Aus der Kinderklinik und Kinderpoliklinik im Dr. von Haunerschen Kinderspital der
Ludwig-
Maximilians-Universität München

Direktor: Univ.-Prof. Dr. Christoph Klein

Amino acid analysis in biofluids using LC-MS/MS: Method development, validation and application in clinical research and dairy science

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
Ulrike Harder
aus
Greifswald
2014

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Berichterstatter:	Prof. Dr. med. Berthold Koletzko
Mitberichterstatter:	Prof. Dr. Dr. Karl-Siegfried Boos Prof. Dr. Dr. Bernhard Olgemöller Prof. Dr. Daniel Teupser
Mitbetreuung durch den promovierten Mitarbeiter:	Dr. med. Wolfgang Peissner
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR
Tag der mündlichen Prüfung:	27.01.2014

List of Abbreviations

AA	amino acid
Ala	alanine
Ap	ante partum
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BCAA	branched chain amino acids
BF	breastfed
Car	carnosine
CE	collision energy
CID	collision induced dissociation
Cit	citrulline
CLA	conjugated linoleic acid
Cntr	control
CXP	collision cell exit potential
Cys	cystine
Cysta	cystathionine
DP	declustering potential
EP	entrance potential
FIA	flow injection analysis
FP	focusing potential
Gln	glutamine
Glu	glutamic acid
Gly	glycine
HLys	hydroxylysine
HP	high protein
HPLC	high performance liquid chromatography
HPro	hydroxyproline
IGF-1	insulin-like growth factor 1
Ile	isoleucine
IS	internal standard
Leu	leucine
LP	low protein
Lys	lysine
Met	methionine

MHis	methylhistidine
mo	month
MS	mass spectrometry
MSUD	maple syrup urine disease
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Orn	ornithine
Phe	phenylalanine
PKU	phenylketonuria
Pp	post partum
Pro	proline
Ser	serine
Thr	threonine
TMR	total mixed ration
Trp	tryptophan
Tyr	tyrosine
Val	valine
Wk	week

1 Introduction

Amino acids (AA) are the building blocks of proteins and also serve as energy sources. They play an important role in the biochemistry of any living organism. Analysis of amino acids profiles is therefore an important tool for the study of metabolic regulation and dysregulation. Acknowledging their central role in energy and protein metabolism, we aimed at developing a high-throughput method for the quantification of all proteinogenic amino acids plus the urea-cycle related amino acids ornithine and citrulline. As part of a targeted metabolomics platform established at the Dr. von Hauner Children´s Hospital, a high-throughput method was developed for a variety of applications in metabolic research, including cohort studies, intervention trials and experimental settings. The desired method had to meet the criteria of being (a) cost-effective for a large number of samples, (b) robust in terms of repeatability over multiple batches, (c) sensitive enough to obtain accurate quantification form very limited samples volumes, (d) inherently selective to enable validation of analyte signals in every sample.

This doctoral thesis project achieved development of a new LC-MS/MS method for AA determination meeting all of these requirements. The presented protocol combines derivatization and ion-pairing to a highly sensitive and robust method which allows quantitative analysis of all proteinogenic AA, citrulline and ornithine. This work describes method optimization and thorough validation according to widely accepted industry standards and subsequent application to diverse projects in clinical research and dairy science. Both applications differed considerably regarding background, sample types and objectives, but AA analysis was performed by the same, broadly applicable new method.

As a first example, samples from the CHOP European Childhood Obesity trial where analyzed, where protein intake in the first year of life was examined for its relation to rapid weight gain in early childhood (*1*). More than 1000 healthy, formula-fed infants were randomly assigned to receive cow-milk–based and follow-on formulas with lower (LP) or higher protein (HP) contents for the first year. Both groups were compared to breast fed (BF) infants. We analyzed serum AA of HP-, LP- and BF groups and compared AA profiles between these nutritional intervention groups.

A second application example of the new methodology was a feeding intervention trial in the field of dairy science. Dairy cows were either supplemented with conjugated linoleic acid (CLA group, n=10) and or with linoleic acid (control group, n=10) from two weeks before to nine weeks after expected calving. The aim of the study was to analyze the relation between CLA supplementation and AA profiles in blood samples taken at eight consecutive time points over the intervention period.

2 Development of a method for quantification of 22 amino acids

2.1 Background

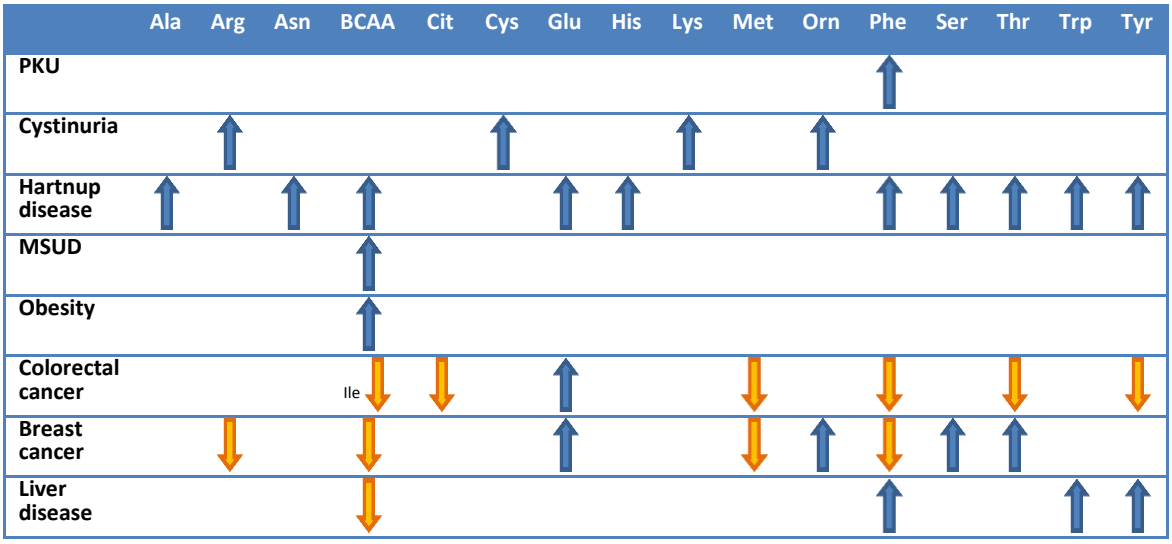
The discovery of AA began with the isolation of asparagine (Asn) from asparagus juice by Louis-Nicolas Vauquelin and Pierre Jean Robiquet in 1806 (*2*). Many years passed until all AA were characterized. Twenty AA are directly encoded in the genetic code and called proteinogenic AA. Asn, a non essential AA, is synthesized by the human body as well as alanine (Ala), arginine (Arg), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), proline (Pro), serine (Ser) and tyrosine (Tyr). Essential AA cannot be synthesized by humans and need to be up taken from dietary sources. Those are histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), trypthophan (Trp) and valine (Val). Arg, Cys and Tyr are semi-essential and need to provided by food during early childhood (*3*). Semi-essential AA can be synthesized by the human body but under certain conditions (growth, strong physical strain) a number of AA are essential and must be taken by food.

AA play an important role in the biochemistry of any living organism. They function as building blocks of proteins and also as energy sources. Some AA in proteins act as catalysts, others serve for storage and transport, while still others are antibodies for our immune system. Tyr is a precursor of adrenaline which regulates moods and stimulates metabolism and the nervous system (*4*). Arg is involved in tumor metabolism (*5*) and His is essential for the growth and regeneration of tissues and needed for the production of red and white blood cells. AA play a central part in the human organism. Published studies have shown changing AA profiles in various diseases e.g. phenylketonuria (*6*), chronic granulomatous disease, cystinuria (*7*), Hartnup disease or maple syrup urine disease, just to name a few monogenetic disorders (*8*).

The usefulness of AA profiling is exemplarily illustrated by newborn screening which is performed after delivery to detect metabolic diseases at an early stage (*9*). During further life, AA profiling is also essential for diseases like metabolic syndrome, obesity and diabetes. Therefore, metabolic profiling is supposed to further our insights into pathophysiology and therapeutic strategies of these disorders. Recent studies show

new possibilities of using plasma AA analysis as biomarker discovery tools by generating diagnostic indices through systematic computation. Insulin resistance has been reported to result in major disturbances of AA profiles (*10*). On the other hand, amino acid uptake has been shown to exert anabolic effects independent form insulin signaling, offering a new rationale for potential therapeutic intervention in patients with reduced insulin secretion (*11*). AA profiling of biological samples could be used to generate indices of diseases (Table 1). Different types of cancer patients (colorectal, breast, lung) show an altered amino acid profile compared to healthy subjects (*12, 13*). In obese humans plasma branched chained AA (BCAA) are markedly increased compared to lean humans (*14*). AA could be useful indicators for facilitating nutritional management of specific physiological and pathological states.

Table 1: Effects of metabolic diseases on AA levels in urine or plasma (↑increased values ↓decreased values)



Analysis of AA profiles is a centrally important tool for studying metabolic regulation and dysregulation. It is essential to develop methods for larger clinical research trials with cost-effectiveness for a high number of samples, robustness in terms of repeatability over multiple batches and inherent selectivity to enable validation of analyte signals in every sample. Additionally, time efficient and comprehensive quantification of AA from small plasma volumes continues to be a challenge. Different determinations of AA are known. The most established methodology, the amino acid analyzer has long run times of 120 min and is not suitable for large number of samples or for large clinical trials (*15*). In the last few years, much effort was spent on developing high-throughput methods (*16-18*). Several analytical HPLC methods have been developed for amino acid quantification, coupling liquid chromatography with optical detection (UV, fluorescence) or mass spectrometry (MS) (*19*). Mass spectrometry (MS) plays an important role as the detector for HPLC due to the

selectivity, which can distinguish analytes by their mass-to-charge ratio (m/z). LC-MS/MS has afforded gains of selectivity and sensitivity by multiple reaction monitoring (MRM). Capillary electrophoresis (CE) does not require derivatization (*20*). Using ion-pairing is another option to separate underivatized AA (*21*). To achieve more sensitivity and selectivity, derivatization reagents like o-phthalaldehyde, phenylisothiocyanate, alkyl chloroformate (*19*) or butanolic HCl (*18, 22*) were applied. Also GC-MS, hydrophilic interaction chromatography (HILIC), NMR and direct infusion have been used for amino acid analysis (*19*).

Acknowledging their central role in energy and protein metabolism, we aimed at developing a high-throughput method for the quantification of AA in clinical trials. To be useful in larger clinical research trials as part of a targeted metabolomics platform, the method should meet the following criteria: cost-effective for a large number of samples, robustness in terms of repeatability over multiple batches, sensitively to obtain accurate quantification from very limited samples volumes, inherent selectively to enable validation of analyte signals (*23*).

Here, we present a protocol combining derivatization and ion-pairing to a highly sensitive and robust method (*23*). The established method provides a reduction of sample volume, ion suppression and analyzing time, while enhancing sensitivity with high resolution chromatographic separation and optimal peak shapes for all analytes. The process allows the quantitative analysis of 22 AA (all proteinogenic AA, citrulline and ornithine) using a LC-MS/MS system (AppliedBiosystems/Sciex API 2000). Over time, seven non proteinogenic AA have been added to the method: Anserine, carnosine, cystathionine, hydroxylysine, hydroxyproline, methylhistidine and sarcosine.

2.2 Experimental

2.2.1 Instrumentation

The HPLC part of the analytical apparatus of a HPLC 1100 system from Agilent combined a binary pump, an autosampler and a column oven with a 10-port column switching valve. Mass spectrometric detection was done with an AppliedBiosystems/Sciex API 2000 triple quadrupole in the sensitive MRM mode, equipped with an Atmospheric Pressure Chemical Ionization (APCI) source which was operated in positive ion mode. Data handling and quantification were done with AppliedBiosystems/MDS Analyst 1.5 software using ’Scheduled MRM’ algorithm, which

allows optimization of effective dwell time by scheduled monitoring of mass transitions only within predefined retention time windows. Chromatographic separation occurred on a single Waters XBridge C18 column, 2.1 mm * 150 mm, 3.5 µm (Waters, Eschborn, Germany).

2.2.2 Reagents

Amino acid standards were purchased from Sigma (Taufkirchen, Germany) in a mix of 17 AA and ammonium chloride. AA in this standard solution are 2.5 µmoles per mL in 0.1 N HCl, except for L-cystine at 1.25 µmoles per mL. L-asparagine anhydrous and L-tryptophan were supplied from Fluka (Taufkirchen, Germany). L glutamine, DL-citrulline and DL-ornithine were supplied from Sigma (Taufkirchen, Germany). Internal standards were obtained from Cambridge Isotope (Andover, MA, USA) in a mix of 12 stable isotope labeled AA (Table 2). Furthermore, L-tryptophan (indole D5) and L-asparagine (U-15N2) were also obtained from Cambridge Isotope (Andover, MA, USA). Methanol and water (both LC-MS grade) were purchased from J.T.Baker (Griesheim, Germany). Heptafluorobutyric acid (HFBA) was purchased from TCI Europe (Zwijndrecht, Belgium). Hydrogene chloride (3M) in 1-butanol and formic acid (LC-MS grade) were supplied from Fluka (Taufkirchen, Germany). Plasma controls (ClinChek® Plasma Control, Lyophilized, for AA, Levels I and II) were obtained from Recipe (Munich, Germany).

Table 2: Composition of labeled internal standard

Name of internal Standard	Labeling of internal Standard
L-Alanine	L-Alanine (2.3.3.3-D4, 98%)
L-Phenylalanine	L-Phenylalanine (ring-13C6, 99%)
L-Leucine	L-Leucine (5.5.5-D3, 98%)
L-Valine	L-Valine (D8, 98%)
L-Arginine	L-Arginine:HCL (5-13C.99%, 4.4.5.5.-D4, 95%)
L-Citrulline	L-Citrulline (5.5-D2, 98%)
DL-Glutamic acid	DL-Glutamic acid (2.4.4-D3, 97%)
L-Tyrosine	L-Tyrosine (ring-13C6, 99%)
L-Ornithine	L-Ornithine:HCL (5.5-D2, 98%)
L-Methionine	L-Methionine (methyl-D3, 98%)
L-aspartic acid	L-aspartic acid (2.3.3-D3, 98%)
Glycine	Glycine (2-13C, 99%, 15N, 98%)
L-Asparagine	L-Asparagine (15N2, 98%)
L-Tryptophan	L-Tryptophan (Indole D5, 98%)

2.2.3 Method Development

Our method based on the chemical derivatization of AA to amino acid butyl esters (*24*). Schulze analyzed amino acid butyl esters in new born screening for inborn errors of metabolism. Figure 1 shows the derivatization with n-butanolic HCl of alanine to alanine butyl ester as an example.

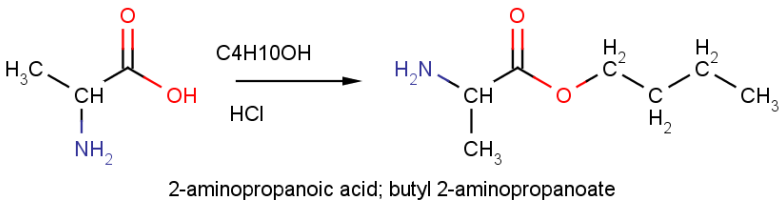


Figure 1: Derivatization of alanine to alanine butyl ester

In the first step of development we optimized several potentials and energies such as declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP), focusing potential (FP) and ion source temperature of MRM compounds with flow injection analysis (FIA) using the quantitative optimization mode of the Analyst software for improving better intensity. Optimized parameters were merged to one method including all MRM transitions and parameters of the analyzed AA. This method is used for chromatographic separation using Xbridge C18 reversed phase column with different gradients of water and acetonitrile. One of the first presentable chromatogram of AA is shown in figure 2. Therefore samples were prepared by Schulze and analyzed with a flow rate of 300 µL/min and a gradient of water and acetonitrile (*24*)(Table 3).

Table 3: Composition of mobile phase

Time [min]	% of water	% of Acetonitrile
0	90	10
3	90	10
17	85	15
45	0	100
47	0	100
49	90	10
54	90	10

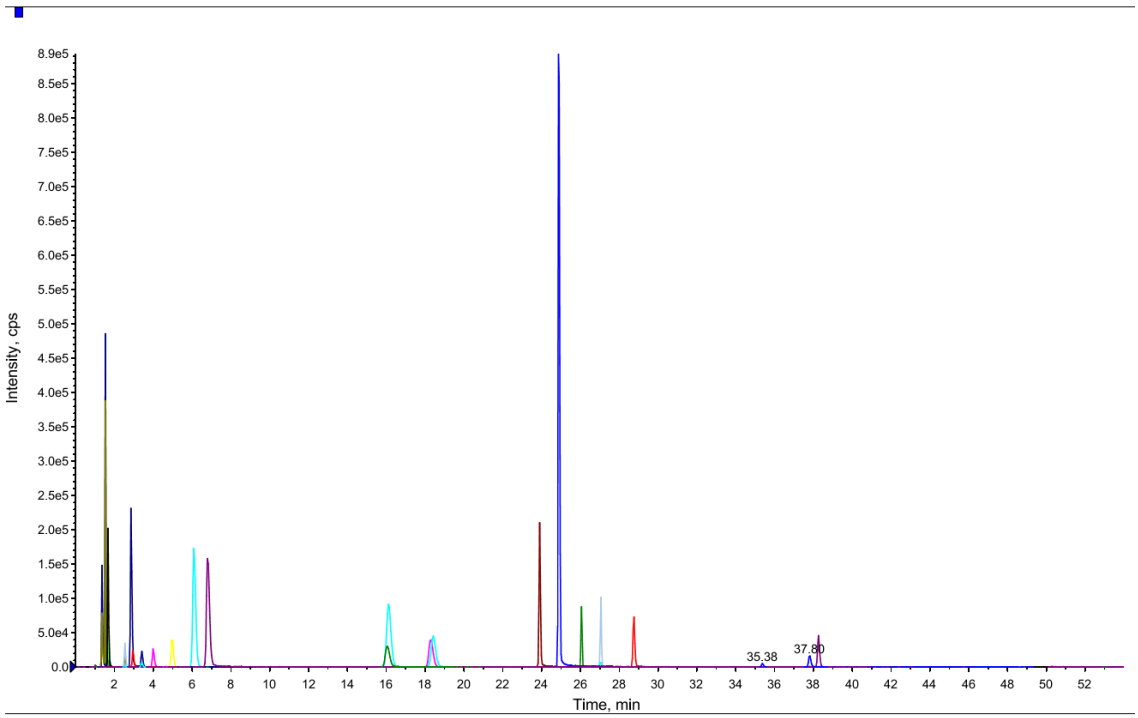
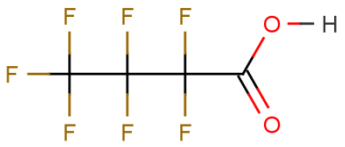


Figure 2: chromatogram of 22 AA using Xbridge C18 with a runtime of 54 min

Figure 2 shows very sharp peaks but polar peaks like Asn, Arg, His and Orn were eluted within 2 min due to insufficient retardation by column. To separate early eluting peaks from the ion suppression zone, peaks have to elute after 3 min. Details about ion suppression are given in section 2.3.3.

