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ANALYTICAL METHODS DEVELOPMENT FOR THE DIAGNOSIS OF INBORN ERRORS OF METABOLISM

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Riassunto

La metabolomica è definita come lo studio del metaboloma ovvero del complesso sistema di metaboliti che, essendo gli intermedi delle reazioni biochimiche, giocano un ruolo importante nella connessione tra i differenti pathways che operano in una cellula vivente.
I metaboliti possono essere visti come il prodotto finale dell'espressione genica o dell'attività proteica (enzimi), che definiscono così il fenotipo biochimico di un sistema biologico nel suo insieme, compreso l'uomo. Mentre la genomica e la proteomica suggeriscono un possibile modo di funzionamento del sistema, la metabolomica dà la rappresentazione reale del sistema.
Similmente a trascrittoma e proteoma, il metaboloma è contesto dipendente e i livelli di ogni metabolita dipendono dallo stato fisiologico, patologico e di sviluppo di una cellula, tessuto o organismo. A differenza, invece, di quanto accade per m-RNA e proteine, è difficile o addirittura impossibile stabilire un link diretto tra geni e metaboliti. La natura convoluta del metabolismo cellulare, dove lo stesso metabolita può partecipare in molti differenti pathways, complica l'interpretazione dei dati metabolici.
Le analisi di genoma, trascrittoma e proteoma sono basate ognuna sul monitoraggio di molecole con simili proprietà chimiche: 4 differenti nucleotidi per genoma e trascrittoma, oppure 22 amminoacidi per il proteoma. Questo facilita lo sviluppo di approcci analitici "high-throughput". Nel caso del metaboloma, invece, esiste una ampia variabilità in termini di struttura chimica e proprietà dei suoi componenti. Difatti, il metaboloma consiste di composti estremamente diversi dal punto di vista chimico che vanno da specie ioniche inorganiche a carboidrati idrofilici, alcoli volatili e chetoni, acidi organici, lipidi idrofobici e prodotti naturali complessi. Questa complessità fa sì che sia impossibile determinare simultaneamente il metaboloma. Pertanto, per ottenere più informazioni possibili riguardanti il metaboloma, è necessario l'ausilio di efficienti preparazioni del campione e tecniche di estrazione selettiva combinate a differenti tecniche analitiche.
Un approccio analitico importante nel campo della metabolomica è rappresentato dall'uso di tecniche di analisi quantitativa come la spettrometria di massa.
I più importanti vantaggi delle spettrometria di massa sono l'alta sensibilità, selettività ed "high-throughput". Inoltre, la sua possibile combinazione con tecniche di separazione, come la cromatografia, consente di migliorare la capacità di analizzare campioni biologici altamente complessi. Generalmente, le applicazioni di spettrometria di massa riguardano l'analisi di piccoli metaboliti come acidi organici, amminoacidi, acidi grassi, steroidi ed i loro coniugati; peptidi, proteine e glicoproteine e oligonucleotidi derivati da biopolimeri (DNA, RNA).
L'analisi del metaboloma ha assunto negli ultimi anni un ruolo clinico importante nell'identificazione di "disordini" nel profilo metabolico di un paziente. Difatti, la valutazione qualitativa e quantitativa, contemporanea ed in funzione del tempo, di un consistente numero di metaboliti, come quelli determinabili mediante spettrometria di massa in fluidi biologici è in grado di fornire, con una accettabile probabilità, la descrizione dello stato biochimico attuale di un organismo, fornendo informazioni sulle interrelazioni tra i diversi processi metabolici che definiscono lo stato. Si possono così determinare attraverso lo studio dei fluidi biologici (come plasma, urine, bile e liquido cefalo-rachidiano) nuovi criteri che definiscono lo stato di salute e lo stato di malattia sulla base di una valutazione integrata della varianza dei livelli dei

metaboliti e dei parametri metabolici sistemici, permettendo di definire lo stato fisio-patologico in termini sistemici o organo specifici. In tale modo più che prendere in considerazione uno o pochi metaboliti con i relativi processi metabolici, la metabolomica esamina l'intero profilo metabolico determinato dall'interconnessione dei diversi processi.
A tale scopo la Spettrometria di Massa, attraverso l'utilizzo di metodi di analisi matematiche multivariate, rappresenta uno dei mezzi più potenti e più utilizzati per l'analisi del metaboloma, permettendo insieme alla genomica funzionale ed alla proteomica di aprire nuove strade conoscitive e applicative nel campo della medicina ed in particolare nella diagnosi e nel follow-up degli errori congeniti del metabolismo (IEMs).
Si definiscono "errori congeniti del metabolismo" disordini genetici causati da alterazioni di una specifica reazione chimica nel metabolismo.
Attualmente si conoscono circa 400 difetti geneticamente determinati e correlati a patologia; singolarmente rari, se considerati nel loro insieme, essi hanno un'incidenza relativamente alta. Al giorno d'oggi si ritiene, infatti, che circa 1 neonato ogni 200 ne sia affetto.
La patogenesi di un errore congenito del metabolismo può essere generalmente attribuita alla mancanza o perdita di funzione di una proteina mutante, con funzione di enzima o di trasporto. La base genetica degli IEMs è estremamente eterogenea e può includere alcuni tipi di difetti genetici: una o più mutazioni puntiformi, delezioni o inserzioni o riarrangiamenti genomici che possono essere a carico di sequenze codificanti o di regolazione. La malattia è generalmente associata al flusso metabolico alterato riguardante il "pathway" che è regolato dalla proteina mutante.
Originariamente la diagnosi degli IEMs veniva fatta attraverso il riconoscimento di anormalità in individui che si ammalavano gravemente o che avevano caratteristiche dismorfiche o ritardo mentale. Tali disturbi venivano classificati come "congeniti" dall'osservazione della ricorrenza nei membri della stessa famiglia. Con l'identificazione di specifici enzimi e pathways metabolici, la comprensione dei processi fisici alla base degli IEMs si è evoluta. Questa conoscenza è stata accompagnata dalla consapevolezza della possibilità di errori a vari livelli nei diversi pathways metabolici.
In particolare il lavoro di tesi del progetto di dottorato si è articolato nei seguenti punti:

1. sviluppo di un metodo "semi-quantitativo" di spettrometria di massa tandem per lo screening neonatale degli errori congeniti del metabolismo;
2. sviluppo e validazione di un metodo rapido di analisi "quantitativa" per il follow-up di pazienti affetti da Fenilchetonuria.

SVILUPPO DI UN METODO "SEMI-QUANTITATIVO" DI SPETTROMETRIA DI MASSA TANDEM PER LO SCREENING NEONATALE DEGLI ERRORI CONGENITI DEL METABOLISMO

L'interesse per lo screening neonatale di massa si è andato progressivamente estendendo in tutto il mondo da quando Robert Guthrie nel 1963 descrisse il metodo per lo screening della Fenilchetonuria (disordine del metabolismo della fenilalanina) mediante l'utilizzo del saggio enzimatico di inibizione della crescita batterica che da lui ha poi preso il nome.

La storia dello screening neonatale delle malattie metaboliche ereditarie ha visto una continua e positiva modificazione dei limiti legati allo screening fino a sovrapporsi ad esame confirmatorio, grazie all'introduzione di nuove tecnologie analitiche, quali la spettrometria di massa tandem (MS/MS), che permettono l'esecuzione di tests quantitativi e non solo qualitativi. Grazie al basso numero di falsi positivi e alla rapidità di analisi, la Spettrometria di Massa Tandem rappresenta oggi l'alternativa più valida al test di Guthrie. Nel corso degli anni, essa, infatti, ha consentito di passare dall'iniziale test singolo per Fenilchetonuria, a test di screening di altre malattie metaboliche quali amminoacidopatie a più bassa incidenza, acidurie organiche e difetti della β-ossidazione degli acidi grassi. E' possibile infatti nella stessa analisi, a partire da una minima quantità di campione, identificare più di trenta errori congeniti del metabolismo.
Il lavoro del progetto di dottorato è stato finalizzato all'introduzione della tecnologia di MS/MS nello screening neonatale degli IEMs. A tale scopo è stato sviluppato un metodo analitico basato sull'accoppiamento di un cromatografo liquido (HPLC) ad uno spettrometro di massa (Tandem Massa), sfruttando le proprietà fisiche e di analisi chimica di entrambi. In particolare, l'uso della tecnica di ionizzazione electrospray (ESI) ha consentito di velocizzare e semplificare ulteriormente l'analisi accoppiando allo spettrometro a triplo-quadrupolo un sistema HPLC senza l'utilizzo di colonne cromatografiche (FIA-MS/MS).
Il vantaggio del sistema MS/MS è legato alla sua elevatissima specificità, difatti l'analita, indotto a frammentare all'interno dello spettrometro, presenta un pattern di frammentazione caratteristico solo di quella molecola. La possibilità di andare a monitorare, in maniera selettiva, solo determinate transizioni consente di abbattere drasticamente la possibilità di falsi positivi durante l'analisi; inoltre, anche in presenza di una matrice molto complessa, questo tipo di indagine strumentale consente di isolare uno o più transizioni per uno o più composti eliminando l'interferenza dei contaminanti. Ulteriori vantaggi sono l'alta velocità di analisi (solo 3 minuti), l'alta riproducibilità e la possibilità di automatizzazione.
Tale metodo è stato utilizzato per analizzare un campione di circa 250 neonati risultati "negativi" allo screening neonatale per la Fenilchetonuria. Dal sangue assorbito su carta da filtro di questi "campioni controllo" sono stati estratti metaboliti quali amminoacidi ed acilcarnitine generalmente coinvolti nel metabolismo degli amminoacidi e degli acidi grassi. Le concetrazioni misurate di ciascun metabolita sono state utilizzate per determinare i valori di "cut-off".
Successivamente, al fine di testare il potenziale diagnostico del metodo analitico sviluppato, è stato analizzato un gruppo di pazienti affetti da diversi IEMs.
Su un totale di 2016 analisi effettuate, solo 33 tests sono risultati "falsi positivi".

SVILUPPO E VALIDAZIONE DI UN METODO RAPIDO DI ANALISI "QUANTITATIVA" PER IL FOLLOW-UP DI PAZIENTI AFFETTI DA FENILCHETONURIA.

La seconda parte del progetto di dottorato è stata finalizzata principalmente allo sviluppo e alla validazione di un metodo analitico per il follow-up di pazienti affetti da Fenilchetonuria. Il metodo si basa su una rapida (3 minuti) separazione cromatografica seguita dall'analisi di spettrometria di massa tandem (LC-MS/MS) degli amminoacidi fenilalanina e tirosina.

Lo studio è stato eseguito su un gruppo di controllo di 24 soggetti e un gruppo di 6 pazienti affetti da Fenilchetonuria (PKU) la cui diagnosi era stata confermata mediante il metodo di analisi “semi-quantitativa” MS/MS su spot per lo screening neonatale.
Il nuovo metodo analitico sviluppato prevede una brevissima preparazione dei campioni (10 minuti) che consiste di un’estrazione da campioni di siero degli amminoacidi fenilalanina e tirosina seguita dall’analisi LC-MS/MS.
E’ stata effettuata una validazione del metodo mediante valutazione della resa e riproducibilità del processo di estrazione, effetto matrice e linearità.
Al fine di valutare la capacità del metodo di discriminare tra un profilo metabolico “normale” e uno “patologico”, è stato analizzato il siero di 6 pazienti PKU; i risultati mostrano una buona risposta “quantitativa” del metodo. Inoltre, messi a confronto i risultati delle concentrazioni di fenilalanina e tirosina degli stessi pazienti ottenute sia con il metodo di analisi “semi-quantitativa” su spot che con l’analisi “quantitativa” su siero liquido, mostrano che entrambi i metodi analitici sono “validi” ad idenitificare la patologia anche se il metodo quantitativo su siero "tal quale" mostra risultati più interessanti in termini di accuratezza e precisione dell’analisi.
Questo, insieme alla semplicità e rapidità di esecuzione della preparazione dei campioni, rende il nuovo metodo LC-MS/MS una potenziale e valida alternativa analitica nel follow-up dei pazienti PKU.

Summary

Metabolomics is defined as the study of metabolome or the complex system of metabolites that, as intermediates of biochemical reactions, play an important role in the connection between the different pathways involved in a living cell. The metabolites can be seen as the end product of gene expression or activity of the protein (enzyme), thus defining the biochemical phenotype of a biological system as a whole, including humans. While genomics and proteomics suggest a possible mode of operation of the system, metabolomics is the real representation of the system.
In common with the transcriptome and the proteome, the metabolome is context-dependent, and the levels of each metabolite depend on the physiological, developmental, and pathological state of a cell, tissue or organism. However, an important difference is that, unlike mRNA and proteins, it is difficult or impossible to establish a direct link between genes and metabolites. The convoluted nature of cell metabolism, where the same metabolite can participate in many different pathways, complicates the interpretation of metabolite data.
The genome, transcriptome and proteome elucidations are based on target chemical analyses of biopolymers composed of 4 different nucleotides (genome and transcriptome) or 22 amino acids (proteome). Those compounds are highly similar chemically, and facilitate "high-throughput" analytical approaches. Within the metabolome, there is, however, a large variance in chemical structures and properties. Thus, the metabolome consists of extremely diverse chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids and complex natural products. That complexity makes it virtually impossible to simultaneously determine the complete metabolome. Therefore, the metabolome has been studied with efficient sample preparation and with selective extractions coupled to a combination of different analytical techniques to achieve as much information as possible.
Mass spectrometry (MS) is the most frequently employed method of detection in the analysis of the metabolome. The most important advantages of MS are its high sensitivity, selectivity and "high-throughput" in combination with the possibility to confirm the identity of the components present in the complex biological samples as well as the detection and, in most of the cases, the identification of unknown and unexpected compounds. Furthermore, the combination of the separation techniques (e.g., chromatography) with MS tremendously expands the capability of the chemical analysis of highly complex biological samples.
Usually, the MS applications are distinguished in three basic groups: small metabolites such as organic acids, amino acids, fatty acids, steroids and their conjugates; peptides, proteins and glycoproteins and oligonucleotides derived from biopolymers (DNA, RNA).
The analysis of metabolomics has taken in recent years an important clinical role in identifying "disorder" in metabolic profile of a patient. In fact, the qualitative, quantitative and contemporary evaluation depending on the time of a significant number of metabolites, such as those determined by mass spectrometry in biological fluids, can provide, with an acceptable probability, the description of the biochemical present a body. It includes information on the interrelationships between different metabolic processes that define the state. You can determine through the study of biological fluids (such as plasma, urine, bile and cerebrospinal fluid) new criteria that

define the health and status of disease on the basis of an integrated assessment of the variance in the levels of metabolites and systemic metabolic parameters, allowing the state to define physio-pathological in terms systemic or organ-specific. In this way rather than consider one or a few metabolites with metabolic processes, metabolomics examine the whole metabolic profile determined through different processes. To that end, the Mass Spectrometry, through the use of mathematical methods of multivariate analysis, is one of the most powerful and most used for the analysis of metabolomics, allowing together with functional genomics and proteomics to open new avenues of knowledge and application in field of medicine, particularly in the diagnosis and follow-up of inborn errors of metabolism (IEMs).
Inborn Errors of Metabolism are the genetic disorders caused by alterations of a specific chemical reaction in the metabolism. Currently about 400 are known defects genetically determined and related pathology. Although individually rare, IEM collectively account for a significant proportion of illnesses. Today it is, in fact, that about 1 infant every 200 is affected.
The pathogenesis of an IEM can generally be attributed to the loss- or gain-of-function of mutant proteins (usually an enzyme or a transporter).
The genetic basis of IEMs is extremely heterogeneous and can involve any type of genetic defect: one or more point mutations, deletions or insertions, or genomic rearrangements that may occur in coding or regulatory sequences. Disease is generally associated with altered metabolite flux through the pathway that is regulated by the mutant protein.
Originally, the diagnosis of inborn errors of metabolism was generally made by observation of recognition of abnormality in people who become ill or had severe dysmorphic features or mental retardation and these disorders were understood to be inherited by observation of recurrence in family members. With the identification of specific enzymes and metabolic pathways, understanding the physical processes underlying these IEMs has evolved. This knowledge has been accompanied by an awareness of the possibility of errors at various levels in different metabolic pathways.
In particular, the thesis work of PhD project was divided into the following points:

1. “Semi-quantitative” method development by tandem mass spectrometry for the newborn screening of inborn errors of metabolism;

2. Development and validation of a rapid method of "quantitative" analysis for the follow-up of patients Phenylketonuria affected.

“SEMI-QUANTITATIVE” METHOD DEVELOPMENT BY TANDEM MASS SPECTROMETRY FOR THE NEWBORN SCREENING OF INBORN ERRORS OF METABOLISM

Interest in the neonatal mass screening has been gradually expanding across the world from when Robert Guthrie in 1963 described the method for screening of Phenylketonuria (disorder of the metabolism of phenylalanine) through the wise use of enzyme inhibition of growth bacterial that he has taken the name.
The history of neonatal screening of inherited metabolic diseases has seen a continuous and positive change limits screening to duplicate the examination confirmation, through the introduction of new analytical technologies, such as tandem

mass spectrometry (MS/MS), which allow testing not only qualitative but also quantitative. Thanks to the low number of false positives and speed of analysis, the Tandem Mass Spectrometry today represents the most viable alternative to the Guthrie test. Over the years, infact it has allowed to move from single test for Phenylketonuria to multiple screening tests for other diseases such as metabolic aminoacidopathies a lower incidence, organic acidurias and defects of fatty acid oxidation.You can, in fact, in the same analysis and with a small amount of sample, identify more than thirty inborn errors of metabolism.
The work of PhD project has been finalized at the introduction of technology MS/MS in the newborn screening of IEMs. For this aim, was developed an analytical method based on the combination of a liquid chromatography (HPLC) with a mass spectrometer (Tandem Mass), exploiting the physical properties and chemical analysis of both. In particular, the use of electrospray ionization (ESI) technique allows for faster and further simplifies the analysis matching the spectrometer to a triple-quadrupole HPLC system without the use of chromatography columns (FIA-MS/MS).
The advantage of the MS-MS system is linked to its high specificity, in fact, the analyte led to fragmentation within the spectrometer has a characteristic pattern of just fragmentation of the molecule. The possibility of going to selectively monitor only certain transitions can drastically cut down the possibility of false positives during the analysis, and, even if there is a very complex matrix this type of survey instrument allows you to isolate one or more transitions to a or more components by eliminating the interference of contaminants. Further advantages are high speed analysis (only 3 minutes), high reproducibility and the possibility of automation. This method was used to analyze a sample of some 250 newborns results "negative" neonatal screening for phenylketonuria. Absorbed by the blood on filter paper of these "control samples" were extracted metabolites such as amino acids and acylcarnitines generally involved in the metabolism of amino acids and fatty acids. Measured concentrations of each metabolite were used to determine the "cut-off" values. Subsequently, in order to test the diagnostic potential of the analytical method developed, was analyzed a group of patients with different IEMs. Out of a total of 2016 tests carried out, only 33 tests are “false positives”.

