.8±1.1	111.9±2.3	103.6±0.9	103.6±1	101.7±0.4	100.2±0.7
Tryptophan	98.6±1.8	103.6±1.1	97.7±0.3	99.2±0.5	97.2±0.2	97.8±0.2
Cystathionine	95.1±4	103.2±3	87.9±2.7	95.5±8.1	83.5±3.5	98.2±9.7
Cystine	104.6±0.8	105.7±1.9	103.4±0.6	103.1±0.6	102.1±0.4	101±0.03

Analytes printed in bold were quantified with a corresponding stable isotope labeled standard.

7.3.3 Saponification of triglycerides

Derivatization with propyl chloroformate, as described in 5.2.4, is performed under alkaline conditions and might also result in transesterfication of fatty acids bound in triglycerides. To investigate whether triglycerides actually are esterified with the propyl chloroformate, the triglyceride trimyristic (C14:0/ C14:0/ C14:0) was dissolved in propanol, derivatized and the amount of free myristic acid was analyzed. The experiment was performed in triplicates with a 0.2 mM and 0.02 mM solution in propanol using 50 µmol each. The recoveries for the free fatty acid ranged from 95 % to 130 %. Due to the unpolar character of triglycerides it was not possible to examine higher triglycerides, which are not soluble in n-propanol. Using high glyceride solutions in chloroform did not result in high glyceride saponification, because the ester in the organic phase is not amenable to the NaOH.

7.3.4 Outlook for the analysis of NEFAs

One major aim in lipidomics is the exclusive analysis of nonesterified fatty acids (NEFA) only without a labour intensive TLC separation prior to the analysis. This might be achieved by modifying the derivatization procedure. Omission of the base should prevent saponitfication or reesterfication. Preliminary experiments were performed on the triglyceride of C10:0. As shown in Figure 33, upon omission of the base no free fatty acid was detected. Further, the yield of the internal standard C14:0 was comparable in both analyses, i.e. with and without the base. Hence, it may be feasible to analyse NEFA by omitting the base.

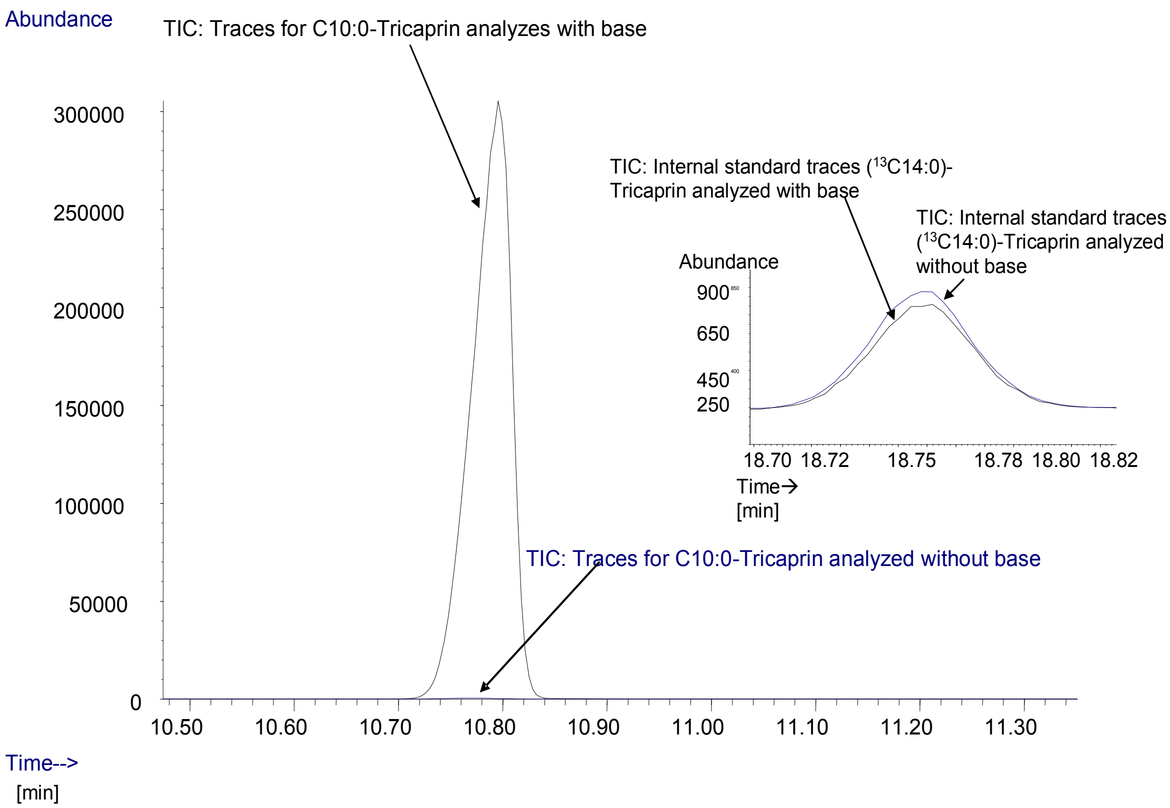


Figure 33: Analysis of the triglyceride tricaprin under two different conditions: with and without base. The ion traces for C10:0 and the internal standard ^{13}C14:0 are shown for the two different derivatization conditions.

8 Quantitative analysis of amino acids and related compounds by LC-MS/MS

8.1 Introduction

Some important amino acids are thermally instable and cannot be quantified by GC-MS, such as arginine, citruline, as well as 1- and 3- methylhistidines. Amino acids are highly polar analytes and, therefore, not suited for conventional reversed-phase high-performance liquid chromatography (RP-HPLC). Therefore, a derivatization is needed. The potential of derivatization with propyl chloroformate, followed by LC-MS/MS analysis for amino acid determination was investiged in this work. The method was expanded to tryptophan metabolites and polyamines, which are of great biomedical interest. Due to their amino or carboxy function they can be derivatized with propyl chloroformate and analyzed by LC-MS/MS. First experiments for this project were performed by Stephan Fagerer. The most important polyamines are spermine, spermidine and putrescine. They can be detected in the cells of all living organisms often in high concentrations.[79] Moreover, they are important factors for cell growth, protect DNA and proteins from damage by active oxygen species,[80, 81] and were suggested as tumor markers. [82-84] Therefore, polyamine analysis is an important extension to the method. Similarly, tryptophan metabolites were implemented in the method. Various articles report that the tumor escape mechanism of cancer cells involves depletion of tryptophan and accumulation of its (toxic) metabolites. [85, 86] The kynurenine pathway is the main pathway of tryptophan metabolism and is activated during inflammatory processes such as immune activation and neurodegenerative disorders. Activation of the pathway decreases the level of tryptophan and increases the concentration of downstream metabolites, including kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid.[87-89] Parts of the tryptophan pathway are shown in Figure 34.

