VU medisch centrum

hogeschool Leiden

Development of an HPLC-MS/MS method for the analysis of phenylalanine and tyrosine in dried blood spots

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Samenvatting

Inleiding: Phenylketonurie is een veel voorkomende aangeboren aandoening die wordt veroorzaakt door een mutatie in het enzym phenylalanine hydroxylase. Hierdoor wordt het animozuur phenylalanine niet afgebroken. De opstapeling van phenylalanine is toxisch en hindert de ontwikkeling van de hersenen. Patienten volgen daarom een eiwit arm dieet en worden regelmatig gecontroleerd door een metabool arts op hun phenylalanine concentratie.

De huidige analyse van phenylalanine in bloed spots wordt gemeten als butyl derivaat met flow injectie analyse gekoppeld aan tandem massa spectrometrie (FIA-MS/MS), hierbij wordt het metaboliet tyrosine ook mee gemeten. Het nadeel van deze methode is dat isomeren met dezelfde massa als phenylalanine en tyrosine niet worden gescheiden en dat de monsteropwerking relatief lang duurt. Het doel van dit onderzoek: de ontwikkeling een methode waarbij gebruik wordt gemaakt van high pressure liquid chromatografie gekoppeld aan tandem massa spectrometrie (HPLC-MS/MS) voor de analyse van phenylalanine en tyrosine in DBS als FMOC derivaat. De chromatographische methode voor de analyse van phenylalanine en tyrosine moet vrij zijn van interferentie, een korte analyse tijd hebben en envoudig genoeg zijn voor het gebruik van routine-samples.

Methode: Voor de analyse van phenylalanine en tyrosine met HPLC-MS/MS werd gebruikt gemaakt van FMOC als derivaat. De analieten werden gedetecteerd met multiple reaction monitoring (MRM). Bij de ontwikkeling van de nieuwe HPLC methode werden verschillende HPLC kolommen getest, op basis van piekvorm werd de geschikte kolom gekozen. Bij deze kolom werd er een factorial design voor het eluens gemaakt. Na de optimalisatie van de samenstelling van het eluens werd de flowrate geoptimaliseerd waarbij gebruik werd gemaakt van peakcapacity. Met de oude methode en de nieuwe geoptimaliseerde methoden werden er 103 samples gekwantificeerd met een kalibratielijn. Om te kijken of waarden van de nieuwe methode overeen komen met die van de oude methode en nog klinisch verantwoord zijn, werden beide methoden ten opzichte van elkaar vergeleken met regressie analyse en Blant-Altman plots.

Resultaten: Tijdens de optimalisatie van de HPLC methode werd er een stoorpiek gescheiden in de overgang van phenylalanine en in de overgang van de interne standaard voor tyrosine. Met de Atlantis kolom vond de beste scheiding plaats. De kolom gaf de beste prestatie bij een flow van 0,4 mL/min met een lineair gradiënt van 3 minuten met een binair eluens dat bestond uit A) 80/20 ACN/H_2O met 125 mg/L ammonium formaat en B) 30/70 ACN/H_2O met 125 mg/L ammonium formaat.

De resultaten van de concentratie phenylalanine en tyrosine met de HPLC-MS/MS als FMOC derivaat correleerde sterk met het resultaat van de FIA-MS/MS als butyl derivaat. Bij de Blant-Altman plots werden wel verschillen opgemerkt. Bij lage concentraties voor phenylalanine gaf de FIA-MS/MS als butyl derivaat veel fouten die de 2 sd grens overschrijden. Dit werd veroorzaakt doordat bij de kalibratielijn voor phenylalanine het hoogste punt afvlakten, hierdoor werd de kalibratielijn onbetrouwbaar. De kalibratielijn van de HPLC-MS/MS als FMOC derivaat bleef over een groter bereik lineaer. Voor tyrosine waren de waarden voor de HPLC significant hoger ten opzichte van de waarden verkregen met FIA-MS/MS maar er was geen duidelijke trend.

Conclusie: Er is een HPLC-MS/MS methode ontwikkeld voor de analyse van phenylalanine en tyrosine als FMOC derivaat. Bij het gebruik van HPLC-MS/MS werd er een stoorpiek gescheiden in de transitie van phenylalanine en de interne standaard voor tyrosine. Daardoor is de nieuwe methode is preciezer. Bovendien is de monstervoorbewerking sneller en heeft de nieuwe methode ook een groter lineair bereik.

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1. Introduction

Phenylketonuria (PKU) is a fairly common inherited disorder, caused by a defect in the phenylalanine metabolism. It was first described by Asbjørn Følling in 1934 [1]. Usually phenylalanine is oxidized by the enzyme phenylalanine hydroxylase (PAH) to form the amino acid tyrosine [2]. There are two variants of PKU that are caused by mutations in PAH gene on chromosome 12q23.2. These are the traditional PKU and hyperphenylalanemia. In the case of traditional PKU, PAH is missing completely. While with the milder form of PKU called hyperphenylalanemia, PAH does function but it is less efficient and therefore not being able to convert all the phenylalanine into tyrosine. The deficiency of PKU results in an elevated phenylalanine level in blood. The accumulation phenylalanine is toxic. Untreated PKU can cause growth failure, seizures and brain damage.

It is impossible to cure PKU, treatment for PKU is focused on reducing the phenylalanine levels in blood. Since phenylalanine is required in order to grow you cannot exclude the phenylalanine intake completely. Patients are required to follow a special diet that reduces the phenylalanine intake. It is important that the treatment for PKU starts as soon as possible. Early diagnosis and treatment can prevent mental retardation. Therefore PKU is one of the diseases that is tested in newborn screening. Routine screening for PKU is performed worldwide [3]

Detection of affected newborns is achieved by the analysis of the ratio of phenylalanine to tyrosine in dried blood spots (DBS). Tandem mass spectrometry is most often used as diagnostic tool for PKU patients. Because a lot of babies needs to be screened every day, a very quick analysis is required. Flow injection analysis coupled to mass spectrometry is the only technique that can cope with the amount of samples that needs to be analyzed each day. This method uses analysis of phenylalanine and tyrosine as their butyl derivative in DBS. [4-6].

After diagnosis patients will be provided a diet made by a dietician and will be in check under a metabolic doctor. To monitor the effect of the treatment, phenylalanine and tyrosine levels of patients are regularly analyzed by a laboratory. Depending on the results the diet can be adjusted. Because of treatment the phenylalanine concentrations for monitoring are much lower than in newborns and a more accurate method is preferred. The amino acids are detected using ion chromatography with a post column derivatization with ninhydrin [7]. This way of analyzing amino acids is considered as the golden standard. Even though its considered as the golden standard because of the long analysis time of 3 hours for each sample prevents its use for screening.

However for the amino acid analyzer needs whole plasma for analysis. Patients are required to visit the hospital for venipuncture. This is very uncomfortable for the patient. Therefore the metabolic laboratory of the VUmc also offer a more patient friendly method for monitoring, which is monitoring from DBS. This way the patient can pinprick puncture at home and send the Guthrie cards to the hospital. The analysis of DBS is based on the newborn screening method. This method is focused on speed while detecting elevated phenylalanine concentrations which is not the best approach for monitoring. Because there is no separation other than the mass spectrometer isobars and isomers with the same mass as phenylalanine and tyrosine will interfere with the analysis.

In order to achieve better accuracy and selectivity a chromatographic separation technique can be used before detection with MS. The goal of this study is to develop an new HPLC-MS/MS method for the analysis of phenylalanine and tyrosine from DBS that can be used for monitoring of PKU patients.

1.1 Background amino acids

Phenylalanine and tyrosine are both α-amino acids. α-amino acids have a central role in metabolism. They serve as building blocks of proteins of peptides and other functional molecules. As their name implies, amino acids are di-functional: they contain both an amino group and a carboxyl group. The functional group that distinguishes this group of metabolites from others such as fats and carbohydrates in the amino group. The general structure of α-amino acids consists a carboxyl group, an amino group and a R-group representing the side chain. The carbon atom next to the carboxyl group on which the amino acid is bonded is called an α-carbon. Hence the name α-amino acids [8]. The role and purpose of each α-amino acids by the different nature of their side chain. In figure 1.1 the structure of phenylalanine and tyrosine are shown, here you can see they differ in their side chains i.e. a phenyl group (phe) vs a hydroxyphenylgroup (tyr).

Fig. 1.1: Structures of phenylalanine (a) and tyrosine (b) .

Humans are not capable of producing all the amino acids they require in order to grow. We can only make 10 of the 20 amino acids ourselves. The remaining 10 are called essential amino acids and must be supplied from food. Failure to obtain enough of even 1 of the 10 essential amino acids, results in degradation of the body's proteins and muscle. Unlike fat, the human body cannot store excess amino acids for later use, so a new supply of the amino acids must be consumed trough food every day.

1.2 Phenylalanine metabolism

One of the essential amino acids is l-phenylalanine [9]. Figure 1.2 shows the processes in the human body that involves phenylalanine. The concentration of phenylalanine in the body is regulated to a steady level. Disturbance of the flux will eventually lead into a change into the regular level of phenylalanine. The main sources of phenylalanine are through dairy and is then recycled through various amino acid pools. Hydroxylation of L-phenylalanine by PAH produces L-Tyrosine. Tyrosine is an nonessential amino acid, it is used to form neurotransmitters such dopamine. Alternative metabolic routes of phenylalanine are decarboxylation into phenylethylamine or transamination into phenylpyruvate [10].

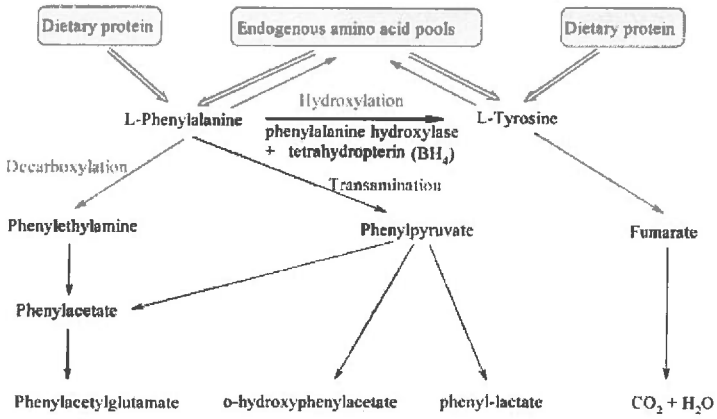

Fig. 1.2: Schematic scheme of the metabolism of phenylalanine in the human body. [10/14]

The hydroxylating system that is responsible for the conversion of phenylalanine to tyrosine consists of:

- Phenylalanine hydroxylase
- Cofactor, tetrahydrobiopterin (BH4)
- Enzymes that regulate BH4. (dihydropterdine reductase and 4α-carbinolamine dehydratase)

Phenylalanine hydroxylase is the enzyme that converts phenylalanine to tyrosine. This reaction occurs in presence of the cofactor tetrahydrobiopterin (BH4) and oxygen. Conversion of phe into tyr involves a para-hydroxylation of the phenylgroup by PAH (figure 1.3). The PAH enzyme is a monomer about 50 kDa in size and consists of 452 amino acids [11].

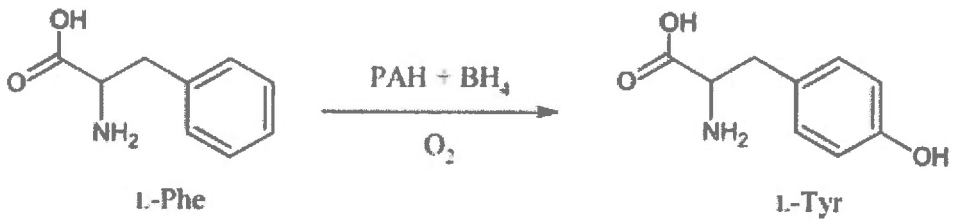

Fig. 1.3: Reaction scheme of the conversion of phenylalanine to tyrosine catalysed by PAH. [10]

When we obtain phenylalanine through food, we suddenly have an increased amount of phenylalanine in our amino acid pool. There are certain mechanisms that regulate this sudden increase in concentration. These mechanisms allow for a fine regulation of the phenylalanine concentration by balancing the levels for the production of protein synthesis while minimizing the exposure to high concentrations of phenylalanine. These control mechanisms work on regulating the BH4 co-factor interaction with PAH.