DEVELOPMENT AND VALIDATION OF A RAPID METHOD OF “QUANTITATIVE” ANALYSIS FOR THE FOLLOW-UP OF PATIENTS PHENYLKETONURIA AFFECTED.

Second part of the PhD project was designed mainly to the development and validation of an analytical method for the follow-up of patients Phenylketonuria affected (PKU). The method is based on a rapid (3 minutes) chromatographic separation followed by tandem mass spectrometry analysis of phenylalanine and tyrosine amino acids.
The study was conducted on a control group of 24 subjects and a group of 6 phenylketonuric patients whose diagnosis had been confirmed previously by the "semi-quantitative" MS/MS analytical method for newborn screening using spot samples.
The new analytical method developed provides a rapid sample preparation (10 minutes) which consists of extraction from serum samples of the amino acid phenylalanine and tyrosine followed by LC-MS/MS.

Also was made a method validation by assessing the recovery and reproducibility of the extraction process, matrix effect and linearity. In order to assess the ability of the method to discriminate between a metabolic "normal" and a "pathological" profile, was analyzed the serum of 6 PKU patients. The results show a good "quantitative" response of the method. Furthermore, comparison of the concentration results of phenylalanine and tyrosine obtained with "semi-quantitative" analysis using spot samples with those obtained by "quantitative" analysis" using liquid serum shows that both analytical methods are "reliale" to identify a disease but the quantitative method using liquid serum shows the most interesting results in terms of accuracy and precision analysis. This, together with the ease and speed of execution of sample preparation, makes the new LC-MS/MS method a potential analytical alternative for the follow-up of PKU patients.

Abbreviations

IEM	Inborn Error of Metabolism
PKU	Phenylketonuria
ESI	Electrospray ionization
MALDI	Matrix Assisted Laser Desorption/Ionization
UV	Ultraviolet
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
FIA	Flow Injection Analysis
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
Q	Quadrupole
QIT	Quadrupole Ion Trap
LIT	Linear Ion Trap
TOF	Time of Flight
FTICR	Fourier Transform Ion Cyclotron Resonance
GC	Gas Chromatography
CE	Capillary Electrophoresis
CID	Collision Induced Dissociation
GD	Gaucher Disease
NBS	Newborn Screening
FAB	Fast Atom Bombardment
NL	Neutral Loss
PI	Precursor Ion
SRM	Single Reaction Monitoring
MRM	Multiple Reaction Monitoring
TPN	Total Parenteral Nutrition
TNT	Transient Neonatal Tyrosinemia
FAO	Fatty Acid Oxidation
PAH	Phenylalanine Hydroxylase
HPA	Hyperphenylalaninemia
LNAA	Large Neutral Amino Acid
CVD	Cardiovascular Disease
LDL	Low Density Lipoprotein

Chapter 1

Introduction

1.1 Metabolomics

The term “metabolomics” has been used to define the “metabolome” that is the complete set of metabolites. Being the intermediates of biochemical reactions, metabolites play a very important role in connecting the many different pathways that operate within a living cell.
The levels of the metabolites are determined by the concentration and the properties of the enzymes, and their level is, therefore, a complex function of many different regulatory processes inside the cell; i.e., regulation of transcription and translation, regulation of protein-protein interactions, and allosteric regulation of enzymes through their interaction with metabolites. Thus, the level of metabolites represents integrative information of the cellular function, and, hence, defines the phenotype of a cell or tissue in response to genetic or environmental changes. Adding further to the complexity is the fact that many intracellular metabolites participate in a large number of different biochemical reactions, and, hereby, the many different parts of the cellular metabolism together as a tightly controlled metabolic network (*Nielsen, 2003*).
Analysis of cellular function at the molecular level requires recruitment of several different analytical techniques. Whereas comprehensive methods for analysis at the transcriptional level (transcriptome) and at the translational level (proteome) are currently in a rapid state of development, and high throughput analytical methods are already in use (*Godovac-Zimmermann & Brown, 2001; Baldi & Hatfield, 2002; Cristoni & Bernardi, 2003*), methods for analysis of the metabolomic approaches are, however, so far less common. Although metabolite profiling has long been applied for medical and diagnostic purposes (*Horning & Horning, 1971; Gates & Sweeley, 1978*) as well as for phenotypic characterization (*Frisvad & Filtenborg, 1983*), it is not until recently that increasing efforts have been undertaken to develop methods to screen a high number of intracellular metabolites in the context of functional genomics (*Fiehn, 2001; Trethewey, 2001*). Increases in mRNA levels do not always correlate with increases in protein levels (*Gygi et al., 1999*), and once translated a protein may or may not be enzymatically active. (*Sumner et al., 2003*) Therefore, changes observed in the transcriptome or in the proteome do not always correspond to phenotypic alterations (Figure 1). Thus, measurement of metabolites synthesized by a biological system, “the metabolome”, constitutes an important complement to assess genetic function (*Oliver, 1997; Oliver et al., 1998; Hellerstein, 2004*).
In common with the transcriptome and the proteome, the metabolome is context-dependent, and the levels of each metabolite depend on the physiological, developmental, and pathological state of a cell, tissue or organism. However, an important difference is that, unlike mRNA and proteins, it is difficult or impossible to establish a direct link between genes and metabolites. The convoluted nature of cell metabolism, where the same metabolite can participate in many different pathways, complicates the interpretation of metabolite data. In addition, for complex organisms such as plants, there are more metabolites than genes in a biological system (*Schwab, 2003*), whereas for microorganisms there are generally fewer metabolites than genes (*Forster et al., 2003*). Multiple mRNAs could be formed from one gene, multiple proteins from one mRNA and multiple metabolites may be formed from one

enzyme, because many enzymes may accept more than one substrate, although enzymes generally have high selectivity. Metabolome analysis covers the identification and quantification of all intracellular and extracellular metabolites with molecular mass lower than 1000 Da, using different analytical techniques. In addition to applications in functional genomics, quantification of metabolite concentrations enables identification of the kinetics that underlies specific intracellular reactions (*Bulchholz et al., 2002; Hellerstein, 2004*). Together with the structure of a metabolic network, the levels of the intracellular metabolites represent key information to gaining understand about how the fluxes through different intracellular metabolites are regulated; information is valuable not only in functional genomics but also in the development of efficient cell factories through metabolic engineering (*Nielsen, 2001, 2003; Hellerstein, 2004*).

The genome, transcriptome and proteome elucidations are based on target chemical analyses of biopolymers composed of 4 different nucleotides (genome and transcriptome) or 22 amino acids (proteome). Those compounds are highly similar chemically, and facilitate high-throughput analytical approaches.

Within the metabolome, there is, however, a large variance in chemical structures and properties. Thus, the metabolome consists of extremely diverse chemical compounds from ionic inorganic species to hydrophilic carbohydrates, volatile alcohols and ketones, amino and non-amino organic acids, hydrophobic lipids and complex natural products. That complexity makes it virtually impossible to simultaneously determine the complete metabolome. Therefore, the metabolome has been studied with efficient sample preparation and with selective extractions coupled to a combination of different analytical techniques to achieve as much information as possible.

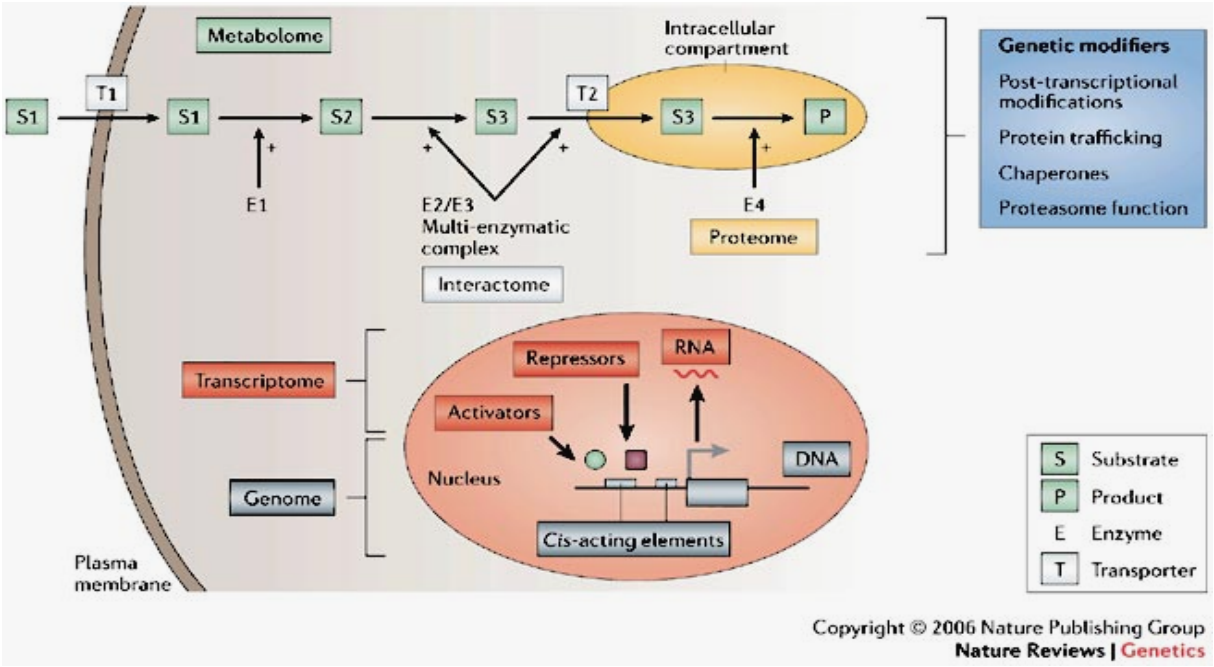


Figure 1. The complex interrelationship between the genome, transcriptome (the relative concentrations of RNA molecules) proteome (the relative concentration of proteins), interactome (the nature of protein–protein interactions) and metabolome (the concentrations of the metabolites that exist in any metabolic and environmental state). Alterations in any of these levels of control might influence the final phenotype.

1.2 Metabolome analysis

1.2.1 Target analysis and metabolite profiling

For the analysis of metabolome, there are basically only two different strategies (*Fiehn, 2002*): target analysis and metabolite profiling (Figure 2).
Generally, target analysis is restricted to quantitative analysis of a class of compounds that are related to a specific pathway or to intersecting pathways. Targeted analysis is very useful for the study of the primary effect of a genetic alteration, and the analytical procedures must include identification and absolute quantification of the selected metabolites in the sample. Metabolite profiling or metabolic profiling involves rapid analysis, often not quantitative, of a large number of different metabolites with the objective to identify a specific metabolite profile that characterizes a given sample. This approach can be sub-divided into (a) metabolic fingerprinting and (b) metabolic footprinting. Fingerprinting covers the scanning of a large number of intracellular metabolites detected by a selected analytical technique or by a combination of different techniques in a defined situation (*Villas-Boas et al., 2005*). Not all metabolites must be identified and quantified, and "raw" data can be used; i.e., one may use the information content from mass spectrometry (MS) profiles or nuclear magnetic resonance (NMR) spectra directly, but the method must give a reproducible profile of the sample.

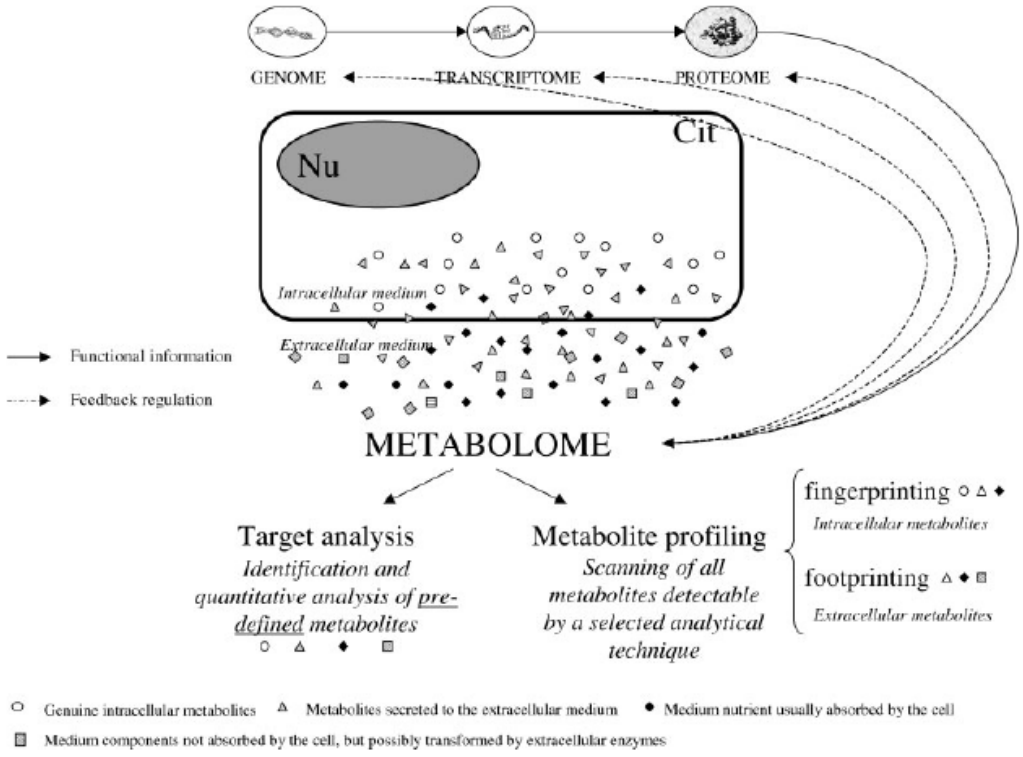


Figure 2. Metabolome analysis in the context of functional genomics. Nu, nucleus; Cit, cytoplasm

Metabolic footprinting is more recently proposed approach (*Allen et al., 2003*), which is technically similar to fingerprinting, but is focused on the measurements of all extracellular metabolites present in a spent culture medium. The compounds

determined are metabolites secreted by the cells into the medium and the medium components biochemically transformed by the organism.Both approaches of metabolite profiling can be used to distinguish between different physiological states of wild-type strains, and between single-gene deletion mutants from even nominally closely related areas of metabolism.Important physiological informations can be extracted using these approaches but it requires identification of the individual metabolites analyzed and it is often laborious and difficult.

1.2.2 Measurement techniques

Mass spectrometry (MS) and NMR are the most frequently employed methods of detection in the analysis of the metabolome.
NMR in particular, is very useful for structure characterization of unknown compounds and has been applied to the analysis of metabolites in biological fluids and cells extracts (*Shockcor et al., 1996*).
However, in certain circumstances, the ^{1}H NMR spectrum is insufficient on its own to provide information that can fully characterize a metabolite. This limitation is obviously the case where analytes contain functional groups that are deficient in protons or where the protons can readily chemically exchange with the solvent; the signals are broadened beyond detection.
Alternatively, other nuclei can also be used; such as ^{13}C NMR for labeled metabolites (*Shockcor et al., 1996; Des Rosiers et al., 2004*). However, ^{13}C NMR presents relatively low sensitivity; i.e., in the range of µmol to mmol.
In addition, ^{13}C NMR analysis may take several hours for a single sample, as a consequence of its low sensitivity (*Des Rosiers et al., 2004*) and the equipment costs are much higher compared to MS based techniques.
The most important advantages of MS are its high sensitivity, selectivity and high-throughput in combination with the possibility to confirm the identity of the components present in the complex biological samples as well as the detection and, in most of the cases, the identification of unknown and unexpected compounds. Furthermore, the combination of the separation techniques (e.g., chromatography) with MS tremendously expands the capability of the chemical analysis of highly complex biological samples.
The basic information of mass spectra is characterized by its simplicity. The spectrum displays masses of the ionized molecule and its fragments reflecting the elemental compositions of both fragments/parent.

1.3 Mass spectrometry for metabolomics

1.3.1 Instrumentation

Mass spectrometry is a well-known analytical tool for measuring the molecular weight of a sample or distinguishing molecules by their mass-to-charge ratios (*Feng et al., 2008*).
Mass spectrometers, that are routinely in used for purposes such as drug discovery, diagnostics and bio-analyses, can measure the mass of a molecule only after it converts the molecule to a gas-phase ion. To do so, imparts an electrical charge to

molecules and converts the resultant flux of electrically charged ions into a proportional electrical current that a data system then reads. The data system converts the current to digital information, displaying a mass spectrum (*Feng et al., 2008*). Mass spectrometers are generally composed of three fundamental parts, namely the ionization sources, the mass analyzer and the detector (Figure 3).
Ionization source is the part of mass spectrometer that ionizes the target materials.