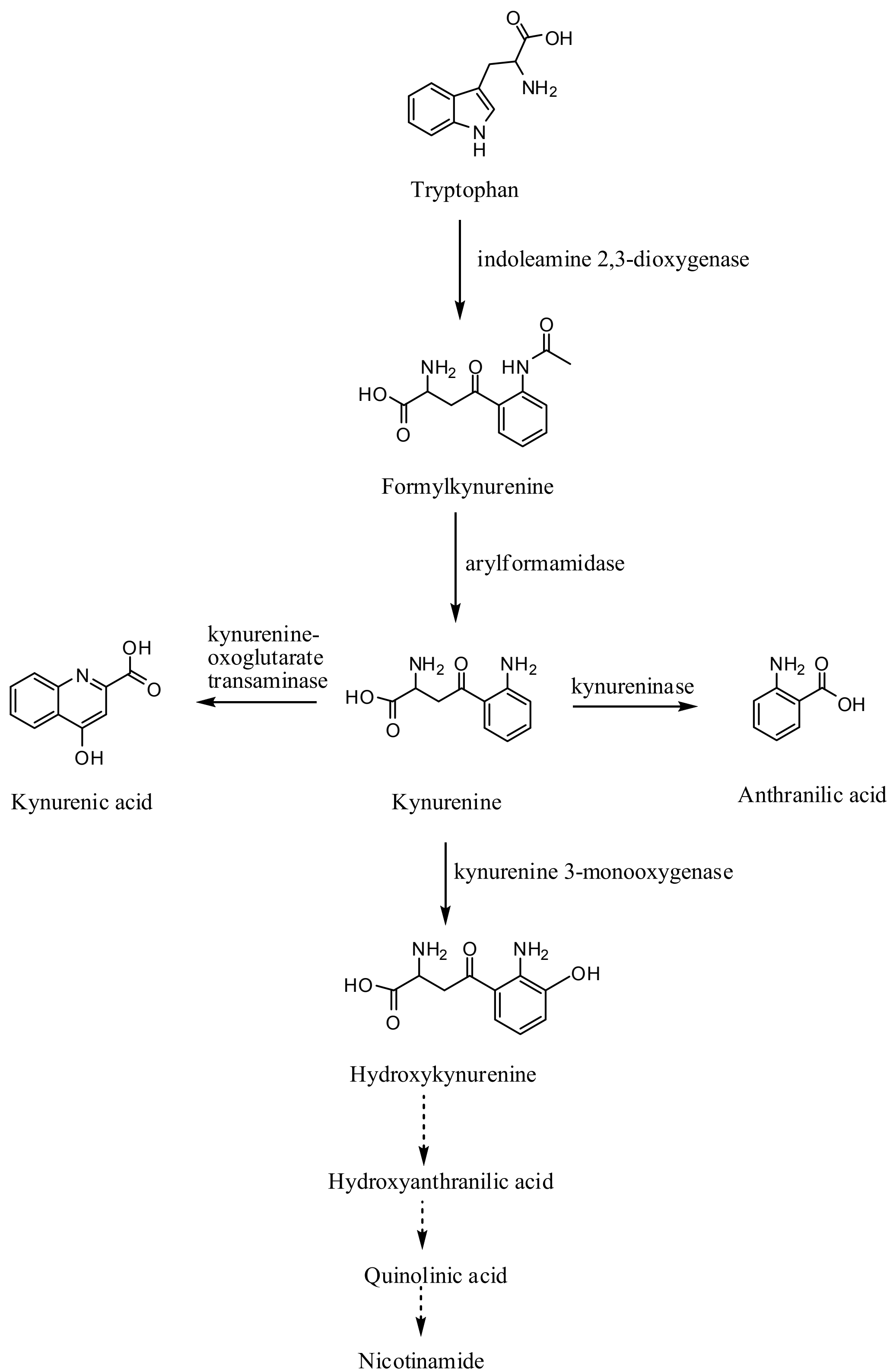


Figure 34: Selected pathways of Tryptophanmetabolism

Yamada *et al.* described the simultaneous measurement of tryptophan and related compounds by liquid chromatography-electrospray ionization tandem mass spectrometry without derivatization by using trifluoroacetic acid as ion pair reagent. [90] The separation was carried on a C18 column. A tandem mass spectrometer operated in MRM with ESI in positive mode was used for detection. Quantification was demonstrated to be reliable for compounds with a dedicated isotope-labeled standard. In contrast, compounds that are not normalized against an isotope-labeled standard, such as polyamines, ornithine and citrulline yield poor linearity and, consequently, are not quantified reliably. Therefore, introduction of isotope-labeled standards for as many analytes as possible should be pursued. Our experiments have already established the importance of internal standards. However, isotope-labeled standards are not available commercially for all metabolites of interest. Instead of synthesizing individual standards for each metabolite, we exploited the derivatization of amino and carboxy functions with propyl chloroformate employing d_3 labeled propanol. Propyl chloroformate (PCF) reacts with carboxylic acids and amines. At room temperature the carboxylic acids are esterified and amines are converted to carbamoyl derivatives and esters, respectively.

8.2 Material and Methods

8.2.1 Chemicals

An amino acid standard (Sigma) containing 27 compounds, mostly amino acids at a concentration of 2.5 mM each (except cystine 1.25 mM), formic acid (puriss p.a.) heptafluorobutyric acid (puriss. p.a.), iso-octane (GC-MS grade) and n-propanol (GC-MS grade), glutamine, arginine, histidine, hydroxylysine, lysine, ornithine, γ-aminobutyric acid, hippuric acid, tryptophan, 3-hydroxy-kynurenine, kynurenine, kynurenic acid, anthranilic acid, agmatine, putrescine, U-13C putrescine, spermine, spermidine, ethanolamine, taurine, norleucine, citrulline, 1- and 3-methyl-histidine, [$^2H_3$] 3-methyl-histidine were purchased from Sigma-Aldrich (Taufkirchen, Germany). The [U-13C, U-15N] cell free amino acid mix was from Euriso-Top (Saint-Aubin Cedex, France). [2, 5, 5-$^{2}H_3$] *α*-aminoadipic acid and [2,3,4,5,6-$^{2}H_5$] hippuric acid were purchased from C/D/N Isotopes (Quebec, Canada). Methanol (LC-MS grade) and chloroform (HPLC grade) were purchase from Fisher (Fisher Scientific GmbH, Ulm, Germany). The EZ:faastTM C18 RP column (250 mm x 2.0 mm, 4 µm) for LC-MS was from Phenomenex .

8.2.2 Instrumentation

An Agilent 1200 series binary SL system with autosampler was used for liquid chromatography. The column was kept at a constant temperature of 50 °C in a column oven . Five µL of sample were injected each run. For separation a binary gradient was used with mobile phase A: water with 1 % (v/v) formic acid and 0.1 % heptafluorobutyric acid and mobile phase B: methanol with 1 % (v/v) formic acid and 0.1 % heptafluorobutyric acid. A C18 RP column (4.6 mm x 150 mm, 5 µm) equipped with a guard cartridge system from Phenomenex® was used for separation to avoid column contamination. The LC-separation was evaluated by Stephan Fagerer. The gradient is shown in Table 21.

Table 21: Gradient for LC separation.

Total time [min]	% Mobile Phase A	% Mobile Phase B
0.0	38	62
12.0	21	79
12.01	2	98
15.0	2	98
15.01	38	62
23.0	38	98

An ABI 4000 QTRAQ mass spectrometer was used for detection. Experiments were performed using the Analyst Software 1.5. The Turbo Ionspray, declustering potential, exit potential and collision energy parameters as well as all precursor and product ion masses for the analytes and internal standards are listed in

Table 22. ESI in positive mode and scheduled MRM were used. The transitions were recorded for one minute at the scheduled retention time. The transitions were adopted from Stephan Fagerer for except 3-methylhistidine IS, hippuric acid IS, putrescine IS, hydroxylysine, agmatine and α-aminoadipic acid IS that were added later to the method.

Table 22: List of derivatized compounds after propyl chloroformate derivatization and their optimized MRM parameters. The numbers in the left column indicate the labeling in the chromatogram in Figure 35.

Compound (number)	Mass [Q1]	Mass [Q3]	RT [min]	DP [V]	CE [V]	CXP [V]
Ethanolamine (1)	148.1	62	2.3	29	14	9
Taurine (2)	212.05	126	2.4	53	17	10
Glutamine (2)	275.15	172.1	3.2	61	19	10
Glutamine IS	282.1	178.1	3.2	61	19	10
3-Methylhistidine (4)	298.2	256.1	3.2	60	18	10
3-Methylhistidine IS	301.2	259.1	3.2	60	18	10
Citrulline (5)	304.2	156.1	3.3	46	24	11
1-Methylhistidine (4)	298.2	210.1	3.6	60	26	12
Hippuric acid (6)	222.1	162.1	3.7	30	13	10
Hippuric acid IS	227.1	167.1	3.7	30	13	10
Serine (7)	234.13	174.08	3.7	50	14	10
Serine IS	238.1	178.1	3.7	50	14	10
Arginine (8)	303.2	70	4.2	88	55	11
Arginine IS	313.2	70	4.2	88	55	11
Asparagine (9)	243.13	157.1	3.9	66	14	11
Asparagine IS	249.13	163.1	3.9	66	14	11
Glycine (10)	204.12	144.07	4.5	56	12	12
Glycine IS	207.1	147.1	4.5	56	12	12
Threonine (11)	248.14	160.1	4.6	53	16	8
Threonine IS	253.14	164.1	4.6	53	16	8
Kynurenic acid (12)	232.1	190.1	4.7	32	21	10
Putrescine (13)	261.2	201.2	5.4	31	13	10
Putrescine IS	265.2	205.2	5.4	31	13	10
ß-Alanine (14)	218.13	158.1	5.6	42	14	10