1.3 Errors in the phenylalanine metabolism

Phenylalanine hydroxylase (PAH) deficiency is often caused by a mutation in the gene on chromosome 12q13.2 This gene helps with the production of the enzyme (PAH). When the enzyme PAH is defective the body cannot break down phenylalanine. Many different mutations in PAH exists and can lead to phenylketonuria (PKU), hyperphenylalaninemia (non-PKU HPA), and variant PKU.

In the case of classical PKU the mutation causes that the enzyme is missing completely. While other mutations causes HPA, which is a milder form of PKU. Here the enzyme is present but it works less efficient. Patients with HPA are able to tolerate larger amounts of phenylalanine in their diets than those with classic PKU.

Varient PKU is a rare form of PKU, here there is a mutation in the gene for the enzyme that aids with the production of BH_4 leading to a aBH_4-Defficientie. BH^4 necessary for proper activity of the enzyme PAH. Because not enough BH_4 is produced PAH cannot work properly and causing build up in phenylalanine.

The metabolism of phenylalanine takes place in the liver and from here transported to the rest of the body through blood. The brain requires phenylalanine to grow. Phenylalanine is a large neutral amino acid, neutral amino acids compete with each other for places for transport across the blood-brain barrier. High concentrations of phenylalanine in blood will saturate the transporter. Therefore other neutral amino acids cannot enter the brain and all these amino acids are necessary for the production of protein. The build up of phenylalanine hinders the development of the brain, causing intellectual /disability impairment.

1.4 Treatment for PKU

Treatment for PKU is focused on reducing the phenylalanine intake so the phenylalanine concentration in the body will stay low, preventing the toxic phenylalanine levels that causes neurologically changes. However phenylalanine is required for the body in order to grow, therefore it can't be avoided completely. PKU patients follow a protein restricted diet, with a limited amount of phenylalanine intake. There are two major risk groups who are particularly vulnerable, children and pregnant woman. In the first 30 years neural development is maximal [12], so it is important to prevent any neurological changes during the time the brain is still developing. Neurological changes can already occur within one month of birth. Therefore it is important that the diary restriction of phenylalanine should start as fast as possible and is maintained throughout childhood. There is an even more important restriction for woman who are pregnant. Because an elevated phenylalanine concentrations will have a teratogenic effect on the developing foetus. It is still discussed if adults should still follow the diet.

The diet starts with small amounts of phenylalanine coming from breast milk. When the child grows up the protein intake is calculated daily, depending on their phenylalanine concentrations. Foods that contains a lot of protein such as eggs, milk, cheese, meat, and fish has to be avoided [13]. However, avoiding these foods will not provide enough protein required for growth. Therefore the diet includes commercially supplements of essential amino acids that are free from phenylalanine. The advised target values of phenylalanine are 40-360 µmol/L.

A metabolic physician checks on the development and growth of the patient and phenylalanine levels are regularly determined by laboratories. It is important phenylalanine levels are monitored because if the phenylalanine concentrations are elevated the diet has to be adjusted.

1.5 Screening and diagnosis of PKU

Disorders in the amino acid metabolism were some of the earliest inborn errors investigated. Since the early 60's amino acids could be detected and identified using ion chromatography using a post column derivatization with ninhydrin [7]. This way of analyzing amino acids still remains the golden standard till this day and is used for monitoring of the PKU patients.

Treatment of PKU is relative easy and can prevent negative effect if detected early. Therefore PKU is one the diseases that is tested by newborn screening. Routine screening for PKU is performed worldwide [4]. Detection of affected newborns is achieved by the analysis of the ratio of phenylalanine to tyrosine in dried blood spots (DBS). Because the concentration of phenylalanine is increased and the concentration of tyrosine decreased, analysis of both amino acids reduce the amount of false positive samples [14].

Even though the amino acid analyzer is considered the golden standard its long analysis times prevents its use in screening methods. Newborn screening involves a lot of samples that needs to be analyzed, so fast analysis time is a must. Flow injection analysis coupled to mass spectrometry is the only technique that can cope with the amount of samples that needs to be analyzed each day. This method uses analysis of amino acids in DBS as their butyl derivative. [4-6].

The DBS is then analyzed with FIA-MS/MS using butyl esters of the amino acids, giving a common loss of butyl formate of 102 DA upon CID. This transitions is highly specific for α-amino acids [15]. Using MS/MS of DBS allows for the screening of 20 metabolic disorders in one run of 90 seconds [16]. The very fast analysis time makes it an efficient method while having a low error rate. Whenever an patient is exceeds the upper limit aadditional tests are performed to confirm the initial results. Then they are tested for the presence of the PAH gene mutation that causes PKU.

1.6 Goal

The metabolic laboratory of the VUmc offers monitoring of PKU. Monitoring is usually achieved with the amino acid analyzer. However for the amino acid analyzer needs whole plasma for analysis. Patients are required to visit the hospital for venipuncture. Which is considered very uncomfortable for the patient. Therefore the metabolic laboratory of the VUmc also offer a more patient friendly method for monitoring of PKU. The analysis of DBS is based on the newborn screening method.

This method is focused on fast analysis while detecting elevated phenylalanine concentrations, which is a good approach for monitoring. Because there is no separation other than the mass spectrometer isobars and isomers with the same mass as phenylalanine and tyrosine will interfere with the analysis. During monitoring, patients are on diet restriction and the phenylalanine concentrations are normalized. For monitoring its required to detect small changes in the concentration of normalized phenylalanine levels. Because the amount of samples entering the laboratory for monitoring is smaller than for screening, it will not harm is the analysis time of the method for monitoring from DBS is a little longer. In order to achieve better accuracy and selectivity a chromatographic separation technique can be used before detection with MS.

The goal of this study is to develop an new HPLC-MS/MS method for the analysis of phenylalanine and tyrosine from DBS that can be used for monitoring of PKU patients. The chromatographic method for the analysis of phenylalanine and tyrosine should be free from interference, maintaining a short run time and be straightforward enough to use for routine samples.

2. Approach and theory of analysis techniques

2.1 Mass spectrometry

Mass spectrometry is an analytical techniques that separates and detects molecules based on their molecular weight. It is widely used in metabolic laboratories with multiple purposes. It can provide both qualitative and quanitative information. When MS is used for quanitative analysis it is often referred to as targeted analysis. With targeted analysis one or a few metabolites are detected by a measuring certain specific transitions. However to be able to measure a transition a second MS is required, this is called tandem MS. Which is further explained in chapter 2.2. The scan mode that is used for targeted analysis is often selective reaction monitoring (SRM) which is explained in 2.3. To be able to detect the molecules they first have to be ionized, this process is explained in the next chapter.

2.1.1 Electron spray ionisation

Before detection, the analyte molecules first need to acquire a charge. This happens in the ionisation chamber, where they are ionised to positive or negative molecules. One technique that can do this is ESI (electron spray ionization). ESI is an ionization technique that is often used for qualitative analysis of metabolites. It uses electrical energy to promote the ions coming from the HPLC as a liquid into the gaseous phase. The conversion to ions from the solution into the gas phase by ESI involves tree steps.

- Dispersal of a fine spray of charge droplets
- Solvent evaporation
- Ion ejection from the highly charged droplets

When the analyte leaves the HPLC column they enter the ionization chamber through the 'Taylor Cone'. The Taylor cone is maintained at a high voltage (e.g. 2,5-6,0 kV). It emits a spray of charged liquid drops. The liquid drops then enter the ionization chamber that is heated. Because of the temperature and the aid of drying gas, the solvent evaporates and the droplets become smaller. However because the charge still remains the same, the electric energy within the charged droplet will become larger compared to it surface area. Till it will become too much, and the ions at the surface of the droplet will be ejected into the gaseous phase. They can than enter the mass spectrometer for analysis.

ESI is 'soft ionization technique', this means that the molecules are not totally destroyed during the ionization process. This can be advantageous in the sense that the molecular- ion is always observed. Because of this the reproducibility of the droplets is very good and therefore extremely useful in qualitative measurements.

2.1.2 Triple quadruple

When multiple mass spectrometers are used in sequence the technique is called Tandem MS (MS/MS). MS/MS allows for control over the formation of fragment ions. In a typical tandem quadruple system there are three quadruples set up in a linear fashion, often called "triple-quad" (Figure 1.4)

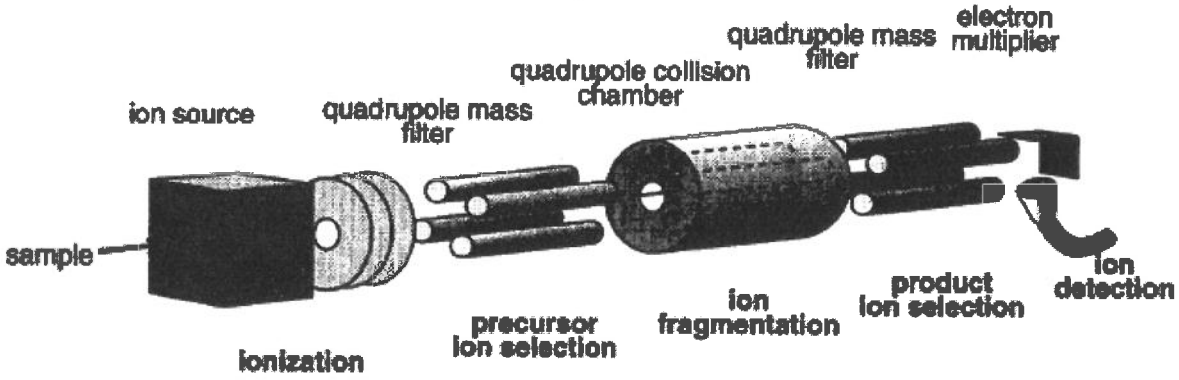

Fig. 2.1: Schematic view of a triple quad.

The first quadruple (Q1) is used to select the analyte ion of interest, also called a precursor ion. The second quadruple is known as a collision cell (Q2). A collision cell is often filled with inert gas, for example argon or nitrogen. When the target molecules collide with the inert gas, the target molecules explodes into multiple fragment ions. This process is known as collision-induced dissociation (CID). The third quadruple (Q3) is used to measure the mass of the ion produced upon CID, usually called fragment ions. The fragment ions analysed in the third mass spectrometer can be connected to the intact molecules in the first mass spectrometer. This process allows for unique scan modes such as product ion scans, precursor ion scans, neutral loss scans and selected reaction monitoring.

2.1.3 Selected reaction monitoring

SRM is one of the possible scans that can be performed with triple quad. Its often used for targeted analysis. As is shown in figure 1.5, the first quadruple is set to only allow on one or more particular masses to enter the collision cell. All other molecules that does not contain these particular masses are filtered out. When the selected molecules enter the collision cell specific fragment ions are formed upon CID. The second mass analyzer select these beforehand chosen m/z values of the fragment. Serving as another filter for the unwanted interference with the same mass that entered the first MS, leading to maximum sensitivity for the targeted molecules. When the fragment ions pass the second quadruple they reach the detector and are measured. Because SRM only measures a set of preselected analyte masses, it allows for very selective and sensitive measurements. It is also known as MRM and is often used with ESI-MS/MS for quantification analysis.

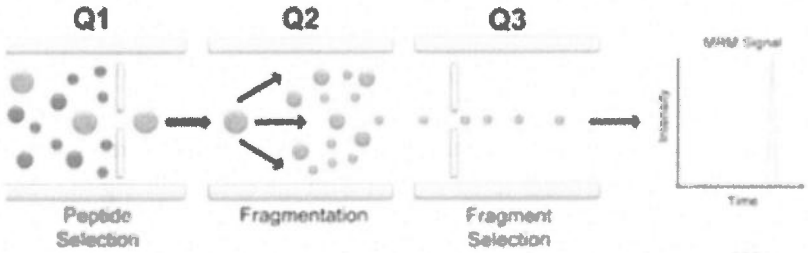

Fig. 2.2: Selected reaction monitoring in MS/MS

SRM is often used in combination with stable isotope dilution mass spectrometry. Stable isotope dilution mass spectrometry is a technique in mass spectrometry that makes use of a stable isotopically-labeled standard (SILS) as internal standard. A SILS is a compound that behaves exactly the same as the analyte. In the SILS some carbon atoms are replaced by ^{13}C atoms or hydrogen atoms are replaced by ^{2}H deuterium atoms. The ^{13}C or ^{2}H atoms containing one extra neutron, making the SILS differ in mass from the analyte. Therefore the SILS can be separated from the analyte by mass spectrometry while it is maintaining the exact same physical and chemical properties as the analyte. This character of the SILS making them perfect as internal standards. When a SILS is added to the same in known quantities, the loss of analyte during the sample preparation and analysis can be corrected with the concentration of the SILS.