The worldwide shortage of acetonitrile in 2009, forced us to use methanol as precipitation reagent instead of acetonitrile. Mobile phases, precipitation reagent was replaced with methanol and coelutions were separated from analytes much better using methanol. Using acetonitrile, we observed coelution in EDTA plasma of Asp. Using methanol coelution was clearly separated from Asp.

A further improvement of chromatography was achieved by the use of ion pair reagent. This chemical can be retarded longer at stationary phase due to the strong bond of the alkyl chain with stationary phase to exclude analytes from the ion suppression zone. Positive loading of reagent is coupled to complex with charged compounds. We used HFBA, an anionic ion pair reagent which is used in reversed phase HPLC (Figure 3). 0.1% is added to the mobile phase A (water) and B (methanol).



2,2,3,3,4,4,4-heptafluorobutanoic acid

Figure 3: Chemical structure of HFBA

Figure 4 shows a chromatogram of one plasma sample using HFBA as ion pair reagent. Compared to figure 2 retention times of analyte shift backward with excellent peak shapes whereby Asn eluted at 4 min as the first peak in chromatogram. During method development, run time of chromatograms was reduced to 16 min for one sample.

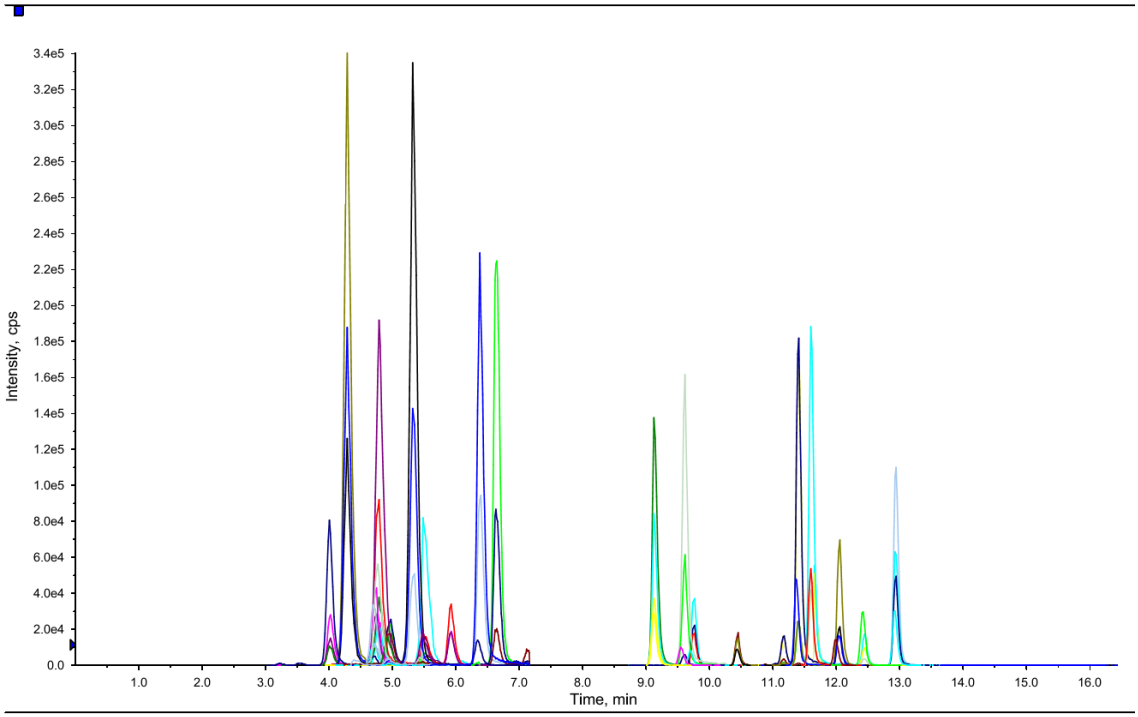


Figure 4: chromatogram of 22 AA using Xbridge C18 and HFBA with a runtime of 16 min

During method development different columns were tested for chromatographic separation of AA. Especially Agilent Zorbax C18 and Phenomenex PFP column were trialed to get better separation results. Agilent Zorbax C18 is well suited for chromatography but the major disadvantage is the inadequate column stability due to very acidic pH levels. The use of PFP column shows moderate retardation and less peak sharpness of some AA. Both columns were not suitable for clinical trials with large number of samples. Advantages of stability and peak sharpness enable the Xbridge column for combination of butylation and ion pair chromatography. In summary, we

measured more than 5000 samples with the same Xbridge column which is profitable for large scale studies.

In some experiments, the addition of acid to the precipitation reagent leads to a better reproducibility. Hence various precipitation reagents have been tried in order to optimize quantification. For testing, calibration curves of all standard concentration were done and correlation coefficient r was determined. Table 4 shows all correlation coefficients less than 0.995. Especially methanol with 2% HCL shows the highest correlation coefficients for all AA (r > 0.995) and is perfectly used as precipitation reagent.

Table 4: Correlation coefficient 0.995 of AA of different precipitation reagents

Precipitation reagent	Correlation coefficient r < 0.995
MeOH	His, Ser, Gln, Lys
MeOH + 2% HCL	-
ACN	Gly, His, Ser, Gln, Lys, Thr
ACN + 2% HCL	His, Pro
MeOH + ACN + H2O + 2% HCL	His, Orn, Trp, Ser, Lys
ACN + TFA (0.05%) + FA (0.1%)	Gly, His, Ser, Gln, Lys

External standard preparation has been optimized and improved in the course of time. Two stock solutions (1 and 2) were prepared. External standard solution 1 consisted of premixed solution of 17 AA (concentration of 2.5 mmol/L). For solution 2, Ala, Cit, Gln, Orn and Trp were weighed and diluted to a concentration of 2.5 mmol/L. It should be emphasize that solution 1 and 2 were mixed in the opposite ratio where low values of S1 are mixed with high values of S2 and the other way around. Finally, following calibration concentration were prepared for quantification of AA (Table 5).

Table 5: External standard concentration of solution 1 and 2

Number of external standard	Ext. Standard Concentration of solution 1 (S1)	Ext. Standard Concentration of solution 2 (S2)
K01	10 µM	200 µM
K02	25 µM	100 µM
K03	50 µM	50 µM
K04	100 µM	25 µM
K05	200 µM	10 µM
K06	CP1	CP1
K07	CP1 + 25 µM	CP1 + 25 µM
K08	CP2	CP2

Figure 5 shows a perfectly measured calibration curve of Ala with r = 0.997 after analyzing 81 unknown samples from a clinical trial.

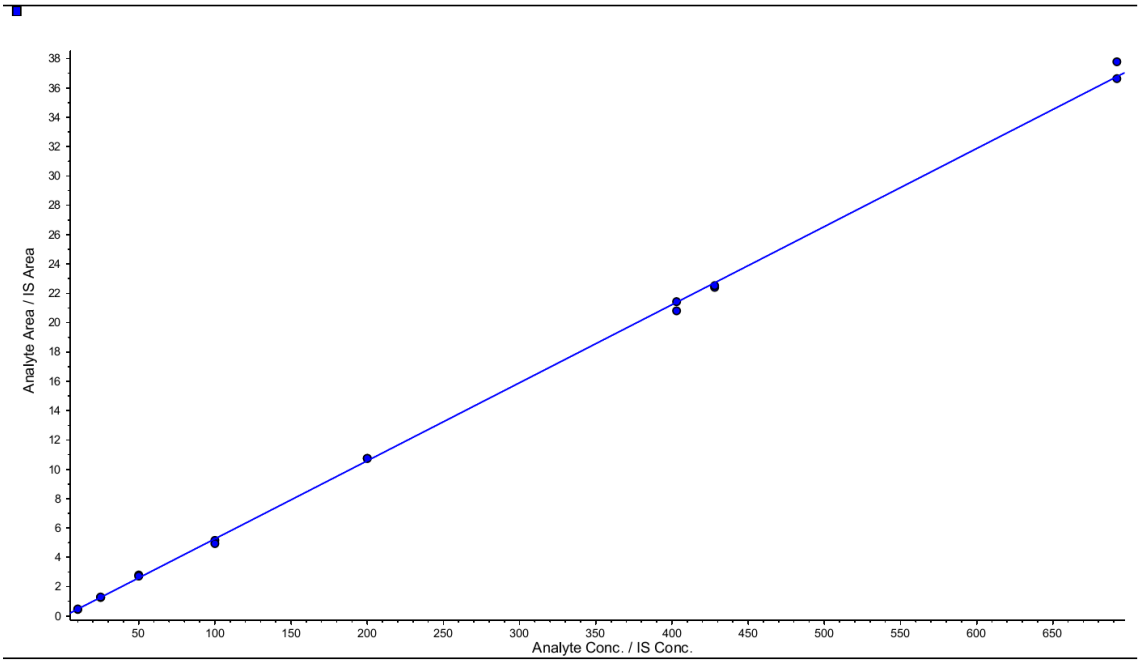


Figure 5: Calibration curve of 8 external standards for Ala, r= 0.997

2.2.4 Preparation of external- and internal standard, elution solvent, QC- and plasma samples

Asparagine-, citrulline-, glutamine-, ornithine- and tryptophan powder were weighed to a concentration of 2.5 mmol/L (solution A).This solution was added to a premixed

solution of 17 AA (concentration of 2.5 mmol/L), purchased from Sigma Aldrich, to a final stock solution of 0.5 mmol/L. The stock solution was diluted in water to final standard calibration concentrations of 10, 25, 50, 100 and 200 µmol/L. Furthermore, 2 plasma control levels, purchased from RECIPE, were prepared. The lower level of plasma control was spike using 25 µmol/L of standard solution. To construct the calibration curve, we need a total of 9 samples including 5 levels of aqueous standard solution, 2 levels of matrix samples, 1 spiked matrix sample and 1 blank.

The internal standard, purchased from Cambridge Isotopes, contains of 12 stable isotope labeled AA and was diluted with 1 ml of methanol/water (1:1). After mixing for 15 min in the ultra sonic bath, the solution was completely transferred in methanol to a final volume of 200 ml. Absolute concentrations of internal standards is about 2.5 µmol/L expect for glycine with 12.5 µmol/L. Aliquots of 10 ml are stored at -85°C in glass bottles.

Anionic ion pair reagents Hepta Fluoro butyric acid (HFBA) was added to the mobile phase to achieve a better interaction between analyte and stationary phase. Therefore, a solution of HFBA (0.5 mol/L), purchased from TCI Europe, was diluted in mobile phase A (water) and mobile phase B (methanol) to a final concentration of 0.5 mmol/L.

For sample preparation a fresh precipitation reagent is used. For a 96 well plate 30 ml methanol was added to 10 ml of internal standard, 4 ml water and 880 µL HCL. 450 µL of this solution was added to a 96 * 1 ml deep well plate using a stepper pipette and 10 µL of sample or standard was added to the precipitation reagent. After shaking for 20 min, the deep well plate is stored for 60 min at +5°C and then centrifuged for 10 min at 2300*g at +5°C. A supernatant of 200 µL was transferred in a 96 * 0.3ml well plate using a multi-channel pipette and dried completely under nitrogen. For derivatization, 100 µL of hydrogene chloride (3M in 1-butanol) were pipetted to each sample and the plate was sealed with PCR adhesive film and incubated for 15 min at 60°C in a thermo shaker. After evaporation, the samples were filled with 100 µL injection solvent (80% water, 20% methanol, 0.1% formic acid) and final shaking for 10 min.

2.2.5 HPLC and MS settings

From the prepared samples, 10 µl were injected into the HPLC system. The gradient system used a single reversed phase column (Waters XBridge C18, 2.1 mm * 150 mm, 3.5 µm) with a flow rate of 0.3 ml/min and a column oven temperature of 40°C. The HPLC gradient was increased linearly from 25 % B to 75% B within 15 min. After isocratic elution for 1.5 min the gradient returned to initial conditions till 16.5 min,

followed by isocratic equilibration until 21 min. Total time from injection to injection accounted for 22 min (including 0.8 min autosampler operation time).

Mass Spectrometric settings were done by direct infusion using a syringe pump (Harvard Apparatus, Holliston, MA, USA) in manual tune mode whereby ion path settings were determined using the compound optimization algorithm of the AppliedBiosystems/MDS Analyst 1.5 software. Three of the most ten intensive MRM (Multiple reaction monitoring) transitions for each analyte were selected and summarized to one final method using the scheduled MRM algorithm which allowed the inclusion of three MRM transition (one quantifier and two qualifier ions) for each AA. Ion source parameters were optimized by FIA for the lower abundance compounds (curtain gas: 30 psi; collision gas: 5 psi; Nebulizer current: 2 µA, temperature: 400 ◦C, Nebulizer gas: 50 psi; desolvation gas: 80 psi; interface heater: on).

2.2.6 Method validation

Method development requires a precise validation using tests for sensitivity, linearity, precision and accuracy, matrix effects and stability. We validated the procedure according to selected guidelines (FDA, Bioanalytical Method Validation 2001; ICH, Validation of Analytical Procedures Q2 (R1) 2005; CDER, Validation of Chromatographic Methods 1994).

For selectivity testing, 2 plasma samples and 2 serum samples were analyzed by using MRM-mode. In addition, 3 blanks and 3 concentration levels of aqueous standards were needed to compare the rate of interferences in chromatograms of samples, blanks and standards. The process was done using one quantifier ion (product ion which is the most abundant fragment of one molecule) and two qualifier ions (product ion of two alternative fragments of the same molecule). Qualifier ion ratio is calculated as the ratio of the signal intensities of quantifier to qualifier. This ratio can indicate possible co-eluting interferences (*23*). Furthermore, more than 700 samples in a clinical trial were measured and ratios of quantifier- and qualifier ions were calculated.

Seven standard samples, each sample prepared three times, were used for calculating calibration linearity (r) of the calibration curve. Accuracy is the quality to being near to the true value. Therefore plasma control samples (level I and II, Recipe) were measured six times with our method. Accuracy was determined by comparison of the calculated concentrations with the set values from the manufacturer. Furthermore, we participated in a collaborative study (Ringversuch) to determine the accuracy of our study. This study is a method of quality assurance for measurement processes.

Basically identical samples were examined by different laboratories. Comparison of the results allows conclusions about the accuracy and quality for measurements of the participating institutes. Therefore, two different plasma samples were prepared and analyzed several times.

Precision is divided into three parts: repeatability, interday and intraday precision. Injection repeatability was determined by injecting a plasma/serum sample ten times each. To evaluate intraday precision, one sample is prepared six times using same conditions by the same technician at the same day. For interday precision, one sample (serum and plasma) is prepared at six different days.

Limit of detection (LOD) is the lowest value of analytes in a sample that can be detected but not quantified. The signal to noise ratio (S/N) should be > 3 (*25*). Lower limit of quantification (LLOQ) is the lowest concentration of analytes in a sample that can be detected and quantified. The determination of LLOQ requires a precision of 20% CV and ± 20 % for accuracy bias (*25*).

Another important part in method validation is the determination of matrix effects in terms of ion suppression (*26*). Therefore we tested for ion suppression using a permanent flow (100 µL/min) of a standard solution which was delivered by a infusion pump and combined via T-piece union with the eluent flow of a HPLC separation of a plasma sample (*27*). Figure 6 shows the construction and the determination of one selected amino acid.

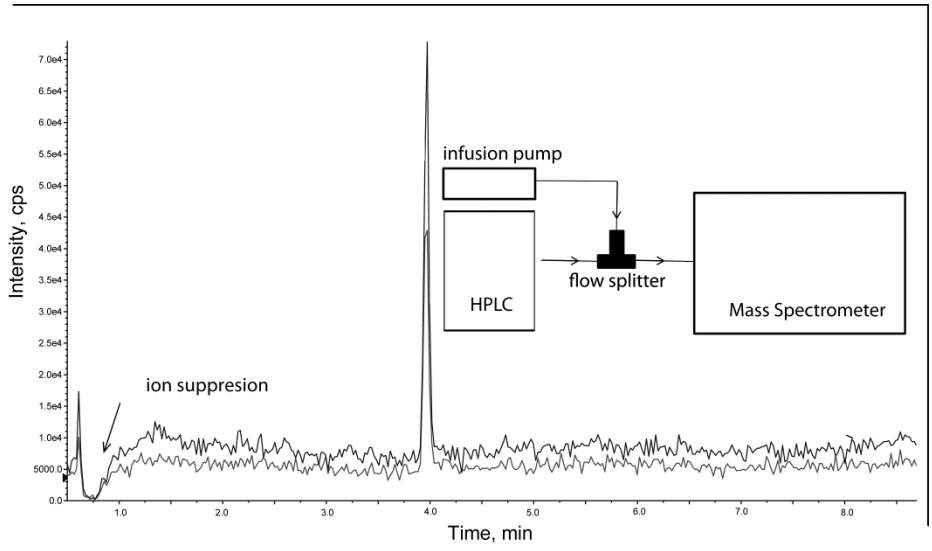


Figure 6: Test for ion suppression exemplified with alanine butyl ester. Derivatized Ala was added at a constant rate (c = 100 M) to the mobile phase via a flow splitter. Simultaneously, a plasma sample was injected and separated by HPLC. Ion suppression is discernible of the continuous AA standard signal at the beginning of the chromatogram.

