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Par

RABTI Hadjira

THÈME

Development, Validation, and Application of Bioanalytical Methods for Phenylketonuria and Tyrosinemia Diagnosis and Monitoring

Soutenue le 29/06/2025 devant le Jury:

EL-KOLLI MERBAH Meriem	**Professeur**	**Univ. Ferhat Abbas Sétif 1**	**Président**
AMRANE Mounira	**Professeur**	**Univ. Ferhat Abbas Sétif 1**	**Directeur de thèse**
BOUNEKHEL Mahmoud	**Professeur**	**Univ. Ferhat Abbas Sétif 1**	**Examinateur**
BENAISSA AKILA	**Professeur**	**Univ. Saleh Boubnider Constantine 3**	**Examinateur**
SOBHI Wided	**Professeur**	**Centre de recherche en biotechnologie (CRBT)**	**Examinateur**
BENSAADA Mostefa	**M.C.A**	**Univ. Mentouri Constantine 1**	**Examinateur**
LALAOUNA Abd Eldjalil	**Professeur**	**Univ. Saleh Boubnider Constantine 3**	**Invité**

الجمهورية الجزائرية الديمقراطية الشعبية

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Presented to the Department of Process Engineering

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Option: Pharmaceutical Engineering

By

RABTI Hadjira

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Supported on 29/06/2025 before the Jury:

El-KOLLI MERBAH Meriem	**Professor**	**Univ. Ferhat Abbas Setif 1**	**President**
AMRANE Mounira	**Professor**	**Univ. Ferhat Abbas Setif 1**	**Supervisor**
BOUNEKHEL Mahmoud	**Professor**	**Univ. Ferhat Abbas Setif 1**	**Examinator**
BENAISSA AKILA	**Professor**	**Univ.Saleh Boubnider Constantine 3**	**Examinator**
SOBHI Wided	**Professor**	**Research center on biotechnology (CRBT)**	**Examinator**
BENSAADA Mostefa	**M.C.A**	**Univ. Mentouri Constantine 1**	**Examinator**
LALAOUNA Abd Eldjalil	**Professor**	**Univ.Saleh Boubnider Constantine 3**	**Invited**

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Dedication

I dedicate this work to the memory of my father Rachid, I miss you father more than words can say. I look forward to this day, I graduate...Thank you for believing in my dream.

To my mother Messaouda, A strong and gentle soul who taught me to trust in Allah and believe in hard work.

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List of abbreviations

4HPD: Hydroxyphenylpyruvate dioxygenase

AA: Amino acid

AAH: Hereditary aminoacidopathies

ACN: Acetonitrile

ANOVA: Analysis of variance

APCI: Atmospheric-pressure chemical ionization

BH4: Tetrahydrobiopterin

BSTFA: N,O-bis-(trimethylsilyl)- trifluoroacetamide

CAA: Chromatography of AAs

CAD: Urinary organic acid chromatography

CCD: Central composite design

CE : Collision energy

CNS: Central nervous system

CPS: Carbamoyl phosphate synthetase I deficiency

CS: Calibration standard

CSF: Cerebrospinal fluid

DBS: Dry blood spot

DOE: Design of experiment

EDTA: Ethylenediaminetetraacetic acid

ESI: Electrospray ionization

FAA: Fumarylacetoacetate

FIA-MS/MS: Flow infusion tandem mass spectrometry

FT-ICR: Fourier transform ion-cyclotron resonance

GC: Gaz chromatography

GMP: Glycomacropeptides

HHH: Hyperornithinemia–Hyperammonemia–Homocitrullinuria syndrome

HILIC: hydrophilic interaction liquid chromatography

HPLC: High performance liquid chromatography

ICH: International conference of harmonization

IEC: Ion exchange chromatography

IEM: Inborn errors of metabolism

ILS: Isotopic labeled standard

IP-LC-MSMS: Ion pair reversed phase liquid chromatography – tandem mass spectrometry

IP-reagent: Ion pairing reagent

LC: Liquid chromatography

LC-MSMS: Liquid chromatography – tandem mass spectrometry

LLE: Liquid–liquid extraction

LLOQ: Lower limit of quantification

LNAA: Large neutral amino acids

LOD: Limit of detection

LOQ: Limit of quantification

MALDI: Matrix-assisted laser desorption/ionization

Meth: Methanol

MLR: Multiple linear regression

MRM: Multiple reaction monitoring

LIST OF ABBREVIATIONS

MS/MS: Tandem mass spectrometry

MS: Mass spectrometry

MSTFA: N-methyl-trimethylsilyltrifluoroacetamide

MSUD: Maple syrup urine disease

MTHFR: Homocystinuria/methylene tetrahydrofolate reductase deficiency

N: Number of theorical plates

NAGS: N-acetylglutamate synthase () deficiency

NBS: Newborn screening

NTBC: Nitisinone

OPA: o-phtalaldehyde

OTC: Deficiency ornithine transcarbamylase

PAH: Phenylalanine hydroxylase

PAL: Phenylalanine ammonia lyase

PDH: Pyruvate dehydrogenase complex

PFPP: Penta fluoro phenyl propyl

PKU: Phenylketonuria

R^2: Determination coefficient

RP-HPLC: Reversed-phase high performance liquid chromatography

rpm: Rotation per minute

RP-UPLC: Reversed-phase ultra-high performance liquid chromatography

Rs: Peaks resolution

RSD: Relative standard deviation

RSM: Response surface methodology

SAA: Sulfosalicylic acid

SFSTP: French Society of Pharmaceutical Sciences and Techniques

SLE: supported liquid extraction

SPE: solid phase extraction

SUA: Succinyl acetone

T: Tailing factor

TAT: Tyrosine aminotransferase

TCA: Trichloroacetic acid

TDFHA: Tridecafluoroheptanoic acid

TOF: Time-of-flight

TRS : Tyrosinemia

TT1: Tyrosinemia type I

TT2: Tyrosinemia type II

TT3: Tyrosinemia type III

ULOQ: Upper limit of quantification

UPLC: Ultra-high performance liquid chromatography

UV: Ultraviolet

VS: Validation standard

β-ETI: β-Expectation tolerance interval

λ max: Maximum absorbance wavelength

Abstract

The aim of this study is to develop and validate fast, accurate, and precise, high performance liquid chromatography with UV detection (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MSMS) methods for phenylketonuria (PKU) and tyrosinemia (TRS) diagnosis and monitoring.

Three methods were developed, HPLC-UV on blood serum, LC-MSMS on blood serum and LC-MSMS on dry blood spots (DBS). Chromatographic conditions in the HPLC-UV technique were optimized using central composite experimental design (CCD). Validation of different methods was accomplished using β-expectation tolerance intervals (β-ETI) for total error measurement that did not exceed 15%. The method applicability was determined using human serum from 442 volunteers and statistical study was conducted to investigate the effect of different factors in phenylalanine (Phe) and tyrosine (Tyr) levels in addition to comparative study with reference method.

Optimal settings for HPLC-UV method were determined. The accuracy profiles were established. Mean analytical bias in spiking levels was acceptable, with relative standards deviation (RSD) below 5% in almost instances. The limit of detection (LOD) and limit of quantification (LOQ) were satisfactory in all methods. The epidemiologic statistical study reveals the most influencing factors on Phe and Tyr concentrations in the blood serum. The suggested approach successfully analyzed Phe and Tyr in human blood samples either using HPLC-UV or LC-MSMS and presented no significant difference with the reference method. The developed methods offer valuable diagnosis and monitoring tool for PKU and TRS.

Keywords: Phenylketonuria, Tyrosinemia, Human blood samples, DBS, HPLC-UV, LC-MSMS, CCD, Epidemiologic study.

GENERAL INTRODUCTION

General introduction

Aminoacidopathies (AAH), also known as inborn errors of AAs metabolism, are metabolic disorders caused by deficiencies in functional enzymes and defective metabolic pathways (Mihali, 2018; Wasim *et al.*, 2018).

Phenylketonuria (PKU) is one of the most frequent AAH characterized by defect in either the phenylalanine hydroxylase (PAH) gene or tetrahydrobiopterin (BH4) cofactor biosynthesis and regeneration, resulting in elevated concentrations of phenylalanine (Phe) in plasma and tissues (Haghighi *et al.*, 2015; Pickens and Petritis, 2020). Moreover, increased plasma levels of Phe can also be detected as a secondary effect of maternal hyperphenylalaninemia (Gu *et al.*, 2008; Pecce *et al.*, 2013). Consequently, simultaneous measurement of Phe and Tyr concentrations in blood, temden with the calculation of the Phe/Tyr ratio, may reduce false positive results in PKU screening (Mo *et al.*, 2013).

Alternatively, tyrosinemia (TRS) is another AAH characterized by defect in breaking down the Tyr and mains to Tyr accumulation in biological fluids and tissues (Fernández-Lainez *et al.*, 2014). If the condition is untreated, Tyr and its metabolites build up in tissues and organs, and lead to serious health problems (*Tyrosinemia*, 2023). Numerous situations can cause hypertyrosinemia like inborn errors of the Tyr degradation pathway, liver failure, blood sampling after eating instead of in the fasting state and rarely vitamin C deficiency and hyperthyroidism (Russo, Mitchell and Tanguay, 2001). Therefore, the distinction of TRS to other causes of hypertyrosinemia is very important.

Various methods have been described for the quantification of Phe and Tyr levels in serum or plasma (Hong and Tang, 2004; Pecce *et al.*, 2013), including enzymatic method (De Silva, Oldham and May, 2010), HPLC method (Neurauter *et al.*, 2013), tandem mass spectrometry (MS/MS) (Dhillon *et al.*, 2011), gas chromatography-mass spectrometry (GC-MS) (Deng *et al.*, 2002) and liquid chromatography-mass spectrometry (LC-MS) (Hardy *et al.*, 2002) . Among these methods, HPLC with fluorescence detection and LC-MSMS methods are usually favored for the analysis of these metabolites.

Nevertheless, these methods have certain disadvantages, such as the use of complex derivatizing agents and sophisticated sample preparation (Hardy *et al.*, 2002; Dhillon *et al.*, 2011; Neurauter *et al.*, 2013; Peat and Garg, 2016) .

The utilization of LC-MSMS has significantly increased in recent years (Liu *et al.*, 2019; Gouda and Nazim, 2020; Pickens and Petritis, 2020). The analysis of different samples for a wide range of inborn errors of metabolism (IEM) was a main primary application of LC-MSMS method. The reasons for choosing LC-MSMS over liquid chromatography (LC) with conventional detectors are basically high specificity and the aptitude to handle complex mixtures (Pitt, 2009).

The MS/MS method remains the preferred choice for screening or diagnostic issue. Despite of their advantages, MS/MS technology is not appropriate for the routine monitoring of Phe and Tyr levels due to its high cost (Mo *et al.*, 2013). Consequently, for the most straightforward and more efficient monitoring of patients with metabolic diseases (Smon *et al.*, 2019), we must use a more economical analytical system such as the HPLC system. The HPLC-UV offers many advantages compared to other detection systems, such as low operation cost, versatility, and simple operation (Flor *et al.*, 2010).

Given the density of population, the rate of IEM occurrence may be expected to be high and preventable complications can be attended to early and before the affected child is debilitated and so timely treatment and intervention can take place. Early detection of those disorders can limit the clinical variations and hence can be used as diagnostic tool in this field (Pourfarzam and Zadhoush, 2013).

Diagnosis of IEM constitutes a real challenge in a developing country with high consanguinity rate and no systematic newborn screening (NBS) (Karam *et al.*, 2013). As a developed country, Algeria is characterized by limited specialized laboratories and genetic centers with lack of NBS infrastructure and limited financial resources. For these reasons, almost patients are still delayed in diagnosis with variable clinical presentations (Pourfarzam and Zadhoush, 2013) and frequently with high cost if samples were analyzed abroad.

Consequently, the objectives of this work are defined as:

- Development and validation of three methods for the quantification of underivatized Phe and Tyr in different types of biological matrices: HPLC-UV in serum, LC-MSMS in serum and LC-MS/MS in DBS.

Methods validation is conducted according to the guidelines provided by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) and adopts the approach suggested by the French Society of Pharmaceutical Sciences and Techniques (SFSTP) guideline.

- Comparison of analytical methods by comparing the performance of the developed methods (HPLC-UV and LC-MSMS for blood serum) to the reference LC-MSMS method to determine its effectiveness in the analysis of AAs in newborns.
- Clinical application of the developed methods in order to test samples of patients in whom the diagnosis of metabolic disorder is suspected or confirmed and this is aimed to validate the clinical applicability of these methods.
- Analysis of the influence of various factors on Phe and Tyr concentrations in samples. Three categories of factors are studied: demographic and genetic factors (age, sex, family history, consanguinity, prematurity), clinical and historical factors (mental retardation, epilepsy (West syndrome), cholestasis, autism, hepatorenal syndrome, developmental delay, acoustic disorders, hydrocephalus, meningitis) and medications factors like associated therapy.

As a result, the indirect aims of the present study are establishment of early and real diagnostic and treatment of those disorders, monitoring known patients, minimizing the number of false-positive results and therefore reducing families stress and anxiety.

This work contains two parts, the first one presents a bibliographical study divided into four chapters. The first chapter is a bibliographic synthesis on AAH, the second is about PKU and TRS. Then after, the third chapter summarizes the most common methods used for quantitative analysis of AAs. The chapter four is about validation of analytical methods. The second part of this manuscript is devoted to practical study which contain two chapters; the material used and the methods followed for the experimental realization are grouped in one chapter and the results obtained accompanied by interpretations are in the other one. We conclude our study by general conclusion and the perspectives.

CHAPTER I.

AMINOACIDOPATHIES

Chapter I. Aminoacidopathies

I.1. Clinical presentations of inborn errors of metabolism

IEMs are rare genetic diseases, generally transmitted by an autosomal recessive manner (Cousin, 2014). These diseases are caused by a defective enzyme, cofactor or transporter in a metabolism pathway, leading to metabolic dysfunction and/or accumulation of toxic intermediate metabolites (Mak *et al.*, 2013; Cousin, 2014). This group of disorders includes amino acid (AA) disorders, urea cycle defects, organic acidemias, fatty acid oxidation defects, mitochondrial disorders, carbohydrate metabolism disorders, purine metabolism disorders and pyrimidines, neurotransmitter and mineral transport disorders, mucopolysaccharidoses, mucolipidoses, lipid metabolism disorders, lipid storage disorders, lysosomal disorders and other defects (Mak *et al.*, 2013).

IEM can present at any age, from the fetal stage to advanced age. It can affect any cell type or organ, or can occur in combinations. Clinical presentations are variable and often nonspecific. IEMs are individually rare, but they are common as a group. Many of these are amenable to dietary intervention and respond to supplementation of deficient metabolites, prevention of metabolic stress, and elimination of toxic metabolites. However, untreated illnesses can be very damaging, leading to serious disability and death (Ricquier, 2005a; Mak *et al.*, 2013) .

Although clinical diagnosis alone is never sufficient, a high index of suspicion is essential as the first step toward a correct diagnosis. It is almost impossible to have a universal and simple clinical protocol for all IEMs. Nevertheless, there are a number of general recommendations for recognizing suspected IEM cases (Mak *et al.*, 2013; Saudubray and Garcia-Cazorla, 2018). Saudubray et al (Saudubray and Garcia-Cazorla, 2018) recommend 12 fundamental principles. They suggest that IEM should be considered in patients of all ages alongside conditions like sepsis, encephalopathy and other diseases with unexplainable symptoms. Treatable IEM should be considered first, particularly in poisoned patients. Family history of neonatal, death, and suggestive autopsy findings are important clues, but are not essential. Genetic counseling should be provided for the family, and specialized centers should be consulted whenever necessary.

The application of spectrometry to NBS and prenatal diagnosis has enabled presymptomatic diagnosis of certain IEMs (El-Hattab, Almannai and Sutton, 2018). However, for most IEMs, screening tests are either too slow, too expensive, or unreliable; therefore, clinical screening is mandatory before beginning the sequence of sophisticated biochemical tests necessary to identify many IEMs (Saudubray and Garcia-Cazorla, 2018).

I.1.1. Clinical classification

According to their pathophysiology, IEMs comprise three large groups: diseases caused by intoxication, diseases by energy deficiency and diseases by deficiency in the synthesis or catabolism of complex molecules (Cousin, 2014; Saudubray and Garcia-Cazorla, 2018) (**Table I.1**). The first two groups constitute a metabolic emergency and include most intermediary metabolism diseases (Ricquier, 2005a; Mak *et al.*, 2013).

I.1.1.1. Endogenous poisoning diseases

The progressive accumulation of one or more compounds (organic acids, AAs, ammonia, metals, etc.) leads to intoxication responsible for acute or chronic clinical symptoms in this group of diseases. Mainly, you can have the following conditions:

- AAHs
- Organic acidurias
- Deficits in the urea cycle are responsible for hyperammonemia, for example ornithine carbamyl transferase deficiency.
- Congenital galactosemia (Cousin, 2014; Saudubray and Garcia-Cazorla, 2018).

I.1.1.2. Energy deficiency diseases

In this type of condition, the symptoms are caused by a failure in the production or use of energy. Their diagnosis is difficult because the clinical manifestations are serious and often variable. This group includes:

- Hepatic or muscular glycogenosis.
- Deficits in mitochondrial oxidation of fatty acids.
- Deficits of the mitochondrial respiratory chain

- Deficits of pyruvate crossroads enzymes, deficits in ketone body metabolism, anomalies of the Krebs cycle and anomalies of creatine biosynthesis (Cousin, 2014; Saudubray and Garcia-Cazorla, 2018).

I.1.1.3. Diseases of complex molecule metabolism

This group corresponds to pathologies of progressive evolution with deficits in the synthesis or catabolism of complex molecules. We essentially find:

- Lysosomal diseases
- Peroxisomal diseases
- Protein glycosylation deficits which are grouped under the name CDG syndrome (Cousin, 2014; Saudubray and Garcia-Cazorla, 2018).

Table I.1. Classification of inborn errors of metabolism (Ezgu, 2016)

Type	Pathophysiology	Examples
By intoxication	Accumulation of toxic substrate	• Aminoacidopathies • Organic acidurias • Carbohydrate metabolism defects
By energy deficiency	Product deficiency	• Carbohydrate metabolism defects • Mitochondrial disorders • Defect in fatty acid oxidation
Default Storage	Chronic accumulation of metabolites from defective catabolism of complex molecules in cellular organelles	• Lysosomal storage disorders • Peroxisomal disorders

I.1.2. Early detection and diagnosis via newborn screening

Neonatal screening offers the possibility of detecting IEMs at an asymptomatic phase and carrying out medical interventions which can positively modify the course of the disease. NBS has had a dramatic effect on improving outcomes in many IEMs, including PKU, maple syrup urinary disease, and medium-chain acyl–coenzyme A dehydrogenase deficiency (Vernon, 2015).

Massachusetts mandated the first test for newborns in the United States in 1963 for PKU using a specific bacterial metabolite inhibition test developed by Guthrie and Susi to detect elevated levels of Phe in DBS (Vernon, 2015).

Since then, more than 150 million newborns have been screened for a growing number of conditions. In the early 2000s, MS/MS was introduced into many NBS programs.

MS allows for rapid detection and quantification of a wide range of metabolites via identification of ion characteristics (mass-to-charge ratios) and comparison with internal standards (Alseekh *et al.*, 2021).

I.2. General information on amino acids

AAs are the basis of the constitution of proteins and other peptides, even if they are not the only constituents (Camus G, 2006). In biological fluids, the concentration of AAs is the result of opposing factors: intake to the body (food intake, endogenous protein catabolism) and their use by the various tissues (protein synthesis, oxidative metabolism) (Thioulouse, Berthe and Couderc, 2010).

I.2.1. Structure

AAs are molecules which have a primary amine function and a carboxylic acid function carried by the same atom of carbon, the carbon α and so these are α-AAs.

The R group corresponds to a variable radical depending on the AA considered. It is therefore who determines the nature of the AA since the rest is invariant (Camus G, 2006). As described in **Figure I.1**, we have a molecule having two ionizable groups: one acidic ($COOH <—> COO^{-}+H^{+}$), the other basic ($NH_2+H^{+}<—> NH_{3+}$). The totally non-ionized form practically does not exist because at acidic pHs for which the COOH function is not ionized, the NH_2 function always is, and vice versa at basic pH for which the NH_2 function is not ionized, the COOH function still is (Camus G, 2006).

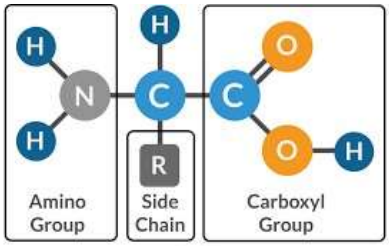

Figure I.1. Amino acid structure (Cousin, 2014)

I.2.2. Nomenclature and classification

AAs differ from each other by their radical. In humans, only twenty different AAs are incorporated into proteins during translation. Their average molecular molar mass is 110 Da**.**

Classically, these twenty AAs are grouped into families, depending on the chemical properties of the radical (**Figure I.2**). Several types of classification are possible because certain AAs can fall into several categories(Camus G, 2006).

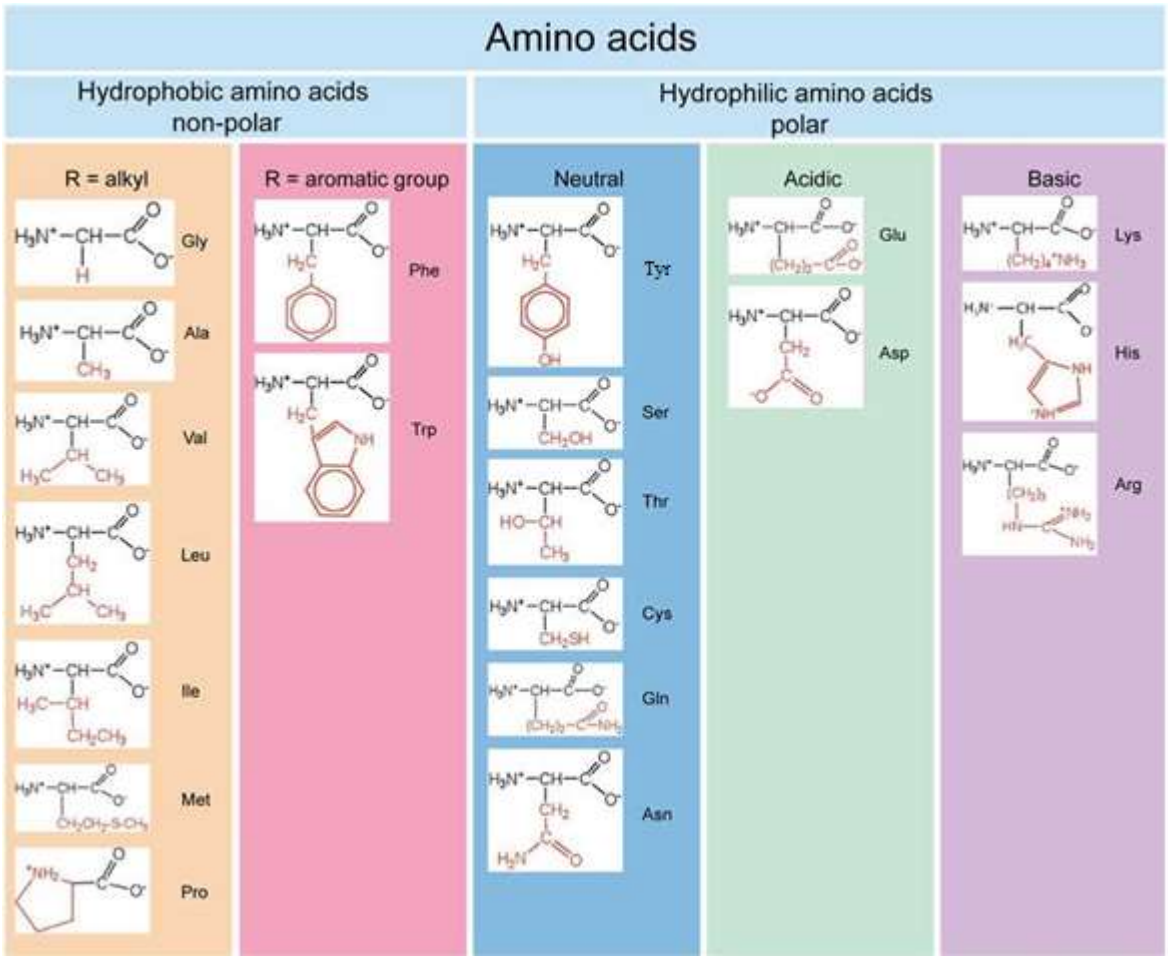

Figure I.2. Structure and classification of the 20 amino acids in their dominant ionic form at intracellular pH (Matthieu C, 2009)

I.2.3. Essential and non-essential amino acids

Based on dietary requirements for nitrogen balance or growth, AAs were traditionally classified as essential (indispensable) and non-essential (Wu, 2009; Cousin, 2014).

The **essential AAs** cannot be synthesized by the body due to the lack of precursor or synthesis enzyme. Food is the only source of intake. These are: Phe, isoleucine, leucine, lysine, threonine, methionine, tryptophan and valine.

The **non-essential AAs** are synthesized by the body and whose intake does not depend exclusively on diet. There are twelve of them: alanine, arginine, asparagine, aspartate, glutamine, glutamate, cysteine, glycine, histidine, proline, serine, and Tyr (Wu, 2009; Cousin, 2014).

Histidine and arginine are said **semi-essential** because only infants need exogenous intake (they can be found in breast milk).

Therefore, AAs come from two sources: an exogenous dietary source and an endogenous source with de novo synthesis or protein turnover (Cousin, 2014).

I.2.4. Biological importance of amino acids

Proteins are composed of AAs that have two essential functions, providing metabolites that play important physiological roles in maintaining the proper functioning of the body, such as glutathione (derived from cysteine) and nitric oxide or NO (arginine derivative), and allow the agglomeration and renewal of body proteins (Hermier and Mariotti, 2018).

The role of AAs is multiple, we can summarize it as follows

- **Structural**: the main role of AAs is protein synthesis. Twenty AAs enter in the composition of proteins and they are called proteinogenic. AAs are the monomers of proteins and play the role of determinants of the structure and function of proteins regarding to their nature, the order in which they are linked and their mutual spatial relationships (Cousin, 2014).
- **Energetic**: AAs can be energetic substances such as glucose, fatty acids and ketone bodies. Their breakdown is a source of energy (Cousin, 2014).
- **Metabolic**: the catabolism of AAs provides atoms and groups of atoms used during synthesis reactions (for example histidine and histamine, aspartate or glycine in synthesis of purine and pyrimidine nucleotides, Tyr in the synthesis of adrenaline). So AAs are more or less direct precursors of molecules of biological interest (Cousin, 2014).

I.3. Aminoacidopathies

Inborn errors of AA metabolism, or hereditary diseases of AA metabolism, or AAH, bring together a set of disorders affecting AA metabolism. These diseases are most often poisoning diseases linked to an enzymatic deficiency in the AAs degradation pathway (Thioulouse, Berthe and Couderc, 2010; Karam *et al.*, 2013). They are rare and often serious; they each affect less than one birth in 10,000 but they can cover a broad phenotypic spectrum and they represent a considerable part of neonatal and pediatric pathology.

Most of these disorders are genetic; they manifest in the neonatal period, often through neurological and metabolic disturbances and may be presented later in the life of the child or even the adult. They are of autosomal recessive inheritance; they are characterized by a free interval of 2 to 5 days between birth and the appearance of the signs (Thioulouse, Berthe and Couderc, 2010). AAHs can be classified into two categories:

- enzymopathies affecting a step in the catabolism of carbon fraction of AAs or the catabolism of amino nitrogen (ureogenesis). If the anomaly is located at the level of one of the first stages of catabolism of AA, it will accumulate (Phe in PKU). If the anomaly is located at the level of the last stages of the metabolic pathway, the product which accumulates is an organic acid;
- membrane transport abnormalities reaching the plasma membrane of cells (renal tubular cells and/or enterocytes) or intracellular membranes (mitochondria, lysosomes).

More than 500 IEMs have been reported so far, with 91 of these disorders potentially treatable if diagnosed early in life. Out of these 91 conditions, 13 disorders are caused by AA disruption which are: PKU, maple syrup urine disease (MSUD), homocystinuria/methylene tetrahydrofolate reductase deficiency (MTHFR), tyrosinemia type II (TT2), citrullinemia type I and type II, argininosuccinic aciduria, carbamoyl phosphate synthetase I (CPS) deficiency, argininemia (arginase deficiency), hyperornithinemia–hyperammonemia–homocitrullinuria syndrome (HHH), N-acetylglutamate synthase (NAGS) deficiency, deficiency ornithine transcarbamylase (OTC) and pyruvate dehydrogenase complex (PDH) deficiency (Wasim *et al.*, 2018).

I.3.1. Diagnosis of aminoacidopathies

Illnesses that manifest in the neonatal period tend to be more severe. Manifestations of many of these disorders include lethargy, insufficient food intake, vomiting and seizures

Conditions that start later tend to affect development more, but can also present with vomiting, convulsions and asthenia (Demczko M, 2024).

To diagnose AAHs, we use a range of clinical signs and basic metabolic screening tests. These are urinary examinations, among others the carrying out of tests by reactive strips indicating the pH and the presence of ketone bodies, and the search for organoleptic characteristics (odor, color), with blood dosages (glycemia, urea, creatinine, uric acid, ionogram , ammonemia, liver test, blood gases) test (Thioulouse, Berthe and Couderc, 2010; Demczko M, 2024).

Rapid colorimetric tests can guide diagnosis without specialized training or advanced analytical equipment but they have low reliability(Wasim *et al.*, 2018).

The ferric chloride and dinitrophenylhydrazine tests have been used for the first diagnosis of different AAHs with a specific color; PKU (green color), Tyrosinuria (blue green color), MSUD (grayish color), and for homocystinuria, the cyanide-nitroprusside test gives pink to beet red color (Wasim *et al.*, 2018).

Additional examinations carried out in specialized laboratories are necessary when AAH is suspected. The biological diagnosis must be biochemical, carried out essentially on three media, blood, urine and cerebrospinal fluid (CSF). Chromatography of AAs (CAA) alone is sufficiently informative in a certain number of cases (Thioulouse, Berthe and Couderc, 2010).

Urinary organic acid chromatography (CAD) is required if the AAHs do not cause any disturbance in the AA concentration but only organic aciduria.

At the end of the exploration, other examinations will be essential and/or will be an integral part of the assessment in the face of any acute situation suspicious of metabolic origin, citing for example lactacidemia, ammoniaemia, blood sugar and pH. Other examinations will be carried out downstream (acylcarnitine profile, urinary orotic acid dosage, etc.) (Thioulouse, Berthe and Couderc, 2010).

In recent years, MS/MS is a cutting-edge technology widely used for the diagnosis of hereditary metabolic diseases, including AAHs (Ricquier, 2005a; Thioulouse, Berthe and Couderc, 2010).

The enzymatic study most often on fibroblasts, supplemented by the genetic study in the case where the responsible mutation is known, are carried out to confirm the disease (Ricquier, 2005; Thioulouse, Berthe and Couderc, 2010). Antenatal diagnosis is possible by measuring metabolites in the amniotic fluid, enzymatic study or the search for the mutation when it is known, are possible in a certain number of pathologies. The development of MS/MS technology gives unprecedented impetus to neonatal screening, but many problems arise, not only technical but also medical (patient care) and ethical (Hamers F, 2020).

I.3.2. Management of aminoacidopathies

Dietary treatments have been used in many developed countries ensuring the development of normal intelligence and preventing the appearance of other complications are of paramount importance for the normal life of the sick child. However, the number of these treatable diseases is small but growing steadily (Demczko M, 2024).

Therefore, the main objective is to standardize the biochemical phenotype of the disease, minimize the products accumulated upstream of the block and increase the defective products downstream of the block while trying to stimulate the residual enzymatic activity by use of alternative metabolic pathways and high-dose vitamin cofactor intake (Demczko M, 2024).

Specific metabolic nutritional therapy and medications can reduce morbidity and improve patient outcomes. For example, leucine and a Phe restricted diet are helpful in cases of MSUD and PKU, respectively. In addition, most AAHs damage the liver (type 1 citrullinemia, type 1 tyrosinemia, etc.) hence the need for early treatment of these disorders to avoid organ transplant (Wasim *et al.*, 2018). The case of forms resistant or not accessible to vitamin treatment is more difficult and requires a strict hypo-protein diet depleted in one or more of the AAs in question(Demczko M, 2024). **Figure I.3** shows the diagnostic guide for AAHs.

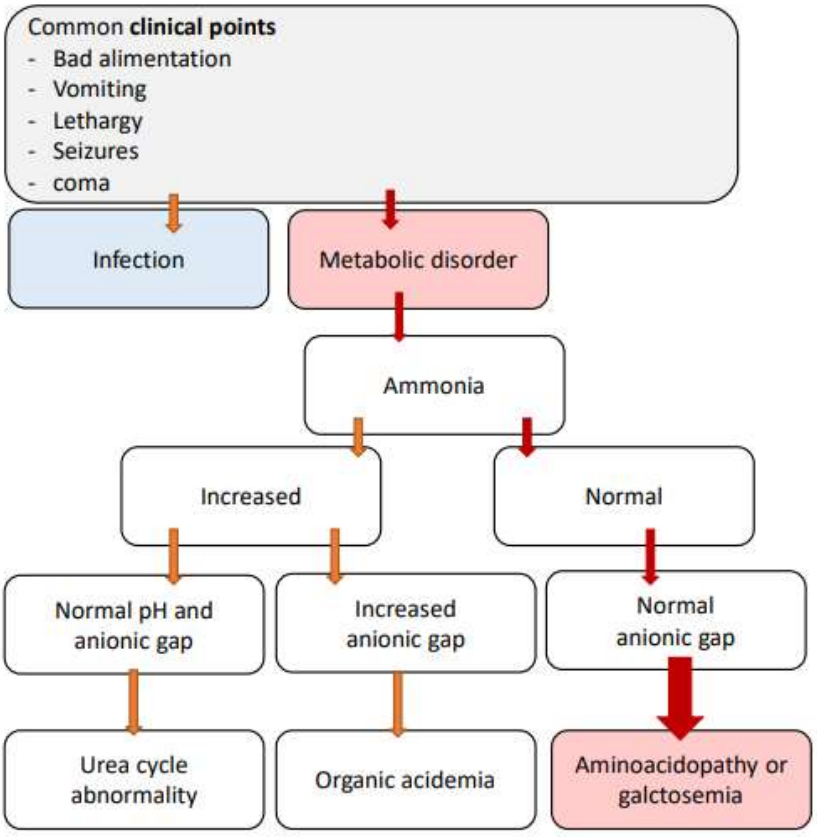

Figure I.3. Diagnostic guide for aminoacidopathies in the newborn (Thioulouse, Berthe and Couderc, 2010)

I.3.3. Prevalence of aminoacidopathies

In most countries, the prevalence of AAHs is low and all developed countries have established NBS programs that allow the collection of prevalence data for such disorders. AAs are caused by genetic mutations in specific genes and most of them are single-gene disorders(Wasim *et al.*, 2018).

In **Table I.2**, thirteen potentially treatable AAHs are summarized with their respective genes, OMIM number and their global prevalence. The majority of these disorders occur due to consanguineous marriages. In the Middle East, the rate of inbreeding is around 40% among first cousins and up to 60% in intermarriage between members of the same family, hence the narrow gene pool which causes an increased incidence of these genetic disorders(Golbahar *et al.*, 2013). In developed countries, the rate of PKU is higher than that of other AAHs (Sherry, Lee and Choi, 2015), like those of the Chinese and Korean population (Golbahar *et al.*, 2013); but this ratio is very high and alarming in Turkey (Khemir *et al.*, 2011; Al Hafid and Christodoulou, 2015).

Table I.2. Causative genes, OMIM number, and prevalence data of common aminoacidopathies

Aminoacidopathy	Treatment options	Respective genes	Worldwide prevalence	References
Phenylketonuria (PKU)	-Phenylalanine and protein restricted diet -BH4 therapy -duration of breastfeeding, fish free diet	PAH (OMIM: 261600)	1:10,000–25,000	(A.E. ten Hoedt, 2012; Al Hafid and Christodoulou, 2015; Banta-Wright *et al.*, 2015)
Maple syrup urine disease (MSUD)	Low leucine diet	BCKDHA, BCKDHB and DBT (OMIM: 248600)	1:185,000	(Van Vliet *et al.*, 2014; Chapman *et al.*, 2018)
HHH syndrome	Low protein diet	SLC25A15 (OMIM: 238970)	More than 100	(Qadri *et al.*, 2016)
N deficient-Acetyl - glutamate Synthesis (NAGS)	NOT-Carbamylglutamate medication	NAGS (OMIM: 237310)	Very rare, unknown incidence	(Caldovic *et al.*, 2010; Kim *et al.*, 2015)
Homocystinuria	-Diet low in methionine -vitamin B6 and cystathionine betasynthase gene therapy (CBS)	MTHFR (OMIM: 236250) and CBS (OMIM: 236200)	1:150,000	(Ozben, 2013; Kumar, Sharma and Singh, 2016)
Tyrosinemia type II	-NTBC -Regime low in phenylalnine and tyrosine	TAT (OMIM: 276600)	1:250,000	(Macchia *et al.*, 2013; Ozben, 2013)
Citrullineemia type I	-Liver transplantation -development of induced pluripotent stem cells (iPSCs)	ASS1 (OMIM: 215700)	1:44,300–200,000	(Ozben, 2013; Venkatesh HA, 2014; Bindi and Eiroa, 2017)

Aminoacidopathy	Treatment options	Respective genes	Worldwide prevalence	References
Citrullineemia type II	-Formula supplemented with medium chain triglycerides (MCT)	SLC25A13 (OMIM: 605814)	1:17,000–230,000	(Ozben, 2013; Woo, Park and Lee, 2014)
Ornithine deficiency Transcarbamylase (OTC)	Adeno-associated virus (AAV) gene therapy	OTC (OMIM: 311250)	1:62,000–77,000	(Shao *et al.*, 2017; Wang *et al.*, 2017)
Pyruvate complex deficiency dehydrogenase (PDH)	-High-fat diet (ketogenic diet) given to mothers during pregnancy	PDHA1 (OMIM: 312170), PDHB (OMIM: 614111), and PDP1 (OMIM: 608782)	Very rare, incidence unknown	(Ciara *et al.*, 2016; Pliss, Jatania and Patel, 2016; Pavlu-Pereira *et al.*, 2020)
Argininosuccinic aciduria	-Arginine therapy and dietary modifications	ASL (OMIM: 207900)	1:70,000	(Baruteau *et al.*, 2017)
Arginineemia (Arginase deficiency)	-Arginine therapy	ARG1 (OMIM: 207800)	1:3,50,000–2,000,000	(Bin Sawad *et al.*, 2022)
Carbamoyl deficiency phosphate synthetase I (CPS I)	-Protein-restriction -Supplementation (citrulline and/or arginine -Gene therapy	CPS1 (OMIM: 237300)	1:62,000–800,000	(Yang *et al.*, 2017)

CHAPTER II.

PHENYLKETONURIA AND TYROSINEMIA

Chapter II. Phenylketonuria and tyrosinemia

II.1. Phenylketonuria

PKU is an autosomal resistive AAH, due to phenylalanine hydroxylase (PAH) deficiency, enzyme allowing the transformation of Phe in Tyr (Al Hafid and Christodoulou, 2015; Banta-Wright *et al.*, 2015).

Mutations in the PAH gene cause the production of a non-functional PAH enzyme, which mains to a reduction in its activity. Untreated PKU is associated with an abnormal phenotype caused by the accumulation of toxic products of Phe metabolism (Williams, Mamotte and Burnett, 2008a). This results in hyperphenylalaninemia, causing severe brain damage with mental retardation, seizures and spasticity in untreated patients. These neurological disorders can be associated overall hypopigmentation (pale skin, blond hair, blue eyes) (Thioulouse, Berthe and Couderc, 2010)

The conversion of Phe to Tyr (**Figure II.1**) is carried out by a hydroxylating system consisting of:

- PAH (Williams, Mamotte and Burnett, 2008a; Thioulouse, Berthe and Couderc, 2010)
- BH4 cofactor which must be recycled after its synthesis so that the hydroxylation reaction can take place .
- The enzymes which serve to regenerate BH4, namely dihydropteridine reductase and 4α-carbinolamine dehydratase (Thioulouse, Berthe and Couderc, 2010).

Para-hydroxylation of Phe is essential for the breakdown of the benzene ring. This alternative pathway of transamination and decarboxylation leads to the formation of metabolites such as phenylpyruvate, phenyllactate, and o-hydroxyphenyl acetate which are excreted into urine. The conversion of Phe to Tyr has two results. First, it drives the endogenous production of the non-essential Tyr. Second, the hydroxylation reaction is the rate-limiting step for the complete oxidation of Phe to CO2 and H2O and contributes to the pool of glucose and 2-carbon metabolites (Williams, Mamotte and Burnett, 2008b).

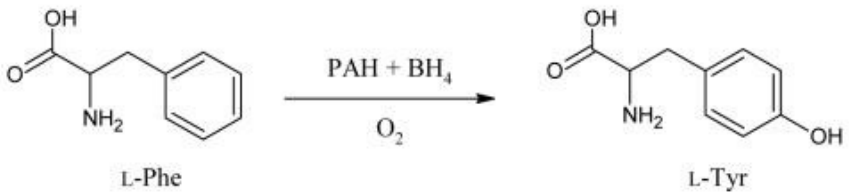

Figure II.1. Conversion of phenylalanine to tyrosine (Williams, Mamotte and Burnett, 2008b)

According to **Figure II.1**, a number of rare and associated disorders due to defects in the BH4 regeneration system may also affect Phe homeostasis and the biosynthesis of catecholamines and serotonin, because this cofactor is common to the hydroxylating enzymes Phe, Tyr and tryptophan (Trp) (Thioulouse, Berthe and Couderc, 2010).

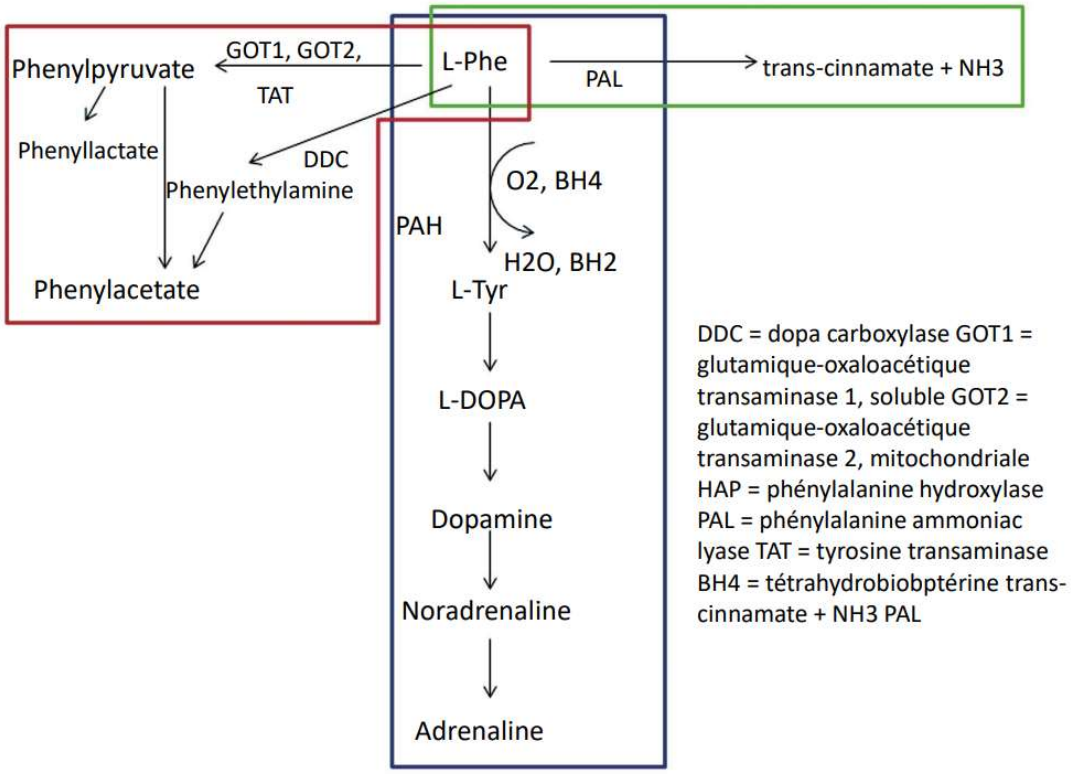

Figure II.2. Phenylalanine metabolic pathway. The major pathway (blue box) is the catalytic conversion of L-Phe to L-Tyr by phenylalanine hydroxylase (PAH). In phenylketonuria (PKU), PAH enzyme deficiency leads to the production of phenyl ketones through an alternative pathway (red box). A third pathway (green box) can be found in plants and yeast involving the enzyme (Al Hafid and Christodoulou, 2015).

II.1.1. Pathophysiology of phenylketonuria

Phe alters neurotransmission and PAH deficiency also leads to a reduction in available Tyr, an AA precursor of certain neurotransmitters (dopamine and catecholamines) including deficiency participates in the pathogenesis of PKU. Rate control of plasma Phe which causes brain damage, constitutes therefore the basis of care. Phe, is an essential AA and the deficiency on its degradation causes PKU, which causes hyperphenylalninemia and a decrease in the synthesis of Tyr. Phe and large neutral AAs (LNAA) (Tyr, tryptophan, threonine, methionine, valine, isoleucine, leucine, histidine) reach the brain *via* the same transporter (LAT1, large neutral AA transporter) competitively. Hyperphenylalninemia leads to an increase in cerebral Phe which has multiple toxic effects on cerebral metabolism and also leads to a deficit in the passage of LNAA (which are all, apart from Tyr, essential AAs) at the cerebral level (Feillet *et al.*, 2010). This LNAA deficiency results in decreased intracerebral protein synthesis and a deficiency in Tyr- and tryptophan-dependent neurotransmitters. These combined abnormalities lead to the neurological, cognitive and neuropsychological deficits of PKU. Indeed, we observe a loss of intelligence quotient (IQ) between 1.9 to 4.1 points per 100 µmol/L of chronic increase in plasma Phe (Wiedemann *et al.*, 2020) .

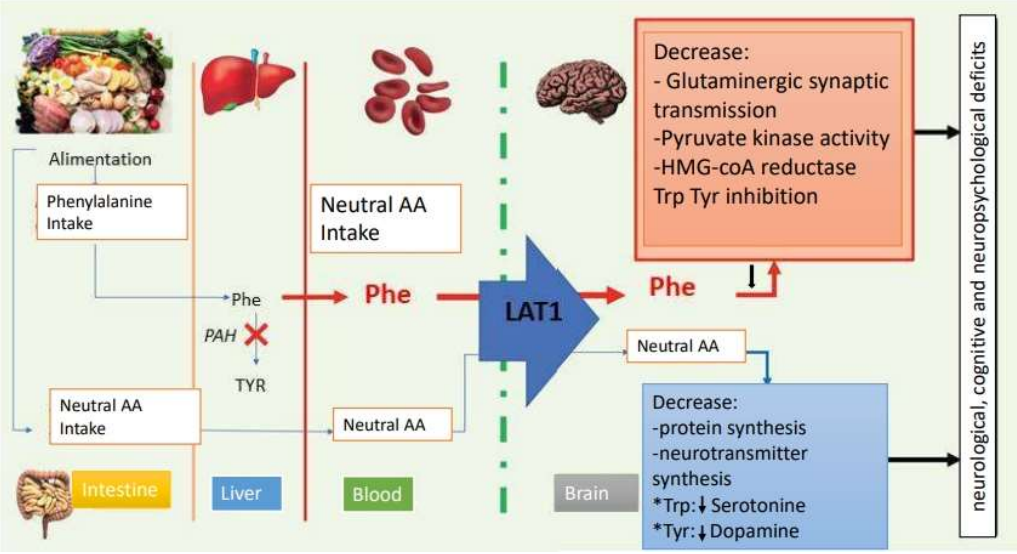

Figure II.3. Pathophysiology of phenylketonuria (Phe: phenylalanie, Tyr: tyrosine, Trp: tryptophane, LAT1: large neutral aminoacids transpoter 1, AA: Aminoacids) (Wiedemann *et al.*, 2020)

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II.1.2. Prevalence

Prevalence of PKU varies worldwide but it is present in every racial and ethnic group (Banta-Wright *et al.*, 2015). The incidence of PKU is highest among Caucasians, occurring in approximately 1 in 10,000 births. PKU can be detected during NBS performed in most Western countries,

The overall prevalence of PKU in Europe and the United States is approximately 1 in 10,000 live births (Williams, Mamotte and Burnett, 2008b; Al Hafid and Christodoulou, 2015) . A higher incidence of the disease is seen in cultures where inbreeding is practiced (e.g. Turkey, Saudi Arabia, or Gaza (1 in 3,500 to 6,500); however, in regions such as Finland, the incidence is low (1 per 100,000) (Dobrowolski *et al.*, 2011).

Table II.1. Phenylketonuria incidence by population(Williams, Mamotte and Burnett, 2008b)

Region/Country		**Incidence of phenylketonuria**
Asian populations	China	1:17,000
	Japan	1:125,000
	Türkiye	1:2,600
	Scotland	1:5,300
	Czechoslovakia	1:7,000
	Hungary	1:11,000
	Denmark	1:12,000
European populations	France	1:13,500
	Norway	1:14,500
	United Kingdom	1:14,300
	Italy	1:17,000
	Canada	1:22,000
	Finland	1:200,000
Arab populations		Up to 1:6,000
Oceania	Australia	1:10,000

II.1.3. Metabolic control (plasma phenylalanine levels)

The prognosis of patients depends on the quality of long-term metabolic control. According to current recommendations, strict metabolic control (Phe level of 120 to 360 µmol/L [2-6 mg/dL]) until the age of 12 is recommended (Grisch-Chan *et al.*, 2019; Wiedemann *et al.*, 2020).

The latest European consensus recommends keeping Phe levels < 600 mol/L [10 mg/dL] for life. The latest French recommendations accept that Phe levels are < 900 µmol/L for adult patients who have completed their studies and who do not experience any problems (Grisch-Chan *et al.*, 2019; Wiedemann *et al.*, 2020).

During pregnancy, the recommendations for metabolic control are very strict (120-360 µmol/L) [2-6 mg/dL], to avoid any risk of hyperphenylalaninemic embryofoetopathy (Feillet *et al.*, 2004; Wiedemann *et al.*, 2020).

II.1.4. Phenylketonuria support

Different therapeutic modalities exist or are in development. They include the reduction of dietary intake of Phe, the degradation of Phe at the digestive level, the inhibition of the passage of Phe at the digestive and cerebral level, the degradation of Phe at the blood level or the restoration of the activity of the PAH at the liver level (Wiedemann *et al.*, 2020).

II.1.4.1. Dietary treatment

The main objective of treatment is to obtain a normal mental, neurological and social outcome in affected patients. Dietary treatment should be started during the neonatal period. PKU patients should strictly limit their intake of protein-rich foods, such as meats, fish, eggs and dairy products. Foods low in protein high in natural starch foods like potatoes, certain vegetables (like peas) can be eaten but only in limited quantities. Due to severe restriction of protein intake, patients with PKU should be supplemented with medical food substitutes containing the right mix of essential AAs, vitamins, minerals and trace elements. Normally, about 90% of dietary Phe intake is converted to Tyr; therefore, a crucial part of treatment is Tyr supplementation (Al Hafid and Christodoulou, 2015).

II.1.4.2. Tetrahydrobiopterin (BH4) therapy

In the 1970s, there was a subset of patients with hyperphenylalaninemia who developed neurological complications despite being placed on diets promptly. This group of patients was found to have mutations that cause defects in BH4 synthesis or recycling. Synthetic biopterin compounds were made available in the late 1970s for patients with atypical PKU who also require precursor neurotransmitters (L-dopa/carbidopa and 5-hydroxytryptophan) as part of their treatment (Al Hafid and Christodoulou, 2015).

The distinction between PKU by PAH deficiency or by BH4 deficiency is clarified regarding to the performance of a BH4 load test, and the quantification of neurotransmitters (dopamine, norepinephrine and adrenaline) and their metabolites and pterins in urine and/or or CSF (Al Hafid and Christodoulou, 2015).

Furthermore, in patients having a PKU by deficiency in PAH and not in BH4, the addition of BH4 diet allows a reduction in plasma Phe of 30 to 70% (Thioulouse, Berthe and Couderc, 2010).

II.1.4.3. Breastfeeding

Since 1960, and the beginnings of screening, the standard of care for infants diagnosed with PKU has recommended breastfeeding and low-Phe formula feedings in conjunction with standard commercial infant formula in an effort to maintain healthy appropriate Phe levels (120–360 µmol/L) for these children (Solverson *et al.*, 2012). Researchers have demonstrated that breastfeeding is a viable option for infants with PKU (Banta-Wright *et al.*, 2015).

II.1.4.4. Neutral amino acid therapy (LNAA)

LNAA, Phe, Tyr, tryptophan and branched-chain AAs share the same transport system across the blood-brain barrier. Therefore, at high concentrations, Phe in the blood will compete with other LNAAs for transport across the blood-brain barrier (Al Hafid and Christodoulou, 2015). Therefore, LNAA supplementation has been shown to reduce brain Phe concentrations despite the observed increase in their plasma levels (Al Hafid and Christodoulou, 2015). However, others have shown decreased blood Phe levels in patients with LNAA supplementation suggesting that LNNAs not only compete with Phe for transport across the blood-brain barrier, but may also exert its effect by competing the Phe for active transport across the intestinal mucosa (Matalon *et al.*, 2006).

II.1.4.5. Glycomacropeptides (GMP)

Glycomacropeptides (GMP) is a protein derived from cheese whey that is naturally low in Phe and high in valine, isoleucine and threonine. GMP, manufactured with sufficient purity and supplemented with the essential AA like Tyr, tryptophan, arginine, cysteine and histidine may be useful in supplementing the Phe restricted diet (MacLeod *et al.*, 2010; Ney, Blank and Hansen, 2013).

II.1.4.6. Degradation of Phe at the digestive level

Another treatment method is to induce the degradation of Phe in the digestive tract. Its interest is to degrade Phe of food origin as well as that coming from the desquamation and degradation of cells and which is reabsorbed by the cells of the digestive tract, thus contributing to the hyperphenylalaninemia observed in PKU patients (Feillet and Bonnemains, 2013; Wiedemann *et al.*, 2020).

II.1.4.7. Gene therapy

Restoration of enzymatic activity in the liver would normalize plasma Phe levels, so PKU is theoretically an ideal model for gene therapy. The first tests were already carried out on mouse models 20 years ago without causing problems. However, multiple problems, especially immunological, encountered in this therapeutic approach, make this type of treatment not possible in PKU (Wiedemann *et al.*, 2020).

Different treatment options of PKU are presented in **Table II.2**.

Table II.2. Therapeutic modalities of phenylketonuria (Wiedemann *et al.*, 2020)

Intestine	Liver	Blood	Brain
Decrease contributions – Hypoprotein diet – Amino acids substitutes – Low protein products – Glycomacropeptides Degradation of Phe – Oral PAL* – Probiotics* – Phelimine* Inhibition of the absorption of the Phe – Neutral amino acids	Increase of PAH activity – BH4 – Sepiapterin* - Genetical therapy* – Genome editing*	Degradation of the blood Phe Enzymotherapy – Subcutaneous: PEG-PAL – Intra-erythrocytic: PAL*	Prevention of cerebral passage of the Phe – Neutral amino acids

*Treatments in development. Phe: phenylalanine, PAH: Phenylalanine hydroxylase; Phelimine: polymers capturing Phe; PAL: phenylalanine ammonia lyase.

II.1.5. Screening and diagnosis of phenylketonuria

In developing countries, the rate of consanguinity is quite high (32%) and the neonatal screening program is lacking, so the diagnosis of PKU is established late, after detection of clinical signs and typical anomalies. For patients with a family history of PKU, new cases are diagnosed at birth (Khemir *et al.*, 2011).

During screening, the Phe dosage is carried out first by fluorimetry where Phe reacts with ninhydrin in the presence of copper to give a fluorescent complex. When monitoring is necessary, the blood Phe concentration is determined by an enzymatic method using Phe dehydrogenase. Confirmatory diagnosis is carried out after screening by centers specializing in the treatment of PKU and consists of monitoring phenylalaninemia and a systematic study of BH4 metabolism. Once the diagnosis is confirmed, therapeutic management depends on the plasma Phe concentration (Thioulouse, Berthe and Couderc, 2010) . PAH deficiency can be classified into classic PKU (Phe > 1200 µmol/L), mild PKU (Phe = 600 to 1200 µmol/L), and mild hyperphenylalaninemia, where blood Phe is above the upper reference limit. , but <600 µmol/L(Williams, Mamotte and Burnett, 2008b; Banta-Wright *et al.*, 2015). The distinction between these groups of patients is very important because they will not have the same level of response to pharmacological doses of BH4 and do not have the same tolerance to Phe in their diet. However, these forms require a controlled restrictive diet (Thioulouse, Berthe and Couderc, 2010).

In the United States, NBS for PKU is performed by MS/MS on DBS. If a newborn has an elevated Phe level (>190 µmol/L) with an increased Phe/Tyr (ratio >3), this is a positive result and diagnostic testing and evaluation is necessary. as soon as possible (Banta-Wright *et al.*, 2015).

Since the late 1960s, and following the development of the Guthrie biochemical test for the diagnosis of PKU and a number of other diseases, the Australian Health Service has conducted NBS programs as part of a global initiative. Filter paper cards have become an accepted facet of newborn care in the modern world (Muchamore, Morphett and Barlow-Stewart, 2006).

More recently, MS/MS was developed as a routine method for NBS tests. The cost-effectiveness of this new approach has made it possible to increase the number of disorders screened in these programs to more than 20 diseases (Millington, 2024).

Filter paper stains have been stable for many years and PKU screening tests have been reported to have a low error rate (David L. Valle *et al.*, 2001). A positive screening result identifies an infant with hyperphenylalaninemia, but it is possible that the increase in Phe concentrations is transient due to another illness and not PKU (e.g., transient 4α-carbinolamine dehydratase deficiency) or whether it is the result of maternal hyperphenylalaninemia. Measuring Phe metabolites in urine is not an accepted method of screening for PKU because it depends on the activity of transaminases (which may be low in newborns) and a large variation between blood concentrations of Phe and urinary metabolites has been demonstrated (Williams, Mamotte and Burnett, 2008).

The differential diagnosis of PKU from disorders of BH4 synthesis or recycling may involve various testing regimens, including BH4 loading testing, measurement of urinary and plasma pterin metabolites and neurotransmitter metabolites, as well as the measurement of dihydropteridine reductase (David L. Valle *et al.*, 2001). Furthermore, tests targeting direct measurement of enzymes (PAH, 4α-carbinolamine dehydratase) would require a tissue biopsy.

The molecular diagnosis of PKU is based on the aberrant metabolic phenotype, disease-causing mutations, and polymorphic haplotypes associated with the PAH locus. For prenatal diagnosis, PKU mutation analysis is particularly useful in carrier detection. A wide variety of molecular genetic techniques have been used (Williams, Mamotte and Burnett, 2008).