Agmatine (15)	217.2	158.1	5.8	48	17	9
Alanine (16)	218.13	130.09	5.9	59	17	10
Alanine IS	222.13	133.09	5.9	59	17	10
γ-Aminbutyric acid (17)	232.2	172.1	6.5	30	13	9
Sarcosine (18)	377	317	6.9	60	17	10
Hydroxylysine (19)	377	125	7	30	14	8
ß-Aminoisobutyric acid (20)	232.2	172.2	7.2	44	14	9
Anthranilic acid (21)	266.1	206.1	7.5	48	11	10
α-Aminobutyric acid (22)	232.3	172.3	7.9	30	13	9
Proline (23)	244.15	184.1	8.5	50	12	10
Proline IS	250.15	190.1	8.5	50	12	10
Ornithine (24)	347.21	287.16	8.6	67	14	8
Methionine (25)	278.13	190.09	8.6	55	15	10
Methionine IS	284.13	195.09	8.6	55	15	10
Aspartic acid (26)	304.17	216.12	9.7	61	18	11
Aspartic acid IS	309.17	220.12	9.7	61	18	11
Histidine (27)	370.19	196.1	9.8	60	31	9
Histidine IS	379.19	204.1	9.8	60	31	9
Valine (28)	246.16	158.12	10	58	16	13
Valine IS	252.16	163.12	10	58	16	13
Lysine (29)	361.23	301.18	10.2	71	14	8
Lysine IS	369.23	309.18	10.2	71	14	8
3-OH-Kynurenine (30)	439.2	336.1	10.2	38	15	9
Glutamic acid (31)	318.18	230.14	10.4	64	18	12
Glutamic acid IS	324.18	235.14	10.4	64	18	12
Tryptophan (32)	333.17	245.13	10.8	68	22	10
Tryptophan IS	346.17	257.13	10.8	68	22	10
Spermidine (33)	404.3	284.3	11	76	23	7
α-Aminoadipic acid (34)	332.3	244.2	11.7	50	18	10
α-Aminoadipic acid IS	335.3	247.2	11.7	50	18	10
Leucine (35)	260.18	172.13	12	58	17	10
Leucine IS	267.18	178.13	12	58	17	10
Phenylalanine (36)	294.16	206.12	12.2	60	16	11
Phenylalanine IS	304.16	215.12	12.2	60	16	11
Isoleucine (37)	260.18	130.08	12.4	53	25	10
Isoleucine IS	267.18	136.08	12.4	53	25	10

Cystine (38)	497.19	248.1	14.5	89	23	6
Cystine IS	505.19	252.1	14.5	89	23	6
Tyrosine (39)	396.19	308.15	14.9	84	19	9
Tyrosine IS	406.19	317.15	14.9	84	19	9
Spermine (40)	547.36	427.26	15.5	95	28	12
Kynurenine (41)	423.21	320.1	16.2	74	14	9

8.3 Sample preparation

8.3.1 General procedure

The sample preparation was carried out as described in section 5.4. This protocol was performed by the MPS-2 Prepsation or manually. In contrast to this protocol 120 µL from the upper organic phase were transferred to a new autosampler vial. The sample was concentrated in an infrared vortexing concentrator and redissolved in 100 µL of mobil phase.

8.3.2 Preparation of the internal standard using d_3-propanol

Two hundred µL of standard mix A and B (mixed equimolar) were added in a 2 mL glass vial followed by the addition of 120 µL of 0.33 M NaOH solution. In the next step 50 µL of a picoline/ d_3-propanol solution were added. The ratio of picoline to d_3-propanol was 23:77. Fifty µL of propyl chloroformate in chloroform/isooctane mix were added to the sample, the solution was mixed for 12 seconds, equilibrated for 1 min and once again mixed for 12 seconds. To extract the derivatized analytes, 250 µL of issooctane were added and the vial was vortexed for 12 seconds. From the upper layer 200 µL were transferred to a new vial. The created internal standard was diluted 1:50 and 10 µL of the solution were added to the samples after transferring of the 120 µL organic phase to a new vial and before the evaporation step. The ratio of propanol to propyl chloroformate is 7:1 in the standard protocol. To reduce the percentage of non-labeled d_3-derivatives the ratio of picoline/ d_3-propanol/propyl chloroformate was varied. The ratio of d_3-propanol to propyl chloroformate of 2.5:1 and 14:1 was also tried. In one experiment the content of the catalyst picoline was increased, so the ratio of d_3-propanol and picoline was 1:1.

8.3.3 Different extraction procedures

Furthermore, the extraction of the derivatives was investigated to increase the yield. Ectraction with isooctane, chloroform, ethyl acetate and isooctane plus addition of a saturated NaCl solution to use the salt out effect was tested. An overview of all tested protocols is shown in Table 23.

Table 23: Different Derivatization protocols.

General procedure: isooctan extration	**Expanded reaction time**	**Isooctane + salt out (sat. NaCl solution)**	**Chloroform extraction**	**Ethyl acetate extraction**
1. Pipette 50 µL standard mixture 2. Complement volume with H_2O to a total of 200 µL 3. Add 120 µL 0.33 *M* NaOH 4. Add 80 µL 3-methyl-pyridine (23% in n-propanol) 5. Add 50 µL propyl chloroformate in chloroform/isooctane (17.4:71.6:11.0) 6. Mix (12 sec), wait 1 min, mix (12 sec) agan 7. Add 250 µL isooctane 8. Transfer 120 µL (organic layer) to a new vial 9. Evaporate solvent (100 mbar, 45 min) 10. Redissolve in 100 µL mobile Phase	Step 1-5 equal to the general procedure 6. Mix (12 sec), wait 2 min, mix (12 sec) again Step 7-10 of the general procedure	Step 1-7 equal to the general procedure 8. Add 50 µL brine Step 7-10 of the general procedure	Step 1-6 equal to the general procedure 7. Add 250 µL chloroform Step 8-10 of the general procedure	Step 1-6 equal to the general procedure 7. Add 250 µL ethyl acetate Step 8-10 of the general procedure

8.4 Quantification

Absolute quantification of compounds was performed by analyzing standard solutions containing equimolar amounts of all amino acids. Three different solutions were used and listed in Table 28. The first solution consisted of 22 compounds in 0.1 M HCl, the second mixture contained 12 compounds, including amino acids not stable in acidic solution, complementary amino acids and tryptophan metabolites, while the third mixture included polyamines, aromatic amino acids and ethanolamine. The first and the second mixture was 2.5 mM, while the third one was 5 mM. For calibration, the three different solutions were mixed at the following ratio: 2:2:1 resulting in a final concentration of 1 mM. For calibratio,n this standard mix was employed in a range of 2.5 pmol to 10 nmol absolute in 16 serial dilutions corresponding to a concentration range of 125 nM to 0.5 mM using 20 µL of biological sample. The calibration and first quantification experiments were performed by using the same standard mix of 20 uniformly ^{13}C and ^{15}N-labeled amino acids as described in 5.4, including arginine and cystine. Arginine was concentrated too low for use as internal standard. During the course of experiments compounds were added to expand the spectrum of internal standards: [2,5,5-$^{2}H_3$] α-aminoadipic acid and [2,3,4,5,6-$^{2}H_5$] hippuric acid, [$^{2}H_3$] 3-methylhistidine, and U-13C labeled putrescine.

8.5 Results and Discussion

8.5.1 LC-MS/MS

The LC-MS/MS method used was adopted from Stephan Fagerer. Previously the tandem mass spectrometer was operated in MRM mode with positive ESI and the separation time was divided into four periods. Now the scheduled MRM modus was used for the analysis. A chromatogram of a standard solution is shown in Figure 35. The separation of the analytes was completed in less than 17 min.