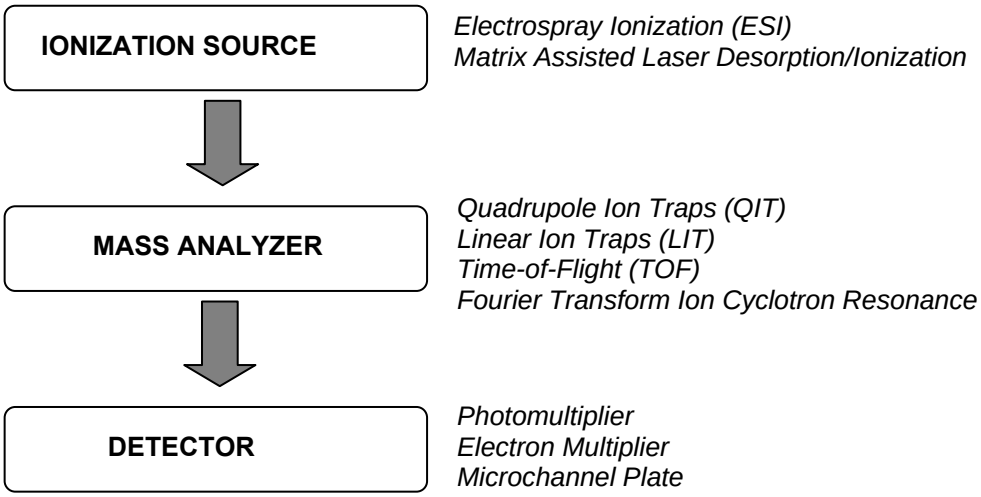


Figure 3. General components of mass spectrometers

The ionization methods employed by the source usually determine what types of samples that can be analyzed. Ionization of the sample molecules were largely produced by electron impact generating radical ions and more could easily break covalent bonds in the molecules generating extensive and reproducible fragmentation. As MS technology advanced, “soft” ionization methods started to emerge especially the electrospray ionization (ESI) (*Fenn et al., 1989*) and matrix assisted laser desorption/ionization (MALDI) (*Karas & Hillenkamp, 1988; Tanaka et al., 1988*), which revolutionized MS techniques. In both methods, sample molecules are ionized with minimal fragmentation.
In MALDI, the analyte is mixed with a solution of matrix which strongly absorbs a UV light and allowed to dry and crystallize. The matrix compound is excited via absorption of a pulsed UV laser and imparts the energy to the analyte, resulting in desorption and ionization. However, increased laser fluence often results in deposition of “excessive” energy, leading to analyte ion fragmentation. The extent of the fragmentation can often be controlled by modulating the power of the laser beam, allowing this phenomenon to be used analytically as a means of producing structurally diagnostic fragment ions. Generally, MALDI surpasses ESI in terms of sensitivity and is more tolerant to salts. Superior sensitivity, relative simplicity of operation and ease of automation made it a top choice as an analytical technique for a variety of proteomics-related applications (*Tanaka K. et al., 1987 & Karas M. et al., 1988*).
ESI, that is considered to be the “softest” ionization methods, uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. This transfer involves three steps: (1)

dispersal of a fine spray of charge droplets, followed by (2) solvent evaporation and (3) ion ejection from the highly charged droplets. Within an ESI source, a continuous stream of sample solution is passed through a stainless steel or quartz silica capillary tube, which is maintained at a high voltage relative to the wall of the surrounding chamber. A mist of highly charged droplets with the same polarity as the capillary voltage is generated. The application of a nebulizing gas (e.g. nitrogen) which shears around the eluted sample solution enhances a higher sample flow rate. The charged droplets, generated at the exit of the electrospray tip, pass down a pressure gradient and potential gradient toward the analyser region of the mass spectrometer. With the aid of an elevated ESI-source temperature and/or another stream of nitrogen drying gas, the charged droplets are continuously reduced in size by evaporation of the solvent, leading to an increase of surface charge density and a decrease of the droplet radius. Finally, the electric field strength within the charged droplet reaches a critical point at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase (*CS Ho et al., 2003*). The emitted ions are sampled by a sampling skimmer cone, placed behind the orifice, and are then accelerated into the mass analyser where they are separated and resolved in according to their mass-to-charge (m/z) ratios. Most commonly used mass analyzers include quadrupole (Q), quadrupole ion trap (QIT), linear ion trap (LIT), Time-of-flight (TOF), Fourier transform ion cyclotron resonance (FTICR-MS) and Orbitrap (*Feng et al., 2008*).

Quadrupole mass analyzer uses quadrupole RF fields to selectively filter ions of interest based on their mass-to-charge ratio and is closely related to QIT where ions are trapped in a 3D quadrupole RF field and then sequentially ejected for separation.

LIT works in the same mechanism as QIT. However, ions are trapped in a 2D quadrupole field instead of a 3D field, which gives LIT higher ion ejection efficiencies and ion storage capacities than the QIT method, this advantage of LIT is especially significant in working with a continuous ionization source such as ESI where increased duty cycles can be achieved. TOF uses electric fields to accelerate ionized molecules and then measures the time they take to reach the detector.

In FTICR-MS, ions are trapped in an analyzer cell composed of electrically conductive walls positioned in a homogenous region of a magnet where they are excited simultaneously and coherently by a large cyclotron radius. The image current produced by the motion of the coherent ions is then recorded and Fourier transformed to produce a mass spectrum. In comparison to other mass analyzers, FTICR-MS has the capability of analyzing high mass bio-molecules with high sensitivity, resolution and mass accuracy. Finally, Orbitrap is a nascent development of mass analyzer, which uses a novel approach to measure the mass-to-charge ratio of ions, bypassing the need for a superconducting magnet in FTICR-MS. But similar to FTICR-MS, Orbitrap also provides high resolving power for each detected peak and exceptional mass accuracy (*Feng et al., 2008*). After ions are separated in the mass analyzer, they are subsequently counted by a detector. Analytical results are presented in a mass spectrum that is a graphical display of the relative abundance of ion signals against the m/z ratios; generally, the highest signal is taken as 100% abundance and all the other signals are expressed as a percentage of this.Usually, MS is employed in combination with prefractionation methods such as gas chromatography (GC), liquid chromatography (LC) and capillary electrophoresis (CE) to reduce the complexity of target samples before MS analysis. GC coupled to MS has been extensively used in metabolome analysis because of its high separation efficiency that can resolve very complex biological mixtures. Only volatile compounds

like ketones and alcohols can be examined directly by GC-MS. Analysis of semi-volatile compounds such as amino acids and lipids require additional chemical derivatization processes; this step adds time to the analysis as well as causes more complex sample-handling and an increased variance in the analysis. Furthermore, heat-labile compounds can not be analyzed and identification of unknown derivatized compounds can be difficult because they are chemically modified (*Feng et al., 2008 & Villas-Boas et al., 2005*). By combining MS with LC, molecular identification and quantification of polar, less-polar and neutral metabolites can be achieved, even when they are present at relatively low concentrations levels and in a complex matrix. LC-MS can provide information on analytes that display chemical diversities, are labile or are difficult to separate at the preparative scale. By using LC-MS, known and unknown compounds that are present in a biological matrix can be detected and identified without any prior knowledge of their exact chemical structure. LC-MS is therefore an important tool for metabolite profiling (*Feng et al., 2008, Villas-Boas et al., 2005 & Katja D. et al., 2007*). CE is a powerful technique for the separation of charged metabolites, offering high-analyte resolution. The combination with mass spectrometry makes CE-MS an ideal tool for the analysis of the metabolome (*Feng et al., 2008, Villas-Boas et al., 2005 & Katja D. et al., 2007*). (Figure 4)

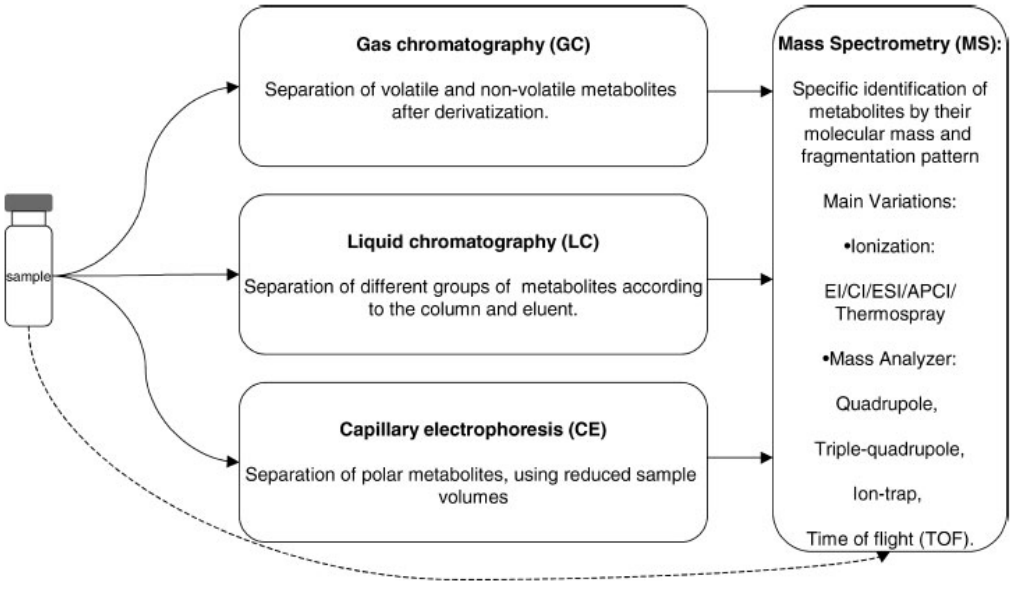


Figure 4. Alternative ways for the most used MS analysis of metabolites. The sample can be directly analyzed by MS or it can be first resolved with different on-line separation techniques.

MS combined with a separation technique offers tremendous opportunities for analysis of complex biological samples because it enables the determination and identification of a large number of metabolites in a single analysis. Furthermore, it often possible to choose between different methods for analysis of a certain group of metabolites (Table 1). However, for metabolome analysis, two issues must be considered in connection with choosing an appropriate analysis method: 1) scope/resolution, because the metabolome comprises different classes of metabolites with distinct physicochemical properties and 2) sensitivity; referring mainly to intracellular analysis, where metabolite concentrations are relatively low (*Katja D. et al., 2007*).

Table 1 Different analytical methods of analysis

Analysis Technique	Application	Advantages	Disvantages	Type of Analysis	Reference
GC-MS	Simultaneous separation, identification and quantification of different classes of metabolites (volatile and non-volatile) in a single analysis. Basically, the type of analysis (targeted or metabolite profiling) is defined by switching the detection between the SIM and SCAN modes.	High chromatographic resolution, ideal to resolve complex biological samples; Enable simultaneous analysis of different classes of metabolites.	Unable to analyze thermo-labile metabolites. Non-volatile metabolites must be derivatized before analysis. Difficult to identify unknown compounds after derivatization.	**Targeted Analysis** Sulfaraphane nitrile **Metabolite Profiling** All different classes of metabolites	Chang et al., 1998 Kube et al., 1999 and others. Nilsson et al., 1996 Baldizsar et al., 1998 Villas-Boas etal.,2003 and others
LC-MS	Separation, identification and quantification of a very broad group of metabolites. Limited potential in identification unless a MS/MS technique is used.	High sensitivity; Average to high chromatographic resolution; No derivatization required; Enable analysis of thermo-labile metabolites.	A few restrictions on LC eluents. De-salting may be needed; Limited structural information; Matrix Effect.	**Targeted Analysis** Glucosinolates **Metabolite Profiling** Glycolytic, Intermediates, Nucleotides, isoprenoids	Mellon et al., 2001 Buchholz et al.,2001; van Darn et al.,2002; Cogne et al., 2003; Novak et al., 2003.
CE-MS	Separation, identification and quantification of polar metabolites, using small sample volumes.	Useful for complex biological samples. Small volumes. High resolution.	Complex methodology and quantification; Buffer incompatibility; Difficulty in interfacingh; Nedd further development.	**Targeted Analysis** Oligosaccharides, amino acids, glycoalkaloids. **Metabolite Profiling** Carboxylic acids, phosphorylated saccharides, nucleotides.	Che et al., 1999. Soga & Heiger 2000 Biance et al., 2003 Scultz & Moini 2003 Soga et al., 2002
MS	The application varies according to the ionization method. Generally applied for determination of metabolites by their molecular mass and fragmentation pattern. Usually applied in metabolite profiling without any quantification and identification of the metabolites.	Rapid screen of metabolites; Negligent sample clean-up for metabolite profiling; High sensitivity; Recommended for identification of unknown compounds (MS-MS).	Identification of metabolites requires MS-MS; Matrix Effect; Incompatibility with higher ionic strength (used in some metabolite extractions).	**Targeted Analysis** Acylcarnitines Amino acids Carbohydrates **Metabolite Profiling** No identification	Chace et al., 1997 Wittmann & Heinzle 2001 Nagy et al., 2003 Castrillo et al., 2003 Allen et al., 2003

1.3.2 Tandem MS

Ion dissociation can often be carried out in the ionization source, for example, by increasing the skimmer potential in the ESI interface or by using high laser power in MALDI. In most cases, however, the sample to be analyzed is a rather heterogeneous mixture and the "in source" fragmentation spectrum would be very difficult to interpret due to the presence of fragment ions derived from different "precursor" ions. A flexible and powerful solution to this problem utilizes a mass spectrometer itself as an ion separation device, which allows the fragmentation of mass-selected precursor ions to be carried out in a variety of ways. This approach, that implies physical separation of the precursor ion prior to fragmentation, was pioneered by McLafferty and is known as *tandem mass spectrometry* or *MS/MS* (*McLafferty F. W., 1980*).
In Tandem MS, two (or more) mass analyzers are incorporated and separated by a collision cell where selectively isolated parent ion from the first analyzer is fragmented for subsequent analysis in the second one. Although ion trap instrument are capable of multiple MS/MS functions (MS^n), the technique is most commonly performed using a triple quadrupole mass spectrometer.
Generally, a quadrupole mass analyser is an assembly of 4 parallel metal rods kepted at equal distance (Figure 5).

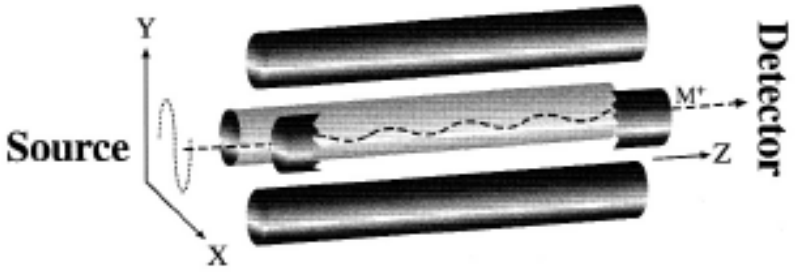


Figure 5. Quadrupole Mass Analyser. The ion (M^+) travels from the source through the 4 metal rods arrangement in the unique oscillating pattern and reaches the detector.

Each pair of opposite rods is connected electrically. The ion stability of certain m/z value depends on RF/DC voltage applied to the quadrupole and masses are analyzed via RF/DC scanning. Single quadrupole MS has only one set ("single") of quadrupole (Q1) and lacks the ability to perform tandem mass spectrometry, whereas in a typical tandem quadrupole system there are three sets ("triple") of quadrupole (Q1, Q2 and Q3) (*Heewon Lee, 2005*) (Figure 6).
In the case of tandem mass spectral analysis using a triple quadrupole mass spectrometer, the analyte ion of interest (usually called the precursor ion) is mass-selected by the first quadrupole (Q1) and allowed to collide with a collision gas (usually argon) in a second RF-only quadrupole collision cell (Q2), where the precursor ions are activated by collision and undergo further fragmentation. This process is known as collision-induced dissociation (CID). The daughter ions resulting from CID are related to the molecular structure of the ions and can be monitored by third quadrupole mass analyser (Q3) providing structural information of the molecular ions. This tandem system is commonly denoted as MS/MS. When Q1 is set to select only one specific m/z ratio, it filters out other molecular ions having different m/z

ratios. This is a “purification” step inside the MS system, eliminating complicated and time-consuming sample purification procedures prior to MS analysis (*CS Ho et al., 2003*).
The following modes of data acquisition are commonly used in a tandem quadrupole system (*CS Ho et al., 2003*):

Product scan (daughter scan): Q1 is static allowing only one ion of specific m/z ratio to pass through and Q3 scans the different Cid product ions. This mode can be used for studying molecular structure; for example, amino acids sequencing of a peptide molecule.

Precursor scan (parent scan): Q1 scans over a range of possible precursor ions and Q3 is static focusing on one unique product ion resulting from CID of a class of precursor ions. For example, m/z ratio of 85 is a common fragment ion from all the butylated acylacrnitines precursor ions (C2-C18).

Neutral loss: both Q1 and Q3 scan together at a constant difference in m/z ratio. This is used to monitor the loss of a neutral fragment for a class of molecules from CID. For example, there is a neutral loss of 102 from most of the butylated amino acids.

Multiple reaction monitoring: both Q1 and Q3 are static for a pre-determined pair of precursor and product ions. This confers the highest selectivity and sensitivity and is commonly used in ESI-MS/MS quantification procedures.

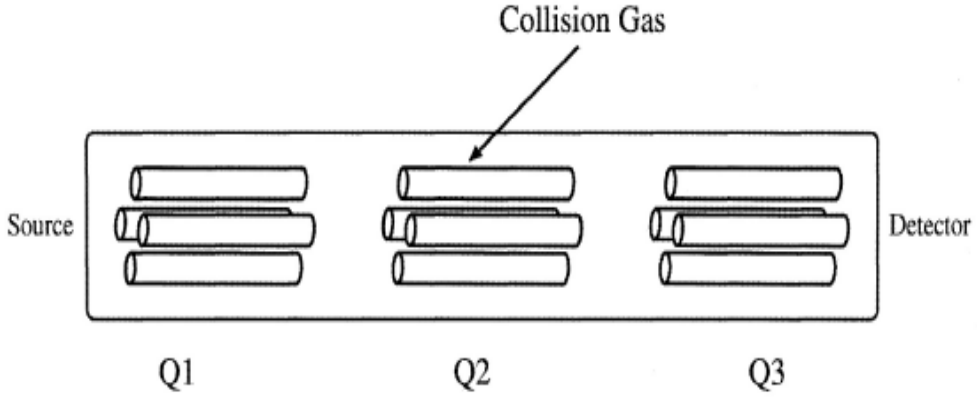


Figure 6. Schematic diagram of a triple quadrupole system. The first (Q1) and the third (Q3) are mass spectrometers and the centre (Q2) is a collision cell.