II.1.6. Progressive complications of phenylketonuria

Complications of PKU are regrouped in the **Table II.3**. During the childhood, when the diet or the treatment is well followed, evolution is usually favorable. Annual nutritional monitoring is essential regardless of patients age (Wiedemann *et al.*, 2020).

Table II.3. Complications of phenylketonuria with age (Wiedemann *et al.*, 2020)

Complication	Age of onset	Etiology	Reversibility	Severity
Mental retardation	0 – 10 years	Late diagnosis ↓↓↓Compliance	No	++++
Autism	0 – 10 years	Late diagnosis ↓↓↓Compliance	No	++++
Epilepsy	0 – 10 years	Late diagnosis ↓↓↓Compliance	No	++++
Depigmentation	0 – 10 years	Late diagnosis ↓↓↓Compliance	Yes	++++

Complication	Age of onset	Etiology	Reversibility	Severity
Mood disorders	10 years old - adult	Relaxation diet	Yes	++
Leukopathy MRI*	Adult	Intracerebral Phe↑↑	Yes	+/- ?
Osteoporosis	10 years old - adult	Protein deficiency	Yes	+
Obesity	10 years old - adult	Multifactorial	+/-	+
Nutritional deficits	All ages	Vegetarian diet Deficiencies	Yes	+/-
B12	All ages	Intake deficiency	Yes	++
Zinc	All ages	Bioavailability problem	Yes	+
Selenium	All ages	Bioavailability problem	Yes	+
Embryofoetopathy	During pregnancy	Lack of metabolic control	No	++++
Nutritional excesses	All ages	High dose of substitute of AA	Yes	**+/-**
Folate	All ages	Excess folate in substitutes	Yes	+/-

*MRI: magnetic resonance imaging; ↓: decrease; ↑: increase

II.2. Tyrosinemia

Tyr is a non-essential aromatic AA resulting from Phe hydroxylation. Tyr breakdown takes place principally in the cytoplasm of hepatocytes, but also in the kidney. Its degradation pathway comprises five enzymatic reactions, and metabolic disorders have been identified in four of these steps (see **Figure II.2**).

TRS is a genetic disorder characterized by defect in breaking down the Tyr and leads to Tyr accumulation in biological fluids and tissues (Fernández-Lainez *et al.*, 2014). If the condition is untreated, Tyr and its metabolites build up in tissues and organs, and lead to serious health problems (*Tyrosinemia*, 2023).

II.2.1. Biochemistry

Numerous situations can cause hypertyrosinemia like inborn errors of the Tyr degradation pathway, liver failure, blood sampling after eating instead of in the fasting state and rarely vitamin C deficiency and hyperthyroidism (Russo, Mitchell and Tanguay, 2001).

The metabolic pathway of Tyr is illustrated in **Figure II.4**.

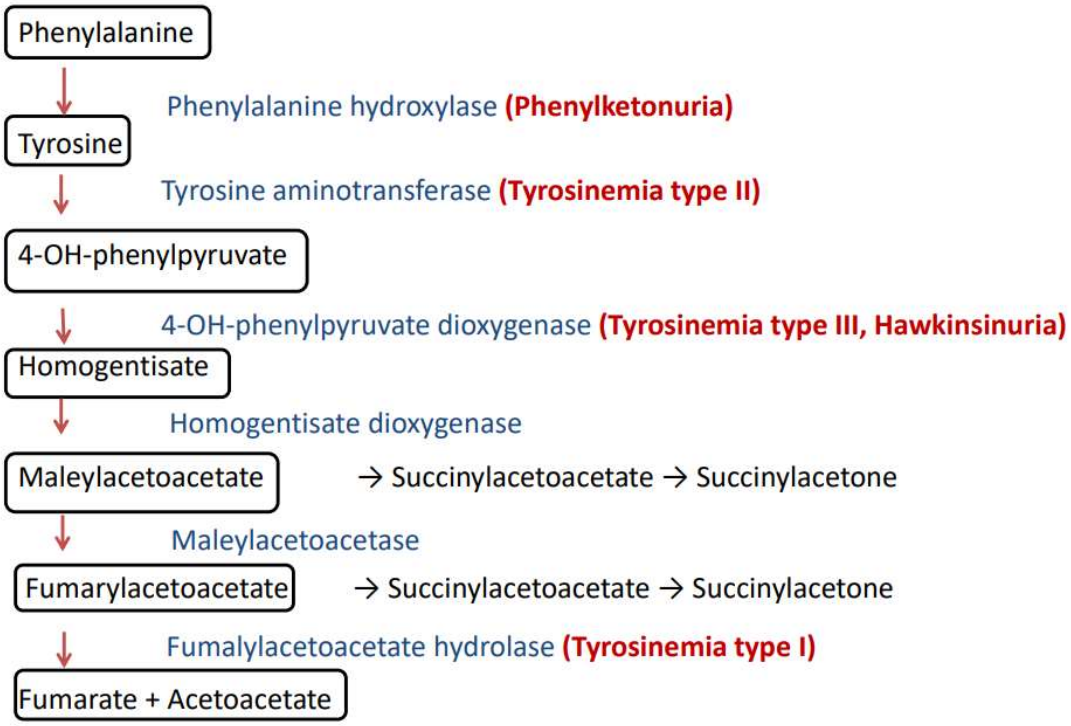

Figure II.4. Metabolic pathway of tyrosine: tyrosine metabolism begins from phenylalanine through tyrosine to fumarate and acetoacetate. Tyrosinemia type I is caused by a deficit of fumarylacetoacetate hydrolase, type II occurs from a deficit of tyrosine aminotransferase, and type III takes place from a deficit of 4-OH-phenylpyruvate dioxygenase (Nakamura *et al.*, 2015)

II.2.2. Classification of tyrosinemia

Three types of TRS are known, distinguished by their genetic cause and symptoms (*Tyrosinemia*, 2023). The various inborn errors of Tyr metabolism with their corresponding enzyme defects are listed in **Table II.4**. Transient TRS of the newborn is not a real IEM, but due to a temporary immaturity in the function of the enzyme 4-hydroxyphenylpyruvate dioxygenase (4HPD), most often with associated vitamin C deficiency or in premature infants fed cow's milk. The disorder resolves spontaneously without significant effects.

TT2, results because of an autosomal recessive deficiency in the enzyme Tyr aminotransferase, named also oculocutaneous TRS since it is characterized by corneal thickening with palmar and plantar hyperkeratosis. Liver and kidney function is usually unaffected. Hawkinsinuria is a rare disorder resultant from abnormal function of the enzyme 4 HPD, and gives a metabolic acidosis and apparent resolution of clinical symptoms as the aptitude to handle with the generated intermediates increases (Russo, Mitchell and Tanguay, 2001).

An associated renal tubular acidosis has also been intermittently observed, which might result in confusion with tyrosinemia type I (TT1). However, a main deficiency of 4 HPD, causes tyrosinemia type III (TT3). Manifestations were ranged from asymptomatic to complicated mental retardation and neurologic anomalies (Russo, Mitchell and Tanguay, 2001).

Blood Tyr can be physiologically high in newborns for a variety of reasons so it is difficult to identify true cases of hereditary TRS. It can be detected using MS/MS method for example. For this reasons, NBS for TRS could be possible by measurement succinylacetone (SUA) in the blood. However, there is a wide variety of genetic and biochemical disease groups that present with TRS. Elevated Tyr (>440 µmol/L; >8 mg/dL) was observed in of 0.5–1.8% newborns in Europe, demonstrating that it is a relatively frequent abnormality, but then, because hereditary TRS is only very rarely encountered, the diagnosis must be made on the foundation of its characteristic clinical manifestations and specific tests (Nakamura *et al.*, 2015).

Table II.4. Major clinical manifestations with enzymatic defects in hypertyrosinemia

(Russo, Mitchell and Tanguay, 2001)

Enzyme	Defect	Major manifestations
Tyrosine aminotransferase	Tyrosinemia type II (oculocutaneous tyrosinemia)	Corneal thickening, developmental delay, hyperkeratosis of palms and soles
4-hydroxy phenylpyruvate dioxygenase	Transient tyrosinemia of the newborn	Transient immaturity of enzyme, usually resolves spontaneously
	Hawkinisinuria	Abnormal function of enzyme results in metabolic acidosis and failure to thrive in some patients

Enzyme	Defect	Major manifestations
	Tyrosinemia type III	Primary deficiency of enzyme; asymptomatic to severe mental retardation and neurologic abnormalities
Homogentisate oxidase	Alcaptonuria	Arthritis in older patients Dark urine when exposed to air
Maleylacetoacetate isomerase		Reported in two siblings with liver failure and renal disease
Fumarylacetoacetate hydroxylase	Hepatorenal tyrosinemia Tyrosinemia type I	Liver, renal, and neurologic disease

II.2.2.1. Hereditary tyrosinemia type I (Hepatorenal Tyrosinemia)

II.2.2.1.1. Genetics

TT1 (OMIM 276700), also called hepatorenal TRS, is an IEM, caused by an autosomal recessive inherited deficiency of the enzyme FAH, the last enzyme in the Tyr catabolic pathway which convert fumarylacetoacetate (FAA) into fumarate and acetoacetate (Scott, 2006; Chakrapani, Gissen and McKiernan, 2012).

The first patients described with TT1 presented with renal tubular defects, liver cirrhosis, , and vitamin D-resistant rickets, although at that time the exact diagnosis was not clear(Williams, Mamotte and Burnett, 2008b; van Ginkel, Rodenburg, Harding, Carla E.M. Hollak, *et al.*, 2019) . Primarily, the main enzyme defect was considered to be a defect of 4HPD (Thioulouse, Berthe and Couderc, 2010; A.E. ten Hoedt, 2012). Few years later, it became clear that the primary enzyme deficiency was located more downstream in the catabolic pathway of Tyr at FAH (see **Figure II.1**) (Nakamura *et al.*, 2015; van Ginkel, Rodenburg, Harding, Carla E. M. Hollak, *et al.*, 2019).

II.2.3.1.2. Clinical Presentation

The clinical manifestations of TT1 are variables, and an affected individual can present symptoms at any time of life, from the neonatal period to adulthood. Thus, among members of the same family, there is large variability of presentation (Scott, 2006; Chakrapani, Gissen and McKiernan, 2012).

TT1 may be classified clinically based on the age at symptoms onset and the disease severity: a severe form presenting before 6 months of age (but rarely in the first 2 weeks of life) with grave liver failure; a subacute form that appears between 6 months and 1 year of age with liver disease (failure to thrive, hepatosplenomegaly, coagulopathy, rickets and hypotonia); and a more chronic form that appears after the first year with chronic liver disease, renal disease, rickets, cardiomyopathy and/or a porphyria- like syndrome. Consequently, the liver is the main organ affected in TT1, and its involvement is a major cause of morbidity and mortality (Scott, 2006; Chakrapani, Gissen and McKiernan, 2012).

II.2.2.1.3. Diagnosis

The detection of high level of SUA in urine or plasma is the pathognomonic metabolite for the confirmation of TT1. SUA can be detected in the biologic fluid of all untreated patients with TT1. Plasma analysis will demonstrate an increase in plasma methionine and Tyr concentrations.

These elevations are produced by generalized liver dysfunction (for methionine elevation) and the inhibition of 4-hydroxyphenylpyruvic dioxygenase by SUA (for Tyr elevation). GC-MS was typically used for SUA detection in extracted organic acids. Elevations of p-hydroxyphenylacetic acid and phydroxyphenyllactic acid can be confirmed by urine organic acid analysis.

Confirmatory testing for TT1 can be performed by the documentation of pathogenic mutations from DNA or the measurement of FAH in cultured skin fibroblasts. Only a limited laboratories are performing DNA mutation analysis of the FAH gene and the enzymatic analysis of FAH is not readily available as a common clinical assay (Scott, 2006).

II.2.2.1.4. Treatment and Prognosis

a) Restrictive Diet

Before 1990s, liver transplantation was the only treatment for TT1. Patients were following a restrictive diet with low Phe and Tyr intake. Even though this diet was helpful at the beginning, it was not fully preventing ulterior liver and renal dysfunction (Morrow and Tanguay, 2017).

b) Liver Transplantation

Liver transplantation is recommended in the most severe TT1with acute liver failure for the reason of the risks associated to the surgery (Scott, 2006). Liver transplantation offers a functional treatment of TT1 and lets a normal unrestricted diet (Chakrapani, Gissen and McKiernan, 2012).

However, it is associated with necessity of lifelong immunosuppressive therapy and approximately 5-10% mortality even in optimal circumstances so it is restricted to patients who fail to respond to nitisinone therapy (Chakrapani, Gissen and McKiernan, 2012).

Even the liver transplantation is curative, however it does not fully correct metabolic perturbations in TT1 because of toxic metabolites excretion in urine by kidneys (Morrow and Tanguay, 2017). The long-term influence of liver transplantation on renal disease in patients with TT1 relates to the age in which they were treated. Prior to nitisinone, wholly patients had tubular dysfunction and some had glomerular dysfunction earlier of receiving transplants. In this group, tubular function will be ameliorated in most patients but they had higher proportion of glomerular dysfunction due to nephrotoxic immunotherapy. Patients pre-treated with nitisinone typically have normal renal function at transplant (Chakrapani, Gissen and McKiernan, 2012).

c) Nitisinone

Nitisinone or 2-(2-nitro-4-trifluoro-methylbenzyol)- 1,3 cyclohexanedione (NTBC), was first used in 1992. NTBC inhibits the second enzyme of the Tyr catabolic pathway (Chakrapani, Gissen and McKiernan, 2012). Thus, the rationale for its use is to block Tyr degradation at an early step so as to prevent the building of toxic downstream metabolite (Morrow and Tanguay, 2017).

The use of NTBC combined to the low Tyr/ Phe diet has confirmed to be very effective in preventing TT1 development, by curing both liver and kidney dysfunctions, particularly when introduced early in life (Scott, 2006; Morrow and Tanguay, 2017). Dietary restriction of Phe and Tyr is crucial to avoid the known adverse effects of hypertyrosinaemia. Consequently, the aim is to maintain Tyr levels between 200 and 400 μmol/l and the Phe level of >30μmol/l (Chakrapani, Gissen and McKiernan, 2012)**.**

In over 90% of patients presenting acutely with hepatic decompensation, rapid clinical improvement occurs, with improvement of prothrombin time within days of starting treatment. Other biochemical parameters of liver function may take more time to normalize. NTBC is recommended in an initial dose of 2 mg/kg body weight per day in liver failure or 1 mg/kg/day else.

Dose adjustment is therefore based on the biochemical response and the object is to sustain a plasma nitisinone concentration of >50 μmol/l or a whole blood concentration of 20–40 μmol/l (Chakrapani, Gissen and McKiernan, 2012).

Supportive treatment is imperative in the acutely ill patient. Vitamin D is essential to treat rickets. Infections should be treated aggressively. Coagulation factors, albumin, electrolytes and acid/base balance should be checked and adjusted as needed (Chakrapani, Gissen and McKiernan, 2012).

II.2.2.1.5. Remaining challenges and future considerations

Future challenges in NBTC treatment will be to develop reliable detection of possible liver cancer, uniform guidelines for treatment and follow-up, and weighing the risks, challenges, and costs of existing and alternative strategies for the treatment of TT1. Almost, three factors are to be consider in the future: (van Ginkel, Rodenburg, Harding, Carla E. M. Hollak, *et al.*, 2019)

a) Pregnancy

There is no available informations about possible teratogenic effect of NTBC. Nevertheless, eleveted dosages of NTBC have been accompanying with corneal lesions, malformations, and reduced survival in of spring of laboratory animals treated with NTBC, while prenatally prescribed normal dosages do prevent early death in TT1 mice and pigs without any teratogenicity.

b) Long-term follow-up

Important clinical symptomatology can be prevented with early introduction of NTBC, although the very long-term effects of NTBC, in terms of effectiveness and toxicity, persist and should be evaluated so strict monitoring of the disease is very important.

c) Considerations from a cost perspective

NTBC is considered as one of the 20 most expensive drugs, with reported annual costs between US $70,000 and US $140,000 for a person of 50 kg. Regarding this, and because of only one cost-effectiveness study has been performed, more studies should be performed in the future.

II.2.2.2. Tyrosinemia type II

TT2 is inherited in an autosomal recessive manner. Genetic counseling should be provided to couples at risk (where both partners are carriers of a disease-causing mutation), informing them that there is a 25% likelihood of having an affected child with each pregnancy (Corinne DE LAET, 2023).

II.2.2.2.1. Clinical description

TT2 involves higher blood Tyr level than TT1 and TT3, but does not exhibit liver and kidney dysfunction as TT1. Serum Tyr level was very high exceeding 20 mg/dL (1100 μmol/L) which is responsible of deposition of needle-like Tyr crystals giving different skin lesions (Nakamura *et al.*, 2015).

In 80% of cases, skin lesions occur, typically beginning after the first year of life but may develop concurrently with ocular symptoms. Common ocular symptoms include redness, photophobia, excessive tearing, and pain. Neurological findings and some degree of intellectual deficit are present in up to 60% of cases. Central nervous system (CNS) involvement is highly variable, with intellectual deficit being the most common manifestation, ranging from mild to severe.

Other signs of CNS involvement include behavioral problems, nystagmus, tremor, ataxia, and convulsions (Corinne DE LAET, 2023).

II.2.2.2.3. Etiology

TT2 is attributed to mutations in the *TAT* gene, which encodes the enzyme tyrosine aminotransferase (TAT). The deficiency of TAT leads to elevated levels of Tyr, which can result in the deposition of Tyr crystals, triggering an inflammatory response and causing oculocutaneous manifestations. Although Tyr crystals have not been identified in skin lesions, it is believed that excess intracellular Tyr may disrupt microtubules and tonofilaments. Additionally, there appears to be a correlation between the severity of CNS involvement and the plasma levels of Tyr (Corinne DE LAET, 2023).

II.2.2.2.3. Diagnostic Methods

Diagnosis of TT2 is based on clinical findings, along with the detection of elevated levels of plasma and urinary Tyr, as well as increased levels of urinary Tyr metabolites, including 4-hydroxyphenylpyruvate, 4-hydroxyphenyllactate, 4-hydroxyphenylacetate, and N-acetyltyrosine. Confirmation of the diagnosis is achieved through mutation analysis, while TAT assays on liver biopsy samples are generally not required (Corinne DE LAET, 2023).

II.2.2.2.4. Management and Treatment

Management primarily focuses on dietary restrictions of phenylalanine and Tyr. Additionally, oral retinoids may be prescribed to treat skin lesions.

Adhering to a controlled diet effectively lowers plasma Tyr levels and leads to a rapid resolution of oculocutaneous manifestations. However, the extent to which this dietary management prevents central nervous system involvement remains uncertain (Corinne DE LAET, 2023).

II.2.2.3. Tyrosinemia Type III

TT3 (OMIM 276710) is an extremely rare genetic disorder transmitted in an autosomal recessive form.

TT3 is produced by mutations in the gene encoding the enzyme 4HPD that catalyzes the second step in the Tyr catabolic pathway meaning the conversion of hydroxyphenylpyruvate to homogentisate. This metabolic disorder is characterized by high levels of serum Tyr and amplified excretion of phenolic metabolites [4-hydroxyphenylpyruvate (4-HPP), 4-hydroxyphenyllactate (4-HPL) and hydrophenylacetate] in the urine (Barroso *et al.*, 2020).

II.2.2.3.1. Clinical description

Manifestations in TT3 are milder than those in TT1 and TT2, and some cases are asymptomatic (Nakamura *et al.*, 2015). A few case reports have been reported in the literature, with a wide clinical phenotype spectrum (Barroso *et al.*, 2020). The elevation in serum Tyr level is moderate, >10 mg/dL (550 µmol/L) as comparing to TT2 (Nakamura *et al.*, 2015). The most prevalent symptoms include mild intellectual disability, seizures, and intermittent loss of balance and coordination (ataxia) (Nakamura *et al.*, 2015; Matt Demczko, 2024). These do not appear in TT1 or TT2, and may be linked to an increase in 4-OH-phenylpyruvate in body fluids (Nakamura *et al.*, 2015). Liver and eye complications are not classically associated with this type of TRS. These symptoms typically manifest during early childhood and can have a significant impact on the individual's development and quality of life (Matt Demczko, 2024).

II.2.2.3.2. Diagnosis

Diagnosis of TT3 is typically done through a NBS test. This test is designed to detect various metabolic disorders. If the screening test is abnormal, further testing may be necessary, such as urine and blood tests, to confirm the diagnosis (Matt Demczko, 2024).

II.2.2.3.3. Treatment

Treatment for TT3 primarily involves dietary restrictions. Individuals with this condition must follow a diet that is low in Tyr and Phe, two AAs that the body struggles to break down. This dietary restriction helps to prevent the buildup of these AAs in the body and can help to manage the symptoms of the disorder. In addition to dietary management, individuals may also require other forms of treatment, such as medication to control seizures or physical therapy to address issues with balance and coordination (Matt Demczko, 2024).

While TT3 is a rare disorder, it is important for individuals and healthcare providers to be aware of its potential symptoms and treatment options. Early diagnosis and proper management can help to improve the quality of life for those affected by this condition (Matt Demczko, 2024).

II.2.3. Frequency of tyrosinemia

About 1 in 10 of all newborns has transitorily elevated levels of Tyr (transient TRS). These cases are not genetic and caused by vitamin C deficiency or an immature liver due to premature birth (*Tyrosinemia*, 2023).

The prevalence of TRS through the world is infrequent. Worldwide, it is estimated to be no more than 1 in 100,000 live births with several exceptions (Russo, Mitchell and Tanguay, 2001).

The prevalence of TT1 I in Europe is reported to be 1 in 125 000 people (Nakamura *et al.*, 2015). TT1 is more common in Norway and Finland, where 1 in 60,000 to 74,000 individuals are affected, and Quebec (Canada), where it occurs in about 1 in 16,000 individuals. It is especially common in the Saguenay-Lac Saint-Jean region of Quebec, where 1 in 1,846 live births results in an affected child (*Tyrosinemia*, 2023). In addition, TT2 occurs in fewer than 1 in 250,000 individuals worldwide (*Tyrosinemia*, 2023), it appears to be more common in Arab and Mediterranean populations.(Corinne DE LAET, 2023). Whereas only a few cases have been reported in TT3 and it is very rare (*Tyrosinemia*, 2023)

II.2.4. Diagnosis of tyrosinemia

High blood Tyr can occur due to other causes besides TT1, TT2 and TT3, which make differentiation and accurate diagnosis necessary. The liver dysfunction associated with type I plays an important role for diagnosis. For distinguishing between types II and III, transient TRS in newborns can pause a problem. Blood AA analysis are useful tools for monitoring blood Tyr. In TT1 and because of liver dysfunction, blood AAs analysis commonly indicates elevated levels of many AAs, including Tyr (>220 µmol/L, 4 mg/dL), methionine (>130 µmol/L, 2 mg/dL), and threonine (>170 µmol/L, 2 mg/dL). In addition, there is increased excretion of Tyr and many other AAs in urinary AAs analysis. A definitive diagnosis requires the confirmation of increased AAs in an analysis of urinary organic acids (**Figure II.5**) (Nakamura *et al.*, 2015).

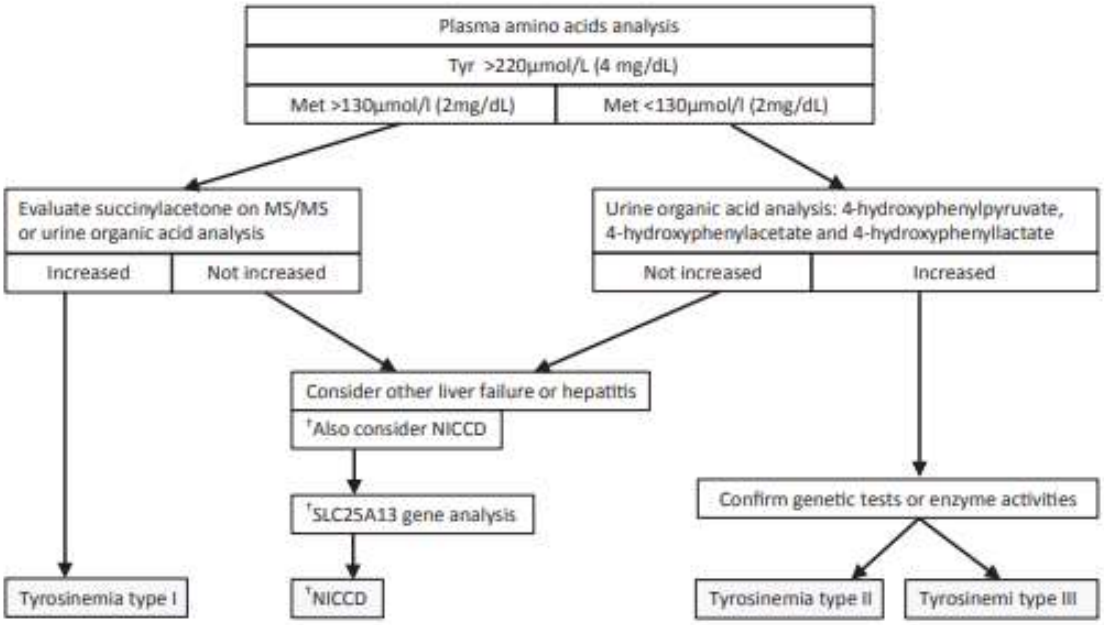

Figure II.5. Diagnosis algorithm of hereditary tyrosinemia (Nakamura *et al.*, 2015). NICCD: Neonatal intrahepatic cholestasis produced by citrin deficiency. MS/MS: mass spectrometry.

CHAPTER III.

AMINO ACIDS ANALYSIS

Chapter III. Amino acids analysis

Quantitative analysis of AAs is an important tool for diagnosis of AAs disorders and nutritional monitoring of individuals with already established diagnosis. Accurate quantification of AAs in biological samples is essential for the early detection and management of these disorders (Sandlers, 2019). Even AAs detection is established in most biological fluids, however, the most known fluids for AAHs diagnostic and monitoring are blood, plasma, and urine. In few cases, CSF was used for analysis. Even though each disorder is biochemically characterized by abnormal levels of one or more AAs, quantitative analysis and interpretation are not restricted to those metabolites and consist from a panel of nearly 40 AAs and specific ratios (Pasquali and Longo, 2022). For example, along with plasma Phe level, it is important also to assess plasma Phe/Tyr ratio that can be used to differentiate between PKU and non-PKU hyperphenylalaninemia.

III.1. Influencing factors

There are different factors affecting the AAs analysis and represent a significant analytical challenge, amongst chemical characteristics, age, groups variability and a wide range of normal physiological levels (Haschke-Becher, Kainz and Bachmann, 2016; Yamamoto *et al.*, 2016).

Some drugs cause signal artifacts by interaction with AAs metabolism. Some anticoagulants used during sample collection contain interfering constituents. For example, blood sodium bisulfate with heparin can produce a peak of S-sulfocysteine, suggesting a false sulfite oxidase deficiency. Ethylenediaminetetraacetic acid (EDTA) additive in collection tube can yied ninhydrin-positive peaks. Consequently, the most preferred for the blood collection are lithium-heparin coated tubes (Sandlers, 2019).

Diet plays a crucial role in influencing AAs levels in the body (Conley *et al.*, 2011; Ottosson *et al.*, 2016). For exemple, the consumption of meat and poultry has been associated with elevated excretion of β-alanine and 1-methylhistidine. Therefore, it is advisable to collect blood samples for AAs analysis after an overnight fast. Additionally, factors like urinary bacterial contamination can significantly affect the urinary AAs profile (Sandlers, 2019).

Hemolysis is another significant factor that can interfere with AAs analysis. It can cause a decrease in arginine levels while simultaneously increasing ornithine due to the activity of arginase in red blood cells.

Additionally, hemolysis can lead to elevated taurine levels released from leukocytes and platelets. Serum is generally not recommended for AAs analysis because the blood must clot at room temperature, which results in the conversion of asparagine to aspartic acid and glutamine to glutamic acid. A 24-hour urine collection is preferred for urine analysis; however, an overnight collection may suffice for diagnostic purposes. To minimize artifacts, no preservatives should be added to the urine sample (Sandlers, 2019).

In general, prolonged storage of samples results in a decrease in glutamine and asparagine levels, while glutamic acid and aspartic acid levels increase concomitantly (Sandlers, 2019).

When analyzing CSF, it is crucial to ensure that the sample is free from blood contamination which can lead to nonspecific increases in various AAs, potentially obscuring important diagnostic results (Sandlers, 2019).

III.2. Analytical methods used in amino acids quantitative analysis

III.2.1. High performance liquid chromatography

HPLC is a sophisticated analytical technique commonly used in biochemistry and analysis to separate, identify, and quantify active chemicals. In HPLC, the solvent is pushed through the column under high pressures of up to 400 atmospheres, allowing the sample to be separated into different constituents based on differences in relative affinities. The HPLC system (**Figure III.1**) generally comprises a column containing a packing material (stationary phase), a pump that drives the mobile phase(s) through the column and a detector that identifies the molecule retention times. This later is affected by the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) utilized. The samples to be analyzed are added in small quantities to the mobile phase stream and are slowed by specific chemical or physical interactions with the stationary phase (Sadaphal and Dhamak, 2022).

Any miscible combination of water or organic liquids can be used as a solvent. Gradient elution has been used to change the mobile phase composition during the analysis. The gradient separates analyte mixtures based on the analyte's affinity for the current mobile phase. The nature of the stationary phase and the analyte influence the choice of solvents, additives, and gradients (Sadaphal and Dhamak, 2022).

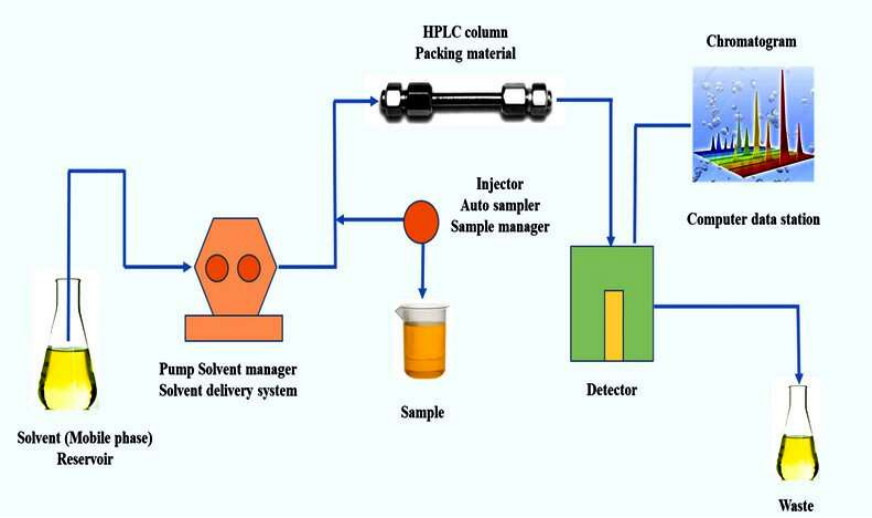

Figure III.1. Principal of high-performance liquid chromatography (Bhati *et al.*, 2022)

HPLC is a widely used analytical technique for the determination of AAs in various samples. Different methods have been developed to optimize the analysis, focusing on aspects such as derivatization, column selection, and mobile phase composition.

HPLC can be classified into several categories based on different criteria: preparative and analytical according to operation scale, affinity chromatography, adsorption chromatography, size exclusion chromatography, ion exchange chromatography and chiral phase chromatography according to separation principle, gradient and isocratic separation regarding the elution technique and finally normal phase and reverse-phase chromatography according to the modes of operation (Deshmukhe, Charde and Chakole, 2021; Sadaphal and Dhamak, 2022).

III.2.2. Mass spectrometry method

Mass spectrometric analysis relies on the separation of ions based on their mass-to-charge ratio (m/z) (Thomas *et al.*, 2022). The plot of ion abundance versus m/z is called mass spectrum, however in various cases the x-axis is labelled ‘mass’ rather than m/z. The spectrum is expressed in terms of Daltons (Da) per unit charge(Glish and Vachet, 2003). For small molecules (<1000 Da), there is classically only a single charge; thus, the m/z value is the same as the mass of the molecular ion. Nevertheless, once larger molecules like proteins or peptides are measured, they classically carry multiple ionic charges, and the z value is a greater than 1 (Garg and Zubair, 2024). Mass spectrometry (MS) primarily provides insights based on the analysis of gas-phase ions.

A mass spectrometer consists of three key components which are ionization source, mass analyzer and detector (**Figure III.2**) (Glish and Vachet, 2003).

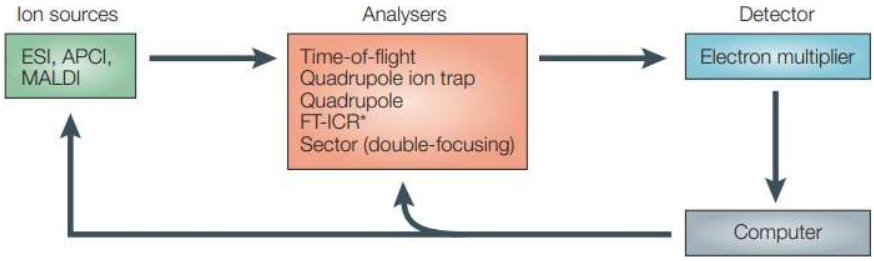

Figure III.2. Components of a mass spectrometer. *FT-ICR does not use an electron multiplier. APCI, atmospheric-pressure chemical ionization; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; FT-ICR, Fourier transform ion-cyclotron resonance(Glish and Vachet, 2003).

Various types of mass analyzers are employed in these analyses, including: triple quadrupole, magnetic sector, radio-frequency ion trap, time-of-flight (TOF), orbitrap, ion cyclotron resonance. In addition to the mass analyzers, there are several methods for generating ions in the gas phase prior to MS analysis. These ionization techniques include: electron ionization, chemical ionization, electrospray ionization (ESI), photoionization, inductively coupled plasma and matrix-assisted laser desorption ionization (MALDI) (Thomas *et al.*, 2022).

III.2.3. Coupling methods in mass spectrometry

III.2.3.1. Tandem mass spectrometry

MS/MS involves combining two or more mass analyzers in series to perform multiple stages of mass analysis. This technique allows for the fragmentation of selected precursor ions and the subsequent analysis of the resulting product ions. One common approach to MS/MS is to use two or more identical types of mass analyzers in series, such as: triple quadrupole analyzer or TOF/TOF analyzer. By combining two or more mass analyzers in series, MS/MS enables researchers to perform more sophisticated and informative analyses, making it a powerful tool in metabolomics analysis (Thomas *et al.*, 2022).

MS/MS offers several advantages such as improved selectivity and specificity so providing more detailed information about the structure and composition of analytes, enhanced sensitivity allowing for the detection of low-abundance compounds and finally better structural elucidation (Thomas *et al.*, 2022).

III.2.3.2. Liquid chromatography coupled to tandem mass spectrometry

Based on differential equilibrium between a mobile phase and a stationary phase, molecules are separated in LC. Using LC-MSMS aids the separation of the analyte(s) of interest from complex matrix components, improving specificity by separating interferences that cannot be differentiated by the mass spectrometer and improving sensitivity and imprecision (Thomas *et al.*, 2022).

A specimen is pretreated by dilution or via purification, and then injected onto the LC system for quantitative analysis (**Figure III.3**). This pretreatment can be simple (for example, dilution or protein precipitation) or more complex (Thomas *et al.*, 2022).

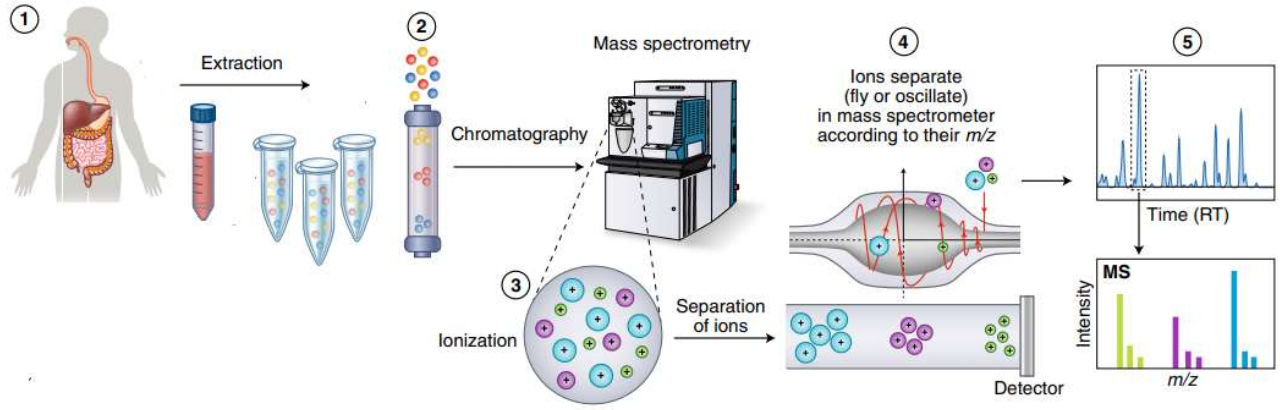

Figure III.3. Analysis workflow in mass spectrometry (Alseekh *et al.*, 2021). (1) sample pretreatment and extraction; (2) metabolite separation on a column (chromatography) (3) ionization of metabolites by ion source; (4) separation on a mass analyzer as ions oscillate on the basis of their mass-to-charge (m/z) ratio; and (5) detection.(with modification)

In order to precipitate the proteins out of solution, an organic solvent or acid are added to a fluid sample such as whole blood or serum/plasma. The supernatant can be evaporated after centrifugation and reconstituted to concentrate the analyte if required (Thomas *et al.*, 2022).

The analyte carried by LC eluent is introduced into the source of the mass spectrometer and subsequently a gas phase ions are produced. The first mass analyser chooses in the incoming ions the precursor ions which move forward towards the collision cell. The precursor ions are fragmented to product ions in the collision cell, which are analysed in the last point of the tandem mass spectrometer (**Figure III.4**). The resultant mass spectrum represents the product ions only; when concentrating only on particular precursor/product ion pairs, quantitative analyses can be achieved using a technique called selected reaction monitoring (MRM) (Thomas *et al.*, 2022).

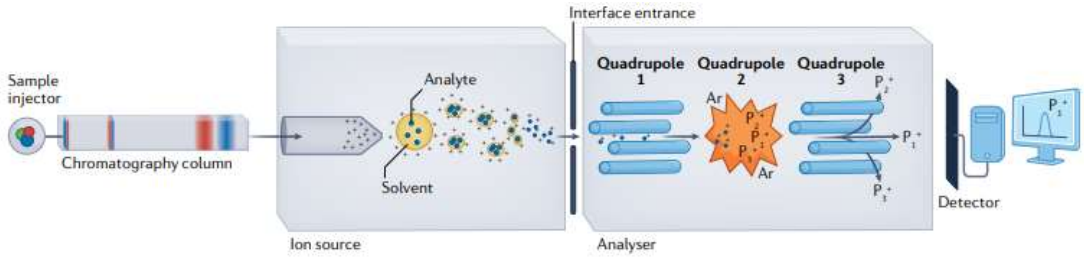

Figure III.4. Principal of liquid chromatography–tandem mass spectrometry. Electrospray ionization shown as the ion source together with a triple quadrupole analyzer (Thomas *et al.*, 2022)

III.2.3.3. Gas chromatography coupled to tandem mass spectrometry

Gas chromatography (GC) separates components of a gases mixture and commands the passage of these molecules according to their physical characteristics such as shape, molecular weight, size, and boiling point. The introduced sample is diluted and vaporized in the chromatograph and subsequently separated. Then after, the gases arrive to the mass spectrometer for analysis. Particularly, a GC sample should be volatile and it does not break down when it was in the mass spectrometer apparatus (Garg and Zubair, 2024).

III.3. Sample preparation in amino acids analysis

The choice of method for AAs analysis depends on the specific requirements of the analysis, including sample type, required sensitivity, and available equipment.

Methods that avoid derivatization tend to simplify the workflow, while those incorporating derivatization can enhance sensitivity and specificity. Each approach has its advantages and limitations, making it essential to select the appropriate method based on the analytical goals.

Sample preparation is essential to successful MS or LC analysis, principally when analyzing complex matrices. This typically involves many steps (Garg and Zubair, 2024). A specimen is pretreated by dilution or via purification, and then injected onto the LC system for quantitative analysis. This pretreatment can be simple (for example, dilution or protein precipitation) or more complex for example, solid phase extraction (SPE), liquid–liquid extraction (LLE) or supported liquid extraction (SLE). Sample extracts are not as clean as with more complex preparation with simple sample preparation. Extracts will still contain significant quantities of sample matrix that could cause issues with the robustness and reproducibility of the LC-MSMS method. As a first step in sample preparation. An internal standard is added in the same amount to all calibrators, quality control materials and patient samples, to ensure that the internal standard goes through all steps of analysis along with the analyte of interest. Before injection, samples can be diluted with a mobile phase or water. The diluted sample should first be centrifuged and/or filtered to eliminate particulates (Thomas *et al.*, 2022).

III.3.1. Direct analysis without derivatization

Detecting AAs using UV absorption typically relies on the carboxyl group (-COOH), which absorbs light in the 200 to 210 nm range. While certain AAs featuring benzene rings can be detected in the 250 to 280 nm range, analyzing them directly often presents challenges in achieving adequate sensitivity and selectivity (Garg and Zubair, 2024).

III.3.2. Derivatization method

To address difficulties associated with direct AAs analysis, derivatization methods have been widely adopted. Given that many AAs possess amino groups (-NH2 and -NHR), a derivatizing reagent that specifically targets the amino group is employed to enhance detection. Derivatization can be defined as the procedure of chemically modifying the target compounds to be more favorably analyzed (Garg and Zubair, 2024).

Derivatization typically involves adding of some well-defined functional group. Derivatization aims to increase volatility and thermal stability, modify chromatographic properties, increase ionization efficiency and favorize fragmentation properties (Garg and Zubair, 2024).

III.4. Examples of analytical methods commonly used

The most commonly used methods used for AAs analysis in laboratory medicine field will be described in the next paragraphs.

There is several LC methods coupled with UV absorbance detection available for the quantification of AAs. The two general approaches are either reversed-phase high performance liquid chromatography (RP-HPLC) or ion exchange chromatography (IEC) followed by post-column derivatization or by pre-column derivatization (Kaspar, 2009).

III.4.1. Ion exchange chromatography

IEC is a separation technique that uses the differences in charge states between the mobile phase and the analytes interacting with the stationary phase within the column. This method is primarily utilized for the analysis of ionic compounds. There are two main types of IEC: anion exchange chromatography and cation exchange chromatography. The choice between these two types depends on the ionic strength of the ion exchange group being used ('Basics of chromatography', 2024).

In the early 1950s, the publication by Moore and Stein on the separation of plasma AAs using a polystyrene resin column marked a significant advancement in quantitative AAs analysis. This development, along with the subsequent automation of the technique, introduced the principle of IEC with post-column derivatization. This method remained the gold standard for clinical AAs analysis for many years. Today, despite numerous methodological advancements, IEC utilizing a lithium buffer system, followed by post-column derivatization with ninhydrin and UV detection, is still employed in clinical settings (Csapó *et al.*, 2008).

The standard sample preparation for IEC in AAs analysis involves deproteinization using 35% (w/v) sulfosalicylic acid (SSA) added to the biological fluid. It is recommended to use a ratio of one volume of SSA to ten volumes of plasma. A fixed amount of a non-physiological AA, which serves as an internal standard, is added to all samples. After a brief incubation period, the sample undergoes centrifugation and filtration, rendering it ready for injection and separation by IEC.

A more traditional approach involves hydrolyzing proteins with hydrochloric acid (HCl) to release AAs, which are then separated using an ion-exchange HPLC column. This method is reliable but may require more complex sample preparation compared to newer techniques. The hydrolysis step is critical, as it can affect the recovery of certain AAs like tryptophan and cysteine (Bartolomeo and Maisano, 2006).

In IEC, the separation of AAs is facilitated by ionic interactions between the AAs and the functional ligands attached to the stationary phase of the column. The chromatographic column is filled with negatively charged resins. When the sample is introduced at a low acidic pH, all AAs carry a positive charge and strongly interact with the column. By manipulating the buffer composition during the separation process, the pH and salt concentration are altered, which changes the charge status of the AAs. As the isoelectric point is reached, the AAs become uncharged and exhibit weaker interactions with the charged column (Sandlers, 2019).

Ionic interactions strength is key factor in the separation of multiple AAs. The rapid of AAs elution depend is observed with the weakest ionic interactions. AAs are mixed with a post-column reagent after column elution and are detected. The most communal post-column derivatization is reaction with ninhydrin that products a purple Ruhemann's chromophore (λmax = 570 nm,) for α-AAs and yellow product with secondary amines (λmax = 440 nm) for like proline and hydroxyproline (Friedman, 2004).

III.4.2. Reverse-phase high performance liquid chromatography

Recently, RP-HPLC appeared as an alternative to the IEC. In this method, hydrophobic interactions between the analyzed AA in the mobile phase and the immobilized hydrophobic ligands attached to the nonpolar column stationary phase represent the base of the separation. RP-HPLC offers a high peaks resolution (Rs) of closely related molecules under a defined range of chromatographic conditions(Sandlers, 2019).

In the case of optical detection, derivatization using o-phthalaldehyde (OPA) as a pre- or post-column reaction, can be used. In the reaction, the presence of thiol such as 2-mercaptoethanol produce a stable fluorescent product which can be detected by UV (340 nm) or fluorescence (excitation 340 nm and emission 410 nm) (Sandlers, 2019).

More advanced Ultra-high performance liquid chromatography (UPLC) systems employing a small particle size (typically 1.7 µM) and a high pH range stable columns is used. High pressure and less solvent are used in these systems which permits an excellent Rs attained in a short time frame. Narayan et al. found that UPLC method is comparable to the reference IEC and hence adaptable to the clinical laboratory (Narayan *et al.*, 2011). A modified reversed-phase ultra-high performance liquid chromatography (RP-UPLC) method was developed by Peake et al. developed and achieved a better Rs for Tyr, glycine, arginine and homocitrulline peaks (Peake *et al.*, 2013).

An effective method involves online precolumn derivatization of AAs using OPA for fluorescence detection. This method is particularly suited for plasma AAs and allows for a linear assay from 5 to 1000 µmol/L. The HPLC system employs a gradient elution method with two mobile phases, enabling the separation of derivatized AAs with high precision and recovery rates between 91% and 108% (Frank and Powers, 2007).

Furthermore, a semi-automated method for AAs analysis includes derivatization with OPA and uses a RP-HPLC setup. This method has been validated for protein biopharmaceuticals and emphasizes the importance of hydrolysis and derivatization for accurate quantification. The method shows high specificity and reproducibility, making it suitable for detailed AA profiling in complex biological samples (Bartolomeo and Maisano, 2006).

Finally, even IEC, RP-HPLC, and RP-UPLC techniques have an acceptable reproducibility and a high sensitivity in the low concentration range, but they all are carried out with optical detection. The main disadvantage of this detection is its low specificity. This can possibly cause to the false findings. For example, in a standard IEC method, amoxicillin and ampicillin co-elute with Phe and it can be reported as falsely elevated (Sandlers, 2019).

III.4.3. Hydrophilic interaction liquid chromatography

An alternative method for separating polar compounds is hydrophilic interaction liquid chromatography (HILIC). This technique utilizes a polar stationary phase, which can include materials such as bare silica, amide, hydroxyl, cyano, amino, and ion-exchange columns.

HILIC is typically paired with RP solvent systems (Sandlers, 2019). The separation process begins with a high percentage of organic solvent, usually ACN, and compounds are eluted by gradually increasing the water content in the mobile phase (Sandlers, 2019).

Langrock et al. demonstrated the successful separation of 16 proteinogenic AAs within 25 minutes using an amide column coupled with ESI-MS/MS. The method enabled the separation of various hydroxyproline isomers (trans-4-Hyp, trans-3-Hyp, and cis-4-Hyp) found in collagen hydrolysates, achieving detection limits below 50 pmol for the hydroxyproline isomers (Langrock, Czihal and Hoffmann, 2006). This highlights the effectiveness of HILIC in analyzing polar compounds, particularly in complex biological samples.

A method developed for the determination of 17 AAs utilizes a HILIC column with a UV detector. This approach allows for the analysis of AAs without the need for derivatization, simplifying sample preparation and reducing costs. The method demonstrates good accuracy and precision (Bhandare *et al.*, 2010).

III.4.4. Flow infusion tandem mass spectrometry (FIA-MS/MS)

Flow infusion tandem mass spectrometry (FIA-MS/MS) method represents an alternative high throughput and specific technique for the AAs analysis. It is also possible to separate AAs by LC prior to the MS analysis, however, it is time-consuming in clinical settings. In addition, MS/MS method is used for the high throughput, cost-effective AAs analysis (Bruno *et al.*, 2016).

Blood samples are collected on filter paper, disks are punched out of the paper and are extracted for NBS. The primary assays required derivatization by butylation to improve detection limits and minimize ion suppression effects in a complex biological matrix (Bruno *et al.*, 2016).

Presently, some clinical laboratories skip on the derivatization step and no chromatographic separation is performed. Extracted and derivatized samples are directly injected to the mass spectrometer. Every analyzed AA is assayed with the corresponding stable isotopic labeled standard (ILS). The ILSs are closely related to the structure of the analyzed AAs and thus have similar physicochemical properties, but can be differentiated by MS analysis as they have a different (m/z) ratios. They are added at a known quantity to normalize the response of each analyzed (Sandlers, 2019). This is aimed to reduces a systematic error due to the poor recovery and decreases multiple matrix effects. The inclusion of ILS also corrects a batch-to-batch variability (Sandlers, 2019).

FIA-MS/MS has a mean limitation in differentiating AAs owing the same m/z such as leucine/isoleucine and hydroxyproline (butylated derivatives m/z 188) and alanine/sarcosine (butylated derivatives m/z 146). In addition, FIA-MS/MS cannot analyze cysteine and homocysteine since these AAs are not stable and react to form cystine and homocysteine which produce double charged molecules during the ionization process and it complicates the analysis (Sandlers, 2019).

III.4.5. Ion pair reversed-phase liquid chromatography – tandem mass spectrometry method

Underivatized AAs can be analyzed by ion pair reversed phase liquid chromatography – tandem mass spectrometry (IP-LC-MSMS). Direct analysis without derivatization reduces sample preparation and minimizes both reagent interferences the errors introduced by reagent and derivative instability. Typically, RP-C18-HPLC columns in combination with charged hydrophobic species used as Ion pairing reagent (IP-reagent) (Kaspar, 2009).

The IP-reagent creates a charged surface with the inorganic counterions after its adsorption at the interface between the stationary and mobile phase, and thus can forming a corresponding diffuse layer. Therefore, the IP-reagent creates an electrostatic surface potential.

Piraud et al. studied HPLC separation on a C18 column with tridecafluoroheptanoic acid (TDFHA) as IP reagent coupled to MS/MS for AAs analysis. MRM was used for quantification and a total of 76 AAs were quantified in less than 20 min (Piraud *et al.*, 2005).

De Person *et al*, studied the influence of five perfluorinated carboxylic acids used as IP reagent on MS response. They concluded that signal intensity depended on type and concentration of IP reagent, as well as MS interface geometry (de Person, Chaimbault and Elfakir, 2008).

III.2.5.6. Gas chromatography mass spectrometry method

Silylation is the derivatization procedure most commonly employed in GC-MS, by using reagents such as N,O-bis-(trimethylsilyl)- trifluoroacetamide (BSTFA) or N-methyl-trimethylsilyltrifluoroacetamide (MSTFA) (Sandlers, 2019).

GC analysis of silylated AAs is possible, but not all produced derivatives are stable; for example, arginine decomposes to ornithine, and glutamic acid form pyro-glutamic acid by rearrangement (Sandlers, 2019).

In addition, the sensitivity of the reagents and derivatives to moisture is another drawback (Halket *et al.*, 2005; Krumpochova *et al.*, 2015).

Another method is the derivatization of AAs with alkyl chloroformates and alcohol. Amino groups are converted directly to carbamate and carboxylic groups to esters. The reaction is catalyzed by pyridine or picoline (Villas-Bôas *et al.*, 2003).

AAs can be derivatized in aqueous solution directly without prior removal of proteins. The AAs react very rapidly with propyl chloroformate and the derivates can be extracted by an organic solvent and the extracted aliquot can be injected directly into the GC-MS apparatus (Villas-Bôas *et al.*, 2003).

Fluorinated alcohols produce more volatile compounds and have been used to the separation of AAs enantiomers. Fluoroalkyl chloroformates were used for the analysis of AAs by GC/MS and more than 30 AAs were separated within 10 min (Zampolli *et al.*, 2007).

III.5. Comparison of different methods used in amino acids quantitative analysis

Table III.1 represents a comparison between different methods available for the quantitative analysis of AAs.

In all LC methods whatever the detection method used, complete automation is difficult because of the necessity of protein precipitation. Even LC methods coupled with optical detection presents high reproducibility, however, pre- and post-column derivatization protocols employing OPA or ninhydrin has extended chromatographic run times, so they are less suited for large clinical and epidemiological studies and presents a lack of analyte specificity compared to MS methods. MS methods are subjected to matrix and ion suppression effects that damage quantitative accuracy and necessitate the use of internal standards. However, HILIC-MS allow the direct analysis of AAs without derivatization (Sandlers, 2019).

GC-MS has excellent reproducibility in retention time, particularly with alkyl chloroformate derivatization. However, thermo-labile derivatives cannot be quantified. Direct flow injection analysis with ESI-MS/MS is widely used in NBS. The one major drawback is the inability to resolve isobaric AAs (Kaspar, 2009).

Commercialized solutions ranging Kits to dedicated instruments reflects the importance of AAs analysis. The development and the improvement of methods is challenging. The focus of research are reduction of sample preparation and analysis time, extension of the analyte spectrum covered, improving sensitivity, robustness and reproducibility (Sandlers, 2019).

Table III.1. Comparison of different methods used in amino acids quantitative analysis (Kaspar, 2009)

Method	LOD/LOQ	Advantages	Disadvantages
LC-methods coupled with optical detection	UV: 5 µM (LOQ)	• Reproducible • Inexpensive • Good linearity in broad range	• Protein precipitation and derivatization required • Poor analyte specificity • No distinction to co-eluting substances
HILIC	5 µM (LOD) 10 µM (LOQ)	• Derivatization not required • Well-suited with MS • Compatible for polar compounds	• Protein precipitation required • Lack of reproducibility • Ion suppression for MS detection
Direct infusion MS/MS		• No separation required • High throughput	• Difficult to automate • Extraction and derivatization needed • Isobaric amino acids cannot be analyzed
IP-LC-MSMS	0.0003 - 9 µM (LOD)	• Derivatization not required • High number of analytes covered especially polar amino acids	• Protein precipitation required • Ion suppression • Contamination of used system with IP reagent
UPLC-MS	1.3 - 5.3 µM (LOQ)	• Rapid separation • High resolution	• Protein precipitation required • Special equipment required • Limited number analytes covered • Ion suppression
GC-MS	0.03 - 19.98 pmol (LOD)	• Robust • Reproducible • High resolution • Rapid separation	• Derivatization required • Not suitable for thermolabile amino acid derivatives

III.6. Methods used for phenylalanine and tyrosine determination

Various methods have been reported for the quantification of Phe and Tyr levels in serum or plasma (Turnell and Cooper, 1982; Hong and Tang, 2004; Pecce *et al.*, 2013), including enzymatic method (De Silva, Oldham and May, 2010), HPLC method (Mo *et al.*, 2013; Neurauter *et al.*, 2013), GC-MS (Laurens *et al.*, 2001; Deng *et al.*, 2002), LC-MS (Hardy *et al.*, 2002; Peat and Garg, 2016) and MS/MS methods (Hardy *et al.*, 2002; Dhillon *et al.*, 2011).

As is shown in **Figure III.5** various detectors used in LC analysis were compared in term of sensitivity. The higher sensitivity is observed with MS detector followed by fluorescence detector and then after by UV detector ('Basics of chromatography', 2024). Therefore, among the previously cited methods, LC-MSMS and HPLC with fluorescence detection methods are commonly preferred for the analysis of these metabolites.

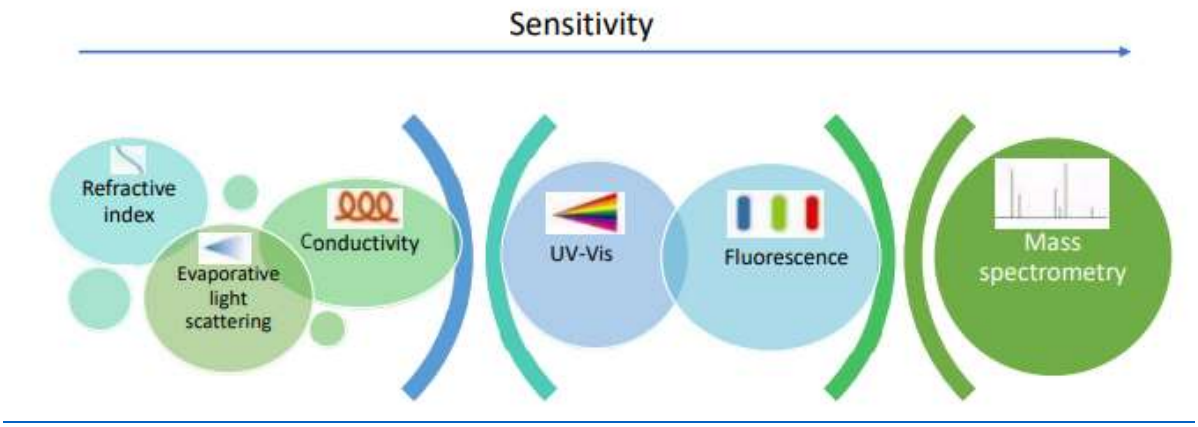

Figure III.5. Various detectors in LC systems along with their increasing sensitivity ('Basics of chromatography', 2024)

However, these methods have certain drawbacks, such as the requirement of using a complex derivatizing agent or sophisticated sample preparation(Hardy *et al.*, 2002; Dhillon *et al.*, 2011; Neurauter *et al.*, 2013; Peat and Garg, 2016).

Recently, the utilization of LC-MSMS has significantly increased (Gouda and Nazim, 2020; Pickens and Petritis, 2020). However, MS/MS technology is not appropriate for the routine monitoring of Phe and Tyr levels in patients because of its high cost (Mo *et al.*, 2013). Consequently, for more effective monitoring of patients with metabolic diseases(Smon *et al.*, 2019), it is recommended to use a more economical analytical system such as the HPLC system. HPLC was used in PKU screening to determine Phe and Tyr in serum (Iwasaki *et al.*, 2012) plasma (Kand'ár and Žáková, 2009) and dried blood-spot specimens (Jeong *et al.*, 2009).