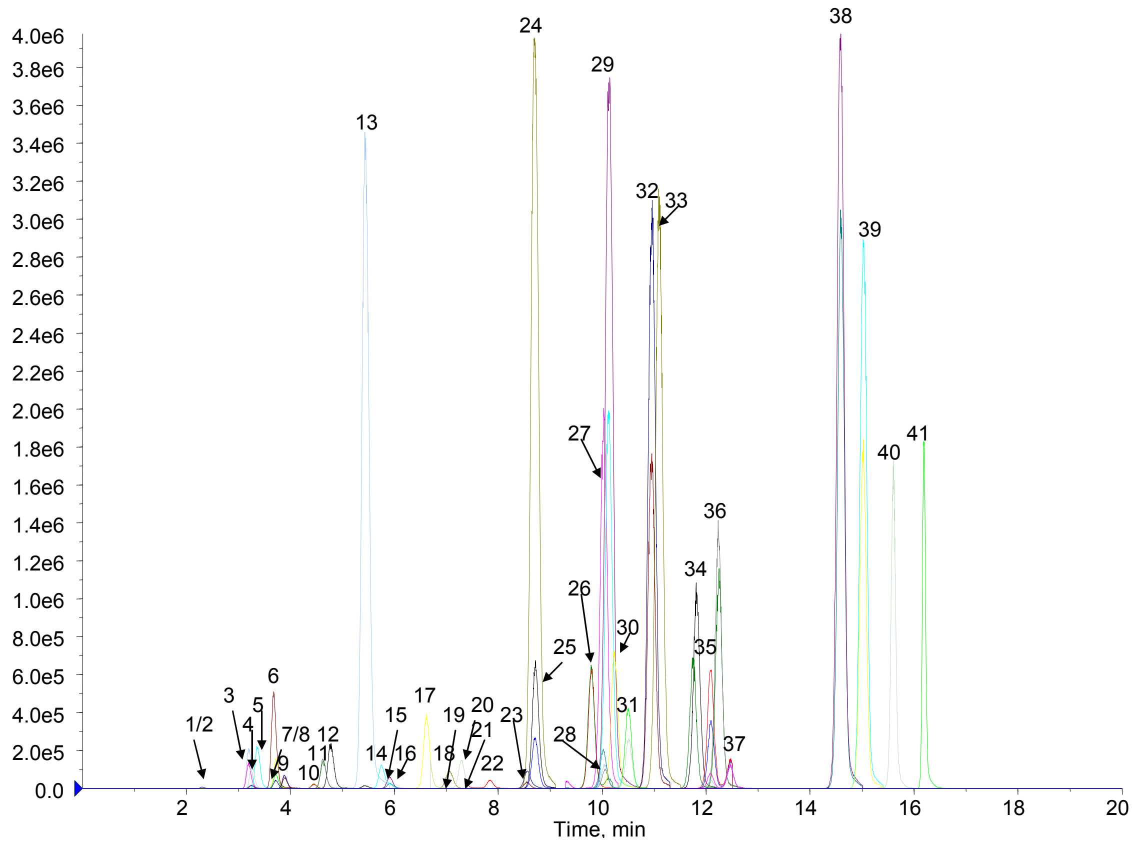


Figure 35: Full chromatogram of the propyl chloroformates obtained by LC-MS/MS. Fourty-one peaks were identified and labeled with numbers. The corresponding compounds are given in Table 22**.**

8.5.2 Calibration

Quantification was carried out as described in 7.2.5 and figures of merit are shown for 38 compounds in table Table 24.The calibration range defined as the LLOQ and ULOQ and the R-square from the calibration are listed. The calibration was linear from 25 pmol to 10,000 pmol for most analytes. R-square-values ≥ 0.99 were obtained for all compounds except kynurenic acid (0.9882), hydroxylysine (0.9877), anthranilic acid and spermidine (0.9862). However, for these amino acids no corresponding stable-isotope had been available. No linear relation between analyte signal and amount was observed for ethanolamine, taurine and agmatine and therefore excluded from Table 24.

Table 24: Calibration parameters of the analytes. LLOQ and ULOQ are given in pmol absolute. Analytes printed in bold were quantified using the internal standard transition of the corresponding stable-isotope labeled amino acid as reference. The internal standard used for the other compounds is given in brackets.

Compound	LLOQ [pmol]	ULOQ [pmol]	R-square
Glutamine	25	10000	0.9997
Methyl-histidine	25	10000	0.9992
Citrulline (Glutamine IS)	25	7500	0.998
Arginine (Glutamine IS)	1000	7500	0.994
Hippuric acid	50	10000	0.9997
Serine	25	10000	0.9998
Asparagine	50	10000	0.9998
Glycine	50	10000	0.9996
Kynurenic acid (Hippuric acid IS)	70	10000	0.9882
Threonine	25	10000	0.9999
Putrescine	25	2500	0.9928
ß-Alanine (Alanine IS)	25	10000	0.9947
Alanine	50	10000	0.9996
γ-Aminobutyric acid (Alanine IS)	100	10000	0.993
Sarcosine (Proline IS)	150	7500	0.991
Hydroxylysine (Threonine IS)	250	10000	0.9877
α-Aminobutyric acid (Aminoadipic acid IS)	25	7500	0.9972
ß-Aminoisobutyric acid (Alanine IS)	100	7500	0.9952
Anthranilic acid (Hippuric acid IS)	500	7500	0.9854
Proline	25	10000	0.9999
Ornithine (Lysine IS)	150	2500	0.9918
Methionine	25	10000	0.9992
Aspartic acid	50	2500	0.9984
Valine	50	10000	0.9997
Histidine	100	2500	0.9967
Lysine	150	2500	0.9927
3-OH-Kynurenine (Hippuric acid IS)	100	7500	0.9965
Glutamic acid	500	10000	0.9985
Tryptophan	100	2500	0.9968
Spermidine (Putrescine IS)	50	2500	0.9862
α-Aminoadipic acid	25	2000	0.9998
Leucine	50	10000	0.9992
Phenylalanine	100	5000	0.9974
Isoleucine	50	10000	0.9997
Cystine	100	2500	0.9943
Tyrosine	100	2500	0.9955
Spermine (Putrescine IS)	100	2500	0.9915
Kynurenine (Hippuric acid IS)	25	5000	0.9965

To prove the reproducibility of the method a standard solution was derivatized and analyzed in triplicates in three different concentrations. An absolute amount of 100 pmol, 250 pmol and 2500 pmol were analyzed and the RSDs are shown in Table 25. The RSDs for the analytes corrected by their own internal standard were between 1 and 5 % except for glycine, hippuric acid, putrescine, alanine, aspartic acid, valine and tyrosine, which yielded higher RSDs at the low concentration level. RSDs higher than 10 % were observed for kynurenic acid, ß-alanine, γ-aminobutyric acid, sarcosine, hydroxylysine, α-aminobutyric acid, ß-aminoisobutyric acid, and anthranilic acid. But these compounds did not have a corresponding internal standard.

Table 25: Reproducibility of LC-MS/MS analysis of propyl chloroformate for independent derivatizations.

Analyte	**Relative standard deviation [%]; n=3**		
	100pmol	250 pmol	2500 pmol
Glutamine	0.61	2.31	0.62
Methyl-histidine	1.05	2.99	2.42
Citruline	15.25	6.53	5.56
Arginine	n.d.	3.93	30.65
Hippuric acid	12.56	5.46	1.08
Serine	10.03	3.62	0.41
Asparagine	2.57	2.25	2.52
Glycine	6.46	0.48	2.70
Kynurenic acid	7.45	7.27	41.77
Threonine	1.48	1.61	0.70
Putrescine	7.45	1.34	2.90
Alanine	17.59	1.99	1.41
ß-Alanine	21.96	7.19	25.55
γ-Aminobutyric acid	13.19	9.85	29.31
Sarcosine	21.74	13.07	12.20
Hydroxylysine	7.61	19.53	17.63
α-Aminobutyric acid	15.93	20.24	10.39
ß-Aminoisobutyric acid	18.06	5.60	25.00
Anthranilic acid	1.52	5.35	33.75
Proline	3.50	0.71	1.07
Ornithine	n.d.	6.85	4.80
Methionine	0.62	0.77	1.73
Aspartic acid	7.27	2.79	2.84
Valine	7.00	1.24	0.71
Histidine	2.65	1.14	3.41
Lysine	n.d.	2.67	4.72
3-OH-Kynurenine	9.44	7.05	4.89
Glutamic acid	3.89	0.82	1.82
Tryptophan	5.86	0.96	1.37
Spermidine	n.d.	1.76	12.15
α-Aminoadipic acid	1.13	1.39	1.71
Leucine	1.07	0.68	0.45
Phenylalanine	3.25	1.76	0.23
Isoleucine	2.09	2.79	1.03
Cystine	1.78	0.98	9.70
Tyrosine	7.27	1.35	0.43
Spermine	8.86	1.91	27.29
Kynurenine	36.95	9.08	7.39

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

8.5.3 Biological samples

For a set of mice serum samples the tryptophan analysis by LC-MS/MS was compared to GC-MS analysis as described in chapter 5. The results are shown in Table 29. Another aim was to detect changes of the tryptophan metabolites kynurenine, kynurenic acid or hydroxykynurenine. In mouse serum the concentrations of kynurenine varied between the LLOQ and two times the concentation of the LLOQ. Kynurenic acid and hydroxykynurenine were not detected above the LLOQ. For some amino acids, e.g. lysine, the measured concentrations were almost outside the calibration range. Therefore, increasing the sample volume or injection volume would exclude some amino acids from the quantification. Up to this point, it proved impossible to quantify the tryptophan metabolites simultaneously with the amino acids.