2.2.1 Derivatization

Derivatization is a technique used in chemistry to change the structure of the analyte into a product with better properties for detection. A specific functional group of the analyte will react with the derivatization reagent to take up the properties of the reagent.

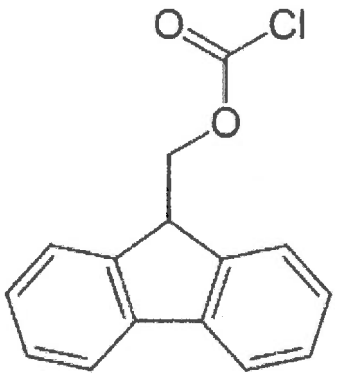

Fig 2.3 Structure of FMOC

Fluorenylmethyloxycarbonyl chloride (FMOC) is an derivate that is often used for the analysis of amino acids with HPLC. In figure 1.3 the structure of FMOC is shown. It is a molecule with a conjugated structure making it a fluorescent compound which can be used to improve sensitivity of amino acids with HPLC. FMOC will also give amino acids a more apolair structure which will results in a better separation in combination with reverse phase chromatography.

FMOC reacts with both primary and secondary amine groups. It reacts through a reaction that is called the Schotten-Baumann reaction. Which is a reaction that is used to synthesis an amide from an amine and chloroacid. The reaction also leads to the formation of acids which will form a salt with the unreacted amine, therefore a base is added in order to let it react with the acid. Making the reaction pH dependent, it generally does not run well below pH 9. Schotten and Baumann made use of a biphasic condition, where the formed amide will stay the organic phase but the acid that is formed will react with the basic aqueous phase. The mechanism of the reaction is shown in figure 2.4.

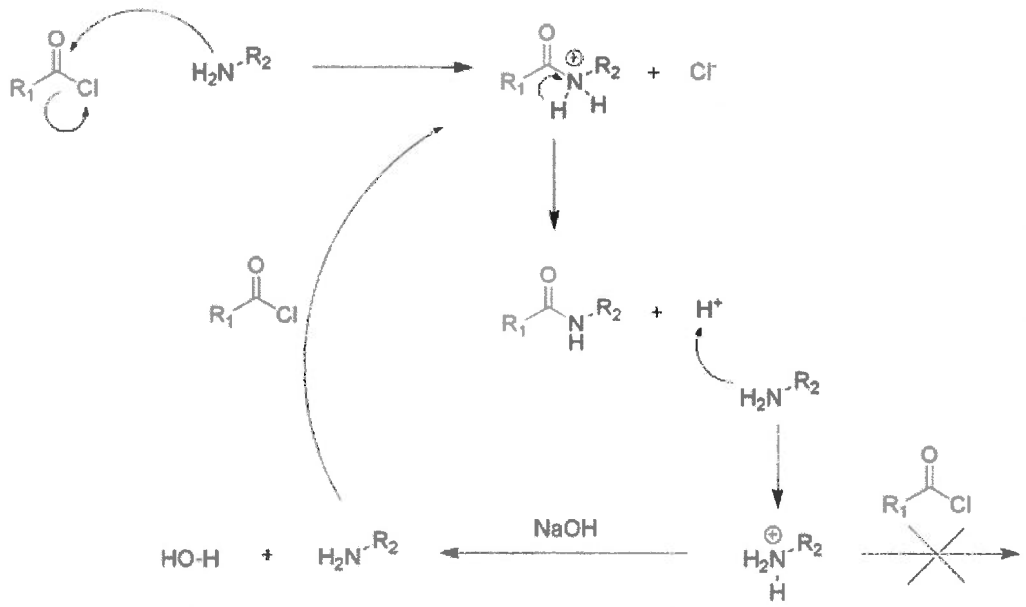

Fig 2.4 Reaction mechanism of the Schotten Bauman reaction.

Because of chloride is a strong electron withdrawing group the carbon becomes a more electrofile and can be attacked by the nucleofile NH_2, forming an amide. The second step is the proton that leaves the amide. The base that is added to the reaction will react with this proton. So the amine will be available for the reaction with the chloric acid.

Butyl derivate requires an esterfication that has to take place in a water free environment and therefore requires two drying steps that are time consuming. The advantages of FMOC over butyl derivative is that it allows for quick reaction in aqueous solution at room temperature and that it will aid the chromatographic properties of the analyte using reserve phase chromatography.

2.2.2 Derivatization of amino acids by MS/MS

FMOC is a derivatization reagent that is popular because of its fluorescent properties. But derivatization is also often used in mass spectrometry to improve sensitivity because a derivative can give a very specific fragmentation and aids with the ionization of the analyte. As reported, FMOC derivatives provides good sensitivity and specificity for the detection of amino acids by HPLC-MS/MS, here a loss of common loss of the elements of FMOC was observed (196 Da) [17]. This process is shown schematically for phenylalanine and tyrosine in figure 2.

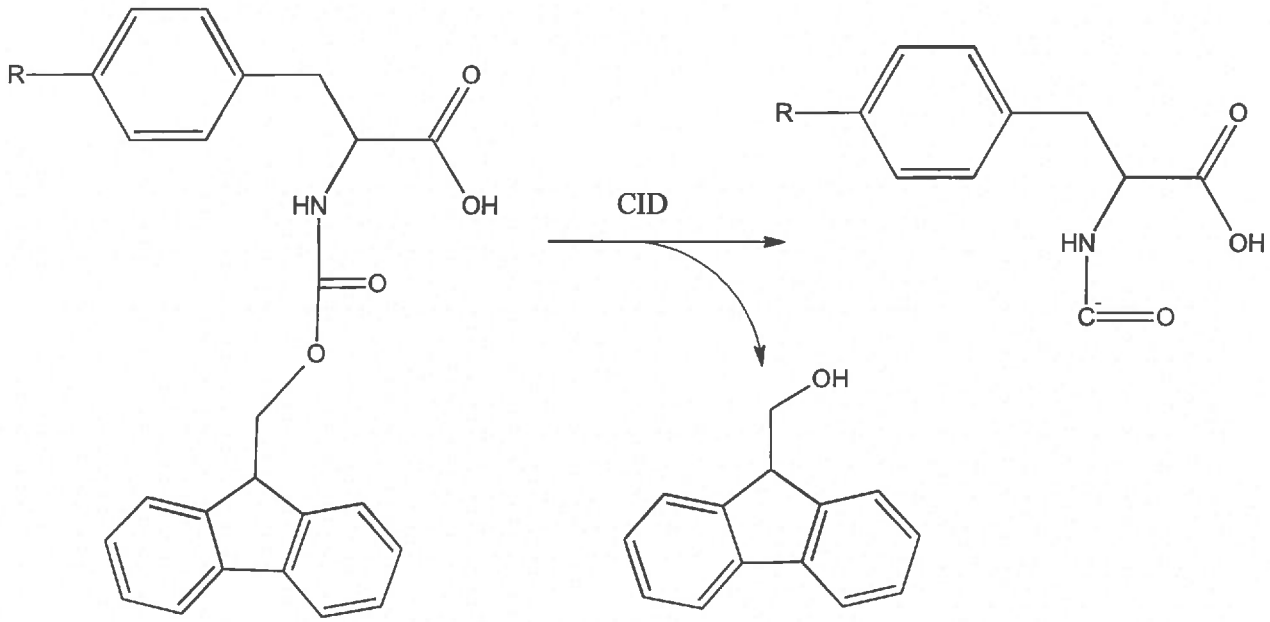

Fig 2.5 Schematic loss of m/z 196 upon CID of phenylalanine (R: H) and tyrosine (R:OH) when derivated with FMOC

The loss of *m/z* 196 using FMOC is specific to all α-amino acids and can thus also be used for the analysis of phenylalanine and tyrosine.

2.3 Development of HPLC method

The primary goal of developing a chromatographic separation is to make sure phenylalanine and tyrosine are free from interference. Reverse phase liquid chromatography will be used because after derivatization with FMOC the analytes become an apolair molecule. Separations with reverse phase liquid chromatography including gradient elution has a lot of parameters that effect its performance. Such as columns, mobile phase and flow rate. Each parameter also affects the other parameters making optimization an complex process. In this paper, three settings were optimized, the column, the mobile phase and lastly the flow rate. Each parameter will be optimized in order before moving on to the next parameter.

2.3.1 HPLC column

The first step in the HPLC method development were to find an appropriate column for the separation of interference from the analyte. The AASA method from which the derivatization is adopted utilizes an Xterra C18 column with 5 µM particle size for separation. However laboratory also has columns with smaller particles, columns with a smaller particle size offer better sensitivity, analytical speed and resolution. Multiple columns with different particle sizes will be tested.

2.3.2 Eluent optimization

The second step in the optimization process will be the optimization of the eluent. Since a gradient elution is used the elution time depend on both eluents. To test for interactions between both eluents an 2x2 factorial design will be designed. Then an 3x2 design can be proposed to optimize the mobile phases and the gradient separation from the significant factors.

2.3.3 Flow rate

The performance of chromatographic separation depends a lot on the flowrate. The van deemter curve is widely used to describe the performance of a chromatographic separation based on the flow rate. However the Van Deemter equation only applies to isocratic separations and does not apply to gradient separations. Because broading of peaks is caused by diffusion in the mobile phase and in a gradient the mobile phase changes and so does diffusion.

Peak capacity is often used to described he performance of a gradient run. It defines the number of peaks that can be separated during a gradient run with a certain resolution. A higher peak capacity means more peaks can be separated during the gradient run. A higher flow rate will improve the peak capacity, since peaks become sharper at higher flow rates. However if you keep increasing the flow rate there will be a moment where a faster flow rate will stop improving the peak capacity, this is point will be the optimal flow rate. The following equation was used to calculate peak capacity.

$$Pc = 1 + \left(\frac{2{,}35}{4} \cdot \frac{Tgradient}{Wh,avg}\right)$$

- $T_{gradient}$: the duration of the gradient
- $W_{h\ avg}$: the average peak with at half-high of all peaks in the chromatogram,.
- P_c: peak capacity

2.4 Method comparison with Bland-Altman

When a new method is developed it needs to be validated. Recovery experiments are often used to test a new methods accuracy. However in clinical chemistry the true values of samples are unknown and the matrix is too complicated for matrix matching. Because it is impossible to provide standards with known values it becomes difficult to determine if a new method gives better results than the regular method. So instead a new method is evaluated by comparing it with the regular method rather than with the true quantities. When two methods are compared to each other it often involves regression analysis and its corresponding scatter plot in combination with a difference plot with its calculated 2sd limits. However recently Bland-Altman plot (BAP) are widely used. BAP is a difference plot of the difference between the two quantitative measurements vs. the mean of the measurements.

A BAP will be explained using an example from hypothetical data [18]. Table 10 in appendix 1 lists the data of two methods that will be compared. The first columns lists data obtained with method A, the second columns shows the values of the same samples measured with method B. Because Bland Altman don't use a reference quantities but instead the mean of both methods is compared to the difference between the methods. The mean is calculated in column 3 with (A+B/2). The difference is calculated (A-B) in column 4. Data can be analyzed as quantity plots or as percentage plots. Often both are used to for better evaluation. The percentage difference is calculated in column 5.

Method comparison with BAP starts with a regular regression plot between the data. Regression plots are really useful in displaying the visual aspect of the relation between the methods. However a high correlation between two methods doesn't necessary mean that the two methods agree. Therefore a regression plot is only used to support BAP. Figure 1 shows the regression analysis between both methods, showing very good agreement.