The advancements in the analysis of Phe and Tyr during the last twenty-five years, through various analytical approaches are highlighted in **Table III.**

Table III.2. Various methods reported in the analysis of phenylalanine and tyrosine in biologic samples

Title	Technique	Sample type	Derivati-zation	LOD/LOQ	Statinnary phase	Mobile phase	Reference
Determination of phenylalanine and tyrosine in plasma and dried blood samples using HPLC with fluorescence detection	HPLC with fluorescence detection	DBS and Plasma	/	Phe: 10.0 µmole/L Tyr: 5.0 µmole/L	LiChroCart 125-4, Purospher RP-18e	EthOH:W (5:95%)	(Kand'ár and Žáková, 2009)
Determination of Phenylalanine and Tyrosine by High Performance Liquid Chromatography-TandemMass Spectrometry	HPLC-MS	Serum	/	/	Supelcosil, LC-18	A: acidified Water B: acidified ACN	(Peat and Garg, 2016)
Quantitative determination of plasma phenylalanine and tyrosine by electrospray ionization tandem mass spectrometry	HPLC-MS	Plasma	Butanolic HCl	1 µmole/L		80%ACN	(Hardy *et al.*, 2002)
Determination of phenylalanine and tyrosine in dried blood specimens by ion-exchange chromatography using the Hitachi L-8800 analyzer	HPLC	DBS	Ninhydrin	/	/	/	(Allard *et al.*, 2004)
Comparison of fluorescence reagents for simultaneous determination of hydroxylated phenylalanine and nitrated tyrosine by high-performance liquid chromatography with fluorescence detection	HPLC with fluorescence detecti	Serum	/	Nitro Phe, Nitro Tyr: 0.1 µmole/L	Capcell Pak MG II (ODS)	A: 20 mM sodium phosphate buffer B: ACN	(Iwasaki *et al.*, 2012)
Determination of phenylalanine in blood by high-performance anion-exchange c hromatography–pulsed amperometric detection	-HPLC anion exchange with amperometric	DBS	Butanol HCl (MSMS)	0,002 mg/dL (LOD)	Carbopac PA1 (HPLC)	300 mM NaOH (HPLC) 80% ACN with 0.01% formic acid (MS/MS)	(Jeong *et al.*, 2009)

Title	Technique	Sample type	Derivati-zation	LOD/LOQ	Statinnary phase	Mobile phase	Reference
	detection - MS/MS			0,07 mg/dL(LOQ)			
Simultaneous determination of phenylalanine and tyrosine in peripheral capillary blood by HPLC with ultraviolet detection	HPLC-UV	Peripheral blood	/	/	Hypersil C8	5% ACN	(Mo *et al.*, 2013)
Simultaneous determination of tyrosine, phenylalanine and deoxyguanosine oxidation products by liquid chromatography–tandem mass spectrometry as non-invasive biomarkers for oxidative damage	LC-MSMS	Urine	N-(heptafluorobutyryl)imidazole (HFBI	0,5µM	CP Sil 8 CB	/	(Orhan *et al.*, 2004)
Reliable analysis of phenylalanine and tyrosine in a minimal volume of blood	LC-MSMS	DBS	Butanol HCl	Phe: 2 µmol/L Tyr: 1 µmol/L	/	90% ACN	(Prinsen *et al.*, 2013)
Rapid quantitative method for the detection of phenylalanine and tyrosine in human plasma using pillar array columns and gradient elution	Microchip with a pillar array and gradient elution	Plasma	NBD-F	/	Phe: 119 Tyr: 87.6 nM (LOD) Phe:395 Tyr: 293 nM (LOQ)	A: water/ACN/TFA (92/8/0.02, v/v/v) B: water/ACN/TFA (8/92/0.02, v/v/v)	(Song *et al.*, 2016)
Development of a simple method for the analysis of phenylalanine in dried blood spot using tandem mass spectrometry	LC-MSMS	DBS	/	0.1 mg/dl	Chromsystem Kits	ACN, Water (1:1 , V:V)	(Gouda and Nazim, 2020)
Amino acid analysis using core–shell particle column	HPLC with fluorescence detection	Mouse Plasma sample	NBD-F	/	core–shell Kinetex C18	water/ACN/TFA (86:14:0.12, v/v/v)	(Song, Funatsu and

Title	Technique	Sample type	Derivati-zation	LOD/LOQ	Statinnary phase	Mobile phase	Reference
							Tsunoda, 2013)
Hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry method for the simultaneous determination of L-valine, L-Leucine, L-isoleucine, L-phenylalanine, and L-tyrosine in human serum	Fluorescence HPLC hydrophilic -MS/MS	Serum	/	0.200 g/mL	Syncronis HILIC	ACN/120 mM ammonium acetate (89:11, v/v)	(Sun *et al.*, 2015)
Simultaneous measurement of phenylalanine and tyrosine by high performance liquid chromatography (HPLC) with fluorescence detection	HPLC +florescence detector	Serum	/	/	RP-C18	Aqueous 15 mM KH2PO4	(Neurauter *et al.*, 2013)
Amino acid analysis of dried blood spots for diagnosis of phenylketonuria using capillary electrophoresis-mass spectrometry equipped with a sheathless electrospray ionization interface	MS electrophoresis (CE-MS)	DBS	/	Phe: 0.03 Tyr: 0.07 mg/L	/	/	(Jeong, Kim and Park, 2013)
Optimization of an HPLC method for phenylalanine and tyrosine quantification in dried blood spot.	HPLC	DBS Serum	OPA	/	Zobrax Eclipse XDB C18	A: (40 mM sodium dihydrogen phosphate monohydrate) B: ACN /methanol/water 150 40:40:20, v/v/v)	(Pecce *et al.*, 2013)
Improved tandem mass spectrometry (MS/MS) derivatized method for the detection of tyrosinemia type I, amino	MS/MS	DBS	n-butanolic-HCl	/	/	ACN: water — 8:2 containing 0.05% formic acid	(Dhillon *et al.*, 2011)

Title	Technique	Sample type	Derivati-zation	LOD/LOQ	Statinnary phase	Mobile phase	Reference
acids and acylcarnitine disorders using a single extraction process							
High resolution mass spectrometry newborn screening applications for quantitative analysis of amino acids and acylcarnitines from dried blood spots	MS/MS	DBS	/	/	/	/	(Pickens and Petritis, 2020)
LC-MSMS method for the quantification of natural amino acids in mouse plasma: Method validation and application to a study on amino acid dynamics during hepatocellular carcinoma progression.	LC-MSMS	Plasma mouse	/	Phe: 5 Tyr: 6,25 µM	Intrada Amino Acid	A: 100 mM ammonium formate B: acetonitrile, water and formic acid (v:v:v = 95:5:0.3	(Liu *et al.*, 2019)
Comparison of liquid chromatography with tandem mass spectrometry and ion-exchange chromatography by post-column ninhydrin derivatization for amino acid monitoring	LC-MSMS	Plasma	Ninhydrin	/	TRAQ Kit	/	(Smon *et al.*, 2019)

Tyr: tyrosine; Phe: phenylalanine; DBS: Dry blood spots; ACN: Acetonitrile.

CHAPTER IV.

VALIDATION OF ANALYTICAL METHODS

Chapter IV. Validation of analytical methods

IV.1. Background

The purpose of validation is to ensure that the analytical method will give sufficiently reliable and reproducible results, taking into account the purpose of the analysis (Peris-Vicente, Esteve-Romero and Carda-Broch, 2015).

Consequently, method validation is the essential step that relies normally on the assessment of several analytical performance characteristics with the aim of verifying method's fit-for purpose.

The ICH has been at the front of generating harmonized guidelines for adoption in the United States, Japan, and the European Community. It issued two significant guidelines titled "Text on Validation of Analytical Procedures (Q2A)" and "Validation of Analytical Procedure Methodology (Q2B)." These guidelines provide a framework for validating analytical methods to ensure they are suitable for their intended purposes, enhancing consistency and reliability across different regulatory regions. In November 2005, these two guidelines were united into one document named Q2(R1), which further elaborates on the validation parameters necessary for various analytical methods (Sahoo1 *et al.*, 2018).

Acceptability limit of a method is used to quantify its objectives. This is an overall threshold value most often expressed as a percentage, preferably set by the user of the result. Uncertainty is a parameter associated with the result of a measurement which characterizes the dispersion of values that can reasonably be attributed to a measurement (Gustavo González and Ángeles Herrador, 2007).

Confidence interval (CI) determined at risk α% is an interval of values which has (1-α)% chance of containing the true value of the estimated parameter: for example, the confidence interval determined at risk of 5 % to 95% chance of containing the true value of the estimated parameter. The tolerance interval is the interval in which we expect to have a proportion (β%) of future results (Belouafa *et al.*, 2017).

Accuracy profile is the combination, in the form of a graph, of several tolerance intervals calculated at different concentration levels and an acceptability limit. Its aim is to estimate, from the results obtained during validation, what guarantee the user will have that the method used routinely will provide acceptable results (Gustavo González and Ángeles Herrador, 2007).

Calibration is the operation which, under specified conditions, establishes in a first step a relationship between the values and associated measurement uncertainties that are provided by standards, and the corresponding responses with their associated uncertainties. In a second step, this information is used to establish a relationship making it possible to obtain a measurement result from a response (Gustavo González and Ángeles Herrador, 2007).

IV.2. Regulatory context

Guidance on the validation of analytical methods is provided in publications such as ICH guidelines: "ICH guideline M10 on bioanalytical method validation and study sample analysis" published in 2022 (ICH, 2022). Its aim is to provide recommendations on how to approach the different characteristics of validation for each analytical method. Furthermore, the document provides an indication on the data that should be presented in a registration document.

There are also many official documents describing the validation criteria to be tested, but they do not propose an experimental protocol and are most often limited to general concepts. This is why two SFSTP commissions successively developed validation guides (in 1992 for analyzes of pharmaceutical specialties and in 1997 for analyzes in a biological environment) with the aim of concretely helping pharmaceutical manufacturers to apply regulatory recommendations. Although these first guides have largely contributed to the application and progress of analytical validations, they nevertheless present weaknesses in terms of the conclusions of the tests carried out and in terms of aiding decision-making with regard to acceptance limits defined for use of an analytical procedure (Hubert *et al.*, 2007).

This is why the SFSTP commission reviews the previous bases of analytical validation for a harmonized approach distinguishing in particular between diagnostic rules and decision rules. The update was published in 2003 and supplemented in 2006 (SFSTP Commission *et al.*, 2006). The latter are based on the use of the accuracy profile, based on the notion of total error (bias + standard deviation), making it possible to simplify the approach to the validation of an analytical procedure while controlling the risk associated with its use. **Tale IV.1** resume some SFSTP publications.

Table IV.1. SFSTP publications and their respective updates (Isabelle Pinguet, 2015).

SFSTP publication		
"Analytical validation guide – Report from an SFSTP commission"	I.	Methodology, published in STP Pharma Prat. (1992)
	II.	Application examples, published in STP Pharma Prat. (1992)
"Chromatographic methods for determination in biological media: validation strategy."	Report of an SFSTP commission" published in STP Pharma Prat. (1997)	
"Validation of quantitative analytical procedures: Harmonization of approaches"	I.	General, published in STP Pharma Prat. (2003)
	II.	Statistics, published in STP Pharma Prat. (2006)
	III.	Application examples, published in STP Pharma Prat. (2006)
	IV.	Application examples, published in Journal of Pharmaceutical and Biomedical Analysis (2008)

The validation protocol described in this section is according to the ICH guidelines (ICH, 2022) and also that of the SFSTP (SFSTP Commission *et al.*, 2006). We only present here the aspects which are related to the validation of a method for measuring a major compound.

IV.3. Accuracy profile

Among various factors determined in validation of analytical methods, precision and accuracy stand out as the most critical, often assessed independently. Therefore, methods are designed to achieve specific goals, such as ensuring repeatability <10% and maintaining an accuracy level within 10% of a reference value. It is important to note that accuracy, which refers to relative bias, can be either positive or negative. Consequently, this could lead to an overall error margin of up to 30% (**Figure IV.1**) (Jitaru *et al.*, 2016).

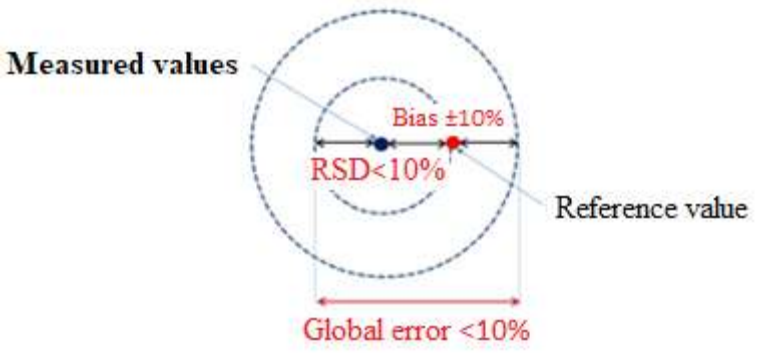

Figure IV.1. Precision and accuracy of an analytical method if interpreted individually (Feinberg, 2007).

The accuracy profile approach centers on a specific criterion known as the acceptability interval. This interval takes into account both the accuracy and precision of the analytical method simultaneously. In this approach, a β-ETI (typically ranging from 80% to 90%) is set a priori when constructing the accuracy profile. This means that the risk of expected results falling outside these limits is less than 10-20%. Additionally, method acceptance limits (λ) are established based on the desired criteria for repeatability and intermediate precision. For multi-element analysis methods, λ is generally set between 20-30%. According to the accuracy profile approach, a method is considered validated when the β-ETI for a given result falls entirely within the pre-determined acceptability limit (λ). **Figure IV.2** provides a graphical representation of a validated result using the defined and λ criteria. (Jitaru *et al.*, 2016).

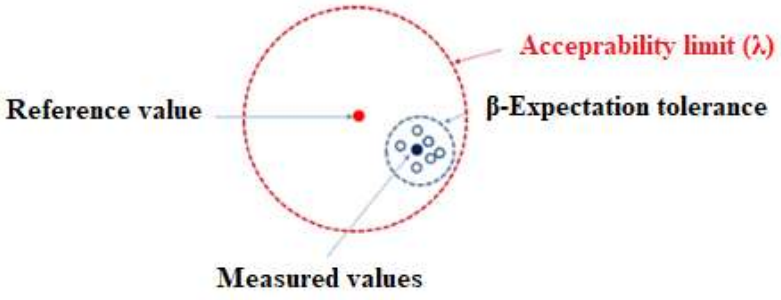

Figure IV.2. Example of valid result using the accuracy profile approach (Feinberg, 2007)

The accuracy profile approach has been extensively employed in the field of separation methods, particularly HPLC. This method involves estimating the bias and intermediate precision through multiple experiments conducted within a single series, typically over one day, and repeating these experiments across several series (days) (Mermet and Granier, 2012).

When multiple studies are performed with varying analyte concentrations, it is possible to construct an accuracy profile that takes into account both the bias of the collaborative study and the interlaboratory reproducibility. This approach relies on data gathered from a large number of participating laboratories. By adapting the accuracy profile method, researchers can gain a more robust understanding of method performance across a wide range of conditions (Mermet and Granier, 2012). **Figure IV.3.** illustrate an example describing an accuracy profile.

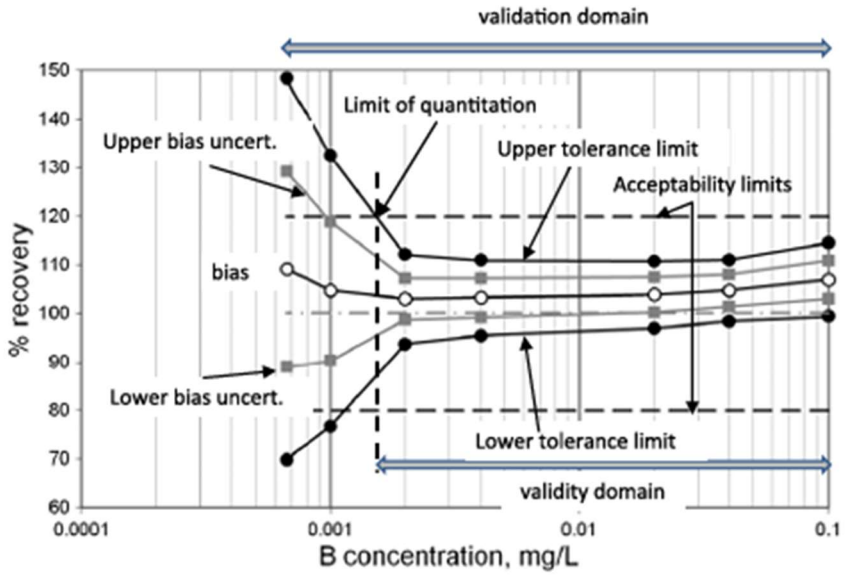

Figure IV.3. Illustration of accuracy profile with various tolerance and acceptability limits (intervals), bias uncertainties, limit of quantitation along with validity domain (Mermet and Granier, 2012)

IV.3.1. Advantages of the Accuracy Profile Approach

The accuracy profile approach offers several advantages (Jitaru *et al.*, 2016):

- It considers both accuracy and precision simultaneously, providing a more comprehensive assessment of method performance.
- The use of β-ETI allows for a quantifiable risk assessment, ensuring that results fall within acceptable limits.
- The graphical representation of the accuracy profile facilitates easy interpretation and communication of method validation results.

- By employing the accuracy profile approach, analysts can have confidence in the reliability and suitability of their analytical methods for specific applications.

IV.3.2. Construction of accuracy profile

To construct the accuracy profile, the steps are as follows (Isabelle Pinguet, 2015):

- Have the operating mode and define the measurand;
- Define the validation domain (range of concentrations) and the expected objective of the method in the form of an acceptability interval;
- Select validation samples whose reference values are known;
- Choose a validation experiment plan;
- For indirect methods, choose the calibration experiment plan;
- Collect data;
- For indirect methods, calculate the concentrations found by inverse calibration;
- Calculate the validation criteria from the concentrations found: mainly the standard deviations of intermediate precision and accuracy biases;
- Calculate the tolerance intervals and construct the accuracy profile;
- Interpret the results and decide whether the method is valid or not.

IV.4. Experiment protocol according SFSTP guidelines

The determination of accuracy, precision and linearity is carried out on the same data coming from a calibration series and a validation series.

The flowchart presented in **Figure IV.4.** presents the procedure to follow to choose the experiment protocol. These different protocols are summarized in **Table IV.2**.

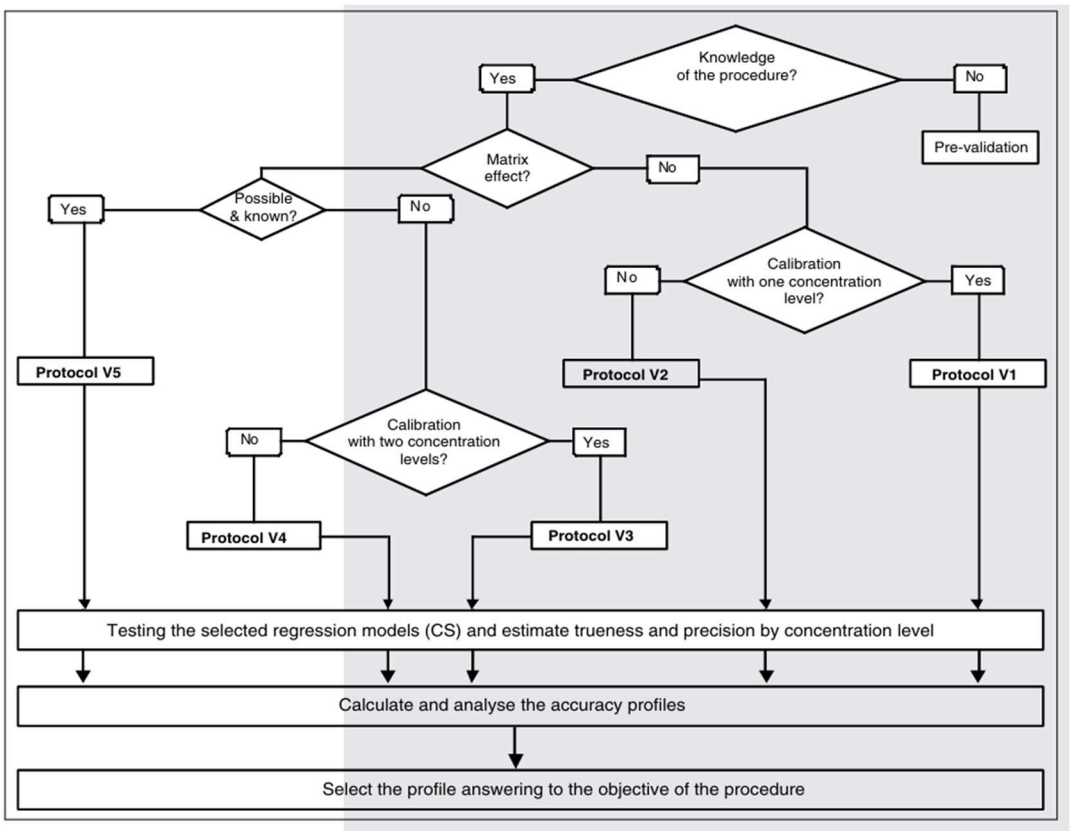

Figure IV.4. Algorithm to select a validation protocol (SFSTP Commission *et al.*, 2006).

CS: calibration standard. VS: validation standards

The total number of tests can be reduced or increased depending on the objective of the analytical procedure.

Table IV.2. Number of calibration and validation standards depending on the chosen protocol(SFSTP Commission *et al.*, 2006)

Standards		**Concentration levels**	**PROTOCOL**				
			V1	**V2**	**V3**	**V4**	**V5**
SE	**Calibration without matrix**	Down		2		2	
		Median	2	2(2)	2	2(2)	
		High	2(1)	2	2(1)	2	
	Calibration with matrix	Down				2	2
		Median			2	2(2)	2(2)
		High			2(1)	2	2
		Additional					2(3)

Standards	Concentration levels	PROTOCOL				
		V1	V2	V3	V4	V5
SV used for validation with matrix	Down	3	3	3	3	3
	Median	3	3	3	3	3
	High	3	3	3	3	3
Minimum number of series		3	3	3	3	3
Total number of attempts (minimum)		33	45	39	63	45

(1)Selecting a concentration level above the target concentration for calibration.

(2)Possible removal of the median concentration level for calibration based on the regression model used to express the response function. In this case, the total number is 39 trials for protocols V2 (without matrix) and V5 (with matrix). The number of trials is 51 for the V4 protocol.

(3)Addition of an additional concentration level for a response function requiring a more complex model (example: four-parameter logistic function).

The evaluation of intermediate precision can be carried out following an experimental plan, as illustrated in **Table IV.3**. This makes it possible to carry out the "p" series of experiments in conditions that are as representative as possible of the variability of routine use (day, operator, equipment, etc.).

Table IV.3. Example of experimental conditions for the evaluation of intermediate precision

	Series					
	1	2	3	4	5	6
Day	1	1	2	2	3	3
Operator	A	B	A	B	A	B

IV.4.1. Response function

The response function relating the response (signal or instrument response) Y and the concentration X is determined based on the calibration series. This relationship is expressed by the following equation:

$$Y = f(X) + \varepsilon \quad \text{(IV.1)}$$

- Or :ε is the residual error.

IV.4.2. Aligning responses and calculating inverse predictions

If, for a given concentration level, the quantities introduced are not identical for all the series, it is essential to align the responses to the average concentration.

The responses (aligned or not) are used to calculate the inverse predictions. The latter represent the concentrations of the solutions of the validation series, calculated by the established response function.

IV.4.3. Estimation and evaluation of validation criteria

IV.4.3.1. Specificity-selectivity

"The specificity of an analytical procedure is its ability to unequivocally establish the existence of the substance to be analyzed in the presence of other potentially present components" (SFSTP Commission *et al.*, 2006).

"Property of an analysis procedure to suit exclusively the characteristic or analyte, with the guarantee that the result of the analysis comes only from the analyte"(SFSTP Commission *et al.*, 2006)

The specificity can be:

- An intrinsic characteristic of the technique (for example: identification by IR spectrometry).
- Obtained by separation (for example: chromatography).
- Obtained mathematically (for example: solving simultaneous equations).

Specificity can be demonstrated indirectly by comparing the results of the analytical procedure in question to another procedure (SFSTP Commission *et al.*, 2006).

IV.4.3.2. Calculation of accuracy, precision and total error

The calculations presented in this protocol are applicable if the number of repetitions is the same in each series. The estimation of the accuracy and precision of the method is carried out with the inverse predictions

a) Accuracy

"The accuracy of an analytical method expresses the closeness of agreement between the average of the test results with the method and the accepted reference value, also called the conventionally true value" (SFSTP Commission *et al.*, 2006).

The accuracy (or bias) is obtained by calculating the difference between the average of the introduced concentrations and the average of the calculated concentrations. The bias can be expressed in absolute, relative or overlapping terms in relation to the quantities introduced:

$\mathbf{biais_j} = \hat{\mu}_j - \bar{x}_{.j.}$ **(IV.2)**	$\mathbf{biais}\ (\%)_j = 100 . \frac{\hat{\mu}_j - \bar{x}_{.j.}}{\bar{x}_{.j.}}$ **(IV. 3)**	$\mathbf{Recovery}\ (\%)_j = 100 . \frac{\hat{\mu}_j}{\bar{x}_{.j.}}$ **(IV.4)**

- $\hat{\mu}_j$**:** the average of the calculated concentrations.
- $\bar{x}_{.j.}$**:** the reference concentration.

b) Precision

The components of the variance parameters (inter-series variances) and (intra-series variances)$\sigma^2_{B,j}\sigma^2_{W,j}$ at each concentration level j are estimated as follows:

$$\mathbf{MSM_j} = \frac{1}{p-1}\sum_{i=1}^{p} n_{ij}\left(\bar{x}_{ij.,calc} - \bar{x}_{j.,calc}\right)^2 \quad \mathbf{(IV.5)}$$

$$\mathbf{MSE_j} = \frac{1}{\sum_{i=1}^{p} n_{ij} - p}\sum_{i=1}^{p}\sum_{k=1}^{n_{ij}}\left(x_{ijk,calc} - \bar{x}_{ij.,calc}\right)^2 \quad \mathbf{(IV.6)}$$

- **If MSEj < MSMj** :

$$\hat{\sigma}^2_{w,j} = \mathbf{MSE_j} \quad \mathbf{(IV.7)} \qquad \text{And} \qquad \hat{\sigma}^2_{B,j} = \frac{MSM_j - MSE_j}{n} \quad \mathbf{(IV.8)}$$

Otherwise :

$$\hat{\sigma}^2_{w,j} = \frac{1}{pn-1}\sum_{i=1}^{p}\sum_{j=1}^{k}\left(x_{ijk,calc} - \bar{x}_{j.,calc}\right)^2 \quad \mathbf{(IV.9)} \qquad \text{And} \qquad \hat{\sigma}^2_{B,j} = 0$$

- **Repeatability variance:** $\hat{\sigma}^2_{Re,j} = \hat{\sigma}^2_{W,j}$ **(IV.10)**

- **Intermediate precision variance:** $\hat{\sigma}^2_{F.I.,j} = \hat{\sigma}^2_{W,j} + \hat{\sigma}^2_{B,j}$ **(IV.11)**

- i: index representing the series.
- j: index representing the concentration level.

- k: index representing the number of concentration levels.
- calc: index representing the calculated concentrations.
- n: the number of repetitions in each series.
- p: the number of series.
- MSM (mean square model): sum of the squares of the model.
- MSE (mean square error): sum of the squares of the residual error. This is the variation attributed to the error.

Precision can be assessed at three levels (Isabelle Pinguet, 2015):

- Repeatability expresses fidelity under identical operating conditions and over a short time interval;
- Intermediate precision expresses intra-laboratory variations: different days, different analysts, different equipment, etc.;
- Reproducibility expresses inter-laboratory reliability.

c) Total error

The total error is the sum of the accuracy (bias) and the intermediate precision. It evaluates the ability of an analytical procedure to produce accurate results.

IV.4.3.3. Accuracy profile

The accuracy profile of the method is obtained by connecting the lower limits Lj to each other on the one hand and the upper limits Uj to each other on the other hand. Limits are calculated as follows:

– Lower limits:

$$\mathbf{L_j = biais(\%)_j - Q_t\left(\nu\,;\frac{1+\beta}{2}\right)\sqrt{1+\frac{1}{p\,n\,B_j^2}}\,CV_{F.I.,j}} \quad \textbf{(IV.12)}$$

– Upper limits:

$$\mathbf{U_j = biais(\%)_j + Q_t\left(\nu\,;\frac{1+\beta}{2}\right)\sqrt{1+\frac{1}{p\,n\,B_j^2}}\,CV_{F.I.,j}} \quad \textbf{(IV.13)}$$

$$\mathbf{R_j = \frac{\hat{\sigma}^2_{B,j}}{\hat{\sigma}^2_{W,j}}} \quad \textbf{(IV.14)}$$

$$\mathbf{B_j = \sqrt{\frac{R_j+1}{n\,R_j+1}}} \quad \textbf{(IV.15)}$$

$$\nu = \frac{(R+1)^2}{\frac{\left(R+\frac{1}{n}\right)^2}{p-1}+\frac{1-\frac{1}{n}}{p\,n}} \qquad \textbf{(IV.16)}$$

Qt: is the quantile β of the Student's t distribution atvdegrees of freedom.

$$Q_t\left(\nu\,;\,\frac{1+\beta}{2}\right)$$

The accuracy profile is a visual tool for evaluating the analytical procedure. The method can be considered accurate at the chance level β for a given concentration level, if the tolerance interval is included in the acceptance limits (-λ%, +λ%).

The acceptance limit (λ) can be variable depending on the objective of the analysis procedure, which is linked to the requirements commonly accepted by professional practice (for example: 1 or 2% on raw materials, 5% on pharmaceutical specialties, 15% in bioanalysis , environment, etc.) (SFSTP Commission *et al.*, 2006).

IV.4.3.4. Linearity

"The linearity of an analysis procedure is its ability, within a certain dosage interval, to obtain results directly proportional to the quantity (e.g. concentration) of analyte in the sample" (SFSTP Commission *et al.*, 2006).

"The linearity requirement applies to results (calculated concentration = f (introduced concentration)), not to responses (signal = f (introduced concentration))" (SFSTP Commission *et al.*, 2006).

IV.4.3.4. Limits of detection and quantification

LOD of an analytical procedure is the smallest amount of the analyte in a sample that can be detected, but not quantified as an exact value under the described experimental conditions of the procedure. The LOQ is the smallest quantity of the analyte in a sample that can be determined under the described experimental conditions with a defined accuracy (Isabelle Pinguet, 2015). The intersection, if it occurs, between the accuracy profile and the acceptance limits defines the low and high quantification limits of the procedure.

Limits of quantification are the extreme values which can be quantified with a defined accuracy and therefore define the dosage interval.

IV.5. Validation criterion according to ICH guidelines

In addition to validation criteria discussed in section **IV.4**, other specific criteria, such as those mentioned here, are also evaluated in the context of a biological analysis and figured in ICH guidelines.

IV.5.1. Matrix effect

A matrix effect is defined as an alteration of the analyte response due to interfering and often unidentified component(s) in the sample matrix. During method validation the matrix effect between different independent sources/lots should be evaluated (ICH, 2022).

IV.5.2. Carry-over

Carry-over is a variation of a measured concentration because of residual analyte from a preceding sample that remains in the analytical instrument (ICH, 2022).

IV.5.3. Selectivity and specificity

According to ICH guidelines, selectivity is the aptitude of an analytical method to distinguish and measure the analyte in the presence of potential interfering agents in the blank biological matrix, whereas, specificity is the ability of a bioanalytical method to detect and differentiate the analyte from other substances, including its related substances (ICH, 2022).

IV.6. Concept of “Fitness-for-purpose”

It is recognized that the goal of analytical method validation is to demonstrate that the method is fit for purpose.

This statement is reflected in the concept of "fitness-for-purpose", of which IUPAC gives the following definition: "degree to which the data produced by a measurement process allows the user to make correct technical and administrative decisions in a specified goal”. We can therefore translate this notion by the suitability of a method for the use for which it is intended. An analytical method will fit into this concept if the results it provides match the requirements of its application (Gustavo González and Ángeles Herrador, 2007).

We will see in this thesis how the application of the protocol proposed by the SFSTP in 2003-2006, based on the use of the accuracy profile and the tolerance interval β, makes it possible to apply this concept of "fitness-for -purpose” (Gustavo González and Ángeles Herrador, 2007) for analytical validation.

CHAPTER V.

MATERIAL AND METHODS

Chapter V. Material and methods

V.1. Material

This section provides a list of instrumentation, materials, chemicals, solvents, and reference/working standards of selected metabolites used during the entire method development, validation and application of the validated method to the sample analysis.

V.1.1. Chemicals and reagents

The details of used chemicals and reagents are provided in **Table V.1**.

Table V.1. Chemicals and solvents used in the study

Chemicals/Solvents	Grade/Reference	Vendor	City, Country
Phenylalanine	HPLC (P2126)	Sigma Chemical Company	USA
Tyrosine	HPLC (T3754)	Sigma Chemical Company	USA
Acetonitrile	LC/MS (75-05-8)	Merk Millipore	Germany
Acetonitrile	HPLC (A 1294)	VWR Chemicals	France
Trichloroacetic	Reagent grade (1201300500)	BIOCHEM	France
Formic acid	Reagent grade (18I054003)	VWR Chemicals	France
Control serum without amino acids	TB Chem, Reagent grade	IMD laboratoires	Algeria
Guthrie paper for DBS	CG012003752	CENTOGENE	Germany
Ammonium acetate	Reagent grade (300981000)	BIOCHEM	France
Hydrochloric acid (HCl)	Reagent grade (J1890)	Honeywell	France
Tetrahydrofuran (THF)	MS/MS (203-726-8)	Merck Millipore	Germany
Ammonium formate	Reagent grade (18J164125)	VWR Chemicals	Germany
Methanol	HPLC (34860)	Honeywell	France

V.1.2. Instrumentation and software

The details of used Instrumentation and software are provided in **Table V.2**.

Table V.2. Instrumentation and equipment used in the study

No.	Instrument	Model	Manufacturer
1	Mass Spectrometer	LCMS 8040	Shimadzu, Japan
2	Liquid Chromatograph	Nexera System	Shimadzu, Japan
3	Analog Heating Stirrer	RSLAB-1C	RS Lab
4	Data integration software	Lab solutions Version 5.65	Shimadzu Corporation, Japan
5	Analytical Balance	Adventurer	OHAUS
6	Micropipettes	10-100µL and 100-1000	Hamburg, Germany
7	Deep freezer	Pharmalow	J.P Selecta
8	Centrifuge machine	Sigma 1-14	Sigma
9	Refrigerator	Fiocchetti	Fiocchetti
10	Water purifier	Aguaplusro	Stakpure
11	pH meter	Starter 3100	OHAUS
12	Vortex	TK3S	Techno Kartell
13	Vacuum pump	NF-WP6122050	Millipore
14	Discovery HS F5-3 Penta fluoro phenyl propyl (PFPP)	2.1 mm × 150 mm, 3 µm column, P/N 567503-U	Sigma-Aldrich
15	Intrada amino acids	3 mm × 50 mm, 3 µm column, Prod# WAA32 Ser# UE27H4J	Imtakt Company
16	Microsoft Excel 2016	Version 2016	Microsoft
17	SPSS	Version 26	IBM SPSS statistics
18	DEO Design of experiment	Trial version 12.0.3.0	State-Ease Inc

- **Discovery HS F5-3 Penta fluoro phenyl propyl (PFPP) column**

The Discovery HS F5-PFPP column is a reversed-phase HPLC column with a 3 µm particle size and typical dimensions of 15 cm length × 4.6 mm internal diameter. It features a pentafluorophenyl bonded phase on high-purity, spherical, fully porous silica. The column is endcapped to reduce silanol activity, providing excellent peak shape and reproducible separations. It operates effectively within a pH range of 2 to 8, withstands temperatures up to 70 °C and up to 400 bars.

The Discovery HS F5-3 offers unique selectivity distinct from C18 phases (Liu and Rochfort, 2014).

- **Intrada amino acids column**

The Intrada Amino Acid column is a specialized LC-MS column designed for the direct, derivatization-free analysis of AAs. It features a unique stationary phase combining normal phase ligand chemistry and ion-exchange properties on pure spherical silica particles of 3 µm size, enabling excellent separation of polar and isobaric amino AAs. The column supports high-throughput analysis, separating up to 55 amino acids within 10 minutes, with options for even faster runs (under 1 minute) using shorter column lengths. It operates under pressures up to 250 bar and is optimized for LC-MS detection, providing superior sensitivity and resolution without the need for pre- or post-labeling steps. This makes it ideal for clinical, metabolomic, and food science applications with reliable amino acid profiling (DeArmond and Bunch, 2022).

V.2. Methods

V.2.1. Deproteinization solvents system and centrifugation parameters

Single parameter optimization was used. ACN, methanol (Meth) and TCA were combined to constitute four solvent systems. Then after, those later with two centrifugation parameters were tested as illustrated in **Table V.3.**

Table V.3. Parameters of deproteinization

Centrifugation parameters	**Rotation speed (rpm)**	**10000**	**12000**	**14000**
	Time (min)	8	10	12
Solvent system	ACN, TCA 5% (V/V)	100:0	75:25	50:50
	Meth, TCA 5% (V/V)	100:0	75:25	50:50

ACN: Acetonitrile; TCA: Trichloroacetic acid; Meth: Methanol

The protocol followed for deproteinization optimization is presented in **Figure V.1.** To optimize centrifugation time, 400 µl of control serum solution was added to 1100 µl of ACN. The preparation was repeated three times and subsequently centrifuged at 10000 rpm for 8,10 and 12 min, respectively. The clearest supernatant was chosen to complete optimization.

When optimizing centrifugation rotation speed, 400 µl of control serum solution was added to 1100 µl of ACN. The preparation was repeated three times and subsequently centrifuged for 10 min at 10000, 12000 and 14000 rpm, respectively.

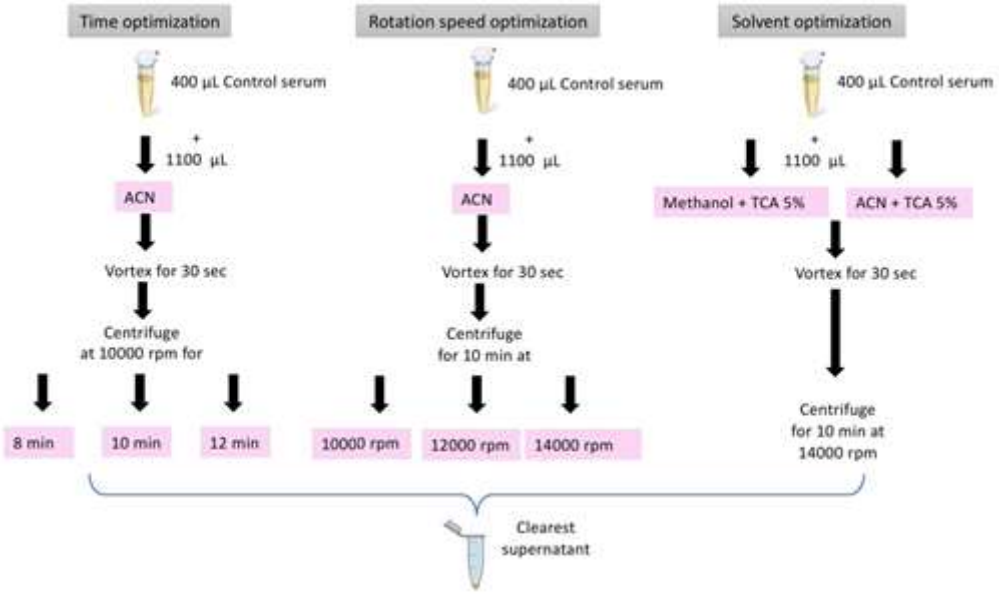

Figure V.1. Work flow for deproteinization optimization

After choosing the clearest supernatant, 400 µl of control serum solution was added to 1100 µl of ACN, Meth, a mixture of ACN with TCA 5% and a mixture of Meth with TCA 5% at different proportions. Different preparations were centrifuged and the clearest supernatant was chosen to complete the work.

V.2.2. Preparation of the spreadsheet

In order to carry out the validation statistical study, we use Excel software from Microsoft Office 2016 registered and installed on the Windows 10 operating system authentic and the spreadsheet was prepared as table format. All the equations included in the statistical study of the validation and construction of the accuracy profile are entered in this table, to have the complete results in tables and graphs, if necessary, just by entering the raw data in terms of test samples and their instrumental responses.

V.2.3. Optimization of HPLC- UV method

V.2.3.1. Solutions for method optimization

16,6 mg of Phe and 18,1 mg of Tyr were weighed accurately and dissolved in a 0.1 M HCl aqueous solution (8,32 ml of HCl 37% made up 1 L with ultrapure water). The volume was made up to 10 ml to get 10 mM stock solution of both compounds. Subsequently, 100µL of the stock standard solution was added to 900µL of ultrapure water to obtain a solution containing 1 mM concentration of both Phe and Tyr.

V.2.3.2. Maximum absorbance wavelength (λ max) determination

A solution of 1 mM of Phe and Tyr was prepared as described in the previous section. The reference cuvette and sample cuvette were filled with solvent (0.1 M HCl aqueous solution) and the baseline was corrected. The desired cell was taken out and filled with the selected sample of high concentration and preceded for scanning. The set UV scanning range was performed from 200 to 350 nm in UV/Vis Spectrometer and the corresponding wavelength with highest peak represents the λ max.

V.2.3.3. Experimental design

The objective of the optimization process was to achieve a complete separation of Phe and Tyr with a target Rs value above 1.5. Based on preliminary studies, three critical factors were identified: buffer concentration, mobile phase composition, and column temperature.

To optimize these factors, a CCD was selected over the Box-Behnken design due to its ability to comprehensively analyze the response under extreme conditions (Siregar *et al.*, 2018). CCD allowed for the evaluation of the interaction effects between the critical factors and the response. The design involved three factors with three levels each (-1, 0, and +1), as showed in **Table V.4**.

Table V.4. Variables and their experimental design levels used in central composite design

Independent variables	**Coded symbols**	**Levels**		
		-1	**0**	**1**
Buffer concentration	X_1	10	30	50
Organic solvent composition	X_2	15	25	35
Column temperature	X_3	20	30	40

A total of 20 experimental runs were performed, including 6 center point runs to assess experimental variability (**Table V.5**). The experiments were conducted in a randomized order to minimize potential bias.

Table V.5. Central composite matrix design with three independent variables

Experimental run	X_1	X_2	X_3	Experimental run	X_1	X_2	X_3
1	-1	-1	-1	**11**	0	-1	0
2	1	-1	-1	**12**	0	1	0
3	-1	1	-1	**13**	0	0	-1
4	1	1	-1	**14**	0	0	1
5	-1	-1	1	**15**	0	0	0
6	1	-1	1	**16**	0	0	0
7	-1	1	1	**17**	0	0	0
8	1	1	1	**18**	0	0	0
9	-1	0	0	**19**	0	0	0
10	1	0	0	**20**	0	0	0

- **Statistical analysis of data**

The experimental data were subjected to statistical analysis using Design Expert 12.0.3.0 software (Trial version, Stat-Ease Inc). Multiple regression analysis was performed to predict the optimized values of buffer concentration, mobile phase composition, and column temperature.

A second-order polynomial model equation was utilized to fit the mathematical quadratic response surface. The model equation is expressed as (Breig and Luti, 2021) :

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \left\{ \sum_{j=1}^{k-1} \sum_{i=j+1}^{k} \beta_{ij} x_i x_j \right\} + \sum_{j=1}^{k} \beta_{jj} x_j^2 + \varepsilon \qquad (\mathbf{V.1})$$

Where:

- y is the response value,
- β_0 is the constant term,
- βj represents the linear regression coefficient for the jth variable,
- βjj represents the quadratic regression coefficient for the jth variable,

- βij represents the interaction regression coefficient between the ith and jth variables,
- xi and xj are the levels of the independent coded variables (ranging from -1 to +1),
- ε represents the error term.

Analysis of variance (ANOVA) was conducted to assess the significance and adequacy of the model, and the Ficher's F test and lack of fit test were used to evaluate the statistical significance of the model and the regression coefficients. A p-value less than or equal to 0.05 was considered statistically significant. Additionally, the coefficient of determination (R^2) and the adjusted coefficient of determination (R^2 adj) were used to assess the goodness of fit of the model equation. To visualize the variations in the response across the three factors, three-dimensional (3D) response surface graphs were plotted. Finally, the experimental conditions that yielded the highest Rs value between the peaks of Phe and Tyr were selected as the optimized chromatographic conditions.

V.2.4. Conditions for analytical methods

V.2.4.1. HPLC conditions

The HPLC conditions for the analysis of Phe and Tyr (**Table V.6**), determined during the optimization phase using the experimental design, were employed, with a specific focus on the three factors investigated.

The analysis was performed on a Shimadzu Nexera XR system, which included binary pumps, an online degassing unit, an autosampler, and a column oven (Shimadzu Corporation, Kyoto, Japan). The separation was conducted using an isocratic elution mode.

Table V.6. Analytical conditions for phenylalanine and tyrosine analysis by HPLC method

Column	Discovery HS F5-3 pentafluorophenylpropyl (PFPP) column (150 x3 mm,3µm)
Mobile phase	Acetonitrile /50mM ammonium acetate at pH 4.5
Elution program	Isocratic
Flow rate	0.2 mL/min
Oven temperature	28 ºC
Injection volume	10µL
Detection system	UV (dual-wavelength monitoring, 280 nm for Tyr and 254 nm for Phe)

V.2.4.2. LC-MSMS conditions

Special conditions of MS/MS of each analyte are imperative for the development of a suitable quantification method by LC–MS/MS. Therefore, each AA solution (10 µM) was directly injected into the mass spectrometer at a rate of 0.6 ml/min.

Then after, the LC-MSMS method was developed for the analysis of Phe and Tyr of serum sample or for DBS. The analysis was performed on a Shimadzu LCMS 8040 triple quadrupole system coupled to the Nexera X2 chromatograph (Shimadzu Corporation). Separation was performed using Intrada Amino Acid 50 mm column (Imtakt Company). The most intense fragments were used for the quantification (m/z= 120 and m/z= 136 for Phe and Tyr, respectively). The separation conditions are tabulated in **Table V.7**.

Table V.7. Analytical conditions for phenylalanine and tyrosine analysis by LC-MSMS method

LC conditions	
Column	Amino Acid column (100 x3 mm,3µm)
Mobile phase	A: ACN/THF/25mM ammonium formate/formic acid: 9/75/16/0.3 B: ACN/100mM ammonium formate: 20/80
Elution program	Gradient elution, 0%B (0-3.0 min), 0 17%B (3.0-9.0 min)
Flow rate	0.6 mL/min
Oven temperature	35 ºC
Injection volume	3 µL
MS conditions	
Interface voltage	ESI MS Voltage : 4,5 kV Temperature: 250°C
Mode	MRM, Positive
Heat block Temp.	400 ºC
Dissolution line (DL) Temp.	300 ºC
Nebulizing gas flow	N2, 1.5 L/min
Drying gas flow	N2, 15 L/min
Argon (collision gas) pressure	230 kPa

V.2.5. Validation of the analytical method

The validation process of the analytical method was conducted using the optimal chromatographic conditions for HPLC method determined through the optimization phase and using the conditions cited earlier for LC-MSMS.

The validation was carried out in accordance with the recommendations of ICH (Feinberg, 2007) and the guidelines outlined by SFSTP (SFSTP Commission *et al.*, 2006). The latter are based on the use of the accuracy profile, which integrates all the essential elements of validation in a statistically correct manner and in a single graph. The following figures overview the workflow for the quantification of Phe and Tyr by HPLC and LC-MSMS methods, respectively.

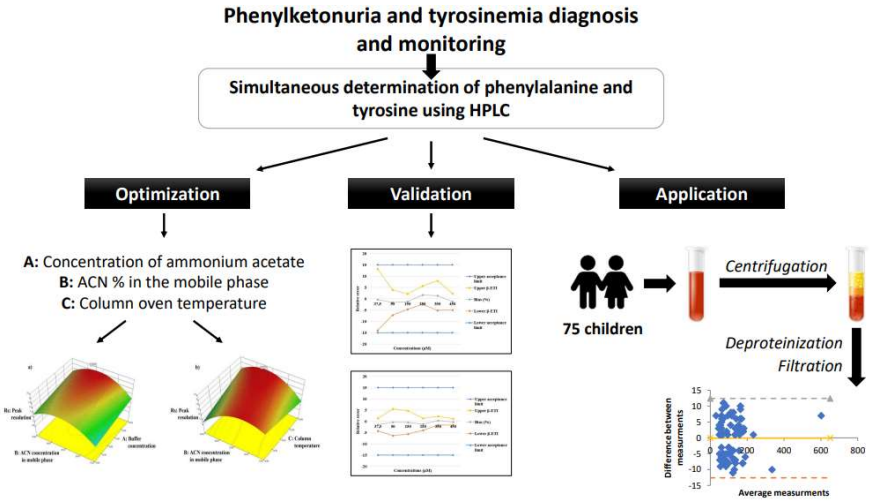

Figure V.2. Workflow overview for the quantification of phenylalanine and tyrosine in human serum by HPLC method

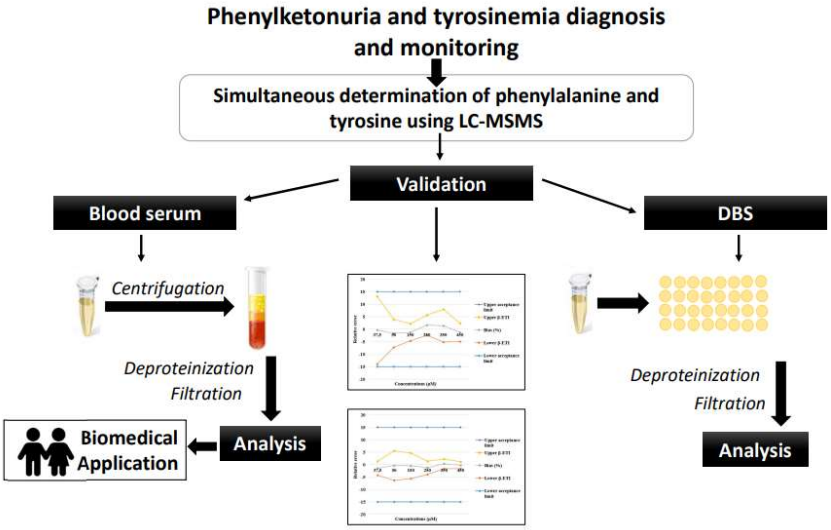

Figure V.3. Workflow overview for the quantification of phenylalanine and tyrosine in human serum by LC-MSMS method

Several parameters were evaluated during the validation process, including specificity, linearity of the response function, linearity of the method, accuracy (trueness and precision), LOD, LOQ and carry-over.

V.2.5.1. Validation protocol

According to the flowchart of the protocols proposed by the SFSTP 2003 (**Figure IV.4** and table **IV.2**) illustrated in the previous chapter, we were able to select the appropriate validation protocol for our study, the dosage procedure is known later and the calibration is done at several concentration levels, we therefore adopted the validation protocol using six concentration levels for the calibration and validation series for their part have six concentration levels according to the last update of ICH guidelines (ICH, 2022) which recommend this number of concentration levels.

Validation parameters were set as follow:

- Acceptability limits (λ): =±15%.
- Probability of confidence (β): = 95%.
- Accepted risk of error (α): 5%.

V.2.5.2. Solutions for method validation

V.2.5.2.1. HPLC

For the method validation, a stock standard solution containing a mixture of Phe (10 mM) and Tyr (10 mM) was prepared. Phe and Tyr powders were dissolved in a 0.1 mol/L HCl aqueous solution to obtain the stock solution. The stock solution was then serially diluted using ultrapure water (calibration series) or control serum (validation series) to create various concentrations ranging from 375 to 4500 µM. These solutions were stored at -30°C, protected from light.

To prepare calibration standards (CS), the stock solutions were further diluted using ultra-pure water, then deproteinizing solvents ACN containing 0.1 % formic acid and TCA 5% (50:50, V/V) were added to the diluted solutions and they were subjected to centrifugation at 14000 rpm for 10 minutes and filtration through a 0.22 µm nylon filter.

For the validation standards (VS), matrices of control serum without AAs were spiked with known concentrations of Phe and Tyr.

These spiked matrices were obtained from independent stock solutions and then diluted using control serum and deproteinized using a mixture of ACN acidified with 0.1 % formic acid and TCA 5% (50:50, V/V). Similar to the CS, the validation solutions were subjected to centrifugation (14000 rpm for 10 minutes) and filtered through a 0.22 µm nylon filter.

The final concentrations of the calibration and validation solutions were 37.5, 50, 150, 250, 350, and 450 µM. All solutions were injected into the HPLC system immediately after preparation to ensure accurate analysis.

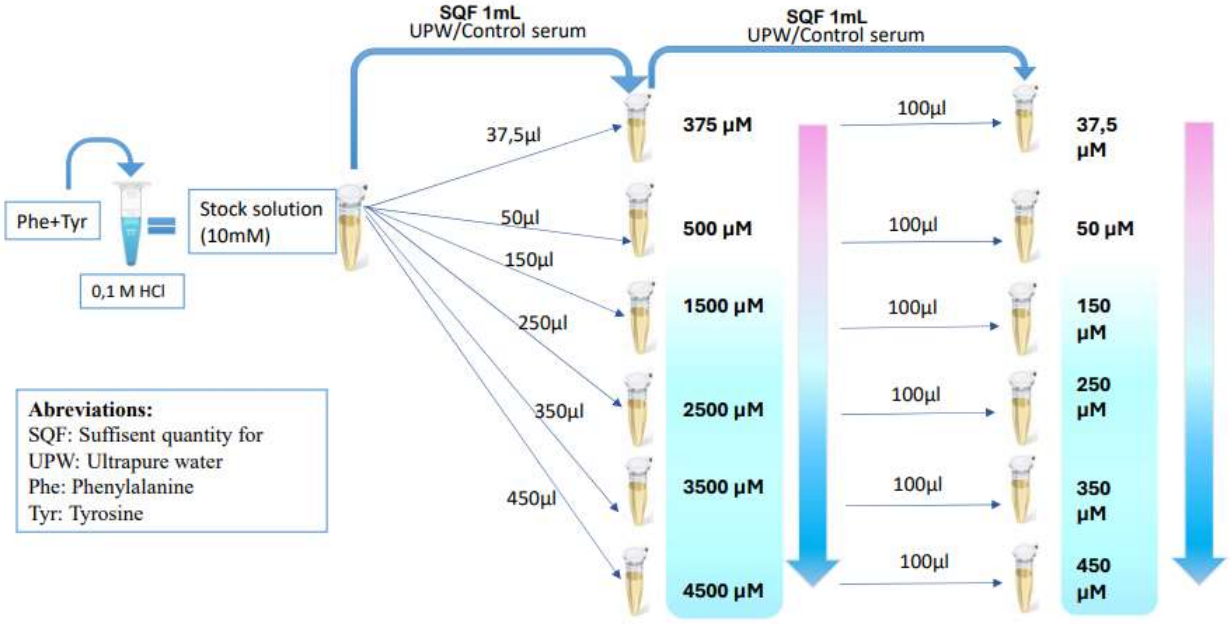

Figure V.4. Different steps followed in the preparation of dilutions in HPLC method for calibration and validation series

V.2.5.2.2. LC MSMS

For LC-MSMS method, the stock solution was prepared by the same manner as described previously and then after it was serially diluted using ultrapure water (calibration series) and with control serum (validation series) to create various concentrations ranging from 50 to 1000 µM and the final concentrations of the calibration and validation solutions were 5 and 100 µM for serum samples (**Figure V.5**).

In the case of DBS, the concentrations of the diluted solutions were ranging from 50 to 450 µM. All dilutions were subsequently deproteinized, centrifuged and filtered for analysis. Different steps followed for the preparation are illustrated in **Figure V.6**.

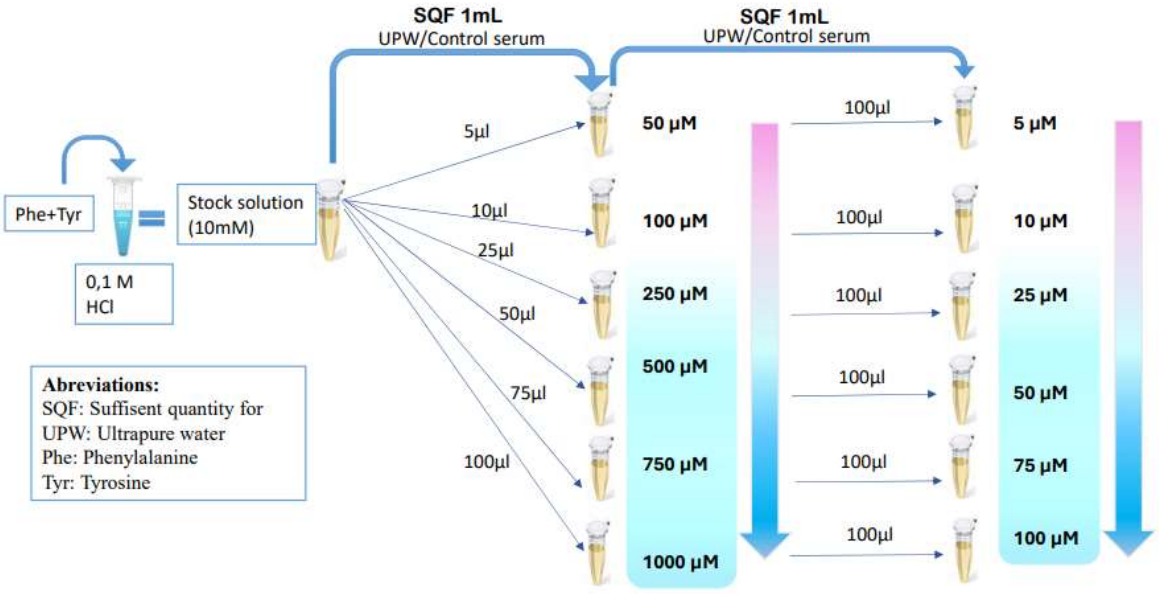

Figure V.5. Different steps followed in the preparation of dilutions for calibration and validation series in LC-MSMS method analyzing blood serum

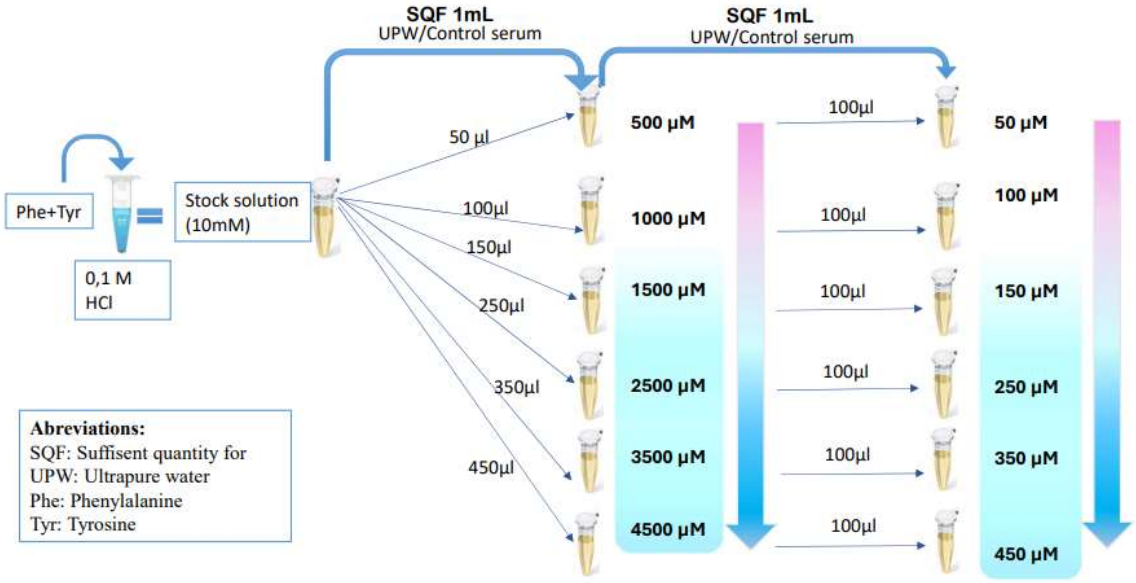

Figure V.6. Different steps followed in the preparation of dilutions for calibration and validation series in LC-MSMS method analyzing DBS samples

V.2.5.2.3. Preparation of DBS

Samples were prepared for LC-MSMS analysis as described in **Figure V.7**. About 50 µl of the previously prepared solutions (50 µM, 100 µM, 150 µM, 250 µM 350 µM 450 µM of Phe and Tyr) were spotted directly on filter paper and less dried for 24 hours. From every filter paper three blood spot discs (each with diameter = 4,5 mm) were punched out and transferred to 1,5 ml Eppendorf tubes for three separated analytical runs so each concentration level was repeated three times (n = 18) to constitute the validation series. Next, 400 µl of ultrapure water was added until complete dissolutions of the spotted solution. Subsequently, 1100 µL of mixture of equal volume of TCA 5% and ACN containing 0.1% formic acid were added to Eppendorf tubes for the extraction of the Phe and Tyr. The samples were extracted under sonication for 30 min at room temperature, and no derivatization was carried out. The extract solution was centrifuged using the optimized conditions of centrifugation, and the resulted supernatant was filtered and used for analysis by LC-MSMS. This protocol was repeated over three different days. For calibration series, there was no need to spot the solutions and they were treated directly after preparation.