8.5.4 Synthesis of internal standards using d_3-propanol

Quantification is more reliable for compounds with a dedicated isotope-labeled standard. For some compounds, no standards are available or very expensive. An elegant way to create an isotope-labeld standard for a large group of compounds in a single reaction would be to derivatize a standard mix with propyl chloroformate in isotope-labeled propanol. The alkoxy group found in the esterfied carboxylic acid corresponds to the alcohol in the reaction medium and not to the alkoxy group of the chloroformate. That was demonstrated by Zampolli and Wang et al.[39, 91] Propyl chloroformate (PCF) reacts with carboxylic acids and amines. At room temperature the carboxylic acids are esterified and amines are converted to carbamoyl derivatives and esters, respectively. Using this approach our aim was to use labeled propanol to produce stabil-isotope labeled derivatives that could be then added to the derivatized samples. A rearrangement of the ester should take place and the obtained product is shown in Figure 36 .

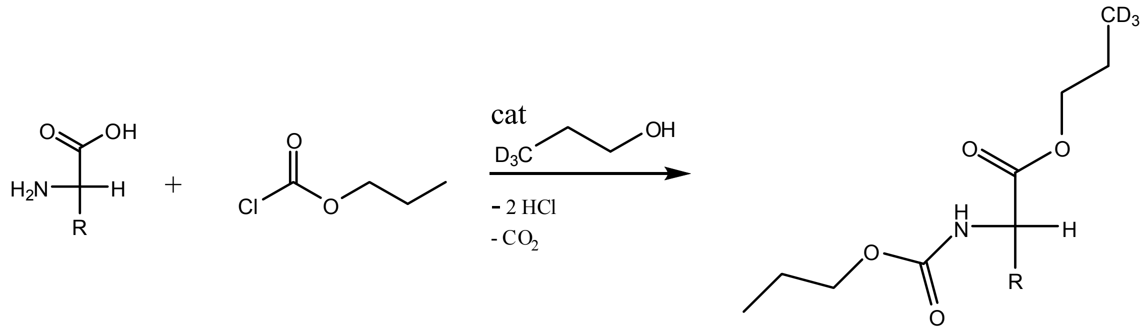


Figure 36: Reaction of the amino acids with d-3 propanol.

Mastermix A and B (compounds listed in Table 28) were mixed equally and 200 µL were subjected to the derivatization procedure as described in chapter 8.3. The rearrangement took place for all compounds but for some compounds also non-labeled derivatives were obtained. This would cause a background signal for the analyte and result in higher LLOQs. Different ratios of propanol to propyl chloroformate were investigated to decrease the content of non-labeled compound. Furthermore, a higher content of the catalyst picoline was tested. The percentage of non-labeled derivative relative to the labeled analyte is shown in Table 26. The amount of non-labeled analyte decreases with increasing d_3-propanol ratio. For glutamine the ratio decreased from 14.4 to 2.8 %. But the yield of the reaction decreased too (data not shown). Therefore, the following experiments were carried out with a ratio of d_3-propanol to propyl chloroformate of 7:1. Here the ratios varied between 1 and 10 %, except for citrulline, sarcosine and glycine.

Table 26: The content of non-labeled analyte relative to the labeled analyte in percent for the different experiments.

Percentage of the non-labeled analyte [%] Analyte	Ratio of d3-propanol to propylchloroformate			Propanol: Picoline=1:1
	2.5:1	**7:1**	**14:1**	
Arginine	4.0	0.4	3.0	0.6
Glutamine	14.4	4.8	2.8	10.6
Citrulline	137.8	37.4	35.1	83.0
Serine	32.5	9.8	5.8	26.3
Asparagine	16.8	5.1	3.1	18.9
Sarcosine	61.8	23.6	10.7	36.9
Kynurenic acid	17.8	5.9	3.4	16.4
Glycine	100.5	32.2	17.7	92.5
Threonine	16.2	4.8	3.1	14.0
Alanine	16.0	4.3	2.2	9.8
β-Alanine	16.2	5.4	2.7	10.8
γ-Aminobutyric acid	31.6	9.1	4.6	18.4
Hydroxylysine	3.6	1.7	0.6	4.0
ß-Aminoisobutyric acid	15.1	4.3	2.1	8.9
α-Aminobutyric acid	16.3	5.1	2.2	10.4
Proline	31.0	9.4	4.9	26.3
Ornithine	30.1	10.6	3.6	12.6
Methionine	18.8	5.9	2.8	13.8
Aspartic acid	3.1	0.3	0.0	1.2
Valine	20.6	6.2	2.9	17.0
Histidine	19.1	7.7	3.3	13.1
Lysine	26.7	9.3	3.6	13.0
3-OH-kynurenine	14.4	5.4	2.5	10.8
Glutamic acid	5.3	0.6	0.2	2.7
Tryptophan	22.9	7.8	3.9	12.9
α-Aminoadipic acid	9.4	2.8	1.7	8.2
Leucine	19.9	6.5	3.2	14.9
Phenylalanine	15.8	5.3	2.6	11.0
Isoleucine	18.0	6.6	2.9	14.8
Cystine	15.6	5.1	3.1	14.2
Tyrosine	16.9	6.5	3.7	12.0
Kynurenine	20.8	5.5	3.3	15.6

Experiments were carried out by using diluted self-made internal standard as described in section 7.3 The internal standard corrected well for injection as investigated for kynurenine. The RSD for a ten-fold injection was 10 % without using an internal standard and below 1 % using the corresponding d_3-labeled internal standard (data not shown). But applying the d_3-labeled internal standard decreased the accuracies during calibration compared to using the ^{13}C and ^{15}N-

labeled amino acids of the algae mix for corrections. In both cases the ratio of analyte and internal standard were applied for the Quantification. A calibration range from 41 -6250 pmol was compared. For threonine the accuracies ranged applying the d_3 standard from 100 to 235 % for the different calibration levels compared to 100 to 119 % using ^{13}C and ^{15}N-labeled threonine. In conlusion, the self-made internal standard could correct for injection and ion suppression, but not for the differences in extraction efficiency. Therefore, this approach was discarded.

8.5.5 Method limitations

There are a few drawbacks of the method. First, the methyl histidines cannot be separated as described by Stephan Fagerer and therefore the method parameters are for the sum of 1-methyl-histidine and 3-methyl-histidine. No linear range was observed for ethanolamine, taurine and agmatine and a high LOQ was observed for arginine compared to the other analytes. Human serum was analyzed and no kynurenine or kynurenine derivatives were deteced. The method is not useful to quantify tryptophan metabolites because the biological concentration is lower than the LLOQ for those analytes.

8.5.6 Extraction experiment

The aim was to test different extraction solvent to increase sensitivity for polar compounds e.g. ethanolamine, citrulline or methylhistidine and to get better yields for all analytes and, consequently, better LLOQs. Changing the extraction medium to more a polar solvent (EtOAc, $HCCl_3$) was tested. Additionally, the expansion of the reaction time as well as an additional step with brine solution was evaluated. The different conditions of the protocol are shown in 8.3, Table 23. The analytes are divided into 6 different groups, which are discussed: neutral amino acids, polar amino acids, tryptophan metabolites and aromatic amino acids, basic compounds as polyamines and basic amino acids and ethanolamine and taurine. The areas of the neutral amino acids are shown in Figure 37.