1.4 Metabolomics and Inborn errors of metabolism

Metabolome analysis is essential to find disorders on a patient’s metabolite profiles and should be added as a clinical tool of diagnosis, follow-up and pathophysiological analysis. Most clinical diagnoses measure enzyme activities in the patient’s blood but the enzyme activity data are not sufficient to detect and predict any disorders in a patient’s metabolite profile.
Suppression or activation in enzyme activity does not always result in the accumulations or reductions of its substrates and products because of the multiplicity of metabolic pathway networks and the robustness of metabolite profiles. Such metabolite profiles can help study the biochemical mechanisms of diseases when they are combined with enzyme activity data. In fact, the combined analysis identifies the enzyme reactions and metabolites that are disordered and is indispensable for

analysis of physiological and environmental perturbations on the metabolite profile of a patient (*Takaaki Nishioka, 2005*). Loss of enzyme activity by genetic or chemical perturbations is critical in the metabolic pathways that have neither bypass nor isozyma. It induces a lack of essential metabolites in the pathway downstream and results in critical disorders on a predetermined metabolite profile. This is easily detected and could be medically treated. However, a loss of one or even more enzyme activities in the middle areas of a metabolic pathway network induces no serious problem because bypass pathways soon compensate the depletion and accumulation of metabolites by supplying and catabolizing them. As the depletions and accumulations spread over a network, they can cause critical disorders on the metabolite profile (Figure 7).
These global disorders are thought to be the origin of lifestyle-related metabolic diseases. Collecting the metabolite profiles of the patients and monitoring their metabolite profiles during follow-up is therefore necessary (*Takaaki Nishioka, 2005*).

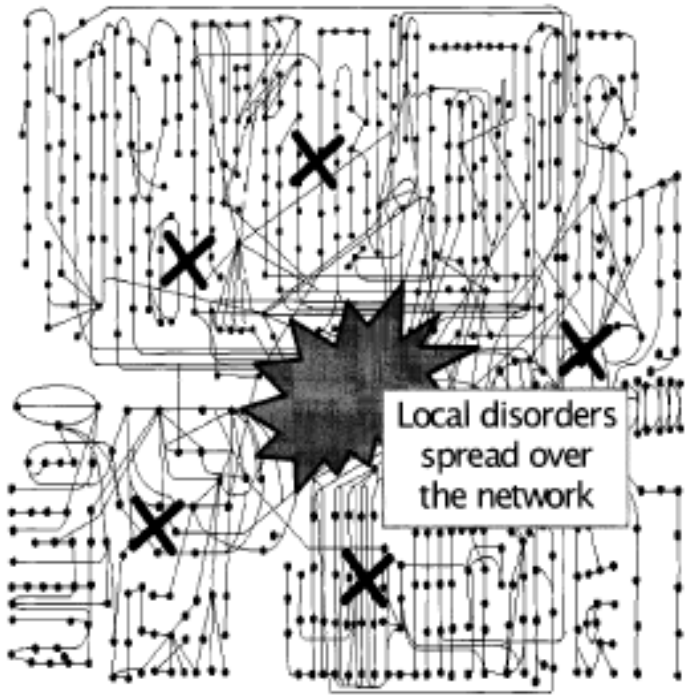


Figure 7. Model of lifestyle related-disease. Crosses are the sites where enzyme reactions are inactivated by internal or external perturbations. When the sites of inactivation are few, they do not affect the metabolite profile because of the multiplicity and regulation of metabolism. When they accumulate and spread over a metabolic pathway network, they affect irreversible, critical disorders on the metabolite profile. Sites of disorders are different from patient *to patient depending on their life style.*

1.4.1 Inborn errors of metabolism (IEMs)

In the early 1900s, Garrod coined the term “Inborn Errors of Metabolism” (IEM) to describe genetic disorders that are caused by alterations of a specific chemical reaction in metabolism (*Garrod A., 1908*). Although individually rare, IEM collectively account for a significant proportion of illnesses, particularly in children. In an Australian study, the prevalence of IEM as detected by newborn screening with tandem mass spectrometry was 15.7 per 100,000 births (*Wilchen B. et al., 2003*).
IEM can be pleiotropic and can involve virtually any organ or system. Initial clinical presentation can occur any time from prenatal development through adulthood, and specific environmental triggers are crucial to determining an individual patient phenotype (*Roe C.R., Ding J, 1995*).

IEM have traditionally been regarded as Mendelian traits that are caused by single-gene mutations, and have served as important models for understanding mechanisms of monogenic disorders. However, as we learn more about the natural histories of IEM, we increasingly recognize that they represent the best examples of complex gene-environment interactions and, more specifically, gene-nutrient interactions that lead to complex disease (*Dipple K.M., McCabe E.R., 2000*). IEMs are therefore a potentially powerful means to dissect both monogenic and common multifactorial diseases.
Many complex diseases can be perceived as specialized cases of IEM that originate from impairment of several steps in different biochemical pathways, each step being less severely affected compared to traditional Mendelian disorders. Therefore, the net effect of a complex IEM on the organ and organism levels is the alteration of one or more metabolite fluxes. A metabolic flux is defined as the production or elimination of a quantity of metabolite per mass of organ or organism over a specific time frame (mole/kg/hr). In a classic IEM, one primary metabolite flux is affected. In a complex disease, a network of metabolite fluxes might be subtly altered to cause a phenotype (*Brendan L. et al., 2006*) (Figure 8).

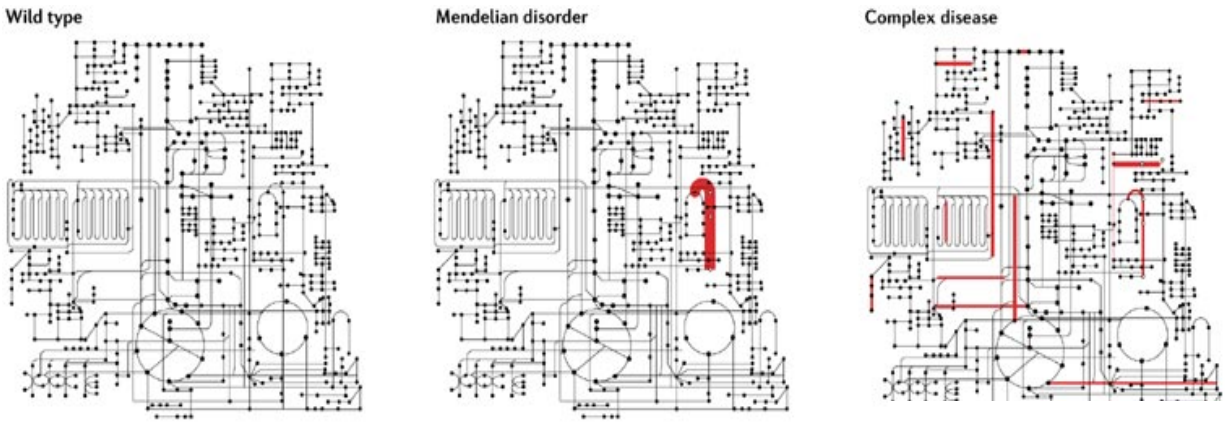


Figure 8. Inborn errors of metabolism and metabolic networks. Affected pathways are shown in red and the degree of involvement is represented by proportional line thickness. In the case of a classic inborn error of metabolism, one primary metabolic pathway is affected. In the case of complex disease, numberous pathways are affected in a more subtle fashion.

1.4.2 IEMs: classification, diagnosis and treatment

IEMs can be categorized according to the affected organ (as in “neurological” or “hepatic” diseases), the affected organelle (for example, “mitochondrial”, “peroxisomal” or “lysosomal” disorders) or the age of presentation (neonatal or adult-onset IEM). Because each of these approaches is informative, no single, universal classification system exists (*Brendan L. et al., 2006*). One of the more illuminating classification systems has been the separation of IEM into disorders that involve large or small molecules (*Applegarth D.A. et al., 1989*) (Table 2). This classification system captures the wide spectrum of pathophysiology, clinical presentation and clinical management of inborn errors. Large-molecule diseases are generally

associated with a gradual onset of disease. By contrast, acute onset with remitting clinical course is often seen in small-molecule diseases.
The pathogenesis of an IEM can generally be attributed to the loss- or gain-of-function of mutant proteins (usually an enzyme or a transporter).
The genetic basis of IEMs is extremely heterogeneous and can involve any type of genetic defect: one or more point mutations, deletions or insertions, or genomic rearrangements. Mutations can occur in coding or regulatory sequences, and mutations in different genes can phenocopy each other by affecting the same pathway. Disease is generally associated with altered metabolite flux through the pathway that is regulated by the mutant protein (*Brendan L. et al., 2006*).
The biological effects of IEM mutations can be mediated by four main processes: direct toxicity of accumulating upstream metabolites; deficiency of downstream metabolites, that is, downstream substrate deficiency; feedback inhibition or activation by the metabolite on the same or different pathway; and diversion of metabolic flux to secondary pathways (Figure 9).
IEM might cause biochemical derangement, the effect of which might be systemic or localized to a specific organ or tissue.
Before an understanding of metabolic pathways developed, originally, the diagnosis of inborn errors of metabolism was generally made by observation of gross abnormalities. Disorders were originally recognized in individuals who became severely ill, or who had dysmorphic features or mental aberrations, and these disorders were understood to be inherited by observation of recurrence in family members. With the identification of specific enzymes and metabolic pathways, our understanding of the physical processes causing these inherited metabolic disorders evolved.
This knowledge was accompanied by the concomitant understanding of the possibility for errors at various stages in those pathways. It was recognized that deficient enzymes could be identified in individuals by analysis of body fluids and measuring the accumulation of substrate for that enzyme, or the accumulation of unusual products of pathways utilized in response to the enzymatic block (*Brendan L. et al., 2006*).
Genomic and proteomic approaches to the primary clinical diagnosis of metabolic disease have been largely disappointing. Clear genotype-phenotype correlations have rarely been seen in IEM, so the primary method for disease evaluation and diagnosis has been through the analysis of metabolites.Genome, transcriptome and proteome profiling technologies each monitor molecules that have similar chemical properties. By contrast, the metabolome is comprised of chemically heterogeneous molecules that vary in different environmental states.
Genomics and proteomics studies are generally carried out using microarrays and 2D gels or mass spectrometry, respectively, but the technique of choice is less obvious in the area of metabolomics, given the chemical heterogeneity.
There are several ways to diagnose an IEM, which depends on the specific disorder or presenting phenotype. Generally, measurements of metabolites in various body fluids are obtained: organic acids in urine, amino acids in the plasma and fatty-acid-conjugation products (acylcarnitines) are commonly used to screen for the accumulation of substrates that are upstream of a biochemical blockage or the deficiency of down-stream products.
Metabolic profiles that are obtained by these means, however, are necessarily incomplete, as each technique might only assay a specific class of compound. These

tests might then lead to assay for more disease-specific enzyme activity, gene sequencing or other diagnostic procedures (*Brendan L. et al., 2006*).

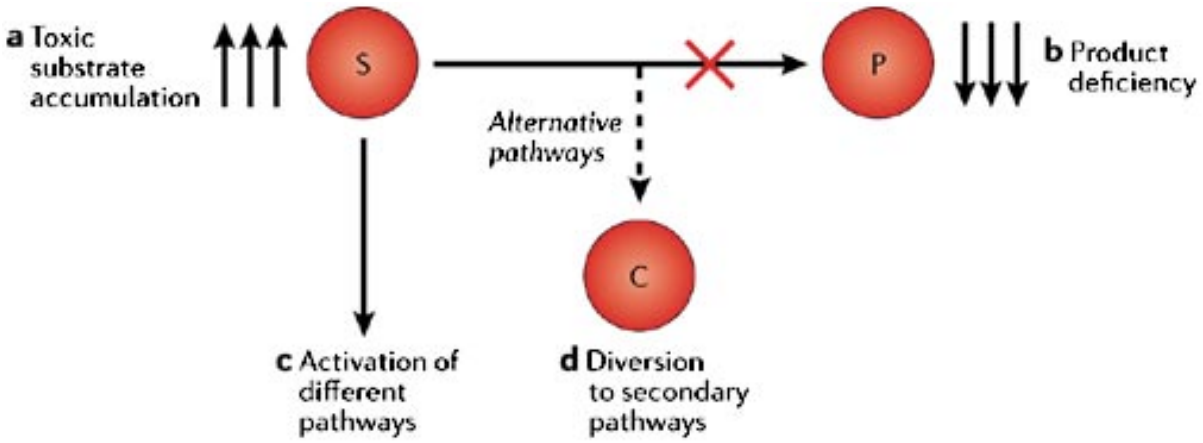


Figure 9. Pathogenetic mechanism in the inborn errors of metabolism. The effect of an enzymatic blockage results in: (**a**) direct toxicity of the accumulating upstream substrate (S); (**b**) deficiency of the downstream product (P); (**c**) activation of alternative pathways; (**d**) diversion of metabolic flux to secondary pathways and alternative metabolite (C) production. Large-molecules diseases often arise from the aberrant synthetic or degradative processing of polymeric molecules, for example, glycoproteins and glycolipids. Small-molecules diseases often involve small organic acids, amino acids, glucose, fatty acids, nucleotides and ammonia. In recent years, it has become clear that deficiencies of intermediary metabolites can also affect the overall activity of a specific biochemical pathway and that this can be caused by the altered transport of metabolites within the cell and/or outside the cell. For example, primary carnitine deficiency which is caused by a defect in a carnitine transporter that is expressed in the kidney can lead to secondary inhibition of the urea cycle and hyperammonaemia.

A broader, metabolomics approach can be obtained by using quantitative techniques such as mass spectrometry and nuclear magnetic resonance spectroscopy (NMR spectroscopy) (*Dunn W.B. et al., 2005*). In particular, the utilization of mass spectrometers in clinical laboratories is undergoing considerable expansion at the outset of the 21st century. This expansion is largely due to extraordinary advances in mass spectrometry (MS) developed in the previous decade. The number and type of clinically significant biomolecules and their analysis using MS are considerable. Usually, the clinical applications of MS is distinguished in three basic groups: small metabolites such as organic acids, amino acids, fatty acids, steroids and their conjugates; peptides, proteins and glycoproteins and oligonucleotides derived from biopolymers (DNA, RNA) (*Chace D.H., 2001*). Furthermore, recently, tandem mass spectrometry has facilitated the rapid and economical evaluation of a broad spectrum of metabolites from small samples, including dried newborn blood spots. This approach has enabled the timely diagnosis of many disorders, thereby facilitating early institution of therapy. The broad application of tandem mass spectrometry to newborn screening will probably also reveal unanticipated intermediate biochemical phenotypes, as well as provide insights into their involvement in more complex phenotypes. In addition to the quantitative assay of multiple metabolites, functional

approaches are needed that incorporate dynamic measurements of the metabolome, ideally under steady-state conditions.
A frequent approach to the diagnosis of IEM is to examine a profile of intermediary metabolites that have been extracted from a biological sample at a given time point. Ideally, if an enzymatic deficiency is present, metabolites upstream of the blockade will accumulate and there will be deficiency of those downstream. However, this approach, although useful and convenient in clinical care, is unrevealing when studying the higher levels of control and the interaction between metabolic networks. This strategy is further complicated by patients with partial, as opposed to null, enzymatic activity. In these patients, the biochemical phenotype might be normal until a specific physiological or catabolic stress causes decompensation (*Brendan L. et al., 2006*). The maturation in our understanding of metabolomics and metabolic flux has allowed a more comprehensive approach to the diagnosis, management and therapy of IEM, leading to the improvements in the prognosis and outcome of certain disorders. Given the extreme variability of IEM, their management and therapy has to be individualized for each patient, based on the patient's diagnosis and phenotype.
Certain IEM can be treated effectively with dietary intervention. A classical example is Phenylketonuria (PKU), a disorder that is caused by phenylalanine hydroxylase deficiency. If phenylalanine is restricted from the diet, the toxic effects of phenylalanine accumulation are prevented and patients can develop with normal intelligence (*Poustie V.J., Rutherford P, 1999*). This discovery was central to the rationale for instituting newborn screening for metabolic disorders (*Guthrie R., Susi A., 1963*). Dietary restriction of metabolites upstream of the block has become a central management strategy for many IEM. Alternatively, dietary supplementation of deficient compounds that are downstream of the block might be useful in IEM. For certain IEMs, replacement of the deficient enzyme is possible. This has been remarkably effective for Gaucher disease (GD) type I, and is now clinically available for other storage diseases, including Fabry disease and some of the mucopolysaccharidoses (*Pastores G.M.,Barnett N.L., 2005*). Another approach to treating the storage diseases as GD type I has been substrate-reduction therapy. Finally, stimulation of alternative pathways for disposal of the accumulation of potentially toxic metabolites upstream of the block might be an effective tool in chronic management.
Therapy for IEMs might also involve the replacement of the defective gene. Currently, this approach is clinically available in solid-organ (*Saudubray J.M. et al., 1999*) or bone-marrow transplants (*Pastores G.M., Barnett N.L., 2005*). IEM have also been an attractive target for virus-mediated gene therapy, as they might respond well to organ-specific transduction, although successful clinical translation has eluted the field (*Mian A., Lee B., 2002 & Brunetti-Pierri N., Lee B., 2005*).