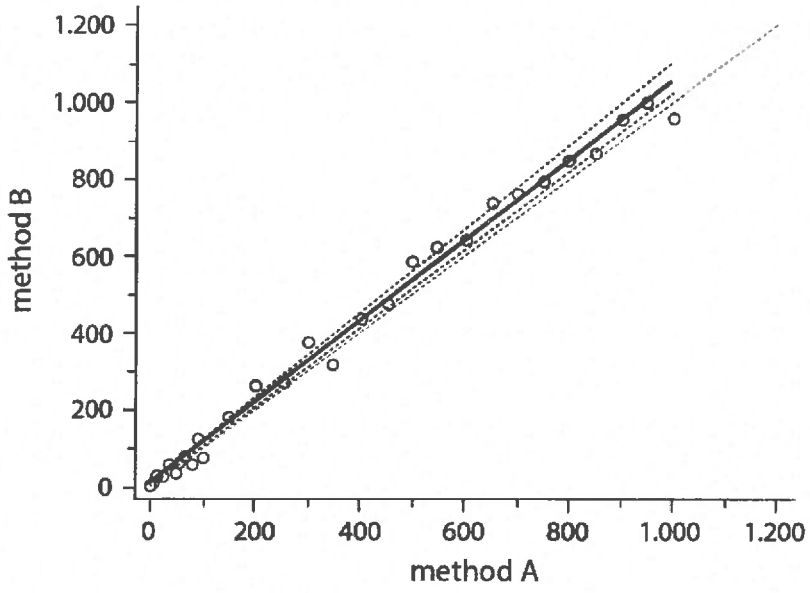

Fig 2.6. Regression analysis between method A and B, r = 0.996
the regression equation is y = 7.08+ 1,06x [18]

Also a BPA was created from the data (fig 2).

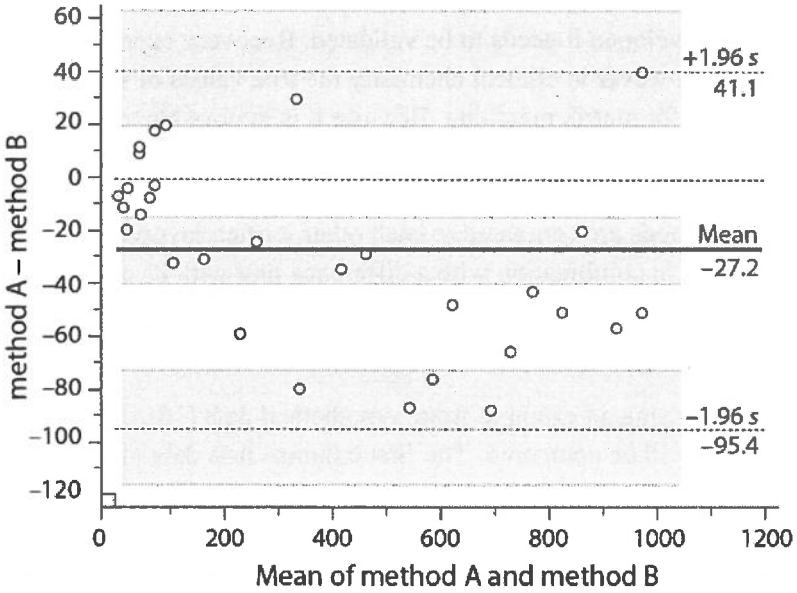

Fig 2.7. Bland-Altman plot of the hypothetical data. [18]

The x-axis shows the mean results of the two methods $\frac{A+B}{2}$
While the y-axis shows the absolute difference between the methods (B-A). The visual information a BAP provides, shows relationships between the differences and the mean. Trends such as, larger differences as the average increases or the variability across the graph, can be detected with BAP.

BAP also involves 2sd statistical limits. These limits are calculated using the mean and the standard deviation of the differences between both measurements, 95% of the data points should lie between these limits. The limits of agreement any systematic difference between the measurements. The mean difference is the estimated bias, and the SD of the differences measures the random fluctuations around this mean.

From figure 2 it was observed that the mean difference between both methods 27, that there is a bias indicates that both method do not give the same results. This bias can be caused in a certain concentration range or it can be constant average. In the BPA the data points in low concentrations are less scatters and an negative trend was observed as the concentration get larger. If the bias of 27 is significant can't be determined with a BAP and has to be calculated with a t-test.

With an BAP the bias between of the mean difference and its interval of 95% are estimated.
However BAP does not determine if these limits are acceptable. To determine if these limits are acceptable is a clinical question not a statistical one. Clinical goals define whether the agreement interval is too wide or if the new method its suitable for its purpose.

3. Materials and methods

3.1 Chemicals and reagents

Phenylalanine and tyrosine were purchased from Merck. D_4-Tyrosine and D_5-phenylalanine were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). Acetonitrile (HPLC) and Methanol were obtained from VWR chemicals. Ammonium formate was purchased from Sigma-Aldrich. FMOC chloride was obtained from Fluca and the reagent was prepared by solving 20 mg FMOC-CL in 10mL acetone. Boric acid was obtained from Merck and buffer was prepared by making a 62,5 mmol/L solution with pH 10.

3.2 Standards

A stock solution of phenylalanine and tyrosine was prepared in methanol (0,21 mM L-PHE and 0,025 mM L-Tyr). This stock was devived in to multiple eppendorf tubes, which were used to prepare the calibration standards in methanol (Table 1). 100 µL internal standard was added to all standards. The internal standard contained 29 µM Phe-D5 and 6 µM Tyr-D4.

Table 1. Calibration standards, total volume was 200 µL.

Volume Stock µL	nmol phe	nmol tyr
0	0	0
5	0,525	0,125
10	1,05	0,25
20	2,1	0,5
50	5,25	1,25
200	21	5

3.3 Sample preparation

Dried blood spots on filter paper were prepared by punching out a 5,5 mm perforator into a vial, corresponding to 8,1 µL blood. Then 100 µL internal standard was added together with 300 µL methanol. Samples were sonicated for 15 min. 25µL of the sample was transferred to a new vial and 50 µL borate buffer was added, samples were derivated with 50 µL FMOC reagent. After which the samples were ready for LC-MS/MS analysis.

3.4 Pilot study of initial chromatographic settings

The development of the HPLC-MS/MS method for the analysis of phenylalanine and tyrosine in DBS started with a pilot experiment. The chromatographic settings of the α-AASA method were used (table 2) Sample preparation and detection with the mass spectrometry are described in 3.3 and 3.5 respectively.

Table 2. Chromatographic settings used for the pilot experiment. Using Xterra RP C18 column, 3.9x150mm, 5 µm. Eluent A: 60:40, ACN:H_2O ; Eluent B: 10:90, ACN: H_2O.

Time (min)	Flow (mL/min)	Eluent A (%)	Eluent B (%)
Initial	0.9	80	20
3	0.9	0	100
3.40	0.9	80	20
4	0.9	80	20

3.5 Mass spectrometry analysis

Quantification of phenylalanine and tyrosine in DBS was performed on a 4000 QTRAP (AB Sciex Instruments) equipped with electrospray ionization (ESI) source operating in negative mode. The ion spray voltage was set to 4,5kV and source temperature to 250 °C. Multiple reaction mode was utilized for the analysis of phenylalanine and tyrosine. The following MRM transitions were monitored: Phenylalanine, *m/z* -386 → *m/z* -200; D5-phenylalanine, *m/z* -391 → *m/z* -205; Tyrosine, *m/z* -402 → *m/z* -206;D4-Tyrosine; *m/z* -406 → *m/z* -208. Dwell time was 0,2s. For collision gas nitrogen was used. Nebulizer gas (GS1) was set to 75 psi and the auxiliary gas (GS2) was set to 60 psi. Curtain gas (CUR) was set to 35 psi. The compound dependent operating parameters (declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) were copied from the α-AASA method. System operation and data acquisition were controlled using Analyst™ software 1.5.

3.6 Column selection

The following three columns were tested for the analysis of phenylalanine and tyrosine as FMOC derivative.

- Ascentis Express RP-amide column (2.1x100mm, 2,7µm): This is a column with alternate reversed phase selectivity to C18 that allows good peak shape for bases while it compatible with a 100% aqueous phase.
- XTerra RP C18 (2.1x100mm, 3,5µm): The Xterra column contains hybrid (inorganic/organic) particles. Because of this the column shares the advantages of both components. Therefore the hybrid particles can be operated at high speeds, high temperatures and at high pH. Which results in that XTerra columns give exceptionally sharp, high-efficiency peaks for basic compounds.
- Atlantis dC18 (2.1x100mm, 3µm): The Atlantis dC18 is a silica based column which is compatible with MS. It allows for an retention of polar and non-polar compounds in reversed phase chromatography. The Atlantis dC18 columns is compatible with a aqueous mobile phases.

Because the all three columns have a smaller particle size than the initial column the flow rate had to be adjusted. The three columns were used by analyzing a DBS with a flow of 0.2 min/mL while maintaining the same chromatographic settings as in table 2.

3.7 2x2 factorial design for eluent levels

For optimization of the eluent an 2x2 factorial design was created. Table 3 shows factorial design. For the organic eluent, the second level was 80%, so that when an 3x3 factorial design was being developed the 3th level could be 100% and there would be three equal spread levels. The second level for the water eluent was chosen to be 30%. The analysis time of the last eluting peak was recorded for each set of settings.

Table 3. Used 2x2 Factorial design for eluent optimization.		Eluent B Organic Eluent	
		60% ACN [-1]	80% ACN [+1]
Eluent A Aqueous eluent	10% ACN [-1]	(-1/-1)	(-1/+1)
	30% ACN [+1]	(+1/-1)	(+1/+1)

3.8 Flowrate optimization using peak capacity

In order to optimize the flow rate, the peak capacity at multiple flowrates between 0,20 and 0,45 mL/min was calculated by using the peak width for each peak in the chromatogram. Peak widths were measured from the chromatogram at peak half-height (wh). Then 4σ peak capacities was calculated according to the equation explained in 2.2.3. For each flowrate the gradient was adjusted such that the gradient volume remained constant. This means when the flow increased 2 times, the gradient was reduced the gradient run 2 times.

3.9 Final settings of the liquid chromatography method

HPLC was performed with a Waters ACQUITY UPLC™ system, equipped with a binary solvent delivery system and an auto-sampler which was maintained at 20 °C. Chromatography was performed on a Waters Atlantis dC18 column (100 mm × 2.1 mm, 3 µm) at room temperature. The mobile phase consisted of (A) 30% ACN in water containing 125 mg/L ammonium formate and (B) 80% ACN in water containing 125mg/L ammonium formate. Phenylalanine and tyrosine were separated under a linear gradient from 100% A to 100B% (0-2min). After elution the column was washed with 100%B for 0,4min and subsequently equilibrated to 100% mobile phase A for 0,6 min.The flow rate was 0,4 ml/min. The total analytical run time was 3min. The injection volume of standards and samples were 5 µL.

3.10 FIA-MS/MS as butyl derivate

Samples were prepared as described in 3.2 and 3.3 till the derivatization step, after sonnicication the sampels were placed in a new vial and dried with N_2 at room temperature. Then 100 µl butyl reagent was added and the vials were placed at 60 °C for 15 minutes. After the derivatization reaction the vials were dried at room temperature again and 1 mL eluent was added. The samples were then ready for analysis. The FIA-MS/MS was operated in positive mode and the following transitions were measured. Phenylalanine, *m/z* 222 → *m/z* 120; D5-phenylalanine, *m/z* 227 → *m/z* 125; Tyrosine, *m/z* 238 → *m/z* 136; D4-Tyrosine; *m/z* 242→ *m/z* 140. The rest of the MS settings were the same as described in 3.5. The samples were introduced to the MS/MS through a capilair using 75% ACN as eluent and a flow of 0.2 ml/min.

3.11 Method comparison

For method comparison 103 dried blood spots of PKU-patients, were reanalyzed with both methods, the regular FIA-MS/MS method and the new HPLC-MS/MS method. The blood spot cards were stored at room temperature. Appendix 2. shows a flowchart of both methods, in which you can see the extraction step remains the same and only the derivatization has to be validated. The mean values for tyrosine and phenylalanine were compared with a paired student's t-test. Linear relationships between both methods were determined from standard correlation coefficients. Lastly Bland-Altman plots were prepared from the mean of both methods vs the difference between the two methods.