The sample solution stability was determined because samples will be in solution for hours in the laboratory environment (cooled autosampler at 10° C) before the last sample injection is completed. After analyzing 111 samples within 48 hours, the first blood plasma was injected six more times. All plasma samples were quantitated and the coefficient of variation and the recovery were defined and compared.

2.3 Results

2.3.1 Efficient chromatography of 22 amino acids combining derivatization and ion-pairng

Requirement for exact quantification of all AA is high resolution chromatographic separation. Because of the zwitterionic character the negatively chargeable carboxyl group can be protected by derivatization. Thus, the retention time of the peaks is changing but the sensitivity can be increased. The positive charged amino group is bounded by the anionic ion pair reagent (Heptafluorobutyric acid, HFBA). This leads to an amplified interaction with the stationary phase, analytes were retarded in a different way and chromatographic resolution and peak shapes are improved. Combining derivatization and ion-pair chromatography, our method enables us to quantify all proteinogenic AA, citrulline and ornithine within a total analyzing time of 22 min using a standard C18 reversed phase column. Figure 7 shows 22 extracted ion chromatograms of a plasma sample. Except for leucine/isoleucine and glutamine/lysine, each analyte shows its own MRM transition. The optimization of Gln and Lys showed very similar fragmentation patterns of the target ion m/z = 203 despite having a different chemical structure. Chromatographic baseline separation allowed their precise quantification. Besides Gln and Lys, also Leu and Ile had the same MRM mass transitions because of their isomeric chemical structure and identical molar mass. Both were baseline separated and could be quantified reliably. Over time, seven non proteinogenic AA have been added to the method: anserine (Ans), carnosine (Car), cystathionine (Cysta), hydroxylysine (HLys), hydroxyproline (HPro), methylhistidine (MHis) and sarcosine (Sar).

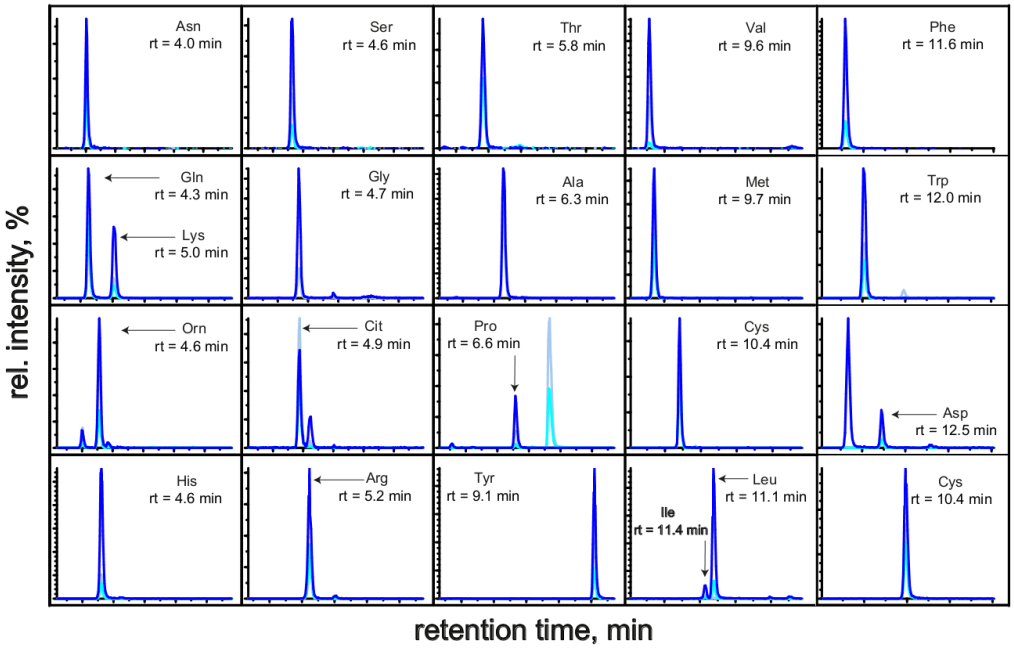


Figure 7: Extracted ions of 22 amino acid butyl esters. Extracted ion chromatogram of a plasma sample illustrating 22 AA.

2.3.2 Sensitivity improvement for minimization of sample volume

Derivatization of the negatively chargeable carboxyl group during butylester formation simplifies the protonation behavior by leaving only positively chargeable groups. Modification of the elution solvents with 0.5 mmol/L HFBA results in a quite acidic pH value of pH 1.5 – 2.0. Chromatographic retention in this setting is both enhanced by increased lipophilicity of butylesters as well as by ion-paring of HFBA with positively charged moieties. In addition, ionization efficiency is enhanced by favoring the positively charged state in acidic solution as well as by promoting the location of lipophilic butylesters to the droplet surface during electrospray desolvation. Butylation and ion-pair chromatography improved peak shape, selectivity and sensitivity. Therefore a sample volume of only 10 µL is adequate for accurate quantification.

2.3.3 Sample clean up and assessment of ion suppression

Sample clean up is important to precipitate interferences like proteins or phospholipids. Inadequate sample preparation of biological samples leads to matrix effects in terms of ion suppression. For a sufficient protein precipitation we added HCL to the precipitation reagents (consists of a mixture of methanol, IS and water). Further, sample preparations like derivatization with butanolic HCL at 60°C leads to degradation of phospholipids. Using post-column infusion system we assessed the ion suppression in

biological matrices. According to Taylor et al. a standard AA solution was added at a constant rate (c = 100µM, flow = 12µl/min) to the mobile phase via a flow splitter (*27*). At the same time a plasma sample was injected and analyzed (Figure 6). MRM transitions of phospholipids commonly encountered in plasma samples were monitored in addition (m/z = 758.6->184 or 786.6->184), but not detected. Figure 6 identified ion suppression within the first 2 min of the chromatogram.

2.3.4 Concept of qualifier ions

Typical performance testing and quality control protocols are based on analysis of periodic calibration check and/or reference samples or synthetic mixes. However, this cannot confirm the actual and real value of an unknown sample. A technique that can determine the quality of analytical results for each analyte is achieved by using qualifier ion monitoring as a fast and specific quality check. The advantage of this concept lies in monitoring the coelution-free, unbiased quantification in every single sample. Our method applies this concept to one of the first for bioanalytical LC-MS/MS for endogenous and small samples. Especially amino acid butyl esters are well suited for the qualifier ion monitoring. The optimization of Q1 and Q3 masses established at least 2 MRM transitions for one analyte. The MRM transitions with the highest intensity were used for quantification, second and/or third transition were used as qualifier ions. Harder et al. show the MRM transitions of all analytes and internal standards (*23*). Due to butylation small molecules like alanine and glycine confirm a second MRM transition. Without butylation only one transition exists.

2.3.5 Validation

Selectivity testing was done without ‘Scheduled MRM’ mode and all MRM transitions were monitored during the entire analyzing time. Asp, Cit and Pro showed several peaks in the chromatogram. Using labeled internal standards and qualifier ion monitoring, all analyte-peaks were correctly assigned and coelution-peaks were excluded. Analyzing Pro standards and samples, two peaks were detected but only one peak showed the quantifier ion (m/z = 172 → 70) and both qualifier ions (m/z = 172 → 116 and m/z = 172 → 57) with their correct ratio. The unknown compound did not include m/z = 172 → 70 and showed a different retention time. An unknown compound is also observed in Cit. In EDTA plasma two peaks for Asp were detected but in serum samples only one peak was observed. The unknown compound was also noted in EDTA blanks (Figure 7+8).

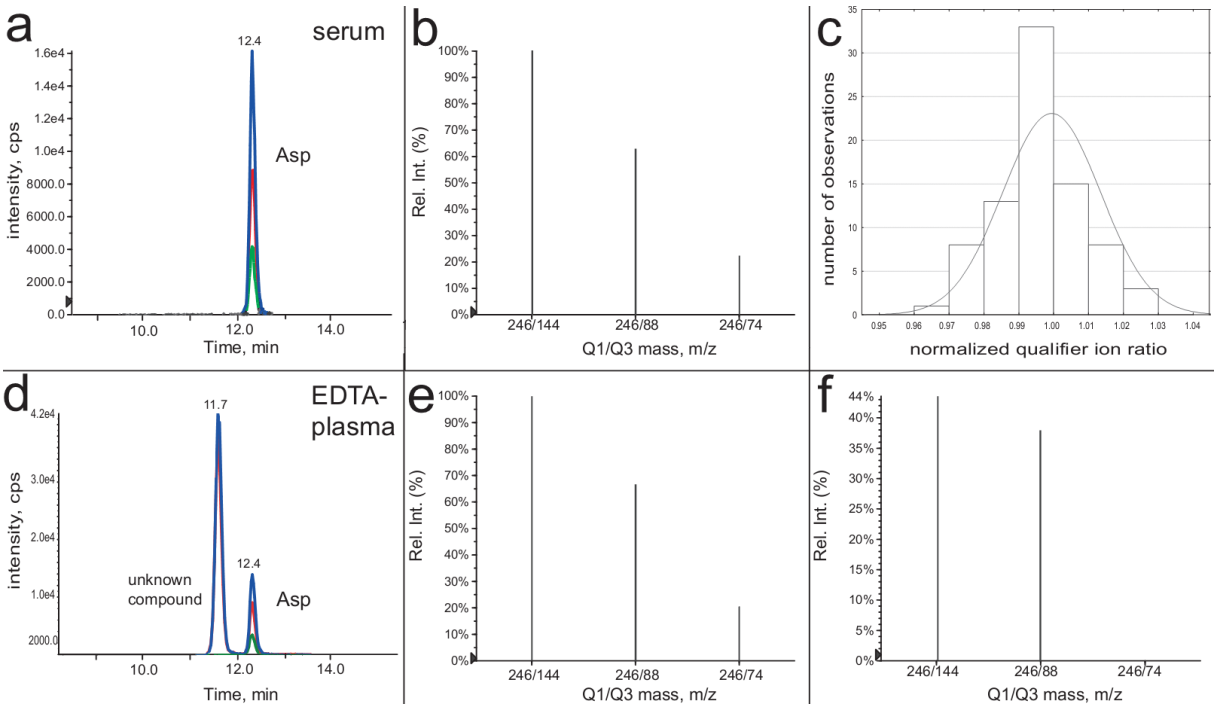


Figure 8: Peak verification of aspartic acid in serum and EDTA plasma. Chromatogram of serum and EDTA-plasma illustrating the selectivity of the MRM transitions for aspartic acid: (a) extracted ion chromatogram of three MRMs for Asp in serum; (b) relative intensity (%) MRM spectrum for Asp of one quantifier ion and two qualifier ions (MRM fingerprint) in serum; (c) distribution of normalized qualifier ion ratio of Asp determined in 81 plasma samples; (d) extracted ion chromatogram of three MRMs

For **linearity** an accurate calibration is prepared combining different matrices, 5 aqueous standards and 3 matrix samples with and without spiking. By adding 0.1 N HCL to the precipitation reagents, reference factors of aqueous standards and matrix samples were equal. Calibration curves of all 22 AA were fitted by weighted (1/x) linear regression. Calibration linearity (r) of an eight point calibration curve were greater than 0.992 (Table 6).

Accuracy of our method was determined using two reference plasma samples. These available lyophilized plasma samples (ClinChek® Plasma Control,Lyophilized, Recipe, Munich, Germany) are a common choice to test internal quality assurance in clinical chemical laboratories. The manufacturer specified 21 AA values (except cystine) with a confidence interval of ± 20%. The accuracy of the control samples compared to the set values were within 82 – 118 % for level I and 96 – 117 % for level II (Table 6). Furthermore, we successfully participated in a collaborative ring trial to verify accuracy of our method.

Table 6: Validation results of all AA

	Linearity	Precision			Accuracy		LLOQ	
AA	calibration linearity (r)	injection repeatability [CV%]	intraday precision [CV%]	interday precision [CV%]	plasma Control 1 [%]	plasma control 2 [%]	LLOQ [µM]	injection repeatability at LLOQ [%]
Ala	0.993	1.6	3.3	3.7	97.3	102.8	1	2.9
Arg	0.992	2.7	6.2	5.3	106.8	97.7	1	4.9
Asn	0.995	1.1	5.5	7.1	101.0	98.4	1	5.2
Asp	0.996	3.3	2.8	7.2	95.9	104.1	1	4.0
Cit	0.994	2.3	5.2	10.2	105.2	98.2	1	12.3
Gln	0.993	1.8	5.1	6.5	107.0	96.0	1	5.5
Glu	0.996	2.1	5.9	10.2	95.1	105.6	1	7.3
Gly	0.992	2.0	3.8	5.9	97.7	100.9	1	6.6
His	0.993	3.2	2.6	10.4	99.4	98.5	1	6.3
Ile	0.997	2.5	5.4	5.9	99.5	101.6	1	9.3
Leu	0.997	2.1	4.0	4.8	99.3	99.3	1	4.8
Lys	0.994	3.1	7.3	10.8	100.5	100.9	1	4.6
Met	0.997	2.7	3.2	6.1	111.2	105.6	1	2.3
Orn	0.994	3.4	10.1	5.7	106.9	96.1	1	8.1
Phe	0.996	2.4	3.7	7.1	101.1	99.1	1	2.8
Pro	0.993	3.0	8.4	6.1	103.5	96.5	1	4.4
Trp	0.991	3.7	8.6	8.0	117.7	103.7	1	5.7
Ser	0.995	2.4	4.3	6.1	99.6	98.9	1	9.6
Thr	0.995	2.7	6.8	8.9	82.0	117.4	1	6.0
Tyr	0.996	1.2	4.3	5.9	102.2	97.6	1	1.9
Val	0.995	3.2	4.3	3.1	98.8	100.2	1	7.7
Cys	0.993	4.8	8.3	10.6	N/A	N/A	1	5.2

For **precision**, injection repeatability, intraday and interday precision were performed. Injection repeatability of 10 consecutive samples was shown in table 6 and average injection repeatability was 2.6% (CV). Intraday precision of all analytes was in the range of 2.6 – 10.1% (CV). Analytes with low concentration (ornithine, CV 10.1%) show greater CVs as highly concentrated analytes (alanine, CV 3.3%). Interday precision of all AA was in the range of 3.1 – 10.8% (CV).

The lower limit of quantification (**LLOQ**), with acceptance criteria of 20% CV for precision and ±20% for accuracy bias (*25*), of all AA analyzed was about 1 µmol/L.

Stability test was monitored for seven days. After seven days the recovery of all analytes was in the range of 94 -102 %, expect for aspartic acid (84%), cystine (74%), and ornithine (112%).

2.4 Discussion

2.4.1 Efficient chromatography combining derivatization and ion-pairing

Due to the instability of Asn, Cys, Gln and Trp, only a few published methods allow the detailed analysis of all proteinogenic AA (*24*). Many procedures do not fully cover all analytes (*28, 29*). A major problem for detecting a huge spectrum of analytes is the insufficient peak sharpness of polar analytes like Asn, Cit, Gln (*17, 18*). Compared to our method, the commercial ITRAQ system for quantification of derivatives AA analyses 44 AA but is less sensitive and the required reagents are toxic and expensive. Using ITRAQ peak sharpness of Asn and Ser is not optimal either (*19*). In comparison, our method presents sharp peaks for all 22 measured analytes and efficient separation of the combined derivatization with ion-pair chromatography. The proposed method delivers high resolution chromatographic separation on standard C18 reversed-phase column which is extremely stable for thousands of samples. Because of a very favorable retention behavior, a separation within 16.5 min is possible using a modern HPLC technology. Improved chromatographic retention of polar AA allows for switching the beginning of the chromatogram to waste to reduce contamination of the mass spectrometers ion source. The flexible use of two ion sources (APCI, atmospheric pressure chemical ionization and ESI, electrospray ionization) provides a further advantage. Normally we used APCI because background signals and matrix effects of APCI have been reported to be often lower than with ESI (*26*). A minor drawback of derivatization with butanolic HCL is the formation of HCL gas during evaporation which may cause corrosion. Metallic parts of the evaporator device can be protected from rust by covering with adhesive film.

2.4.2 Sensitivity improvement for minimization of sample volume

By improving the sensitivity, a sample volume of 10 µL is used. Other procedures use considerably more sample volume (100 µL) (*18, 28*). The fact that the developed method requires only 10 µL of plasma or serum renders it well suitable for clinical studies. Especially in newborns or infants, sample collection is extremely difficult due to

limited sample volume. For the newborn screening laboratory, the new method may be suited as a second-tier analysis to reconfirm results from direct infusion analysis.

2.4.3 Sample cleanup and assessment of ion suppression

The addition of HCL (final concentration 0.1N) to the precipitation reagents (including, methanol, IS and water) enhanced protein elimination. Organic deproteination in methanol has been commonly used in previous publications (*18, 28*). Also phospholipids, which are known as a major source of ion suppression in biological matrices, can be inactivated using derivatization at 60°C. The ion suppression test showed a decrease of basal intensity in the first two min of chromatogram. Therefore ion suppression is negligible because the first peak eluted after 4 min. The absence of ion suppression was also proved by the construction of one linear calibration curve mixing aqueous standards and matrix samples. A higher risk for ion suppression provides methods with faster gradients, early eluting peaks and less comprehensive sample clean up (*17, 21, 30*).

2.4.4 Concept of qualifier ions

Amino acid butyl esters confirmed high-intensity useful CID fragmentation patterns ideally suited for qualifier ion monitoring, since multiple product ions from complementary parts of the AA molecule were observed. Their intensity relative to the most abundant product was high enough to allow accurate calculation of qualifier ion ratio. Other derivatization processes often exhibit the loss of the derivatization group which makes this approach not well suited for qualifier ion monitoring. Dansyl chloride, another common derivatization reagent, produces a CID spectrum with uniform and unspecific fragments. The AA derivates are unstable and the fragmentation preferred the development of product ion 171 m/z (*31*). In our method, we use a number of fragments from CID but the common neutral loss 102 m/z appeared for 12 from 22 AA and is therefore used for quantification. Second and third transitions were used as qualifier ions depending on their intensity.