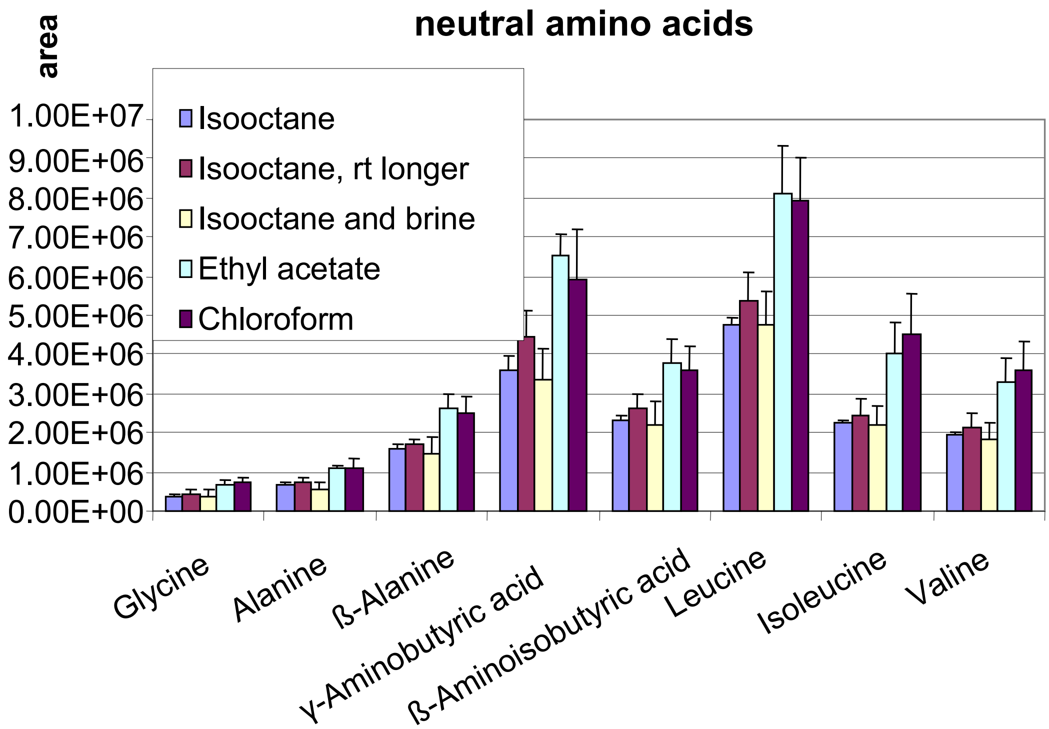


Figure 37: Area obtained by different extraction protocols for derivatives of neutral amino acids.

The areas for the polar amino acids are compared in Figure 38. All those amino acids contain a functional group e.g. threonine and serine have a hydroxyl group, aspartic acid and glutamic acid a second carboxy function, glutamine and asparagine an amide function, and cystine and methionine contain sulfur. Apart from methionine the higher extraction yields were obtained with ethyl acetate and chloroform compared to isooctane, with chloroform showing the best performance.

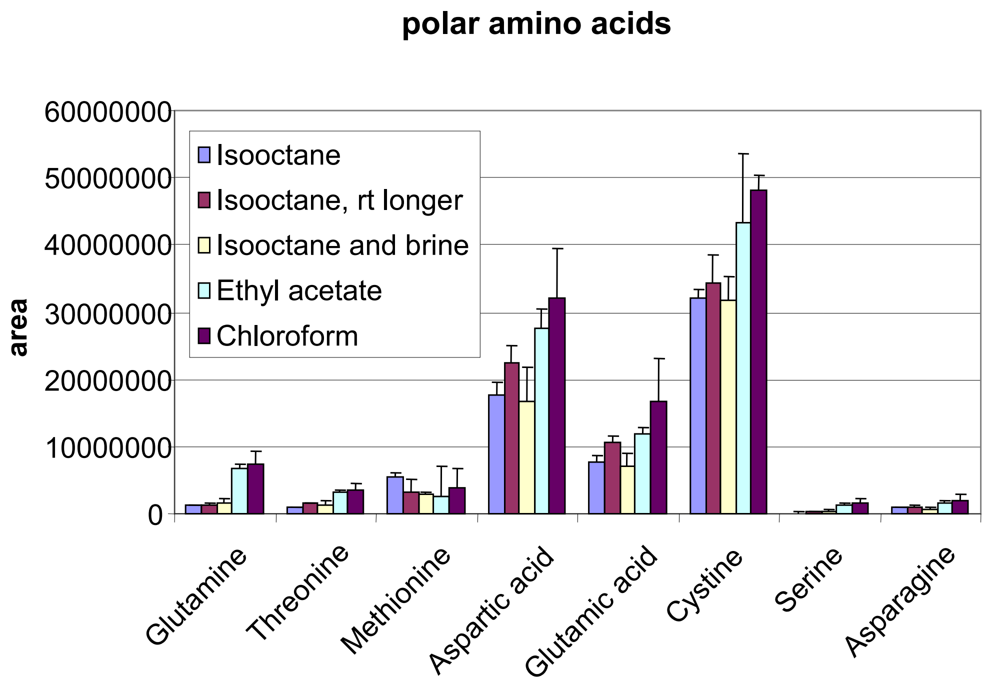


Figure 38: Area obtained by different extraction protocols for derivatives of polar amino acids.

Ethyl acetate is the best solvent for the extraction of polyamines, lysine and ethanolamines (data not shown). For methylhistidine the yield was almost 5 or 9 times higher using ethylacetate and chloroform, respectively. For arginine the yield was 30 or 25 times higher using ethylacetate and chloroform, respectively. Both are shown in Figure 39

Methylhistidine and arginine

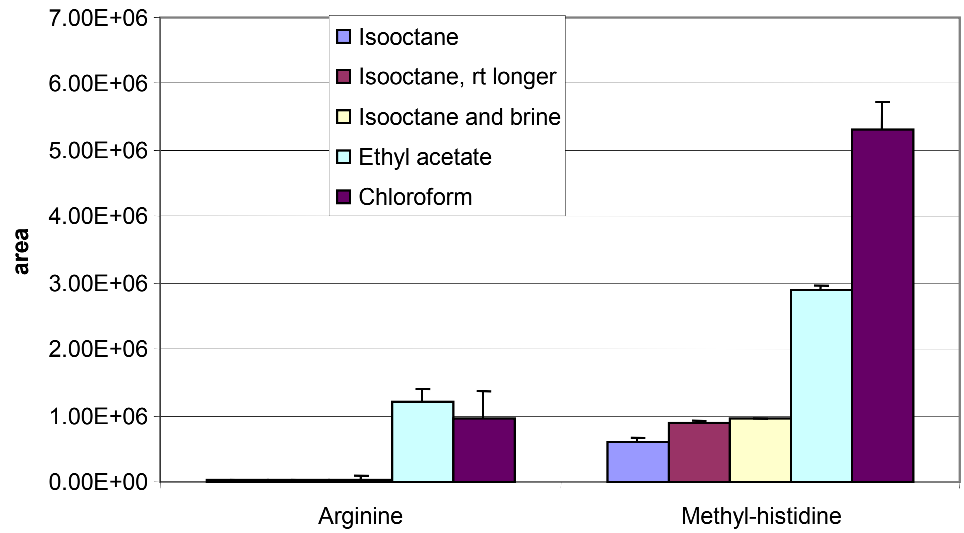


Figure 39: Area obtained by different extraction protocols for arginine and methylhistidine

The same trend was observed for aromatic amino acids and tryptophan metabolites (data not shown). In conclusion, using either ethyl acetate or resulted in higher yield and improved LLOQs. In particular, for the analysis of the tryptophan derivates improved LLOQs are needed.