4. Results and discussion

4.1 General chromatography

Figure 4.1 shows a chromatograph of a patient sample. The sample was analyzed using four different MRM transitions as described in 3.2. Blue shows the transition of phenylalanine, which was detected at RT 3.39min and its interference at 2.59 min. In red is the transition of the internal standard for phenylalanine. The green peak shows the signal for tyrosine at 2.9 min and in grey is the internal standard for tyrosine.

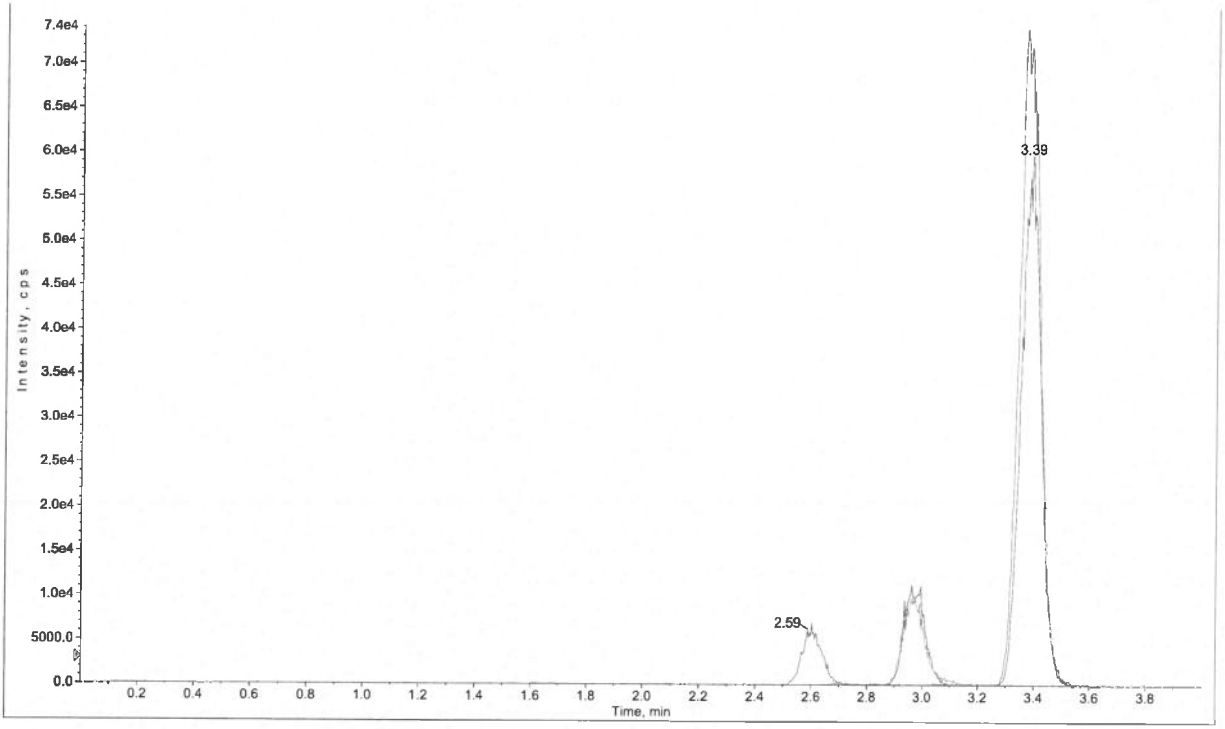

Fig. 4.1: Chromatogram of DBS for the analysis of phenylalanine and tyrosine with all four transitions. Performed on an Xterra RP C18 column, 3.9x150mm, 5 µm.

The separation between interference and phenylalanine is a good indication for the purpose of using HPLC-MS/MS instead of FIA-MS/MS.

4.2 Column selection

Three different columns were tested for the analysis of phenylalanine and tyrosine with HPLC-MS/MS. For each column the chromatogram will be displayed.

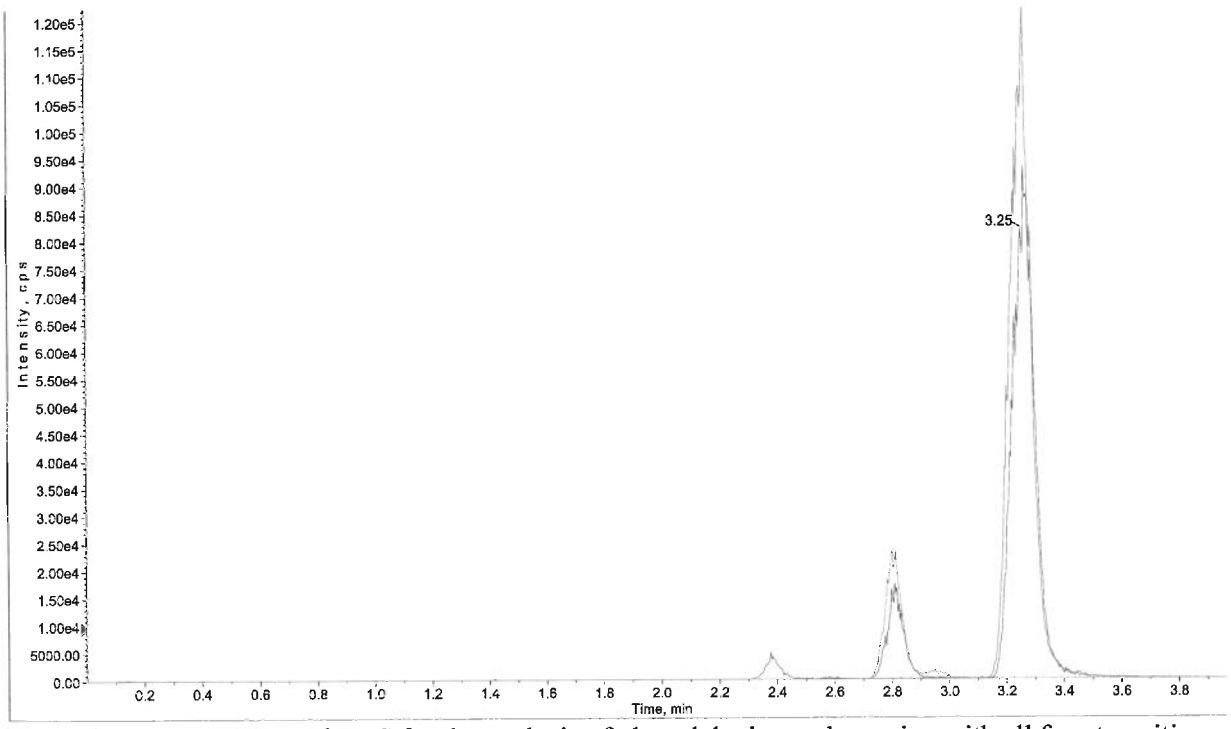

Fig. 4.2. Chromatogram of DBS for the analysis of phenylalanine and tyrosine with all four transitions. Performed on Ascentis Express RP-amide column (2.1x100mm, 2,7µm)

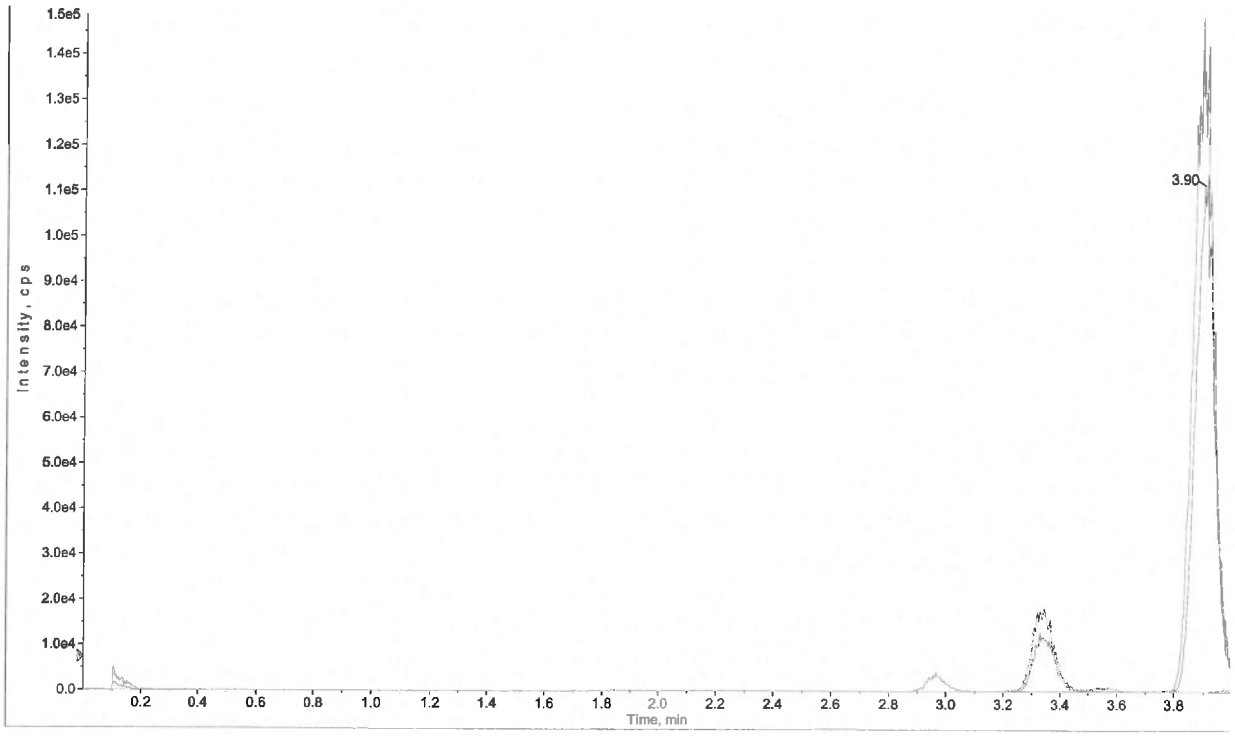

Fig. 4.3. Chromatogram of DBS for the analysis of phenylalanine and tyrosine with all four transitions. Performed on XTerra RP C18 (2.1x100mm, 3,5µm):

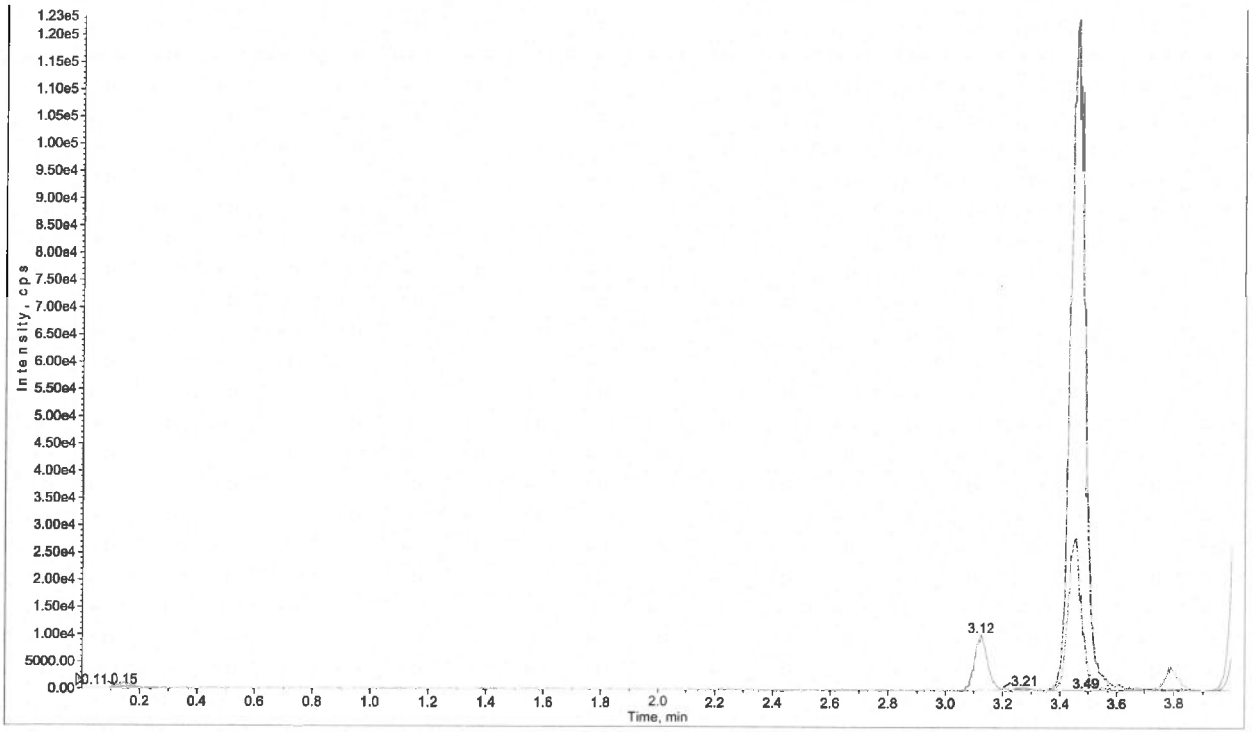

Fig. 4.4. Chromatogram of DBS for the analysis of phenylalanine and tyrosine with all four transitions. Performed on Atlantis dC18 (2.1x100mm, 3µm):

From figure 4.4 you can see that the Ascentis column eluted phenylalanine as FMOC derivative within 3.4 minutes. Which is the same as when an Xterra column of µm was used. The other two column showed a longer retention times. However, while the Atlantis column showed the longest retention time, using this column very sharp peaks were obtained and there was a baseline separation in the transition of interference in the path of the internal standard for tyrosine (grey). The other two columns weren't capable of that. Table 4. lists the peak width for tyrosine and the asymmetry obtained with Analyst™ software. From which you can see that the Atlantis column obtained the sharpest peaks and most symmetrical peaks. The interference peak showed a peak height of 5000 counts and the IS of tyrosine had a peak height of 25000 counts. So the effect of the interference is 20%. Therefore the Atlantis column was selected to perform the analysis for phenylalanine and tyrosine.