2.4.5 Validation

Based on the qualifier ion concept, interferences e.g. in Asp, Cit and Pro can be detected reliably and rapidly within one analytical procedure. The typical fingerprint for

Asp in EDTA plasma is completely different to the unknown interfering compound. The interference has not only been identified in EDTA plasma, also in EDTA blank. A possible explanation of this finding may be that EDTA is degraded during derivatization and esterified to form an isomer of aspartic acid butyl ester. The impurity shares two of three transitions with aspartic acid but can clearly be separated by absence of the third transition. Unknown compounds in Cit and Pro cannot be explained but separation of analyte and impurity was obvious and clear.

Calibration linearity for all analytes was greater than 0.992 using eight points from different matrices. The more calibration points from different matrices were used, the more precise was the calibration curve.

Mean values for accuracy should be within ± 20% of the target values. Our method fulfilled the specified values for all analytes of all measured samples.

Intraday and interday precision for all analytes was less than 11% and comparable to other methods (*32*).

The LLOQ, with acceptance criteria of 20% CV for precision and ±20% for accuracy bias of all AA analyzed was about 1 µmol/L. Langrock et al have reported quantification limits of above 10 µM and Booger et al have reported similar values of LLOQ but using 100 µL of sample volume. Our methods required a sample volume of only 10 µL with a LLOQ of 1µmol/L.

Standard sample solutions were stable over seven days. Application of 14 stable isotopes justified the sample solution stability. Recovery of all compounds shows a variety of 94–102%, except for Cys (74%), Asp (84%) and Orn (112%). Cys is well known to be unstable. Low abundance AA like aspartic acid (normal concentration is about 20 µM) and ornithine (about 45 µM) exhibited higher variation. Therefore it is recommended to measure the samples as soon as possible.

2.5 Conclusion

Ion pair chromatography and derivatization to butyl esters have been combined to a new methodology for determination of plasma AA by LC–MS/MS. Several published methods are often less sensitive (*17, 18*). The developed method requires only 10 µL of sample volume, while other previously proposed methods used 100 µL of plasma (*18, 28*). The very small sample volume needed enables studies in infants, as well as the use of capillary (fingerstick) sample. Within 36 hours, 96 samples can be measured. Thus, our method is suitable for clinical trials with large number of samples. All proteinogenic AA, Cit and Orn can be measured in the same run with baseline chromatographic separation of Leu and Ile. Over time, seven non proteinogenic AA

have been added to the method: Ans, Car, Cysta, HLys, HPro, MHis and Sar. In comparison, existing methods often did not fully cover all proteinogenic AA (*28, 29*) or exhibited problems with appropriate chromatographic separation (insufficient peak sharpness, early eluting peaks) (*17, 18*). Further advantage of the new method is the modified sample preparation using HCl in methanol which results in markedly improved sample cleanup. Improved sample preparation enabled us to avoid ion suppression bias, which was proved by equal detector response of matrix-free calibrators and serum/plasma samples. Other published methods with less efficient sample cleanup and too short retention of early eluting analytes risked significant ion suppression (*17, 21, 30*). The proposed protocol is one of the first introducing the concept of qualifier ion monitoring to quantitative analysis of endogenous metabolites. Using this approach, we have shown that the well-known selectivity problem of small molecule bioanalytical mass spectrometry can be addressed in automated highthroughput analysis, ensuring unbiased quantitation of every single sample. Short run time and low costs per sample allow for analysis of large sample numbers, e.g. from epidemiological studies.

3 Amino acid profiles in formula-fed and breastfed infants from a randomized clinical trial

3.1 Background

Obesity is a more and more common problem in our world. Especially in industrialized countries, obesity is dramatically increasing due to less physical work and an oversupply of food. Over-nutrition is not the only major cause, also physical inactivity, genetic factors, eating disorders, metabolic disorders or food intolerance contribute to the pathology of obesity. In modern times, while children prefer to watch television and play computer games, advertisements for sweets, soft drinks, alcohol and fast food support causes for obesity. Within recent years a lot of work was done to find further reasons for the increasing incidence of obesity.

Major problems of obesity are the consequence for health and economy. Obesity is strongly linked to adult disease like Type II diabetes, metabolic syndrome and cardiovascular diseases syndrome (*14, 33-36*). Bray et al. claimed obesity as a chronic, relapsing, neurochemical disease (*37*). Increasing obesity also results in the appearance of heart disease, hypertension and cancer (*38*) and has large implications for healthcare costs. In times of abundance, more and more people get obese and for many researchers it is a challenge to unravel the mechanism and the causes of obesity. Therefore, it is very important to understand causes of obesity. Recent studies in humans have suggested that there may be an association between rapid weight gain in infants and the later risk of obesity (*39*). A positive association of protein intake and early growth was observed by Stunkard and co-workers (*40*). He compared children of obese mothers, which have a high risk for overweight, with infants of lean mothers during the first 2 years of life. He proposes that growth and development of infants dependent on energy intake (*40*). In other studies, no effect of infant formula on growth in the first 4 month was observed (*41*).

Early programming, the process by which different factors act during early life, may have long-term consequences on health. For example, specific questions could be: How does the diet of the mother, of the newborn or of the child affect the risk of later obesity? What is the impact of parental education, ethnic origin or infant gender on later obesity? In the context of a large EU funded research project, early programming is applied in the CHOP European Childhood Obesity trial where protein intake in the first year of life is examined for rapid weight gain in early childhood (*1*). More than 1000 healthy, formula-fed infants were randomly assigned to receive cow-milk–based and

follow-on formula with lower (LP) or higher protein (HP) contents for the first year. Both groups were compared to breast fed (BF) infants. With our new developed method which is described above, we analyzed and quantified serum AA of HP-, LP- and BF group and compared AA profiles in the infants to predict diet induced weight gain or obesity.

In some studies, protein intake is associated with insulin-like growth factor (IGF-1) (*14, 42*). IGF is a hormone with 3 possible peptide ligands (IGF I, IGF II, Insulin) and is involved in the regulation of growth and development (*43*). AA, the building blocks of protein, act as stimulators of insulin secretion suggesting a possible relationship between AA and IGF. In the late seventies, Felig et al. already observed an increase of Ile, Leu, Val, Phe and Tyr in obese subjects (*44*). Exactly 40 years later; Newgard confirmed the results and showed that BCAA supports insulin resistance in obesity (*14*). Furthermore, the group of Newgard investigated the association of special AA with the mammalian target of rapamycin (mTOR) (*14*). mTOR is responsible for cell growth, cell proliferation, cell motility, cell survival, protein synthesis and transcription (*45*). mTOR complex 1 (mTORC1) activation promotes cell growth and cell proliferation. mTORC1 acts as metabolic sensor and is regulated by availability of AA, growth factors, energy store and oxygen (*46*). Especially, Arg, Gln and Leu are important for mTORC1 activation (*47, 48*). Recent findings of the activation of mTORC1 showed that Leucyl tRNA synthetase plays a crucial role in AA induced mTORC1 activation. A significant association with development of diabetes is observed for BCAA and aromatic AA (*49*).

Three different types of milk were fed to the infants, breast milk and two formula milks with low and high protein categories (*1*). The hypothesis was raised that high protein intake in infants can affect higher BCAA concentrations and leads to faster growth, elevated adipose tissue and elevated risk of later obesity. We investigated the effect of milk intake on the serum AA concentration in infants.

3.1.1 Study design and population

The study was a double blind, randomized intervention trial. Infants were fed with cow milk based formula with either high protein or low protein levels within the first year of life. Formula feeding in each of these two groups was started with an infant formula preparation and was switched to follow-on formula after 4 months. 1757 healthy newborns, born between October 2002 and July 2004, participated in the study and were recruited in 5 countries (Belgium, Germany, Italy, Poland and Spain). Newborns

of mothers with hormonal, metabolic diseases and drug addiction during pregnancy were excluded from the study. During the first eight weeks after birth, all infants were breastfed. Formula-fed infants were totally formula-fed after eight weeks up to the fifth month of age and breastfed infants had to be breastfed up to three months of age. Standard formulas were replaced by follow-up formula (from 4th month of age). Standard formula contained lower cow milk protein levels compared to follow-up formula but had an identical energy density. Differences in protein content were balanced by fat content. Details of formula nutrition and study population were reported by Koletzko et al. 2009 (*1*). Of 1757 healthy newborn recruited at birth, 1138 newborn were formula fed and 619 were breastfed. The group of formula fed infants was divided by protein intake level, low protein diet contains 1.8 g and 2.2 g protein/100kcal protein and high protein diet comprises 2.9 and 4.4 g protein/100kcal. Eight European study centers (Reus, Spain; Tarragona, Spain; Warsaw, Poland; Brussels, Belgium; Liege, Belgium; Milan, Italy; Nuremberg, Germany and Munich, Germany) collected serum samples at 6 month after birth of 726 infants. Amino acid analysis was performed from 275 low protein infant samples, 277 high protein infant samples and compared to 174 breastfed infant samples (Figure 9). Longitudinal follow up was done for anthropometry, nutrition, neuropsychology and sample collection.

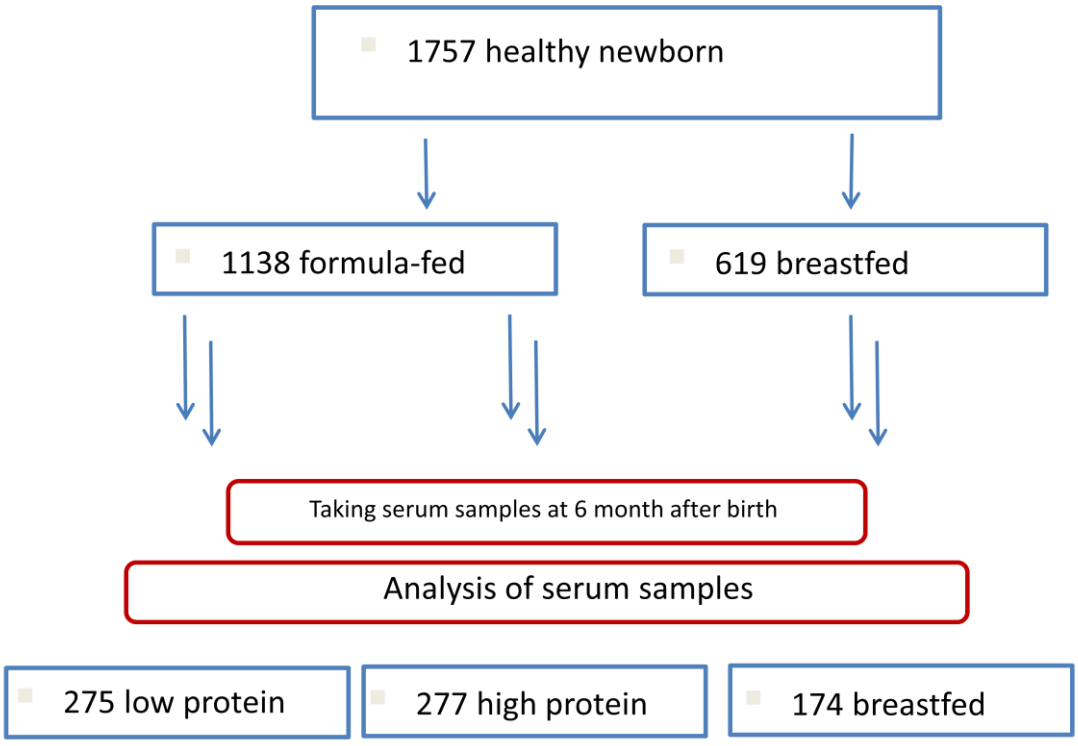


Figure 9: Flow chart of the CHOP study subjects and numbers of group classification

3.1.2 Amino acid analysis

At the age of sixth months, venous blood samples were taken and serum was stored at -70°C. 726 samples were transported on dry ice to Germany, Dr. von Hauner Childrens Hospital for AA analysis. Determination of AA was performed by HPLC (Agilent, 1100) coupled with mass spectrometric detection (Applied Biosystems, API 2000). Equipment for AA analysis is described in detail in chapter 2.2.1. A detailed description of sample preparation is given in chapter 2.2.5. 10 µL of plasma is added to a mixture of methanol, internal standard and HCL. After protein precipitation and centrifugation, a supernatant of 200 µL is dryed under nitrogen. For derivatization, 100 µL butanolic HCL is added and incubated for 15 min at 60°C. After evaporation, 100 µL mobile phase is added and 10 µL of sample is injected into HPLC.

3.1.3 Sample definition for clinical trials in a 96 well plate

In the morning before sample preparation, 81 samples were thawed and registered in a sample registration template. This template was developed by Wolfgang Peissner and is suitable for automatically batch writing for 81 unknown samples and standard calibrators. For clinical studies of more than hundred samples, it is advisable to use 96 well plate samples due to the total high throughput measurement of 81 unknown samples in very short time. Figure 10 shows the arrangement of samples, standards, matrix and QC-samples and blank.

	1	2	3	4	5	6	7	8	9	10	11	12
A 01-12	unknown sample 1	unknown sample 2	unknown sample 3	unknown sample 4	unknown sample 5	unknown sample 6	unknown sample 7	unknown sample 8	unknown sample 9	unknown sample 10	unknown sample 11	unknown sample 12
B 13-24	unknown sample 13	unknown sample 14	unknown sample 15	unknown sample 16	unknown sample 17	unknown sample 18	unknown sample 19	unknown sample 20	unknown sample 21	unknown sample 22	unknown sample 23	unknown sample 24
C 25-36	unknown sample 25	unknown sample 26	unknown sample 27	unknown sample 28	unknown sample 29	unknown sample 30	unknown sample 31	unknown sample 32	unknown sample 33	unknown sample 34	unknown sample 35	unknown sample 36
D 37-48	unknown sample 37	unknown sample 38	unknown sample 39	unknown sample 40	unknown sample 41	unknown sample 42	unknown sample 43	unknown sample 44	unknown sample 45	unknown sample 46	unknown sample 47	unknown sample 48
E 49-60	unknown sample 49	unknown sample 50	unknown sample 51	unknown sample 52	unknown sample 53	unknown sample 54	unknown sample 55	unknown sample 56	unknown sample 57	unknown sample 58	unknown sample 59	unknown sample 60
F 61-72	unknown sample 61	unknown sample 62	unknown sample 63	unknown sample 64	unknown sample 65	unknown sample 66	unknown sample 67	unknown sample 68	unknown sample 69	unknown sample 70	unknown sample 71	unknown sample 72
G 73-84	unknown sample 73	unknown sample 74	unknown sample 75	unknown sample 76	unknown sample 77	unknown sample 78	unknown sample 79	unknown sample 80	unknown sample 81	aqueous standard **10 µM** 82	aqueous standard **25 µM** 83	aqueous standard **50 µM** 84
H 85-96	aqueous standard **100 µM** 85	aqueous standard **200 µM** 86	spiked sample 87	matrix sample CP1 88	matrix sample CP2 89	QC sample 90	QC sample 91	QC sample 92	QC sample 93	QC sample 94	QC sample 95	blank 96

Figure 10: Arrangement of a 96 well plate for standards and sample. Well 1-81 is used for unknown samples (brown squares), well 82-89 is used for standard samples (blue squares), well 90-95 is used for QC-samples (green squares) and well 96 is used for the blank

3.1.4 Statistical analysis

Statistical analyses were performed with Statistica (Version 10) (StatSoft GmbH, Hamburg, Germany) and medians (25 th ,50th ,75 th percentile) were calculated with Microsoft Office Excel 2007.

3.2 Results

3.2.1 Amino acid concentration in serum

Measuring AA concentration in infant serum was finished for all proteinogenic AA besides cystine. Cystine is known as an unstable compound and only fresh aliquots with short storage time can be reliably qualified. Additionally, Cit and Orn were analyzed. Comparison of 21 AA levels indicated a clear difference between groups of high and low protein. All AA concentrations of BF, LP and HP are shown in table 7. P-values were calculated between LP and HP group. In general, LP and BF group differ only marginally and are roughly comparable. Differences were observed between the LP and the HP groups. All essential AA were significantly elevated in HP. Especially the BCAA (Leu, Ile and Val) showed differences between LP and HP. In contrast, most non-essential AA (Ala, Arg, Asn, Asp, Cit, Gln, Glu, His, Orn, Ser) were not affected by different protein intake. For Gln and Glu, we observed a tendency towards higher levels in BF compared to LP and HP. Gln, Glu and Gly showed a slight elevation in LP group. Non-essential AA, Gly, Pro and Tyr also differed significantly between LP and HP group. A representation of the results is revealed as box plots in figure 11. Based on the plots, the influence of protein intake on concentration of the essential AA is clearly depicted. In the box plot of Phe, we observed an outlier in HP group with 502 µmol/L. In physiological concentrations, the average of Phe is around 100 µmol/L. In figure 12, the outlier of Phe is first included and marked with an arrow and secondly excluded.

Table 7: AA concentration of infants in BF-, LP-, HP-group. Blood samples were collected 6 month after birth. All results presented as medians (25th, 75th percentile). P values, between LP and HP, were calculated with the use of independent t-tests.