Table 2 Classification system of IEMs

Molecule/pathway affected	Representative disease	OMIM #	Clinical presentation	Management options
Large-molecule diseases				
Complex lipid degradation	Gaucher disease Type I	230800	Hepatomegaly, splenomegaly, bony lesions, acid β-glucosidase deficiency.	Enzyme replacement; substrate reduction.
Mucopoly-saccharidoses	MPS I (Hurler syndrome)	607014	Corneal clouding, mental retardation, hernias, dysostosis multiplex, hepatosplenomegaly. Elevated urine excretion of glycosaminoglycans, α-L-iduronidase-activity deficient.	Bone marrow transplantation. Enzyme replacement.
Glycogen storage diseases	GSD1A (Von Gierke disease)	232200	Hepatomegaly, pancreatitis, hypoglycaemia, hyperlipidaemia, hyperuricaemia, lactic acidosis. Liver adenomas and hepatocellular carcinomas.	Avoid hypoglycaemia and acidosis with continuous feeding or supplementation with uncooked cornstarch. Liver transplantation for carcinoma or adenoma.
Peroxisomal diseases	Zellweger syndrome	214100	Dysmorphic features, hepatomegaly, polymicrogyria, severe mental retardation. Pipecolic acidaemia, elevated long-chain fatty acids. Death usually in first year.	Symptomatic.
Small-molecule diseases				
Amino-acid-metabolism disorders	Phenylketonuria	261600	Microcephaly, pale pigmentation, mental retardation (if untreated). Teratogenic effects on foetuses of affected mothers (if untreated).	Phenylalanine-restricted diet.
Urea-cycle disorders	Ornithine transcarbamylase deficiency	311250	Hyperammonaemia, vomiting, coma. Episodic hyperammonaemia. X-chromosome-linked inheritance.	Acute: detoxification with dialysis, nitrogen scavengers. Long-term: protein restriction, nitrogen supplementation, arginine supplementation, nitrogen scavengers. Liver transplantation.
Organic acid-metabolism disorders	Methylmalonic acidaemia	251000	Mental retardation, episodic acidosis, hyperammonaemia, stroke-like episodes, progressive renal failure, neutropenia, cardiomyopathy.	Dietary restriction of propiogenic amino acids; carnitine supplementation. Consider liver transplantation.
Monosaccharides	Galactosaemia	230400	Progressive symptoms after start of milk feeding. Vomiting, jaundice, liver failure, sepsis, cataracts.	Lactose free, galactose-restricted diet.
Pyruvic-acid and lactic-acid metabolism disorders	Pyruvate carboxylase deficiency	266150	Psychomotor retardation, lactic acidosis, hypotonia.	Carbohydrate restricted diet. Cofactor supplementation.
Fatty-acid metabolism	Medium-chain acyl-CoA dehydrogenase deficiency	201450	Episodic hypoketotic hypoglycaemia. Decompensation with fasting or illness.	Avoid fasting and catabolic stress. Carnitine supplementation.
Purine- and pyrimidine-metabolism disorders	Adenosine deaminase deficiency	102700	Severe combined immune deficiency (SCID).	Bone marrow transplant.
Cholesterol biosynthetic disorders	Smith-Lemli-Opitz syndrome	270400	Dysmorphic features, midline malformations, severe psychomotor retardation.	Cholesterol supplementation. Consider HMG-CoA reductase inhibitors.
Vitamin and cofactors-metabolism defects	Biotinidase deficiency	253260	Metabolic acidosis, neurologic impairment, seizures, skin and hair defects.	Biotin supplementation.
Heme synthesis	Acute intermittent porphyria	176000	Abdominal colic, episodic events mimicking acute abdomen, polyneuropathy.	Avoid triggers. Treat acute attacks with IV glucose and hemin preparations.
Copper- and iron-transport disorders	Wilson disease	277900	Chronic liver disease, cirrhosis, psychiatric disease. Neurological deterioration.	Avoid copper in diet. D-penicillamine. Consider liver transplantation.

*A more extensive version of this table is available online. HMG-CoA, high mobility group-coenzyme A.

1.5 Objectives of the research project

The work of thesis project was focused on the analytical methods development for the diagnosis of metabolic diseases and was divided as follows:

√ "SEMI-QUANTITATIVE" METHOD DEVELOPMENT BY TANDEM MASS SPECTROMETRY FOR THE NEWBORN SCREENING OF INBORN ERRORS OF METABOLISM

The tandem mass spectrometry (MS/MS) enables multianalyte detection for a large number of metabolic disorders in a single analytical run in order to dramatically increase the duty cycle. The metabolic profile has clearly shown improvements in the detection of diseases such as phenylketonuria and several new disorders arising from errors in fatty acid oxidation and organic acid metabolism. MS/MS is a powerful tool for accessing the metabolic status of a newborn and can detect both inborn metabolic errors as well as examine the effect of acquired diseases or pharmacologic intervention on intermediary metabolism.
A new analytical method has been developed for the rapid determination of amino acids and acylcarnitines in dried blood spots and in serum spots by FIA-MS/MS; the most important feature of this analytical method is the ability to detect and quantify a very wide range of different metabolites from a single sample preparation and injection.

√ DEVELOPMENT AND VALIDATION OF A RAPID METHOD OF "QUANTITATIVE" ANALYSIS FOR THE FOLLOW-UP OF PATIENTS WITH PHENYLKETONURIA

Phenylketonuria (PKU) is a metabolic disorder caused by a deficiency of phenylalanine hydroxylase (PAH), the hepatic enzyme that catalyses the conversion of phenylalanine (Phe) to tyrosine (Tyr), using tetrahydrobiopterin (BH4) as a coenzyme. Tandem mass spectrometry has been widely advocated in screening for Phenylketonuria using dried blood samples. Although dried blood spots can be used for quantification of phenylalanine, they are not ideal for the precise quantification required for dietary management because of the imprecision of the sample volume (*Holub M et al., 2006*).
Because of analytical limitations, the analysis of metabolites extracted from spot samples has not sufficient statistical power to generate high confidence in the precision of the result; therefore, this assay have to consider a semi-quantitative analysis (*Chace et al., 2001*).
A new analytical method has been developed for the quantification of Phe and Tyr levels in serum by liquid chromatography coupled with electrospray tandem mass spectrometry. This quantitative LC-MS/MS analysis offers the better precision and accuracy, in comparison with the commonly FIA-MS/MS method for neonatal screening of Phenylketonuria. This method represents a valide alternative for evaluation of therapy in the follow-up of patients.

Chapter 2

Introduction

2.1 Newborn screening

Mass newborn screening (NBS) via blood spot began in the 1960s when Guthrie and Susy developed a method for estimation of phenylalanine in blood samples collected on a filter paper using a bacterial inhibition assay named “Guthrie test” (*Guthrie R., Susy A. 1963 & MacCready R.A., Hussey M.G., 1964*).
On the day the newborn is to be discharged from the nursery, a blood specimen, obtained from the heel by capillary puncture, is directly impregnated into a filter-paper card, and, when dry, mailed to the central laboratory where is execute the “Guthrie test” (Figure 1).

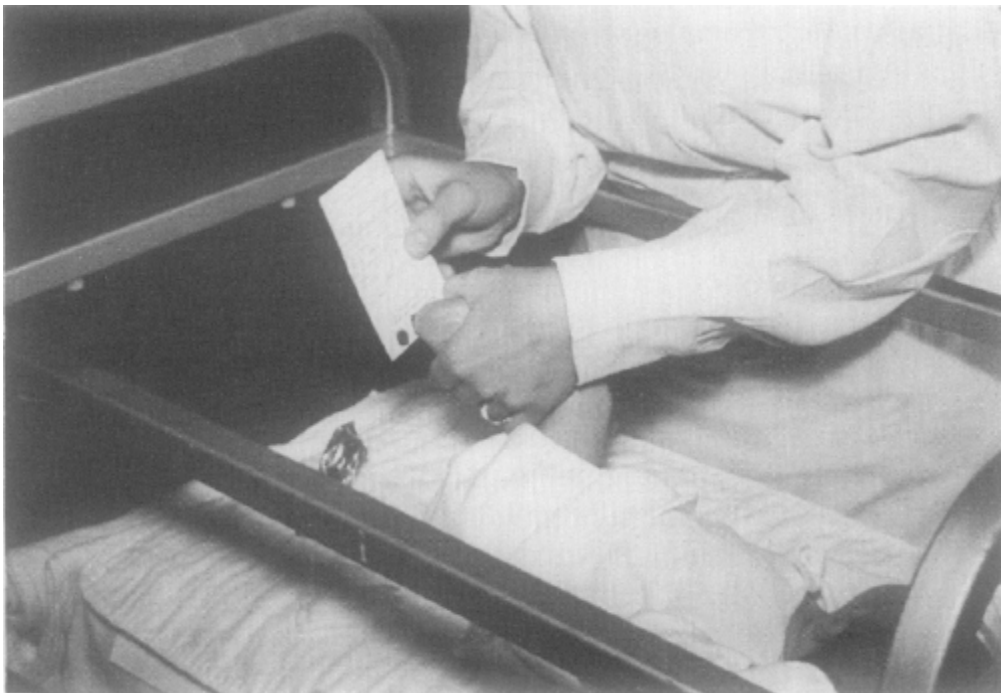


Figure 1. Impregnation of blood from the heel of a newborn into a filter-paper card.

The principle of this assay is that bacterial growth, which would normally occur in a proper medium, is inhibited by an analogue (e.g. B2-thienylalanine) of the amino acid in question (e.g. phenylalanine). However, the analogue can itself be counteracted by the amino acid so that growth occurs in the presence of the specific amino acid (Figure 2).
An important aspect of this assay is that when the concentration of the inhibiting analogue is kept constant, the amount of bacterial growth is directly proportional to the concentration of the amino acid.
In practice, the assay is performed in plastic trays containing agar, a medium consisting of various inorganic and organic compounds, bacteria (Bacillus subtilis) and a specific inhibitor (analogue). After these materials have been pipetted into the

trays and the agar solidified, a disc from each filter-paper blood specimen is punched onto the agar.

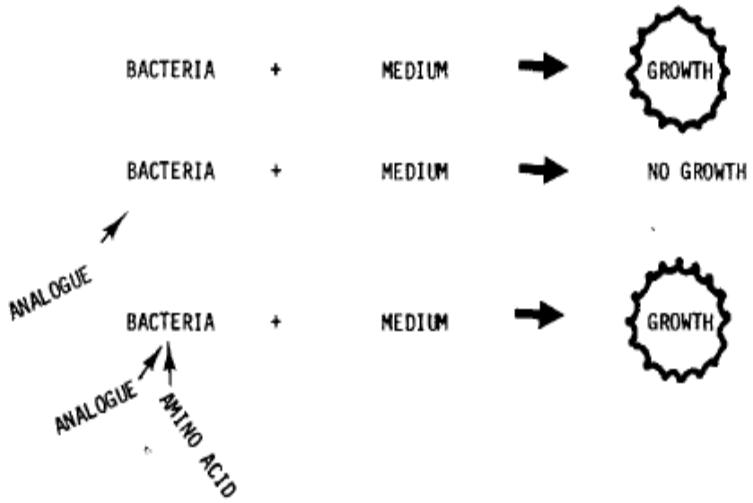


Figure 2. Diagrammatic representation of the mechanism of the "Guthrie" bacterial inhibition assay. In this depiction it is suggested that the amino acid competes with the analogue at the level of cellular transport. This may be true though it is equally plausible that the competition is at the intracellular metabolic level.

Each tray contains, in addition to the newborn blood discs, a row of control blood discs containing varying amounts of the amino acid. After complete preparation, the trays are incubated at 37°C overnight, following which the growth around each disc is observed (*Guthrie R., Susy A. 1963*) (Figure 3).

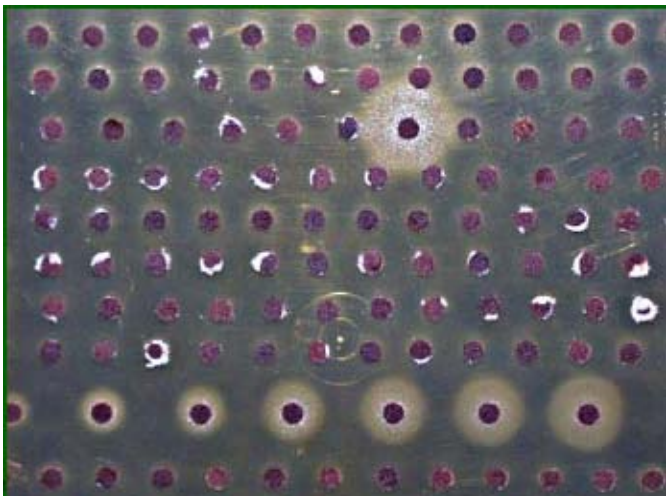


Figure 3. A typical "Guthrie' plate for phenylalanine. The second row from the downstairs contains dried horse blood filter-paper discs used as controls with concentrations of phenylalanine varying from less than 2 mg/100 ml to 20 mg/100 ml. In the third row from the top is a blood specimen from a neonate indicating marked hyperphenylalaninaemia (phenylalanine concentration almost 20 mg/100 ml). This infant was later proved to have phenylketonuria.

This approach was introduced as a way to detect and start early treatment for phenylketonuria (PKU), a disorder of amino acid phenylalanine metabolism, to prevent the neurodevelopmental problems found in untreated patients. It was so

successful that soon thereafter, the screening for PKU was instituted in the entire US and many other developed countries (*Paul D.B., 1997*).
In the 1970s, the ability to test for congenital hypothyroidism allowed another addition to the neonatal blood spot panel. Since then, various disorders were added to state NBS panels, one disorder at a time. Each disorder required the development of new testing materials and each test was run separately (*Paul DB, 2008*). Since 1992, in Italy it is a routine component of neonatal care to screen infants for congenital hypothyroidism, phenylketonuria and cystic fibrosis; the Italian law (*law n.104/92*) provides that the newborn infant is to be subject to newborn screening for these diseases between forty eight and seventy second hour of life. The primary aim is the early detection and treatment of clinically important disorders in order to minimize morbidity and mortality in early childhood. Among the disorders that may be diagnosed, some cause severe illness or death within the first few days of life and newborn screening may serve only to suggest a diagnosis that might otherwise have been missed. However, most of the disorders are treatable if they are diagnosed early. With early diagnosis and appropriate treatment, some problems can be avoided; these include biochemical disturbances such as hyperammonemia in patients with urea-cycle disorders that present after the newborn period, severe metabolic acidosis in patients with disorders of organic acids, or hypoketotic hypoglycemia, cardiomyopathy or rhabdomyolysis in patients with disorders of fatty-acid oxidation; if left untreated, these disorders may lead to brain damage, other organ damage or death.
In the 1990s, with the introduction and development of electrospray tandem mass spectrometry (ESI-MS/MS) into the metabolic screening laboratories, the paradigm of analyzing one analyte per disorder changed, it has become possible to use a single test to screen for a wide range of very rare disorders that have not been screened for previously. Tandem mass spectrometry is used in many screening programs to analyze amino acids and acylcarnitines in blood to detect disorders of amino acids, organic acids and fatty-acids metabolism. With a single and “2-3 min” long analysis of a small blood spot, MS/MS allows the determination of multiple acylcarnitines and amino acids (multiplex testing) characteristic of several metabolic disorders (*Carpenter KH, Wiley V., 2002 & McCandless SE., 2004*) (Figure 4). Where previously one blood spot could detect one disorder, it is now possible to detect upwards of 80 disorders on a single blood spot (*Copeland S, 2008*).

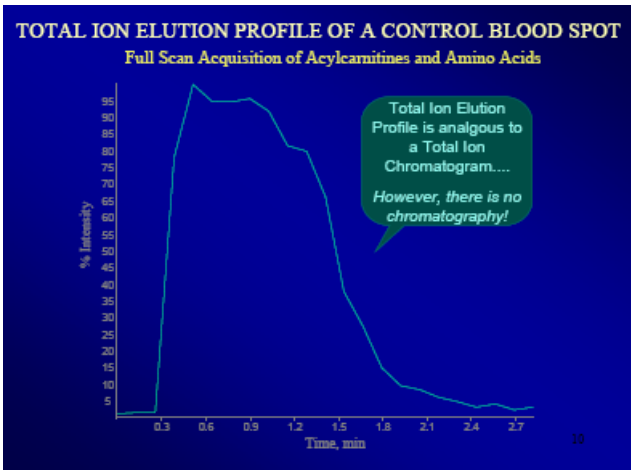


Figure 4. Total ion elution profile of amino acids and acylcarnitines extracted from dried blood spot.