9 Conclusion and Outlook

9.1 GC-MS method

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, 31 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 other available methods is the complete automation and a very robust quantification. By limiting manual sample preparation steps, the sample throughput is increased, which is of high importance in metabolomics studies. For the analysis of blood or urine a sample amount of 20 - 50 µL is necessary. The introduction of stable-isotope labeled amino acids as internal standards immensely improved the method reproducibility over using only norvaline as internal standard, which allowed the accurate and robust quantification of amino acids in large sample batches. The method was validated for the analysis of amino acids using certified amino acid standard and reference plasma, and its applicability was shown by matrix spike experiments. The application for metabolomic studies with large sample numbers was demonstrated by anaylyzing 2 blinded sets. The method was adapted to allow the combined analysis to the total fatty acid content of 17 fatty acids and 25 free amino acids in a single gas chromatographic run. The chromatographic run time increased from 12 min to 50 min. Modification of the derivatization protocol may allow the analysis of free fatty acids as a subset of the lipidome. There is also the possibility to expand the method for the analysis of additional compounds e.g. polyamines or other dipeptides.

9.2 LC-MS/MS method

Limitations of the GC-MS 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. Therefore, the potential of derivatization with propyl chloroformates, followed by LC-MS/MS analysis for amino acid determination was investigated. The method was expanded to tryptophan metabolites and polyamines. In total 41 analytes were investigated. Due to their amino function they can be derivatized with propyl chloroformate and analyzed by LC-MS/MS. The main focus was the evaluation of a novel strategy to generate a stable-isotope labeled standard by using d_3-labeled propanol. Experiments showed that the created standard was not suitable for quantification purposes. Therefore, isotope-labeled analogs have to act as internal standards. In total 23 stable-isotope labeled amino acids were used as internal standards but for many analytes no stable-isotope labeled standard was available. e.g. kynurenine. Means of synthesizing labeled compounds include chemical synthesis or the use of enzymes. Matin et al. demonstrated the enzymatic conversion of tryptophan to kynurenine using indoleamine 2,3-dioxygenase (IDO).[92] Changing tryptophan to labeled tryptophan would lead to labeled kynurenine. It was not possible to detect all analytes e.g. tryptophan metabolites above the LLOQ in biological samples. Therfore the sensitivity of the method has to be increased. The sample preparation contain a extraction with isooctane. Experiments with different solvents showed that there is a way to increase the yield of extraction using chloroform or ethyl acetate. Due to the better handling applying the ethyl acetate approach would be the best way for the future.

10 References

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11 Appendix

Table 27: [U-13C, U-15N] labeled cell free amino acid mix extracted from algae. Molar % and weight % as provided from the manufacturer (determined by HPLC). The standard was dissolved in 10 mL water (resulting concentrations given in the table).

AA	Molar %	Weight %	M [g/mol]	m [mg]	conc [µmol/10 ml]	conc [nmol/µl]
Aspartic acid	7.84	8.25	138.1	2.327	16.846	1.6846
Glutamic acid	10.04	11.67	153.13	3.291	21.491	2.1491
Asparagine	4.34	4.56	138.12	1.286	9.310	0.9310
Serine	4.26	3.53	109.09	0.995	9.125	0.9125
Glutamine	4.43	5.15	153.15	1.452	9.483	0.9483
Histidine	0.41	0.51	164.16	0.144	0.876	0.0876
Glycin	9.33	5.53	78.07	1.559	19.975	1.9975
Threonine	4.77	4.48	124.15	1.263	10.176	1.0176
Alanine	13.29	9.35	93.09	2.637	28.324	2.8324
Arginine	4.9	6.78	174.2	1.912	10.98	1.098
Tyrosine	2.13	3.04	191.19	0.857	4.484	0.4484
Valine	6.53	6.04	123.15	1.703	13.831	1.3831
Methionine	1.63	1.92	155.21	0.541	3.488	0.3488
Tryptophan	1.81	2.92	217.23	0.823	3.791	0.3791
Phenylalanine	2.41	3.15	175.19	0.888	5.070	0.5070
Isoleucine	4.71	4.88	138.18	1.376	9.959	0.9959
Leucine	8.66	8.97	138.18	2.530	18.306	1.8306
Lysine	3.98	4.6	154.19	1.297	8.413	0.8413
Proline	3.9	3.55	221.13	1.001	4.527	0.4527
Cysteine		not det.				

Table 28: The three differt Mastermixsolutions for the analysis by LC-MS/MS as propyl chloroformates

Mastermix A **Conc: 2.5 µM**	**Mastermix B** **Conc: 2.5 µM**	**Mastermix C** **Conc: 5 µM**
ß-alanine	Glutamin	hippuric acid
Alanine	Arginine	3-methyl-histidine
A-aminoadipic acid	γ-aminobutyric acid	Anthranilic acid
α-aminobutyric acid	Histidine	Spermine
ß-aminoisobutyric acid	Hydroxylysine	Spermidine
Asparagine	Kynurenine	Putrescine
Aspartic acid	Kynurenic acid	Agmatine
Citrulline	Hydroxykynurenine	Ethanolamine
Cystine (1.25 µM)	Lysine	
Glutamic acid	1-methyl-histidine	
Glycine	Ornithine	
Isoleucine	Tryptophan	
Leucine		
Methionine		
Phenylalanine		
Proline		
Sarcosine		
Serine		
Taurine		
Threonine		

Tyrosine		
Valine		

Table 29: Comparison of tryptophan values analyzes as propyl chloroformates by GC-MS and LC-MS/MS

μM	GC-MS	LC-MS/MS
C1	78.45	74.5
C2	101.8	97.5
C3	135.25	124.5
C4	93.35	88.5
C5	143.9	130.5
C6	132.55	120.5
N1	158.75	142
N3	126.35	112.5
N4	182.65	145
N5	129.4	120.5
M1	190.95	168
M2	120.9	113
M3	132	122
M4	129.95	121
M5	124.3	114.5
M6	145.45	133.5

12 Curriculum Vitae

Personal Data:

Name:	Hannelore Kaspar
Nationality:	German
Date of birth:	28.03.1980

Education:

10/2005 – present:	Natural Sciences Graduate Student at the Institute of Functional Genomics, University of Regensburg, Germany (Advisor: Prof. Dr. PJ. Oefner)
02/2005 – 07/2005:	Teacher at the gymnasium in Olching for chemistry
10/2004:	Diplom in Chemistry
02/2004 – 09/2004:	Diploma thesis at the Ludwig-Maximilians-University of Munich, on 'Total synthesis of *rac* Curcutetraol' (Advisor: Prof. Dr. Th. Lindel)
11/1999 – 09/2004	Chemistry studies at the Ludwig-Maximilians-University of Munich
1990 – 1999:	High school in Fürstenfeldbruck
1986 – 1990:	Primary school in Fürstenfeldbruck

Stipends and Awards:

2006 Scholarship for the ISC 2006 (International symposium on chromatography)

2008 Scholarship for the ISCC 2008 (International symposium on capillary chromatography)

13 Publications and Presentation

13.1 Publications

1. Mülhaupt T, **Kaspar H**, Otto S, Reichert M, Bringmann G, Lindel T. Isolation, Structural Elucidation, and Synthesis of Curcutetraol. **EurJOC** 2004.
2. Timischl B, Dettmer K, **Kaspar H**, Thieme M, Oefner PJ. Development of a quantitative, validated capillary electrophoresis-time of flight-mass spectrometry method with integrated high-confidence analyte identification for metabolomics. ***Electrophoresis*** 2008;29:2203-14.
3. **Kaspar H**, Dettmer K, Gronwald W, Oefner PJ. Automated GC-MS analysis of free amino acids in biological fluids. ***J Chromatogr B Analyt Technol Biomed Life Sci*** 2008;870:222-32.
4. Popp FC, Eggenhofer E, Renner P, Slowik P, Lang SA, **Kaspar H**, Geissler EK, Piso P, Schlitt HJ, Dahlke MH. Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. ***Transpl Immunol*** 2008;20:55-60.
5. **Kaspar H**. 32nd International Symposium on Capillary Chromatography and 5th GCxGC Symposium. ***Anal Bioanal Chem*** 2008;392:773-4.(Congress report)
6. **Kaspar H**, Dettmer K, Gronwald W, Oefner PJ. Advances in amino acid analysis. ***Anal Bioanal Chem*** 2009;393(2):445-52. (Review)
7. Gronwald W, Klein MS, **Kaspar H**, Fagerer SR, Nurnberger N, Dettmer K, Bertsch T, Oefner PJ. Urinary Metabolite Quantification Employing 2D NMR Spectroscopy. ***Anal Chem*** 2008;80: 9288–9297.
8. **Kaspar H**, Dettmer K, Oefner PJ: Automatisierte Aminosäureanalytik in biologischen Matrices mittels GC-MS. ***GIT Spezial – Separation*** 2008, pp 27-31.
9. **Kaspar H**, Dettmer K, Chan Q, Daniels S, Nimkar S, Daviglus ML, Stamler J, Elliott P, Oefner PJ. Urinary Amino Acid Analysis: A Comparison of iTRAQ®-LC-MS/MS, GC-MS, and Amino Acid Analyzer. ***J Chromatogr B Analyt Technol Biomed Life Sci*** 2009; Epub ahead of print on May 15.