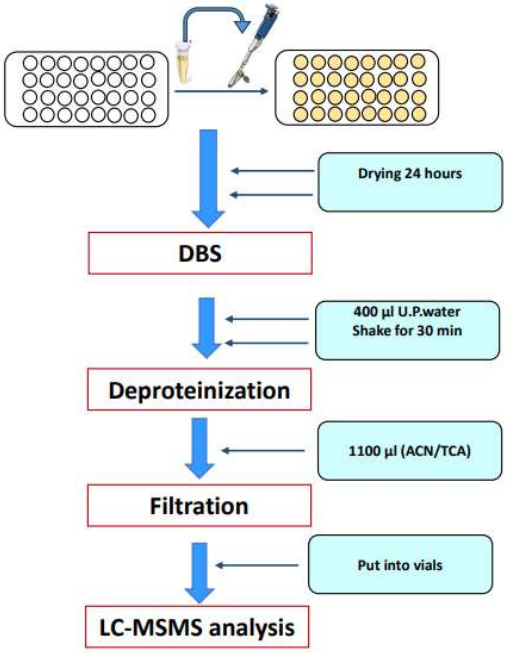

Figure V.7. Preparation and analysis of dry blood spots (DBS) for method validation (ACN: acetonitrile, TCA: trichloroacetic acid, U.P.water: ultrapure water)

V.2.5.3. Validation design

V.2.5.3.1. Evaluation of matrix effect

To determine the presence or absence of matrix effect, the lines L1 and L2 should be compared, the line L1 obtained with the CS (absence of the matrix) and the line L2 obtained with the VS (presence of the matrix). The comparison of the lines is based on the Student's t-test according to a statistical strategy which allows the search for matrix effect.

We compare the slopes obtained for the analyte without and with matrix; if their difference is significant, we conclude the presence of matrix effect, otherwise we can conclude that there is no matrix effect. This therefore involves comparing an observed value (b1 - b2) to a theoretical value (zero) knowing the total error (the standard deviation) made on b1 and b2

$$t_{calc} = \frac{|b1-b2|}{\sqrt{\sigma_{b1}^2+\sigma_{b2}^2}} \qquad (V.2)$$

With:

b1 and b2 respective slopes of the regression lines L1 (obtained from the analyte without matrix) and L2 (obtained from the analyte with matrix);

σ_{b1}^2 and σ_{b2}^2 respective variances of the slopes b1 and b2;

α: accepted risk of error (5%);

n1 and n2: number of samples produced for the analyte without and with matrix;

n1+n2-4: degree of freedom (dof).

Then the calculated t (t_{calc}) should be compared to the value t_0 (α; n1+n2-4) read from the Student's table.

$t_{calc} < t_0$ means that there is no significant difference between the slopes b1 and b2 .

If the slopes are not significantly different, we can also compare the intercepts using a similar approach:

$$t_{calc} = \frac{|a1-a2|}{\sqrt{\sigma_{a1}^2+\sigma_{a2}^2}} \qquad (V.3)$$

With:

a1 and a2 respective intercepts of the regression lines L1 (obtained from the analyte without matrix) and L2 (obtained from the analyte spiked with matrix);

σ_{a1}^2 and σ_{a2}^2 respective variances of the intercepts a1 and a2;

α: accepted risk of error (5%);

n1 and n2: number of samples produced for the analyte without and with matrix;

n1+n2-4: degree of freedom (dof).

After calculating the t-statistic for intercepts and if $t_{calc} < t_0$ that's means no significant difference between the intercepts a1 and a2, and therefore there is no matrix effect (SFSTP Commission *et al.*, 2006).

V.2.5.3.2. Validation phase

After verifying the matrix effect of the analysis method, we operate on two sets of samples:

- CS: can be carried out without the matrix (if the absence of matrix effect has been demonstrated) or with the matrix, used to evaluate the different response functions y = f(x) in order to perform the opposite predictions.
- VS: should always be carried out with the matrix, used for the purpose of determining the total error at each concentration level, calculating the tolerance interval and plotting the accuracy profile and determining the lower and upper limits quantification (LLOQ and ULOQ) (assay interval) (SFSTP Commission *et al.*, 2006)

Regarding the preparation of calibration and validation series, this depends on the validation protocol chosen (see **Table IV.2**). After the pre-validation phase, which confirmed the absence of any matrix effect, we proceeded with the validation design following the **V2** protocol based on the SFSTP guidelines (Feinberg, 2007; Hubert *et al.*, 2007; Isabelle Pinguet, 2015).

In protocol **V2**, calibration is conducted without a matrix but includes multiple concentration levels (SFSTP Commission *et al.*, 2006).

CS at six concentration levels (m = 6) were performed in duplicate (n = 2) in three different days, for each series of experiments (k = 3). Each level was repeated twice. The number of levels was adequate to produce regression models.

The VS were performed in triplicate (n = 3) for each series of experiments (k = 3), and each level was analyzed three times. **Table V.8** summarizes different concentrations levels for validation and calibration series.

Table V.8. Concentration levels for validation and calibration series

Method	Level	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
HPLC (Blood serum)	Level %	15%	20%	60%	100%	140%	180%
	Concentration (µM)	37,5	50	150	250	350	450
LC-MSMS (Blood serum)	Level %	10%	20%	50%	100%	150%	200%
	Concentration (µM)	5	10	25	50	75	100
LC-MSMS (Dry blood spot)	Level %	20%	40%	60%	100%	140%	180%
	Concentration (µM)	50	100	150	250	350	450

V.2.5.4. Validation criterions

V.2.5.4.1. Suitability system, specificity, and carry-over

a) Suitability system

The system suitability test involved performing six replicate injections of the 100% CS for each developed method. The parameters assessed during this test were the retention time and peak area. The precision of the HPLC and the LC-MSMS conditions intended for the analysis was utilized for evaluating the system suitability. The RSD should be less than 2% (Siregar *et al.*, 2018) and the tailing factor (T) should be ≤ 2, while the number of theoretical plates (N) should typically be greater than 2000 (Shabir, 2003).

b) Specificity

Specificity can be demonstrated either by comparing the chromatograms obtained from the standard solutions of the analytes of interest and the blank solution which should have not peak at the same retention time as the analyte.

To assess the specificity of the method in both the absence and presence of the matrix (Van Dam *et al.*, 2014), the following solutions were prepared and injected following the optimized procedures: a blank solution of the mobile phase, standard solutions of the analytes of interest (AAs), and standard solutions spiked with the matrix (control serum without AAs).

The criterion for determining the absence of interfering components is when the response is below 20% of the LLOQ for the analyte (Smith, 2012). This threshold ensures that any potential interferences do not significantly affect the accurate quantification of the analyte.

c) Carry-over

Carry-over was evaluated by injecting blank samples immediately after injecting the higher concentration CS at the ULOQ. The presence of carry-over in the blank sample should not exceed 20% of the LLOQ (Smith, 2012). This criterion ensures that any residual analyte from the previous injection does significantly impact subsequent sample analysis and quantification (Clarin, Barclay and Östervall, 2020).

V.2.5.4.2. Linearity of the response function

The linearity of the response function, also referred to as the calibration curve, is crucial for assessing the relationship between the response (signal) and the concentration (quantity) of the analyte in the sample. It demonstrates the method's ability to accurately measure the analyte within a specific concentration range.

To evaluate linearity, a set of six standard solutions with different concentrations were prepared and analyzed. The responses obtained from these standard solutions were plotted against their corresponding concentrations. The curve fitting was then performed using the least squares method to establish a linear fit curve for each analyte of interest (Van Dam *et al.*, 2014).

V.2.5.4.3. Trueness, precision, and accuracy

Trueness refers to the degree of agreement between an accepted value and the mean experimental value (Zacharis and Vastardi, 2018).

In this study, the concentration of the VS was back-calculated to determine the trueness, which is expressed as the relative bias (%) between the calculated and accepted values.

Precision measures the agreement among measurements obtained from different samplings of a homogeneous sample under specific conditions (Zacharis and Vastardi, 2018). The precision is assessed by calculating the RSD of repeatability and intermediate precision for each concentration level studied (Zacharis and Vastardi, 2018). To evaluate repeatability, three injections of six different solutions containing concentrations of Phe and Tyr (their concentrations are previously cited in **Table V.8**) were performed on the same day, and the RSD values were calculated. The inter-day precision (intermediate precision) was determined by repeating these injections over three different days.

Accuracy represents the agreement between the obtained value and an accepted reference value or an actual conventional value. It accounts for both systematic and random errors, which contribute to the total error associated with the result (Isabelle Pinguet, 2015). Therefore, accuracy is expressed as the sum of trueness and precision, combining both the closeness of agreement and the degree of scattering observed in the results.

V.2.5.4.4. Linearity of the method

The linearity of an analytical procedure, or the linearity of the results, refers to its ability to provide measurements that are directly proportional to the concentrations of the analyte in the sample within a specified range (Isabelle Pinguet, 2015).

In this study, the linearity of the method was evaluated by comparing the actual concentrations of the prepared validation solutions with the concentrations determined by the method.

In the context of testing for linearity, Ficher's test can be used to estimate whether the variance of residuals from a linear regression model with different levels of the independent variable remains constant. If the variances are not homogeneous, it proposes that the linearity assumption may be violated (Sayago and Asuero, 2004).

V.2.5.4.5. Limit of detection and limit of quantification

The LOD is the smallest analyte quantity that can be detected but not accurately quantified. At the same time, the LOQ is referred to the lowest amount of analyte in a sample which can be determined quantitatively with suitable precision and accuracy (Zacharis and Vastardi, 2018). The LOD and the LOQ may be expressed as described in the equations (Shabir, 2003):

$$\mathbf{LOD = 3.3\ \sigma/S} \qquad (\mathbf{V.4})$$

$$\mathbf{LOQ = 10\ \sigma/S} \qquad (\mathbf{V.5})$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

V.2.5.4.6. Accuracy profiles

The accuracy profiles were used to determine the most appropriate response function for the analysis. Various response functions, such as linear, with or without transformation, weighted, or unweighted, were considered. In this thesis, three response functions were studied, Y=bX and Y=bX+a and Y = k(100%) X The selection was based on the accuracy profiles and the β-ETI.

The β-ETI is a parameter that defines the interval within which a certain proportion β of future measurements is expected to fall within the acceptance limits. In this study, β-ETIs expressed as relative % bias were estimated for each concentration of Phe and Tyr. A fixed β-ETI of 95% was used. If the results were not satisfied, β-ETI of 80% could be used.

To validate the analytical method, it is required that the β-ETI falls within the range of ±λ (±15%) conditions. If the β-ETI satisfies this criterion, it confirms the validity of the analytical method (Zacharis and Vastardi, 2018).

V.2.5.4.7. Impact of storage duration and temperature on phenylalanine and tyrosine concentrations

To assess the impact of time and storage temperature on the levels of Phe and Tyr, serum samples and acidified aqueous solutions were stored at -30°C, 6°C, and room temperature (25°C) for one month. Next, they were analyzed by both HPLC and LC-MSMS methods.

V.2.6. Biomedical application of the developed method

V.2.6.1. Sample size estimation

In order to calculate the sample size for a study based on the prevalence of a disease, we use the following formula derived from Cochran's method (Naing *et al.*, 2022; Sadiq *et al.*, 2024):

$$n = \frac{Z^2 \times P\,(1 - P)}{d^2} \qquad (\mathbf{V.6})$$

With:

n: required sample size

Z: statistic for the desired confidence level (1.96 for a 95% confidence level)

P: expected prevalence (expressed as a proportion, e.g., 0.136 for 13.6%)

d: margin of error (precision), which is the acceptable difference between the sample estimate and the population parameter (e.g., 0.05 for 5% precision)

V.2.6.1. Epidemiologic study

a) Descriptive study

We conducted a retrospective descriptive study. Data were collected from the information sheets (**Appendix I)** of volunteers either undergoing disease (PKU or TRS) monitoring or were being evaluated for a suspected diagnosis. They were attending from 2020 to 2024. The following data were recorded: age, gender, age at diagnosis, familial background (including hereditary diseases, sibling deaths, consanguinity, and prematurity), complications, developmental milestones, follow-up of dietary treatment and area of residence. Additionally, information on the onset of symptoms, whether present from birth, during the neonatal period, or later, was obtained.

The collected data were filtered, and coded according to the required needs and specifications, and it was stored in an Excel format with a protection arrangement. Variables are presented as numbers and percentages. A p-value less than 0.05 was considered as significant.

b) Statistical study

The data were analyzed using the SPSS statistical program and three groups were tested, suspected volunteers' group, PKU patients' group and all volunteers group.

First, we estimated the effect of each qualitative factor (gender, history, associated therapy…) on the response represented by Phe and Tyr concentrations obtained by the developed methods and their respective ratios. This is through using Levene's test to evaluate the equality of variances and Student's t-test to evaluate the equality of means.

Second, we have assessed the effect of each quantitative factor (volunteers age, fasting duration, age of symptom onset…) on the already cited responses by using regression equations and spearman correlation test.

Then, the difference between suspected volunteers' group and PKU patients' group was estimated using Chi square test and Fisher's test for qualitative factors and by Levene's test and Student's t-test for quantitative factors.

The last step was the evaluation of interactions and complex relationships between variables using multi linear regression (MLR) with stepwise selection. The MLR model can be expressed mathematically(Smalheiser, 2017) as follows:

$$Y_i = b_0 + b_1X_1 + b_2X_2 + \cdots + b_iX_i + \varepsilon \qquad \textbf{(V.7)}$$

Where:

- Y: dependent variable (the outcome we are trying to predict)
- Xi: independent variables (predictors)
- b0 : Y-intercept (constant term)
- bi: coefficients for each independent variable
- ε: error term (residuals)

V.2.6.3. Sample collection and conservation

An agreement with private laboratory was established in order to facilitate sample collection, conservation and transportation. The collected blood samples primarily served other testing purposes, and only 0.2 mL of residual blood was utilized for this particular study.

Upon collection, the blood samples were promptly centrifuged, and the resulting serum was separated and stored at -30°C, shielded from light, until further analysis.

To prepare the samples, deproteinization was performed using a solvent mixture consisting of ACN and trichloroacetic acid (TCA) in a 50:50 (v/v) ratio. The solutions were subsequently subjected to centrifugation at 14000 rpm for 10 minutes, and the resulting supernatant was filtered through a 0.22 µm nylon filter.

V.2.6.4. Sample analysis

The pretreated samples from 442 volunteers amongst 45 PKU patients, were analyzed by the previously developed HPLC-UV and LC-MSMS methods. However, the method utilizing DBS was not applicated and this is because of lack in the Guthrie paper.

Before each analysis, a quality control test was performed to ensure the validity of the model by injecting 100 µM of standard solution prepared in matrix, we used the total error as limit value.

The concentrations of Phe and Tyr in the prepared serums, as well as the Phe/Tyr ratio, were calculated. The established cut-off values for Tyr were from 207 to 226 µmol/L(UK NSC, 2014) and for Phe were 200 µmol/L with Phe/Tyr ratio of 2 (Eastman *et al.*, 2000; Ceglarek *et al.*, 2002) and. If the Phe/Tyr ratio is less than 2 and the Phe concentration exceeds 400 µmol/L, the child is recalled to obtain a second sample. Additionally, positive cases with a Phe level exceeding 200 µmol/L and a Phe/Tyr ratio greater than 2 are also recalled for further confirmation. Moreover, the developed HPLC-UV and LC-MSMS methods were compared together using Bland-Altman plot and Spearman correlation.

To assess the accuracy and applicability of the developed HPLC and LC MSMS method, the Phe and Tyr levels of 75 samples were compared to the values obtained using the reference method, which involved the analysis of the samples using LC-MSMS of results of another laboratory.

This comparison allows for the evaluation of agreement and concordance between the methods, providing further validation for both the accuracy and applicability of the developed methods in the biomedical application.

CHAPTER VI.

RESULTS AND DISCUSSION

Chapter VI. Results and discussion

The following section is devoted to the results obtained accompanied by interpretations and discussion according to previous studies in the same field.

VI.1. Deproteinization solvents system and centrifugation parameters

Deproteinization is a critical step that significantly influences the types and yield of metabolites to be extracted. Two kinds of extraction are conventionally used, monophasic and biphasic. The first one consists of organic solvent addition at known proportion in a single step. Thus, it is more adapted because of its simplicity (Chan *et al.*, 2013).

To access the efficiency of monophasic extraction and to optimize parameters used for deproteinization, two solvent systems (at different proportions) and two centrifugation parameters (rotation speed and time) were tested.

Both the rotation times of 10 min and 12 min showed clear supernatant as compared to the duration of 8 min which was insufficient to separate different constituents of serum samples, therefore 10 min was chosen for investigation of rotation speed. The clearest supernatant was shown with highest rotation speed of 14000 rpm among the tested speeds and it was fixed to optimize the solvent system. Furthermore, the solvent system containing ACN, TCA 5% (50:50 V/V) showed the best limpidity. Consequently, using ACN, TCA 5% (50:50 V/V) in tandem with rotation of 14000 rpm for 10 min ensure maximum limpidity of treated samples, hence those parameters were used to complete the work. Additionally, previous study by Chan and colleagues (Chan *et al.*, 2013) reported that the number of extracted metabolites increase when using monophasic deproteinization. Therefore, according to our finding and to the results of Chan et al previously cited, we conclude that increasing the rotation speed and the aqueous proportion in the solvent system, in one single step increase the deproteinization efficiency.

In this study, using a high proportion of TCA 5% is avoided because of its acidity that may affect column useful lifetime. With long term use, deterioration by acid may decrease retention by breaking alkyl groups and reduce separation efficiency (Osaka Soda, 2024).

Evans and colleagues reported in previous study, that 0.1% formic acid plays a role in extraction of metabolites and is ideal for formation of the positive ions (Evans *et al.*, 2009), which are necessary for the LC-MSMS analysis. For this reason, ACN used for the deproteinization was acidified by 0,1 % formic acid.

VI.2. Optimization and validation of HPLC- UV method

VI.2.1. UV absorbance wavelengths

Figure VI.1 illustrate the UV spectrum of Phe and Tyr. As is shown in the figure, Phe has a maximum UV absorbance at 254 nm, however Tyr has two maximum UV absorbance, at 254 nm and at 280 nm. This is in accordance with previously published study of Hazra et al (Hazra, Samanta and Mahalingam, 2014). Therefore, we have used dual mode for the UV detection, 254 nm for Phe detection and 280 nm for Tyr detection.

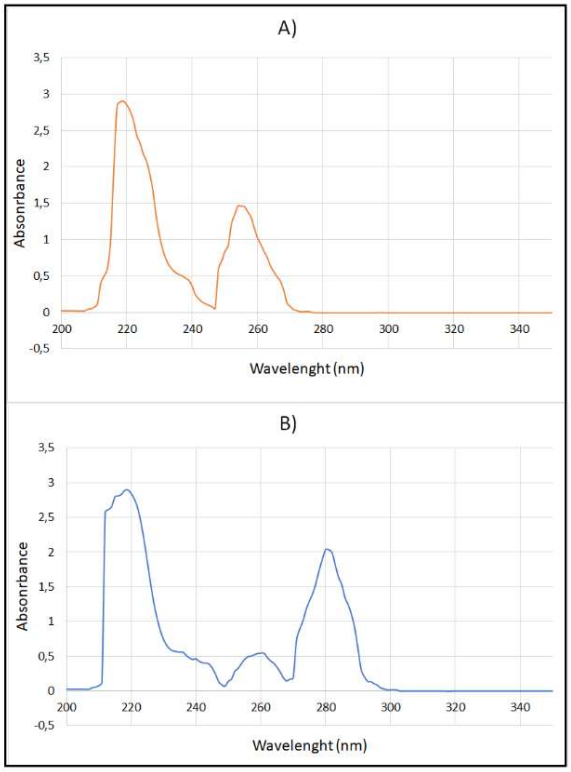

Figure VI.1. UV absorption spectra of A) phenylalanine and B) tyrosine

VI.2.2. Experimental design

The Rs between Phe and Tyr peaks was optimized using response surface methodology (RSM). The results of experimental design used in this study is summarized in **Table VI.1**, which includes the three independent variables: buffer concentration, organic solvent proportion, and column temperature. A total of 20 experiments were conducted based on this CCD matrix design.

Table VI.1. Central composite matrix design with three independent variables and measured response

Run	Coded variables			Uncoded variables			Resolution (Rs)
	X_1	X_2	X_3	A:Buffer concentration (mM)	B:ACN proportion (%)	C:Column temperature (%)	
1	-1	-1	-1	10	15	20	1,059
2	1	-1	-1	50	15	20	1,369
3	-1	1	-1	10	35	20	1,513
4	1	1	-1	50	35	20	1,745
5	-1	-1	1	10	15	40	1,536
6	1	-1	1	50	15	40	1,745
7	-1	1	1	10	35	40	0,743
8	1	1	1	50	35	40	1,165
9	-1	0	0	10	25	30	1,373
10	1	0	0	50	25	30	1,889
11	0	-1	0	30	15	30	1,369
12	0	1	0	30	25	30	1,148
13	0	0	-1	30	25	20	1,994
14	0	0	1	30	25	40	1,906
15	0	0	0	30	25	30	1,838
16	0	0	0	30	25	30	1,513
17	0	0	0	30	25	30	1,944
18	0	0	0	30	25	30	1,93
19	0	0	0	30	25	30	1,895
20	0	0	0	30	25	30	1,906

VI.2.2.1. Statistical analysis

To develop the predictive model, a multiple regression analysis was performed on the obtained experimental results.

The polynomial equation was:

$$Rs = +1.82 + 0.17\,X_1 - 0.076\,X_2 - 0.059X_3 + 0.017\,X_1X_2 + 0.011X_1X_3 - 0.28\,X_2X_3 - 0.14\,X_1^2 - 0.48\,X_2^2 + 0.18\,X_3^2 \quad (\mathbf{VI.1})$$

The statistical analysis results, summarized in Table **VI.2**, considered a variable as non-significant when its p-value is higher than 0.05.

Based on ANOVA, the results indicate that the buffer concentration (X1) is significant ($p < 0.05$), as well as the interaction between the organic solvent proportion and column temperature (X2 X3), and the quadratic terms of the organic solvent proportion ($X2^2$).

Additionally, insignificant terms were eliminated from the model. The proposed model for Rs in terms of coded factors was:

$$Rs = 1.82 + 0.17\,X_1 - 0.059\,X_3 - 0.28\,X_2X_3 - 0.48\,X_2^2 \quad (\mathbf{VI.2})$$

where Y represents the predicted response variable, and X_1, X_2, and X_3 correspond to the buffer concentration, organic solvent proportion, and column temperature, respectively.

Table VI.2. ANOVA analysis for the reduced quadratic model of the resolution between phenylalanine and tyrosine peaks

	Sum of squares	**df**	**Mean square**	**F-value**	**p-value**
Model	2,135	5	0,427	20,349	< 0.0001
X_1	0,285	1	0,285	13,598	0.0024
X_2	0,058	1	0,058	2,782	0.1175
X_3	0,034	1	0,034	1,631	0.2223
X_2X_3	0,607	1	0,607	28,916	< 0.0001
X_2^2	1,150	1	1,150	54,819	< 0.0001
Residual	0,294	14	0,021		
Lack of Fit	0,161	9	0,018	0,669	0.7172
Pure Error	0,133	5	0,027		
Total	2,428	19			
$*R^2$	0.879				
$*R^2$. Adj	0.836				

■ Significant effect, df: degree of freedom., X_1: buffer concentration (mM); X_2: organic solvent proportion (%); X_3: column temperature (°C), $*R^2$ and $*R^2$. Adj are calculated for the reduced model.

The determination coefficient (R^2) or regression coefficient ($R^2 = 0.879$) obtained from the regression analysis for the Rs (Eq. **VI.2**) is satisfactory (> 0.8) (Lestari, Rohman and Martono, 2019), exhibiting that the experimental model was a good fit using the polynomial equation. The adjusted R-squared value of 0.836 and the difference between predicted R^2 with the adjusted R^2 was less than 0.2 (Lestari, Rohman and Martono, 2019) indicates that the model explains approximately 83.6% of the variance. These results suggest that the established model satisfactorily fits the experimental data and can be used for predicting responses. Fobs (20.349) is greater than critical F (5,14) = 2.96 taken from the table in **Appendix II**, which reveals that the model is valid and effective in navigating the design space.

A comparison of the actual and predicted Rs values was carried out and the results are illustrated in **Figure VI.2**. We note that the actual and predicted values are almost similar, so it can be inferred that the suggested mathematical model effectively reproduced the observed Rs values.

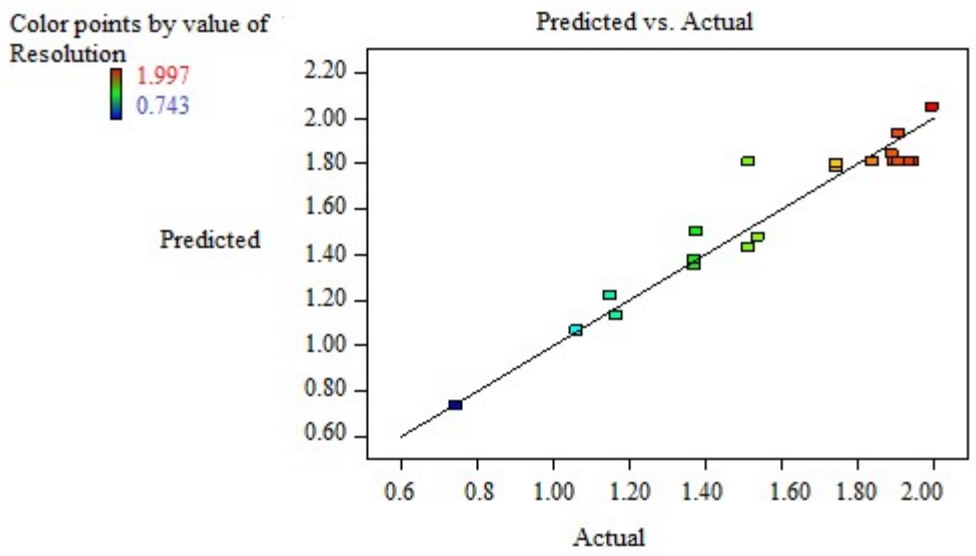

Figure VI.2. Comparison of predicted and actual values of resolution

VI.2.2.2. Graphical analysis

VI.2.2.2.1. Principal effects plots

As shown in **Figure VI.3**, the variable of buffer concentration (A) has a positive effect on Rs, while ACN concentration in mobile phase (B) and the column oven temperature (C) both have negative insignificant effect.

Variable A affected positively, meaning that the increased levels of buffer concentration would increase the Rs between Phe and Tyr peaks, while the increasing in column oven temperature or in ACN percentage in mobile phase could decreases the Rs.

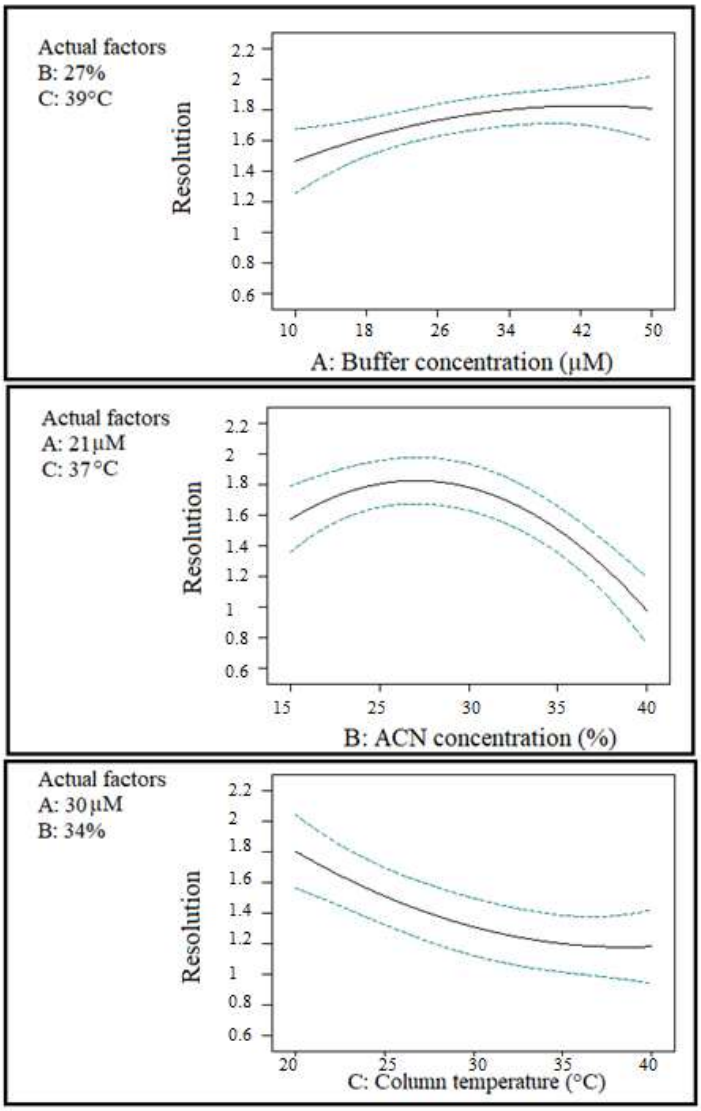

Figure VI.3. Plots of principal effects of the different factors on peak resolution

The composition of the mobile phase is crucial for achieving optimal Rs in chromatography. Key factors that significantly influence Rs, are the mobile phase composition and the ratio of aqueous to organic solvents, the pH of the mobile phase, and the ionic strength of the buffer. By fine-tuning these parameters, one can markedly enhance Rs (Stephanie Koczur, 2023).

In this study, it was found that increasing the buffer ionic strength positively impacts separation; however, this increase must be carefully managed to prevent column clogging.

The elution strength of the sample solvent influences the peak shape because using a sample solvent with an elution strength inferior to that of the mobile phase can sharpen the peak shape by suppressing the diffusion of the analyte in the column. On the other hand, using a sample solvent with elution strength superior to that of the mobile phase can broaden the peak shape, especially if the injection volume is increased (*Effects of Sample Solvents on Peak Shape*, 2023). In our work, we have used ACN with TCA for sample pretreatment to have similar elution strength between the sample solvent and the mobile phase in order to improve the peak shape. Co-injection of a diluent solvent, such as water, can help to preserve a consistent peak shape even when using a sample solvent with a higher elution strength. This technique is mainly useful in reversed-phase analysis (*Effects of Sample Solvents on Peak Shape*, 2023).

Changing the mobile phase composition can also help in resolving closely eluting peaks. For example, switching from Meth to ACN as the organic modifier can improve peak spacing in certain separations (Stephanie A. Schuster *et al.*, 2013).

Increasing the column temperature can lower mobile phase viscosity and enhance solvent diffusion rates, ultimately improving column efficiency and Rs (Dong, 2006). For example, temperature decrease water viscosity as was reported previously (Yang *et al.*, 2002). However, in our study, column temperature has a negative effect on the separation between Phe and Tyr. We suppose that not only mobile phase viscosity is influenced by temperature but other parameters are implemented also.

The effects of temperature on analyte retention and selectivity vary for each analyte, depending on the properties of the mobile and stationary phases. Typically, raising the column temperature reduces the retention time of polar compounds but increases the retention time of non-polar compounds (‘Basics of chromatography’, 2024).

Phe is classified as a non-polar AA. Its hydrophobic nature is attributed to the benzyl side chain, which lacks polar functional groups. Tyr is generally classified as a polar AA due to its hydroxyl (-OH) group, which contributes to its polar nature and allows it to form hydrogen bonds.

While Tyr has a non-polar aromatic side chain, the presence of the hydroxyl group gives it polar characteristics, making it capable of interacting with water and other polar molecules (*BIS 105: Biomolecules and Metabolism (Murphy): Amino Acids*, 2024). The temperature could affect the retention of those analytes because of their polarity proprieties.

Temperature is a key element in chromatographic separation, impacting the process via several mechanisms. Primarily, it impacts the thermodynamic properties of both solvents and solutes, including pressure, viscosity, and diffusivity. These factors, in turn, affect the equilibrium between the mobile and stationary phases. Secondly, temperature influences the kinetics of adsorption and desorption of solutes on the stationary phase, which can alter retention times and peak shapes. In addition, temperature can affect any chemical reactions that may occur during the separation process, such as oxidation or hydrolysis (Heidorn, 2024).

Consequently, to achieve reliable and reproducible chromatographic results, it is crucial to maintain the temperature within a narrow range throughout the chromatographic run. Fluctuations in temperature can alter the physical and chemical properties of both the stationary and mobile phases, leading to variations in retention times, selectivity, Rs, and peak shape (Heidorn, 2024).

The selection of the column stationary phase is important for Rs. Using columns with small solid-core particles and smaller particle sizes can increase Rs by improving column efficiency (Stephanie Koczur, 2023). This is the advantage added by PFPP column which own a small particle size.

The injection volume should be optimized to avoid overloading the column, which can lead to peak broadening and decreased Rs. A general rule is to inject 1-2% of the total column volume (Stephanie Koczur, 2023).

In addition, the flow rate should be optimized to achieve the best Rs. Lowering the flow rate can narrow peaks and improve resolution, while increasing the flow rate can broaden peaks and decrease resolution (Stephanie Koczur, 2023). In our word we have used a mobile phase flow rate of 0.3 ml/min according to the instructions of column manufacturer.

Proper system and autosampler preparation, including the selection of appropriate containers for light-sensitive analytes and the use of actinic vials, can prevent analyte degradation and improve peak resolution (Stephanie Koczur, 2023).

In summary, optimizing the sample solvent, mobile phase composition, column selection, injection volume, flow rate, and system preparation are all critical steps in attaining good Rs in HPLC.

VI.2.2.2.2. Interaction plots

The purpose of the interaction graph is to indicate how the relationship between a factor and a response depends on the value of another factor. This graph shows the levels of the first factor on the "x" axis and separate lines for each level of the other factor. The larger the gap between the start and end of the lines, the stronger the interaction between the factors.

Figure VI.4 shows the interactions between different factors. According to this figure, we can notice a large gap between the lines corresponding to the interaction ACN concentration in mobile phase × column oven temperature, and a small gap between the lines corresponding to the interaction ACN concentration in mobile phase × buffer concentration and the interaction buffer concentration × column oven temperature. This finding confirms the results of the ANOVA table where only the interaction ACN concentration in mobile phase × column oven temperature was significant. For example, the interaction effect indicates that the Rs depends on ACN concentration in mobile phase on one side, and the column oven temperature of the other side. If ACN was is used at 35% concentration (+1 in coded value), a heigh Rs is obtained with temperature cited at 20°C (-1 in coded value). However, a very low Rs is observed when temperature cited at 40°C (+1 in coded) value together with ACN concentration of 35% (+1 in coded value).

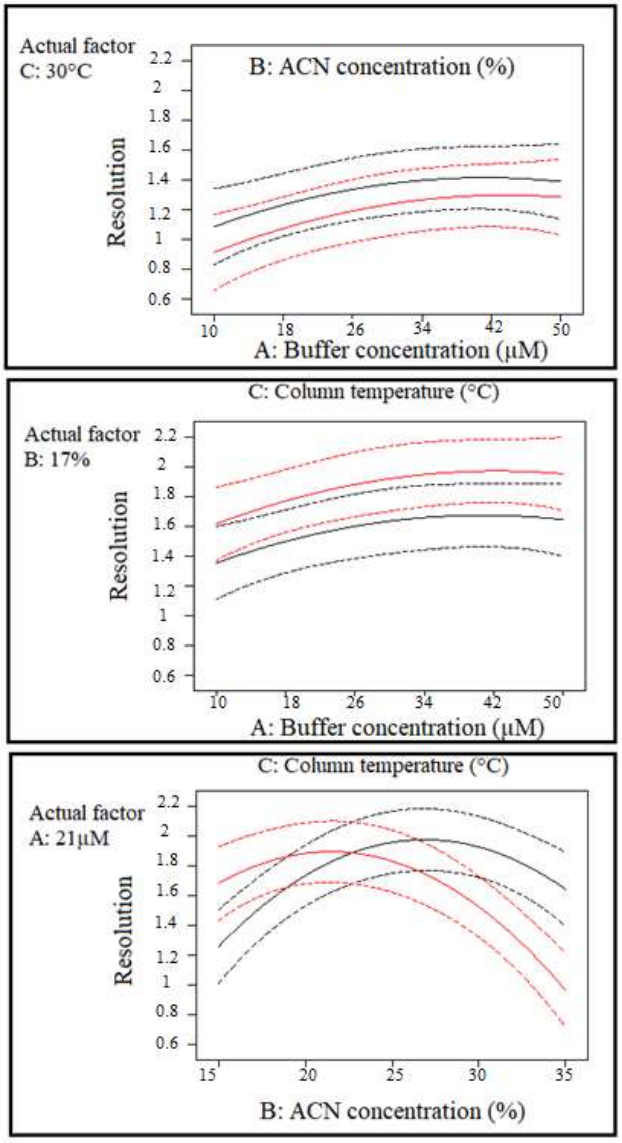

Figure VI.4. Plots of the interaction effects of the different factors on peak resolution

VI.2.2.2.3. Contour and 3D surface plots

Contour plots of different interactions are represented in **Figure VI.5**. According to this figure, we conclude the higher Rs (up to 2) was obtained when using high level of buffer concentration (50 mM) with high column oven temperature (40°C) or also by medium level of ACN concentration (25-30%) together with low column oven temperature (20°C). In the case of using medium or high levels of buffer concentration (25-50 mM) with low concentration of ACN in mobile phase (25%), the Rs will not exceed 1.8.

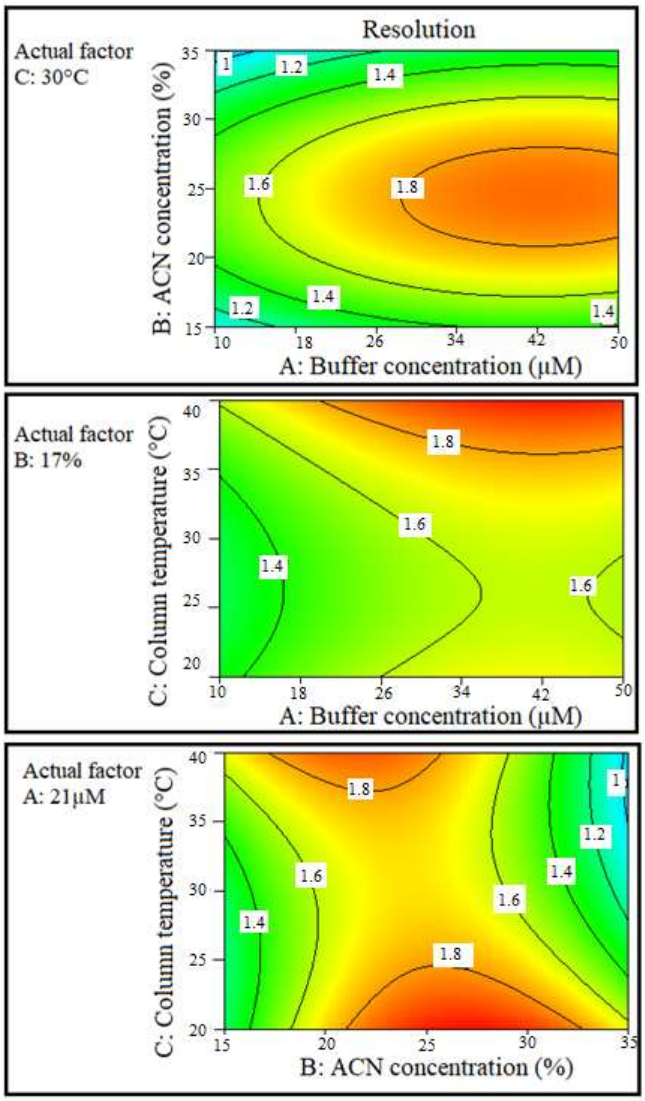

Figure VI.5. Contour plots of the interaction effects of the different factors on peak resolution

The 3D surface plot of the resolution graph is shown in **Figure VI.6**. Such graphical representations show the effects of two factors on the investigated response, while the third factor remains set at the center of the design. The responses surface plots enable to understand the interaction effects between two parameters or more, and to determine the optimal value of each variable in order to minimize or maximize a given response, as reported in the literature (Breig and Luti, 2021).

It revealed that increasing the buffer concentration and the % of organic solvent (ACN) in the mobile phase under different levels of column temperature had different resolution values.

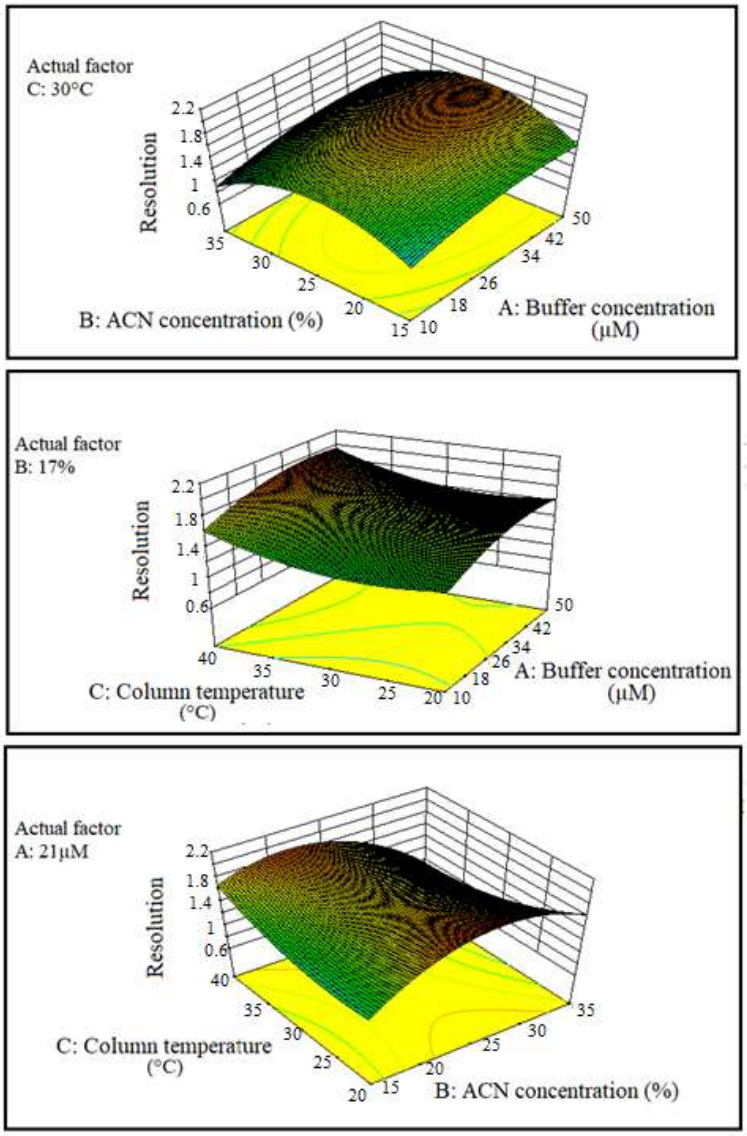

Figure VI.6. 3D surface plot of the resolution graph

VI.2.2.3. Determination of optimal parameters

The optimized method utilized a mobile phase consisting of ammonium acetate buffer (50 mM, pH 4.5 adjusted with formic acid) supplemented with 24% ACN, and the column oven temperature was set at 28°C.The desirability value for the precited conditions was 0.971 **(Figure VI.7**).

This approach successfully achieved the desired objective of method optimization, aiming for a Rs range of 1.5-2, which ensures baseline separation of all key analytes (Dong, 2006).

To validate the proposed optimal conditions obtained from CCD, the experiment was repeated six times using different preparations under the same conditions. The resulting Rs was determined to be 1.99 ± 0.03 (n=6).

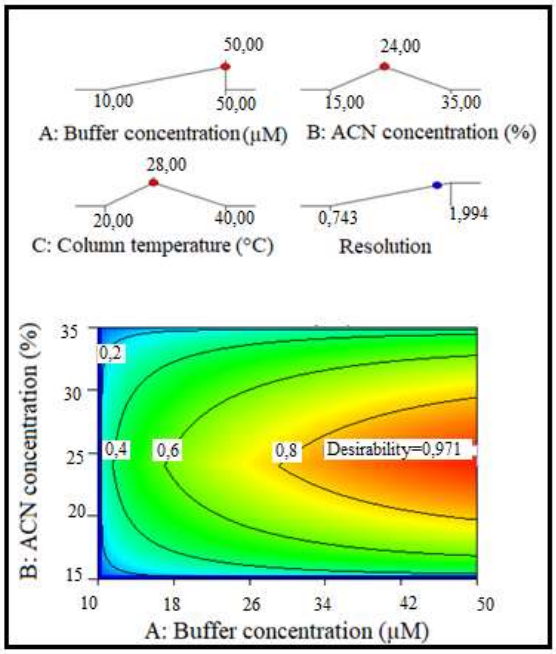

Figure VI.7. Optimized conditions for chromatographic separation and corresponding desirability

Figure.VI.8 displays the HPLC chromatogram obtained using the optimized conditions suggested by CCD, resulting in a Rs of 1.99 for both Phe and Tyr. The plate number (N) (as shown in **Figure VI.8**) was determined to be 2133 for Phe and 4241 for Tyr, indicating an acceptable column efficiency, surpassing the recommended value of 2000 (Shabir, 2003) .The T factor for both analytes was determined. The calculated T values for Phe and Tyr were less than 2, indicating accurate quantitative measurements based on peak area values and symmetric peak shapes (Shabir, 2003). This suggests that both chromatographic peaks exhibit a Gaussian shape.

As demonstrated by Mo et al in their study (Mo *et al.*, 2013), Phe and Tyr were well separated (Rs >1.5), however the retention time of Phe and Tyr was about 5.9 min and 8.4 min, respectively.

In addition, Neurauter G and collaborators (Neurauter *et al.*, 2013) reported that the analysis time was about 7 min.

Our described HPLC-UV method for the simultaneous determination of Phe and Tyr implicates decrease in retention time to achieve more convenient conditions of separation and so allows considerably shorter separation time with high sensitivity. The method works fast as one single run is completed within 4 min.

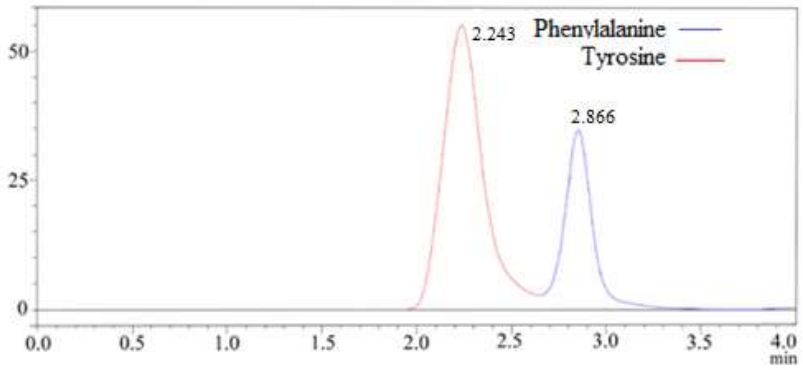

Figure VI.8. HPLC chromatogram obtained using the optimized condition suggested by central-composite design

VI.2.3. Method validation

VI.2.3.1. Raw data

The raw data obtained for the CS and VS for Phe and Tyr, are mentioned in the following tables (**Table VI.3**, **IV.4**, **VI.5** and **VI.6**)

Table VI.3. Raw data for phenylalanine calibration series of HPLC-UV method

Conc level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (15%)	1	37,515	21546	37,503	21651	37,503	21001

Conc level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
	2	37,515	20609	37,503	20437	37,503	20923
2 (20%)	1	50,02	28324	50,005	27543	50,005	27435
	2	50,02	27876	50,005	28392	50,005	26564
3 (60%)	1	150,06	87045	150,015	89432	150,015	85473
	2	150,06	82603	150,015	84543	150,015	88432
4	1	250,1	150384	250,025	148435	250,025	149903
(100%)	2	250,1	147068	250,025	149325	250,025	149774
5	1	350,14	213005	350,035	213514	350,035	209152
(140%)	2	350,14	209157	350,035	206132	350,035	207317
6	1	450,18	258886	450,045	257407	450,045	259552
(180%)	2	450,18	255302	450,045	265583	450,045	264552

Table VI.4. Raw data for phenylalanine validation series of HPLC-UV method

Conc-level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (15%)	1	37,515	19937	37,503	21563	37,503	21305
	2	37,515	21683	37,503	21901	37,503	21711
	3	37,515	20398	37,503	20546	37,503	22314
2 (20%)	1	50,02	28689	50,005	28122	50,005	27625
	2	50,02	28068	50,005	28546	50,005	27998
	3	50,02	27534	50,005	27958	50,005	29454
3 (60%)	1	150,06	88673	150,015	86324	150,015	87023
	2	150,06	84983	150,015	87519	150,015	85756
	3	150,06	86723	150,015	85398	150,015	86543
4 (100%)	1	250,1	146205	250,025	148530	250,025	147324
	2	250,1	147643	250,025	150674	250,025	146435
	3	250,1	153112	250,025	149476	250,025	151432
5 (140%)	1	350,14	217365	350,035	211101	350,035	210543
	2	350,14	207568	350,035	212015	350,035	208991
	3	350,14	199763	350,035	201010	350,035	206994
6 (180%)	1	450,18	261571	450,045	263348	450,045	261439
	2	450,18	252920	450,045	259035	450,045	265515
	3	450,18	265098	450,045	261689	450,045	258426

TableVI.5. Raw data for tyrosine calibration series of HPLC UV method

Conc-level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (15%)	1	37,526	107432	37,503	107342	37,53	104262
	2	37,526	105994	37,503	108021	37,53	106213
2 (20%)	1	50,035	151217	50,005	153125	50,05	152987
	2	50,035	153987	50,005	153524	50,05	153241
3 (60%)	1	150,105	525160	150,015	520465	150,15	512547
	2	150,105	519043	150,015	523463	150,15	509817
4 (100%)	1	250,175	894356	250,025	874532	250,25	867416
	2	250,175	875345	250,025	880932	250,25	897915
5 (140%)	1	350,245	1247382	350,035	1230720	350,35	1253560
	2	350,245	1264845	350,035	1263471	350,35	1273971
6 (180%)	1	450,315	1623245	450,045	1616546	450,45	1623109
	2	450,315	1601253	450,045	1594460	450,45	1593728

Table VI.6. Raw data for tyrosine validation series of HPLC-UV method

Conc-level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (15%)	1	37,526	103998	37,503	107123	37,53	103907
	2	37,526	107001	37,503	108343	37,53	104321
	3	37,526	105432	37,503	104984	37,53	105009
2 (20%)	1	50,035	151013	50,005	151312	50,05	153543
	2	50,035	153311	50,005	152324	50,05	153523
	3	50,035	152901	50,005	153451	50,05	151342
3 (60%)	1	150,105	509012	150,015	535075	150,15	512817
	2	150,105	512968	150,015	520980	150,15	512547
	3	150,105	527621	150,015	511296	150,15	499394
4 (100%)	1	250,175	882986	250,025	871224	250,25	857417
	2	250,175	866586	250,025	883216	250,25	867915
	3	250,175	869435	250,025	873619	250,25	874622
5 (140%)	1	350,245	1257865	350,035	1240654	350,35	1269693
	2	350,245	1244785	350,035	1234023	350,35	1248121
	3	350,245	1265865	350,035	1254419	350,35	1259164
6 (180%)	1	450,315	1603214	450,045	1609847	450,45	1619113
	2	450,315	1612344	450,045	1599122	450,45	1596434
	3	450,315	1621346	450,045	1615493	450,45	1607942

VI.2.3.2. Matrix effect

Table VI.7 and **Table VI.8** summarize the results of comparison of different lines obtained by analyzing Tyr and Phe. By application of Student t-test, no matrix effect was detected when comparing the prepared solutions with and without matrix. We proceed to compare the two slopes and the two intercept at the origin. t calc over the repeated series (three days) was found less than critical t (0,05; 26) which was equal to 2,06 (read on the Student Table, **Appendix III**). This is the same for both analytes, therefore we conclude the absence of matrix effect by the absence of significant difference in the slopes and in the intercepts (SFSTP Commission *et al.*, 2006).

These results lead to using the protocol **IV** of validation according to SFSTP guidelines (**Figure VI.4** and **Table VI.2**)

Table VI.7. Comparison of slopes and intercepts of calibration and validation series of phenylalanine analyzed by HPLC-UV method

Day	**Day 1**		**Day 2**		**Day 3**	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	586,56	584,957	588,44	590,647	588,92	591,563
s (b)	7,51	8,884	5,06	7,121	4,22	5,112
a	-475,07	-421,874	-462,12	-722,925	-449,04	-1112,472
s (a)	1973,85	2334,403	1328,73	1870,733	1108,40	1342,797
R	1,00	0,9988	1,00	0,9992	1,00	0,9996
R²	1,00	0,9976	1,00	0,9985	1,00	0,9992
t calc	0,14	Equal slopes	0,25	Slope equal	0,40	Slope equal
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	0,02	Equal intercepts	0,11	Equal intercepts	0,38	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

Table VI.8. Comparison of slopes and intercepts of calibration and validation series of tyrosine analyzed by HPLC-UV method

Day	**Day 1**		**Day 2**		**Day 3**	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	3655,55	3654,018	3634,05	3631,921	3652,70	3659,707
s (b)	13,56	16,0615	11,60	18,010	17,50	24,836
a	-32320,01	-28868,641	-28412,07	-27210,568	-34763,97	-32029,129
s (a)	3563,37	4221,489	3048,02	4730,881	4600,48	6529,690

R	1,00	0,9999	1,00	0,9998	1,00	0,9997
R²	1,00	0,9998	1,00	0,9997	1,00	0,9995
t calc	0,07	Equal slopes	0,10	Equal slopes	0,23	Equal slopes
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	0,62	Equal intercept	0,21	Equal intercepts	0,34	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

VI.2.3.3. Validation criterion

VI.2.3.3. Suitability system

The results of the system suitability test demonstrated consistent and satisfactory performance of the HPLC method. The RSD values for the retention time and peak area were represented in **Table VI.9** and were found to be below 2%, indicating repeatable measurements. The T factor was also within the acceptable limit of ≤ 2 (Shabir, 2003), implying symmetrical and well-defined peaks. Moreover, the number of theoretical plates (N) exceeded 2000 (Shabir, 2003), confirming efficient chromatographic separation.

These results verify that the HPLC method employed for the analysis possesses good system suitability, meeting the necessary criteria for precision, peak shape, and column efficiency.

Table VI.9. Results of the suitability system

Parameter	Phenylalanine	Tyrosine	Result
Retention time (min)	2,869±0,005	2,244±0,007	Repeatable measurements
RSD of retention time (%)	0,18	0,32	
Tailing factor (T)	1,39	1,25	Peak symmetry
Number of theoretical plates (N)	2133	4241	Column efficiency

The results are expressed as Mean±SD

VI.2.3.4. Selectivity

The results of the specificity test illustrated in **Figure VI.9**, demonstrated that the blank solution exhibited negligible response, indicating the absence of interfering components. The standard solutions of the analytes of interest displayed distinct and well-defined peaks, confirming their selectivity and accurate detection.

Furthermore, the standard solutions spiked with the matrix exhibited responses within the acceptable range, indicating that the presence of the matrix did not significantly interfere with the quantification of the analytes.

These findings verify that the developed method is specific and capable of accurately quantifying the analytes of interest, even in the presence of the matrix. The established criterion of a response below 20% of the LLOQ (Area of matrix = 1150) ensures the reliable determination of the analytes, free from significant interferences.