2.2 Historical development leading to the application of MS/MS to newborn screening

Before 1980, diagnosis of inborn errors of organic acid or fatty acid metabolism usually required gas chromatography (GC) analysis of urine extracts with relatively non-specific detectors. Identification of a metabolite in urine was based solely on its retention time. The addition of mass spectrometry (MS) to GC applications in the late 1970s dramatically improved the analysis of organic acids by providing mass spectral identification of each compound at a particular retention time. This combined technique, known generally as GC/MS, became a gold standard for identification of metabolic disorders from urine specimens (*Chalmers RA et al., 1982 & Niwa T., 1986*).
In the mid-1980s, recognition that patients with carnitine deficiency were frequently diagnosed with a metabolic disorder heightened suspicion of a possible connection between carnitine and disorders of fatty and organic acid metabolism (*Chalmers RA et al., 1984 & Di Donato S. et al., 1984*). At that time, routine investigation of a possible metabolic disorder included measurement of plasma and urine carnitine concentrations.
Methods used to measure carnitine, acetylcarnitine (C2) and total carnitine often involved enzymatic and radioenzymatic assays, whereas measurement of acylcarnitines usually involved HPLC (*Marzo A et al., 1997*). Carnitine measurement generally included free, nonesterified and total carnitine (free plus esterified carnitine). Quantification of esterified carnitine was imprecise, calculated by substraction of the free carnitine (FC) concentration from total carnitine. Efforts to understand carnitine deficiencies, metabolic disorders and Reye syndrome and their correlation with free, total and esterified carnitine led to recognition that esterified carnitine may be diagnostic. Efforts were made to further investigate carnitine and acylcarnitine deficiencies to gain a better understanding of the role of carnitine and its fatty acid esters in metabolic diseases (*Bieber LL et al., 1986 & Roe CR et al., 1983*). As a result, the establishment of new methods for carnitine and acylcarnitine measurement became important. Eventually, this search for new methods led to the development of MS/MS for newborn screening.
Before the availability of MS/MS, two approaches to the investigation of carnitine and acylcarnitines included either hydrolysis of fatty acyl residues from carnitine with subsequent analysis by GC/MS, or extensive sample preparation for chemical modification of acylcarnitines to facilitate their analysis by GC/MS. Although both approaches presented sample preparation and time limitations, they nonetheless served to provide integral information about the role of acylcarnitines in metabolic diseases.
The limitations of previous methods led to the search for a simpler and more direct method for the measurement of acylcarnitines. As a result, a new form of MS for non-volatile specimens, known as fast atom bombardment (FAB) MS emerged (*Chai WG et al., 1987 & Millington DS et al., 1984*). With FAB MS, samples are ionized after bombardment by a stream of atoms. Unlike the electron impact ionization used in GC/MS, FAB MS produces little fragmentation during the ionization process. This feature is often referred to as “soft ionization”. After ionization, the mass spectrometer detects and quantifies ions in a complex mixture. For the analysis of complex biological extracts, however, overlapping and suppressed masses lead to mass spectra that are not interpretable and thus uninformative. Consequently, further modifications led to the addition of liquid chromatography for separation of

acylcarnitines from one another and from other compounds present in a sample (*Millington DS et al., 1989*). The fundamental technologic developments in MS/MS application to metabolite analysis initiated in the early 1990s still exist today.

2.3 MS/MS detectable disorders

Currently, nation-wide neonatal screening programs in North America, Europe and Australia are using automated ESI-MS/MS for metabolic profiling of amino acids and acylcarnitines from blood spots. State-of-the-art laboratories can screen several hundred samples per day by sampling of blood spots on the Guthrie card, extraction of analytes, formation of derivatives, ESI-MS/MS analysis and electronic data processing with computer-alert of abnormal results.
Disorders detected by most MS/MS newborn screening programs can be divided into three major categories: amino acid disorders including urea cycle defects, organic acid disorders and fatty acid oxidation defects (*Garg U et al., 2006*) (Table 1).

Table 1. *Inherited metabolic disorders detected by newborn screening with tandem mass spectrometry*

Aminoacidopathies
Phenylketonuria and hyperphenylalaninemias
Tyrosinemia
Maple Syrup urine disease
Homocystinuria
Hypermethioninemia
Nonketotic hyperglycinemia
Hyperornithinemia-hyperammonemia-hyperhomocitrullinuria syndrome
Argininosuccinic acid synthetase deficiency
Argininosuccinate lyase deficiency
Argininemia
Organic Acidemias
Glutaric acidemia type I
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency
Propionic acidemia
Methylmalonic acidemia
Isovaleric acidemia
2-Methylbutyryl-CoA dehydrogenase deficiency
3-Methylcrotonyl CoA carboxylase deficiency
Multiple carboxylase deficiency
Defects of Fatty Acid Oxidation
Short chain acyl-CoA dehydrogenase deficiency
Isobutyryl-CoA dehydrogenase deficiency
Medium chain acyl-CoA dehydrogenase deficiency
Long chain hydroxy acyl-CoA dehydrogenase deficiency
Very long acyl-CoA dehydrogenase deficiency
Multiple acyl-CoA dehydrogenase deficiency
Ethylmalonic acidemia
B-ketothiolase deficiency
Carnitine palmitoyltransferase I and II deficiency
Carnitine transport defect

2.3.1 Disorders of amino acid metabolism

Tandem mass spectrometry technology can measure the amount of various amino acids present in the blood spot. The amino acids on the panel were chosen for their ability to indicate the presence of specific disorders. In general, they should be detectable after 24 hours of age despite feeding status and, when disease is present, will continue to increase with time. External factors can cause false elevations including hyperalimentation and liver dysfunction. The presentation of primary disorders of amino acid metabolism in general is insidious and slow, and patients do not commonly have major metabolic decompensations. However, when untreated, the symptoms may present to neurologists as ataxia, mental retardation and seizures. Treatment consists of limiting the offending amino acid in the diet and, in the case of tyrosinemia type I, blockade of the proximal enzyme step.
The biochemistry of amino acids is both complex and interdependent (*Christenson R et al., 1999 & Scriver CR 1995*). Physiologically important amino acids, known as α-amino acids, have many functions in metabolism, serving as building blocks of peptides and proteins, precursors to hormone mediators and other functional molecules, and sources of energy production.
Many amino acids are readily interconverted to other amino acids by transamination. Metabolism includes conversion of amino acids to ammonia and organic acids by deamination. Many inherited disorders of amino acid metabolism are characterized by a significant elevation in the concentration of certain amino acids in blood (amino acidemias) and urine (amino aciduria), ammonia and organic acids (organic acidemias). Abnormal concentrations of amino acids may also be found in blood or urine as a result of organ failure and impaired function from diseases or immature development.
In the MS/MS analysis, α-amino acids fragment in a reproducible manner, producing a neutral molecule, formic acid. This analysis requires derivatization to enhance ionization. The most common derivative is formed by esterification of the carboxylic function groups with butanol. These butyl ester derivatives of amino acids fragment as a butyl formate with a mass of 102 Da (Figure 5). Hence, the MS/MS analysis using an NL 102 Da scan will selectively detect amino acids (*Chace D.H., Kalas T.A., 2005*).

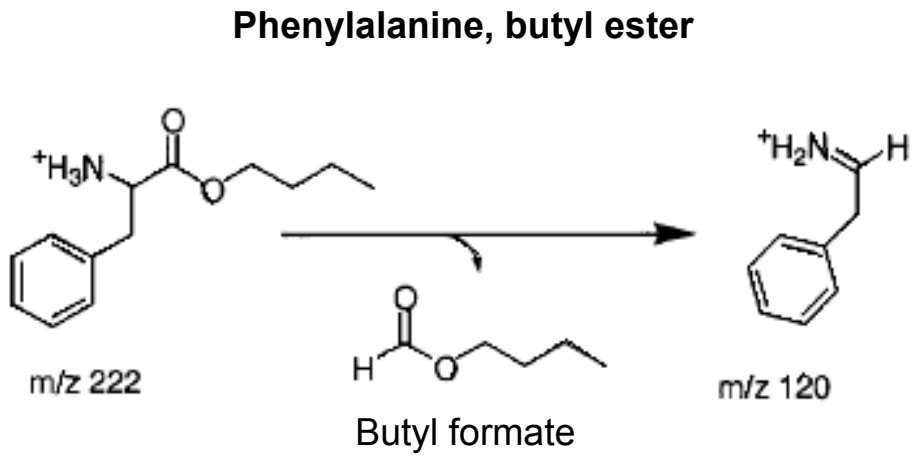


Figure 5. Schematic of collision-induced dissociation (CID) of the protonated butyl esters of phenylalanine

NL 102 amino acid analysis acquires the data for phenylalanine (Phe) and other important amino acids such as tyrosine (Tyr), leucine (Leu) and methionine (Met) simultaneously (*Chace D.H. et al., 1993, Chace D.H. et al., 1995 & Chace D.H. et al., 1996*) (Figure 6).

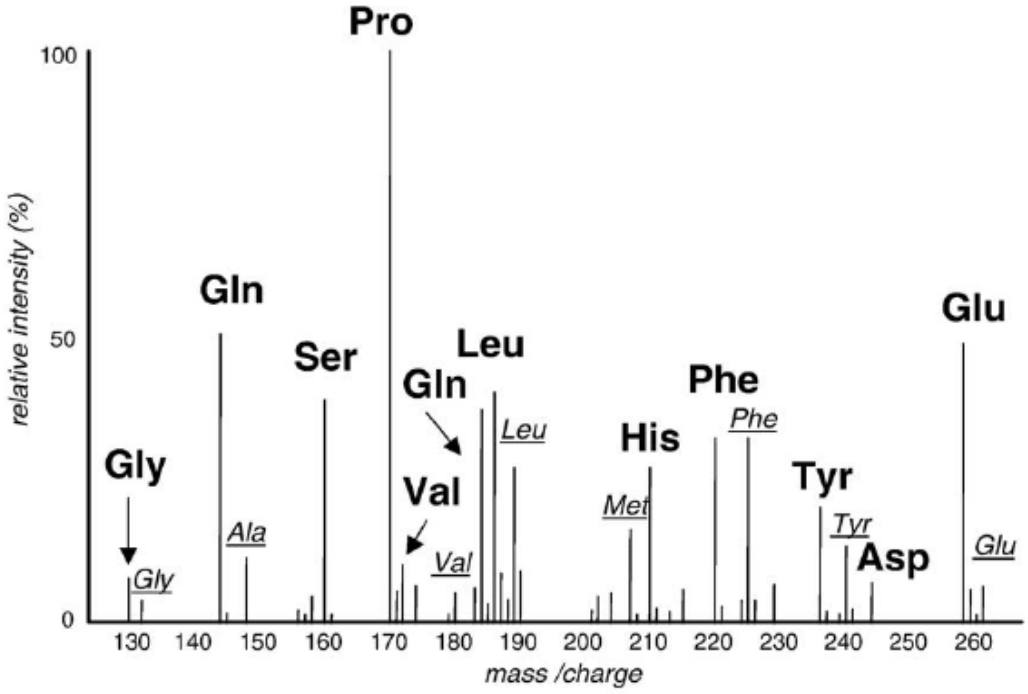


Figure 6. Tandem mass spectra of amino acid butyl esters using an NL 102 scan. Profile obtained from a blood spot of a normal newborn. The horizontal axis represents mass to charge ratios while the vertical axis represents ion intensity expressed as a % of the largest peak in the spectrum. Internal standards are represented as smaller italic fonts and underscored.

A complete amino acid analysis requires the use of another type of MS/MS scan that selectively detects basic amino acids such as glycine (Gly), ornithine (Orn), arginine (Arg) and citrulline (Cit) (Figure 7).

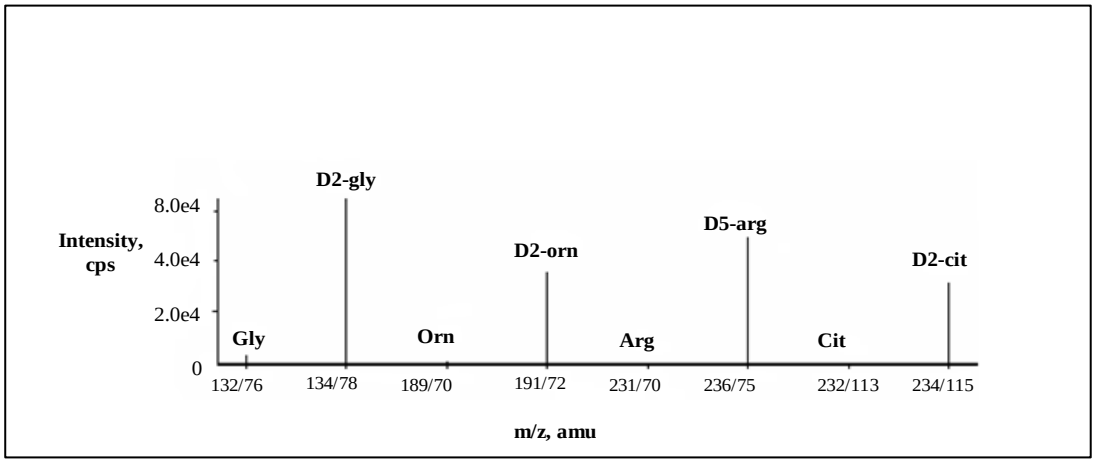


Figure 7. Tandem mass spectra of basic amino acid butyl esters using a Selected Reaction monitoring scan (SRM). Profile obtained from a blood spot of a normal newborn.

This scan, also known as selected reaction monitoring (SRM), is useful in choosing a limited set of amino acids by selecting their specific mass values and fragment ions. Essentially, the difference between SRM and NL scans is that NL scan displays an entire mass range while SRM scans choose only a few mass values (Figure 8).
Final, the advantage is that no secondary injections, separate sample analysis or modifications in sample preparation are required. Neutral Loss and Selected Ion Monitoring are all incorporated within the 2-min amino acid analysis (*Chace D.H., Kalas T.A., 2005*).

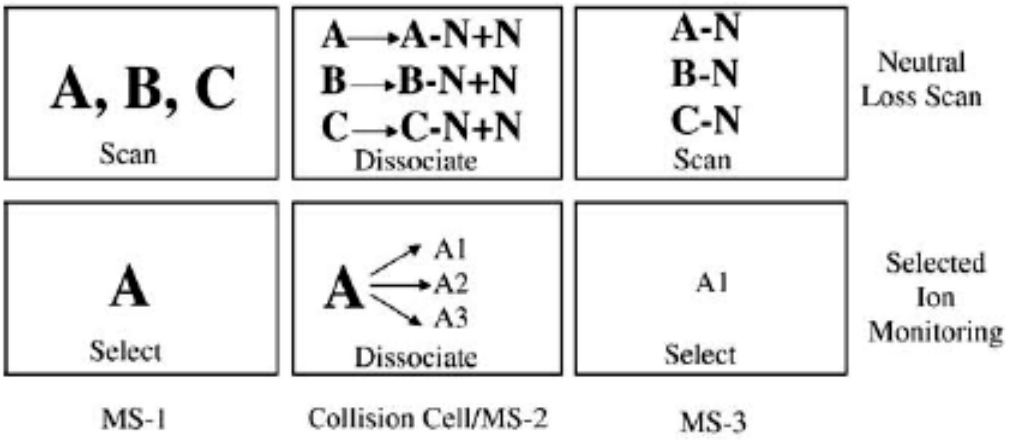


Figure 8. Schematic representation of different scan modes for MS/MS. Neutral loss scan: in this scan, first and third mass spectrometers analyze those ions which differ by a certain number of mass units (equivalent to a neutral fragment, N). This scan is particularly useful for monitoring a group of compounds producing a common neutral molecule such as neutral α-amino acids. Selective reaction monitoring: in this scan, all the mass spectrometers are static as they are analyzing only preselected ions (A, A1). This scan is particularly useful for quantification as this is the most sensitive of all the scans.

2.3.2 Fatty acid and organic acid disorders

The oxidation of fat plays a major role in energy metabolism especially during fasting periods. Fatty acids with carbon chain lengths of primarily 18 carbons or less are metabolized in the mitochondria by a process known as β-oxidation (*Scriver C.R., 1995 & Rinaldo P. et al., 1998*). They are transported through the cellular membrane into the cell cytosol and are translocated across the outer mitochondrial membrane to form fatty acylCoA thioesters. The fatty acyl group is transferred to carnitine and transported into the inner mitochondrial matrix, where it is transferred back to CoA to reform a fatty acyl-CoA plus free unesterified carnitine. The fatty acyl-CoA metabolites undergo oxidation by a complex of membrane-bound and matrix-soluble enzymes (that are “size” specific), producing acetyl-CoA and fatty acyl-CoA thioesters. Each round of metabolism produces a single molecule of acetyl-CoA and a fatty acyl-CoA that is two carbons shorter. Acetyl-CoA is converted in the liver to other substrates (ketone bodies) that are utilized by other tissues for energy metabolism. Very long-chain fatty acids, primarily 20-26 carbons, are metabolized by peroxisomal β-oxidation (*Blau N. et al., 1996 & Verhoeven N.M. et al., 1995*) (Figure 9).

Intermediates of mitochondrial β-oxidation or peroxisomal β-oxidation are important clinical diagnostic markers for many disorders of fatty acid and organic acid metabolism. These mitochondrial fatty acids can be found in blood and plasma and detected using methods such as MS/MS.
This analysis is rapidly becoming the method of choice in newborn and clinical screening of inherited disorders of fatty acid oxidation, an important class (20-30 disorders) of metabolic diseases (*Scriver C.R., 1995 & Rinaldo P. et al., 1998*).
These inherited defects can produce hypoglycemia, vomiting, liver disease, cardiomyopathy, developmental delay, hypotonia, seizures, coma and premature sudden death. Symptoms can occur early in the newborn period through adult life in varying degrees of severity. Alternatively, these diseases may be asymptomatic until a life-threatening episode. Exacerbation of the fatty acid oxidation disorders occurs especially during fast or inadequate caloric intake whereas organic acidemias are exacerbated by high protein intake.
Several disorders in mitochondrial β-oxidation have been characterizated and include very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, short-chain acyl-CoA dehydrogenase (SCAD) deficiency, multiple acyl-CoA dehydrogenase deficiency (MADD, GA-II), carnitine transporter defects, long and short-chain hydroxyl-acyl-CoA dehydrogenase (LCHAD and SCHAD, respectively) deficiencies, carnitine-palmitoyl transferase type I and II deficiencies (CPT-I and CPT II), HMG CoA-lyase and 2,4-dienoyl-CoA reductase deficiencies (*Scriver C.R., 1995*) (Figure 10).
Disorders of fat metabolism produce significant elevations of free fatty acids in plasma. These fatty acids are eliminated in urine predominantly as a conjugate with glycine (an acylglycine). Fatty acids in the mitochondria form acylcarnitines and are exported into the cell cytosol and plasma. Acylcarnitines are eliminated in bile and urine.
In metabolic disorders, carnitine deficiency results in many cases from the continued formation of fatty acylcarnitine and its subsequent loss in urine and bile. Amelioration of these deficiencies may include enhancing elimination of toxic fatty acids as acylglycines or acylcarnitines by administration of glycine and carnitine or reducing metabolism of fatty acids by restoring and maintaining blood glucose at normal levels.
The MS/MS analysis of acylcarnitines is characterized by a stable product ion at m/z 99 or m/z 85 for methyl or butyl esters, respectively (Figure 11). On the basis of the product ion spectra, all acylcarnitines of the same derivatization technique share a common mass for that product ion, e.g. m/z 99 or m/z 85 for methyl or butyl esters of acylcarnitines, respectively. Precursor ion (Figure 12) scans are therefore used to generate a selective MS/MS analysis of acylcarnitines (Figure 13).
One of the most important developments that occurred during the 1990s was multiple metabolite analysis in a single sample injection. For example, both butyl esters of amino acids and acylcarnitines are analyzed concurrently (Figure 14). Furthermore, additional scan functions have been developed for free carnitine and basic amino acids (*Chace D.H. et al., 1999 & Chace D., Naylor E., 1999*). These multiple scan functions have enabled a comprehensive multiple metabolic profile in newborn screening (*Naylor E. Chace D.H., 1999*). Disorders of amino acid metabolism, organic acid metabolism and fatty acid metabolism are measured in a single test.