AS	BF	LP	HP	P value
	n=174	n=275	n=277	
Ile	70 (59, 91)	75 (63, 88)	101 (80, 128)	0.000000
Leu	132 (112, 170)	143 (123, 173)	201 (160, 253)	0.000000
Lys	190 (161, 232)	195 (162, 234)	230 (186, 270)	0.000000
Phe	81 (68, 101)	96 (78, 111)	110 (93, 131)	0.000000
Trp	64 (56, 74)	60 (52, 72)	74 (60, 87)	0.000000
Tyr	84 (69, 103)	101 (85, 126)	125 (94, 154)	0.000000
Val	213 (181, 274)	256 (220, 299)	367 (291, 458)	0.000000
Pro	284 (242, 364)	296 (243, 368)	339 (267, 446)	0.000002
Thr	144 (123, 181)	145 (125, 178)	164 (138, 205)	0.000002
Met	28 (23, 35)	35 (28, 41)	38 (30, 49)	0.000005
Gly	287 (236, 334)	334 (270, 409)	296 (244, 361)	0.000042
Asn	50 (40, 63)	50 (40, 62)	56 (44, 68)	0.001704
Cit	25 (19, 34)	30 (25, 39)	34 (28, 39)	0.003912
Asp	44 (34, 55)	42 (34, 52)	45 (37,57)	0.016384
Gln	505 (420, 612)	484 (384, 588)	441 (365, 551)	0.018738
His	101 (89, 123)	108 (93, 128)	113 (98, 132)	0.051140
Glu	306 (256, 403)	273 (225, 348)	263 (211, 327)	0.075365
Ala	467 (402, 567)	476 (385, 601)	466 (387, 551)	0.167310
Ser	233 (203, 270)	214 (183, 252)	214 (187, 259)	0.704893
Orn	111 (84, 143)	105 (85, 138)	104 (83, 137)	0.890628
Arg	174 (142, 218)	174 (141, 222)	173 (140, 213)	0.997504

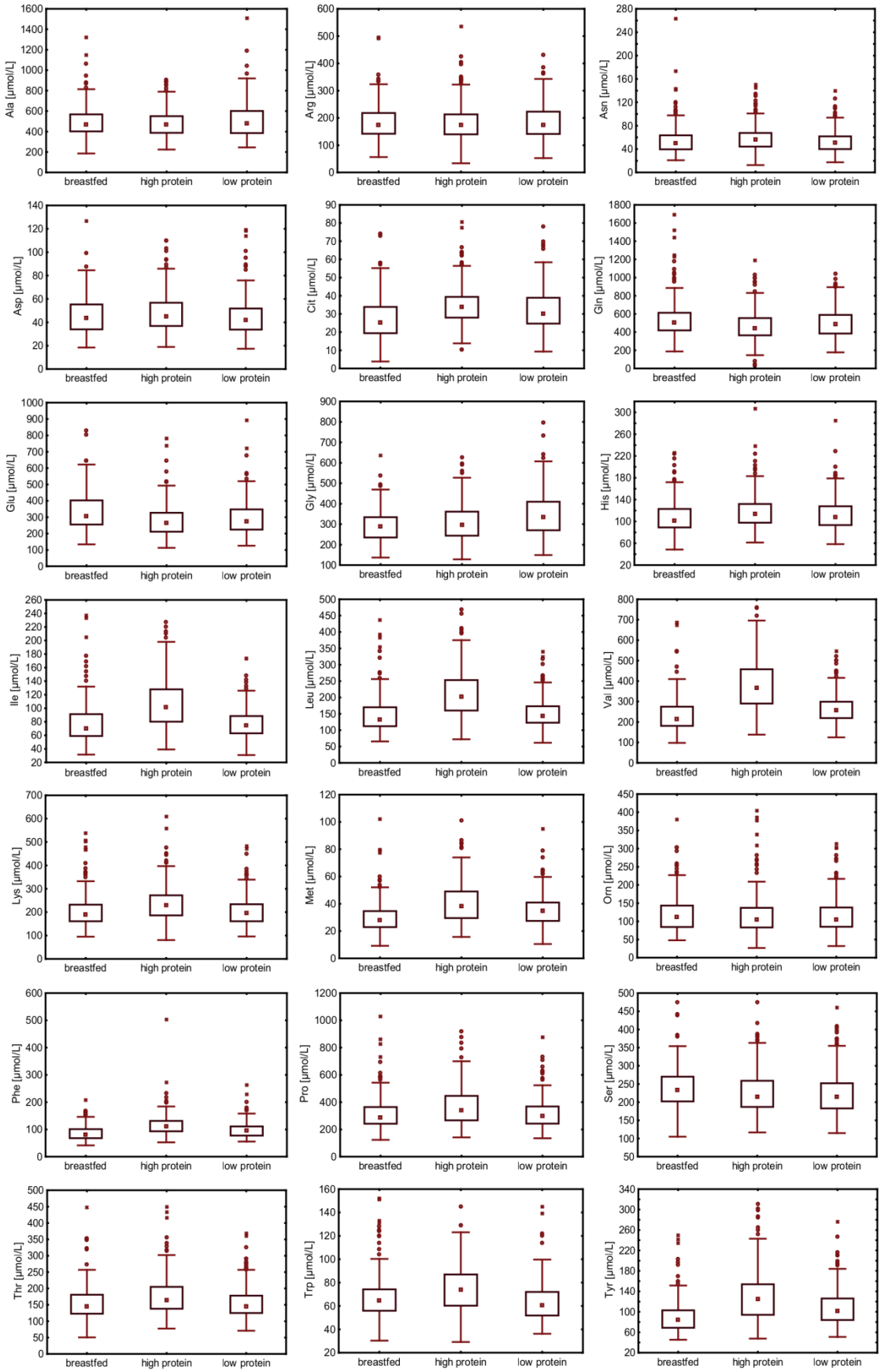


Figure 9: Box plots of serum AA concentrations in formula-fed and breastfed infants at 6 mo of age.

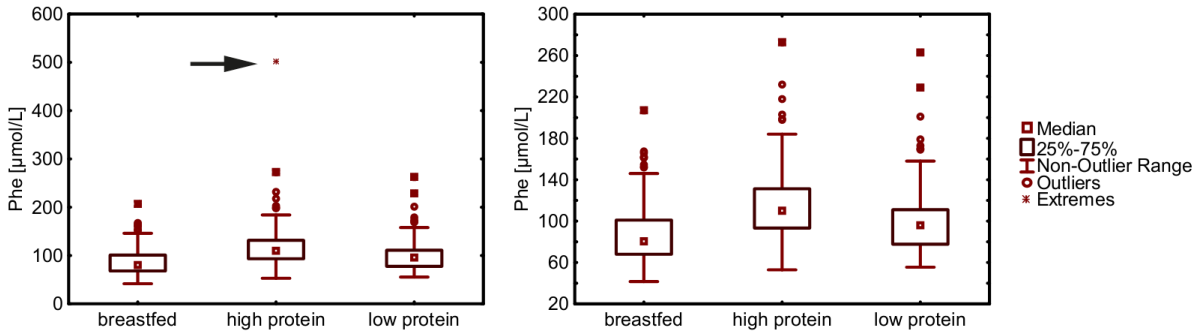


Figure 10: Box plots of Phe concentrations in formula-fed and breastfed infants at 6 mo of age (with outlier, marked with a black arrow, and without outlier)

3.3 Discussion

We found that HP intake in formula affected serum AA concentrations which markedly differed from the breastfed infants. HP group show increased serum AA levels especially for the essential AA Ile, Leu and Val. Our findings confirm the results of studies with Socha et al. 2011 (*50*). The group of Socha analyzed the AA from the same study using amino acid analyzer with showing the same major results. Further analyses are necessary for detailed information about later risk of weight gain or obesity. High values of essentials AA in HP group in early infancy might be a risk for weight gain in early infancy (*50*). To reflect on reasons for infancy weight gain feasible explanation can be given in detail in further chapter.

3.3.1 Transport of AA

AA are supplied as proteins to the diet. Digestion of these proteins starts in stomach but happens mainly in the small intestine to AA. The small intestine is the absorption organ for AA coming from dietary protein and the first pass for AA to circulation. Influenced by protein hydrolysis and microflora in the intestine, AA pass through active transport to the portal vein blood and free AA enter liver cells. In the long run, AA reach circulation (*8*). Except Ile, Leu and Val, all AA are degraded in the liver for systematic circulation. The BCAA (Ile, Leu and Val) contribute to different metabolic processes such as acting as substrates for protein synthesis, precursors for Ala and Gln synthesis, as modulator of muscle protein synthesis and as central nervous system control of food intake. Due to their important role in metabolism, it is interesting that BCAA are not degraded in the liver (*51*). Liver is not suitable for degradation of BCAA due to lack of the responsible aminotransferase, which is involved in the first degradation step of BCAA (*51*). BCAA are preferentially oxidized in muscle, fat, kidney

and brain tissue (*8*). The group of Herman depicted an increased oxidation rate of BCAA in adipose tissue from normal mice compared to skeletal muscle (*52*).

AA transport across the plasma membrane is mediated by AA transport systems (*53*). The literature presents different AA transport systems where AA can enter circulation. At least seven transport systems from blood to somatic cells are known, but a lot more are assumed (Table 8) (*8*).

Table 8: Transport systems of AA (this table was constructed after Löffler et al. (*8*))

Amino acid transport system	Amino acids
A	Ala, Gly, Ser, Pro, Met
ASCP	Ala, Ser, Cys, Pro
L	Leu, Ile, Val, Phe, Tyr, Trp, Met
Ly	Lys, Arg, Orn, His
Dicarboxylat	Asp, Glu
ß	Taurin, ß-Ala
N	Gln, Asn, His

Among these systems, the AA transport system L, an Na^+ independent transport system, is a major nutrient transport system responsible for the transport of neutral AA including several essential AA such as BCAA and aromatic AA (*54*). During HP intake we observed significantly higher serum values in Ile, Leu, Val, Phe, Tyr, Trp, Met, Gly and Lys. It is obvious that BCAA, aromatic AA and Met are transported by system L into circulation.

For Gln we observed high serum concentrations in the lowest protein group (BF) and Gln behaved differently than other AA. Gln is the transporter unit for amino groups and tissue exhibits higher concentration of Gln. It is interesting that high protein intake leads to decreasing levels of Gln. It seems that an oversupply of protein, Gln values decreases.

Non-essential AA are catabolized in the intestine and de novo synthesis is the major determining factor of systemic concentration.

3.3.2 Activation of mTOR by AA

The mammalian target of rapamycin (mTOR) is an serine/threonine protein kinase and belongs to the phosphoinositide-3-kinase (PI3K) related kinase protein family (*55*). mTOR is a key component of two multi protein functional complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Each complex consists of different proteins, some are found in both subtypes and each complex provides diverse functions exclusively. mTORC1 activation supports many intra and extra cellular processes such as cell growth, cell proliferation and is regulated by growth factors, nutrients (especially availability of AA), oxygen and energy sensing signals. mTORC2 subserves for cell processes such as survival and polarity of cells and cytoskeletal organization (*46, 48*). Moreover, an inappropriately high level of mTOR activity plays a central role in the development of several diseases including metabolic syndrome, cancer and diabetes (*45, 56, 57*).

AA are necessary for activation of mTOR (*58*). The presence of AA, especially Leu and Glu, regulated mTORC1 (*46*). Leucine is included in protein synthesis steps and serves as regulator of protein synthesis. mTORC1 consists of mTOR, regulatory associated protein of mammalian target of rapamycin (Raptor), G protein b subunit-like protein (GbL), proline-rich Akt substrate of 40 kDa (PRAS40), and Deptor (*58*). For activation of mTORC1, AA are directly acting on Rheb. Rheb is identified as a gene that is rapidly induced in brain neurons (*48*). Activation of mTOR by AA is mediated by the Rag family including heterodimers RAGA or RAGB and RAGC or RAGD. Figure 13 shows a very simplified scheme of mTOR activation by AA or growth factors. These activators are two essential aspects for mTOR activation. Moreover, Leu and Gln are the most important AA for activation process. During a protein rich diet, Leucyl tRNA synthetase is involved in mTORC1 activation. The Rag anchored by ragulators (MP1, p14, p18) on the lysosome membrane. This membrane can activate Rheb which is activating mTORC1. Rheb activity is improved by TSC complex. If the activation of mTOR is caused by AA, a high protein intake could promote cell growth and therefore weight gain. Various authors support this hypothesis. Hoppe and coworkers analyzed 142 Danish infants fed with high protein diet and seems that protein intake stimulate early growth (*59*). Further authors confirm weight gain in infants whose compared breastfed infants and formula fed infants (*60-62*).

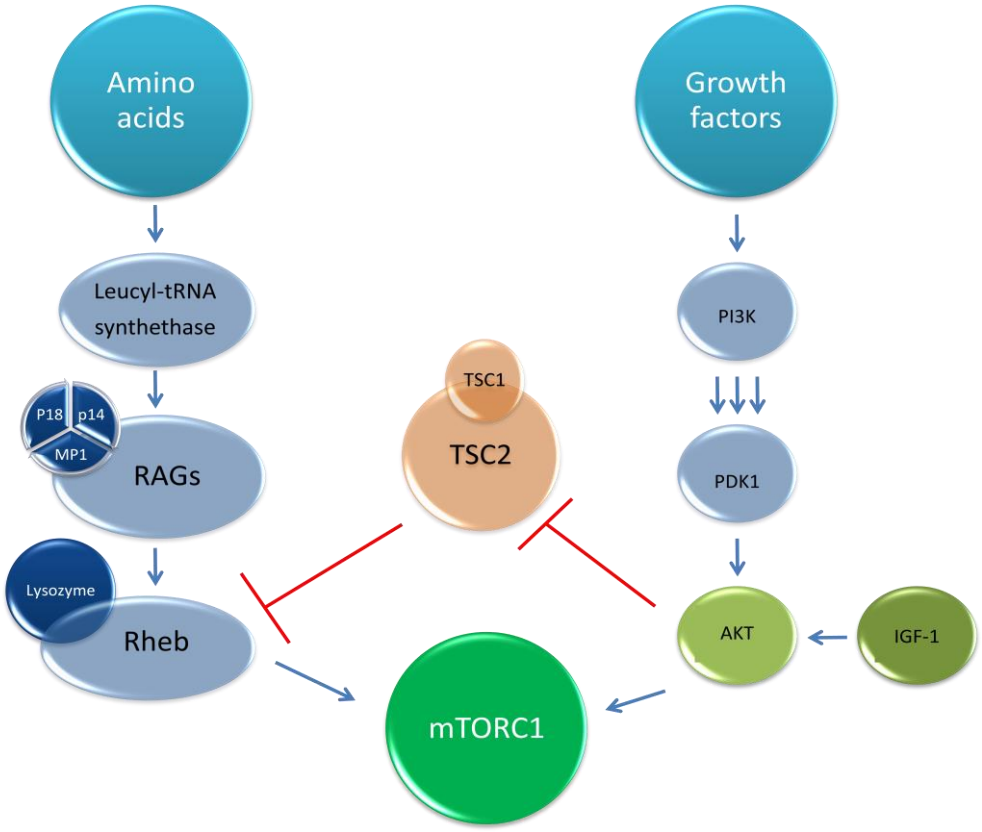


Figure 11: Overview of mTORC1 activation by AA and growth factors (Different publications were used to illustrate a simplified representation of mTORC1 activation (*46, 48, 58*))

3.3.3 Activation of IGF-1

Insulin like growth factor (IGF) is a complex protein with high resemblance to insulin. This complex molecule consists of two ligands (IGF-1 and IGF-2), four receptors (IGF-1R-IGF-4R), six IGF binding proteins (IGFBP1-6) and the acid-label subunit (ALS) of the circulating IGFBP complex (*63*). IGF-1 and 2 consists of single chain peptides with 7.5 kDa where IGF-1 is composed of 70 AA and IGF-2 consists of 67 AA. The IGF molecule showed four domains (A-D), domain A and B are equal to the A and B chains of insulin (*42*). IGF-1 displays a major role in growth and development as a function of disposable energy and essentials nutrients (e.g. AA) from body reserves and diet (*64*).

Experimental studies have implicated IGF-1 blood concentration differs by changing the diet (especially protein intake). AA, in particular BCAA are closely linked to stimulation of insulin secretion. Furthermore, IGF axis is known to regulate early growth and affect adipose tissue (*50*). IGF concentration in LP and HP can provide information about stimulation of insulin secretion. In this study IGF parameters were not measured in my doctoral thesis but the publication of Socha et al. illustrate the measurement of

IGF-1, IGFBP2 and IGFBP3 in the same study. The group concludes that HP intake stimulates IGF axis and insulin release in infancy. IGF-1 improves growth during the first 6 month of life (*50*).

3.3.4 Effects of protein intake in formula-fed infants

Protein intake in the first year of life may exert several effects. In addition to preferential uptake from BCAA into the muscle, increased BCAA concentrations result in activation of mTOR and IGF-1 (*50, 58*). On the one hand mTOR, activation supports many intra and extra cellular reactions such as cell growth, cell proliferation. On the other hand, Newgard supported the association to insulin resistance (*14*). AA are correlated to insulin release and insulin is known to increase cellular glucose uptake and inhibition of lipolysis. This metabolic alteration is linked with weight gain and the achieved body mass index. Rapid weight gain is a risk factor for development of overweight in later life (*65, 66*) and risk of obesity is also known from IGF-1 activation (*50*). In addition, an increased protein intake is associated with early puberty (*67*). High values of BCAA, Tyr and Phe correlates with diabetes and might be a predictor for future diabetes (*49, 68*). In comparison with BF infants, high protein diets with higher concentrations of numerous AA especially BCAA observed increasing BCAA concentration in serum and could lead to a number of effects on later life (Figure 14).