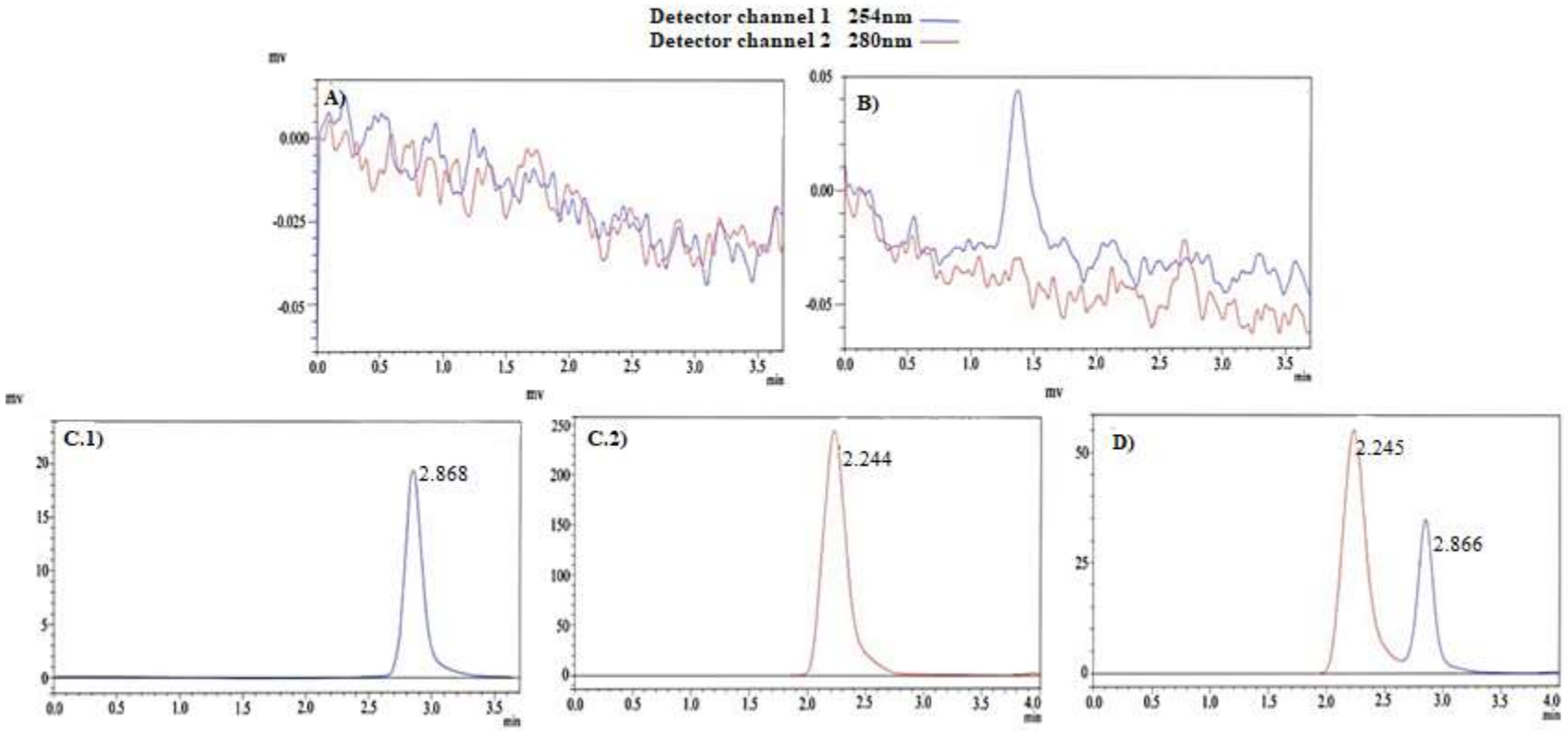

Figure VI.9. Representative chromatogram of (A) a procedure blank; (B) a standard matrix solution; (C) each tested amino acid (C.1 phenylalanine and C.2 tyrosine); and (D) a mixture of phenylalanine and tyrosine in matrix solution, all run under optimized conditions as suggested by central-composite design

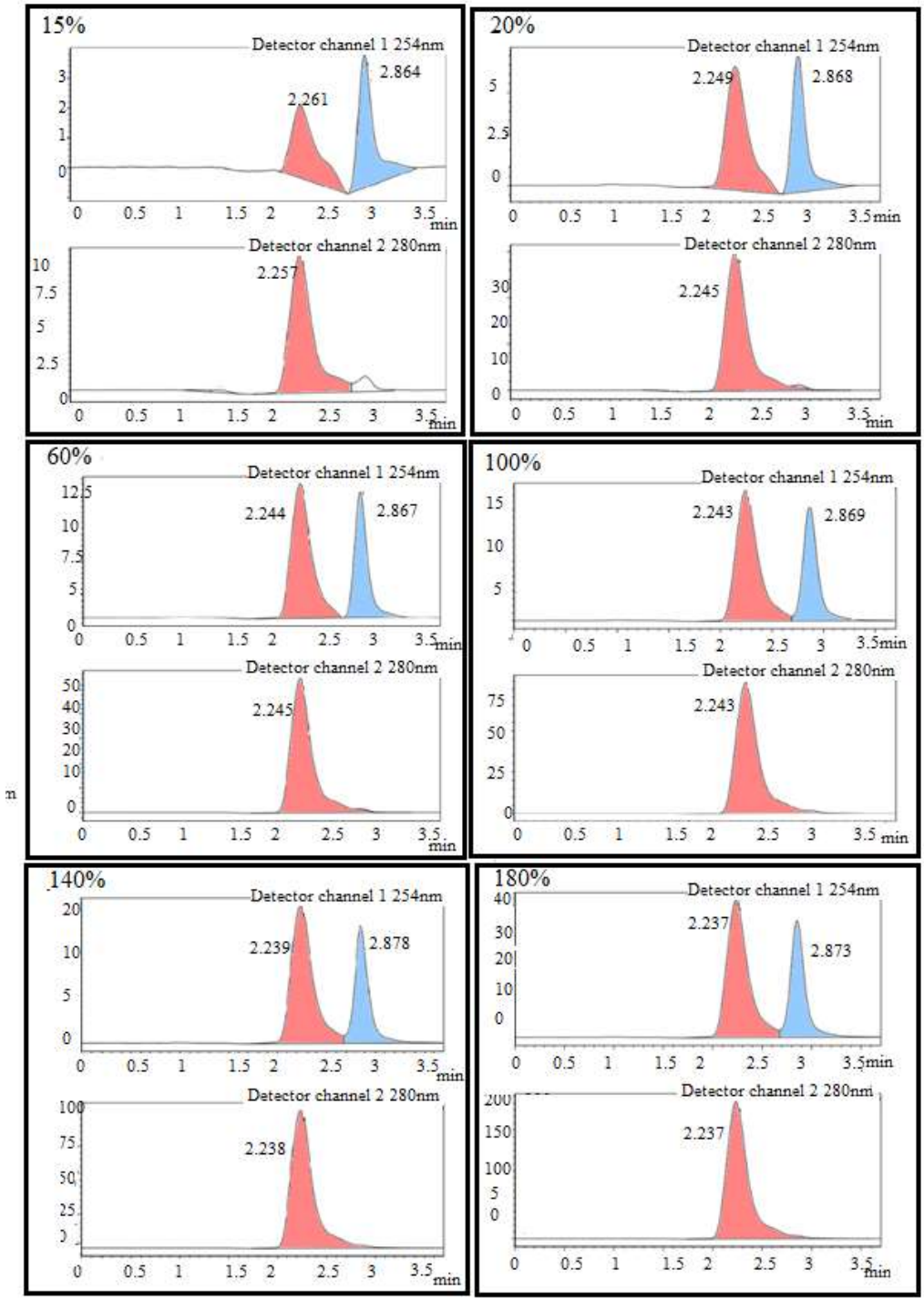

Figure VI.10. Representative chromatograms for phenylalanine (blue) and tyrosine (red) at different concentration levels, analyzed by HPLC-UV

IV.2.3.5. Carry-over

The results of the carry-over evaluation indicated that the observed response in the blank samples was significantly below the 20% threshold of the LLOQ (Area of matrix = 1150), suggesting that any residual analyte from the previous injection did not have a significant impact on subsequent sample analysis.

These findings validate the effectiveness of the developed method in controlling carry-over, ensuring accurate and reliable quantification of analytes in consecutive injections. The integrity of the analytical results is thus maintained, indicating the effectiveness of the "between-injection" cleaning processes.

VI.2.3.6. Linearity of the response function

VI.2.3.6.1. Response functions

The linearity assessment results revealed that the calibration curves for the analytes of interest exhibited excellent linearity over the tested concentration range. It is verified over the validation interval, using data from the repetitions of each concentration level, for the calibration series.

A statistical analysis of the straight lines obtained is performed, the slope is compared to the reference value 0, and the fit is checked. The results obtained for the comparison of the lines [Y = f (X)] for calibration series are summarized in **Tables VI.10** and **VI.11**.

Table VI.10. Response functions of phenylalanine analyzed by HPLC-UV method

Response function		**Day 1**	**Conclusion**	**Day 2**	**Conclusion**	**Day 3**	**Conclusion**
Y = b X + a	b	584,957	Significant slope	590,647	Significant slope	591,563	Significant slope
	s (b)	8,884		7,121		5,112	
	a	-421,874		-722,925		-1112,472	
	s (a)	2334,403		1870,733		1342,797	
	R	0,9988		0,9992		0,9996	
	R^2	0,9976		0,9985		0,9992	
	F	4335,055		6878,136		13391,204	
	F (0,05 ;1;10)	4,964		4,964		4,964	

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X	b	583,645	Significant slope	588,399	Significant slope	588,103	Significant slope
	R	0,9996		0,9997		0,9998	
	R^2	0,9992		0,9995		0,9997	
	F	14230,151		22248,476		40970,766	
	F (0,05 ;1;11)	4,844		4,844		4,844	
Y = k(100%) X	k	594,666	/	595,460	/	599,294	/

Table VI.11. Response functions of tyrosine analyzed by HPLC-UV method

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X + a	b	3654,018	Significant slope	3631,921	Significant slope	3659,707	Significant slope
	s (b)	16,0615		18,010		24,836	
	a	-28868,641		-27210,568		-32029,129	
	s (a)	4221,489		4730,881		6529,690	
	R	0,9999		0,9998		0,9997	
	R^2	0,9998		0,9997		0,9995	
	F	51756,947		40665,510		21713,3013	
	F (0,05 ;1;10)	4,964		4,964		4,964	
Y = b X	b	3564,282	Significant slope	3547,288	Significant slope	3560,177	Significant slope
	R	0,9998		0,9998		0,9997	
	R^2	0,9996		0,9996		0,9994	
	F	28699,281		29787,296		19957,080	
	F (0,05 ;1;11)	4,844		4,844		4,844	
Y = k(100%) X	k	3536,926	/	3510,576	/	3527,134	/

The comparison of the lines [Y = f (X)] for the CS and VS is satisfactory. The chosen calibration system is satisfactory. The R^2 obtained from the linear regression analysis was close to 1 ($R^2 \geq 0.999$ for both analytes) for Phe and Tyr, indicating a strong linear relationship between the response and concentration inside the studied calibration range. F calculated over the repeated series (three days) was found more than critical F and all slopes were significant.

In addition, the slope is comparable to 1 at 5% risk was significant in studied function Y=bX and Y=bX+a. The method is linear for the validation interval.

Similar results were reported by Mo Xi Ming and collaborators (Mo *et al.*, 2013) when the range of concentrations was from 6 µM to 1512 µM for Phe and 5.5µM to 1250 µM for Tyr and the R^2 was found to be equal to 0.9989 for Phe, and equal to 0.9999 for Tyr. In a second work cited by Neurauter. G and collaborators, R^2 was found to be equal to 0.996 and 0.968 for Phe and Tyr, respectively (Neurauter *et al.*, 2013).

For the choice of the response function, which is capable of producing a sufficient proportion of future results which will be within the acceptability limit, we can proceed by two methods, either by calculating the indices: of dosage interval, of accuracy, of trueness and of precision; or by comparing the obtained the accuracy profiles.

VI.2.3.6.2. Alignment of response (inverse predicted quantity)

When the quantities introduced are not identical, the alignment of the responses on the mean of the concentrations introduced is required and this for each concentration level. The alignment of the responses obtained with the validation samples is according to the inverse functions X=(Y-a)/b, X=Y/b and X=Yk(100%). The back calculated concentrations are summarized in the following tables (**Table VI.12** and **VI.13**).

Table VI.12. Back calculated concentrations of phenylalanine analyzed by HPLC-UV method

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
37,5	34,8	37,7	37,9	34,1	36,6	36,2	33,5	36,2	35,5
	37,8	38,3	38,6	37,1	37,2	36,9	36,4	36,8	36,2
	35,6	36,0	39,6	34,9	34,9	37,9	34,3	34,5	37,2
50	49,7	48,8	48,6	49,1	47,8	47,0	48,2	47,2	46,1
	48,7	49,5	49,2	48,1	48,5	47,6	47,2	47,9	46,7
	47,8	48,6	51,7	47,2	47,5	50,1	46,3	46,9	49,1
150	152,3	147,4	149,0	151,9	146,7	148,0	149,1	145,0	145,2

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	145,9	149,4	146,8	145,5	148,7	145,8	142,8	147,0	143,1
	148,9	145,8	148,2	148,5	145,1	147,1	145,8	143,4	144,4
250	250,6	252,7	250,9	250,4	252,4	250,5	245,8	249,4	245,8
	253,0	256,3	249,4	252,9	256,0	249,0	248,2	253,0	244,3
	262,4	254,3	257,8	262,2	254,0	257,5	257,4	251,0	252,7
350	372,2	358,6	357,8	372,3	358,7	358,0	365,4	354,5	351,3
	355,4	360,1	355,1	355,5	360,3	355,3	348,9	356,0	348,7
	342,1	341,5	351,8	342,1	341,6	351,9	335,8	337,5	345,4
450	447,7	447,0	443,8	448,0	447,5	444,5	439,7	442,2	436,2
	432,9	439,7	450,7	433,2	440,2	451,4	425,1	435,0	443,0
	453,7	444,2	438,7	454,0	444,7	439,4	445,6	439,4	431,2

Table VI.13. Back calculated concentrations of tyrosine analyzed by HPLC-UV method

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
37,5	36,3	37,0	37,1	29,2	30,2	29,2	29,4	30,5	29,4
	37,2	37,3	37,2	30,0	30,5	29,3	30,2	30,9	29,5
	36,7	36,4	37,4	29,6	29,6	29,5	29,8	29,9	29,7
50	49,2	49,1	50,7	42,3	42,7	43,1	42,7	43,1	43,5
	49,8	49,4	50,7	43,0	42,9	43,1	43,3	43,4	43,5
	49,7	49,7	50,1	42,9	43,3	42,5	43,2	43,7	42,9
150	147,1	154,8	148,7	142,7	150,8	143,9	143,8	152,4	145,2
	148,2	150,9	148,7	143,8	146,9	143,8	144,9	148,4	145,2
	152,2	148,3	145,1	147,9	144,1	140,1	149,1	145,6	141,4
250	249,4	247,3	242,8	247,6	245,6	240,6	249,5	248,1	242,8
	244,9	250,6	245,7	243,0	249,0	243,5	244,8	251,6	245,8
	245,7	248,0	247,5	243,8	246,3	245,4	245,6	248,8	247,7
350	351,9	349,1	355,3	352,7	349,7	356,3	355,4	353,4	359,6
	348,3	347,2	349,4	349,0	347,8	350,2	351,7	351,5	353,5
	354,1	352,8	352,5	354,9	353,6	353,3	357,7	357,3	356,6
450	446,3	450,7	450,7	449,5	453,8	454,3	453,0	458,5	458,6
	448,8	447,7	444,5	452,0	450,8	29,2	455,5	455,5	452,2
	451,3	452,3	447,7	454,6	455,4	29,3	458,1	460,1	455,4

VI.2.3.7. Trueness, precision, and accuracy

Processing collected data according to the previously described protocol makes it possible to build up different profiles by applying different calibration models. Indeed, it is possible to compute regression models using the calibration data between 15 and 180% of the nominal concentration. We can thus propose three accuracy profiles based on three calibration functions for both Phe and Tyr. In addition, it is possible to build up **Tables VI.14** and **VI.15** that contain all the performance criteria for both analytes at each concentration level.

As can be derived from the data of **Tables VI.14** and **VI.15**, all values did not exceed the acceptable limits of $\lambda \pm 15\%$. Most of the evaluated criteria are expressed in relative values.

The trueness, precision, and upper and lower limits of β-ETI, were determined by analyzing the back-calculated concentrations of the VS using already cited models. **Tables VI.14** and **VI.15** present the performance criteria for both analytes at each concentration level.

The results presented in **Tables VI.14** and **VI.15** demonstrate that the recoveries of Phe are within the acceptance range for all response functions. The recovery values for Phe were ranged from 98.35% to 101.66% for the response function Y=b X + a, from 96,18% to 101,55% for the response function Y = b X and from 94,61% to 99,89% for the response function Y= k(100%). For Tyr, the recoveries were ranged from 98.57% to 100.34% for the response function Y=b X + a, from 79,09% to 100,56% for the response function Y = b X and from 79,82% to 100,48% for the response function Y= k(100%). Therefore, only the response function Y=b X + a gives acceptable recovery among the tested functions.

These findings demonstrate the accuracy of the developed method in measuring the target analytes within the investigated concentration range, with recoveries closely aligned with the desired value of 100% for the response function Y=b X + a for both analytes. The average recoveries of Phe and Tyr were about 98% using the response function Y=b X + a, as is cited by Mo et al (Mo *et al.*, 2013). These findings are similar to our results for the first response function. In addition, the average recoveries of Phe and Tyr were 97–108% as reported in another study (Galba *et al.*, 2016). As comparing to this study, our findings were more accurate with higher recoveries and lower RSDs.

Furthermore, the relative biases are ≤ 10% for the response function Y=b X + a, either when analyzing Phe or Tyr, indicating minimal systematic errors in the measurement process when using this function for data analysis.

As seen in **Table VI.14**, the relative biases for each concentration level in all studied models for Phe ranged from – 5,39 to +1,65 %, which are acceptable and below the established limits of 10% (Zacharis and Vastardi, 2018). On the other hand, relative biases for Tyr were acceptable only for the first studied model and ranged from -1,43 to +1,25 % (**Table VI.15**).

The results for repeatability and intermediate precision showed that all RSDs were found to be ≤ 5% for all tested functions either for Phe or for Tyr. RSDs were ranging from 1,35% to 4.44% for Phe and from 0,56% to 2,30% for Tyr. These findings indicate excellent precision across all concentration levels (Shabir, 2003).

The results mentioned by Mo et al (Mo *et al.*, 2013) showed intraday and inter-day RSDs less than 4% and 6%, respectively. This is similar to our results, however in this study only one function was tested (Y = b X + a). Moreover, Inter-day precision (n = 5 days), expressed as % RSD, was in ranged from 1 to 13% in the study conducted by Galba G and collaborators (Galba *et al.*, 2016).

The first response function was the most appropriate because of its simplicity and ease of implementation in routine analysis (Anandhi and Nathiya, 2023).

Based on the data presented in **Tables VI.14** and **VI.15**, the accuracy profiles for Phe (**Figure VI. 11**) and Tyr (**Figure VI.12**) were constructed. The results demonstrate that the upper and lower limits of the interval of tolerance for each concentration level remain within the acceptable range of λ ± 15% (Papavasileiou *et al.*, 2022). These findings indicate that the method demonstrates accuracy and meets the specified criteria for trueness and precision. As a consequence, the obtained results demonstrate that the method is valid for the measurement of both analytes within the investigated concentration range.

Table VI.14. Performance criteria for phenylalanine analyzed by HPLC-UV method

Performance criteria		Response function	Concentration (µM))					
			Level 1 (15%)	Level 2 (20%)	Level 3 (60%)	Level 4 (100%)	Level 5 (140 %)	Level 6 (180%)
			37,5	50	150	250	350	450
Trueness	Relative bias (%)	Y = b X + a	-0,37	-1,64	-1,21	1,65	1,41	-1,27
		Y = b X	-3,38	-3,81	-1,67	1,55	1,45	-1,16
		Y = k(100%) X	-4,96	-5,39	-3,28	-0,11	-0,21	-2,78
	Recovery yield (%)	Y = b X + a	99,63	98,35	98,79	101,66	101,42	98,73
		Y = b X	96,61	96,18	98,32	101,55	101,45	98,84
		Y = k(100%) X	95,03	94,61	96,72	99,89	99,79	97,22
Precision	RSD repeatability (%)	Y = b X + a	3,29	2,24	1,35	1,61	2,63	1,44
		Y = b X	3,41	2,08	1,41	1,63	2,64	1,45
		Y = k(100%) X	3,41	2,06	1,38	1,68	2,62	1,46
	RSD precision (%)	Y = b X + a	4,44	2,24	1,35	1,61	2,63	1,44
		Y = b X	3,57	2,08	1,41	1,63	2,64	1,45
		Y = k(100%) X	3,59	2,06	1,38	1,68	2,62	1,46
Accuracy	Tolerance lower limit	Y = b X + a	-13,95	-7,24	-4,59	-2,37	-5,16	-4,87
		Y = b X	-12,37	-9,01	-5,21	-2,53	-5,13	-4,77
		Y = k(100%) X	14,02	10,54	6,72	4,32	6,74	6,42
	Tolerance higher limit	Y = b X + a	13,21	3,94	2,17	5,69	7,99	2,33
		Y = b X	5,59	1,37	1,85	5,63	8,04	2,45
		Y = k(100%) X	4,08	0,24	0,15	4,10	6,33	0,86

Table VI.15. Accuracy profiles parameters for tyrosine analyzed by HPLC-UV method

Performance criteria		Response function	Concentration (µM))					
			Level 1 (15%)	Level 2 (20%)	Level 3 (60%)	Level 4 (100%)	Level 5 (140%)	Level 6 (180%)
			37,5	50	150	250	350	450
Turness	Relative bias (%)	Y = b X + a	-1,43	0,35	-0,45	1,25	0,33	-0,24
		Y = b X	20,91	-14,30	-3,40	-2,01	0,55	0,48
		Y = k(100%) X	-20,18	-13,51	-2,51	-1,11	1,48	1,40
	Recovery yield (%)	Y = b X + a	98,57	99,64	99,55	98,75	100,34	99,75
		Y = b X	79,09	85,69	96,60	97,98	100,56	100,48
		Y = k(100%) X	79,82	86,48	97,49	98,88	101,48	101,41
Precision		Y = b X + a	1,00	0,65	1,83	0,88	0,79	0,57
		Y = b X	1,27	0,71	1,93	0,92	0,82	0,56

Performance criteria		Response function	Concentration (µM))					
			Level 1 (15%)	Level 2 (20%)	Level 3 (60%)	Level 4 (100%)	Level 5 (140%)	Level 6 (180%)
			37,5	50	150	250	350	450
	RSD repeatability (%)	Y = k(100%) X	1,27	0,75	1,93	0,91	0,80	0,592
	RSD precision (%)	Y = b X + a	1,08	1,22	1,97	0,99	0,79	0,57
		Y = b X	1,74	0	2,25	1,07	0,81	0,57
		Y = k(100%) X	1,81	0	2,30	1,13	0,80	0,60
Accuracy	Tolerance lower limit	Y = b X + a	-4,25	-6,31	-5,62	-3,87	-1,64	1,66
		Y = b X	-26,24	-16,08	-9,67	-5,01	-1,48	-0,91
		Y = k(100%) X	-25,73	-15,41	-8,92	-4,27	-0,51	-0,07
	Tolerance higher limit	Y = b X + a	1,40	5,60	4,71	1,37	2,31	1,17
		Y = b X	15,58	12,52	2,86	0,96	2,59	1,87
		Y = k(100%) X	14,63	11,62	3,89	2,04	3,47	2,88

The relative values allow us to build up **Figures VI.11** and **VI.12**. The accuracy profile for Phe, if setting a ±15% acceptability limit, will enable us to conclude that the method is valid for all the tested functions, as shown in **Figure VI.11**. Nevertheless, for Tyr, it was valid for only the first function response Y = b X + a, for the all studied range (**Figure VI.12**). For response function Y= b X and Y = k(100%) X the method is not valid for 15% and 20 % of the Tyr nominal value, but achieves its objective between 60% and 180%.

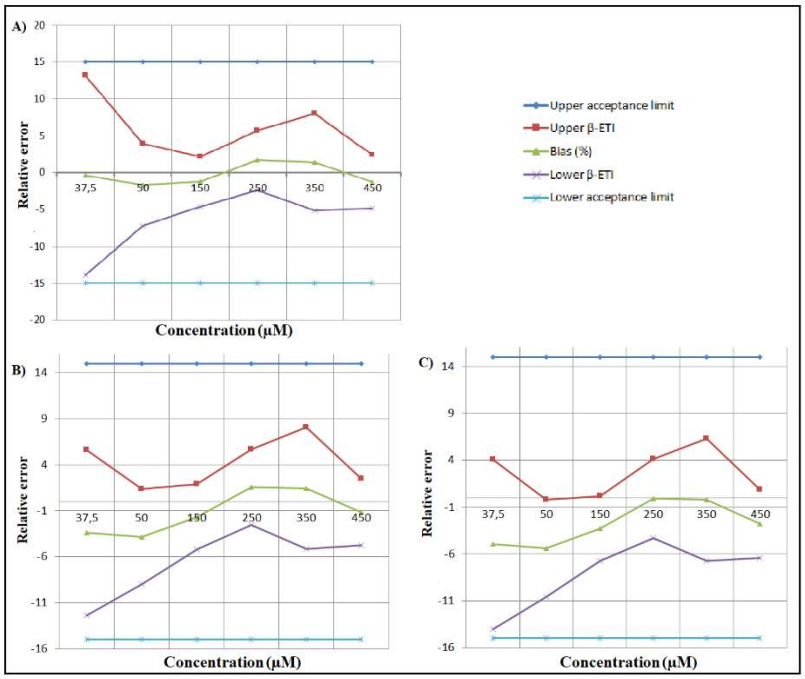

Figure VI.11 HPLC-UV accuracy profiles of phenylalanine (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

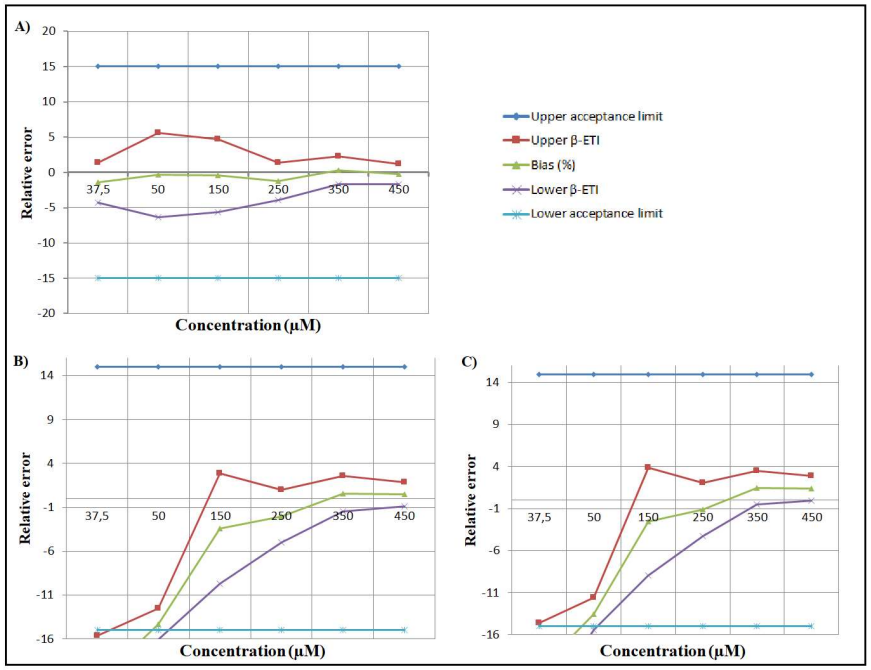

Figure VI.12. HPLC accuracy profiles of tyrosine (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

The choice of the accuracy profile is based on the objective of finding biases closer to zero, with a narrowest tolerance interval and located within the limits of acceptability, to have a valid method in the concentration interval studied. After carefully examining the accuracy profiles of the different models and according to these reasons for choice, we have selected the linear regression model Y = bX + a. On the other hand, the relative total errors are calculated for each concentration level, the results obtained are illustrated in **Figure VI.13**.

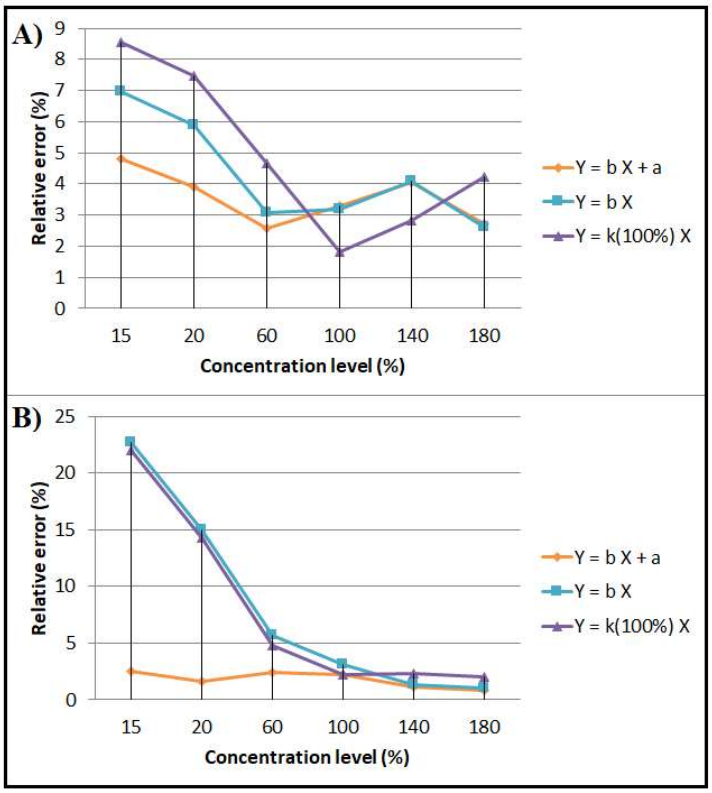

Figure VI.13. Relative error for (A) phenylalanine and (B) tyrosine analyzed by HPLC-UV method

According to this result, all concentration levels are judged to be included in the acceptability interval for Phe (all response functions) and for Tyr if we use the response function Y = b X + a. Referring to the accuracy profile already elucidated, the method developed in the present work is considered as valid for the assay interval where the accuracy profile is within the acceptance limits since the upper and lower tolerance limits do not intercept with the upper and lower acceptability limits.

This means that the method is capable of producing a proportion of 95% of acceptable results and only 5% of future measurements of unknown samples may be outside these limits. The validity range of the method is therefore between the concentration levels 80% and 120%.

VI.2.3.8. Linearity of the method

By comparing the actual concentrations of the prepared validation solutions with the concentrations determined by the method, the linearity of the method can be evaluated.

As can be seen, all calibration points are found within the prediction interval (**Figure VI.14**) and R2 present a value of 0.9989 and 0,9999 with slope values of 1,0008 and 0,9997 for Phe and Tyr respectively. These results indicate the strong relationship between actual and calculated concentrations.

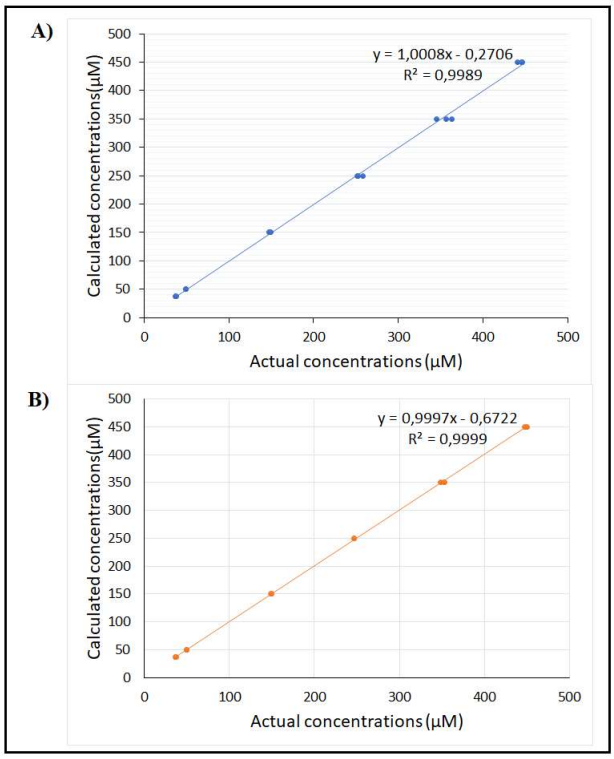

Figure VI.14. Linearity of HPLC method for (A) phenylalanine and (B) tyrosine determination in blood serum

VI.2.3.9. Limit of detection and limit of quantification

LOD and LOQ for Phe and Tyr were determined using the slope b of the calibration curve and the standard deviation of the intercept S(a), by application of the equations **V.4** and **V.5** described in section (**V.2.5.4.5**); for respectively calculating LOD and LOQ.

For Phe, the means of LOD and LOQ were calculated to be 10,4 µM and 31,4 µM, respectively. Similarly, for Tyr, the means of LOD and LOQ were found to be 4,7 µM and 14,1 µM, respectively.

The LOD were 1.5 µmol/Land 1 µmol/L of Phe and Tyr, respectively as cited by Mo Xi Ming and collaborators (Mo *et al.*, 2013). These results are slightly different to our results. This can be attributed to the difference in the tested concentration rang. In this study, authors have used concentrations ranging from 6 µM to 1512 µM for Phe and from 5.5 µM to 1250 µM for Tyr however in our study the concentration were ranging from 37,5 µM to 450 µM.

Table VI.16. Limit of detection and limit of quantifications of phenylalanine and tyrosine measured by HPLC-UV method

Analyte		Day 1	Day 2	Day 3	Mean
Phenylalanine	S (a)	2334,4	1870,7	1342,8	/
	Slope (b)	585,0	590,6	591,6	/
	LOD (µM)	13,2	10,5	7,5	10,4
	LOQ (µM)	39,9	31,7	22,7	31,4
Tyrosine	S (a)	4221,5	4730,9	6529,7	/
	Slope (b)	3654,0	3631,9	3659,7	/
	LOD (µM)	3,8	4,3	5,9	4,7
	LOQ (µM)	11,6	13,0	17,8	14,1

VI.2.3.10. Impact of storage duration and temperature on phenylalanine and tyrosine concentrations by HPLC method

To assess the impact of time and storage temperature on the levels of Phe and Tyr, serum samples and acidified aqueous solutions containing Phe and Tyr were stored at -30°C, 6°C, and room temperature (25°C) for one month.

As depicted in **Figure VI.15**, both Phe and Tyr levels remained stable for at least 30 days when serum samples or acidified aqueous solutions were stored at -30°C. They remained stable for 7 days when stored at 6°C. However, all specimens showed instability when stored at room temperature. Mo et al. obtained similar results when testing Phe and Tyr levels in blood samples after 7 days of storage (Mo *et al.*, 2013). Nevertheless, in the present study, we examined both acidified aqueous solutions and serum samples over a more extended period of time.

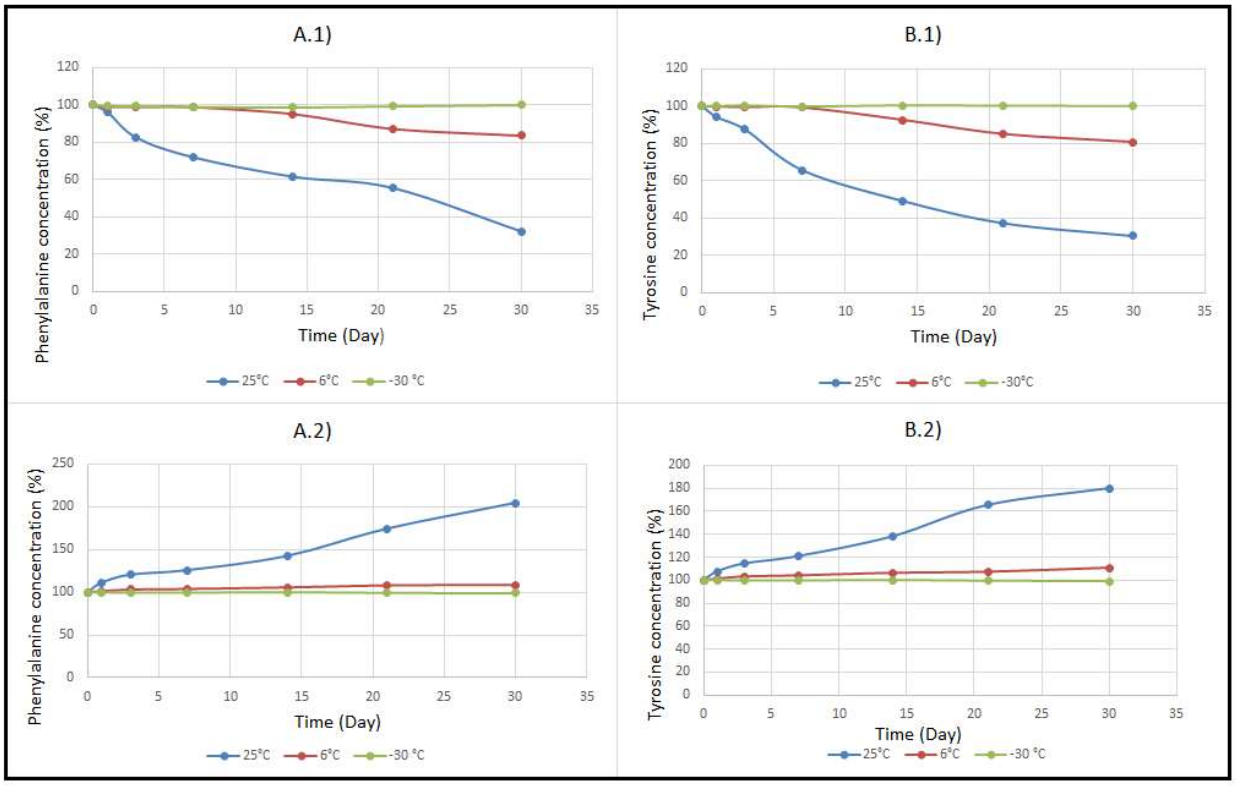

Figure VI.15. Impact of storage duration and temperature on phenylalanine (Phe) concentrations (A.1) in prepared acidic aqueous solutions and (A.2) in serum specimens and on tyrosine (Tyr) concentrations (B.1) in prepared acidic aqueous solutions and (B.2) in serum specimens (All analyzed by HPLC-UV method)

VI.3. Validation of LC-MSMS method

MRM measurements were performed using cone voltage and collision energy values derived from preliminary fragmentation studies with a continuous infusion of a 100 µM solution of each analyte within 0,5 min.

MS/MS analysis was done in positive ion mode and the ion transition for MS/MS detection in MRM were 166>120 and 182>136 with confirmatory ions 166>103 and 182>91 for Phe and Tyr, respectively. MRM is specific for well-targeted compounds. It is a selective and sensitive targeted MS technique used for quantifying specific analytes in a sample, unlike full scan which records all the ions present in the sample (Han *et al.*, 2020). Therefore, the mass spectra (**Figure VI.16**) for each analyte exhibit only two peaks. Collision energies were -15 and -29 (eV) for Phe ions and they were -15 and -30 (eV) in the case of Tyr ions.

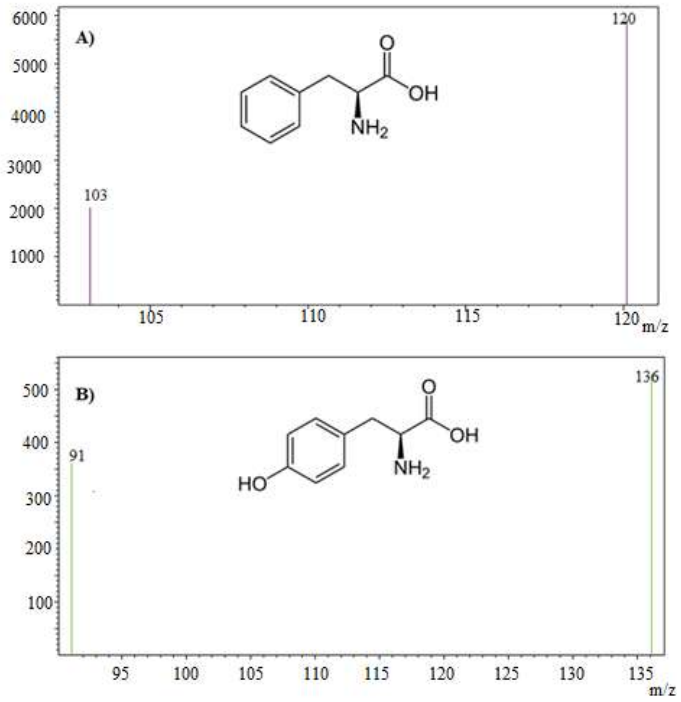

Figure VI.16. Mass spectrum of (A) phenylalanine and (B) tyrosine with their respective molecular formula

The ions with m/z 166 and m/z 182 are most likely a protonated Phe and Tyr molecules, $[M+H]^+$. For Phe fragmentation, the molecular peak M+ (absent in the spectrum) normally comes out at m/z = 165, odd because of the nitrogen atom. Also, the peak at m/z = 120 is the result of the cleavage of the CO – CNH2 bond and which gives the fragment C6H5 – CH2 – CHNH2 + and therefore loss of COOH. Similarly, the peak at m/z = 103 is the result of the cleavage of the CH – NH2 bond and which gives the fragment C6H5 – CH – CH + and therefore loss of NH3.

In the case of Tyr, the M+ molecular peak (absent in the spectrum) normally comes out at m/z = 181: odd because of the nitrogen atom. Also, the peak at m/z = 136 is the result of the cleavage of the CO – CNH2 bond and which gives the fragment C6H4OH – CH2 – CHNH2 + and therefore loss of COOH. Also, the peak at m/z = 91 can in our opinion only symbolize the tropylium ion [C7H7]+ known to be very stable. In **Figure VI.17**, we will propose possible fragmentation patterns that can explain the formation of these different fragments.

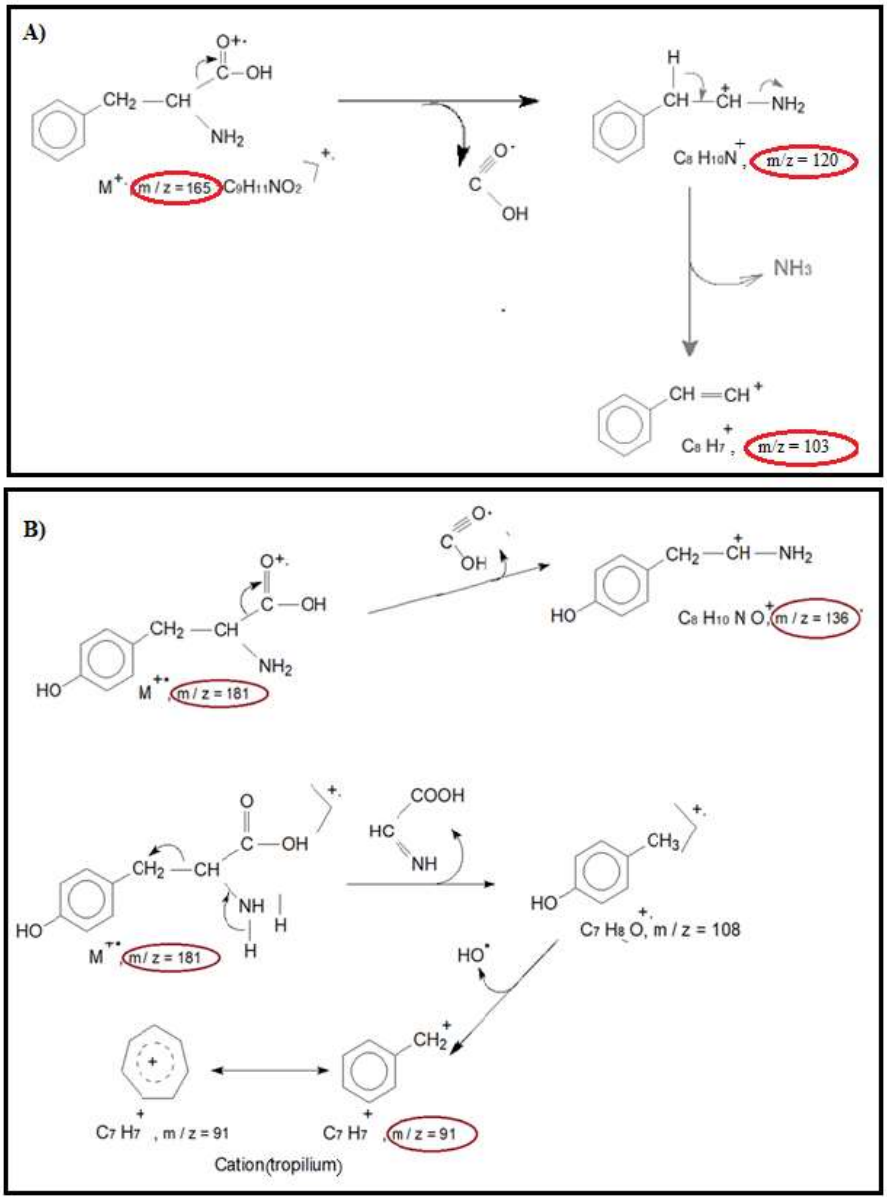

Figure VI.17. Proposed fragmentation mechanism of (A) phenylalanine and (B) tyrosine

VI.3.1. Raw data

VI.3.1.1. LC-MSMS for blood serum

The raw data obtained for the CS and VS are mentioned in the tables below (**Tables VI.17, VI.18**, **VI.19** and **VI.20**).

Table VI.17. Raw data for phenylalanine calibration series of LC-MSMS method (blood serum)

Conc-level	Repeti-tion	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (10%)	1	5,006	13543	5,005	13423	5,001	13639
	2	5,006	13890	5,005	13932	5,001	12911
2 (20%)	1	10,012	20543	10,020	21095	10,001	21658
	2	10,012	21876	10,020	22432	10,001	20965
3 (50%)	1	25,030	41987	25,050	42645	25,003	41989
	2	25,030	43654	25,050	42946	25,003	42970
4 (100%)	1	50,060	81761	50,100	79132	50,005	78072
	2	50,060	79432	50,100	82817	50,005	82329
5 (150%)	1	75,090	117546	75,150	120934	75,008	116978
	2	75,090	120432	75,150	114552	75,008	120765
6 (200%)	1	100,120	151549	100,200	155746	100,010	153654
	2	100,120	156283	100,200	154913	100,010	155432

Table VI.18. Raw data for phenylalanine validation series of LC-MSMS method (blood serum)

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (10%)	1	5,006	12988	5,005	13254	5,0005	13421
	2	5,006	13943	5,005	13656	5,0005	12867
	3	5,006	14032	5,005	12534	5,0005	13142
	1	10,012	20958	10,02	20749	10,001	21780

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
2 (20%)	2	10,012	20749	10,02	20654	10,001	20531
	3	10,012	22265	10,02	21546	10,001	20958
3 (50%)	1	25,03	40989	25,05	42432	25,0025	42314
	2	25,03	42432	25,05	42335	25,0025	41591
	3	25,03	43670	25,05	43234	25,0025	40489
4 (100%)	1	50,06	78072	50,1	79932	50,005	80219
	2	50,06	80932	50,1	77954	50,005	78499
	3	50,06	79329	50,1	79456	50,005	81072
5 (150%)	1	75,09	111978	75,15	117956	75,0075	115712
	2	75,09	119956	75,15	122655	75,0075	119112
	3	75,09	120765	75,15	115453	75,0075	117978
6 (200%)	1	100,12	151654	100,2	152437	100,01	150430
	2	100,12	157437	100,2	154213	100,01	154430
	3	100,12	150432	100,2	156463	100,01	156654

Table VI.19. Raw data for tyrosine calibration series of LC-MSMS method (blood serum)

Conce level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (10%)	1	5,006	4931	5,005	5004	5,0005	5122
	2	5,006	5134	5,005	5132	5,0005	5213
2 (20%)	1	10,012	7365	10,02	7354	10,001	7665
	2	10,012	7442	10,02	7554	10,001	7532
3 (50%)	1	25,03	14409	25,05	14543	25,0025	14675
	2	25,03	14854	25,05	14789	25,0025	14662
4 (100%)	1	50,06	26343	50,1	25858	50,005	28934
	2	50,06	25932	50,1	26574	50,005	25343
5 (150%)	1	75,09	37765	75,15	37897	75,0075	38712
	2	75,09	38564	75,15	38232	75,0075	38998
6 (200%)	1	100,12	51432	100,2	49031	100,01	50652
	2	100,12	49322	100,2	51435	100,01	51375

Table VI.20. Raw data for tyrosine validation series of LC-MSMS method (blood serum)

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (10%)	1	5,006	4998	5,005	5143	5,0005	4989
	2	5,006	4936	5,005	4963	5,0005	5387
	3	5,006	5221	5,005	5078	5,0005	5299
2 (20%)	1	10,012	7426	10,02	7417	10,001	7654
	2	10,012	7364	10,02	7547	10,001	7776
	3	10,012	7854	10,02	7394	10,001	7645
3 (50%)	1	25,03	14832	25,05	14417	25,0025	14902
	2	25,03	14156	25,05	14915	25,0025	14128
	3	25,03	13543	25,05	13622	25,0025	14574
4 (100%)	1	50,06	26998	50,1	26693	50,005	26084
	2	50,06	25178	50,1	26121	50,005	27684
	3	50,06	25858	50,1	27964	50,005	25419
5 (150%)	1	75,09	37653	75,15	38113	75,0075	36977
	2	75,09	36426	75,15	39434	75,0075	38977
	3	75,09	37897	75,15	37942	75,0075	37723
6 (200%)	1	100,12	50132	100,2	49348	100,01	51312
	2	100,12	49675	100,2	50985	100,01	49044
	3	100,12	49031	100,2	51435	100,01	49657

VI.3.1.2. LC-MSMS for dry blood spots

The raw data obtained for the CS and VS for Phe and Tyr analyzed by LC-MSMS for DBS, are mentioned in the tables below (**Table VI.21, VI.22**, **VI.23** and **VI.24**).

Table VI.21. Raw data for phenylalanine calibration series of LC-MSMS method (dry blood spots)

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (20%)	1	50,020	34513	50,010	32135	50,005	33973

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
	2	50,020	31867	50,010	34124	50,005	32312
2 (40%)	1	100,040	78960	100,020	79473	100,010	76342
	2	100,040	75165	100,020	76956	100,010	78266
3 (60%)	1	150,060	117226	150,030	117303	150,015	120981
	2	150,060	115934	150,030	119321	150,015	118612
4	1	250,100	202886	250,050	202152	250,025	202868
(100%)	2	250,100	205543	250,050	211886	250,025	205536
5	1	350,140	299515	350,070	299254	350,035	301854
(140%)	2	350,140	289515	350,070	286556	350,035	287465
6	1	450,180	389288	450,090	371483	450,045	373483
(180%)	2	450,180	369643	450,090	391823	450,045	395643

Table VI.22. Raw data for phenylalanine validation series of LC-MSMS method (dry blood spots)

Conc level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (20%)	1	50,02	33110	50,01	32432	50,005	32342
	2	50,02	34231	50,01	32956	50,005	32213
	3	50,02	31913	50,01	35161	50,005	34546
2 (40%)	1	100,04	77054	100,02	79023	100,01	76991
	2	100,04	76423	100,02	78187	100,01	76892
	3	100,04	79035	100,02	77456	100,01	77824
3 (60%)	1	150,06	112869	150,03	129723	150,015	121343
	2	150,06	118543	150,03	116593	150,015	121451
	3	150,06	117989	150,03	111924	150,015	120239
4	1	250,1	206435	250,05	208939	250,025	205342
(100%)	2	250,1	200989	250,05	191991	250,025	212453
	3	250,1	203546	250,05	214371	250,025	207711
5	1	350,14	294934	350,07	291439	350,035	298182
(140%)	2	350,14	301098	350,07	297265	350,035	300023
	3	350,14	297584	350,07	292142	350,035	301423
6	1	450,18	385463	450,09	371305	450,045	391375
(180%)	2	450,18	379349	450,09	391145	450,045	378821
	3	450,18	375876	450,09	382314	450,045	389945

Table VI.23. Raw data for tyrosine calibration series of LC-MSMS method (dry blood spots)

Conc level	Repetition	Series 1		Series 2		Series 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (20%)	1	50,02	6319	50,01	6762	50,005	6523
	2	50,02	6521	50,01	6313	50,005	6554
2 (40%)	1	100,04	12870	100,02	12987	100,01	13451
	2	100,04	13213	100,02	13441	100,01	12734
3 (60%)	1	150,06	19358	150,03	19547	150,015	19823
	2	150,06	20451	150,03	20545	150,015	20611
4 (100%)	1	250,1	34105	250,05	34716	250,025	35143
	2	250,1	33076	250,05	33425	250,025	34048
5 (140%)	1	350,14	48822	350,07	46756	350,035	46113
	2	350,14	46322	350,07	47172	350,035	47638
6 (180%)	1	450,18	59896	450,09	62109	450,045	61453
	2	450,18	61708	450,09	59328	450,045	62343

Table VI.24. Raw data for tyrosine validation series of LC-MSMS method (dry blood spots)

Conc level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
J	k	x	y	x	y	x	y
1 (20%)	1	50,02	6515	50,01	6657	50,005	6276
	2	50,02	6574	50,01	6576	50,005	6798
	3	50,02	6306	50,01	6832	50,005	6723
2 (40%)	1	100,04	13268	100,02	13654	100,01	12956
	2	100,04	13370	100,02	13121	100,01	12922
	3	100,04	12964	100,02	12745	100,01	13745
3 (60%)	1	150,06	21564	150,03	19651	150,015	19956
	2	150,06	20257	150,03	20783	150,015	20813
	3	150,06	20816	150,03	20992	150,015	19645
4 (100%)	1	250,1	35297	250,05	33591	250,025	35043
	2	250,1	34565	250,05	35423	250,025	34321
	3	250,1	33212	250,05	33192	250,025	33088
5 (140%)	1	350,14	49961	350,07	45167	350,035	48401
	2	350,14	47503	350,07	48582	350,035	48756
	3	350,14	46398	350,07	46573	350,035	44924

Conc level	Repetition	Day 1		Day 2		Day 3	
		Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)	Introduced concentration (µM)	Response (Area)
6 (180%)	1	450,18	60178	450,09	60238	450,045	61993
	2	450,18	58349	450,09	61827	450,045	63093
	3	450,18	61989	450,09	61281	450,045	60212

VI.3.2. Matrix effect

VI.3.2.1. LC-MSMS for blood serum

The results are illustrated in **Tables VI.25** and **VI.26**. In this part, we applicate Student t-test in order to evaluate matrix effect. The results showed that no matrix effect was detected when comparing the prepared solutions with and without matrix. We proceed to compare the two slopes and the two intercept at the origin.

By comparison of the two slopes and tow intercepts for both analytes, t calc over the repeated series (three days) was found to be lower than critical t (0,05; 26) which was equal to 2,06 (read on the Student's table, **Appendix III**).

We conclude that there is no significant difference between the slopes and the intercepts of different preparations spiked or not with matrix. Therefore, there is no matrix effect.

These results lead to using the protocol **IV** of validation according to SFSTP guidelines (see **Figure VI.4** and **Table VI.2**

Table VI.25. Comparison of slopes and intercepts of calibration and validation series of phenylalanine analyzed by LC-MSMS method for blood serum

Day	Day 1		Day 2		Day 3	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	1471,60	1483,40	1487,80	1485,77	1485,24	1491,93
s (b)	16,14	13,49	11,75	14,56	10,70	12,78
a	6180,69	6279,06	5653,63	6295,86	5573,00	5880,19
s (a)	906,44	757,74	660,60	818,41	600,10	716,85
R	1,00	1,00	1,00	1,00	1,00	1,00

R²	1,00	1,00	1,00	1,00	1,00	1,00
t calc	0,56	Equal slopes	0,11	Equal slopes	0,40	Equal slopes
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	0,08	Equal intercepts	0,61	Equal intercepts	0,33	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

Table VI.26. Comparison of slopes and intercepts of calibration and validation series of tyrosine analyzed by LC-MSMS method for blood serum

Day	**Day 1**		**Day 2**		**Day 3**	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	475,17	465,94	472,76	478,93	482,66	469,66
s (b)	4,59	2684,09	4,90	2612,49	6,96	2878,47
a	2612,72	3,94	2695,14	4,51	2753,88	4,88
s (a)	257,90	221,46	275,27	253,22	390,61	273,47
R	1,00	1,00	1,00	1,00	1,00	1,00
R²	1,00	1,00	1,00	1,00	1,00	1,00
t calc	0,56	Equal slopes		Equal slopes		Equal slopes
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	1,53	Equal intercepts	0,93	Equal intercepts	1,53	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

VI.3.2.2. LC-MSMS for dry blood spots

For further confirmation of the findings cited in the previous section and based on the results presented in the **Tables VI.27** and **VI.28**, no matrix effect was observed when comparing the prepared solutions with and without the matrix after application of the Student's t-test and this is by comparison the two slopes and the two intercepts at the origin.

The calculated t-value over the repeated series (three days) was found to be less than the critical t-value of 2.06 (read from the Student's t-distribution table, **Appendix III**). Accordingly, there is no matrix effect, as indicated by the absence of significant differences between the slopes and the intercepts.

The absence of significant differences in the intercepts and the slopes suggests that there is no matrix and contributes to the adoption of protocol **IV** of SFSTP guidelines to continue the technique validation.

Table VI.27. Comparison of slopes and intercepts of calibration and validation series of phenylalanine analyzed by LC-MSMS method for dry blood spots

Day	**Day 1**		**Day 2**		**Day 3**	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	872,61	867,99	867,93	868,99	885,78	875,84
s (b)	6,23	11,01	10,98	12,25	5,02	12,85
a	-11614,05	-11204,37	-10081,35	-10355,31	-11590,92	-11472,62
s (a)	1652,75	2923,48	2915,12	3252,08	1331,73	3409,48
R	1,00	1,00	1,00	1,00	1,00	1,00
R²	1,00	1,00	1,00	1,00	1,00	1,00
t calc	0,37	Equal slopes	0,06	Equal slopes	0,72	Equal slopes
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	0,12	Equal intercepts	0,06	Equal intercepts	0,03	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

Table VI.28. Comparison of slopes and intercepts of calibration and validation series of tyrosine analyzed by LC-MSMS method for dry blood spots

Day	**Day 1**		**Day 2**		**Day 3**	
Series	Validation	Calibration	Validation	Calibration	Validation	Calibration
b	136,54	135,06	135,42	135,52	137,71	137,71
s (b)	1,62	104,64	1,59	-116,40	1,51	-451,52
a	-511,24	1,94	-217,79	1,53	-450,93	1,74
s (a)	429,79	515,30	421,27	405,02	401,12	462,70
R	1,00	1,00	1,00	1,00	1,00	1,00
R²	1,00	1,00	1,00	1,00	1,00	1,00
t calc	0,59	Equal slopes		Equal slopes		Equal slopes
t (0,05 ; 26)	2,06		2,06		2,06	
t calc	0,92	Equal intercepts	0,17	Equal intercepts	0,00	Equal intercepts
t (0,05 ; 26)	2,06		2,06		2,06	

VI.3.3. Suitability system

The results of the system suitability test (**Table VI.29**) indicated that the LC-MSMS method performed consistently and satisfactorily. The RSDs values for both retention time and peak area were below 2%, demonstrating repeatable measurements. Additionally, T≤ 2, suggesting that the peaks were symmetrical and well-defined. Furthermore, N> 2000, confirming efficient chromatographic separation (Shabir, 2003) . Overall, these findings validate that the LC-MSMS method used for analysis exhibits excellent system suitability, satisfying the required criteria for precision, peak shape, and column efficiency. A slight difference between the retention time was observed. The peak integration is challenging when using full scan conditions, also the peaks shapes of the FIA-MS/MS results are frequently irregular and precision of peak integration is less perfect. In addition, chromatographic process producing Gaussian shape of peaks with numerous data points whose area can be more thoroughly measured (Casetta *et al.*, 2000). Consequently, using HPLC in combination with MS/MS can resolve those problems. The LC-MSMS technology used in this study offer the most appropriate peaks shape specially when it is combined with MRM mode.