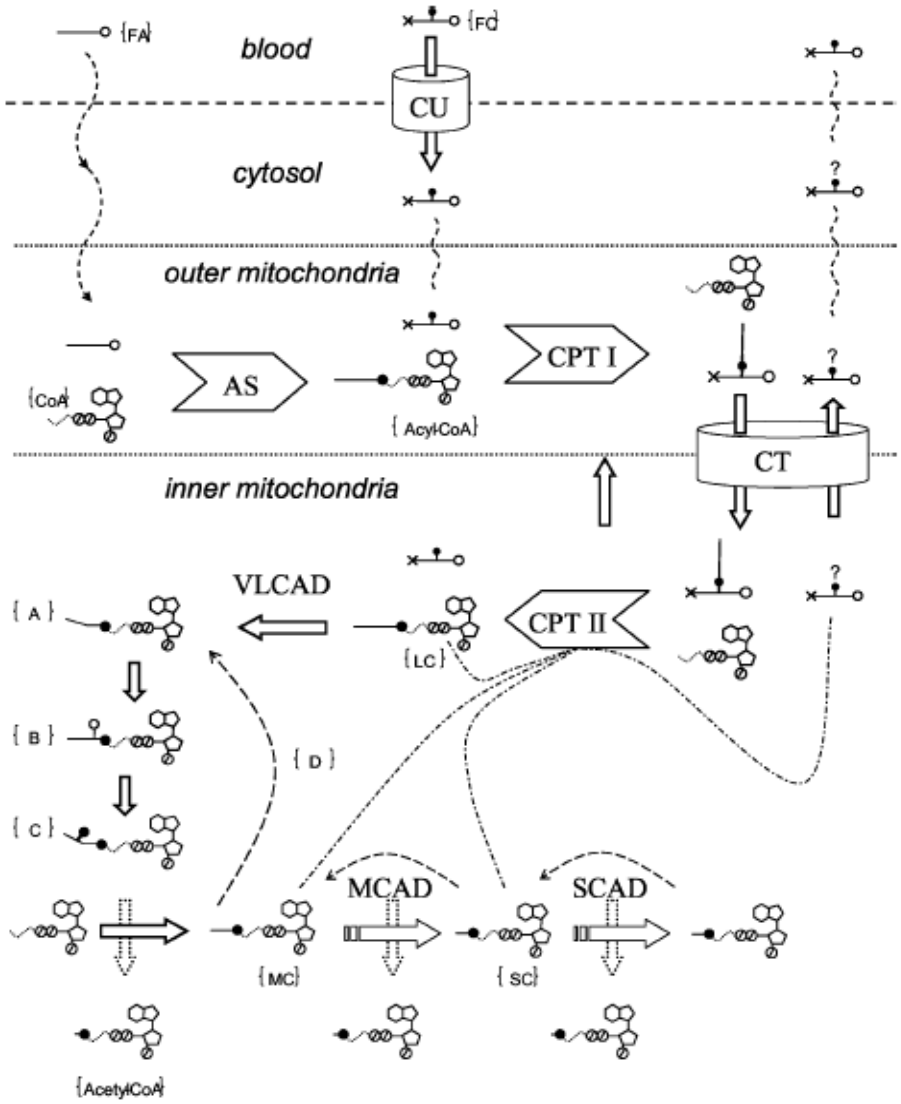


Figure 9. Illustration of β-oxidation and the roles of carnitine and acylcarnitines in metabolism. FA, fatty acids; LC, long-chain acyl-CoA; MC, medium-chain acyl-CoA; SC, short-chain acyl-CoA; A, product of acyl-CoA dehydrogenase; B, product of enoyl-CoA hydratase; C, product of 3-hydroxyacyl-CoA dehydrogenase; D, product of β-ketothiolase; CU, carnitine uptake; AS, acyl-CoA synthase; CT, carnitine translocase.

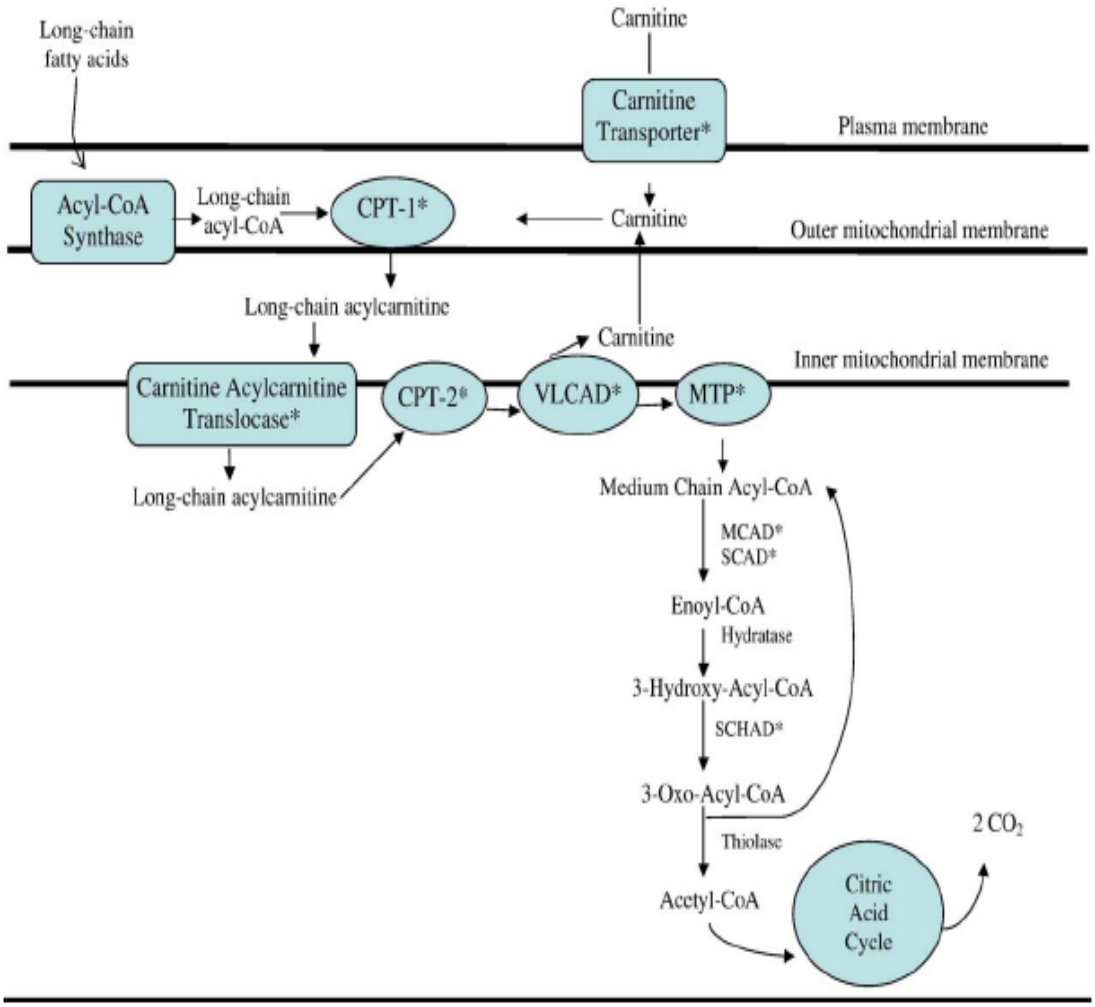


Figure 10. Mitochondrial fatty acid oxidation pathways. Abbreviations: CPT-1 = carnitine palmitoyl transferase-1; CPT-2 = carnitine palmitoyl transferase-2; MCAD = medium chain acyl-CoA dehydrogenase; MTP = mitochondrial trifunctional protein; SCAD = short chain acyl-CoA dehydrogenase; SCHAD = short chain 3-hydroxy acyl-CoA dehydrogenase; VLCAD = very long chain acyl-CoA dehydrogenase. MTP, a membrane bound enzyme, has three enzyme activities: long-chain 3-hydroxyacyl-CoA dehydrogenase, long chain enoyl-CoA hydratase and long-chain 3-oxo-acyl-CoA thiolase, and functions to convert long chain fatty acids to medium chain fatty acids. Enzymes/ transporters marked with * are generally targeted for detection by MS/MS newborn screening.

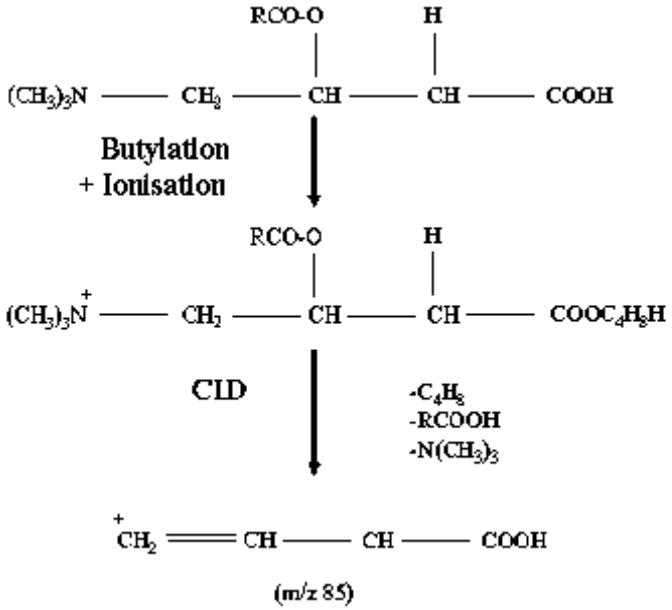


Figure 11. Butylation and fragmentation of acylacarnitines to yeld a common fragment at m/z 85.

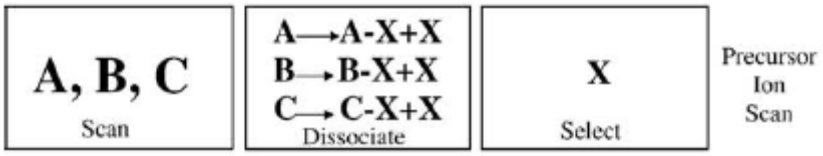


Figure 12. Schematic representation of Precursor ion scan: In this scan, the first mass spectrometer allows the transmission of selected ions (A–C) while the third mass spectrometer is set to analyze a specific fragment ion (X) generated from the selected ions (A–C). This scan is particularly useful for monitoring a group of compounds producing a common ion such as acylcarnitines.

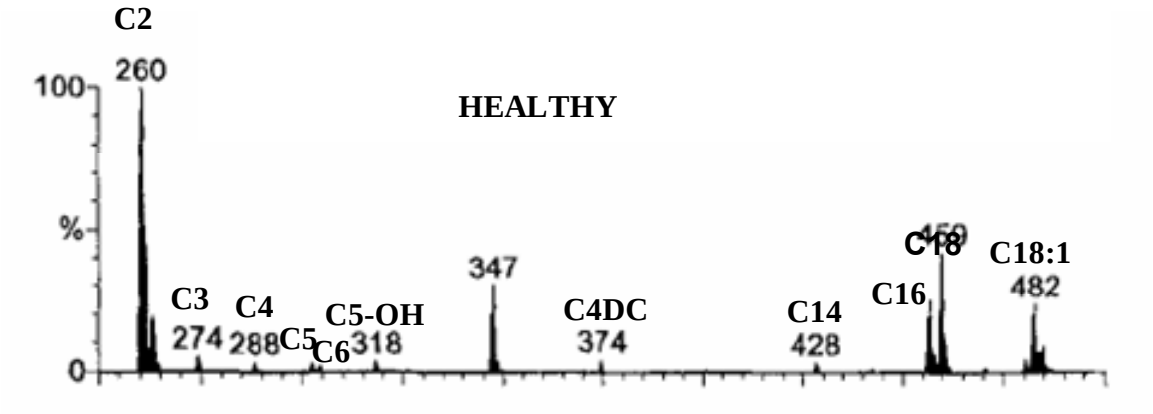


Figure 13. Normal blood acylcarnitine profile obtained by ESI-MS/MS analysis using precursor-ion scanning of m/z 85.

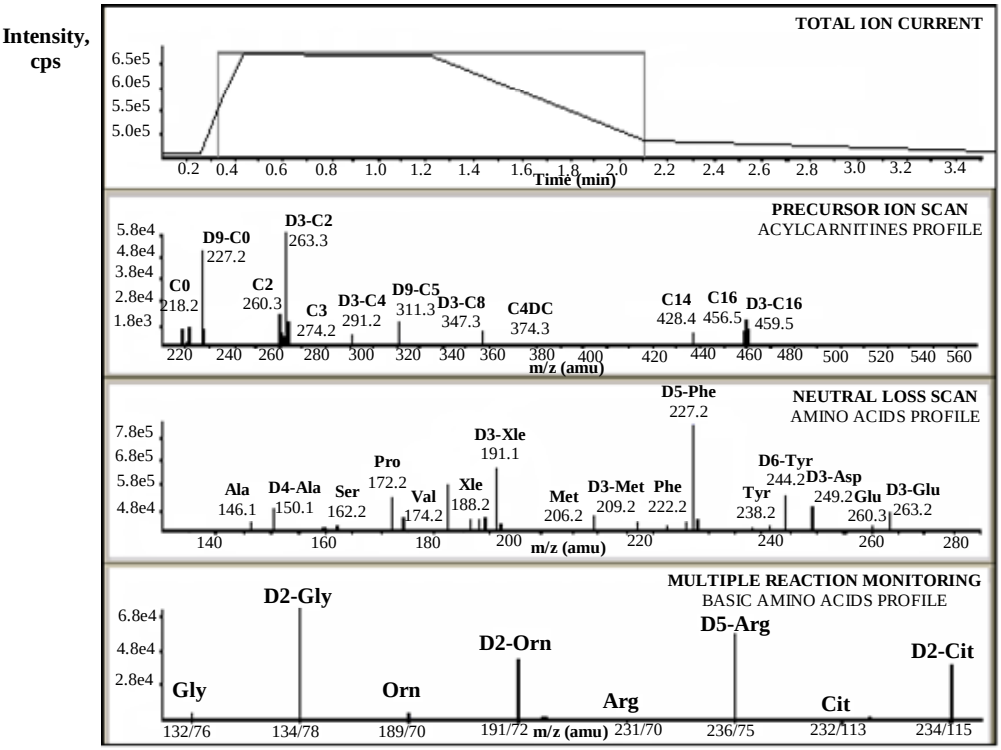


Figure 14. Multiple metabolic profile of amino acids and acylcarnitines.

Materials and Methods

2.4 Expanded Newborn Screening programs

Analytical method development, optimization and validation by MS/MS for an "expanded newborn screening pilot program" were the first fundamental aim of this research project.
Blood spot samples obtained at 48 to 72 hours of life from all healthy infants born in Campania region were tested by tandem mass spectrometry for amino acids and acylcarnitines detection. The cut-off values were determined for each analyte.
After that, was assessed the diagnostic potential of MS/MS method developed. For this aim were tested the blood spot samples of patients that were under treatment in the pediatric department of polyclinic hospital "Federico II" in Naples. These subjects were divided in children that had 1 month of life and in children that are over 1 month year old.
For the first group was necessary a blood spot sample for MS/MS analysis but for child over 1 month of life is to be did a venous blood drawing that is to be mailed to laboratory where is to be centrifuged and then the serum is to be impregnated on filter paper. The treatment of serum specimen for extraction of amino acids and acylcarnitines is the same of the blood spot specimen and the same analytical method was applied. The cut-off values for amino acids and acylcarnitines from dried serum spot were calculated.
However, it is necessary to consider that, although for most of the conditions screened, the sensitivity and specificity of MS/MS is very high, there are false positives causing parental stress and consuming health care resources. That is because there are different factors contributing to false positive results include prematurity or low birth weight, the use of total parenteral nutrition (TPN), nutritional modifications and use of medications such as carnitine and pivalic acid. For this reason, also the cutoff values for amino acids and acylcarnitines extracted by dried blood spots from healthy preterm neonates were determined.