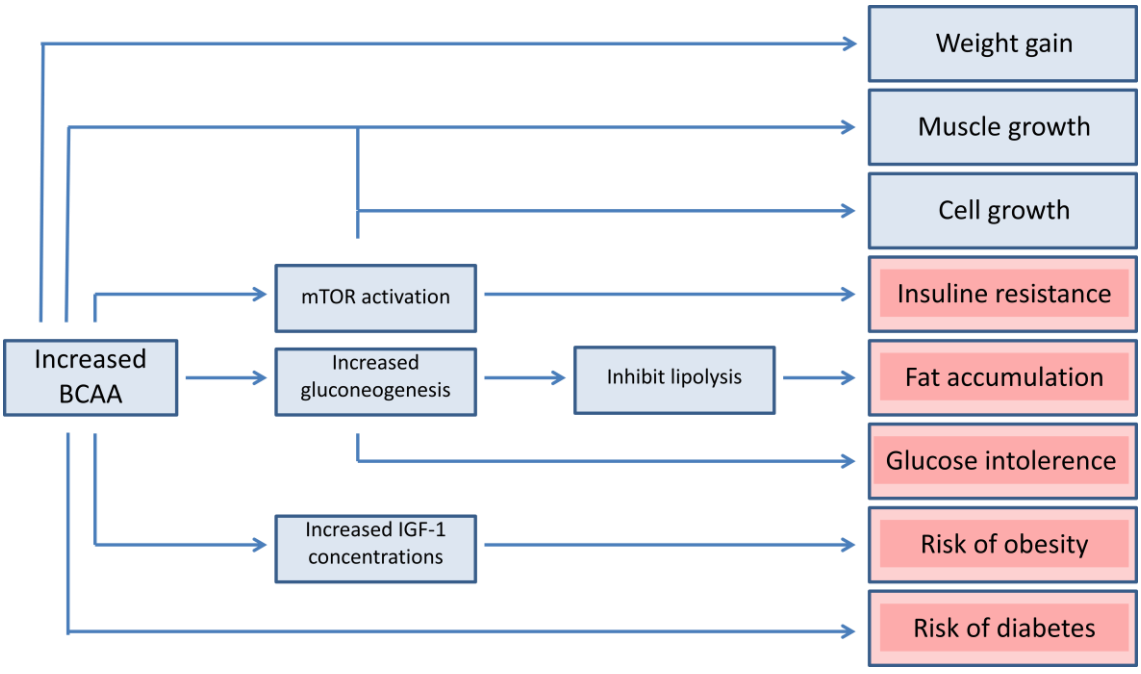


Figure 12: Effects of increased blood BCAA on human body (information has been assembled from several references and summarized in one figure (*14, 46, 49, 50, 57, 65, 68-70*))

Different effects of increased blood BCAA concentrations are reported in several publications. Increased BCAA values leads to an enhanced activation of growth hormones like mTOR, IGF-1 and gluconeogenesis (*14*). mTOR activation is associated with insulin resistance (*14*) and increased IGF-1 values show a higher risk for obesity and diabetes (*14, 49*). The connection between the directly observed growth factor activation (mTOR, IGF-1) and the subsequent increase in risk of several diseases is not described in detail (*71, 72*). By reducing the protein content in infant formula, blood BCAA concentrations are lower and possible effects (such as listed in figure 14) maybe reduced. The same energy density with a reduced amount of protein (available in LP and BF) could show a reduced activation of growth hormones (mTOR, IGF-1). These results support the imprinting of feeding breast milk. This statement is supported by other authors by diminishing obesity trough lower protein intake in early infants (*1*).

3.4 Conclusion

With our novel method development, we quantified serum AA of HP-, LP- and BF group and compared AA profiles in the first year of life to predict diet induced weight gain or obesity. Results of formula-fed infants were evaluated with breast fed infants. This showed a significant difference in serum AA profiles such as BCAA, Gly, Lys, Met, Phe, Pro, Thr, Trp and Tyr. Essential AA were absorbed from diet and probably released into circulation via L-transport system. Non essential AA are mainly catabolized in the intestine and de novo synthesis is the major determining factor of systemic concentration. AA are necessary for activation of mTOR and IGF-1. By the presence of high AA concentration in HP group, mTOR can be activated resulting in cell growth and cell proliferation. However, diseases such as insulin resistance, diabetes, fat accumulation can be caused by increased protein intake. Based on the observations of potential risk, a reduced protein intake in early life and/or breast milk feeding infants could contribute to health.

4 Plasma Amino acids of CLA supplemented cows

4.1 Background

Conjugated linoleic acid (CLA) refers to a group of geometric and positional conjugated dienoic isomers produced during biological or industrial hydrogenation of linoleic acid [18:2(n-6), Δc9, c12] (*73*). The biologically important isomers are cis9,trans11-CLA and trans10,cis12- CLA. Cis9,trans11-CLA, as a so-called rumen acid, is mainly isomerized from linoleic acid in the rumen of ruminants during the microbial biohydrogenation by the rumen bacterium *Butyrivibrio fibrisolvens (73)*. Trans10,cis12-CLA is also formed in the rumen by bacteria, but in lower concentrations. Therefore, milk and milk products, meat and meat products from ruminants and partially in hydrogenated vegetable oils contain CLA isomers. The total CLA content in milk is about 2-30 g/kg fat, while values in beef are differing between 3.1- 9.9 g/kg fat (*74*). In the rumens of sheep and cattle, microbial bioconversion mainly produces the cis9,trans11-CLA isomer, whereas dietary supplements and functional foods contain cis9,trans11-CLA and trans10,cis12-CLA isomers in approximately equal amounts (*75*).

There is great interest in CLA and conjugated linolenic acid isomers because of their supposed health-promoting properties. This is surprising because most trans fatty acids in food are usually undesirable due to their formation in technical processing (heating, hardening). Nevertheless, CLA is becoming more popular since 1987 when Michael Pariza discovered this compound (*73*). CLA has been implicated to a lot of beneficial biological effects on human health. Supplementation of CLA for 4 weeks reduces body fat in human men with signs on metabolic syndroms (*76*), benefit effect on immune function was observed in 28 healthy human participants (*77*) and reduce of body fat was also observed in 62 obese children (*78*). Furthermore, CLA supplementation in humans is associated with reduction of cancer incidence in rats and mice, reduction of severity of atherosclerosis and enhancement of lean mass (*79-81*). According these effects, supplements of CLA are available for sale and manufacturer promote rapid weight gain.

The conviction of beneficial CLA effect is also utilize in animal breeding. Benefits of CLA supplementation have been refuted by different working groups (*82, 83*). In contrast, the thesis of milk fat synthesis reduction is confirmed by several researchers

in cows (*84*) and goats (*85*). In the study of Lock milk yield, dry matter intake and protein content were not affected by treatment of CLA (*85*).

During the last 15 years isomers of CLA has received considerable scientific interests after the demonstration of beneficial effects on cardiovascular and oncological diseases in laboratorial animals (*86*). According to position and configuration of the conjugated double bonds, various isomers of CLA with differing effects have been identified (*87*). Park et al. initially observed a decreasing effect on body fat in mice (*88*). Later studies reported that fat loss was stronger in growing than in mature animals (*89*). However, CLA may negatively affect insulin resistance and leptin concentration in mice (*90*). Terpstra et al. summarized CLA effects on insulin, leptin, glucose and plasma lipids in humans (*91*). Effects of milk composition or metabolic key parameters on CLA supplementation were also mentioned in cows (*92, 93*). Supplementation with CLA - in particular the t10, c12 CLA isomer - reduces milk fat and causes changes in fat metabolism of dairy cows. Lanna et al. discussed the effect of CLA supplementation on fatty acids (*94*). However, CLA effects on AA concentrations are generally unknown. In energy deficiency, as it occurs during early lactation in high producing dairy cows, the energy spared by CLA-induced milk fat depression might be repartitioned towards synthesis of milk protein. This could be reflected in different time courses of free AA during early lactation in plasma and milk. Therefore, CLA supplementation on amino acid profiles was investigated as, to our knowledge, this relationship was not investigated at this time. With our new developed method which is described in section 2, we quantified AA of plasma and milk samples of dairy cows and compared AA profiles in CLA supplemented and non CLA supplemented cows. For example, specific questions could be: Does CLA supplementation change AA concentration in dairy cows? Apart from the milk fat reduction there may be a change in AA concentrations. During lactation, gluconeogenesis could be influenced by CLA, whereby propionate from ruminal metabolism is the major substrate for generation of glucose (*95*). One further idea is to investigate whether CLA supplementation in cows takes another way to generate glucose. Because of transamination and deamination processes of AA, the carbon skeleton could be used for gluconeogenesis. Maybe CLA supplementation affects AA metabolism, which could result in changed glucose production.

4.2 Materials and Methods

4.2.1 Animals and Treatment

Twenty German Holstein cows (first lactation >9.000 kg milk in 305 d) were kept in a tie stall at Leibniz Institute for farm animal biology (FBN) and were fed a total mixed ratio (TMR) ad libitum based on grass and corn silage and had free access to water (Table 9). They were randomly assigned to 2 groups three weeks (wk) prior to parturition. From 2 wk before to 9 wk after expected calving one group (CLA, n=10) was supplemented with 50 g/d Lutrell pure, (BASF, Ludwigshafen, Germany) containing 11.8 g/100g trans-10 cis-12 linoleic acid. The control group (Ctrl, n=10) was supplemented with 50 g/d control fat (linoleic acid; cis-9 cis-12) preparation (BASF; Ludwigshafen, Germany).

Table 9: Ingredients and chemical composition of diets

Composition	Diet	
g/kg dry matter	**Close-up[1]**	**Lactation[2]**
Corn silage	335	315
Grass silage	255	165
Wheat straw	80	20
Grass hay	50	50
Concentrate MLF 2000[3]		320
Concentrate Universal 18/3[4]	170	
Extracted rapeseed meal	100	40
Dryed beet pulp		80
Minerals[5,6]	10	10
Chemical composition		
Utilizable protein[7]	140	165
NE_L[7]	6.5	7.1
ADF[7]	220	160
NDF[7]	390	300

1 Close-up diet was fed between 2 wk before until calving

2 Lactation diet was fed after parturition.

3 Concentrate MLF 2000 contained: 33% soya, 20% corn, 17% wheat gluten, 13% wheat, 8% rape seed expeller, 5% low sugar beet pulp chips, 2% Sodium hydrogen carbonate, 1.3% Calcium carbonat, 0.2% sodium chloride

4 Concentrate Universal 18/3 contained: 20-40% Cereals (triticale, rye, wheat, barley), < 25% rape seed expeller, malt germs, wheat gluten, wheat bran, peeled oat-bran, beet pulp chips, molasses, glycerol, minals, vitamins

5 Minerals in close-up feeding contained: 14% Calcium, 12.5% sulphur, 12% chlorine, 10 % magnesium, 2% phosphorus

6 Minerals in lactation feeding contained: 92% crude ash , 8% phosphorus 6 % magnesium, 5% calcium

7 German Society of Nutrition Physiology. 2001.

4.2.2 Data recording, sample collection and analysis

Cows were milked twice daily and dry mater intake (DMI) and milk yield were recorded daily, BW and milk composition were measured weekly. Feed analysis was performed according to Weender Standard Procedure (*96*) at the Agricultural Faculty of the University of Rostock and at the Landwirtschafliche Untersuchungs- und Forschungsanstalt, Rostock, Germany. Milk samples were analysed for fat, lactose, and protein in milk by the Landeskontrollverband für Leistungs- und Qualitätsprüfung Mecklenburg-Vorpommern e.V. (Güstrow, Germany). Energy corrected milk (ECM) and energy balance was calculated as described (*97*). Aliquot milk samples of wk 1, 2, 3, 4, 6, 9 and 12 were collected.

Furthermore blood samples were taken by venipunture in Li-heparinate monovettes (Sarstedt, Nümbrecht, Germany) 2 wk before expected calving and after delivery at wk 1, 2, 3, 4, 6, 9 and 12. Samples were stored at -20°C until the analysis of free plasma AA. For the analysis of samples a new standard for external calibration was purchased (Fluka, amino acid standard, physiological) which includes more AA with a carboxyl group. We quantified twenty six AA (all proteinogenic AA plus Car, Cit, Cysta, HLys, HPro, MHis and Orn) by LC-MS/MS combining derivatization and ion-pair chromatography as described in detail in section 2.2.5.

4.2.3 Statistical analysis

Data were analyzed using the ANOVA (PROC MIXED) procedure of SAS with diet and lactation wk as fixed effect, and diet x time interaction. Plasma AA concentrations were adjusted for basal plasma AA values prior to CLA supplementation and parturition. Values are expressed as means ± SE. Pairwise Pearson correlation and scatter plots of all AA were created using Excel. Pearson correlation coefficients between plasma and milk AA concentrations within group and lactation wk were calculated. The

significance level was set at $P < 0.05$, and a trend was considered when $0.05 \leq P \leq 0.10$.

4.3 Results

4.3.1 Amino acid concentrations in plasma and milk

Plasma AA concentrations from late pregnancy until to the twelfth wk after calving are shown in the Appendix table 1 for the CLA group and Appendix table 2 for the control group. The table shows the averaged absolute values of all measured free plasma AA with standard errors (SE). All concentrations were lying in the range between 0 µmol/L and 687 µmol/L. Cysta and HLys was detectable but determined concentrations were below lower limit of detection (LLOD). In contrast to Cysta and HLys, Gly showed the highest plasma concentration of all analytes (261 µmol/L – 687µmol/L). In general, the largest changes of AA concentrations occurred between the second wk before and the third wk after calving. Onwards, only slow increases or decreases were observed.

Free milk AA concentrations behaved totally different from plasma AA. In milk, only Ala, Arg, Asn, Asp, Gly, Glu, HPro, Lys, Pro, Ser, Thr and Val could be reliably quantified with extremely high values for Asp and Glu. Concentrations of other free milk AA could not detected within the LLOQ of 1µM. Compared to plasma, concentrations of free Glu and Asp in milk were 11-fold and 5-fold increased, respectively. AA like Gly, HPro, MHis, Pro, Ser and Val decreased over time, while other AA did not change appreciably with advancing lactation.

4.3.2 Correlation of plasma AA

Table 10 shows selected pair-wise Pearson correlations of all plasma amino acid concentrations without consideration of the longitudinal gradient sorted by R-squared values in decreasing order. Especially Ile and Leu (figure 15A) and Ile and Val showed similar correlations with R-squared values of $R^2 = 0.8600$ and $R^2 = 0.8068$, respectively. Arg concentrations were positively related to Orn concentrations with $R^2 = 0.8030$ (15B). Positive linear relations were also observed between Arg and Asp ($R^2 = 0.7167$), Asp and Lys ($R^2 = 0.6322$), Thr and Lys ($R^2 = 0.6879$) and Phe and Tyr ($R^2 = 0.7406$). The correlation between the pairs Asn/Asp (figure 15D) and Gln/Glu (figure 15C) is completely different where Asn/Asp show a moderate positive correlation of

0.6193 but Gln/Glu only show a R-squared value of 0.3376. No significant correlation was observed between Asp and Glu (R^2 = 0.1158), Glu and Pro (R^2 = 0.0610), Ser and Trp (R^2 = 0.1658) and Thr and Gly (R^2 = 0.1006). Interestingly, positive linear relations between two AA with high R-squared values also show similar time courses trends.

Table 10: Pairwise Pearson correlation of selected AA

Amino acid	Correlated Amino acid	R-squared values
Leu	Val	0.8655
Ile	Leu	0.8600
Ile	Val	0.8068
Arg	Orn	0.8030
Phe	Tyr	0.7406
Asp	Arg	0.7167
Thr	Lys	0.6879
Asp	Thr	0.6660
Asp	Lys	0.6322
Asn	Asp	0.6193
Asp	Ile	0.5476
Orn	Pro	0.5280
Cit	Orn	0.5098
Arg	Cit	0.5091
Asp	Met	0.4810
Ala	Asp	0.4611
Thr	Ile	0.3836
Gln	Glu	0.3376
Ile	Trp	0.3248
Gln	Ser	0.2535
Ser	Trp	0.1658
Asp	Glu	0.1158
Thr	Gly	0.1006
Glu	Pro	0.0610

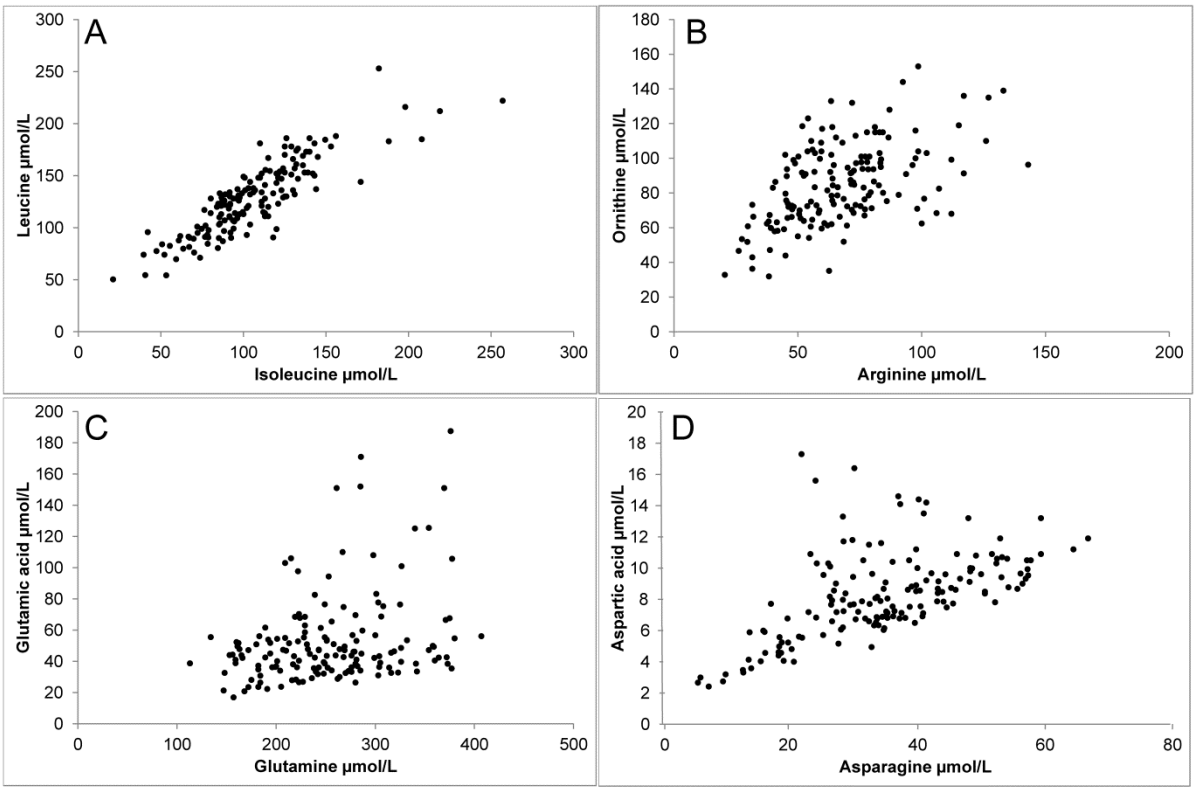


Figure 13: pair-wise metabolite scatter plot for selected AA pair concentrations in plasma measured with LC-MS/MS; A Ile/Leu pair, which are high correlated (0.8600, Pearson), B Arg/Orn pair, which are high correlated (0.8030, Pearson), C Gln/Glu pair, which are uncorrelated (0.3376, Pearson) and D Asn/Asp which are moderate correlated (0.6193, Pearson).