The Atlantis column has the second smallest particle size of the three columns. The RP amide columns had smaller particle size and also had fastest retention time. Both columns were produced for eluting bases while compatible in aqueous mobile phases. It's not clear why the peaks of the atlantis column are sharper while having longer retention times.

Table 4. Peak asymmetry of tyrosine using the three different columns
Eluent A: 60:40, ACN:H_2O ; Eluent B: 10:90, ACN: H_2O.

Column	Tyrosine width at 50%	IS width at 50%	Peak asymmetry
Ascentis	0,0571	0,0630	1,25
Xterra	0,0901	0,0837	1,42
Atlatis	0,0569	0,0519	1,19

4.3 Eluent optimization

For eluent optimization an 2x2 factorial design was made. Table 5 shows factorial design. The retention time of the last eluting peak was chosen as response.

Table 5. Used 2x2 Factorial design for eluent optimization.		Eluent B Organic Eluent	
		60% ACN [-1]	80% ACN [+1]
Eluent A Aqueous eluent	10% ACN [-1]	(-1/-1)	(-1/+1)
	30% ACN [+1]	(+1/-1)	(+1/+1)

The results of the optimization is displayed in figure 4.5

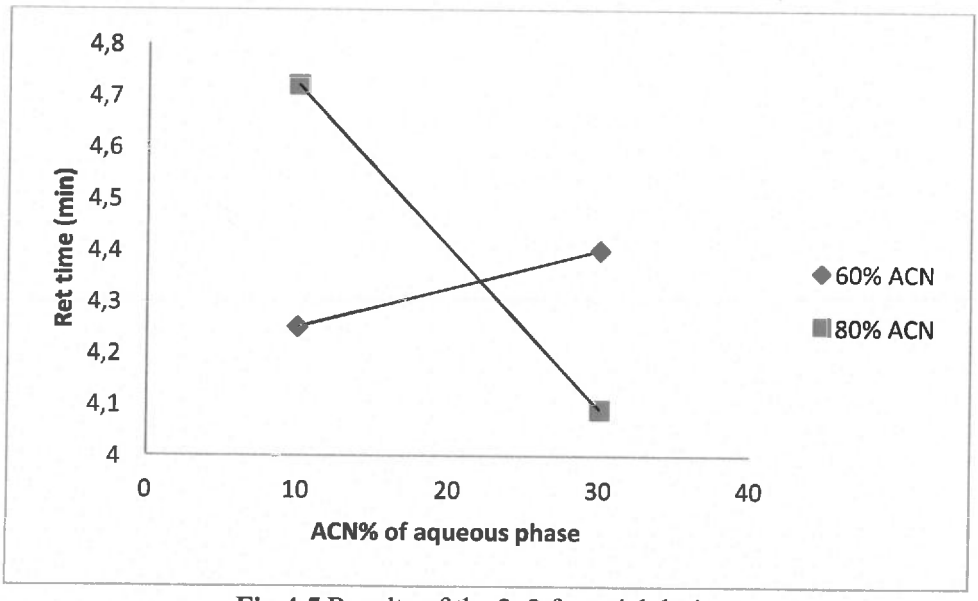

Fig 4.5 Results of the 2x2 factorial design.

From figure 4.5 we can observe that there is an interaction between the amount of acetonitrile in the organic eluent and the amount of acetonitrile in the aqueous eluent. When the aqueous eluent with 10% was used in combination with an organic eluent of 80% acetonitrile it performed worse when only 60% acetonitrile was used in the organic eluent. However each setting was only measured one time, so it is not certain if these new settings are significantly better than the regular settings. Because its expected that whenever more organic phase is added to the eluent the elution times will be faster. Since the design didn't meet the hypothesis based on theory it was decided not to create a 3x3 design. The optimal setting for eluent were the organic eluent containing 80% ACN was in combination with an aqueous eluent of 30% gave the shortest retention time.

4.4 Flow rate optimization

To optimize the flow rate the peak capacity for each flow rate between 0.2 and 0.45 was calculated. The results are listed and table 6 and the data is plotted in figure 4.6. Raw data can be found in appendix 3.

Table 6. Peak capacities of flow optimization.

Flow (mL/min)	Peak capacity
0,2	36,0
0,25	45,2
0,3	48,3
0,35	57,9
0,4	62,3
0,45	61,2

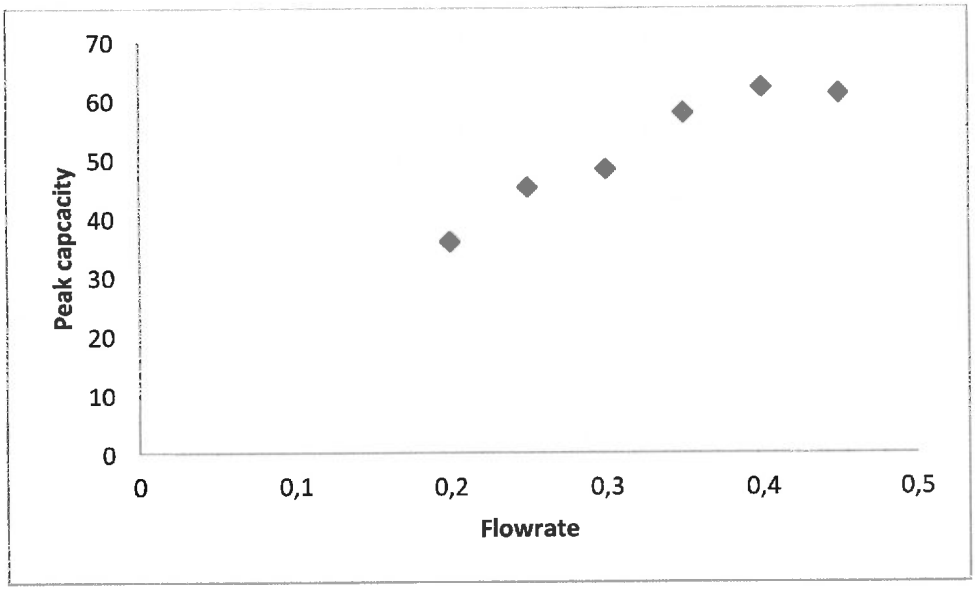

Fig. 4.6: Results of the optimization for the flow rate using peak capacity

Before flow optimization a flow of 0.2 mL/min was used. From figure 4.6 you can see this gave a peak capacity of 36, but a peak capacity of 62 was achieved for a short 3 minute gradient by increasing the flowrate up to 0.4 mL/min. At a flowrate of 0.45 mL/min you can see the peak capacity decreasing, also at this point the back pressure was too high to continue increasing the flowrate.

4.5 Final settings

With the selection of the best column and optimization of the eluent and flowrate the development of the new HPLC method was finished. A chromatogram using the optimized setting is shown in figure 4.7. The settings are shown in table 7.

Table 7. Chromatographic settings of the optimized method.
Eluent A: 80:20, ACN:H_2O ; Eluent B: 30:70, ACN: H_2O.

Time (min)	Flow (mL/min)	Eleunt A (%)	Eluent B (%)
Initial	0.4	100	0
2	0.4	0	100
2.40	0.4	100	0
3	0.4	100	0

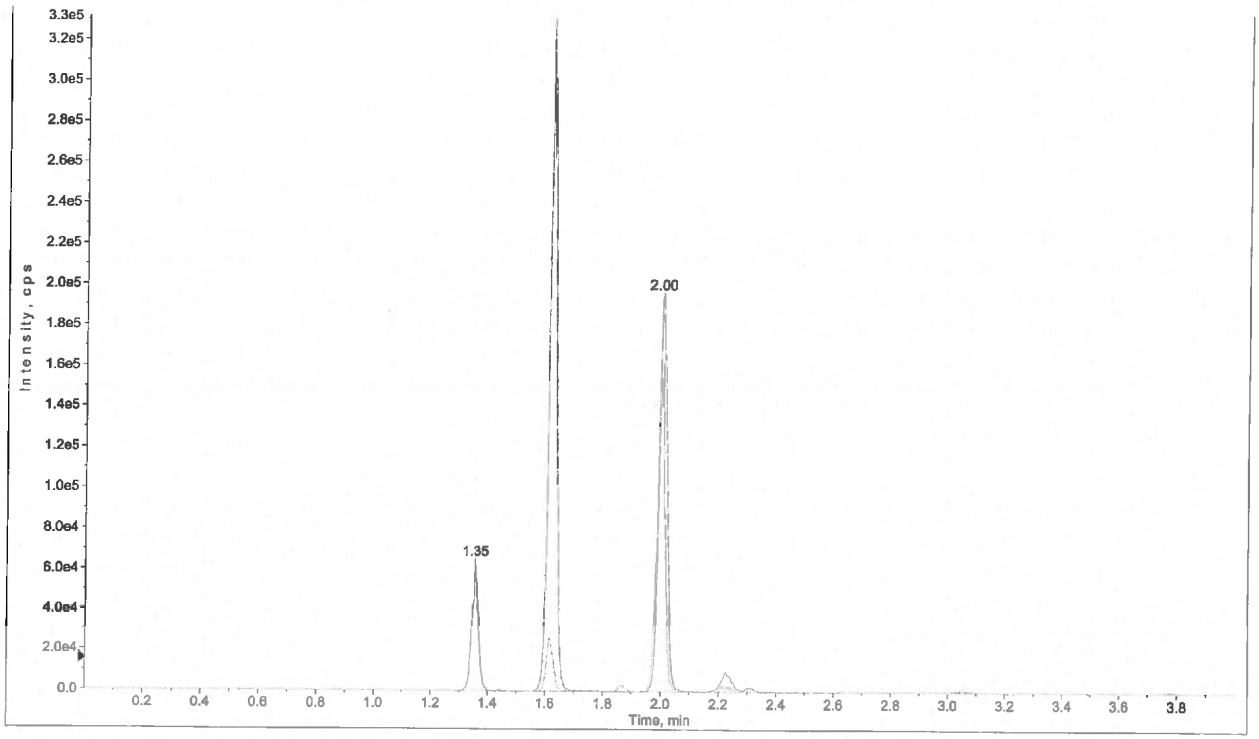

Fig. 4.7. Chromatogram of DBS for the analysis of phenylalanine and tyrosine with all four transitions. Performed on Atlantis dC18 (2.1x100mm, 3µm) using the settings listed in table 5.

Figure 4.7 shows a chromatogram of a DBS. Here we can see that tyrosine as FMOC derivative eluted at 1.6 min and was separation from interference that eluted at 1.9 min. Phenylalanine as FMOC derivative eluted at 2 min and was separated from interference that eluted at 1.35 min.