Table VI.29. Results of the suitability system of LC-MSMS method

Parameter Analyte	**Blood serum**		**Dry blood spots**		**Result**
	Phenylalanine	Tyrosine	Phenylalanine	Tyrosine	
Retention time (min)	1,771±0,02	1,987±0,01	1,816±0,03	2,008±0,022	Repeatable measurements
RSD of retention time (%)	1,13	0,5	1,65	1,1	
Tailing factor (T)	1,451	1,648	1,441	1,702	Peak symmetry
Number of theoretical plates (N)	2359	2983	2212	2843	Column efficiency

The results are expressed as Mean±SD

VI.3.4. Selectivity

The blank solution showed a negligible response, indicating that there were no interfering components present, as illustrated in **Figure VI.18**.

The selectivity and accurate detection of the analytes of interest were confirmed by the standard solutions, which produced distinct and well-defined peaks.

Additionally, the standard solutions spiked with the matrix demonstrated responses within the acceptable range, suggesting that the matrix's presence did not significantly interfere with the quantification of the analytes. Therefore, the developed method is specific and effectively quantifies the analytes of interest, even in complex matrices. The criterion of maintaining a response below 20% of the LLOQ (Area of matrix = 385) guarantees the reliable measurement of the analytes, ensuring that significant interferences are minimized.

Representative MRM-chromatograms for Phe and Tyr at different concentration levels in blood serum or in DBS are presented in **Figures VI.19** and **VI.20**, respectively.

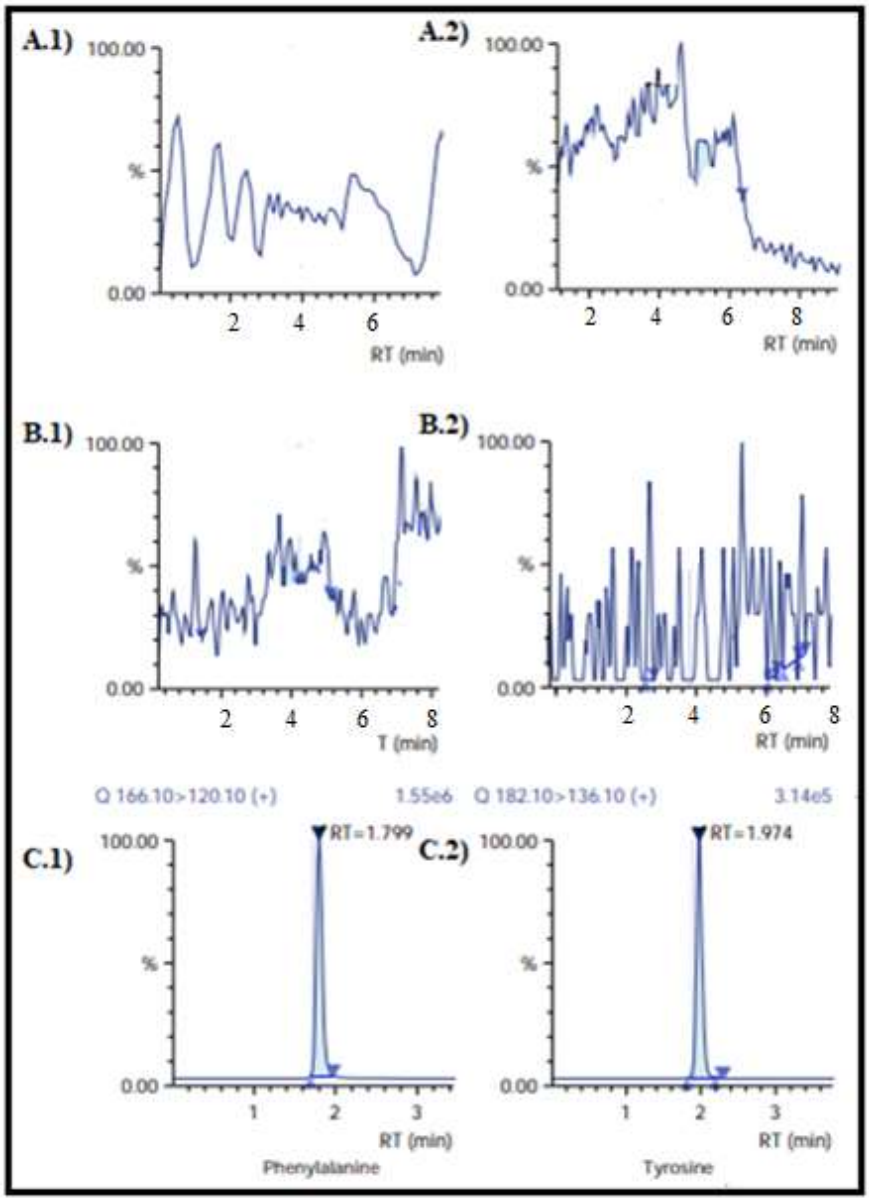

Figure VI.18. Representative chromatogram of (A) a procedure blank (A.1 with phenylalanine detection and A.2 with tyrosine detection); (B) a standard matrix solution (B.1 with phenylalanine detection and B.2 with tyrosine detection) and (C) each tested amino acid (C.1 phenylalanine and C.2 tyrosine), all analyzed by LC-MSMS method

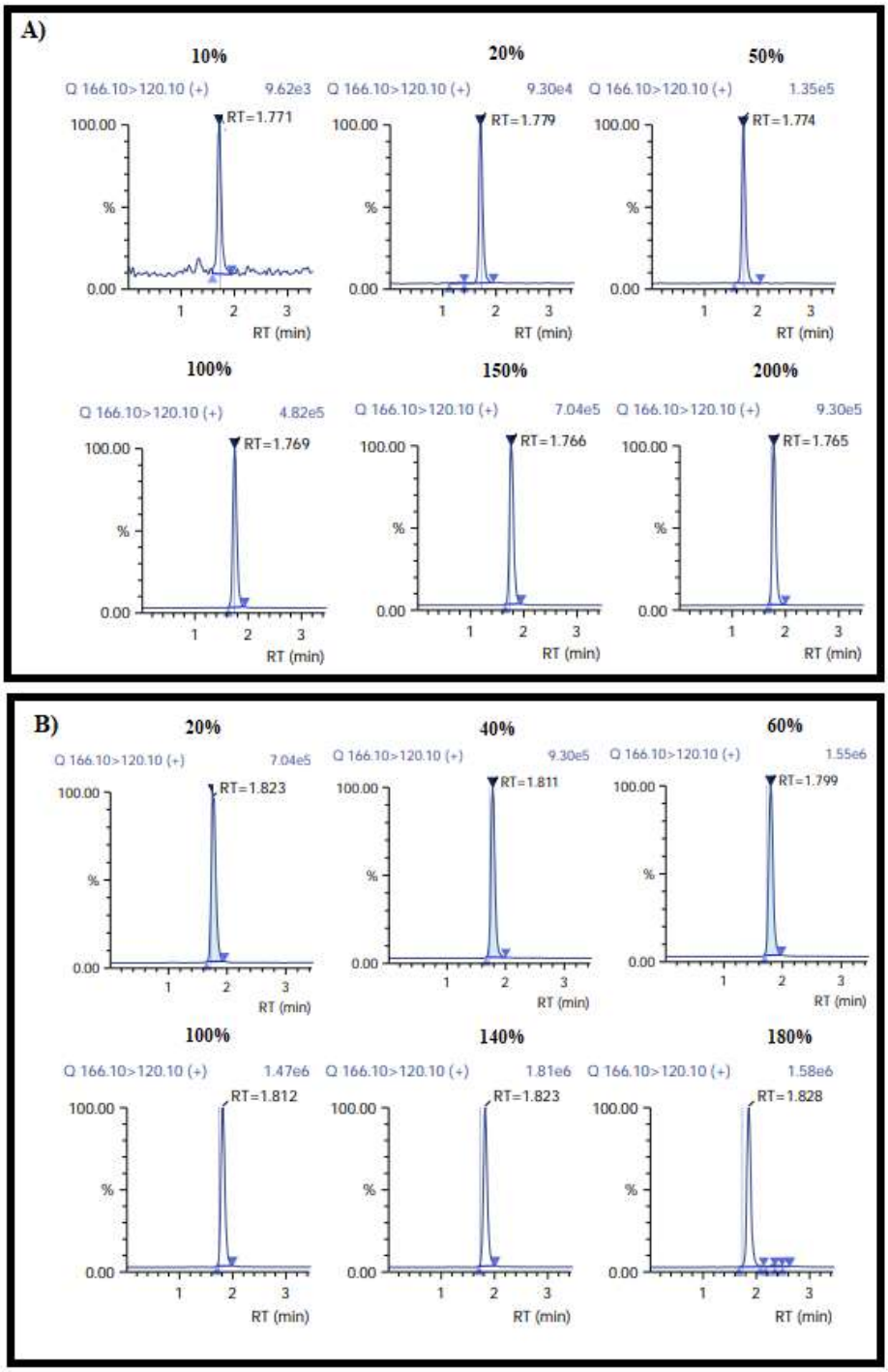

Figure VI.19. Representative MRM-chromatograms for phenylalanine at different concentration levels in A) blood serum, B) dry blood spots

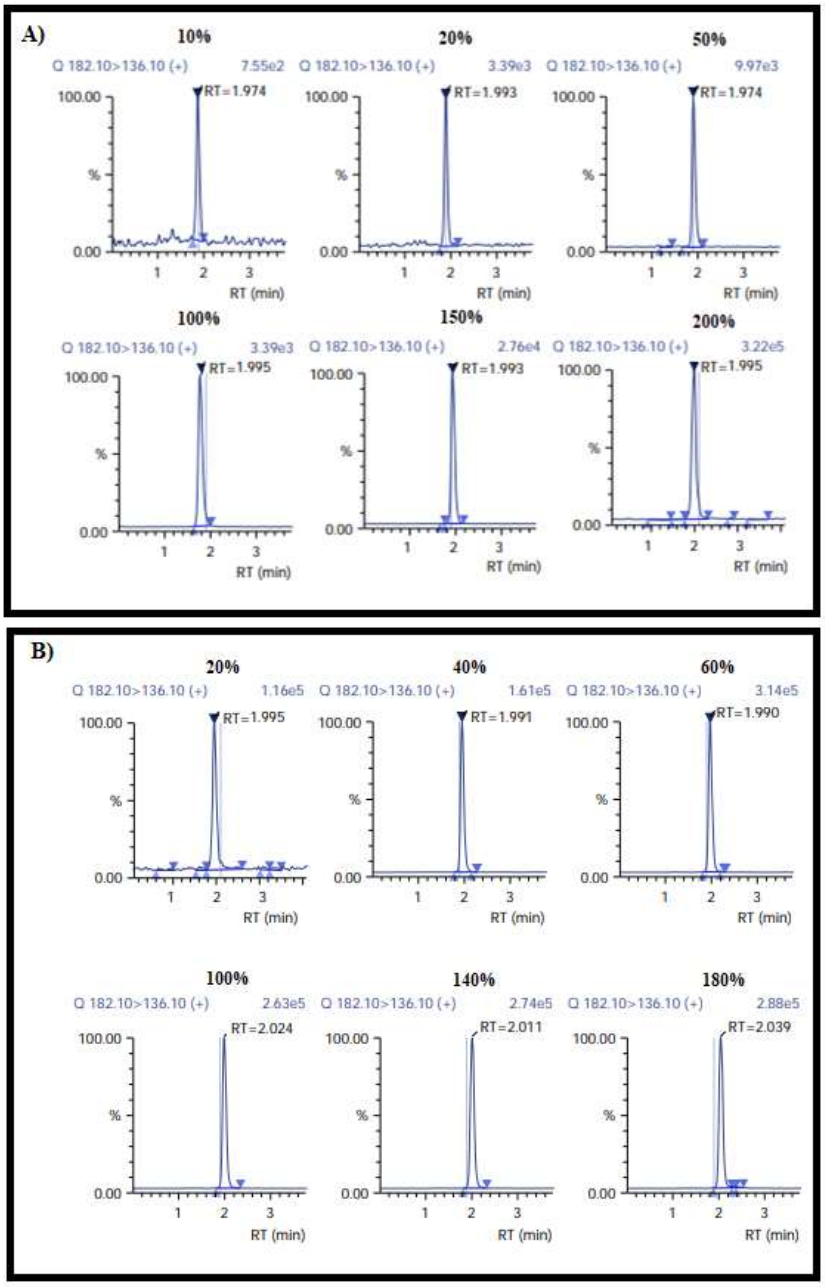

Figure VI.20. Representative MRM-chromatograms for tyrosine at different concentration levels in A) blood serum, B) dry blood spots

VI.3.5. Carry-over

The evaluation of carry-over effect demonstrated that the observed response in the blank samples was well below the 20% of the LLOQ (Area of matrix = 385). This suggests that any residual analyte from the previous injection did substantially affect the subsequent sample analysis. These findings validate the method's ability to control carry-over, ensuring accurate and reliable quantification of analytes across consecutive injections. The analytical results' integrity is maintained and the "between-injection" cleaning procedures employed in the method, is effective.

VI.3.6. Linearity of the response function

The results of the linearity assessment demonstrated that the calibration curves for the analytes of interest displayed excellent linearity throughout the tested concentration range. This was confirmed over the validation interval by analyzing data from the repeated measurements at each concentration level within the calibration series.

VI.3.6.1. LC-MSMS for blood serum

VI.3.6.1.1. Response functions

A statistical analysis of the resulting straight lines was conducted through comparison of the slope to the reference value of 1 and the intercept to the reference value of 0, while also assessing the overall fit. The results of the comparison of calibration series lines Y=f(X) are summarized in **Tables VI.30** and **VI.31**.

Table VI.30. Response functions of phenylalanine analyzed by LC-MSMS method for blood serum

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X + a	b	1483,40	Significant slope	1485,77	Significant slope	1491,93	Significant slope
	s (b)	13,49		14,56		12,78	
	a	6279,06		6295,86		5880,19	
	s (a)	757,74		818,41		716,85	
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	12085,22		10409,40		13628,86	

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
	F (0,05 ;1;10)	4,96		4,96		4,96	
Y = b X	b	1571,45	Significant slope	1573,98	Significant slope	1574,47	Significant slope
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	4991,80		4889,20		5686,50	
	F (0,05 ;1;11)	4,84		4,84		4,84	
Y = k(100%) X	k	1610,00	/	1616,26	/	1603,85	/

Table VI.31. Response functions of tyrosine analyzed by LC-MSMS method for blood serum

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X + a	b	465,94	Significant slope	478,93	Significant slope	469,66	Significant slope
	s (b)	2684,09		2612,49		2878,47	
	a	3,94		4,51		4,88	
	s (a)	221,46		253,22		273,47	
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	13958,34		11299,00		9280,79	
	F (0,05 ;1;16)	4,49		4,49		4,49	
Y = b X	b	511,80	Significant slope	510,53	Significant slope	521,32	Significant slope
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	3192,48		2971,22		2717,98	
	F (0,05 ;1;11)	4,84		4,84		4,84	
Y = k(100%) X	k	522,12	/	523,27	/	542,72	/

The comparison of the calibration series lines Y=f(X) yielded satisfactory results, indicating that the chosen calibration system is appropriate. The R^2 obtained from the linear regression analysis was equal to 1,00 for both analytes, demonstrating a strong linear relationship between the response and concentration within the studied calibration range for both Phe and Tyr (Dong, 2006). Furthermore, the slope was statistically different to 0 at a 5% risk level in the studied functions Y=bX and Y=bX+a. (F calculated> F ctitical). These findings collectively confirm the linearity of the method over the validation interval.

VI.3.6.1.2. Alignment of response (inverse predicted quantity)

When the introduced quantities are not equivalent, it is necessary to align the responses to the mean of the introduced concentrations. This alignment is performed for each concentration level. For the validation samples, the alignment of the obtained responses is carried out using the inverse functions; X=(Y-a)/b, X=Y/b and X=Yk(100%). The back calculated concentrations are summarized in the following tables.

Table VI.32. Back calculated concentrations of phenylalanine analyzed by LC-MSMS method for blood serum

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5	4,5	4,7	5,1	8,3	8,4	8,5	8,1	8,2	8,4
	5,2	4,9	4,7	8,9	8,7	8,2	8,7	8,4	8,0
	5,2	4,2	4,9	8,9	8,0	8,3	8,7	7,7	8,2
10	9,9	9,7	10,7	13,3	13,2	13,8	13,0	12,8	13,6
	9,7	9,6	9,8	13,2	13,1	13,0	12,9	12,8	12,8
	10,8	10,2	10,1	14,2	13,7	13,3	13,8	13,3	13,1
25	23,4	24,3	24,4	26,1	26,9	26,9	25,4	26,2	26,4
	24,3	24,2	23,9	27,0	26,8	26,4	26,3	26,1	25,9
	25,2	24,8	23,2	27,8	27,4	25,7	27,1	26,7	25,2
50	48,3	49,5	49,8	49,6	50,7	50,9	48,4	49,4	50,0
	50,3	48,1	48,7	51,4	49,4	49,9	50,2	48,1	48,9
	49,2	49,1	50,4	50,4	50,4	51,5	49,2	49,1	50,5
75	71,2	75,0	73,6	71,2	74,8	73,5	69,5	72,8	72,1
	76,5	78,2	75,9	76,2	77,8	75,6	74,4	75,7	74,3

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	77,1	73,3	75,1	76,8	73,2	74,9	74,9	71,3	73,6
100	97,9	98,2	96,9	96,4	96,6	95,5	94,1	94,1	93,8
	101,8	99,4	99,6	100,1	97,8	98,1	97,7	95,2	96,3
	97,1	100,9	101,0	95,6	99,2	99,5	93,3	96,6	97,7

Table VI.33. Back calculated concentrations of tyrosine analyzed by LC-MSMS method for blood serum

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
5	5,0	5,2	4,6	9,8	10,1	9,6	9,6	9,8	9,2
	4,9	4,8	5,5	9,6	9,7	10,3	9,4	9,5	9,9
	5,5	5,0	5,3	10,2	9,9	10,2	10,0	9,7	9,8
10	10,1	10,0	10,2	14,5	14,5	14,7	14,2	14,2	14,1
	10,0	10,2	10,4	14,4	14,8	14,9	14,1	14,4	14,3
	11,0	9,9	10,1	15,3	14,5	14,7	15,0	14,1	14,1
25	25,7	24,7	25,2	28,9	28,2	28,6	28,4	27,5	27,5
	24,3	25,8	23,6	27,6	29,2	27,1	27,1	28,5	26,0
	23,0	23,1	24,5	26,4	26,6	28,0	25,9	26,0	26,9
50	51,3	50,7	48,3	52,7	52,2	50,0	51,6	50,9	48,1
	47,4	49,5	51,6	49,1	51,1	53,1	48,2	49,8	51,0
	48,9	53,3	47,0	50,5	54,7	48,8	49,5	53,3	46,8
75	73,7	74,8	70,9	73,5	74,5	70,9	72,0	72,7	68,1
	71,1	77,6	75,0	71,1	77,1	74,8	69,7	75,2	71,8
	74,2	74,4	72,4	74,0	74,2	72,4	72,5	72,4	69,5
00	99,9	98,5	100,6	97,8	96,5	98,4	95,9	94,1	94,5
	98,9	101,9	95,9	96,9	99,7	94,1	95,0	97,2	90,4
	97,6	102,9	97,2	95,7	100,5	95,2	93,8	98,1	91,5

VI.3.6.2. LC-MSMS for dry blood spots

VI.3.6.2.1. Response functions

As was shown for blood serum, a statistical analysis of the DBS straight lines was performed, comparing the slope to the reference value of 1 and the intercept to the reference value of 0. The results of the comparison of the CS lines Y=f(X) are summarized in **Tables VI.34** and **VI.35**.

Table VI.34. Response functions of phenylalanine analyzed by LC-MSMS method for dry blood spots

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X + a	b	867,99	Significant slope	868,99	Significant slope	875,84	Significant slope
	s (b)	11,01		12,25		12,85	
	a	-11204,37		-10355,31		-11472,62	
	s (a)	2923,48		3252,08		3409,48	
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	6212,27		5029,82		4647,68	
	F (0,05 ;1;10)	4,96		4,96		4,96	
Y = b X	b	832,20	Significant slope	835,90	Significant slope	839,19	Significant slope
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	9052,59		9044,47		7831,54	
	F (0,05 ;1;11)	4,84		4,84		4,84	
Y = k(100%) X	k	816,53	/	827,91	/	816,73	/

Table VI.35. Response functions of tyrosine analyzed by LC-MSMS method for dry blood spots

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
Y = b X + a	b	135,06	Significant slope	135,52	Significant slope	137,71	Significant slope
	s (b)	104,64		-116,40		-451,52	
	a	1,94		1,53		1,74	
	s (a)	515,30		405,02		462,70	
	R	1,00		1,00		1,00	
	R^2	1,00		1,00		1,00	
	F	4841,06		7887,32		6238,89	
	F (0,05 ;1;16)	4,49		4,49		4,49	
Y = b X	b	135,39	Slope significant	135,15	Significant slope	136,27	Significant slope
	R	1,00		1,00		1,00	

Response function		Day 1	Conclusion	Day 2	Conclusion	Day 3	Conclusion
	R^2	1,00		1,00		1,00	
	F	18343,88		29500,45		21795,90	
	F (0,05 ;1;1)	4,45		4,45		4,45	
Y = k(100%) X	k	134,31	/	136,25	/	138,37	/

The chosen calibration system is appropriate. The robust linear relationship between the response and concentration across the calibration range is confirmed by linear regression analysis which yielded a R^2 of 1,00 for both Phe and Tyr (Dong, 2006).

Additionally, the slope was found to be statistically significant and different to 0 at a 5% significance level for the functions Y=bX and Y=bX+a (F calculated> F critical). Together, these results affirm the linearity of the method throughout the validation interval.

VI.3.6.2.2. Alignment of response (inverse predicted quantity)

For the validation series, the alignment of the obtained responses is carried out using the inverse functions; X=(Y-a)/b, X=Y/b and X=Yk(100%). The back calculated concentrations are presented in **Table VI.36** and **VI.37**.

Table VI.36. Back calculated concentrations of phenylalanine analyzed by LC-MSMS method for dry blood spots

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
50	51,0	49,2	50,0	39,8	38,8	38,5	40,5	39,2	39,6
	52,3	49,8	49,9	41,1	39,4	38,4	41,9	39,8	39,4
	49,7	52,4	52,5	38,3	42,1	41,2	39,1	42,5	42,3
100	101,6	102,8	101,0	92,6	94,5	91,7	94,3	95,4	94,3
	100,9	101,9	100,9	91,8	93,5	91,6	93,6	94,4	94,1
	103,9	101,0	101,9	94,9	92,6	92,7	96,8	93,5	95,3
150	142,9	161,2	151,6	135,6	155,2	144,6	138,2	156,7	148,6

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
	149,4	146,1	151,8	142,4	139,5	144,7	145,1	140,8	148,7
	148,8	140,7	150,4	141,7	133,9	143,3	144,4	135,2	147,2
250	250,6	252,3	247,5	248,0	249,9	244,7	252,7	252,3	251,4
	244,4	232,8	255,6	241,4	229,6	253,1	246,0	231,8	260,1
	247,3	258,6	250,2	244,5	256,4	247,5	249,2	258,9	254,3
350	352,6	347,2	353,5	354,3	348,6	355,3	361,1	351,9	365,1
	359,7	353,9	355,6	361,7	355,6	357,5	368,6	359,0	367,3
	355,6	348,0	357,2	357,4	349,4	359,1	364,3	352,8	369,0
450	456,8	439,1	459,9	463,0	444,1	466,3	471,9	448,4	479,2
	449,8	461,9	445,6	455,7	467,8	451,4	464,4	472,4	463,8
	445,8	451,8	458,3	451,5	457,3	464,6	460,2	461,7	477,4

Table VI.37. Back calculated concentrations of tyrosine analyzed by LC-MSMS method for dry blood spots

C (µM)	X = (Y-a)/b			X = Y/b			X=Y/k(100%)		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
50	51,4	50,8	48,8	48,3	49,4	46,1	48,5	48,8	45,4
	51,9	50,2	52,6	48,7	48,8	49,9	48,9	48,3	49,1
	49,9	52,0	52,1	46,7	50,7	49,3	46,9	50,1	48,6
100	100,9	102,4	97,3	98,3	101,3	95,1	98,7	100,2	93,6
	101,6	98,5	97,1	99,1	97,4	94,8	99,5	96,3	93,4
	98,7	95,7	103,1	96,1	94,6	100,9	96,5	93,5	99,3
150	161,6	146,7	148,2	159,8	145,8	146,4	160,5	144,2	144,2
	152,0	155,0	154,4	150,1	154,2	152,7	150,8	152,5	150,4
	156,1	156,6	145,9	154,2	155,8	144,2	154,9	154,0	142,0
250	262,2	249,6	257,7	261,5	249,3	257,1	262,7	246,5	253,2
	256,8	263,1	252,5	256,1	262,9	251,8	257,3	259,9	248,0
	246,9	246,7	243,5	246,1	246,3	242,8	247,2	243,6	239,1
350	369,5	335,1	354,7	370,2	335,2	355,2	371,8	331,4	349,8
	351,5	360,3	357,3	352,0	360,5	357,8	353,5	356,5	352,3
	343,4	345,4	329,5	343,8	345,6	329,6	345,3	341,7	324,6
450	444,3	446,3	453,4	445,9	447,0	454,9	447,9	442,0	448,0
	430,9	458,1	461,4	432,3	458,8	463,0	434,3	453,7	455,9
	457,6	454,0	440,5	459,3	454,8	441,8	461,4	449,7	435,1

VI.3.7. Trueness, precision, and accuracy

As discussed previously, two methods can be employed to select a response function that yields a sufficient proportion of future results within acceptable limits. These approaches involve either calculating indices (such as dosage interval, accuracy, trueness, and precision) or comparing accuracy profiles to ensure that the selected response function satisfies the required performance criteria.

VI.3.7.1. LC-MSMS for blood serum

Different profiles were built up by applying different calibration models and processing collected data according to the previously described protocol. Indeed, it is possible to compute regression models using the calibration data between 10 and 200% of the nominal concentration.

We can thus propose three accuracy profiles based on three calibration functions for both Phe and Tyr. Performance criteria of both analytes at each concentration level are grouped in **Table VI.38** and **Table VI.39**.

As can be derived from the data of **Table VI.38** and **Table VI.39**, all values did not exceed the acceptable limits of $\lambda \pm 15\%$. Most of the evaluated criteria are expressed relative values.

Analyzing the back-calculated concentrations of the VS using already cited models mains to the determination of trueness, precision, and upper and lower limits of β-ETI.

For the study of trueness through recovery and bias and as is shown in **Table VI.38** and **Table VI.39**, the recoveries of Phe are within the acceptance range for all response functions. The recovery values for Phe ranged from 96,27% to 100,63% for the response function Y=b X + a, from 97,64% to 169,19% for the response function Y = b X and from 95,41% to 165,32% for the response function Y= k(100%). For Tyr, the recoveries were ranged from 97,66% to 102,15% for the response function Y=b X + a, from 97,20% to 198,63% for the response function Y = b X and from 94,50 to 193,09% for the response function Y= k(100%). Therefore, only the response function Y=b X + a, gives acceptable recoveries among the tested functions either for Phe or for Tyr.

These findings demonstrate the accuracy of the developed method in measuring the target analytes within the investigated concentration range, with recoveries closely aligned with the desired value of 100% for the response function Y=b X + a for both analytes.

In our study, we have improved the recoveries of the method when using the first response function, as comparing to those found by Cassetta B and collaborators. In this later study, recovery of Phe was ranged from 98% to 110% and that of Tyr was ranged from 106% to 108% (Casetta *et al.*, 2000).

As seen in **Tables VI.38**, the relative biases for the first model for Phe are ranged from – 3,73 to +0,63 %, which are acceptable and below the established limits of 10%. On the other hand, relative biases for Tyr were acceptable also and ranged from -2,33 to +2,15 % (**Table VI.39**). These findings indicate minimal systematic errors in the measurement process when using the function Y=b X + a for data analysis. This later is known to be simple and more appropriate for routine analysis(Anandhi and Nathiya, 2023).

The results for repeatability and intermediate precision showed that almost RSDs were found to be ≤ 5% for all test functions either for Phe or for Tyr. For Phe , RSDs were ranging from 1,62% to 6.86 % for the response function Y = b X + a, from 1,47 to 3,72% for the response function Y = b X and from 1,6 to 3,71% for the response function Y = k(100%)X. The first concentration level (10%) when tested by the function Y = b X + a, presents a high RSD (6,86%) On the other hand, in the case of Tyr, RSDs were ranging from 2,6% to 5,73 % for the response function Y = b X + a and from 1,95 to 3,82% for other functions. These findings indicate excellent precision across all concentration levels.

Similar results were found by Cassetta B *et al* and Peng M *et al*, intraday and interday reproducibility tests using plasma samples were found to be within the acceptable range for both analytes (Casetta *et al.*, 2000; Peng *et al.*, 2019).

Table VI.38. Performance criteria for phenylalanine analyzed by LC-MSMS method for blood serum

Performance criteria		**Response function**	**Concentration (µM))**					
			Level 1 (10%)	**Level 2 (20%)**	**Level 3 (50%)**	**Level 4 (100%)**	**Level 5 (150%)**	**Level 6 (200%)**
			5	**10**	**25**	**50**	**75**	**100**
Trueness	Relative bias (%)	Y = b X + a	-3,73	0,63	-3,23	-1,46	0,13	-0,82
		Y = b X	69,19	34,2	7,09	0,94	-0,149	-2,35
		Y = k(100%) X	65,32	31,14	4,64	-1,35	-2,42	-4,58
	Recovery yield (%)	Y = b X + a	96,27	100,63	96,76	98,53	100,13	99,17
		Y = b X	169,19	134,21	107,09	100,94	99,85	97,64
		Y = k(100%) X	165,32	131,14	104,64	98,64	97,57	95,41
Precision	RSD repeatability (%)	Y = b X + a	6,86	4.12	2,60	1,62	2,86	1,81
		Y = b X	3,72	2,83	2,33	1,47	2,70	1,73
		Y = k(100%) X	3,71	2,86	2,2	1,6	2,69	1,77
	RSD precision (%)	Y = b X + a	6,86	4.12	2,60	1,62	2,86	1,81
		Y = b X	3,80	2,83	2,88	1,47	2,70	1,73
		Y = k(100%) X	3,75	2,86	2,2	1,64	2,69	1,77
Accuracy	Tolerance lower limit	Y = b X + a	-20,83	9,63	9,72	5,52	-6,99	5,33
		Y = b X	59,67	27,13	1,11	-2,73	-6,90	-6,69
		Y = k(100%) X	55,93	23,99	-0,86	-5,47	-9,13	-9,01
	Tolerance higher limit	Y = b X + a	13,37	10,89	3,25	2,59	7,26	3,69
		Y = b X	78,70	41,28	13,07	4,629	6,603	1,97
		Y = k(100%) X	74,71	38,29	10,14	2,763	4,279	-0,16

Table VI.39. Accuracy profiles parameters for tyrosine analyzed by LC -MSMS method for blood serum

Performance criteria		Response function	Concentration (µM))					
			Level 1 (10%)	Level 2 (20%)	Level 3 (50%)	Level 4 (100%)	Level 5 (150%)	Level 6 (200%)
			5	10	25	50	75	100
Trueness	Relative bias (%)	Y = b X + a	1,64	2,15	-2,33	-0,45	-1,62	-0,73
		Y = b X	98,63	46,89	11,39	2,68	-1,87	-2,79
		Y = k(100%) X	93,09	42,79	8,28	-0,16	-4,61	-5,49
	Recovery yield (%)	Y = b X + a	100,64	102,15	97,66	99,54	98,37	99,26
		Y = b X	198,63	146,89	111,39	102,68	98,12	97,20
		Y = k(100%) X	193,09	142,79	108,28	99,83	95,39	94,50
Precision	RSD repeatabi-lity (%)	Y = b X + a	5,73	3,28	4,31	4,24	2,48	2,06
		Y = b X	2,75	2,00	3,52	3,82	2,31	1,95
		Y = k(100%) X	2,65	2,12	3,60	3,82	2,30	1,95
	RSD precision (%)	Y = b X + a	5,73	3,28	4,31	4,24	2,93	2,37
		Y = b X	2,75	2,00	3,52	3,82	2,72	2,24
		Y = k(100%) X	2,65	2,12	3,60	4,15	3,15	2,82
Accuracy	Tolerance lower limit	Y = b X + a	-6,90	-2,74	-8,76	-6,79	-6,31	-4,43
		Y = b X	/	/	6,13	-3,01	-6,23	-6,27
		Y = k(100%) X	/	/	2,90	-6,57	-9,93	-10,29
	Tolerance higher limit	Y = b X + a	10,19	7,06	4,09	5,87	3,06	2,95
		Y = b X	/	/	16,64	8,39	2,47	0,69
		Y = k(100%) X	/	/	13,66	6,23	0,71	-0,70

Based on upper and lower limits of β-ETI, the accuracy profiles for Phe (**Figure VI.21**) and Tyr (**Figure VI.22**) were constructed.

The results concerning the first model analyzing Phe demonstrate that the upper and lower limits of the interval of tolerance for concentration levels from 20% to 200% remain within the acceptable range of λ ± 15% (Papavasileiou *et al.*, 2022). For the concentration level 10%, the tolerance lower limit was -20,83 and exceeds the acceptable range of λ ± 15%. So, the method was accurate from 20 to 200% for Phe when using the first response function. For other functions, the method was accurate from 50% to 200%.

In the case of Tyr analysis, the method was accurate from 10 to 200 % for the response function Y=b X + a, from 100% to 200% for the response function Y = b X and from 50 to 200% for the response function Y= k(100%).

These findings indicate that the method demonstrates accuracy and meets the specified criteria for trueness and precision for the response function Y=b X + a for both Phe and Tyr.

The choice of the accuracy profile is based on the objective of finding a narrowest tolerance interval and located within the limits of acceptability and biases closer to zero and to have a valid method in the concentration interval studied. After carefully examining the accuracy profiles of the different models and according to these reasons for choice, we have selected the linear regression model Y = b X + a.

As a result, the obtained results demonstrate that the method is valid using the function Y = b X + a, for the measurement of both analytes within the investigated concentration range exceptionally for the level 10% (5 µM) of Phe. By extrapolation on the **Figure VI.21,** we conclude that the method is valid using the function Y = b X + a, for the measurement of Phe from 15% to 200% meaning from 7,5 µM to 100 µM. In addition, as shown in **Figure VI.23**, if using β set at 80% we deduce a validity from 10% to 200% for the first function when analyzing Phe. On the other hand, relative errors were calculated for both analytes and illustrated in **Figure VI.24.**

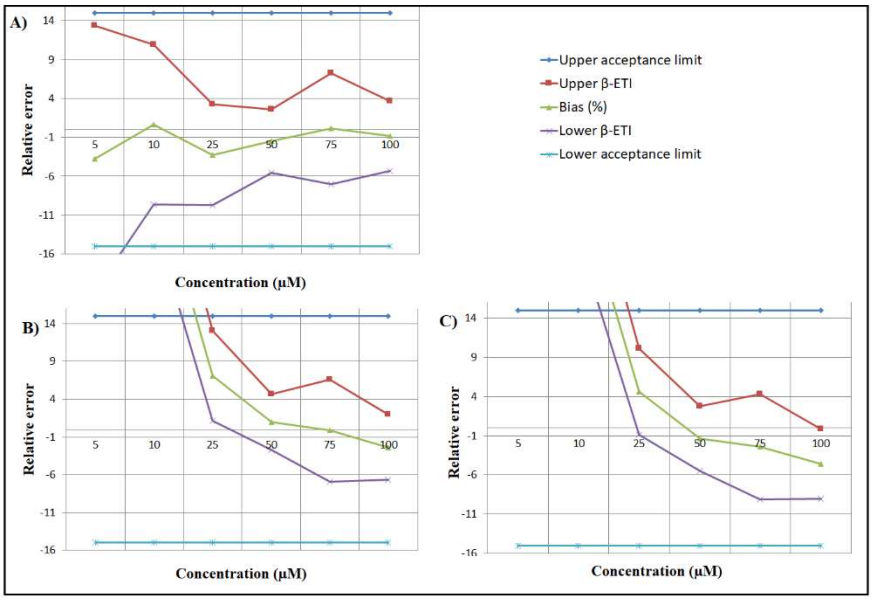

Figure VI.21. LC-MSMS accuracy profiles of phenylalanine in blood serum (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

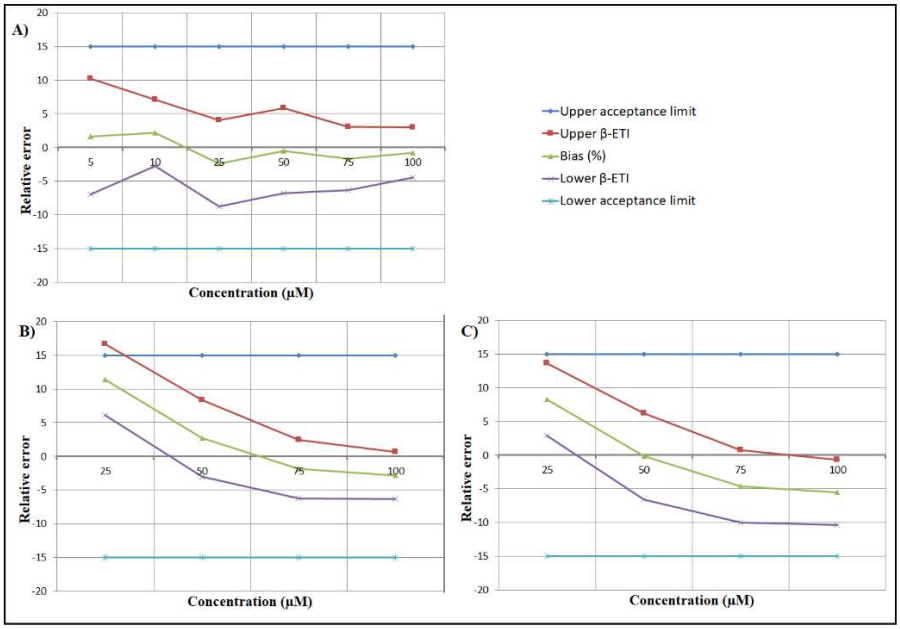

Figure VI.22. LC-MSMS accuracy profiles of tyrosine in blood serum (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

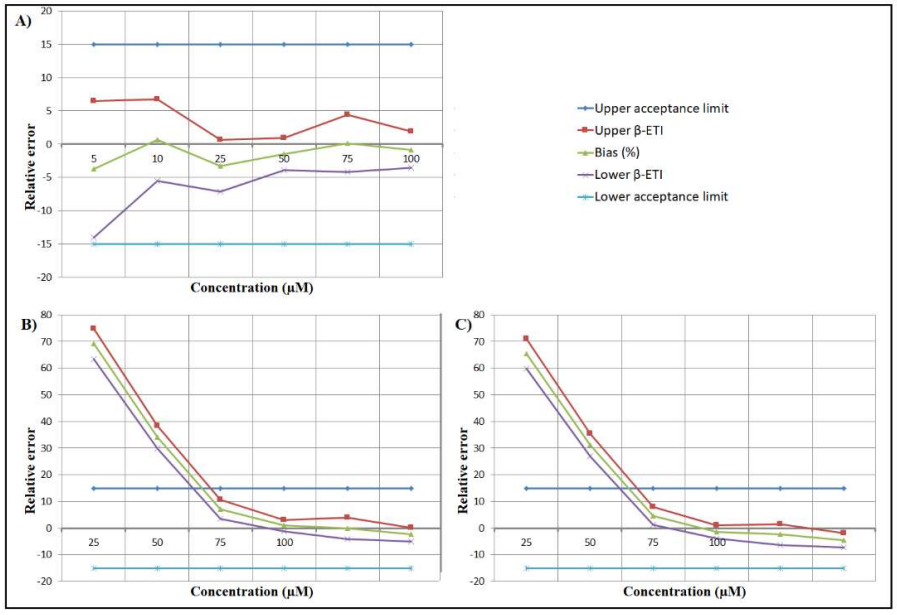

Figure VI.23. LC-MSMS accuracy profiles of phenylalanine in blood serum (± 15% acceptability limits, beta 80%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

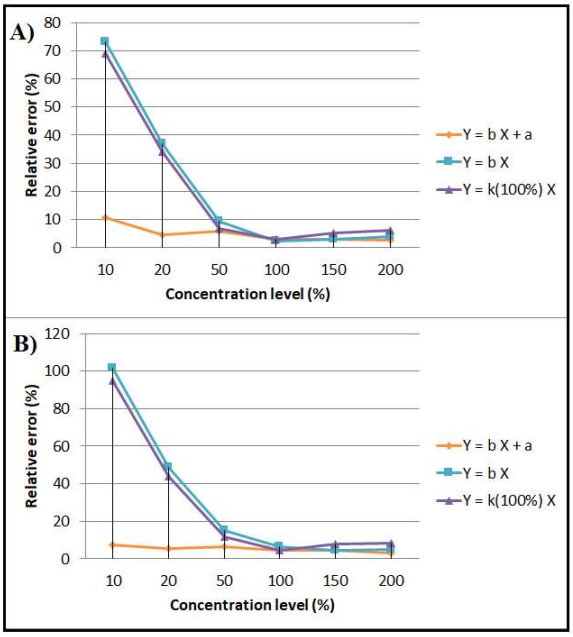

Figure VI.24. Relative error for (A) phenylalanine and (B) tyrosine analyzed by LC-MSMS method for blood serum

VI.3.7.2. LC-MSMS for dry blood spots

Regression models can be computed using the calibration data between 50 and 180% of the nominal concentration. This allows for the proposal of three accuracy profiles based on three calibration functions for both Phe and Tyr. Furthermore, **Tables VI.40** and **VI.41** can be generated, encompassing all the performance criteria for both analytes at each concentration level. The trueness, precision, and the β-ETI were established by analyzing the back-calculated concentrations of validation series, utilizing the previously mentioned models. The analysis of the data presented in **Tables VI.40** and **VI.41** reveals that all values remained within the acceptable limits of λ ± 15%.

The results presented in **Tables VI.40** and **VI.41** demonstrate that the recoveries of Phe are within the acceptance range for only the first response function. The recovery values for Phe were ranged from 99,46% to 101,78% for the response function Y=b X + a, from 79,45% to 101,77% for the response function Y = b X and from 80,94% to 103,68% for the response function Y= k(100%). For Tyr, the recoveries were ranged from 99,47% to 102,16% for the response function Y=b X + a, from 97,3% to 101,06% for the response function Y = b X and from 96,58% to 100,33% for the response function Y= k(100%). Therefore, only the response function Y=b X + a, gives acceptable recoveries among the tested functions for all studied concentrations. These findings demonstrate the accuracy of the developed method in measuring the target analytes within the investigated concentration range, with recoveries closely aligned with the desired value of 100% when using response function Y=b X + a for both analytes. Similar results have been reported previously (Anandhi and Nathiya, 2023), when almost recoveries were superior to 90% and RSDs are below 6%.

As seen in **Table VI.40**, the relative biases for the first model for Phe are ranged from – 0,53 % to +1,52 %, which are acceptable and below the established limits of 10% (Zacharis and Vastardi, 2018), unlike to other functins where the relatives biases were more than 10%.

On the other hand, the relative biases of Tyr were acceptable for all tested functions in the acceptable limits (**Table VI.41**). These findings indicate minimal systematic errors in the measurement process when using the functionY=b X + a for data analysis of Phe and when using any function for analyzing Tyr.

The results for repeatability and intermediate precision showed that all RSDs were found to be ≤ 5% for all test functions either for Phe or for Tyr. RSDs were ranging from 0,88% to 4,42% for Phe and from 2,03% to 4,02% for Tyr. These findings indicate excellent precision across all concentration levels whatever the model used.

The first response function by its simplicity, interpretability and ease of implementation in routine analysis implicate that the linear regression model is the most adequate (Anandhi and Nathiya, 2023).

Table VI.40. Performance criteria for phenylalanine analyzed by LC-MSMS for dry blood spots

Performance criteria		**Response function**	**Concentration (µM))**					
			Level 1 (20%)	Level 2 (40%)	Level 3 (60%)	Level 4 (100%)	Level 5 (140%)	Level 6 (180%)
			50	**100**	**150**	**250**	**350**	**450**
Trueness	**Relative bias (%)**	Y = b X + a	1,52	1,78	-0,53	-0,47	1,05	0,46
		Y = b X	-20,54	-7,10	-5,13	-1,55	1,55	1,77
		Y = k(100%) X	-19,05	-5,36	-3,34	0,30	3,46	3,68
	Recovery yield (%)	Y = b X + a	101,52	101,78	99,46	99,52	101,05	100,46
		Y = b X	79,45	92,89	94,86	98,44	101,55	101,77
		Y = k(100%) X	80,94	94,63	96,65	100,30	103,46	103,68
Precision	**RSD repeatability (%)**	Y = b X + a	2,61	1,06	3,96	2,97	0,88	1,70
		Y = b X	3,51	1,23	4,31	3,12	0,91	1,76
		Y = k(100%) X	3,41	1,08	4,42	3,32	0,91	1,95
	RSD precision (%)	Y = b X + a	2,61	1,06	3,96	2,97	1,21	1,70
		Y = b X	3,51	1,31	4,31	3,12	1,27	1,76
		Y = k(100%) X	3,41	1,08	4,42	3,32	1,98	2,10
Accuracy	**Tolerance lower limit**	Y = b X + a	-4,97	-0,74	-10,41	-7,89	-2,66	-3,78
		Y = b X	-29,29	-10,42	-15,9	-9,34	-2,37	2,62

Performance criteria		Response function	Concentration (µM))					
			Level 1 (20%)	Level 2 (40%)	Level 3 (60%)	Level 4 (100%)	Level 5 (140%)	Level 6 (180%)
			50	**100**	**150**	**250**	**350**	**450**
		Y = k(100%) X	-27,57	-8,07	-14,36	-7,97	-6,21	-1,81
	Tolerance higher limit	Y = b X + a	8,03	4,30	9,33	6,95	4,78	4,71
		Y = b X	-11,79	-3,79	5,63	6,24	5,47	6,16
		Y = k(100%) X	-10,53	-2,66	7,66	8,57	13,14	9,18

Table VI.41. Accuracy profiles parameters for tyrosine analyzed by LC-MSMS for dry blood spots

Performance criteria		Response function	Concentration (µM))					
			Level 1 (20%)	**Level 2 (40%)**	**Level 3 (60%)**	**Level 4 (100%)**	**Level 5 (140%)**	**Level 6 (180%)**
			50	**100**	**150**	**250**	**350**	**450**
Turness	**Relative bias (%)**	Y = b X + a	2,16	-0,52	1,97	1,28	-0,10	-0,08
		Y = b X	-2,69	-2,50	0,98	1,07	-0,01	0,19
		Y = k(100%) X	-3,41	-3,21	0,25	0,33	-0,72	-0,54
	Recovery yield (%)	Y = b X + a	102,16	99,47	101,97	101,28	99,89	99,91
		Y = b X	97,30	97,49	100,98	101,06	99,99	100,19
		Y = k(100%) X	96,58	96,78	100,25	100,33	99,27	99,45
Precision	**RSD repeatability (%)**	Y = b X + a	2,44	2,61	3,17	2,79	3,60	2,19
		Y = b X	2,98	2,63	3,23	2,82	3,64	2,18
		Y = k(100%) X	2,87	2,87	3,23	3,15	3,99	2,03
	RSD precision (%)	Y = b X + a	2,44	2,61	3,48	2,79	3,60	2,19
		Y = b X	3,04	2,63	3,50	2,82	3,64	2,18
		Y = k(100%) X	2,87	2,87	4,21	3,15	4,02	2,03
Accuracy	**Tolerance lower limit**	Y = b X + a	1,47	-4,43	-3,40	-2,88	-5,48	-3,36
		Y = b X	-7,25	-6,43	4,41	-3,15	-5,44	-3,06
		Y = k(100%) X	-7,69	-7,50	-6,82	-4,37	-6,73	-3,58
	Tolerance higher limit	Y = b X + a	5,81	3,38	7,34	5,46	5,27	3,18
		Y = b X	1,86	1,42	6,38	5,28	5,43	3,45
		Y = k(100%) X	0,87	1,07	7,34	5,03	5,28	2,49

Based on the data presented in **Tables VI.40** and **VI.41**, the accuracy profiles for Phe (**Figure VI.25**) and Tyr (**Figure VI.26**) were constructed.

The results concerning the first model analyzing Phe demonstrate that the upper and lower limits of the interval of tolerance for concentration levels from 20% to 180% remain within the acceptable range of λ ± 15% (Papavasileiou *et al.*, 2022). Consequently, the method was accurate from 20 to 180% for Phe when using the first response function, from 60% to 100 % for the response function Y = b X and from 40% to 180% for the third function. Additionally, the method was accurate from 20% to 180 % for all the response functions when analyzing Tyr.

These findings indicate that the method demonstrates accuracy and meets the specified criteria for trueness and precision for the response function Y=b X + a for Phe and and for all tested functions in the case of Tyr.

In the study reported by Bloom K et al, it was reported that inter-assay imprecision observed with DBS is a little higher than that of plasma. This result was attributed to lack of sample homogeneity across the DBS or to changes in the environment of the laboratory or in calibration (Bloom, Ditewig Meyers and Bennett, 2016).Therefore in our study, using the same stock solutions to prepare the calibration and the validation series together with operating in the same conditions reflect the precision found among all the developed methods.

The development of accuracy profile is based on the objective of finding minimal values of biases, with a narrowest tolerance interval and located within the limits of acceptability and to achieve finally valid method over the studied concentration interval. As was shown in **Figures VI.25** and **VI.26**, the method was valid from 20 to 180% for the first function for both analytes, and for other functions in the case of Tyr.

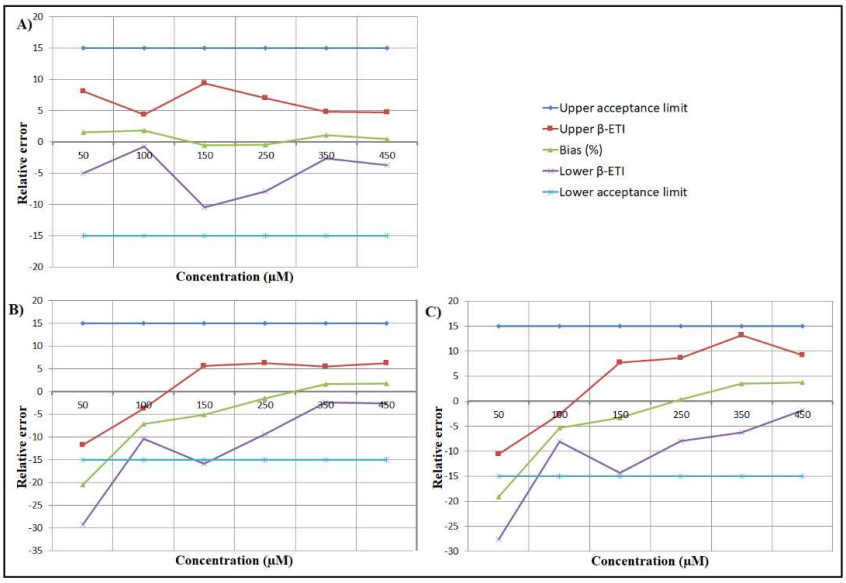

Figure VI.25. LC-MSMS accuracy profiles of phenylalanine in dry blood spots (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

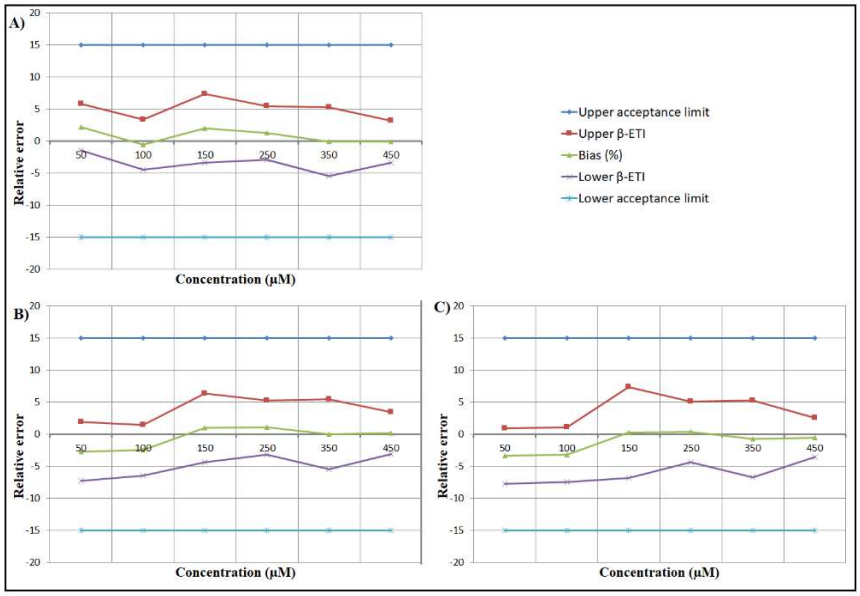

Figure. VI.26. LC-MSMS accuracy profiles of tyrosine in dry blood spots (± 15% acceptability limits, beta 95%) for different response functions, A) Y=bX+a, B) Y=bX, and C) Y = k(100%) X

Furthermore, **Figure VI.27** represent the relative error for Phe and Tyr analyzed by LC-MSMS method for DBS.

According to this result, all concentration levels are judged to be included in the acceptability interval for Tyr (all response functions) however for Phe if only the response function Y = b X + a was used.

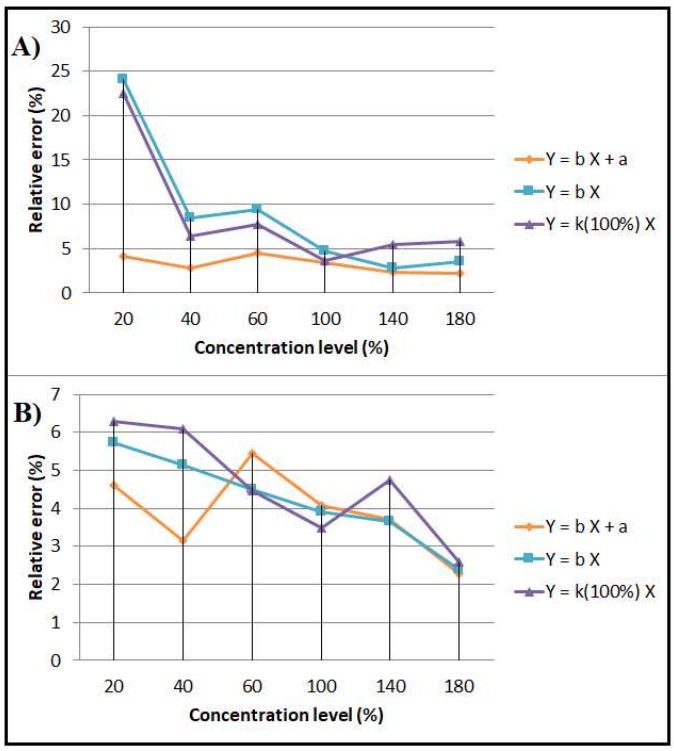

Figure VI.27. Relative error for phenylalanine (A) and tyrosine (B) analyzed by LC-MSMS method for dry blood spots.

VI.3.8. Linearity of method

The linearity of the method can be evaluated by comparing the actual concentrations of the prepared validation solutions with the concentrations determined by the method.

VI.3.8.1. LC-MSMS for blood serum

As can be seen, all calibration points lie within the prediction interval of the regression curve **(Figure VI.28)** and R^2 present a value of 0.9994 and 0,9998 with slope values of 1,0029 and 1,011 for Phe and Tyr, respectively. These results designate the strong relationship between actual and calculated concentrations. all calibration points.

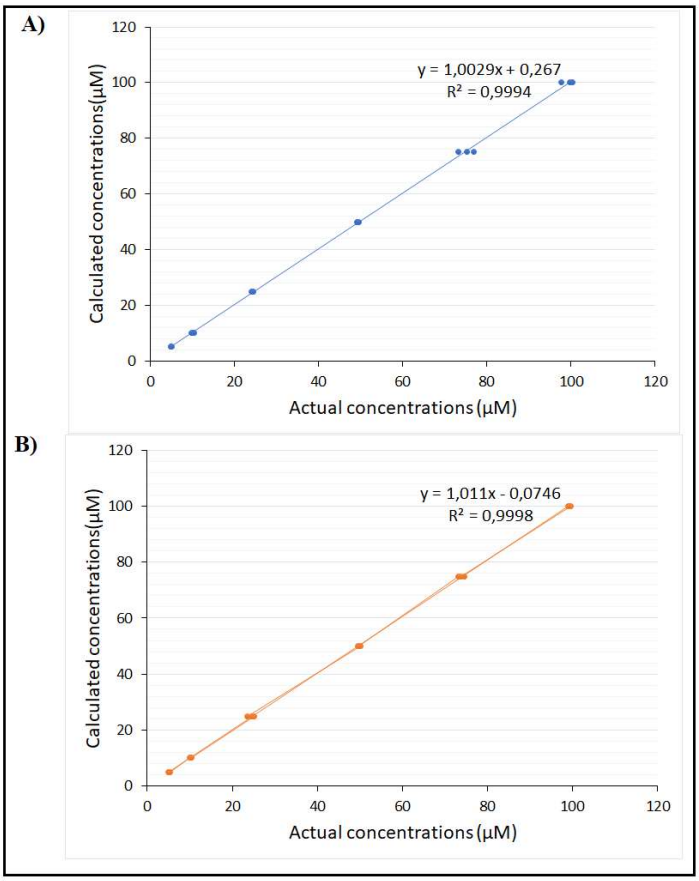

Figure VI.28. Linearity of LC-MSMS method for (A) phenylalanine and (B) tyrosine determination in blood serum

VI.3.8.2. LC-MSMS for dry blood spots

As can be seen, all calibration points are found within the prediction interval of the regression curve (**Figure VI.29**) and R^2 present a value of 0.9997 and 0,9992 with slope values of 0,9946 and 1,0025 for Phe and Tyr, respectively. These results designate the strong relationship between actual and calculated concentrations.