13.2 Oral presentations

1. Automated high-throughput analysis of amino acids by GC-MS

   18. Doktorandenseminar des AK Separation Science, Hohenroda (2008)

2. Automated high-throughput analysis of amino acids by GC-MS
   ISC 2008 - 27th International Symposium on Chromatography, Münster (2008)

3. GC-MS versus LC-MS/MS and amino acid analyzer for high-throughput amino acid analysis; invited talk at the Institut of Epidemiology, Public Health and Primary Care, Imperial College London, South Kensington Campus, London, UK (2007)

13.3 Poster presentations

1. High-throughput amino acid profiling in biological samples by GC-MS

   ISC 2006 - 27th International Symposium on Chromatography, Kopenhagen, DK (2006)

2. High-throughput amino acid profiling in biological samples by GC-MS

   ISCC 2008 - 32nd International Symposium on Capillary Chromatography and 5th GCxGC Symposium

14 Summary

Amino acids are intermediates in cellular metabolism and their quantitative analysis plays an important role in disease diagnostics. A gas chromatography-mass spectrometry (GC-MS) based method was developed for the quantitative analysis of free amino acids as their propyl chloroformate derivatives in biological fluids. Derivatization with propyl chloroformate could be carried out directly in the biological samples without prior protein precipitation or solid-phase extraction of the amino acids, thereby allowing for automation of the entire procedure, including addition of reagents, extraction and injection into the GC-MS. The total analysis time was 30 minutes, including sample preparation and 31 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 certified amino acid standard and reference plasma, and its applicability to different biological fluids was shown. Intraday 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%.

Using two blinded sets of urine specimens containing replicates, the GC-MS method was further validated and the results were compared with those obtained for iTRAQ® derivatization HPLC-tandem mass spectrometry and ion exchange chromatography with postcolumn ninhydrin detection of amino acids. The technical error (TE), as determined by repeated aliquot measurements of various urine specimens was calculated to prove that the method was suitable for the quantitative analysis of amino acids in large clinical and epidemiological studies. The quantitative results obtained by the three methods were compared by regression analysis and Bland-Altman plotting.

The method was further expanded to fatty acids. Due to the carboxy function fatty acids can be derivatized with propyl chloroformate and included in the develpoped GC-MS method. To resolve isobaric fatty acids the GC program had to be expanded and the analysis time increased to 50 min for one GC run. LODs for the fatty acids ranged from 0.08 µM to 39 µM. To that end, the method was adapted to allow the combined analysis of the total fatty acid content of 17 fatty acids and 25 free amino acids in a single gas chromatographic run.

The number of amino acids amenable to GC analysis is limited and therefore, the potential of derivatization with propyl chloroformates, followed by LC-MS/MS analysis for amino acid determination was investigated. The method was expanded to tryptophan metabolites and polyamines that are of great interest in several biological projects. The intention to use an in-house synthesized internal standard for each analyte failed as experiments showed that the created standard is not suitable for quantification purposes. Therefore, isotopes labeled analytes have to act as internal standards. Using 23 stable-isotope labeled compounds as internal standards, the method aims the quantification of 41 analytes comprising amino acids, tryptophan metabolites and polyamines. It was not possible to detect tryptophan metabolites above the LLOQ in biological samples. Preliminary experiments were performed to improve the method by evaluating choice of the extraction solvent.

15 Zusammenfassung

Aminosäuren sind Zwischenprodukte im zellulären Stoffwechsel und ihre quantitative Analyse ist speziell bei der Diagnose von Krankheiten von enormer Bedeutung. Zur Bestimmung von Aminosäuren in unterschiedlichen biologischen Proben wurde eine gaschromatographische mit Massspektrometer gekoppelte Methode entwickelt, welche auf der Derivatisierung von Aminosäuren mit Chlorameisensäurepropylester beruht. Diese Art der Derivatisierung kann ohne vorgeschaltete Proteinfällung oder Festphasenextraktion direkt in biologischen Proben durchgeführt werden, wodurch eine Automatisierung des gesamten Prozesses - Zugabe der Reagenzien, Extraktion und Injektion ins GC-MS - ermöglicht wird. Die Gesamtanalysenzeit inklusive Probenvorbereitung beträgt 30 min, wobei durch die Verwendung von 19 stabile isotopenmarkierten Aminosäuren als interner Standard 31 Aminosäuren und Dipeptide quantifiziert werden konnten. Die Nachweisgrenzen (LOD) lagen zwischen 0,03 und 12 µM und die unterste Quantifizierungsgrenze (LLOQ) zwischen 0,3 und 30 µM. Die Methode wurde durch die Analyse eines zerifizierten Standards und Referenzplasma validiert und die Anwendbarkeit für verschiedene biologische Proben getestet. Die relative Standardabweichung für eine Zehnfachbestimmung am selben Tag lag zwischen 2,0 und 8,8% für menschlichen Harn, zwischen 0,9 und 8,3% für menschliches Plasma und zwischen 1,3 und 9,1% für Mäuseharn, während die Standardabweichung für eine Zehnfachbestimmung für menschlichen Harn über mehrere Tage verteilt zwischen 1,5 und 14,1% lag.

Die GC-MS Methode wurde weiterhin durch die Analyse von zwei verdeckten Probensets validiert, welche Splitproben enthielten. Dieselben Proben wurden zusätzlich mit der iTRAQ® Derivatisierung gefolgt von HPLC -Tandemmassen-spektometrie und einer Nachsäulenderivatisierung mit Ninhydrin mittels eines Aminosäurenanalysator gemessen. Um die Eignung der Methode für einen hohen Probendurchsatz zu zeigen, wurde der technische Fehler für die

Splitproben berechnet. Die quantitativen Ergnisse aller drei Methoden wurden durch Regressionsanalyse und Bland-Altman Auftragungen miteinander verglichen.

Die Methode wurde zusätzlich für die Analyse von Fettsäuren erweitert, welche aufgrund ihrer Carboxylgruppe mit Chlorameisensäurepropylester derivatisiert werden können. Um isobare Fettsäuren trennen zu können mußte die GC-trennung auf von 11 auf 50 min erweitert werden. Der Bereich der Nachweisgrenzen (LOD) lag zwischen 0.08 und 39 µM. Mit der erweiterten Methode ist es möglich eine vereinte Analyse von Aminosäurenkonzentration und totalen Fettsäurenkonzentration für 17 Fettsäuren und 25 Aminosäuren durchzuführen.

Da die Anzahl der Aminosäuren die mittels GC bestimmt werden können limitiert ist, wurde zusätzlich die Möglichkeit zur Aminosäurenanlytik mittels LC-MS/MS Chlorameisensäurepropylesterderivate getestet. Tryptophanmetabolite und Polyamine sind in mehreren biologischen Projekten von großem Interesse und wurden deshalb in die Methode integriert. Da der eigens synthetisierte Standard nicht zu Quantifizierungszwecken eingesetzt werden konnte wurden erneut isotopenmarkierten Aminosäuren als interner Standard verwendet. Insgesamt wurden 23 isotopenmarkierte Verbindungen für die Quantifizierung 41 Analyten (Aminosäuren, Tryptophanderivate und Polyamine) verwendet. Mit dieser Methode war es nicht möglich Konzentrationen für Tryptophanmetabolite oberhalb der unteren Quantifizierungsgrenze in biologischen Proben zu bestimmen. Zur Verbesserung der Nachweisgrenzen wurden erste Experimente durchgeführt, die eine bessere Extraktion der Analyten ermöglichen.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Regensburg, 04.06.2008

Hannelore Kaspar