5. Validation

5.1 Method comparison with t-test

DBS from PKU-patients (n = 103) were reprocessed and reanalyzed with both, the new and old method. For convenience the old method will be referred to as FIA-MS/MS and the new method will be referred to as HPLC-MS/MS. Phenylalanine and tyrosine were quantified on five different days, where each day a new calibration curve was prepared, these values are found in Appendix 4. The difference between both methods was calculated as followed: Values HPLC-MS/MS– Values FIA-MS/MS. These calculated values were tested with a t-test. The results of the difference between the methods for phenylalanine are listed in table 8.

Table 8. Difference found in the values for phenylalanine between both methods

Subjects	Phe ratio (Mean + SD)	Phe conc (Mean + SD)
FIA-MS/MS	0.752 ± 0.409	252 ± 130
HPLC-MS/MS	0.693 ± 0.388	248 ± 133
Paired difference (A-B)	-0.059 ± 0.045	-4.49 ± 24.4
Difference, %	-8.19 ± 6.23	-1.79 ± 9.74
Tstat (Tcrit: 1.98)	-13.27	-1.86

From table 8 it was observed that the average ratio for phenylalanine with HPLC-MS/MS was 8.19% lower than the ratios obtained with FIA-MS/MS, which was significant different. The difference in ratio resulted in a average of 1,79% lower concentration of phenylalanine when analyzed with HPLC-MS/MS, however this was not significant different from the values found with FIA-MS/MS. That the ratios show a significant difference but the concentration does not can be explained by the different responses of the linear regression, this will be further explained on the next page. Table 9 shows the difference between both methods for tyrosine

Table 9. Difference found in the values for tyrosine between both methods

Subjects	Tyr ratio (Mean + SD)	Tyr conc (Mean + SD)
FIA-MS/MS	0.879 ± 0.431	67.7 ± 31.4
HPLC-MS/MS	0.747 ± 0.357	57.4 ± 26.7
Paired difference (A-B)	0.132 ± 0.118	10.3 ± 8.4
Difference, %	16.2 ± 14.5	16.5 ± 13.5
Tstat (Tcrit: 1,98)	11.30	12.40

The average ratio for tyrosine obtained with HPLC-MS/MS was 16.2 ± 14.5% higher (table 9). Resulting that the concentration for tyrosine measured with HPLC-MS/MS is 16,5 ± 13,5% higher. So, both the ratio and concentration with HPLC-MS/MS are significantly higher than the concentration with FIA-MS/MS.

5.2 Method comparison with Bland-Altman

The ratio and concentrations of phenylalanine were plotted in a linear graph (figure 5.1.) Regression analysis showed strong correlation for both metabolites across the entire range. Because of the high correlation it seems like the two methods are related. However because a high correlation doesn't mean that both methods give the same results, Bland-Altman plots were also made. The degree of agreement is indicated by calculating the bias, estimated by the mean and the SD of the differences.

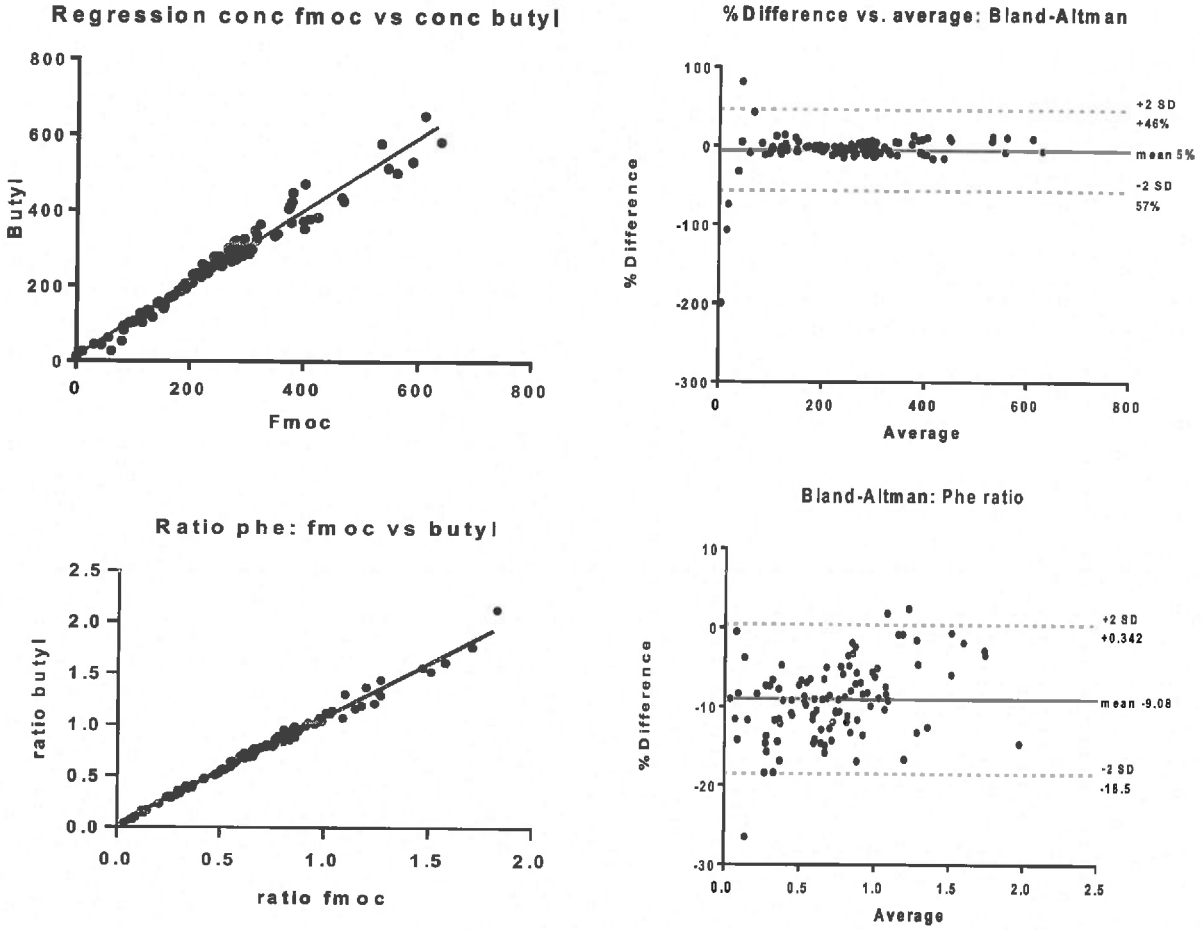

Fig.5.1: Scatter plot of phenylalanine values between both methods (left) and the corresponding Bland-Altman plot (right).

From the Bland-Altman plot of phenylalanine it was observed that the differences between both methods become larger at lower concentrations. The 2sd border is crossed multiple times. This is caused by the calibration of FIA, because sometimes the calibration curves off. An example is shown in figure 5.2. Therefore the calibration of the two methods give different results.

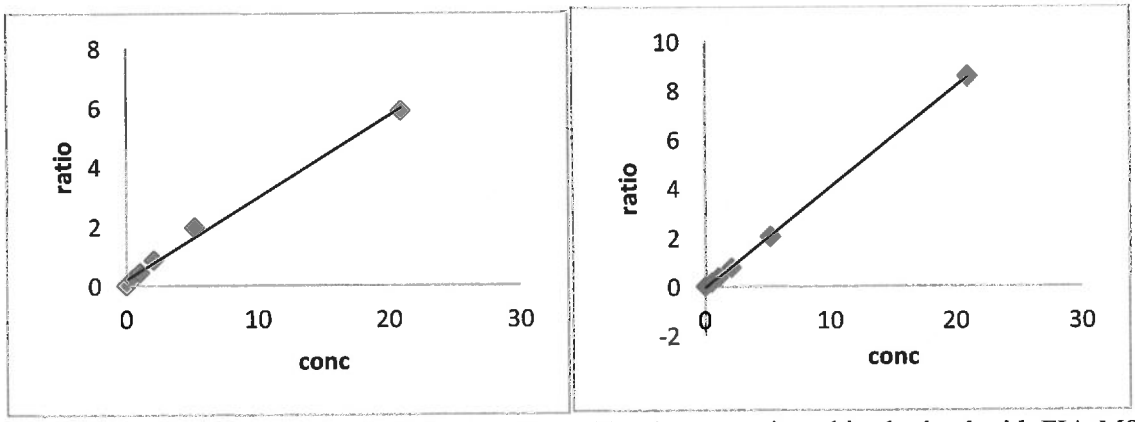

Fig.5.2: Example of the difference in linearity. The calibration curve in red is obtained with FIA-MS/MS as butyl derivative. The calibration curve in blue is obtained with HPLC-MS/MS as FMOC derivative. Both calibration curves were analyzed from the same standards.

From figure 5.2 it was observed that when the calibration of FIA-MS/MS starts to curve off the calibration of HPLC-MS/MS still stays linear. Thus the error in the calibration at a low concentration for the FIA-MS/MS becomes larger than the HPLC-MS/MS.

Bland-Altman analysis was conducted the same way on the results for tyrosine (figure 5.3)

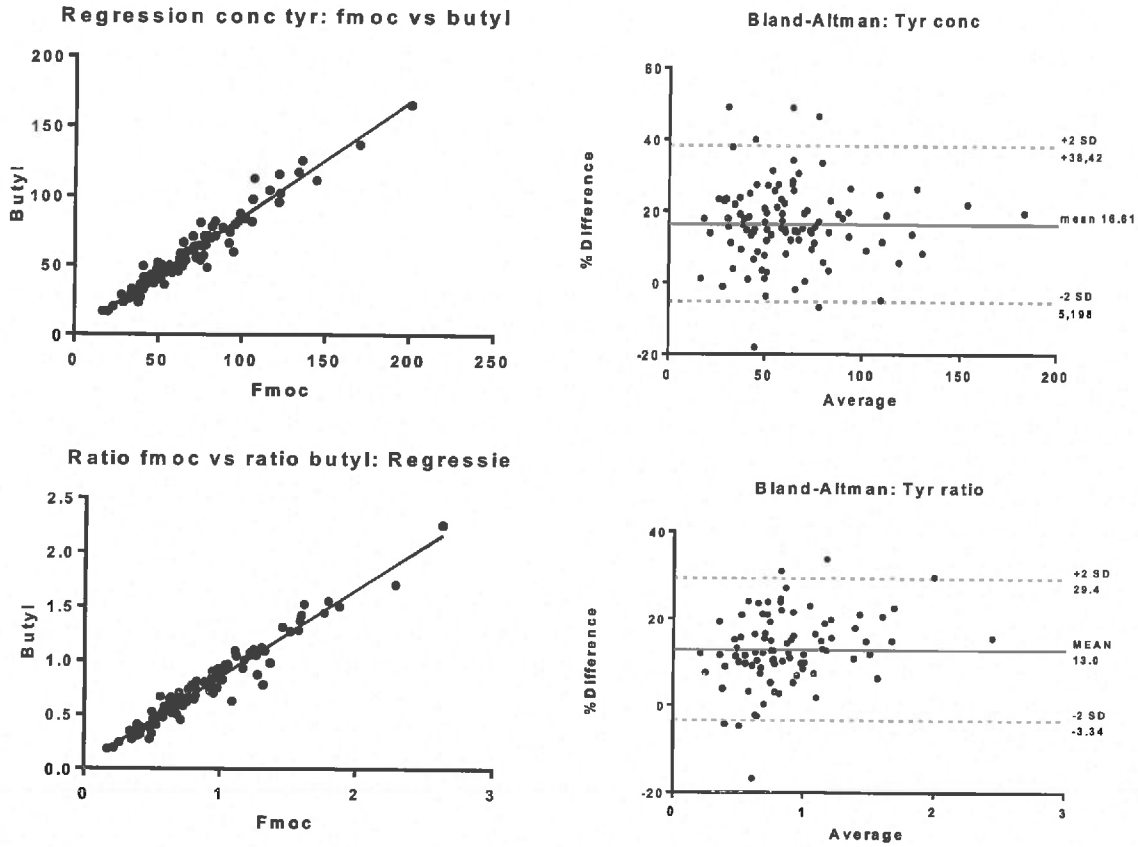

Fig.5.3. Scatter plot of tyrosine concentration between both methods (left) and the corresponding Bland-Altman plot (right).