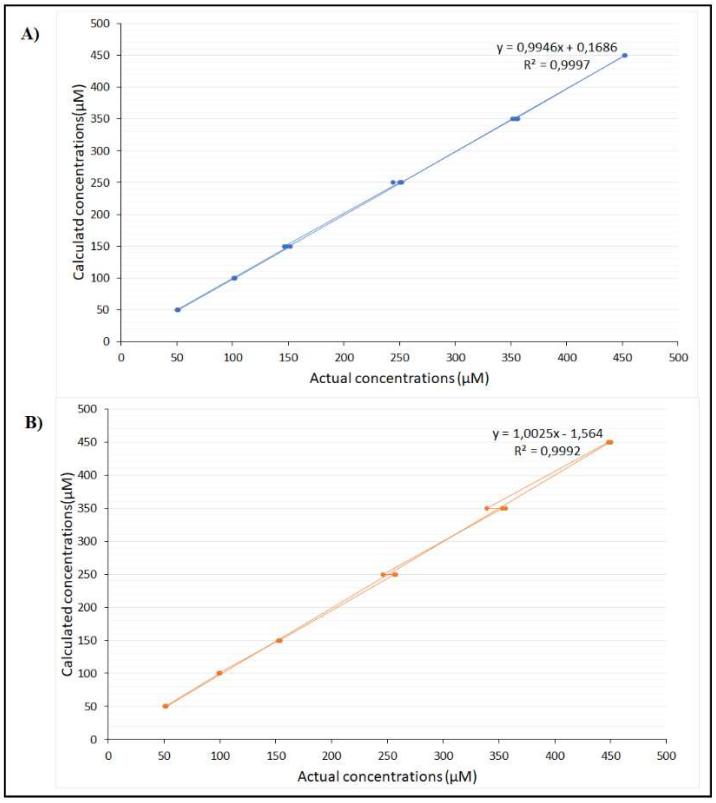

Figure VI.29. Linearity of LC-MSMS method for (A) phenylalanine and (B) tyrosine determination in dry blood spots

VI.3.9. Limit of detection and limit of quantification

LOD and LOQ for Phe and Tyr were determined for LC-MSMS method either in blood serum or in DBS. This was accomplished by using the equations **V.4** and **V.5** described in section (**V.2.5.4.5**) to respectively calculate LOD and LOQ.

For LC-MSMS method of blood serum, the LOD and LOQ of Phe were calculated to be 1,7 µM and 5,1 µM, respectively. Similarly, for Tyr, the LOD and LOQ were found to be 2,1 µM and 6,4 µM, respectively. In previously reported work, LOQs were found to be equal to 0.1 µM for both Phe and Tyr within a studied range of 0,1-100 µM and 0,05-50 µM, respectively (Peng *et al.*, 2019). The difference between our results and the already cited one is attributed to the difference between the studied ranges and also to the difference in the analytical instrument performance especially because the authors used an AB Sciex 3200 Q TRAP mass spectrometer (Peng *et al.*, 2019), which is known by its high sensitivity and performance.

For LC-MSMS method of DBS, the LOD and LOQ of Phe were calculated to be 12,1 µM and 36,7 µM, respectively. Similarly, for Tyr, the means LOD and LOQ were found to be 10,1 µM and 30,6 µM, respectively (**Table VI.42**).

Table VI.42. Limits of detection and quantification for phenylalanine and tyrosine determined by LC/MS-MS method

Analyte	**Day**	**Day 1**		**Day 2**		**Day 3**		**Mean**	
	Medium	Serum	DBS	Serum	DBS	Serum	DBS	Serum	DBS
Phenylalanine	**S (a)**	757,7	475,2	818,4	472,8	716,9	482,7		
	Slope (b)	1483,4	257,9	1485,8	275,3	1491,9	390,6		
	LOD (µM)	1,7	11,1	1,8	12,3	1,6	12,8	1,7	12,1
	LOQ (µM)	5,1	33,7	5,5	37,4	4,8	38,9	5,1	36,7
Tyrosine	**S (a)**	2923,5	136,5	3252,1	135,4	3409,5	137,7		
	Slope (b)	868,0	429,8	869,0	421,3	875,8	401,1		
	LOD (µM)	1,8	10,4	1,9	10,3	2,7	9,6	2,1	10,1
	LOQ (µM)	5,4	31,5	5,8	31,1	8,1	29,1	6,4	30,6

VI.3.10. Impact of storage duration and temperature on phenylalanine and tyrosine concentrations by LC-MSMS method

Serum samples and acidified aqueous solutions of Phe and Tyr were stored at -30°C, 6°C, and room temperature (25°C) for one month in order to assess the impact of time and storage temperature on the levels of Phe and Tyr. Every period of time, the stored specimens were filtered and analyzed by LC-MSMS. As described by **Figure VI.30**, all specimens showed instability when stored at room temperature, stability for 7 days when stored at 6°C and stability for at least 30 days when serum samples or acidified aqueous solutions of analytes were stored at -30°C.

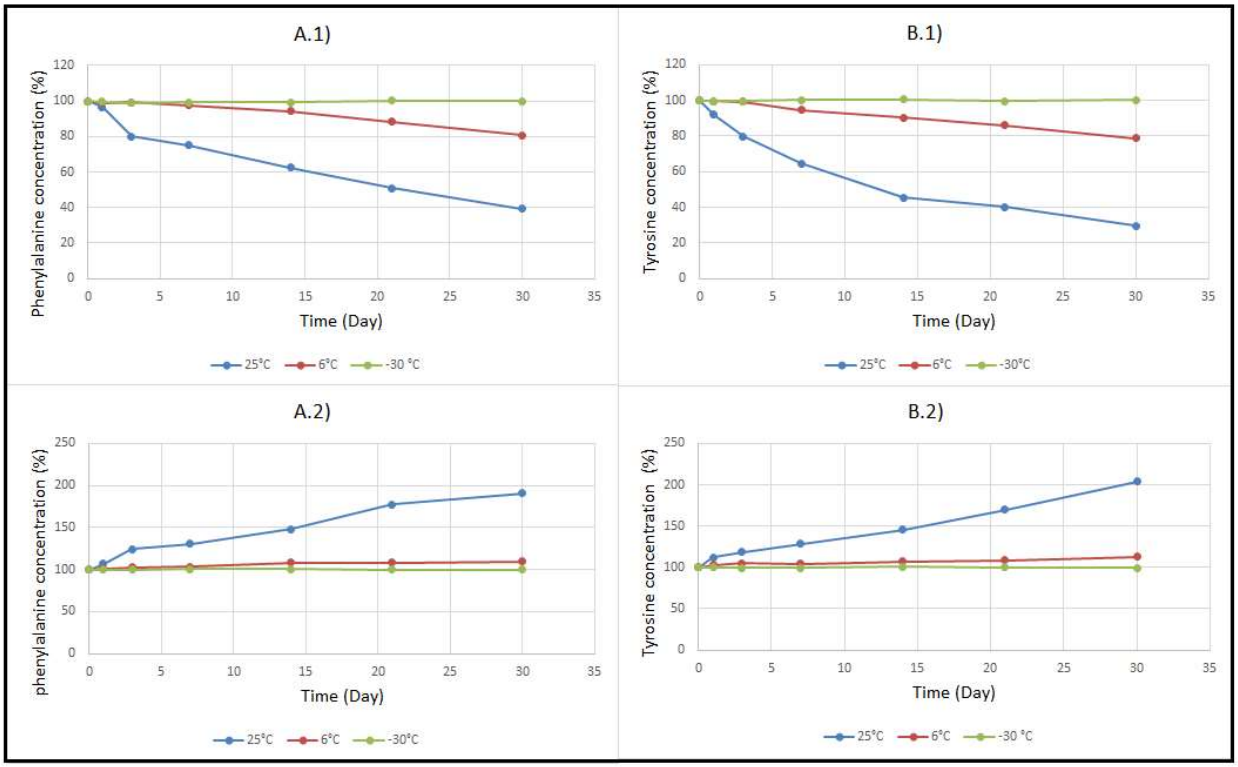

Figure VI.30. Impact of storage duration and temperature on phenylalanine (Phe) concentrations (A.1) in prepared acidic aqueous solutions and (A.2) in serum specimens and on tyrosine (Tyr) concentrations (B.1) in prepared acidic aqueous solutions and (B.2) in serum specimens (all analyzed by LC-MSMS method)

VI.5. Biomedical application of the developed methods

VI.5.1. Descriptive epidemiologic study

This comprehensive epidemiologic overview highlights the consanguinity rates, complications, developmental milestones, and geographic distribution of volunteers in the study.

VI.5.1.1. Sample size

When the prevalence of a disease is well known, we can use the equation **V.6** to estimate the sample size, otherwise, researchers typically use a conservative approach by assuming a prevalence rate of 50% to calculate the sample size. This is because a prevalence of 50% maximizes the required sample size, providing a more robust estimation for various scenarios (Naing *et al.*, 2022; Sadiq *et al.*, 2024).

Therefore, in our study as the prevalence is unknown, we set P=0.5. This is a standard practice to ensure that the sample size is sufficiently large (Naing *et al.*, 2022; Sadiq *et al.*, 2024).

We set the confidence level at 95% which corresponds to a Z value of 1.96. A common precision choice is 5% (0.05). After substitution of values into the formula, the calculation gives n=384. Thus, the required sample size would be approximately 384 participants.

VI.5.1.2. Patient demographics and characteristics

The demographic information about the participated volunteers provides valuable insights into the characteristics of the patient population involved in the study.

During the study period, a total of 459 volunteers participated. Of all the patients, one died and 16 others lacked adequate data in their medical records. Ultimately, 442 volunteers were included in the present study amongst 397 were suspected to be PKU or TRS positive and 45 were already known to be PKU patients.

In terms of sex distribution, over half of the suspected volunteers were males (276 children, 69,5%), while 121 participants (31,5%) were females. For PKU patients, 30 (66%) were males and 14 were females.

A total of 44 PKU patients (97,8 %) were diagnosed more than 30 days after birth. Only one patient (2, 2%) received his diagnosis within the first month of life. The onset of their symptoms was after birth for 8 patients (17,77%). Suspected volunteers share the same percentage in their symptom onset.

All volunteers were of Algerian nationality. Geographically, all the PKU patients and the majority of suspected volunteers resided in Setif city (390 patients represents 98,2% of patients), followed by Djidjel (0,5%), M'sila and Constantine comprising the remaining percentage.

VI.5.1.3. History of volunteers

In the context of analysis request, 88 volunteers (22,16%) were participated to the study because of their family history and 71 volunteers (17,88%) were participated to control their abnormal metabolic profile. For PKU patients, the main context of analysis request was the monitoring of their PKU. In addition to this, 16 PKU patients (35,55%) has family history leading the analysis together with 19 patients (42,22%) had abnormal metabolic profile.

In term of history of suspected volunteers, three factors are investigated (**Figure VI.31**). Consanguinity was the most prevalent among this population, with 128 volunteers (32,25%) having a consanguineous background. Amongst, 101 patients (78,9%) were classified as having second-degree consanguinity, while 27 patients (21,1%) had third-degree consanguinity. Conversely, 269 volunteers (29,25%) were not consanguineous. Two other factors were studied, death in siblings which represent 9,07% of population (36 patients) and prematurity at 28 patients (7,05%).

In addition, among PKU patients only one case was premature and 25 (55,55%) were consanguineous with 20 patients (46,67%) having second-degree consanguinity and 4 patients (8,89%) had third-degree consanguinity.

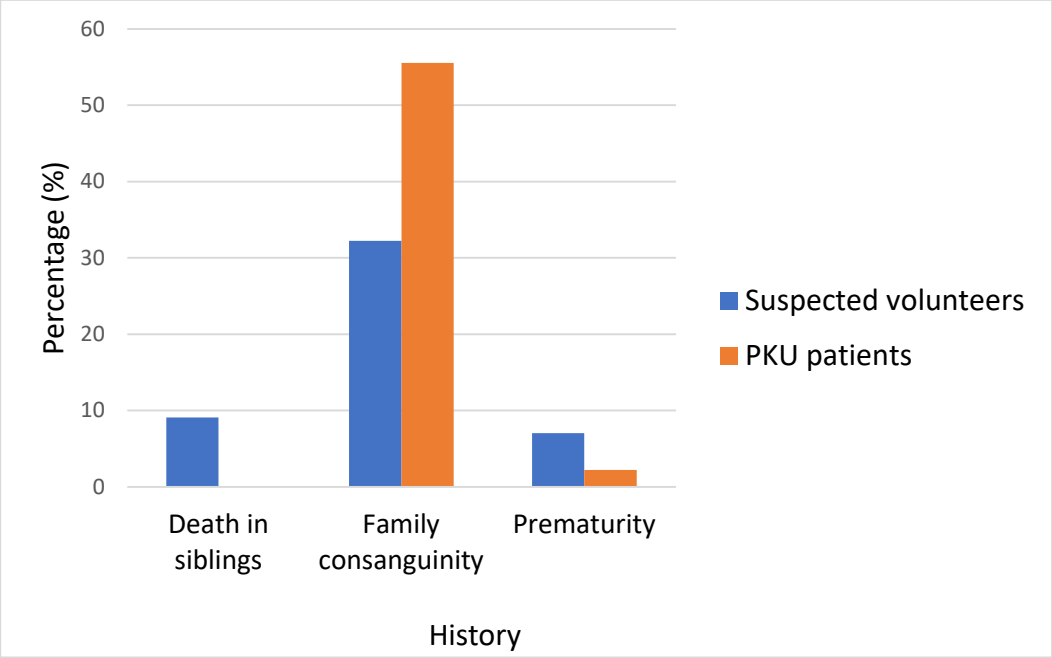

Figure VI.31. History of volunteers participated in the study

VI.5.1.4. Complications

The most frequently observed complication in the suspected volunteers was mental retardation, affecting 72 volunteers (18,13%). Other complications included epilepsy in 23 volunteers (5,79%), high bilirubin contents in 14 volunteers (3,52%), hepatorenal complications together with acoustic problems were observed in 11 volunteers (2,77%), and autism in 37 volunteers (9,31%). Notably, 49,37 % of volunteers reported no complications.

For developmental milestones, 373 patients (93,95%) exhibited no developmental delays, while 24 patients (6,04 %) experienced developmental delays.

On the other hand, the most frequently observed complication in the PKU patients was mental retardation, affecting 10 patients (22,22%). Other complications such as epilepsy and acoustic problems were observed in only 1 patient (2,2%). In addition, 33 PKU patients (73,33%) reported no complications (**Figure VI.32**).

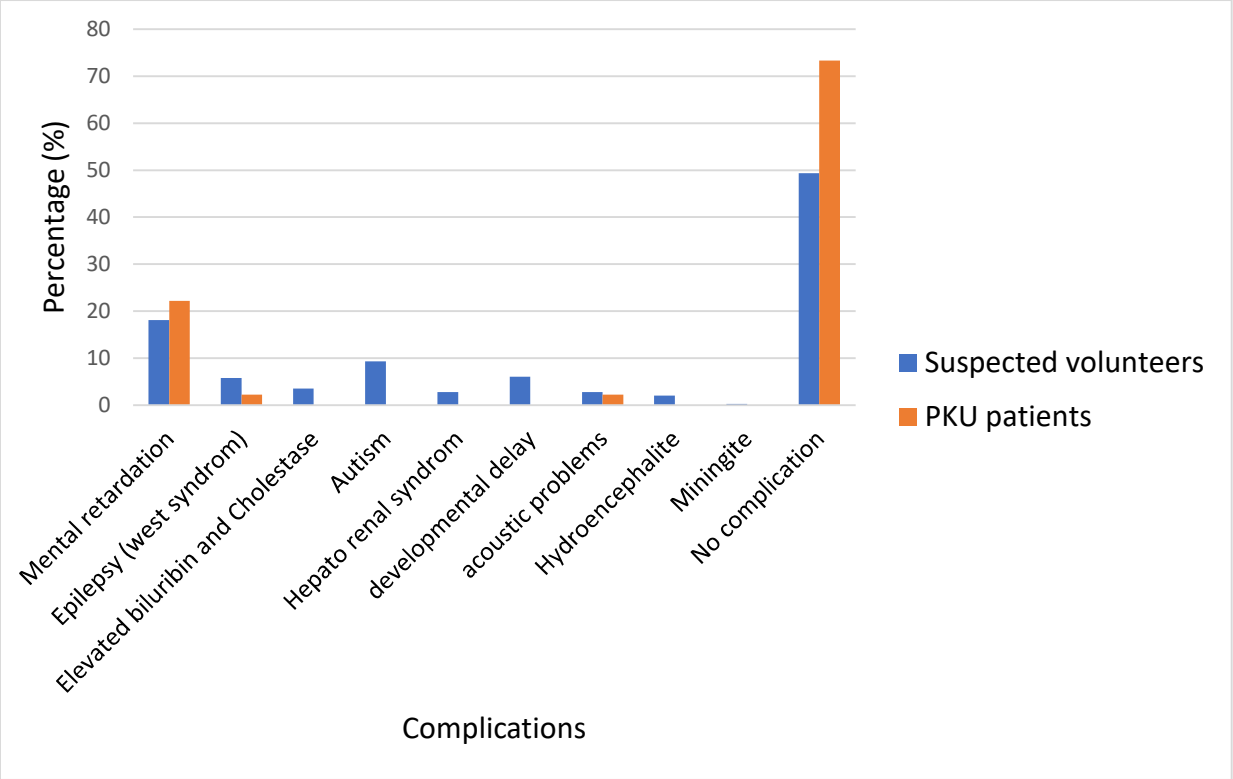

Figure VI.32. Associated complications of volunteers participated in the study

VI.5.1.5. Prevalence of phenylketonuria and tyrosinemia

The prevalence of PKU and TRS varies significantly across different ethnic groups and countries worldwide, with rates of 1 in 4,500 in Italy and 1 in 125,000 in Japan for PKU (El-Metwally *et al.*, 2018) and of 1 in 100 000 to 1 in 120 000 for TRS with most of the reported cases clustering in two regions, Scandanavia and Quebec (Canada) (El-Karaksy *et al.*, 2011). A systematic review examining the prevalence of PKU in Arab countries reported notably high rates in Saudi Arabia, the United Arab Emirates, the Gaza Strip, and Iraq (El-Metwally *et al.*, 2018).

It indicated that the prevalence of classical PKU in Arab countries, ranges from 0.005% to 0.0167% based on data from comprehensive national NBS programs.

The findings revealed that Turkey has the highest prevalence of classical PKU at 0.067%, while the UAE reported the lowest at 0.005%. Only a few countries in the MENA region, such as Qatar, Saudi Arabia, the UAE, and Turkey, have implemented comprehensive NBS programs. Other reviews assessing NBS programs globally indicate that only a limited number of Middle Eastern countries, including Bahrain, Kuwait, and Egypt, have developed extensive NBS initiatives (El-Metwally *et al.*, 2018). However, there is currently no data available regarding the prevalence of PKU in Algeria.

Specific prevalence data for Algeria alone is not extensively detailed in the available literature, it is included in the broader regional statistics. The review emphasizes the need for effective NBS programs to identify PKU early, which is crucial for managing the condition and preventing associated health issues (El-Metwally *et al.*, 2018).

In addition, TRS is unwell understood in our country and very few studies on this disease have been reported. Hence, its prevalence in Algeria is unknown (Reda Belbouab *et al.*, 2024). In Arabic countries the prevalence of TT1 demonstrates significant variation, influenced by cultural, genetic and healthcare factors. In Lebanon, a study covering 12 years stated 22 cases of TT1 among a population of about 6.8 million, indicative of a prevalence around 0.32 cases per 100,000 individuals. The study emphasized the challenges in diagnosis and treatment due to limited resources (Daou *et al.*, 2021). Another research was conducted in Egypt from Cairo University which recognized 76 cases of hepatorenal TRS from 2006 to 2019, with a significant consanguinity rate of 80% amongst affected families. This proposes a higher prevalence in the context of genetic factors (Reda Belbouab *et al.*, 2024). In Turkey, with studies indicating around 1 in 100,000 births, similar to global estimates and so the prevalence of TT1 is higher, but local variations exist because of consanguinity and genetic factors (Daou *et al.*, 2021).

Therefore, it is assumed that the prevalence of TT1 and PKU in Algeria is notably high, mainly due to the country's high rate of consanguinity, which is reported to be around 23% (Moussouni *et al.*, 2024).

A study conducted at Mustapha University Hospital in Algiers analyzed 35 cases of TT1 diagnosed between 2010 and 2022. The mean age of clinical onset was approximately 6.5 months, with a mean diagnosis age of 11 months. Key clinical findings included hepatomegaly in 92% of cases, splenomegaly in 23%, and liver failure in all patients. The study highlights the importance of early diagnosis and treatment with NTBC to manage the condition effectively.

Our study suggests a potentially high prevalence of PKU in Algeria when compared to TRS; however, we cannot provide an exact figure due to the lack of information from the NBS program in our country. The high rate of consanguineous marriages (Moussouni *et al.*, 2024), may contribute to an increased prevalence. Additionally, our findings indicated a significant degree of consanguinity among patients. Therefore, the high prevalence of consanguineous marriages in Algeria has contributed to an increase in autosomal recessive conditions, including IEMs. Consequently, it is essential to establish a comprehensive NBS service in Algeria to facilitate early diagnosis and appropriate management of these disorders.

Additional study investigating the spectrum of IEM in Jordan found that 137 out of 151 families had consanguineous parents, which aligns with our results and those from other Arab countries. For instance, in Kuwait, 8 out of 9 PKU patients and in Oman, 9 out of 11 patients had consanguineous parents (Dababneh *et al.*, 2022).

NBS program is designed to identify congenital and heritable disorders early. When treated, individuals with metabolic disorders can lead relatively normal lives, resulting in reduced long-term medical costs. Conversely, if left untreated, these disorders can lead to severe consequences (Ma *et al.*, 2020). Overall, we suppose that the high prevalence of TRS and PKU in Algeria underscores the need for improved screening and early intervention strategies to mitigate the disorder's impact on affected individuals.

VI.5.1.6. Dietary and therapy in progress

In terms of dietary management, 64,44% of PKU patients (29 patients) are adhered to the prescribed dietary treatment and 24,5 % (11 patient) receive the sapropretin dihydrochloride (Kuvan) which is a synthetic formulation of BH4, a naturally occurring cofactor for PAH, effectively lowers blood Phe levels in a proportion of patients with PKU (Sanford and Keating, 2009).

Information about therapy or dietary management of 5 patients were lacked, so they were declared as unknown dietary.

In this group, associated therapy is mainly represented by antiepileptics such as valproic acid, levetiracetam and vigabatrin, with 8,89% (4 patients), followed by vitamins, minerals and omega 3 supplementation with a percentage of 2,22% (1 patients). Antipsychotics are represented essentially by chlorpromazine chlorohydrate and risperidone and share the same percentage as like as supplementation therapy.

Other group of volunteers (suspected volunteers) are not adherent yet to dietary treatment neither to synthetic cofactor PHA (sapropretin dihydrochloride). A total of 93 children (23,5%) are subjected to different therapies. Anti-epileptics such as valproic acid, levetiracetam and vigabatrin, were in the first line of associated treatment with a highest percentage of 8,82% followed by supplementation therapy (15 patients with 3,78%) especially omega 3, potassium, calcium and vitamins. Corticosteroid therapy occupied 1,07% (4 patients), whereas antibiotic therapy occupied only 0,5% (2 patients) and finally, cerebral oxygenators with cerebral myorelaxants share the same percentage of 0,25% (1 patient each) (**Figure VI.33**).

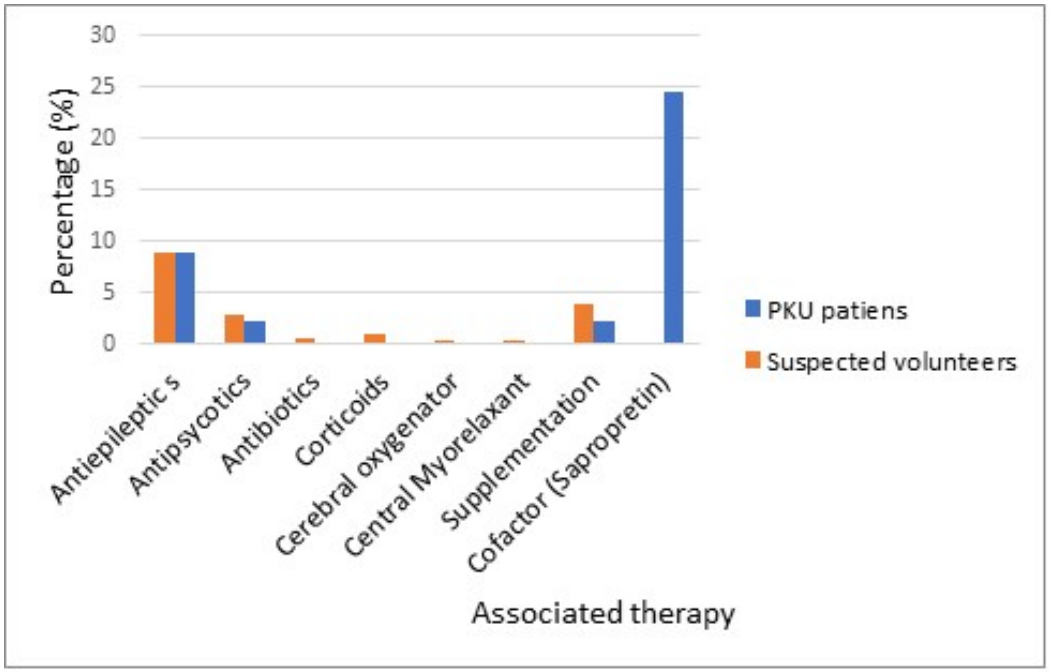

Figure VI.33. Therapy in progress of volunteers participated in the study

VI.5.2. Statistical analysis

VI.5.2.1. Qualitative Analysis

In order to study the qualitative factors effect on Phe or Tyr levels in the blood, we have used at first the Levene's test to determine the equality of variances among two groups of data. It is one of the most common statistical techniques used to test whether mean difference between two groups is statistically significant (Zhou, Zhu and Wong, 2023). Secondly, we have used the Student's t-test, also called independent samples t-test or umpaired t-test which is an inferential statistical test that determines if there is a statistically significant difference between the means in two unrelated (independent) groups (Mishra *et al.*, 2019).

Significance level of Levene's test is computed and when it is insignificant ($p > 0.05$, equal variances) simple t-test is used, otherwise ($p < 0.05$ unequal variances), corrected t-test (unequal variances *t*-test) is used (Mishra *et al.*, 2019).

Effect of different factors on responses including Phe and Tyr levels obtained by HPLC-UV method or by LC-MSMS method and their respective ratios R1 and R2, was investigated.

VI.5.2.1.1. Sex distribution

As shown in **Table VI.43**, all p values of t-test are >0,05 which confirm that there is no significant difference between males and females (variable X), either in suspected group or confirmed group of patients (PKU patient). This finding aligns with other reports and confirms that gender is not contributing to PKU (Harvey Levy, 2012) or to TRS (*Tyrosinemia*, 2023) in terms of their genetic standpoint.

Table VI.43. Effect of sex distributions on phenylalanine and tyrosine concentrations and ratios

Volunteers	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
PKU patients	15	30	Phe-HPLC-UV	0.037	Different variances	0.054	Equal means
			Tyr-HPLC-UV	0.539	Equal variances	0.542	Equal means
			R1-HPLC-UV	0.424	Equal variances	0.351	Equal means

Volunteers	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
			Phe-LC-MSMS	0.030	Different variances	0.050	Equal means
			Tyr-LC-MSMS	0.709	Equal variances	0.313	Equal means
			R2-LC-MSMS	0.317	Equal variances	0.365	Equal means
Suspected volunteers	121	276	Phe-HPLC-UV	0.802	Equal variances	0.807	Equal means
			Tyr-HPLC-UV	0.962	Equal variances	0.975	Equal means
			R1-HPLC-UV	0.559	Equal variances	0.904	Equal means
			Phe-LC-MSMS	0.809	Equal variances	0.763	Equal means
			Tyr-LC-MSMS	0.908	Equal variances	0.938	Equal means
			R2-LC-MSMS	0.540	Equal variances	0.954	Equal means
All Volunteers	136	306	Phe-HPLC-UV	0.471	Equal variances	0.763	Equal means
			Tyr-HPLC-UV	0.890	Equal variances	0.972	Equal means
			R1-HPLC-UV	0.303	Equal variances	0.674	Equal means
			Phe-LC-MSMS	0.465	Equal variances	0.790	Equal means
			Tyr-LC-MSMS	0.832	Equal variances	0.975	Equal means
			R2-LC-MSMS	0.243	Equal variances	0.724	Equal means

VI.5.2.1.2. Context of the request

In this section, two reasons of the analysis request are studied, family history and control of abnormal metabolic profile. The results are detailed in **Table VI.44**.

For PKU patients' group, there is no significant contribution of request context to the Phe or Tyr levels (t-test $p > 0,05$).

However, when studying suspected volunteers' group and all volunteers group, we observe a significant contribution of family history and the second cause of the request (controlling abnormal metabolic profile) in R1, R2 and Phe concentrations obtained by both developed methods.

We suppose that since of the genetic source of the studied diseases and their contribution to anomalous profile of Phe and Tyr, almost doctors request the analysis of Phe and tyr when finding positive family history of the child and also if an abnormal metabolic profile appears in the patient. Consequently, in our study the context of analysis request is mainly influencing on Phe concentration, R1, R2. Tyr concentrations are not affected because almost suspected volunteers (384 volunteers, 96,72%) were supposed to be PKU positives.

Table VI.44. Effect of context of analysis request on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
PKU patients	Family history	29	16	Phe-HPLC-UV	0.242	Equal variances	0.545	Equal means
				Tyr-HPLC-UV	0.019	Different variances	0.929	Equal means
				R1-HPLC-UV	0.829	Equal variances	0.466	Equal means
				Phe-LC-MSMS	0.229	Equal variances	0.560	Equal means
				Tyr-LC-MSMS	0.039	Different variances	0.763	Equal means
				R2-LC-MSMS	0.636	Equal variances	0.536	Equal means
	Control of abnormal metabolic profile	26	19	Phe-HPLC-UV	0.757	Equal variances	0.520	Equal means
				Tyr-HPLC-UV	0.530	Equal variances	0.886	Equal means
				R1-HPLC-UV	0.256	Equal variances	0.307	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Phe-LC-MSMS	0.821	Equal variances	0.478	Equal means
				Tyr-LC-MSMS	0.252	Equal variances	0.704	Equal means
				R2-LC-MSMS	0.584	Equal variances	0.360	Equal means
Suspected volunteers	Family history	309	88	Phe-HPLC-UV	0.000	Different variances	0.004	Different means
				Tyr-HPLC-UV	0.000	Different variances	0.109	Equal means
				R1-HPLC-UV	0.000	Different variances	0.017	Different means
				Phe-LC-MSMS	0.000	Different variances	0.004	Different means
				Tyr-LC-MSMS	0.000	Different variances	0.114	Equal means
				R2-LC-MSMS	0.000	Different variances	0.014	Different means
	Control of abnormal metabolic profile	326	71	Phe-HPLC-UV	0.000	Different variances	0.003	Different means
				Tyr-HPLC-UV	0.000	Different variances	0.173	Equal means
				R1-HPLC-UV	0.000	Different variances	0.007	Different means
				Phe-LC-MSMS	0.000	Different variances	0.003	Different means
				Tyr-LC-MSMS	0.000	Different variances	0.183	Equal means
				R2-LC-MSMS	0.000	Different variances	0.005	Different means
All volunteers	Family history	338	104	Phe-HPLC-UV	0.000	Different variances	0.001	Different means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Tyr-HPLC-UV	0.001	Different variances	0.103	Equal means
				R1-HPLC-UV	0.000	Different variances	0.004	Different means
				Phe-LC-MSMS	0.000	Different variances	0.001	Different means
				Tyr-LC-MSMS	0.000	Different variances	0.100	Equal means
				R2-LC-MSMS	0.000	Different variances	0.004	Different means
	Control of abnormal Metabolic profile	352	90	Phe-HPLC-UV	0.000	Different variances	0.001	Different means
				Tyr-HPLC-UV	0.002	Different variances	0.151	Equal means
				R1-HPLC-UV	0.000	Different variances	0.003	Different means
				Phe-LC-MSMS	0.000	Different variances	0.001	Different means
				Tyr-LC-MSMS	0.001	Different variances	0.152	Equal means
				R2-LC-MSMS	0.000	Different variances	0.002	Different means

p-value <0,05

VI.5.2.1.3. Family history

In term of family history, three factors are studied, consanguinity degree (no consanguinity is designed as 0, second consanguinity as 2, and third consanguinity as 3), death in siblings and prematurity. The results are grouped in **Table VI.45**.

In PKU patients' group, no significant contribution of consanguinity degree to the Phe or Tyr levels or ratios (t-test p > 0,05). In addition, data from two other factors are not sufficient so they were not analyzed.

For other groups, consanguinity degree 0 and 3, have an effect on R1, R2 and Phe concentrations obtained by both developed methods. Death in siblings has also a significant effect of Tyr levels when analyzed by HPLC-UV or by LC-MSMS method.

Consanguineous marriages heighten the likelihood that both parents have identical recessive alleles, which in turn increases the risk that their children will inherit two copies of the mutated gene linked to PKU or TRS. This phenomenon is especially pronounced in populations where such unions are prevalent, resulting in a greater occurrence of autosomal recessive disorders (Shawky *et al.*, 2013).

In addition, since PKU and TRS are inherited in an autosomal recessive manner, siblings have a 25% chance of being affected if both parents are carriers of the mutated gene, a 50% chance of being a carrier, and a 25% chance of being unaffected. This genetic predisposition can main to multiple siblings being diagnosed as positives within the same family, increasing the risk of complications and potential mortality, particularly in populations with high rates of consanguinity (Matt Demczko, 2024).

In a study involving Mexican patients with TT1, the rate of mortality was reported to be as high as 78%. The study noted also that five families had a history of a deceased sibling with analogous symptoms, suggesting a pattern of inherited risk within families (Fernández-Lainez *et al.*, 2014). These results are in accordance to ours and Tyr levels are affected by sibling death.

Table VI.45. Effect of family history on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
PKU Patients	Family consanguinity 0	25	20	Phe-HPLC-UV	0.037	Different variances	0.188	Equal means
				Tyr-HPLC-UV	0.300	Equal variances	0.203	Equal means
				R1-HPLC-UV	0.409	Equal variances	0.518	Equal means
				Phe-LC-MSMS	0.037	Different variances	0.193	Equal means
				Tyr-LC-MSMS	0.310	Equal variances	0.322	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R2-LC-MSMS	0.541	Equal variances	0.527	Equal means
	Family consanguinity 2	24	21	Phe-HPLC-UV	0.010	Different variances	0.158	Equal means
				Tyr-HPLC-UV	0.334	Equal variances	0.805	Equal means
				R1-HPLC-UV	0.158	Equal variances	0.247	Equal means
				Phe-LC-MSMS	0.008	Different variances	0.163	Equal means
				Tyr-LC-MSMS	0.282	Equal variances	0.650	Equal means
				R2-LC-MSMS	0.070	Equal variances	0.255	Equal means
	Family consanguinity 3	41	4	Phe-LC-UV	0.467	Equal variances	0.935	Equal means
				Tyr-HPLC-UV	0.155	Equal variances	0.072	Equal means
				R1-HPLC-UV	0.507	Equal variances	0.372	Equal means
				Phe-LC-MSMS	0.405	Equal variances	0.944	Equal means
				Tyr-LC-MSMS	0.855	Equal variances	0.352	Equal means
				R2-LC-MSMS	0.085	Equal variances	0.378	Equal means
Suspected volunteers	Death in siblings	361	36	Phe-HPLC-UV	0.001	Different variances	0.092	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.044	Different means
				R1-HPLC-UV	0.241	Equal variances	0.165	Equal means
				Phe-LC-MSMS	0.000	Different variances	0.079	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.044	Different means
				R2-LC-MSMS	0.283	Equal variances	0.206	Equal means
	Family consanguinity 0	127	270	Phe-HPLC-UV	0.000	Different variances	0.015	Different means
				Tyr-HPLC-UV	0.014	Different variances	0.152	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R1-HPLC-UV	0.000	Different variances	0.023	Different means
				Phe-LC-MSMS	0.000	Different variances	0.015	Different means
				Tyr-LC-MSMS	0.014	Different variances	0.166	Equal means
				R2-LC-MSMS	0.000	Different variances	0.020	Different means
	Family consanguinity 2	298	99	Phe-HPLC-UV	0.105	Equal variances	0.234	Equal means
				Tyr-HPLC-UV	0.001	Different variances	0.144	Equal means
				R1-HPLC-UV	0.947	Equal variances	0.797	Equal means
				Phe-LC-MSMS	0.129	Equal variances	0.247	Equal means
				Tyr-LC-MSMS	0.001	Different variances	0.155	Equal means
				R2-LC-MSMS	0.956	Equal variances	0.868	Equal means
	Family consanguinity 3	371	26	Phe-HPLC-UV	0.000	Different variances	0.033	Different means
				Tyr-HPLC-UV	0.530	Equal variances	0.791	Equal means
				R1-HPLC-UV	0.000	Different variances	0.031	Different means
				Phe-LC-MSMS	0.000	Different variances	0.031	Different means
				Tyr-LC-MSMS	0.488	Equal variances	0.764	Equal means
				R2-LC-MSMS	0.000	Different variances	0.027	Different means
	Prematurity	369	28	Phe-HPLC-UV	0.145	Equal variances	0.431	Equal means
				Tyr-HPLC-UV	0.935	Equal variances	0.739	Equal means
				R1-HPLC-UV	0.243	Equal variances	0.842	Equal means
				Phe-LC-MSMS	0.146	Equal variances	0.408	Equal means
				Tyr-LC-MSMS	0.920	Equal variances	0.743	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R2-LC-MSMS	0.261	Equal variances	0.773	Equal means
All volunteers	Death in siblings	406	36	Phe-HPLC-UV	0.007	Different variances	0.166	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.047	Different means
				R1-HPLC-UV	0.081	Equal variances	0.069	Equal means
				Phe-LC-MSMS	0.004	Different variances	0.147	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.047	Different means
				R2-LC-MSMS	0.091	Equal variances	0.087	Equal means
	Family consanguinity 0	152	290	Phe-HPLC-UV	0.008	Different variances	0.016	Different means
				Tyr-HPLC-UV	0.041	Different variances	0.170	Equal means
				R1-HPLC-UV	0.000	Different variances	0.010	Different means
				Phe-LC-MSMS	0.010	Different variances	0.016	Different means
				Tyr-LC-MSMS	0.038	Different variances	0.174	Equal means
				R2-LC-MSMS	0.000	Different variances	0.009	Different means
	Family consanguinity 2	322	120	Phe-HPLC-UV	0.460	Equal variances	0.262	Equal means
				Tyr-HPLC-UV	0.005	Different variance	0.134	Equal means
				R1-HPLC-UV	0.851	Equal variances	0.971	Equal means
				Phe-LC-MSMS	0.517	Equal variances	0.271	Equal means
				Tyr-LC-MSMS	0.005	Different variances	0.148	Equal means
				R2-LC-MSMS	0.856	Equal variances	0.891	Equal means
	Family consanguinity 3	412	30	Phe-HPLC-UV	0.000	Different variances	0.038	Different means
				Tyr-HPLC-UV	0.470	Equal variances	0.632	Equal averages

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R1-HPLC-UV	0.000	Different variances	0.019	Different means
				Phe-LC-MSMS	0.000	Different variances	0.036	Different means
				Tyr-LC-MSMS	0.476	Equal variances	0.689	Equal means
				R2-LC-MSMS	0.000	Different variances	0.018	Different means
	Prematurity	413	29	Phe-HPLC-UV	0.458	Equal variances	0.552	Equal means
				Tyr-HPLC-UV	0.671	Equal variances	0.851	Equal means
				R1-HPLC-UV	0.150	Equal variances	0.642	Equal means
				Phe-LC-MSMS	0.425	Equal variances	0.523	Equal means
				Tyr-LC-MSMS	0.684	Equal variances	0.841	Equal means
				R2-LC-MSMS	0.164	Equal variances	0.592	Equal means

p-value <0,05

VI.5.2.1.4. Associated complications

When testing effect of associated complications to the studied diseases, many factors are implicated counting, mental retardation, bilirubinemia and hepatic cholestasis, autism, acoustic problem, hydrocephaly, development delay, hepato-renal syndrome and epilepsy (west syndrome).

Table VI.46 represents the effect of associated complications on Phe and Tyr concentrations and ratios.

In PKU patients' group, mental retardation and no complication have insignificant effect in the studied diseases and this in all responses (t-test $p > 0,05$). Furthermore, data from other factors are not sufficient so they were not analyzed.

Severe intellectual disability is result of untreated or late-treated PKU. The accumulation of Phe during critical periods of brain development interrupts neurotransmitter synthesis and normal myelination, leading to significant cognitive deficits (van Vliet *et al.*, 2018).

Studies indicate that untreated PKU can cause microcephaly, seizures, and various neuropsychiatric issues like behavioral disturbances and mood disorders (van Vliet *et al.*, 2018). Despite mental retardation is frequented complication in PKU, however it is not the only complication and large clinical spectrum can be associated. Therefore, in our group of patients, this factor has no significant contribution to Phe levels in the blood. In addition, some suspected volunteers were not developed any complication.

For other groups, a significant effect on Tyr levels by HPLC-UV or by LC-MSMS method was observed with bilirubinemia and hepatic cholestasis as complication. No associated complication also has a significant contribution to Tyr concentrations analyzed by the developed methods.

In TRS, the liver's ability to process bilirubin and bile flow is impaired, leading to the retention of bile acids and other substances in the liver and bloodstream due to damage from toxic metabolites like SUA. Cholestasis and direct hyperbilirubinemia are commonly observed, with studies showing that approximately 36% and 46% of patients present with cholestasis and elevated bilirubin levels at diagnosis, respectively (Daou *et al.*, 2021). According to our study, some suspected volunteers were not developed any complication related to TRS, whereas some of them were significantly affected by cholestasis and bilirubinemia as liver's dysfunction result. We suppose that the absence of complications related to TRS could be explained as the suspected diagnosis of almost participant was PKU and not TRS.

Table VI.46. Effect of associated complications on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
PKU Patients	Mental retardation	35	10	Phe-HPLC-UV	0.902	Equal variances	0.493	Equal means
				Tyr-HPLC-UV	0.678	Equal variances	0.872	Equal means
				R1-HPLC-UV	0.817	Equal variances	0.525	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Phe-LC-MSMS	0.981	Equal variances	0.447	Equal means
				Tyr-LC-MSMS	0.741	Equal variances	0.783	Equal means
				R2-LC-MSMS	0.823	Equal variances	0.256	Equal means
	No complications	12	33	Phe-HPLC-UV	0.822	Equal variances	0.820	Equal means
				Tyr-HPLC-UV	0.265	Equal variances	0.955	Equal means
				R1-HPLC-UV	0.745	Equal variances	0.864	Equal means
				Phe-LC-MSMS	0.836	Equal variances	0.767	Equal means
				Tyr-LC-MSMS	0.226	Equal variances	0.692	Equal means
				R2-LC-MSMS	0.801	Equal variances	0.556	Equal means
Supsected volunteers	Mental retardation	325	72	Phe-HPLC-UV	0.525	Equal variances	0.822	Equal means
				Tyr-HPLC-UV	0.387	Equal variances	0.615	Equal means
				R1-HPLC-UV	0.001	Different variances	0.274	Equal means
				Phe-LC-MSMS	0.548	Equal variances	0.842	Equal means
				Tyr-LC-MSMS	0.375	Equal variances	0.590	Equal means
				R2-LC-MSMS	0.001	Different variances	0.286	Equal means
	Epilepsy (west syndrome)	374	23	Phe-HPLC-UV	0.337	Equal variances	0.616	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Tyr-HPLC-UV	0.354	Equal variances	0.706	Equal means
				R1-HPLC-UV	0.548	Equal variances	0.481	Equal means
				Phe-LC-MSMS	0.357	Equal variances	0.728	Equal means
				Tyr-LC-MSMS	0.394	Equal variances	0.743	Equal means
				R2-LC-MSMS	0.496	Equal variances	0.581	Equal means
	Bilirubinemia and hepatic cholestasis	383	14	Phe-HPLC-UV	0.000	Different variations	0.118	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.047	Different means
				R1-HPLC-UV	0.424	Equal variances	0.164	Equal means
				Phe-LC-MSMS	0.000	Different variances	0.112	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.047	Different means
				R2-LC-MSMS	0.458	Equal variances	0.153	Equal means
	Autism	360	37	Phe-HPLC-UV	0.276	Equal variances	0.361	Equal means
				Tyr-HPLC-UV	0.457	Equal variances	0.540	Equal means
				R1-HPLC-UV	0.331	Equal variances	0.340	Equal means
				Phe-LC-MSMS	0.241	Equal variances	0.380	Equal means
				Tyr-LC-MSMS	0.473	Equal variances	0.593	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R2-LC-MSMS	0.220	Equal variances	0.295	Equal means
	Hepato-renal syndrom	386	11	Phe-HPLC-UV	0.000	Different variances	0.095	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.040	Different means
				R1-HPLC-UV	0.482	Equal variances	0.118	Equal means
				Phe-LC-MSMS	0.000	Different variations	0.089	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.040	Different means
				R2-LC-MSMS	0.572	Equal variances	0.135	Equal means
	developmental delay	373	24	Phe-HPLC-UV	0.767	Equal variances	0.736	Equal means
				Tyr-HPLC-UV	0.419	Equal variances	0.430	Equal means
				R1-HPLC-UV	0.310	Equal variances	0.522	Equal means
				Phe-LC-MSMS	0.813	Equal variances	0.734	Equal means
				Tyr-LC-MSMS	0.430	Equal variances	0.423	Equal means
				R2-LC-MSMS	0.203	Equal variances	0.436	Equal means
	acoustic problem	386	11	Phe-HPLC-UV	0.440	Equal variances	0.554	Equal means
				Tyr-HPLC-UV	0.524	Equal variances	0.565	Equal means
				R1-HPLC-UV	0.505	Equal variances	0.553	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Phe-LC-MSMS	0.485	Equal variances	0.590	Equal means
				Tyr-LC-MSMS	0.505	Equal variances	0.517	Equal means
				R2-LC-MSMS	0.482	Equal variances	0.437	Equal means
	Hydroen cephalitis	389	8	Phe-HPLC-UV	0.120	Equal variances	0.377	Equal means
				Tyr-HPLC-UV	0.320	Equal variances	0.412	Equal means
				R1-HPLC-UV	0.440	Equal variances	0.982	Equal means
				Phe-LC-MSMS	0.135	Equal variances	0.389	Equal means
				Tyr-LC-MSMS	0.355	Equal variances	0.407	Equal means
				R2-LC-MSMS	0.703	Equal variances	0.815	Equal means
	No complication	201	196	Phe-HPLC-UV	0.029	Different variations	0.156	Equal means
				Tyr-HPLC-UV	0.000	Different variaances	0.045	Different means
				R1-HPLC-UV	0.250	Equal variances	0.523	Equal means
				Phe-LC-MSMS	0.029	Different variations	0.158	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.042	Different means
				R2-LC-MSMS	0.385	Equal variances	0.704	Equal means
All volunteers	Mental retardation	360	82	Phe-HPLC-UV	0.285	Equal variances	0.530	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Tyr-HPLC-UV	0.392	Equal variances	0.640	Equal means
				R1-HPLC-UV	0.000	Different variances	0.174	Equal means
				Phe-LC-MSMS	0.276	Equal variances	0.521	Equal means
				Tyr-LC-MSMS	0.369	Equal variances	0.580	Equal means
				R2-LC-MSMS	0.000	Different variances	0.140	Equal means
	Epilepsy (western syndrome)	418	24	Phe-HPLC-UV	0.180	Equal variances	0.437	Equal means
				Tyr-HPLC-UV	0.323	Equal variances	0.671	Equal means
				R1-HPLC-UV	0.298	Equal variances	0.326	Equal means
				Phe-LC-MSMS	0.195	Equal variances	0.533	Equal means
				Tyr-LC-MSMS	0.368	Equal variances	0.715	Equal means
				R2-LC-MSMS	0.251	Equal variances	0.390	Equal means
	Bilirubinemia and Cholestasis	428	14	Phe-HPLC-UV	0.000	Different variances	0.145	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.048	Different means
				R1-HPLC-UV	0.255	Equal variances	0.109	Equal means
				Phe-LC-MSMS	0.000	Different variances	0.139	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Tyr-LC-MSMS	0.000	Different variances	0.048	Different means
				R2-LC-MSMS	0.267	Equal variances	0.101	Equal means
	Autism	405	37	Phe-HPLC-UV	0.130	Equal variances	0.186	Equal means
				Tyr-HPLC-UV	0.481	Equal variances	0.505	Equal means
				R1-HPLC-UV	0.117	Equal variances	0.153	Equal means
				Phe-LC-MSMS	0.108	Equal variances	0.196	Equal means
				Tyr-LC-MSMS	0.498	Equal variances	0.554	Equal means
				R2-LC-MSMS	0.065	Equal variances	0.127	Equal means
	Hepato-renal syndrom	431	11	Phe-HPLC-UV	0.000	Different variances	0.112	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.041	Different means
				R1-HPLC-UV	0.314	Equal variances	0.086	Equal means
				Phe-LC-MSMS	0.000	Different variances	0.106	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.041	Different means
				R2-LC-MSMS	0.369	Equal variances	0.097	Equal means
	Development delay	417	25	Phe-HPLC-UV	0.446	Equal variances	0.899	Equal means
				Tyr-HPLC-UV	0.417	Equal variances	0.439	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R1-HPLC-UV	0.137	Equal variances	0.263	Equal means
				Phe-LC-MSMS	0.479	Equal variances	0.886	Equal means
				Tyr-LC-MSMS	0.429	Equal variances	0.430	Equal means
				R2-LC-MSMS	0.083	Equal variances	0.200	Equal means
	acoustic problem	430	12	Phe-HPLC-UV	0.562	Equal variances	0.580	Equal means
				Tyr-HPLC-UV	0.522	Equal variances	0.656	Equal means
				R1-HPLC-UV	0.562	Equal variances	0.804	Equal means
				Phe-LC-MSMS	0.575	Equal variances	0.603	Equal means
				Tyr-LC-MSMS	0.506	Equal variances	0.596	Equal means
				R2-LC-MSMS	0.809	Equal variances	0.975	Equal means
	Hydrocephaly	434	8	Phe-HPLC-UV	0.084	Equal variances	0.296	Equal means
				Tyr-HPLC-UV	0.311	Equal variances	0.383	Equal means
				R1-HPLC-UV	0.309	Equal variances	0.814	Equal means
				Phe-LC-MSMS	0.096	Equal variances	0.306	Equal means
				Tyr-LC-MSMS	0.347	Equal variances	0.377	Equal means
				R2-LC-MSMS	0.503	Equal variances	0.962	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
	No complication	148	294	Phe-HPLC-UV	0.084	Equal variances	0.209	Equal means
				Tyr-HPLC-UV	0.000	Different variances	0.049	Different means
				R1-HPLC-UV	0.472	Equal variances	0.724	Equal means
				Phe-LC-MSMS	0.072	Equal variances	0.203	Equal means
				Tyr-LC-MSMS	0.000	Different variances	0.049	Different means
				R2-LC-MSMS	0.439	Equal variances	0.766	Equal means

p-value < 0,05

VI.5.2.1.5. Associated therapy

In this section, the associated therapy of different group of volunteers participating in this study, was investigated. This is comprised antiepileptics, antipsychotics, corticosteroid therapy, antibiotics therapy, cerebral oxygenator, cerebral muscle relaxant, supplementation with omega 3, vitamins and minerals and dietary treatment with cofactor sapropretin. The results of this section are presented in **Table IV.47**.

For PKU patients' group, there is no significant contribution of neither associated therapy by antiepileptic drugs nor by associated dietary the Phe or Tyr levels or ratios (t-test $p > 0,05$). Nevertheless, cofactor associated therapy using synthetic BH4 (sapropretin), has significant effect on both R1 and R2.

Clinical studies have shown that sapropterin significantly decreases blood Phe concentrations. In a 6-week study, PKU patients treated with sapropterin (10 mg/kg/day) showed a mean decrease in blood Phe levels of 236 ± 257 µmol/L as compared to an increase of 2.9 ± 240 µmol/L in the placebo group ($p < 0.001$). Another reported study showed that after 3 weeks of treatment with 20 mg/kg/day, the mean diminution in blood Phe levels was 149 ± 134 µmol/L ($p < 0.001$) (EMA, 2024).

In addition, as it is reported in another study, BH4 introduced in the neonatal period is associated with less blood Phe fluctuation before 6 years (Theron *et al.*, 2023). Consequently, sapropretin ensure satisfactory control of blood Phe levels and nutritional balance as it is confirmed by our study when it influences R1 and R2 so it influences directly Phe levels in the blood.

In suspected volunteers' group and all volunteers group, we observe a significant contribution of global associated therapy affect Tyr concentrations obtained by both developed methods. Thus, when patients are under associated therapy, this is may influence their Tyr blood concentrations. In addition, when studying each type of therapy alone, antiepileptics have significant effect on Phe concentrations obtained by both developed methods as compared to antipsychotics, corticosteroids and supplementation drugs which has no significant effect on all studied responses.

Studies have confirmed that adult PKU mice with lower serum Phe levels were less disposed to seizures compared to those with elevated levels, suggesting that high Phe concentrations may aggravate seizure activity. Therefore, high serum Phe levels have been related to increased predisposition to seizures in individuals with PKU. In the same study, stabilization of serum Phe levels within 12 hours of dietary intervention significantly decreased the occurrence of seizures in PKU mice (Martynyuk *et al.*, 2007). Consequently, low-Phe diet can normalize serum Phe levels, which in turn may prevent seizure susceptibility. Those findings may explain the relationship between antiepileptic drugs and Phe levels. This indicates that maintaining optimal Phe levels is crucial for managing epilepsy in PKU patients.

In the third group, there is no contribution to all studied therapies in all responses. In addition, data from some studied factors like antibiotics therapy, cerebral oxygenator, cerebral muscle relaxant, supplementation with omega 3, vitamins and minerals are insufficient and they could not be analyzed.

Table VI.47. Effect of associated therapy on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
PKU Patients	Antiepileptic	44	1	Phe-HPLC-UV	0.459	Equal variances	0.824	Equal means
				Tyr-HPLC-UV	0.919	Equal variances	0.791	Equal means
				R1-HPLC-UV	0.639	Equal variances	0.936	Equal means
				Phe-LC-MSMS	0.469	Equal variances	0.803	Equal means
				Tyr-LC-MSMS	0.828	Equal variances	0.553	Equal means
				R2-LC-MSMS	0.901	Equal variances	0.861	Equal means
	Dietary	16	29	Phe-HPLC-UV	0.512	Equal variances	0.975	Equal means
				Tyr-HPLC-UV	0.726	Equal variances	0.643	Equal means
				R1-HPLC-UV	0.979	Equal variances	0.583	Equal means
				Phe-LC-MSMS	0.518	Equal variances	0.948	Equal means
				Tyr-LC-MSMS	0.732	Equal variances	0.283	Equal means
				R2-LC-MSMS	0.788	Equal variances	0.324	Equal means
	Cofactor (Sapropretin)	34	11	Phe-HPLC-UV	0.011	Different variances	0.066	Equal means
				Tyr-HPLC-UV	0.652	Equal variances	0.690	Equal means
				R1-HPLC-UV	0.004	Different variances	0.016	Different means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Phe-LC-MSMS	0.015	Different variances	0.065	Equal means
				Tyr-LC-MSMS	0.559	Equal variances	0.496	Equal means
				R2-LC-MSMS	0.045	Different variances	0.010	Different means
Suspected volunteers	Associated Therapy	303	94	Phe-HPLC-UV	0.009	Different variances	0.059	Equal means
				Tyr-HPLC-UV	0.023	Different variances	0.037	Different means
				R1-HPLC-UV	0.320	Equal variances	0.408	Equal means
				Phe-LC-MSMS	0.008	Different variances	0.066	Equal means
				Tyr-LC-MSMS	0.021	Different variances	0.027	Different means
				R2-LC-MSMS	0.577	Equal variances	0.668	Equal means
	Antiepileptic	362	35	Phe-HPLC-UV	0.025	Different variances	0.003	Different means
				Tyr-HPLC-UV	0.104	Equal variances	0.346	Equal means
				R1-HPLC-UV	0.350	Equal variances	0.222	Equal means
				Phe-LC-MSMS	0.024	Different variances	0.005	Different means
				Tyr-LC-MSMS	0.103	Equal variances	0.353	Equal means
				R2-LC-MSMS	0.442	Equal variances	0.285	Equal means
	Antipsychotic	386	11	Phe-HPLC-UV	0.961	Equal variances	0.898	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Tyr-HPLC-UV	0.778	Equal variances	0.685	Equal means
				R1-HPLC-UV	0.674	Equal variances	0.589	Equal means
				Phe-LC-MSMS	0.944	Equal variances	0.885	Equal means
				Tyr-LC-MSMS	0.706	Equal variances	0.669	Equal means
				R2-LC-MSMS	0.827	Equal variances	0.470	Equal means
	Corticosteroid therapy	393	4	Phe-HPLC-UV	0.845	Equal variances	0.968	Equal means
				Tyr-HPLC-UV	0.484	Equal variances	0.569	Equal means
				R1-HPLC-UV	0.015	Different variances	0.541	Equal means
				Phe-LC-MSMS	0.801	Equal variances	0.985	Equal means
				Tyr-LC-MSMS	0.491	Equal variances	0.560	Equal means
				R2-LC-MSMS	0.003	Different variances	0.523	Equal means
	Supplementation	382	15	Phe-HPLC-UV	0.204	Equal variances	0.467	Equal means
				Tyr-HPLC-UV	0.254	Equal variances	0.388	Equal means
				R1-HPLC-UV	0.418	Equal variances	0.532	Equal means
				Phe-LC-MSMS	0.207	Equal variances	0.440	Equal means
				Tyr-LC-MSMS	0.263	Equal variances	0.355	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				R2-LC-MSMS	0.133	Equal variances	0.312	Equal means
All volunteers	Associated Therapy	332	110	Phe-HPLC-UV	0.309	Equal variances	0.850	Equal means
				Tyr-HPLC-UV	0.017	Different variances	0.026	Different means
				R1-HPLC-UV	0.149	Equal variances	0.257	Equal means
				Phe-LC-MSMS	0.317	Equal variances	0.892	Equal means
				Tyr-LC-MSMS	0.018	Different variances	0.025	Different means
				R2-LC-MSMS	0.075	Equal variances	0.161	Equal means
	Antiepileptic	403	39	Phe-HPLC-UV	0.078	Equal variances	0.211	Equal means
				Tyr-HPLC-UV	0.104	Equal variances	0.334	Equal means
				R1-HPLC-UV	0.329	Equal variances	0.279	Equal means
				Phe-LC-MSMS	0.073	Equal variances	0.244	Equal means
				Tyr-LC-MSMS	0.123	Equal variances	0.392	Equal means
				R2-LC-MSMS	0.464	Equal variances	0.321	Equal means
	Antipsychotic	430	12	Phe-HPLC-UV	0.721	Equal variances	0.830	Equal means
				Tyr-HPLC-UV	0.707	Equal variances	0.646	Equal means
				R1-HPLC-UV	0.436	Equal variances	0.935	Equal means

Volunteers	Variable X	Count No (0)	Count Yes (1)	Variable Y	Levene p-value	Equality of variances	t-test p-value	Conclusion t-test
				Phe-LC-MSMS	0.626	Equal variances	0.853	Equal means
				Tyr-LC-MSMS	0.649	Equal variances	0.636	Equal means
				R2-LC-MSMS	0.552	Equal variances	0.809	Equal means
	Corticosteroid therapy	438	4	Phe-HPLC-UV	0.966	Equal variances	0.855	Equal means
				Tyr-HPLC-UV	0.476	Equal variances	0.544	Equal means
				R1-HPLC-UV	0.040	Different variances	0.596	Equal means
				Phe-LC-MSMS	0.926	Equal variances	0.897	Equal means
				Tyr-LC-MSMS	0.482	Equal variances	0.534	Equal means
				R2-LC-MSMS	0.010	Different variances	0.571	Equal means
	Supplementation	426	16	Phe-HPLC-UV	0.159	Equal variances	0.395	Equal means
				Tyr-HPLC-UV	0.224	Equal variances	0.334	Equal means
				R1-HPLC-UV	0.475	Equal variances	0.558	Equal means
				Phe-LC-MSMS	0.151	Equal variances	0.368	Equal means
				Tyr-LC-MSMS	0.229	Equal variances	0.308	Equal means
				R2-LC-MSMS	0.246	Equal variances	0.407	Equal means

p-value <0,05

VI.5.2.2. Quantitative analysis

In order to determine the influence of quantitative factors on Phe and Tyr concentrations and on their respective ratios, regression equations with their p-values, R-squared and R-residuals of normality test of each variable were calculated and the significance was confirmed by using Spearman correlation test which can be used either with normal or non-normal data distribution. Statistical significance was considered at $p < 0,05$. Influencing degree of significant factors was expected by using the value and sign of regression coefficient

VI.5.2.2.1. Age of volunteers

As shown in **Table VI.48**, the first tested group of volunteers is PKU patients. For this group there is no significant effect of volunteers age on all tested responses. However, in suspected volunteers' group and all volunteers' group, Tyr concentration calculated by both methods, was significantly affected by age of volunteers. The regression model is valid ($R^2 < 0,3$). We can observe also that increasing the factor (age of volunteers) by one unit (one day) decreases the response by 0,01 units. All other tested responses are not affected by volunteers age and there is no correlation between them.