4.3.3 Time courses of plasma AA

Time courses of plasma AA were classified in 3 different groups. Figure 16 shows time courses of all AA. In the largest group of AA (Ala, Arg, Asp, Asn, Cit, His, Ile, Leu, Lys, Met, Orn, Pro, Thr, Trp, Tyr, Val), concentrations were decreasing shortly after calving and then up-regulated again approaching basal or higher values at the end of the observation period (all black marked AA in figure 16). In the second group (Gly, HPro, MHis and Ser) concentrations increased after delivery and then moved back to basal values (all green and orange marked AA in figure 16). Plasma concentrations of MHis peaked around wk 1 after calving and gradually decreased below starting values in the following weeks (highlighted in orange in figure 16). The last group of AA (Gln, Glu, Phe) was characterized by post-parturition decrease of plasma concentrations, which was sustained until wk 12 (all grey marked AA in figure 16).

In general, data points around delivery take extreme values. Concentrations of Arg, Orn, Thr, Trp, and Val were strongly reduced from ap to first wk pp and increased thereafter, mostly returning to ap levels in lactation wk 3-4. In contrast, Phe dropped from ap to first wk pp and remained at this level until wk 10. Some AA slowly increased to the ap level within twelve wk. A time effect of free milk AA concentrations is only

observed for Arg, Asp, Glu, Gly, HPro, Ser and Val with a significance value p=0.01. Plasma AA like Arg, Asn, Asp, Car, Gly, HLys, HPro, MHis, Orn, Thr, Trp and Val concentrations were affected by lactation wk (p≤0.05). None of the free AA differed significantly between the CLA group and the control group at any time point.

The supplementation with CLA did not affected plasma concentrations of AA. It seems as if AA concentrations are higher in the control group compared to the CLA group. It is also interesting, that basal values in the control group are higher which is not significant and might be purely accidental. An intervention effect of free milk AA concentrations is not observed.

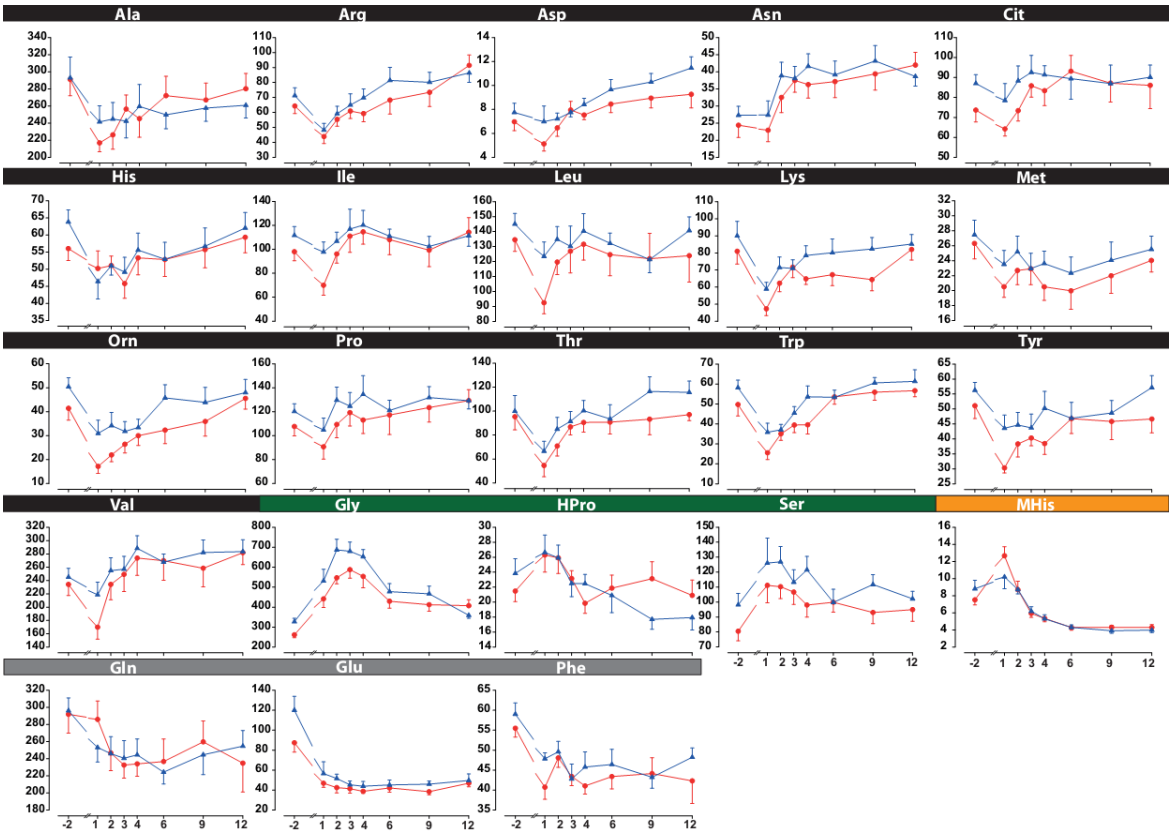


Figure 14: Time courses profiling of mean plasma AA concentration with Standard Error from week -2 ap to wk 12 pp The x-axis represents 8 data points of time in weeks from -2 to 12 weeks and the y-axis the absolute mean plasma concentration of both groups (red line: CLA group; blue line: control group). Data are presented as mean ± SE

4.4 Discussion

4.4.1 Correlation of AA pairs are dependent on equilibrium reaction

Same functional groups show high relations between the corresponding AA. Especially Ile, Leu and Val (BCAA) are highly correlated in functional groups. One possible explanation for high correlations between the BCAA can be explained by close proximity of metabolic pathways and involved enzymes in equilibrium reactions that convert BCAA obviously directly in one and another with plasma levels changing in parallel. This statement is in agreement to the results of Camacho et al (*98*). This group explained the origin of correlation in the metabolic network. To their opinion, correlations dependent on equilibrium reaction, mass conservation, single enzymes and asymmetric control distribution (*98*). Arg concentrations correlated positively with Orn concentrations due to the conversion of Arg to Orn. Correlation between Arg and Cit (R^2 = 0.5091) and Orn and Cit (R^2 = 0.5098) is lesser but a positive tendency is observed. Lys, Met, Thr and Ile are synthesized from Asp resulting in high correlation. Positive high correlation in AA metabolism is observed between Phe and Tyr where Phe is oxidized to Tyr. Hydrolysis from Asn to Asp shows a correlation of 0.6193 (Figure 15D). The metabolic neighbor-pair Gln and Glu are less correlated where Asp and Glu are subjects to hydrolysis reactions due to aparaginase and glutaminase, respectively. Camacho et al confirmed the lesser correlation between Gln and Glu with the statement of a not necessarily correlation of neighbor metabolites. There are a lot of neighbor analytes pairs in the metabolic map, which have low correlation e.g. Gln and Glu. In summary we can explain some metabolic reactions by correlations. Measuring correlation can give information about the biological reactions between metabolites.

4.4.2 Classification of time courses

4.4.2.1 *Stress and negative energy balance leads to decreased AA levels after delivery*

Figure 16 shows time courses of all plasma AA which were classified in 3 different groups. In the largest group of AA (Ala, Arg, Asp, Asn, Cit, His, Ile, Leu, Lys, Met, Orn, Pro, Thr, Trp, Tyr, Val), concentrations were decreasing shortly after calving and then up-regulated again approaching basal or higher values at the end of the observation period. Low concentrations of AA might be explained by stress of delivery and negative energy balance. Meijer et al. determined the peripartal concentrations of 23 AA in 36 dairy cows and observed very similar time curves (*99*). Compared to the group of

Meijer our results are more detailed due to more measured AA, the lesser variance and thus the more precise time courses. The author observed the rapid recovery of Lys, Thr, Trp, Tyr and the branched chain AA (BCAA). Furthermore, absolute plasma AA concentrations of Arg, Asp, Cit, Gln, His, Ile, Leu, Lys, Met, Phe, Orn, Ser, Thr, Trp, Tyr and Val were comparable, but for absolute AA concentrations of Ala, Asn, Glu, Gly, Pro and Trp results differ. This may be explained by breed, food intake, age or analytics. Furthermore, plasma Arg seems to be a marker for hypothalamic feed intake regulation in rats (*100*). Laeger 2012 observed an elevation of plasma arginine level after feed restriction in dairy cows (*101*). In our study plasma arginine is also increasing with. Thus, plasma arginine seems to be a less critical signal for feed regulation in cows. Plasma Lys did not show significant time dependence but a tendency to increase in later samples. Laeger explained that elevated Lys concentrations originate from protein breakdown in lactating cows (*101*).

4.4.2.2 *Increased MHis levels as a marker for muscle protein breakdown*

As high producing dairy cows are in a state of energy deficiency during early lactation, breakdown of muscle protein occurs and plasma concentration of released methylated AA increases. This has previously been observed in goats (*102*). Free AA except Car, Gly, HPro, MHis and Ser can be used for synthesis of lactose and casein. However, Gly, HPro, MHis and Ser showed a strong increase during the first wk after delivery and leads to anabolic state. Gly (both groups) and MHis (CLA group) increased by 60 %. HPro (CLA group) and Ser (both groups) rose after birth up to 38 % from the base value. Plasma MHis (the sum of 1- and 3-MHis) is a derivative of histidine which is found in contractile muscles, when His is methylated after incorporation into actin and myosin (*103*). Its plasma and urine concentrations are considered as markers of muscle protein breakdown. MHis was measured as the sum of 1- and 3-methylhistidine due to insufficient chromatographic separation by the LC-MS/MS method used. Our study showed a high and fast increase of MHis ap, which peaked in the first wk after delivery, decreased sharply until wk 6 and remained stable from that time onwards. This observation is in line with results of Blum et al., 1985 who determined 3-methylhistidine in lactating cows (*103*) and observed the same time course of the AA profile but with higher absolute concentrations of MHIs than in our study. This observation can be explained by its release from catabolized muscle protein during negative energy balance. Also feed restriction causes increased plasma leves of MHis in lactating cows (*101*). During delivery and milk production, AA requirements exceeding intake are compensated by muscle protein breakdown, which leads to

elevated 3-methylhistidine levels as MHis is not a precursor for protein synthesis. Almeida et al., 2008, reported similar MHis profiles in lactating goats (capra hircu) (*102*). A further reason for the high plasma concentration of MHis and also HPro, might be related to their non proteinogenic character. These AA can not be used for protein building or synthesis of lactose and casein which is a major task after delivery. The increase after calving around the first wk was also noticed for Gly, HPro and Ser. It seems that the degradation of muscle proteins and the release of free AA also influenced Gly, HPro and Ser levels. In collagen, Gly and HPro are known to be an major AA and increased plasma levels might indicate as an indicator of collagen breakdown (*104*). Increases of Gly and Ser are known but not fully explained. Some authors explained the increase of Gly with mobilization of extrahepatic protein (*105, 106*). Studies with low protein diet also showed elevated Gly concentration after calving (*106*). The excess of Gly may be needed for milk protein production (*107*). Ser is a precursor of glycerate-3-phosphate and can create Gly and Cys. After delivery, Gly and Ser are concentrated in plasma and may be not be used for gluconeogenesis. Onwards, concentrations decreased, so Ser and Gly may be utilized for production of glycerate-3-phosphate during gluconeogenesis.

4.4.2.3 *Plasma Glu decreased dramatically after delivery*

The pronounced decrease of Glu in plasma (around 50% in both groups) may possibly be associated with the very high concentrations of free Glu in milk (300 – 650 µmol/L). Interestingly, plasma Glu concentrations show high values before parturition and decrease strongly after parturition. The reason is unknown, but it might be that the fetus is supplied with free Glu from plasma. After parturition, free Glu concentration in plasma strongly dropped which may indicate selective transport of Glu into milk. Glu is required for the synthesis of casein and whey proteins, as it is one of the major components of casein (25 -30%) (Kaufmann et al. 1987). The strong decrease in plasma agrees with the hypothesis that Glu and Gln are the limiting AA for casein synthesis (*108*). Windmueller et al., 1974 explained the drop off Gln by the increased demand from the gut (*109*). Key processes like the transfer of the amino group of an AA, in which the amino group is mostly transferred to alpha-ketoglutarate. Maybe an increased requirement of the calf makes high Glu values necessary in milk.

4.5 Conclusion

With our new developed method, we quantified AA of plasma and milk samples in dairy cows of CLA supplemented cows. CLA interventions have been studied in some parameters e.g. milk fat in dairy cows whereby the effect on AA is unknown. We classified time courses in three different time groups where most AA (Ala, Arg, Asn, Asp, Cit, His, Ile, Leu, Lys, Met, Orn, Pro, Thr, Trp, Tyr, Val) decreased up to delivery and increased slowly to their basal values. The energy deficit during early lactation leads to a catabolic state characterize by fat loss and breakdown of tissue protein. Especially muscle protein is degraded, which becomes obvious by increasing plasma MHis levels. Released AA especially Glu is used as substrates for synthesis of casein and lactose which is needed for the calf. Gly, HPro and Ser showed a strong increase during the first wk after delivery and leads to anabolic state. Increased plasma levels of Gly and HPro might indicate as an indicator of collagen breakdown. Our analysis shows no intervention effect on free plasma and milk AA. Due to stressful delivery we only observed a time effect which influence plasma AA concentrations. This suggests that CLA supplement did not affect AA precursors for gluconeogenesis and AA synthesis during early lactation.

5 Summary

Clinical studies show that the composition of circulating free AA in blood, are a marker for monogenetic and multigenetic diseases. The analysis of a large number of subjects in clinical trials is often limited by complicated and long sample preparation steps. As part of the metabolomics platform established at the Dr. von Hauner Children´s Hospital, a high-throughput method was developed which allows a selective, sensitive, precise and robust quantification of 22 AA from very small sample volumes. Over time further AA were added to the methodology. All AA of 96 samples can be measured within 36 hours. Using an internal standard in methanolic solution proteins were precipitated from only 10 µL sample volume. For AA quantification, the evaporated supernatant is derivatized and analyzed with an ion-pair reagent. The methodology is based on a comprehensive and detailed validation with an interday precision of 3.1-10.8% for all analytes. Every year we participate in a collaborative study which ensures an accurate determination of all analytes.

In the context of early programming, the newly developed AA method was used for quantifying 726 serum samples in a randomized clinical trial. Here, the relation between different protein intake (formula with high or low protein content) and AA profiles at 6 month old infants was analyzed. A significant alteration in serum concentration was found in the HP group for following AA: BCAA, Gly, Lys, Met, Phe, Pro, Thr, Trp und Tyr. Essential AA were taken by nutrients and probably released from the L-transport system into circulation. In comparison, non essential AA are catabolized in the intestine and regulated by de novo synthesis in equal concentrations. Therefore, no significant difference of non essential AA was observed between HP and LP group. High protein diet leads to an activation of growth hormones (mTOR, IGF-1) by enhanced availability of AA which is manifested in increased cell growth and proliferation. Nevertheless, the increased hormone activation can causes diseases such as insulin resistance, diabetes or obesity. Because of the increased risk for diseases a high protein diet in early infancy is not recommended but breast feeding or low protein diet can have beneficial effects for health.

Within a cow supplementation project, the newly developed AA method has proved effective. From two weeks before to nine weeks after expected calving one group (CLA group, n=10) was supplemented with CLA and one group (control group, n=10) was supplemented with linoleic acid. The aim of the study was to analyze the relation between CLA supplementation and AA profiles in blood samples. These were taken at

a total of eight times before and after delivery. We observed three different time courses of AA. In most cases AA levels decreased after delivery and afterwards concentrations increased slowly to their initial values. Glu is decreased before delivery which may indicate that the fetus is supplied sufficient with Glu. After delivery Glu is increasingly transported in the milk. Gly, HPro, MHis and Ser increased after delivery and then moved back to basal values where MHis did not increase in the first 12 weeks due to muscle protein breakdown. CLA supplementation showed no significant difference between the two groups on the AA concentrations and thus showed no effect on AA synthesis.

6 Zusammenfassung

Klinische Studien zeigen, dass die Zusammensetzung von zirkulierenden, freien AS im Blut, ein Marker für monogene und multigenetische Krankheiten ist. Die Analyse von hohen Probandenzahlen in klinischen Studien wird oftmals durch aufwendige und lange Probenaufarbeitungsschritte begrenzt. Im Rahmen der Metabolomics Plattform, die im Dr. von Haunerschen Kinderspitals etabliert wurde, wurde eine Hochdurchsatzmethode entwickelt, die eine selektive, sensitive, präzise und robuste Quantifizierung von 22 AS aus kleinen Probenvolumina ermöglicht. Im Laufe der Zeit konnten noch weitere Aminosäuren zur Methodik hinzugefügt werden. Dabei können innerhalb von 36 Stunden 96 Proben analysiert werden. Mit Hilfe eines deuterierten, interenen Standards in methanolischer Lösung werden Proteine aus nur 10 µL Probenvolumen gefällt. Zur Quantifizierung der AS wird anschliessend der eingedampfte Überstand derivatisiert und in Kombination mit einem Ionen-Paar-Reagenzes chromatographiert. Der Methodenaufarbeitung liegt eine umfassende und ausführliche Validierung zugrunde, die einer Interday Precision von 3.1 -10.8 % für alle Analyten erzielt. Zusätzlich unterzieht sich unsere Methode jedes Jahr an einem Ringversuch, der eine exakte Bestimmung aller Analyten gewährleistet.