Table 2 suggested there was a significant difference in the concentration of tyrosine between both methods. This suggests that there is a bias in one of the methods. Which is also shown in the Bland-Altman plot of tyrosine (figure 5.3). Where most of the data points lie above the 0 axis. Therefore also more points cross the 2SD limit. Though the effects we see can be explained because with the HPLC interference is separated from the internal standard. Therefore we expected the concentrations of tyrosine found with HPLC higher than those of the FIA-MS/MS.

The concentration of both metabolites with HPLC-MS/MS displayed strong correlation compared to FIA-MS/MS. However the Blant-Altman plots showed some lack in agreement. In the Bland-Altman plot for phenylalanine we could observe that the HPLC-MS/MS gave more reliable results when the concentration with low values for phenylalanine. Generally the values of HPLC-MS/MS for phenylalanine were in line with those obtained with FIA-MS/MS. For tyrosine however the values of the HPLC-MS/MS were significant higher that those obtained with FIA-MS/MS. But this difference can be explained by the chromatographic separation.

4. Conclusion

The purpose for this research project was to develop an HPLC-MS/MS method for routine analysis of phenylalanine and tyrosine in DBS as FMOC derivative. The first step was to develop and optimize the chromatographic settings of the HPLC method. The Atlantis column showed the best performance with a flow of 0.4 mL/min using a binary eluent of A) 80/20 ACN containing 125 mg/L ammonium formate and B) 30/70 ACN containing 125 mg/L ammonium formate. With these chromatographic settings an interfering peaks were separated in the transition for phenylalanine and of D4-Tyrosine as well.

For method comparison 103 samples were analyzed with both the new HPLC-MS/MS method and the previous used FIA-MS/MS method. Upon comparison the concentration of both metabolites showed strong correlation between both methods. However the BAP showed some lack in agreement. From the BAP of phenylalanine it was observed that the HPLC-MS/MS gave more reliable results in the low concentration area. Generally the values of HPLC-MS/MS for phenylalanine were in line with those obtained with FIA-MS/MS. For tyrosine however the values of the HPLC-MS/MS were significant higher that those obtained with FIA-MS/MS. But this difference can be explained by the chromatographic separation. Therefore it was decided to accept the new HPLC-MS/MS method for routine use of the analysis of phenylalanine and tyrosine in DBS.

An new HPLC-MS/MS method has been developed to analyze phenylalanine and tyrosine in DBS. This new method may be used for the quantification of phenylalanine and tyrosine to monitor clinical treatment of PKU. The new method offers better selectivity and also reduces the sample preparation by 60 min.

5. Recommendations

During the optimization of the eluent an 2x2 factorial design was made. However the factorial design was based on one measurement. Making it vulnerable to errors. The next time it is suggested that multiple measurements are done for one data point. Also its important that the column is well equibrilated before the gradient run starts.

In this research project we developed an HPLC-MS/MS to replace the FIA-MS/MS for the analysis of phenylalanine and tyrosine. There is still one more assay that is currently using FIA-MS/MS. This assay is the analysis of acylcarnitines. Acylcarnitines are also measured in the newborn screening because they detect possible defects in the beta-oxidations. By applying an HPLC column before detection of the acyl carnitines they can be analyzed more selective and an improvement in accuracy can be obtained.

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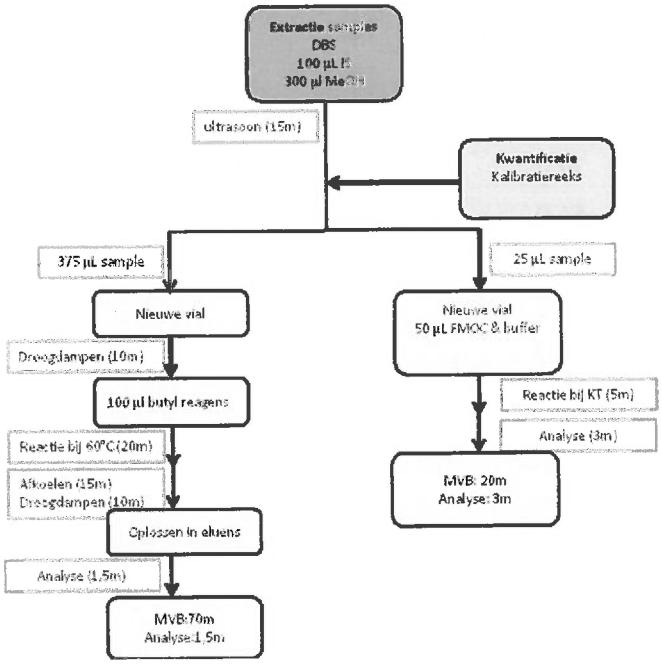
Appendix 1. hypothetical data for the Bland-Altman plot

Table 10. Hypothetical data of an agreement between two methods (Method A and B).

	Method A (units)	Method B (units)	Mean (A+B)/2 (units)	(A – B) (units)	(A – B)/ Mean (%)
	1.0	8.0	4.5	-7.0	-155.6%
	5.0	16.0	10.5	-11.0	-104.8%
	10.0	30.0	20.0	-20.0	-100.0%
	20.0	24.0	22.0	-4.0	-18.2%
	50.0	39.0	44.5	11.0	24.7%
	40.0	54.0	47.0	-14.0	-29.8%
	50.0	40.0	45.0	10.0	22.2%
	60.0	68.0	64.0	-8.0	-12.5%
	70.0	72.0	71.0	-2.0	-2.8%
	80.0	62.0	71.0	18.0	25.4%
	90.0	122.0	106.0	-32.0	-30.2%
	100.0	80.0	90.0	20.0	22.2%
	150.0	181.0	165.5	-31.0	-18.7%
	200.0	259.0	229.5	-59.0	-25.7%
	250.0	275.0	262.5	-25.0	-9.5%
	300.0	380.0	340.0	-80.0	-23.5%
	350.0	320.0	335.0	30.0	9.0%
	400.0	434.0	417.0	-34.0	-8.2%
	450.0	479.0	464.5	-29.0	-6.2%
	500.0	587.0	543.5	-87.0	-16.0%
	550.0	626.0	588.0	-76.0	-12.9%
	600.0	648.0	624.0	-48.0	-7.7%
	650.0	738.0	694.0	-88.0	-12.7%
	700.0	766.0	733.0	-66.0	-9.0%
	750.0	793.0	771.5	-43.0	-5.6%
	800.0	851.0	825.5	-51.0	-6.2%
	850.0	871.0	860.5	-21.0	-2.4%
	900.0	957.0	928.5	-57.0	-6.1%
	950.0	1001.0	975.5	-51.0	-5.2%
	1000.0	960.0	980.0	40.0	4.1%
mean ($\bar{d}$)				-27.17	-17.40%
standard deviation (s)				34.81	-12.64%

Appendix 2. Flowchart of the sample preparation for FIA-MS/MS as butyl derivate (left) and the new HPLC-MS/MS (right)



Appendix 3. Raw values of the flow optimization using peak capacity

Table 11. Raw values of flow optimization using peak capacity.

Flow mL/min	Peak 1 wh	Peak 2 wh	Peak 3 wh	Peak 4 wh	Peak 5 wh	Peak 6 wh	Avg wh	Tgradient Tg	Peak capacity
0,2	0,048	0,050	0,051	0,050	0,054	0,050	0,050	3,0	36,0
0,25	0,040	0,037	0,038	0,039	0,043	0,043	0,040	3,0	45,2
0,3	0,033	0,037	0,037	0,040	0,039	0,039	0,037	3,0	48,3
0,35	0,028	0,029	0,031	0,034	0,031	0,033	0,031	3,0	57,9
0,4	0,028	0,027	0,028	0,030	0,027	0,031	0,029	3,0	62,3
0,45	0,024	0,027	0,026	0,029	0,037	0,033	0,029	3,0	61,2

Appendix 4. Raw values validation

Table 12. Values used for Bland-Altman plots and t-test

Phenylalanine		Tyrosine	
Conc fmoc	Conc butyl	Conc fmoc	Conc butyl
381,3	447,2	84,0	81,2
324,1	363,5	70,8	70,5
278,3	317,6	51,6	47,8
252,5	277,4	79,0	68,6
294,3	319,6	65,1	66,3
317,5	336,7	53,7	47,7
188,3	197,3	107,1	112,4
534,8	577,8	45,7	42,8
282,9	301,8	75,2	80,5
205,5	230,2	49,6	51,5
314,8	346,7	49,4	47,7
224,5	244,6	82,1	77,5
402,4	470,6	41,0	49,1
376	412,2	78,6	67,9
267,5	299,4	95,8	79,0
213,7	233,4	62,0	45,2
222,6	257,3	84,1	70,9
267,8	295,3	92,9	73,8
243,1	276,9	66,5	53,2
296,8	324,8	88,5	76,8
248,4	271,7	62,8	53,8
282	320,4	62,9	54,0
379,5	423,3	67,0	59,4
225	255,8	61,8	47,7
244,8	255,3	99,0	87,0
200,1	205	39,4	35,9
251,4	259,6	136,1	125,3
216	231,1	170,4	136,8
200,7	204,6	106,0	81,3
61,9	26,1	134,0	116,9
134,8	117,2	55,6	45,7
154,7	139,4	45,7	40,0
80,8	52,4	116,5	103,9
116,6	103,1	81,5	69,6
156	148	122,1	115,4
146	153,4	64,3	53,0
194,1	193,2	54,0	45,6
171,8	173,1	75,2	53,2
562,9	499,8	73,1	54,9
639,8	582	54,7	47,1
547,5	513	76,8	63,2

Table 12 (continued). Values used for Bland-Altman plots and t-test

Phenylalanine		Tyrosine	
Conc fmoc	Conc butyl	Conc fmoc	Conc butyl
236,8	251,1	32,9	26,0
113,3	125,7	20,1	16,8
165,3	168,2	64,9	56,3
180,3	186,6	23,1	20,1
146	156,3	42,8	35,8
31,4	43,5	69,9	60,4
401,4	351,7	56,6	49,5
251,4	255,7	54,8	44,5
466,8	434	65,0	51,4
424,5	381,4	63,8	53,7
292,9	278,7	74,6	64,0
378,3	368,2	144,6	111,0
92,9	101,7	58,4	44,4
470,6	424,1	43,5	37,5
220,6	223,1	47,3	36,8
143,7	153,7	78,6	64,2
589,9	529,4	63,2	58,3
349,5	332	41,8	35,5
354,5	338,6	48,2	44,2
0	11,9	200,9	165,2
275,2	265,6	51,7	50,2
305,2	296,5	64,5	48,9
31,4	43,5	72,6	55,0
11,3	24,7	77,2	56,7
83,3	92,8	72,9	56,2
7,2	23,7	122,9	101,7
304,5	284,6	122,1	95,2
276	274,1	64,7	55,6
233,3	234,6	101,9	83,6
309,9	296,5	64,1	55,2
184,1	184,9	47,3	40,9
256,6	251,8	61,9	50,1
289,8	278,3	45,6	37,9
259,1	263,6	50,2	49,6
273	264,8	71,3	61,8
117,8	120,1	77,4	70,5
44,2	42,1	71,3	63,2
239,5	247,9	33,4	27,9
192,7	205,8	41,5	41,1
297,1	302,1	97,8	81,6
283,1	270,5	47,3	41,0
122,2	128	34,0	30,4
410,5	377,8	106,6	97,4
128,6	132,2	34,1	32,8
95	101,5	79,5	71,1

Table 12 (continued). Values used for Bland-Altman plots and t-test

Phenylalanine		Tyrosine	
Conc fmoc	Conc butyl	Conc fmoc	Conc butyl
83,8	81,1	51,8	39,3
400	371,8	79,3	48,1
101	104,8	40,7	33,6
113,6	121,3	28,1	28,4
317	320,7	29,1	23,0
287,5	295,4	38,0	30,5
126,6	134	92,3	65,8
374,1	404,9	41,3	32,5
279,1	301,5	33,5	28,6
101	103	32,1	25,5
218,4	230,5	39,2	26,7
198,3	207,3	33,4	26,4
56,5	61,5	16,9	16,7
129,5	126,5	95,0	59,2
205,4	207,6	48,0	41,3
610,9	651,5	38,1	23,1
271,3	282,8	53,7	35,8