The relationship between patient age and Phe or Tyr concentrations in the blood for patients with PKU is important. As patients age, their mean blood Phe and Tyr levels tend to increase (Kouchiwa *et al.*, 2012), and the percentage of these levels that fall within therapeutic target ranges failures. So, the suggested therapeutic target range for blood Phe is 120-360 µmol/L for patients below 12 years and 120-600 µmol/L for those aged 12 years and older (Theron *et al.*, 2023). However, in our study there was no contribution of patients age to Phe concentrations. This can be explained as small fluctuations in Phe concentrations can be observed in the tested range of patients age which was up to 15 years and almost volunteers were aged less than 12 years.

Table VI.48. Effect of volunteers age on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
PKU Patiens	Phe-HPLC-UV	-0.011	231,879	0.555	0.008	Not-normal	Invalid model	0.057	0.709	Not significant
	Tyr-HPLC-UV	0.002	109,306	0.743	0.003	Not-normal	Invalid model	0.097	0.525	Not significant
	R1-HPLC-UV	0.000	2,010	0.747	0.002	Not-normal	Invalid model	-0.008	0.957	Not significant
	Phe LC-MSMS	-0.009	227,800	0.633	0.005	Not-normal	Invalid model	0.092	0.547	Not significant
	Tyr-LC-MSMS	-0.001	116,019	0.859	0.001	Not-normal	Invalid model	0.036	0.815	Not significant
	R2-LC-MSMS	0.000	1,873	0.874	0.001	Not-normal	Invalid model	0.084	0.584	Not significant
Suspected volunteers	Phe-HPLC-UV	-0.006	124,971	0.260	0.003	Not-normal	Invalid model	-0.018	0.716	Not significant
	Tyr-HPLC-UV	-0.012	130,143	0.039	0.011	Not-normal	Valid model	-0.047	0.348	Not significant
	R1-HPLC-UV	0.000	1,022	0.211	0.004	Not-normal	Invalid model	-0.018	0.720	Not significant

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
	Phe-LC-MSMS	-0.006	124,673	0.259	0.003	Not-normal	Invalid model	-0.018	0.719	Not significant
	Tyr-LC-MSMS	-0.012	130,707	0.034	0.011	Not-normal	Valid model	-0.059	0.237	Not significant
	R2-LC-MSMS	0.000	1,028	0.229	0.004	Not-normal	Invalid model	-0.007	0.882	Not significant
All volunteers	Phe-HPLC-UV	-0.006	134,316	0.277	0.003	Not-normal	Invalid model	-0.010	0.836	Not significant
	Tyr-HPLC-UV	-0.010	127,460	0.048	0.009	Not-normal	Valid model	-0.040	0.407	Not significant
	R1-HPLC-UV	0.000	1,111	0.268	0.003	Not-normal	Invalid model	-0.015	0.759	Not significant
	Phe-LC-MSMS	-0.005	133,568	0.299	0.002	Not-normal	Invalid model	-0.005	0.915	Not significant
	Tyr-LC-MSMS	-0.011	128,701	0.037	0.010	Not-normal	Valid model	-0.058	0.222	Not significant
	R2-LC-MSMS	0.000	1,101	0.191	0.004	Not-normal	Invalid model	0.003	0.947	Not significant

p-value <0,05

VI.5.2.2.2. Age of support

The effect of support age on Phe and Tyr concentrations and ratios are grouped in **Table VI.49**. For PKU Patients, the age of patient support was relevant in Phe concentrations. For example, the regression model was valid with low R^2 (< 0,3), and increasing the support age by one unit (one month) increases the response by 6,36 units and by 6,56 (µM) for HPLC and LC-MSMS method, respectively. The ratios R1 and R2 are also significantly affected by support age. The regression model was valid with low R^2 (< 0,3), and increasing the support age by one unit (one month) increases the response 0,07 units. Correlation is average 0,3-0,7 according to Spearman correlation coefficient.

In suspected volunteers' group, the age of patient support was relevant in both Phe and Tyr concentrations if it analyzed by HPLC-UV or by LC-MSMS method. In the case of Phe, regression model was valid with low R^2 (< 0,3), and increasing the support age by one unit (one month) decreases the response by 1,73 units and by 1,78 (µM) for HPLC and LC-MSMS method, respectively with weak significant correlation (<0,3). Similar results were obtained when analyzing Tyr, regression model was valid with low R^2 (< 0,3), and increasing the support age by one unit (one month) decreases the response by 2,23 units and by 2,21 (µM) for HPLC and LC-MSMS method, respectively with insignificant correlation. A weak significant correlation (<0,3) also was observed with R1 and R2 ratios. These findings could be explained as the metabolism in general differs with age and the contents in AAs vary also however we should not delay the support of the disease.

When gathering all volunteers in the third tested group, Phe, Tyr and R2, had both significant weak correlations. In addition, Tyr concentration calculated by both developed methods had a valid model (R^2 < 0,3) and increasing the factor by one unit decreases the response by 2,08 units.

Initiating intervention as early as possible, preferably within the first week of life, is crucial for preventing irreversible neurological damage. Phe concentrations in the blood should be closely monitored to achieve levels within the recommended target range from infancy forward (Muntau *et al.*, 2017). We can conclude that the relationship between age and blood Phe or Tyr concentrations is particularly relevant in the context of metabolic disorders such as PKU and TRS. Monitoring Phe and Tyr levels is indispensable for handling these conditions effectively, as age can influence both dietary requirements and metabolic responses.

Table VI.49. Effect of support age on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
PKU patients	Phe-HPLC-UV	6,357	118,980	0.036	0.098	Not-normal	Valid model	0.205	0.177	Not significant
	Tyr-HPLC-UV	-0.717	123,100	0.427	0.015	Not-normal	Invalid model	-0.159	0.296	Not significant
	R1-HPLC-UV	0.071	0.897	0.002	0.203	Not-normal	Valid model	0.381	0.010	Significant
	Phe-LCMSMS	6,559	116,183	0.030	0.104	Not-normal	Valid model	0.223	0.140	Not significant
	Tyr-LC-MSMS	-0.803	125,625	0.364	0.019	Not-normal	Invalid model	-0.168	0.269	Not significant
	R2-LC-MSMS	0.072	0.891	0.001	0.213	Not-normal	Valid model	0.399	0.007	Significant
Suspected Volunteers	Phe-HPLC-UV	-1,733	141,756	0.034	0.011	Not-normal	Valid model	-0.131	0.009	Significant
	Tyr-HPLC-UV	-2,229	143,199	0.013	0.015	Not-normal	Valid model	-0.090	0.075	Not significant

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
	R1-HPLC-UV	-0.002	1,143	0.664	0.000	Not-normal	Invalid model	-0.103	0.041	Significant
	Phe-LC-MSMS	-1,776	142,259	0.028	0.012	Not-normal	Valid model	-0.147	0.003	Significant
	Tyr-LC-MSMS	-2,213	142,959	0.014	0.015	Not-normal	Valid model	-0.072	0.152	Not-significant
	R2-LC-MSMS	-0.004	1,178	0.404	0.002	Not-normal	Invalid model	-0.120	0.017	Significant
All volunteers	Phe-HPLC-UV	-1,157	142,075	0.156	0.005	Not-normal	Invalid model	-0.126	0.008	Significant
	Tyr-HPLC-UV	-2,079	140,963	0.011	0.015	Not-normal	Valid model	-0.111	0.020	Significant
	R1-HPLC-UV	0.003	1,141	0.588	0.001	Not-normal	Invalid model	-0.089	0.061	Not-significant
	Phe-LC-MSMS	-1,175	142,209	0.146	0.005	Not-normal	Invalid model	-0.136	0.004	Significant
	Tyr-LC-MSMS	-2,077	141,078	0.010	0.015	Not-normal	Valid model	-0.098	0.040	Significant

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
	R2-LC-MSMS	0.001	1,171	0.821	0.000	Not-normal	Invalid model	-0.099	0.039	Significant

p-value <0,05

VI.5.2.2.3. Age of symptoms onset

Table VI.50 represent the effect of symptoms onset age on Phe and Tyr concentrations and ratios.

In PKU patients, the correlation is significant and average 0,3-0,7 for R1 and R2. Additionally, when increasing age of symptoms onset by one unit (one month), the response increases (R1 or R2) by 0,1 units.

For suspected volunteers, and when analyzing Tyr, regression model was valid with low R^2 (< 0,3), and increasing the onset symptoms age by one unit (one month) decreases the response by 3,70 units and by 3,74 (µM) for HPLC and LC-MSMS method, respectively with weak correlation.

For Phe analyzed by LC-MSMS method, **r**egression model was also valid with low R^2 (< 0.3), and increasing the support age by one unit (one month) decreases the response by 2,34 units weak correlation (< 0,3).

Similar results were obtained when testing all volunteers as one united group, the Tyr **r**egression model was valid with low R^2 (< 0,3), and increasing the factor by one unit (one month) decreases the response by 3,42 units and by 3,51 units (µM) for HPLC and LC-MSMS method, respectively with weak correlation (<0,3). However, Phe by LC-MSMS had a non-valid regression model with significant weak correlation.

These findings confirm the importance of early detection of metabolic disease. If treatment begins within the first few weeks of life, many children can keep normal cognitive function and evade the severe symptoms associated with high Phe or Tyr levels.

Table VI.50. Effect of symptom onset age on phenylalanine and tyrosine concentrations and ratios

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
PKU patients	Phe-HPLC-UV	7,601	161,648	0.082	0.069	Not-normal	Invalid model	0.336	0.024	Significant
	Tyr-HPLC-UV	-0.720	117,422	0.577	0.007	Not-normal	Invalid model	-0.026	0.866	Not-significant
	R1-HPLC-UV	0.091	1,337	0.006	0.163	Not-normal	Valid model	0.426	0.004	Significant
	Phe-LC-MSMS	7,850	160,153	0.072	0.073	Not-normal	Invalid model	0.325	0.030	Significant
	Tyr-LC-MSMS	-1,346	122,686	0.287	0.026	Not-normal	Invalid model	-0.126	0.411	Not-significant
	R2-LC-MSMS	0.101	1,277	0.002	0.207	Not-normal	Valid model	0.447	0.002	S ignificant
Suspected volunteers	Phe-HPLC-UV	-2,251	131,111	0.051	0.010	Not-normal	Invalid model	-0.083	0.100	Not-significant

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
	Tyr-HPLC-UV	-3,699	135,901	0.004	0.021	Not-normal	Valid model	-0.150	0.003	Significant correlation
	R1-HPLC-UV	0.010	1,024	0.169	0.005	Not-normal	Invalid model	0.004	0.936	Not-significant
	Phe-LC-MSMS	-2,335	131,570	0.040	0.011	Not-normal	Valid model	-0.116	0.021	Significant
	Tyr-LC-MSMS	-3,737	136,249	0.003	0.022	Not-normal	Valid model	-0.166	0.001	Significant
	R2-LC-MSMS	0.008	1,045	0.292	0.003	Not-normal	Invalid model	-0.009	0.859	Not-significant
All volunteers	Phe-HPLC-UV	-1,665	136,104	0.147	0.005	Not-normal	Invalid model	-0.094	0.049	Significant
	Tyr-HPLC-UV	-3,405	133,698	0.003	0.020	Not-normal	Valid model	-0.164	0.001	Significant
	R1-HPLC-UV	0.015	1,074	0.057	0.008	Not-normal	Invalid model	0.002	0.965	Not-significant

Volunteers	Variable Y	Regression Coefficient	Intercept	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
	Phe-LC-MSMS	-1,716	136,333	0.131	0.005	Not-normal	Invalid model	-0.119	0.012	Significant
	Tyr-LC-MSMS	-3,506	134,651	0.002	0.021	Not-normal	Valid model	-0.186	0.000	Significant
	R2-LC-MSMS	0.014	1,085	0.078	0.007	Not-normal	Invalid model	-0.003	0.957	Not-significant

p-value <0,05

VI.5.2.2.4. Fasting duration

As shown in **Table VI.51**, PKU patients' and all volunteers' groups had non-sufficient data, so they were not analyzed.

For suspected volunteers and when analyzing Phe, **r**egression model was valid with low R^2 (< 0,3), and increasing the fasting duration by one unit (one hour) decreases the response by 7,01 units and by 7,12 (µM) for HPLC and LC-MSMS method, respectively with insignificant correlation for Phe analyzed by HPLC method.

When analyzing Tyr, regression model was valid with low R^2 (< 0,3), and increasing the fasting duration by one unit (one hour) decreases the response by 8,98 units and by 8,99 units (µM) for HPLC and LC-MSMS method, respectively with insignificant correlation.

In a study reported earlier (Uaariyapanichkul *et al.*, 2018), univariate analysis of fasting duration showed significant association with alanine, glycine, methionine, and citrulline and after, multivariate models adjustment $p < 0.1$, Phe is affected also by fasting duration.

Previous studies have typically fasted children aged over two years for 8 to 10 hours before blood collection, while some did not specify fasting durations. However, no research has thoroughly clarified the impact of fasting on AAs levels in humans. Therefore, it is recommended that fasting should not be a mandatory practice for children (Uaariyapanichkul *et al.*, 2018).

However, it was reported that taking blood samples after an overnight fast demonstrate less day-to-day variation in AA concentrations, making them more reliable for monitoring (van Dam *et al.*, 2017).

Accordingly, comprehension of the effects of fasting on these AAs is crucial for managing conditions like PKU and TRS, where monitoring blood Phe and Tyr levels is crucial for dietary management. The fluctuations in AAs concentrations during fasting underscore the critical role of timing in both blood sampling and dietary interventions for individuals with metabolic disorder.

Table VI.51. Effect of fasting duration on phenylalanine and tyrosine concentrations and ratios

Variable Y	Regression Coefficient	Interpretation	p-value regression	R-squared	Residuals Normality	Regression Conclusion	Spearman Correlation Coefficient	Spearman Correlation p-value	Correlation Conclusion
Phe-HPLC-UV	-7,009	175,901	0.005	0.020	Not-normal	Valid model	-0.088	0.082	Not-significant
Tyr-HPLC-UV	-8,980	187,056	0.001	0.027	Not-normal	Valid model	-0.088	0.084	Not-significant
R1-HPLC-UV	-0.012	1,211	0.471	0.001	Not-normal	Invalid model	-0.064	0.208	Not-significant
Phe-LC-MSMS	-7,117	176,649	0.004	0.021	Not-normal	Valid model	-0.105	0.038	Significant
Tyr-LC-MSMS	-8,992	187,168	0.001	0.027	Not-normal	Valid model.	-0.089	0.079	Not-significant
R2-LC-MSMS	-0.015	1,239	0.354	0.002	Not-normal	Invalid model	-0.081	0.108	Not-significant

p-value <0,05

VI.5.2.3.1. Comparison of qualitative factors

Chi squared test is a statistical method used to determine if there is a significant association between categorical variables (McHugh, 2013).

According to **Table VI.52**, some differences are existed between PKU patients' group and suspected volunteers' group which are essentially, context of the analysis request with its two studied factors, family history including family consanguinity second degree and death in siblings. For associated complications, there was difference in autism between the tested groups.

Those differences could be explained as the context of analysis is confirmation for the presence of PKU or TRS for suspected volunteers' group so it is for diagnosis wile for PKU patients group the main reason for analysis is monitoring of their PKU. In addition, the number of participants in each group is quite different and influence directly the results.

Table VI.52. Comparison of qualitative factors of phenylketonuria patients' group and suspected volunteers' group

Variable type	Variable analyzed	Test	p-value	Conclusion
Gender	Sex distribution	Chi-square test	0.694	No difference
Context of request	Family history	Chi-square test	0.044	Difference
	Control of abnormal metabolic profile	Chi-square test	0.000	Difference
Family history	Death in siblings	Fisher's exact test	0.038	Difference
	Family consanguinity 0	Chi-square test	0.001	Difference
	Family consanguinity 2	Chi-square test	0.002	Difference
	Family consanguinity 3	Fisher's exact test	0.536	No difference
	Prematurity	Fisher's exact test	0.341	No difference
	Mental retardation	Chi-square test	0.503	No difference
Associated complications	Epilepsy (western syndrome)	Fisher's exact test	0.494	No difference
	Bilirubinemia and haptic Cholestasis	Fisher's exact test	0.379	No difference
	Autism	Fisher's exact test	0.023	Difference
	Hepato-renal syndrome	Fisher's exact test	0.612	No difference
	Developmental delay	Fisher's exact test	0.495	No difference
	Acoustic problems	Fisher's exact test	1	No difference
	Hydrocephly	Fisher's exact test	1	No difference
	Miningite	Fisher's exact test	1	No difference

Variable type	Variable analyzed	Test	p-value	Conclusion
	No complications	Chi-square test	0.306	No difference
	Associated Therapy	Chi-square test	0.080	No difference
Associated therapy	Antiepileptic	Fisher's exact test	1	No difference
	Antipsychotic	Fisher's exact test	1	No difference
	Antibioc therapy	Fisher's exact test	1	No difference
	Corticosteroid therapy	Fisher's exact test	1	No difference
	Cerebral oxygenator	Fisher's exact test	1	No difference
	Central myorelaxant	Fisher's exact test	1	No difference
	Supplementation	Fisher's exact test	1	No difference

p-value <0,05

VI.5.2.3.2. Comparison of quantitative factors

According to **Table VI.53**, a significant difference was found in Phe concentrations calculated by both methods together with R1 and R2 for the tested groups, while there was no significant difference in Tyr levels. This could be explained as that almost suspected volunteers are mainly supposed to be PKU positive and not TRS. Moreover, PKU patients has generally normal Tyr concentrations. Thus, Tyr levels are not different between the tested groups.

Table VI.53. Comparison of quantitative factors of phenylketonuria patients' group and suspected volunteers' group

Variable Y	Levene's test for equality of variances (p-value)	Conclusion	Student's test (p-value)	Conclusion
Phe-HPLC-UV	2,036E-02	Different variances	3,439E-04	Different means
Tyr-HPLC-UV	4,071E-01	Equal variances	7,853E-01	Equal means
R1-HPLC-UV	8,733E-05	Different variances	1,157E-04	Different means
Phe-LC-MSMS	1,286E-02	Different variances	3,288E-04	Different means
Tyr-LC-MSMS	3,954E-01	Equal variances	7,402E-01	Equal means
R2-LC-MSMS	1,302E-05	Different variances	8,977E-05	Different means

p-value <0,05

VI.5.2.4. Multivariate analysis

In order to understand interactions and complex relationships between variables, MLR was utilized. It is a method that spreads simple linear regression by permitting for multiple explanatory variables to predict the response variable outcome.

The primary goal of MLR is to find a linear relationship between the independent variables and the dependent variable, allowing predictions based on the values of these independent variables (Smalheiser, 2017).

ANOVA p-value was used to determine the model validity then, the intercept was employed in order to determine the effect degree. The results are tabulated in **Table VI.54**.

In this study, factors that together have a significant influence on Phe (HPLC-UV or LC-MSMS) are family history, patient fasting duration and family consanguinity third degree. History of participant together with consanguinity affect positively the response. Increasing the variable by one unit, increase slightly the Phe concentrations. However, negative contribution of fasting duration was observed.

Whereas, factors that have a significant influence on Tyr (HPLC-UV or LC-MSMS) are death in siblings with positive effect and patient fasting duration with negative effect Tyr levels either by HPLC-UV or by LC-MSMS. Therefore, death in siblings' and fasting duration if they will be present in the same time, Tyr levels will be affected. However, the effect of death in siblings' factor has more pronounced. We conclude that patient fasting is common factor which influence both Phe and Tyr concentrations, and it is essential to avoid false positive cases and maintain a correct results.

For other tested factors, ANOVA p-values >0,05 therefore, their models were not valid.

Table VI.54. Multivariate analysis of different factors effect on analyte concentrations

Variable X	Variable Y	ANOVA p-value	Intercept
Family history	Phe-HPLC-UV	0,001	0,173
	Phe-LC-MSMS	0,001	0,173
Fasting duration	Phe-HPLC-UV	0,001	-0,158
	Phe-LC-MSMS	0,001	-0,162
	Tyr-HPLC-UV	0,013	-0,123
	Tyr-LC-MSMS	0,013	-0,123

Variable X	Variable Y	ANOVA p-value	Intercept
Consanguinity 3	Phe-HPLC-UV	0,009	0,133
	Phe-LC-MSMS	0,008	0,134
Death in sibling	Tyr-HPLC-UV	0,000	0,248
	Tyr-LC-MSMS	0,000	0,253

VI.5.2. Clinical application

VI.5.2.1. Result of clinical application

The validated methods were utilized to analyze Phe and Tyr concentrations in the blood samples of 442 children. The results of for 442 children obtained by HPLC-UV method and by LC-MSMS method were compared.

Incorporating quality control tests involving standard solutions prepared in relevant matrices before each analysis is a best practice that enhances the reliability and validity of analytical results. This proactive approach not only ensures compliance with regulatory standards but also reinforces confidence in the data produced, ultimately supporting product safety and efficacy.

The LOQs were apparently lower than Phe or Tyr physiological levels, therefore samples could be diluted before pretreatment.

A study of Peng M and collaborators, identifies the age-related reference intervals for plasma AAs. A slight difference was observed between different samples (Peng *et al.*, 2019). Therefore, in our study we used the refence interval close to that's of Mustapha BACHA hospital (Algeria) to avoid any difference or errors in the results.

Normality test by Kolmogorov-Smirnova and Shapiro-Wilk was applicated and indicate non normal distribution of data with p-values < 0,05, therefore we use Spearmann's non-parametric correlation. Values closer to – 1 and + 1 designate stronger relationships while values closer to 0 indicate weaker relationships (Zar, 2005).

r values were 0, 984 for Phe and 0,985 for Tyr when using Spearmann's non-parametric correlation. Consequently, and the correlation is significant with a p-value < 0.01.

The Bland-Altman plot (**Figure VI.34**) illustrates that the average difference between measurements obtained from the HPLC- UV and the LC-MSMS method is nearly zero (mean = 0.17 for Phe and 0,04 for Tyr). This indicates a strong consistency between the two techniques. The dispersion of differences in the data is unbiased, with most values clustered around the mean.

As indicated by p-value > 0.05, there is no statistical difference between the differences and 0 at 5% risk for the tested methods. Consequently, both developed methods present a viable option for Phe and Tyr analysis in clinical laboratories.

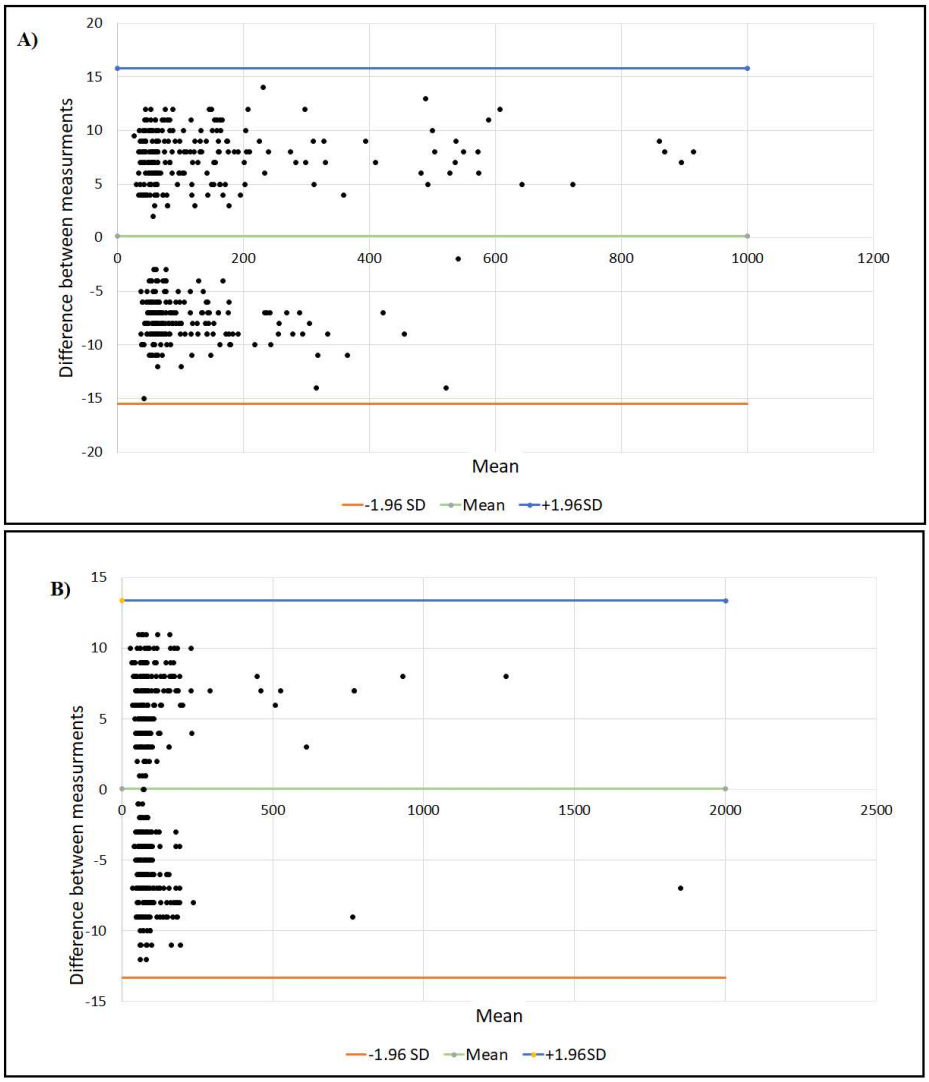

Figure VI.34. Bland-Altman plot to evaluate results determined by the LC-MSMS method and HPLC-UV for (A) phenylalanine and (B) tyrosine measurements in 442 blood samples

For PKU diagnosis, the Phe/Tyr ratio was calculated by means of the values obtained from the developed methods (HPLC and LC-MSMS). When only Phe screening was employed, the percentage of presumptive positives was 11 %, indicating that 45 children had Phe concentrations above 200 µmol/L when either using HPLC-UV or LC-MSMS method.

However, when the Phe/Tyr ratio was utilized, the rate of recalls was reduced to 5,7% (23 children). 15 cases exhibited a Phe concentration above 200 µmol/L and a Phe/Tyr ratio above 2, resulting in a positive outcome and recall for confirmation.

Another 8 cases had a Phe concentration above 400 µmol/L but a Phe/Tyr ratio below 2, making the decision uncertain and requiring a recall. 22 cases identified through Phe-only screening were a false positive, displaying a Phe concentration above 200 µmol/L but a normal Phe/Tyr ratio, leading to a negative determination.

All other cases yielded negative results, as they exhibited both normal Phe concentrations and Phe/Tyr ratios. Regarding the recalled children, 15 children were confirmed as positive for PKU with frequency of 3,77% in the tested population and necessitated further confirmatory tests. The eight undecided children, however, were determined to be negative, and it is suspected that they had not fasted for a sufficient period of time. These results affirm the utility of the Phe/Tyr ratio in PKU diagnosis.

Moreover, calculating the Phe/Tyr ratio can serve as a valuable tool in diagnosing PKU and TRS, effectively reducing the likelihood of false positive results.

For TRS diagnosis, 15 children had high level of Tyr. All of them were recalled for a second sampling and analysis. After recall, only 4 perform a second analysis and they still exhibit a high level of Tyr (above 226 µmol/L). Thus, they were advanced to further confirmatory tests and the frequency of TRS among the tested population was 1,01%.

VI.5.2.2. Comparison of developed methods and reference method

The developed methods were compared to a reference LC-MSMS method for 75 samples.

Normality test by Kolmogorov-Smirnova and Shapiro-Wilk tests was applicated and indicate non normal distribution of data with p-values < 0,05, therefore we use Spearmann's non-parametric correlation. As seen in the previous section, values closer to – 1 and + 1 designate stronger relationships while values closer to 0 indicate weaker relationships (Zar, 2005). R values were 0, 878 and 0,979 for Phe and Tyr, respectively when comparing HPLC-UV method with reference method and they were 0,995 and 0,996 for Phe and Tyr respectively when comparing LC-MSMS method with reference method.

The Bland-Altman plot (**Figure VI.35**) reveals that the mean difference between measurements from the developed methods and the reference method is approximately zero (-0,08 and 0,77 for Phe and Tyr, respectively when comparing HPLC-UV method with reference method and 0,23 and 0,11 for Phe and Tyr, respectively when comparing LC-MSMS method with reference method). This close agreement suggests that the two techniques are highly consistent with the reference method. Besides, the differences in data dispersion are unbiased, with the majority of values distributed evenly around the mean. Consequently, the both developed methods emerge as a practical alternative for Phe and Tyr analysis in clinical settings.

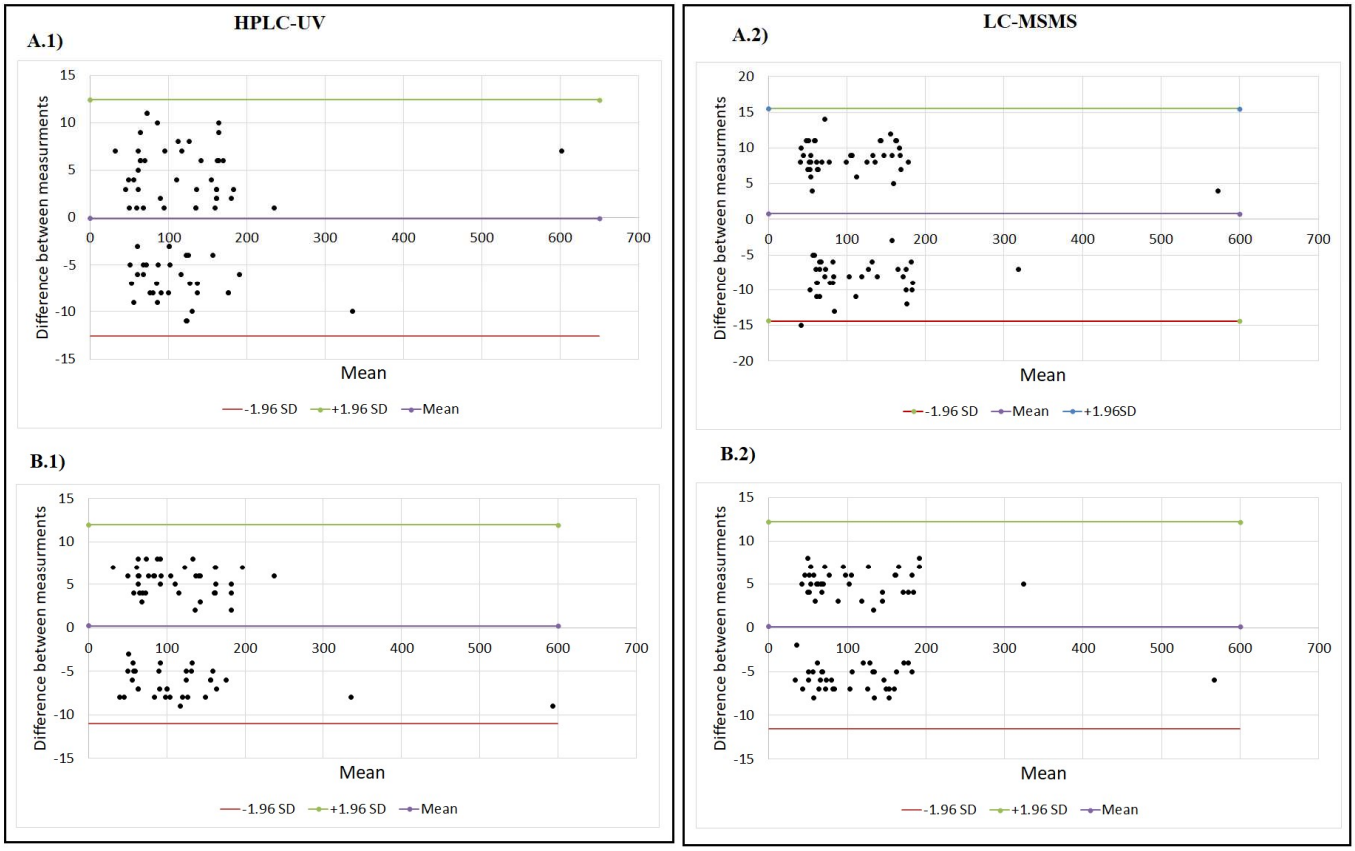

Figure VI.35. Bland-Altman plot to evaluate results for phenylalanine determined by (A.1) LC-MSMS, by (A.2) HPLC-UV and for tyrosine determined by (B.1) LC-MSMS, by (B.2) HPLC-UV; all compared to reference method in 75 samples

GENERAL CONCLUSION

General conclusion

We have developed and implemented three analytical methods for simultaneous quantitative analysis of Phe and Tyr for the diagnosis of monitoring of patients with PKU or TRS. HPLC-UV method will be devoted to determination of Phe and Tyr on blood serum samples whereas LC-MSMS will be dedicated to Phe and Tyr analysis in both blood serum samples and DBS samples.

Development and optimization of HPLC-UV method was in the first line of this work. Statistical tools and the DOE concept enabled finding the critical factors that affected the method and their optimization, which finally led to the exact conditions required for the desired response. Validation of the method following SFSTP guidelines, demonstrated its linearity, selectivity, trueness, precision, and high sensitivity. The developed HPLC-UV method can be used for routine analysis and patient monitoring. In addition, the developed LC-MSMS method for both analyzing blood serum and DBS was linear and exhibited selectivity, trueness, precision, and high sensitivity. Consequently, it can be used for screening or diagnosis of either PKU or TRS. The method validation criteria have highlighted the robustness and reliability of the technique either by HPLC-UV or by LC-MSMS.

The epidemiologic study demonstrates that the PKU and TRS are increasingly recognized among Algerian children and this may be explicated by the high rate of consanguinity. It reveals also the influence of various factors especially consanguinity, fasting duration, age of symptom onset, age of support and some associated complications on Phe and Tyr concentrations in blood samples.

The biomedical application of the developed methods reveal that the developed methods offer a valuable tool for PKU of TRS diagnosis or monitoring and represent an alternative for suspected or affected individuals.

Comprehensive analysis of Phe and Tyr in blood has the potential of yielding information on the pathophysiological status of affected children. The present study opens the access to prevention and reduction of health costs by early detection of PKU ad TRS which are rapidly progressive and cause irreversible damage and therefore, reduction of long-term health expenses. It offers also an improvement of the quality of life of patients and their families and lead to development of local expertise in diagnostic technology.

Skilled health care during pregnancy, neonatal and postnatal period prevents complications for mother and newborn, and allows for early detection and management of such problems.

The proposed study has a great impact in the scientific and socio-economical domains and it can resolve a public health problem. By this study, we can establish a valuable precautionary medicine and enable diagnosis and treatment before the onset of symptoms and on the other hand improve another research in the same field.

Finally, an agreement with private laboratory as part of service provision was very supportive in samples collection, conservation and transport and in standards and reagents acquisition. However, there are some points which need more exploration, the first one is the necessity of establishing age reference interval to correctly interpret the results. Secondly, for confirmation of TRS type, the analysis of SUA is crucial and should be taken into account. In addition, national program of systematic neonatal screening is of a great importance and represent a public health emergency to avoid consequences of such IEM. Moreover, biomedical application of the developed LC-MSMS on DBS samples is crucial and allows both specific and rapid management of the newborn. We suggest that the implement of private clinics and laboratories in such studies will advance the research in this field and improve the quality of results.

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APPENDIX

Appendix I (Clinical and biological information sheet)

PATIENT

NOM : …… **Prénom :** ……
Date de naissance : …… Hôpital : ……
Service : …… Médecin : ……..
Téléphone : …… Mail : ……..……..……..

CONTEXTE DE LA DEMANDE

- ☐ Suivi de maladie héréditaire du métabolisme connue : ……
- ☐ ATCD familial de maladie héréditaire du métabolisme : ……
- ☐ Diagnostic suspecté :……
- ☐ Contrôle de profil métabolique anormal

ANTECEDENTS

- ☐ Décès dans la fratrie
- ☐ Consanguinité familiale
- ☐ Prématurité

DEBUT DES SYMPTOMES

- ☐ Dès la naissance
- ☐ En période néonatale
- ☐ Plus tard (préciser) :……

CIRCONSTANCES DU PRELEVEMENT

- ☐ Patient à jeun : dernier repas à ……..

COMPLICATIONS

- ☐ Retard mental
- ☐ Retard de croissance
- ☐ Autisme
- ☐ Autres (préciser)
- ☐ Pas de complication

THERAPEUTIQUE EN COURS

……
……

MALADIE METABOLIQUE SUSPECTEE / BUT DE LA DEMANDE

……
……

Appendix II (Ficher Snedecor's table)

The table gives the value x such that P(F ≥ x) = 0.05 as a function of the numbers of degrees of freedom νA and νB

ν_B \ ν_A	1	2	3	4	5	6	7	8	9
1	161,446	199,499	215,707	224,583	230,160	233,988	236,767	238,884	240,543
2	18,513	19,000	19,164	19,247	19,296	19,329	19,353	19,371	19,385
3	10,128	9,552	9,277	9,117	9,013	8,941	8,887	8,845	8,812
4	7,709	6,944	6,591	6,388	6,256	6,163	6,094	6,041	5,999
5	6,608	5,786	5,409	5,192	5,050	4,950	4,876	4,818	4,772
6	5,987	5,143	4,757	4,534	4,387	4,284	4,207	4,147	4,099
7	5,591	4,737	4,347	4,120	3,972	3,866	3,787	3,726	3,677
8	5,318	4,459	4,066	3,838	3,688	3,581	3,500	3,438	3,388
9	5,117	4,256	3,863	3,633	3,482	3,374	3,293	3,230	3,179
10	4,965	4,103	3,708	3,478	3,326	3,217	3,135	3,072	3,020
11	4,844	3,982	3,587	3,357	3,204	3,095	3,012	2,948	2,896
12	4,747	3,885	3,490	3,259	3,106	2,996	2,913	2,849	2,796
13	4,667	3,806	3,411	3,179	3,025	2,915	2,832	2,767	2,714
14	4,600	3,739	3,344	3,112	2,958	2,848	2,764	2,699	2,646
15	4,543	3,682	3,287	3,056	2,901	2,790	2,707	2,641	2,588
16	4,494	3,634	3,239	3,007	2,852	2,741	2,657	2,591	2,538
17	4,451	3,592	3,197	2,965	2,810	2,699	2,614	2,548	2,494
18	4,414	3,555	3,160	2,928	2,773	2,661	2,577	2,510	2,456
19	4,381	3,522	3,127	2,895	2,740	2,628	2,544	2,477	2,423
20	4,351	3,493	3,098	2,866	2,711	2,599	2,514	2,447	2,393
21	4,325	3,467	3,072	2,840	2,685	2,573	2,488	2,420	2,366
22	4,301	3,443	3,049	2,817	2,661	2,549	2,464	2,397	2,342
23	4,279	3,422	3,028	2,796	2,640	2,528	2,442	2,375	2,320
24	4,260	3,403	3,009	2,776	2,621	2,508	2,423	2,355	2,300
25	4,242	3,385	2,991	2,759	2,603	2,490	2,405	2,337	2,282
26	4,225	3,369	2,975	2,743	2,587	2,474	2,388	2,321	2,265
27	4,210	3,354	2,960	2,728	2,572	2,459	2,373	2,305	2,250
28	4,196	3,340	2,947	2,714	2,558	2,445	2,359	2,291	2,236
29	4,183	3,328	2,934	2,701	2,545	2,432	2,346	2,278	2,223
30	4,171	3,316	2,922	2,690	2,534	2,421	2,334	2,266	2,211
40	4,085	3,232	2,839	2,606	2,449	2,336	2,249	2,180	2,124
60	4,001	3,150	2,758	2,525	2,368	2,254	2,167	2,097	2,040
120	3,920	3,072	2,680	2,447	2,290	2,175	2,087	2,016	1,959
∞	3,842	2,997	2,606	2,373	2,215	2,099	2,011	1,939	1,881

Ficher Snedecor's table (Continued)

ν_B \ ν_A	10	12	15	20	24	30	40	60	120	∞
1	241,882	243,905	245,949	248,016	249,052	250,096	251,144	252,196	253,254	254,313
2	19,396	19,412	19,429	19,446	19,454	19,463	19,471	19,479	19,487	19,496
3	8,785	8,745	8,703	8,660	8,638	8,617	8,594	8,572	8,549	8,527
4	5,964	5,912	5,858	5,803	5,774	5,746	5,717	5,688	5,658	5,628
5	4,735	4,678	4,619	4,558	4,527	4,496	4,464	4,431	4,398	4,365
6	4,060	4,000	3,938	3,874	3,841	3,808	3,774	3,740	3,705	3,669
7	3,637	3,575	3,511	3,445	3,410	3,376	3,340	3,304	3,267	3,230
8	3,347	3,284	3,218	3,150	3,115	3,079	3,043	3,005	2,967	2,928
9	3,137	3,073	3,006	2,936	2,900	2,864	2,826	2,787	2,748	2,707
10	2,978	2,913	2,845	2,774	2,737	2,700	2,661	2,621	2,580	2,538
11	2,854	2,788	2,719	2,646	2,609	2,570	2,531	2,490	2,448	2,405
12	2,753	2,687	2,617	2,544	2,505	2,466	2,426	2,384	2,341	2,296
13	2,671	2,604	2,533	2,459	2,420	2,380	2,339	2,297	2,252	2,206
14	2,602	2,534	2,463	2,388	2,349	2,308	2,266	2,223	2,178	2,131
15	2,544	2,475	2,403	2,328	2,288	2,247	2,204	2,160	2,114	2,066
16	2,494	2,425	2,352	2,276	2,235	2,194	2,151	2,106	2,059	2,010
17	2,450	2,381	2,308	2,230	2,190	2,148	2,104	2,058	2,011	1,960
18	2,412	2,342	2,269	2,191	2,150	2,107	2,063	2,017	1,968	1,917
19	2,378	2,308	2,234	2,155	2,114	2,071	2,026	1,980	1,930	1,878
20	2,348	2,278	2,203	2,124	2,082	2,039	1,994	1,946	1,896	1,843
21	2,321	2,250	2,176	2,096	2,054	2,010	1,965	1,916	1,866	1,812
22	2,297	2,226	2,151	2,071	2,028	1,984	1,938	1,889	1,838	1,783
23	2,275	2,204	2,128	2,048	2,005	1,961	1,914	1,865	1,813	1,757
24	2,255	2,183	2,108	2,027	1,984	1,939	1,892	1,842	1,790	1,733
25	2,236	2,165	2,089	2,007	1,964	1,919	1,872	1,822	1,768	1,711
26	2,220	2,148	2,072	1,990	1,946	1,901	1,853	1,803	1,749	1,691
27	2,204	2,132	2,056	1,974	1,930	1,884	1,836	1,785	1,731	1,672
28	2,190	2,118	2,041	1,959	1,915	1,869	1,820	1,769	1,714	1,654
29	2,177	2,104	2,027	1,945	1,901	1,854	1,806	1,754	1,698	1,638
30	2,165	2,092	2,015	1,932	1,887	1,841	1,792	1,740	1,683	1,622
40	2,077	2,003	1,924	1,839	1,793	1,744	1,693	1,637	1,577	1,509
60	1,993	1,917	1,836	1,748	1,700	1,649	1,594	1,534	1,467	1,389
120	1,910	1,834	1,750	1,659	1,608	1,554	1,495	1,429	1,352	1,254
∞	1,832	1,753	1,667	1,572	1,518	1,460	1,395	1,319	1,223	1,025

Appendix III (Student's table)

Critical values of *t* for two-tailed tests

Significance level (α)

Degrees of freedom *(df)*	.2	.15	.1	.05	.025	.01	.005	.001
1	3.078	4.165	6.314	12.706	25.452	63.657	127.321	636.619
2	1.886	2.282	2.920	4.303	6.205	9.925	14.089	31.599
3	1.638	1.924	2.353	3.182	4.177	5.841	7.453	12.924
4	1.533	1.778	2.132	2.776	3.495	4.604	5.598	8.610
5	1.476	1.699	2.015	2.571	3.163	4.032	4.773	6.869
6	1.440	1.650	1.943	2.447	2.969	3.707	4.317	5.959
7	1.415	1.617	1.895	2.365	2.841	3.499	4.029	5.408
8	1.397	1.592	1.860	2.306	2.752	3.355	3.833	5.041
9	1.383	1.574	1.833	2.262	2.685	3.250	3.690	4.781
10	1.372	1.559	1.812	2.228	2.634	3.169	3.581	4.587
11	1.363	1.548	1.796	2.201	2.593	3.106	3.497	4.437
12	1.356	1.538	1.782	2.179	2.560	3.055	3.428	4.318
13	1.350	1.530	1.771	2.160	2.533	3.012	3.372	4.221
14	1.345	1.523	1.761	2.145	2.510	2.977	3.326	4.140
15	1.341	1.517	1.753	2.131	2.490	2.947	3.286	4.073
16	1.337	1.512	1.746	2.120	2.473	2.921	3.252	4.015
17	1.333	1.508	1.740	2.110	2.458	2.898	3.222	3.965
18	1.330	1.504	1.734	2.101	2.445	2.878	3.197	3.922
19	1.328	1.500	1.729	2.093	2.433	2.861	3.174	3.883
20	1.325	1.497	1.725	2.086	2.423	2.845	3.153	3.850
21	1.323	1.494	1.721	2.080	2.414	2.831	3.135	3.819
22	1.321	1.492	1.717	2.074	2.405	2.819	3.119	3.792
23	1.319	1.489	1.714	2.069	2.398	2.807	3.104	3.768
24	1.318	1.487	1.711	2.064	2.391	2.797	3.091	3.745
25	1.316	1.485	1.708	2.060	2.385	2.787	3.078	3.725
26	1.315	1.483	1.706	2.056	2.379	2.779	3.067	3.707
27	1.314	1.482	1.703	2.052	2.373	2.771	3.057	3.690
28	1.313	1.480	1.701	2.048	2.368	2.763	3.047	3.674
29	1.311	1.479	1.699	2.045	2.364	2.756	3.038	3.659
30	1.310	1.477	1.697	2.042	2.360	2.750	3.030	3.646
40	1.303	1.468	1.684	2.021	2.329	2.704	2.971	3.551
50	1.299	1.462	1.676	2.009	2.311	2.678	2.937	3.496
60	1.296	1.458	1.671	2.000	2.299	2.660	2.915	3.460
70	1.294	1.456	1.667	1.994	2.291	2.648	2.899	3.435
80	1.292	1.453	1.664	1.990	2.284	2.639	2.887	3.416
100	1.290	1.451	1.660	1.984	2.276	2.626	2.871	3.390
1000	1.282	1.441	1.646	1.962	2.245	2.581	2.813	3.300
Infinite	1.282	1.440	1.645	1.960	2.241	2.576	2.807	3.291

Critical values of *t* for one-tailed tests

Significance level (α)

Degrees of freedom *(df)*	.2	.15	.1	.05	.025	.01	.005	.001
1	1.376	1.963	3.078	6.314	12.706	31.821	63.657	318.309
2	1.061	1.386	1.886	2.920	4.303	6.965	9.925	22.327
3	0.978	1.250	1.638	2.353	3.182	4.541	5.841	10.215
4	0.941	1.190	1.533	2.132	2.776	3.747	4.604	7.173
5	0.920	1.156	1.476	2.015	2.571	3.365	4.032	5.893
6	0.906	1.134	1.440	1.943	2.447	3.143	3.707	5.208
7	0.896	1.119	1.415	1.895	2.365	2.998	3.499	4.785
8	0.889	1.108	1.397	1.860	2.306	2.896	3.355	4.501
9	0.883	1.100	1.383	1.833	2.262	2.821	3.250	4.297
10	0.879	1.093	1.372	1.812	2.228	2.764	3.169	4.144
11	0.876	1.088	1.363	1.796	2.201	2.718	3.106	4.025
12	0.873	1.083	1.356	1.782	2.179	2.681	3.055	3.930
13	0.870	1.079	1.350	1.771	2.160	2.650	3.012	3.852
14	0.868	1.076	1.345	1.761	2.145	2.624	2.977	3.787
15	0.866	1.074	1.341	1.753	2.131	2.602	2.947	3.733
16	0.865	1.071	1.337	1.746	2.120	2.583	2.921	3.686
17	0.863	1.069	1.333	1.740	2.110	2.567	2.898	3.646
18	0.862	1.067	1.330	1.734	2.101	2.552	2.878	3.610
19	0.861	1.066	1.328	1.729	2.093	2.539	2.861	3.579
20	0.860	1.064	1.325	1.725	2.086	2.528	2.845	3.552
21	0.859	1.063	1.323	1.721	2.080	2.518	2.831	3.527
22	0.858	1.061	1.321	1.717	2.074	2.508	2.819	3.505
23	0.858	1.060	1.319	1.714	2.069	2.500	2.807	3.485
24	0.857	1.059	1.318	1.711	2.064	2.492	2.797	3.467
25	0.856	1.058	1.316	1.708	2.060	2.485	2.787	3.450
26	0.856	1.058	1.315	1.706	2.056	2.479	2.779	3.435
27	0.855	1.057	1.314	1.703	2.052	2.473	2.771	3.421
28	0.855	1.056	1.313	1.701	2.048	2.467	2.763	3.408
29	0.854	1.055	1.311	1.699	2.045	2.462	2.756	3.396
30	0.854	1.055	1.310	1.697	2.042	2.457	2.750	3.385
40	0.851	1.050	1.303	1.684	2.021	2.423	2.704	3.307
50	0.849	1.047	1.299	1.676	2.009	2.403	2.678	3.261
60	0.848	1.045	1.296	1.671	2.000	2.390	2.660	3.232
70	0.847	1.044	1.294	1.667	1.994	2.381	2.648	3.211
80	0.846	1.043	1.292	1.664	1.990	2.374	2.639	3.195
100	0.845	1.042	1.290	1.660	1.984	2.364	2.626	3.174
1000	0.842	1.037	1.282	1.646	1.962	2.330	2.581	3.098
Infinite	0.842	1.036	1.282	1.645	1.960	2.326	2.576	3.090

Appendix IV (Scientific Production)

Research articles

- **Hadjira Rabti**, Mounira Amrane, Abdeldjalil Lalaouna, Abdenacer Flilissa, Yacine Benguerba; *Optimization and validation of a bioanalytical HPLC–UV technique for simultaneous determination of underivatized phenylalanine and tyrosine in the blood for phenylketonuria diagnosis and monitoring* (Biomedical chromatography journal, 2023)

International communications

- **Rabti.H**, Amrane.M, Touabti.A, Bioud.B, Bouaoune.N; *Study of GC/MS technique for Newborn screening of organic acid disorders* (IWCE 2017)
- **Rabti H**, Amrane M, Boutrid N, Rahmoune H, Bioud B, Touabti A *; Diagnostic et suivi des phenycétonuries pour la prévention des atteintes neurologiques* (Journées d'hiver 2021 Tunis)

National communications

- **Rabti H**, Amrane M, Lalaouna A, Flilissa A; *Optimization and validation of a bioanalytical HPLC-UV technique for simultaneous determination of phenylalanine and tyrosine in the blood for phenylketonuria diagnosis and monitoring* (SAGBM, Algiers 2024)
- **Rabti H**, Lalaouna A, Flilissa A, Amrane M, Boutrid N, Rahmoune H, Touabti A ; *Rapport phénylalanine/tyrosine dans le diagnostic et le suivi des phénylcétonuries* (SAGBM, Oran 2022).
- **Rabti H**, Lalaouna A, Flilissa A, Amrane M, Boutrid N, Rahmoune H, Touabti A., *Diagnostic et suivi des tyrosinémies pour la prévention des atteintes hépato-rénales* (SAGBM, Oran 2022)

Abstract

The aim of this study is to develop and validate fast, accurate, and precise, high-performance liquid-chromatographic with UV detection (HPLC-UV) and liquid chromatography tandem mass spectrometry (LC-MSMS) methods for phenylketonuria (PKU) and tyrosinemia (TRS) diagnosis and monitoring. Three methods were developed, HPLC-UV on blood serum, LC-MSMS on blood serum and LC-MSMS on dry blood spots (DBS). Chromatographic conditions in the HPLC-UV technique were optimized using central composite experimental design (CCD). Validation was accomplished using β-expectation tolerance intervals (β-ETI) for total error measurement that did not exceed 15%. The method applicability was determined using human serum from 442 volunteers and statistical study was conducted to investigate the effect of different factors in phenylalanine (Phe) and tyrosine (Tyr) levels in addition to comparative study with reference method. Optimal settings for HPLC-UV method were determined. The accuracy profiles were established. Mean analytical bias in spiking levels was acceptable, with relative standards deviation (RSD) below 5% in almost instances. The limit of detection (LOD) and limit of quantification (LOQ) were satisfactory in all methods. The epidemiologic statistical study reveals the most influencing factors on Phe and Tyr concentrations in the blood serum. The suggested approach successfully analyzed Phe and Tyr in human blood samples either using HPLC-UV or LC-MSMS and presented no significant difference with the reference method. The developed methods offer valuable diagnosis and monitoring tool for PKU and TRS.

Keywords: Phenylketonuria, Tyrosinemia, Human blood samples, DBS, HPLC-UV, LC-MSMS, CCD, Epidemiologic study.

Résumé

L'objectif de cette étude est de développer et valider des méthodes rapides, exactes et précises de chromatographie liquide à haute performance avec détection UV (HPLC-UV) et de chromatographie liquide en tandem avec spectrométrie de masse (LC-MSMS) pour le diagnostic et la surveillance de la phénylcétonurie (PKU) et de la tyrosinémie (TRS). Trois méthodes ont été développées, HPLC-UV sur sérum sanguin, LC-MSMS sur sérum sanguin et LC-MSMS sur taches de sang séchées (DBS). Les conditions chromatographiques dans la technique HPLC-UV ont été optimisées à l'aide d'un plan d'expérience composite central (CCD). La validation a été réalisée à l'aide d'intervalles de tolérance d'attente β (β-ETI) pour une mesure d'erreur totale ne dépassant pas 15 %. L'applicabilité de la méthode a été déterminée à l'aide de sérum humain de 442 volontaires et une étude statistique a été menée pour étudier l'effet de différents facteurs sur les niveaux de phénylalanine (Phe) et de tyrosine (Tyr) en même temps qu'une étude comparative avec la méthode de référence. Les paramètres optimaux pour la méthode HPLC-UV ont été déterminés. Les profils d'exactitude ont été établis. Le biais analytique moyen dans les niveaux de concentration était acceptable, avec un écart type relatif (RSD) inférieur à 5 % dans la plupart des cas. La limite de détection (LOD) et la limite de quantification (LOQ) étaient satisfaisantes dans toutes les méthodes. L'étude statistique épidémiologique révèle les facteurs les plus influents sur les concentrations de Phe et Tyr dans le sérum sanguin. L'approche suggérée a permis d'analyser avec succès Phe et Tyr dans des échantillons de sang humain en utilisant soit HPLC-UV soit LC-MSMS et n'a présenté aucune différence significative avec la méthode de référence. Les méthodes développées offrent un outil précieux de diagnostic et de surveillance pour la PKU et la TRS.

Mots clés : Phénylcétonurie, Tyrosinémie, Échantillons de sang humain, Taches de sang séchées, HPLC-UV, LC-MSMS, CCD, Étude épidémiologique.

ملخص

الهدف من هذه الدراسة هو تطوير طرق سريعة، دقيقة وعالية الأداء للكروماتوغرافيا السائلة (HPLC-UV) و(LC-MSMS) لتشخيص ومراقبة الفينيل كيتونوريا (PKU) والتيروزينيميا (TRS). تم تطوير ثلاث طرق، HPLC-UV على مصل الدم، وLC-MSMS على مصل الدم وLC-MSMS على بقع الدم الجافة (DBS). تم تحسين الظروف الكروماتوغرافية في تقنية HPLC-UV باستخدام التصميم التجريبي المركزي المركب (CCD). تم التحقق من صحة كل الطرق باستخدام فترات تحمل التوقع β (β-ETI) لقياس الخطأ الكلي الذي لم يتجاوز 15٪. تم تحديد قابلية تطبيق الطريقة باستخدام مصل بشري من 442 متطوعًا وأجريت دراسة إحصائية للتحقيق في تأثير العوامل المختلفة في مستويات الفينيل ألانين (Phe) والتيروزين (Tyr) بالإضافة الى المقارنة مع الطريقة المرجعية. تم تحديد الإعدادات المثلى لطريقة HPLC-UV. تم تحديد ملفات الدقة. كان الانحراف المعياري النسبي (RSD) أقل من 5٪ في معظم الحالات وكانت مستويات LOD وLOQجيدة. تكشف الدراسة الإحصائية الوبائية عن العوامل الأكثر تأثيرًا على تركيزات Phe وTyr في مصل الدم. نجح النهج المقترح في تحليل Phe وTyr في عينات الدم البشرية إما باستخدام HPLC-UV أو LC-MSMS ولم يظهر أي فرق كبير مع الطريقة المرجعية. تقدم الطرق المطورة أداة تشخيص ومراقبة قيمة لـ PKU وTRS.

الكلمات المفتاحية: الفينيل كيتونيوريا، التيروزينيميا، عينات دم بشرية، بقع الدم الجافة، HPLC-UV، LC-MSMS، CCD، دراسة وبائية.