Im Zusammenhang mit der Programmierung des Stoffwechsels durch die Ernährung im Säuglingsalter wurde die neu entwickelte AS-Methode zur Quantifizierung von 726 Serum Proben in einer randomizierten klinischen Studie eingesetzt. Dabei wurde der Bezug zwischen Proteinzufuhr (Formelnahrung mit hohem Eiweißanteil bzw. niedriegem Eiweißanteil) und AS-Profil bei 6 Monate alten Säuglingen analysiert. Eine signifikante Veränderung der Plasmakonzentrationen zeigte sich in der Gruppe der formelernährten Kindern mit hohem Proteinanteil für folgende AS: BCAA, Gly, Lys, Met, Phe, Pro, Thr, Trp und Tyr. Essentielle AS werden über die Nahrung aufgenommen und vermutlich über das L-Transportsystem in die Zirkulation freigesetzt. Im Vergleich dazu werden nicht essentielle AS im Darm katabolisiert und mit Hilfe der de novo Synthese in gleichbleibenden Konzentrationen reguliert, sodass kein signifikanter Unterschied in beiden Gruppen beobachtet wurde. Durch eine proteinreiche Nahrung können AS vermehrt an der Aktivierung von Wachstumshormonen (mTOR, IGF-1) teilhaben, was sich im vermehrten Zellwachstum und –proliferation manifestiert. Nichts desto trotz kann die vermehrte Wachstumshormonaktivierung Krankheiten wie Insulinresistenz, Diabetes oder auch Übergewicht hervorrufen. Aufgrund des erhöhten Krankheitsrisikos ist einerseits von einer proteinreichen Ernährung im frühen Säuglingsalter abzuraten, andererseits kann

das Stillen bzw. eiweißänhnliche Brustmilchzusammensetzung gesundheitsunterstützend sein.

Auch im Rahmen eines Supplementierungsprojektes bei Kühen, hat sich die neu entwickelte AS-Methode bewährt. Dazu wurden jeweils 10 Kühe kurz vor und nach der Geburt mit CLA (CLA-Gruppe) oder mit Linolsäure (Kontroll-Gruppe) supplementiert. Ziel der Studie war es, den Zusammenhang zwischen CLA Supplementierung und AS-Profil im Blut zu analysieren. Dazu wurden zu insgesamt 8 Zeitpunkten vor und nach der Geburt, Blutproben entnommen. Es kristallisierten sich 3 verschiedene Zeitverläufe heraus, wobei die meisten AS-Konzentrationen nach der Geburt abfallen und langsam wieder auf ihre Ausgangswerte ansteigen. AS wie Glu sinken vor der Geburt stark ab, was dafür sprechen könnte, dass der Fetus mit ausreichend Glu versorgt wird und nach der Geburt vermehrt in die Milch transportiert wird. Gly, HPro, MHis und Ser steigen bis zur Geburt an und fallen dann wieder ab wobei Mhis durch den Muskelproteinabbau keinen Konzentrationsanstieg in den ersten 12 Laktationswochen erfährt. Die Supplementierung mit CLA zeigte keinen signifikanten Unterschied beider Gruppen auf das AS-Profil und zeigte somit keine Auswirkungen auf die AS-Synthese.

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8 Appendex

Table 1: AA concentration of CLA supplemented cows. All results presented as mean

CLA group	week -2			week 1			week 2			week 3			week 4			week 6			week 9			week 12		
AA	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]	Mean [µmol/L]		SE [µmol/L]
Ala	291	±	18.9	216.9	±	10.4	226.3	±	16.9	256.4	±	16.5	245.3	±	21.8	272.1	±	22.6	267.1	±	19.7	280.2	±	17.9
Arg	64.3	±	5.1	43.9	±	4.7	55.3	±	4.6	60.9	±	5	59.2	±	5.4	68.3	±	9.4	73.4	±	9.4	91.4	±	6.9
Asn	24.4	±	3.6	22.9	±	3.3	32.5	±	4.5	37.5	±	3.5	36.3	±	4	37.1	±	4.7	39.4	±	4.8	42	±	3.7
Asp	7	±	0.7	5.1	±	0.6	6.5	±	0.7	8	±	0.7	7.5	±	0.4	8.4	±	0.7	8.9	±	0.8	10.3	±	0.5
Car	5.8	±	1.9	9	±	3	8.8	±	2.9	9.8	±	3.3	10	±	3.3	10.5	±	3.5	12	±	4	11.9	±	4
Cit	73.7	±	5.9	64.2	±	3.5	73.4	±	5.2	85.9	±	5.7	83.4	±	7.5	93.2	±	7.9	87.2	±	9.5	95.6	±	7.4
Cysta	2.1	±	0.3	2.8	±	0.1	2.8	±	0.1	3	±	0.1	2.8	±	0.1	2.6	±	0.3	3.1	±	0.1	3.2	±	0.1
Gln	292	±	22.2	286	±	21.6	246.7	±	20.6	232.6	±	15.4	234	±	14.6	236.7	±	26.3	259.7	±	24.5	260.9	±	23.9
Glu	87.5	±	9.4	46.8	±	4.1	42.5	±	5.3	41.4	±	4.4	38.8	±	2.1	42.2	±	4.2	38.4	±	3.1	46.9	±	3.5
Gly	261.6	±	14.4	441.7	±	43	547.2	±	30.6	588.1	±	42.7	554	±	57.4	429.6	±	34.3	412.4	±	32.6	407.4	±	29.5
His	56	±	5.1	50.2	±	8	51.1	±	5	45.8	±	5.5	53.3	±	5.6	52.8	±	7.7	55.7	±	5.6	59.3	±	4.5
HLys	0.8	±	0.1	0.8	±	0.2	0.4	±	0	0.4	±	0.1	0.4	±	0	0.4	±	0.1	0.4	±	0	0.4	±	0.1
HPro	21.5	±	1.4	26.3	±	2.3	25.9	±	2.1	23.2	±	1.1	19.9	±	1.3	21.9	±	1.7	23.1	±	2.3	20.9	±	2.1
Ile	98	±	7.6	69.9	±	8.3	96.1	±	7.7	111	±	13.6	114.6	±	10.3	108	±	12.6	99.3	±	13.8	114.3	±	12.1
Leu	134.5	±	7.7	92.5	±	7.4	119.6	±	8.3	126.9	±	14.5	131.5	±	10.6	124.6	±	14.1	121.9	±	17	137.7	±	12.3
Lys	81	±	7.5	47.3	±	3.9	62.3	±	5	71.7	±	6.1	64.8	±	3.3	67.3	±	6.5	64.4	±	6.6	82	±	6.2
Met	26.3	±	2.1	20.5	±	1.4	22.7	±	1.9	22.9	±	2.1	20.5	±	1.8	20	±	2.5	22	±	2.3	24	±	1.5
MHis	7.5	±	0.6	12.7	±	1.1	8.7	±	1	5.9	±	0.5	5.4	±	0.4	4.3	±	0.3	4.3	±	0.3	4.3	±	0.3

Orn	41.4	±	4.9	17.2	±	2.9	21.9	±	2.8	26.4	±	3.6	30	±	4.1	32.3	±	5.7	35.9	±	6.1	45.5	±	4.5
Phe	55.5	±	2.2	40.7	±	3	48.1	±	2.4	43.4	±	2.3	41	±	2	43.4	±	3.1	44.1	±	4	47	±	3.6
Pro	107.7	±	7.8	90.7	±	10.3	109.3	±	11.1	119.2	±	11.1	113	±	11.2	117.2	±	16.3	123.5	±	12.2	129.3	±	9.1
Ser	80.5	±	6.4	111.1	±	11.6	110.2	±	8	106.6	±	8.2	98	±	8.1	99.9	±	6.7	93	±	7.4	94.9	±	7.7
Thr	95.3	±	11	54.6	±	9.4	70.9	±	8.3	86.8	±	6.8	90.5	±	8.1	90.8	±	10	93.2	±	13	97.1	±	5
Trp	49.7	±	5.7	25.6	±	3.5	35.1	±	3 3.3	39.5	±	3.8	39.6	±	4.6	53.6	±	3.7	55.9	±	4	56.7	±	3.1
Tyr	51.1	±	4.3	30.3	±	1.7	38.3	±	4.3	40.3	±	2.6	38.4	±	3.6	46.8	±	4.9	45.8	±	6.1	46.7	±	4.6
Val	234.2	±	16.7	169.9	±	18.1	234.2	±	23	249.3	±	25.7	273.8	±	25.9	269.9	±	29.4	258.5	±	27.9	281.9	±	17.9

Table 2: AA concentration of cows in control group. All results presented as mean

Control group	**week -2**			**week 1**			**week 2**			**week 3**			**week 4**			**week 6**			**week 9**			**week 12**		
AA	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**	**Mean [µmol/L]**		**SE [µmol/L]**
Ala	293.1	±	24.3	241.4	±	18.7	244.8	±	19.4	242.4	±	19.7	259.5	±	25.6	249.8	±	16.3	257.6	±	15.5	260.9	±	14.9
Arg	71.2	±	5.2	48.3	±	4.5	59.2	±	4.9	64.9	±	7.5	69.8	±	5.8	81.4	±	8.6	80.2	±	6.6	86.4	±	6.3
Asn	27.3	±	2.6	27.4	±	4.1	38.9	±	3.9	38.1	±	3.5	41.6	±	3.6	39.1	±	4	43.2	±	4.5	38.6	±	2.8
Asp	7.8	±	0.8	7	±	1.3	7.2	±	0.5	7.8	±	0.4	8.4	±	0.5	9.7	±	0.8	10.3	±	0.7	11.5	±	0.9
Car	7.6	±	0.8	8.8	±	1.1	11.7	±	1.2	13.5	±	1.1	12.3	±	1	11.3	±	1.2	12.9	±	1	13.2	±	1.6
Cit	87	±	4.4	78.5	±	8.4	88.4	±	7.4	92.6	±	8.5	91.4	±	4.5	89.4	±	10.3	87	±	9.2	90.2	±	6.1
Cysta	2.2	±	0.3	2.6	±	0.3	3	±	0.1	3.1	±	0.1	3.2	±	0.1	3.3	±	0.2	3.3	±	0.1	2.9	±	0.3
Gln	296.2	±	14.9	253.2	±	17.1	245.9	±	19.8	240.6	±	20.5	244.5	±	18.7	224.3	±	13.9	244.7	±	23.3	254.7	±	18.3
Glu	120.1	±	13.9	56.6	±	11.7	51.6	±	4.3	45.3	±	3.8	43.9	±	4.7	45.1	±	5	46	±	3.1	49.7	±	6.6
Gly	327.2	±	16.8	531.6	±	57.7	687.3	±	53.3	680	±	45.9	653.1	±	35.1	477.8	±	40.3	467	±	38.4	359.4	±	15
His	63.8	±	3.5	46.4	±	5.1	51	±	2.7	49.2	±	4.3	55.6	±	4.9	52.9	±	4.9	56.7	±	5.3	62.1	±	4.5

HLys	0.8	±	0.1	0.7	±	0.1	0.6	±	0.1	0.6	±	0.1	0.4	±	0.1	0.3	±	0	0.3	±	0.1	0.4	±	0.1
HPro	23.8	±	2	26.6	±	2.3	25.9	±	1.7	22.5	±	1.8	22.5	±	1.2	20.9	±	2.3	17.7	±	1.3	17.9	±	1.7
Ile	111.6	±	7.3	97.8	±	8.4	106.8	±	7.4	116.9	±	16.6	120.2	±	12.2	110.9	±	5.8	102.4	±	8.2	111.3	±	8.8
Leu	145	±	7.1	123.5	±	9.6	134.8	±	8.5	130.2	±	13.5	140.4	±	11.5	132.2	±	6.9	121.3	±	8.8	140.7	±	9.1
Lys	90	±	8.5	58.9	±	3.9	71.5	±	6	70.9	±	5.1	78.5	±	5.6	80.1	±	8	82.3	±	6.7	85.2	±	5.5
Met	27.5	±	1.9	23.5	±	1.8	25.2	±	2.1	23	±	2	23.6	±	1.7	22.3	±	2.1	24.1	±	2.4	25.5	±	1.8
MHis	8.8	±	1	11.3	±	0.9	8.7	±	0.5	6.2	±	0.5	5.3	±	0.5	4.3	±	0.3	3.9	±	0.3	4	±	0.3
Orn	50.4	±	3.8	31	±	5.4	34.2	±	5.5	31.8	±	4.1	33.4	±	3.4	45.8	±	5.5	43.9	±	6.2	48	±	5.5
Phe	59	±	2.8	47.9	±	1.5	49.6	±	2.6	42.8	±	3.7	45.8	±	3.8	46.4	±	3.8	43.2	±	2.7	48.2	±	2.3
Pro	120.2	±	6.3	104.8	±	9.9	129.7	±	10.7	124.7	±	11.3	134.5	±	15.5	121	±	8.6	131.8	±	9.2	129.2	±	6.9
Ser	98.2	±	7.5	126	±	16.7	126.9	±	10.2	113.3	±	8.3	121.5	±	8.9	99.8	±	8.8	111.7	±	6.6	102.2	±	5
Thr	100	±	13	66.6	±	8.2	85	±	9.7	91.5	±	8.2	100.4	±	8.5	93.5	±	11.7	116.4	±	12.3	115.6	±	9.4
Trp	58.2	±	3.8	35.9	±	4.5	37.1	±	2.7	45.5	±	3.3	48.3	±	3.4	53.4	±	3.6	60.6	±	2.6	61.4	±	5.8
Tyr	56.3	±	2.6	43.6	±	4.4	44.7	±	4.2	43.7	±	4.6	50.3	±	5.6	46.9	±	5.3	48.7	±	4.1	57.2	±	4
Val	245.3	±	13.2	218.7	±	18.7	255	±	19.2	257.1	±		288.4	±	19	267.7	±	12.2	282.2	±	18.9	283.5	±	17.7

9 Danksagung

Ich danke meinem Doktorvater Herrn Professor Koletzko,der keine Kosten und Mühen gescheut hat, mich über die gesamte Zeit zu motivieren und respektieren. Der Zuspruch und das Vertrauen offenbarten sich mit Stolz und Selbstbewusstsein.

Zugleich danke ich meinem Betreuer Herrn Doktor Peissner, der mir mit seinem Fachwissen zur Seite stand, viele seiner freien Abende und Nächte für mich opferte und mir nicht zuletzt auch durch private Gespräche und anregende Diskussionen zu einem wertvollen und freundschaftlichen Wegbegleiter wurde.

Ich danke Herrn Doktor Demmelmair, der mir stets Ansprechpartner war und mein Forschungsprojekt durch Ideen, Anregungen und konstruktive Kritik bereicherten.

Ich danke Frau Doktor Metges, die ein interessantes Versuchsprojekt zur Verfügung stellte und mir Einblicke in die Welt der Tierforschung zeigte.

Ich danke all meinen Kollegen insbesondere Frau Nießer, die mir viele Aufgaben abnahm und mich als Banknachbarin auch in angespannter und gestresster Lage ertrug und Herrn Hellmuth für überzeugende Ideen und für die Korrektur meiner Arbeit.

Ich danke meinem Freund, der mir stets Mut zugesprochen und mich in meiner Arbeit bestärkt hat. Hätte er mir nicht den Rücken freigehalten, wäre meine Arbeit in dieser Form nicht möglich gewesen.

Und nicht zuletzt danke ich meinen Eltern, die in jeglicher Hinsicht die Grundsteine für meinen Weg gelegt haben und die es wohl gar nicht fassen können, dass ich jetzt endlich fertig bin.

10 Publications and presentations

Publication

U. Harder, B. Koletzko, W. Peissner. Quantification of 22 plasma amino acids combining derivatization and ion-pair LC-MS/MS. Journal of Chromatography B, 879 (2011) 495–504

W. Peissner, **U. Harder**, N.N.-Reinehr-Gruppe, B. Koletzko, T. Reinehr. Metabolomic profile in obese children before and after weight loss (Manuscript in preparation)

U. Harder, W. Peissner, K. Hötger, A. Tröscher H. M. Hammon, B. Koletzko, C. C. Metges. Plasma amino acid of CLA supplemented cows (Manuscript in preparation)

U. Harder, L. Wauters, B. Koletzko, W. Peissner. Determination of plasma metabolites using Selected Optimized Flow Injection Analysis (SOFIA) (Manuscript in preparation)

Presentation

U. Harder, W. Peissner, B. Koletzko. Development of a sensitive method for the analysis of amino acids from 10 µL plasma using LC-MS/MS. Metabolomics & More; The Impact of Metabolomics on the Life Sciences; Freising-Weihenstephan, March 10-12, 2010 (poster presentation).

U. Harder, B. Koletzko, W. Peissner. Sensitive Quantification of 22 Amino Acids from 10 µL Plasma Combining Derivatization and Ion-Pair LC-MS/MS. METABOLOMICS 2010, Amsterdam, June 27-July 1, 2010 (poster presentation).

U. Harder, W. Peissner, K. Hötger, A. Tröscher, H. M. Hammon, B. Koletzko , C. C. Metges . Free plasma and milk amino acids in dairy cows supplemented with rumen-protected conjugated linoleic acid. Oskar Kellner Symposium 2011, September 09-11. 2011 (poster presentation, nominated for best poster).

U. Harder, Quantification of 22 plasma amino acids combining derivatization and ion pair LC-MS/MS. Young Investigator Forum. 30 May-2June 2012, The Netherlands (Bergen) (oral presentation).

11 Selbstständigkeitserklärung

Ich, Ulrike Harder, erkläre ausdrücklich, dass es sich bei der von mir eingereichten schriftlichen Arbeit um eine von mir selbsständig und ohne fremde Hilfe verfasste Arbeit handelt. Ich erkläre ausdrücklich, dass ich sämtliche verwendete fremde Quellen als solche durch Zitate kenntlich gemacht habe. Ich habe bisher noch keinen Promotionsversuch unternommen, und die vorliegende Dissertation wurde nicht in gleicher oder ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht.

Mein Anteil an den praktischen Tätigkeiten:
Methodenentwicklung und Validierung der gesamten Aminosäure Methode u.a.

- Literaturrecherche
- Derivatisierungsoptimierung für alle zu messenden Aminosäuren
- Optimierung verschiedener stationären und mobilen Phasen
- Optimierung von Probenvolumen, Fällungsreagenzien
- Optimierung des Ionenpaar-Reagenzes, der Kalibriergerade
- Aufarbeitung verschiedener Probenmatrices
- Aufarbeitung und Quantifizierung von insgesamt 5000 Analysenproben

Bedienung, Instandhaltung des LC-MS/MS Systems u.a.

- Optimierung verschiedener Gase und Potentiale für alle zu messenden Aminosäuren
- kleine Reparaturen z.B. von Verschleißteilen

Teilnahme und Durchführung am jährlichen Ringversuch zur Aminosäureanalytik

Messung, Auswertung und Quantifizierung der Aminosäurekonzentration in beiden vorgestellen Studien (siehe Kapiel 3 und 4)