2.4.1 Sample preparation

From each dried blood filter card a 3 mm spot, containing 3.3 uL of blood, was punched and transferred into tubes, where 200 uL of methanol containing deuterated amino acids and acylcarnitines standards was added. The concentrations of the internal standards were as follows: ^{15}N, 2-^{13}C-Glycine 12.5 µmol/L, $^{2}H_4$-Alanine 2.5 µmol/L, $^{2}H_8$-valine 2.5 µmol/L, $^{2}H_3$-Leucine 2.5 µmol/L, $^{2}H_3$-Methionine 2.5 µmol/L, $^{2}H_5$-Phenylalanine 2.5 µmol/L, $^{13}C_6$-Tyrosine 2.5 µmol/L, $^{2}H_3$-Aspartate 2.5 µmol/L, $^{2}H_3$-Glutammate 2.5 µmol/L, $^{2}H_2$-Ornithine 2HCl 2.5 µmol/L, $^{2}H_2$-Citrulline 2.5 µmol/L, $^{2}H_4$ ^{13}C-Arginine HCl 2.5 µmol/L; $^{2}H_9$-carnitine (free carnitine, CN) 0.76 nmol/mL, $^{2}H_3$-acetylcarnitine (C2) 0.19 nmol/mL, $^{2}H_3$-propionylcarnitine (C3) 0.04 nmol/mL, $^{2}H_3$-butyrylcarnitine (C4) 0.04 nmol/mL, $^{2}H_9$-isovalerylcarnitine (C5) 0.04 nmol/mL, $^{2}H_3$-octanoylcarnitine (C8) nmol/mL, $^{2}H_9$-myristoylcarnitine (C14) nmol/mL, $^{2}H_3$-palmitoylcarnitine (C16) 0.08 nmol/mL (stable isotope standards, CIL, Andover, MA, USA). These standards were used to quantify amino acids and acylcarnitines that are extracted into the methanol. It is important to note that quantification of the

compounds in a dried blood spot (DBS) is different from clinical analyses that use liquid blood, plasma or urine samples. Traditional isotope dilution technique requires addition of a stable isotope standard to a liquid blood sample where it can uniformly mix with the unlabeled compounds in the original sample. Because the chemical properties of the labeled standards and the unlabeled compound are nearly identical, all extraction and derivatization procedures will be nearly the same for batch species. Because the blood spot is a dry sample, a liquid standard cannot be uniformly mixed with the original sample. As a result, adding the standard to the methanol extracts causes the loss of a certain degree of accuracy and precision because of variations in both blood volumes in the disk and extraction efficiency (*Adam B.W. et al., 2000*). The remainder of the sample preparation after extraction, however, is identical for both standard and metabolite. Therefore, this technique is a partial isotope dilution, often indicated as “pseudoisotope dilution” (*Chace D.H., 2001*).
The samples were vortexed and shaked for 20 minutes. After that, the solvent was evaporated under a gentle stream of N_2. The most common method for extracted metabolite preparation requires esterification that facilitates ionization and improves sensitivity. The derivatization requires addition of 75 µL of acidified butanol (3N HCl in n-butanol) for butyl esters formation. After incubation at 65°C for 25 minutes, the excess derivatization agent was removed under a gentle stream of N_2. Just prior to MS/MS analysis the specimen was reconstituted in 300 µL of solvent containing acetonitrile/water/formic acid (70:30:0.05 by volume) that assist in protonation and ionization (Figure 15).
The same sample preparation was used for dried serum spot specimens.

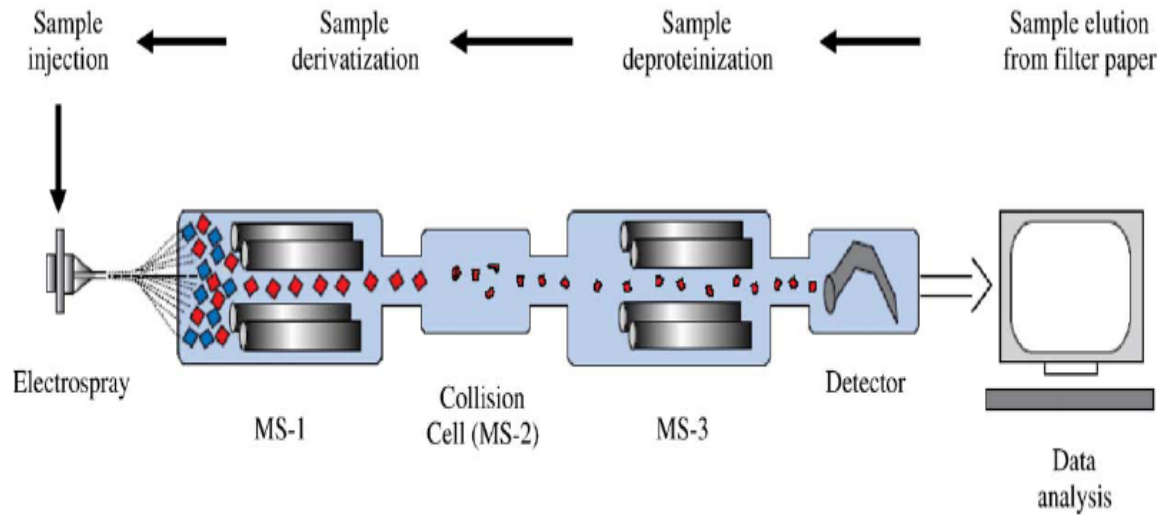


Figure 15. Illustration of sample preparation from newborn screening filter paper spot and analysis by MS/MS. The sample is prepared and then injected into the MS/MS. In MS-1 ions are separated based on their mass to charge ratio. The selected ions enter the collision cell (another mass spectrometer with inert gas flow for collision), where fragmentation of selected ions takes place. MS-3 separates fragmented ions and presents them to the detector. Data analysis involves ion identification and quantification, and flagging of abnormal results.

2.4.2 Acylcarnitines and amino acids profile

Acylcarnitines and amino acids were analyzed as butyl esters on an API 2000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Sciex, Concord, Ontario, Canada) with an electrospray source. For analysis, of each reconstituted specimen were injected 60 µL into a flowing stream of solvent (50 µL/min).
Solvent delivery is via high performance liquid chromatography binary pump (HPLC Agilent 1100) with no chromatography, a method called "flow injection analysis" (FIA). The stream teminated in a spray that was charged at 5500 volts.
All data were acquired in positive ion mode with temperature source at 300°C, nebuliser gas at 40 psi, desolvation gas at 50 psi. The declustering potential and the collision energy were adjusted with specific optimum values determined for each compound by infusion of standard solution. All acylcarnitines were measured by positive precursor ion scan of m/z 85 (scan range m/z 200-560). The specific isotope of each acylcarnitine was used for quantification.
For amino acid profiling, different neutral loss scan functions were used as follows: neutral loss of m/z 102 for alanine (Ala), serine (Ser), proline (Pro), valine (Val), leucine/isoleucine (Ile), methionine (Met), histidine (His), phenylalanine (Phe), tyrosine (Tyr), aspartic acid (Asp) and glutamic acid (Glu), and special multiple reaction monitoring experiments for glycine (Gly), (132→76), ornithine (Orn) (189→70), arginine (Arg) (231→70), citrulline (Cit) (232→113). The specific isotope of each amino acid was used for quantification. The total time for 1 complete analysis of acylcarnitines and amino acids was 3 minutes per sample.
Individual amino acid and acylcarnitine species were quantified by comparing their yield to the respective deuterated standard. In particular, the concentrations were automatically transferred into a *ChemoView* software that was developed to assist in data processing and quantitative calculations.
This program determines the amino acid and acylcarnitine concentration under the following calculation:

$$C_{analyte} = (I_a * V_{IS} * C_{IS}) / (V_{BS}\, I_{IS} * RRF)$$

Where $C_{analyte}$ is the analyte concentration; I_a is the m/z signal intensity of analyte; V_{IS} is the internal standard volume; C_{IS} is the internal standard concentration; V_{BS} is the blood spot volume; I_{IS} is the m/z signal intensity of internal standard; RRF is a correcting factor. The same calculation was applied for dried serum spot containing 1.2 µL of serum.

2.5 Results and Discussion

By ESI-MS/MS method developed, the following amino acids were analyzed: alanine (Ala), serine (Ser), proline (Pro), valine (Val), isoleucine (Ile), methionine (Met), histidine (His), phenylalanine (Phe), tyrosine (Tyr), aspartatic acid (Asp), glutamic acid (Glu) (Figure 16) and basic amino acids as glycine (Gly), ornithine (Orn), arginine (Arg), citrulline (Cit) (Figure 17).
The following acylcarnitines were analyzed: free carnitine (FC), acetylcarnitine (C2), propionylcarnitine (C3), Malonylcarnitine (C3DC), butyrilcarnitine (C4), methylmalonylcarnitine (C4DC), Isovalerylcarnitine (C5), Glutarylcarnitine (C5DC), Tiglylcarnitine (C5:1), 3-hydroxyisovalerylcarnitine (C5OH), hexanoylcarnitine (C6), Adipylcarnitine (C6DC), ocatnoylcarnitine (C8), Octenoylcarnitine (C8:1), decanoylcarnitine (C10), Decenoylcarnitine (C10:1), dodecanoylcarnitine (C12), Dodecenoylcarnitine (C12:1), tetradecanoylcarnitine (C14), Tetradecenoylcarnitine (C14:1), Tetradecadienoylcarnitine (C14:2), palmitoylcarnitine (C16), hexadecenoylcarnitine (C16:1), 3-hydroxyhexadecanoylcarnitine (C16OH), stearoylcarnitine (C18), oleylcarnitine (C18:1), 3-idroxyoleilcarnitine (C18:1 OH) (Figure 18).
A decision limit (cutoff) for each analyte or analyte ratio was set on the 99.5th (0.05th) percentile, based on data collected and analyzed from 1200 healthy neonates and from 500 healthy neonates preterm (<1600 gr of weight) (Table 2).
In addition, the ability to set flags (high and low limits) facilitated more rapid and accurate interpretation. Furthermore, calculation of molar ratios (concentration ratios) and raw ions intensities have served to improve the sensitivity of diagnostic screening for metabolic diseases. All cut-off values have been continuously updated until today in order to also minimize false-positive cases and avoid false negatives.
A decision limit (cutoff) for each analyte or analyte ratio was set on the 99.5th (0.05th) percentile, based on data collected and analyzed on the serum spot samples based on measurements from 250 healthy subjects (Table 3).
The diagnostic potential of this analytical method was evaluated by analysis of serum spot samples of patients with various types of inborn errors of metabolism.
First example reported is a case of Phenylketonuria (Figure 19).
As you can see in the figure 19, the amino acid profile in the bottom panel is characterized by a very prominent ion at m/z 222 corresponding to protonated molecular ion of butylated phenylalanine and a very high ratio of phenylalanine to tyrosine (MH^+=238). Further, the concentration of phenylalanine calculated by Chemoview software is very high in comparison with the cut-off value. Therefore, these results were clearly consistent with a diagnosis of Phenylketonuria (PKU), a defect in the metabolism of phenylalanine caused by a deficiency of the enzyme phenylalanine hydroxylase or its cofactor. The concentration of phenylalanine in blood is affected by the influx of phenylalanine into the blood stream through dietary absorption, i.v. administration and protein breakdown. The rate of elimination or turnover of phenylalanine is affected primarily by the enzyme phenylalanine hydroxylase (irreversible enzyme) and secondarily through phenylalanine transaminase (reversible enzyme) (Figure 20). The products of phenylalanine that may be important in the assessment of phenylalanine metabolism include tyrosine, phenylpiruvic acid, phenylethalamine, phenylacetate, phenylacetylglutamine and hydroxyphenylacetate (*Chace DH et al., 2005*).

As with phenylalanine, a NL 102 scan function is used in measuring tyrosine (Figure 21). The amino acid profile in the Figure 21 showed an abundant ion at m/z 238 corresponding to the butyl derivative of tyrosine. These data were consistent with a diagnosis of tyrosinemia. But increased tyrosine may indicate several metabolic states, including three variations of tyrosinemia, types I, II and III (Figure 22) and a transient form, transient neonatal tyrosinemia (TNT). Therefore, additional investigations, using other methods, were needed to distinguish the different types of tyrosinemia. In this case, the diagnosis of tyrosinemia type III was confirmed by the detection of a significant elevation of 4-OH-phenylpiruvate and 4-OH phenyllactate in the patient's urine by GC/MS analysis. This form of tyrosinemia is caused by deficiency of tyrosine aminotransferase enzyme (Figure 23) (*Chace et al., 2005*).
The abnormal acylcarnitine profile was showed in the bottom panel of Figure 24. In this case there is a very prominent ion at m/z 302 corresponding to isovalerylcarnitine (C5), a key metabolite in the isovaleric aciduria (IVA), a disorder of intermediate metabolism characterized by deficiency of mitochondrial isovaleryl-CoA dehydrogenase (Figure 25).
This diagnosis was confirmed by GC/MS detection of high concentration of isovalerylglycine in the urine of patient (Figure 26).
The abnormal acylcarnitine profile in the bottom panel of Figure 27 showed an abundance of ion at m/z 274 corresponding to propionylcarnitine (C3), a key metabolite in two organic acidurias: Propionic aciduria (PA) and Methylmalonic aciduria (MMA), categorized as defects in the metabolism of the organic acyl CoA compounds resulting from a deficiency of either propionyl CoA carboxylase (PA) or methylmalonyl CoA mutase (MMA) (Figure 26).
In order to distinguish among PA and MMA, is important to calculate the methylmalonylcarnitine (C4DC) concentration that, generally, is abnormal just in MMA. In the profile of Figure 27, the C4DC value is not alterated and therefore the preliminary diagnosis was PA. This result was further confirmed by GC/MS detection of high concentration of 3-OH-propionic acid and methylcitric acid in the urine of patient (Figure 29).
As with propionic acidemia, the principle metabolite related to detection of methylmalonic acidemia is propionylcarnitine, as measured using MS/MS (Figure 28). Other metabolites such as free carnitine (C0), acetylcarnitine (C2) and palmitoylcarnitine (C16) are important and in fact, it is quite difficult to distinguish a severe case of MMA from PA using MS/MS.
The median concentration of C3 in cases of PA is generally greater than that for MMA, but variation is great enough to make distinction of the two particular disorders poor. The main features of the acylcarnitine profile that make recognition of this group of disorders easier are the same, however, namely, an elevated C3, C3/C2 ratio and C3/C16 ratio.
In the most positive cases, the increase in the concentration of the diagnostic metabolite, C3, is quite high. In late onset or less severe cases, however, the concentration of C3 may only be moderately elevated.
Our laboratory has used and tested numerous ratios and cut-offs for C3. In our experience, in addition to the concentration of C3, the ratio of C3/C2 has proven to be the most important diagnostic measure in newborns and older infants. The ratio of C3/C16 is also helpful in newborns but declines in utility with age, in part because of the rapid decrease over time in long-chain acylcarnitines relative to short-chain acylcarnitines. In the diagnosis reported was very discriminate factor among PA and MMA the concentration of C4DC that is very high in comparison with the normal

value, alteration characteristic of MMA disease. This preliminary diagnosis was confirmed by GC/MS detection of high concentration of methylmalonic acid, 3-OH-propionic acid and methilcitric acid in the urine of patient (Figure 29).
In all analysis, each analyte crossing the cutoff was flagged automatically. Samples in which 1 or more parameters were flagged underwent a repeated analysis from the same sample spot.
33 tests (1.6%) on 2016 performed until today were classified as false-positive. These were eliminated by repeat analysis by MS–MS on the same or a second sample spot and by GC–MS analysis of urine for organic acids.

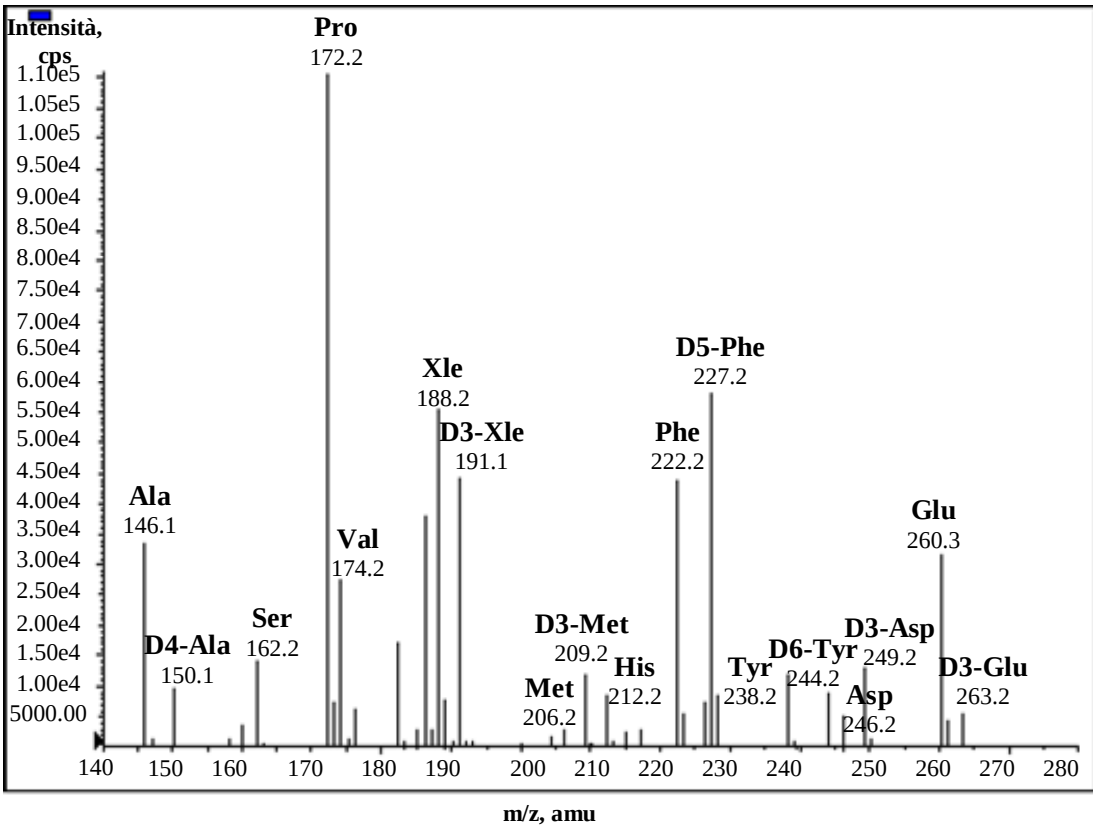


Figure 16. Amino acids metabolic pathway analysed in our laboratory by MS/MS detection.

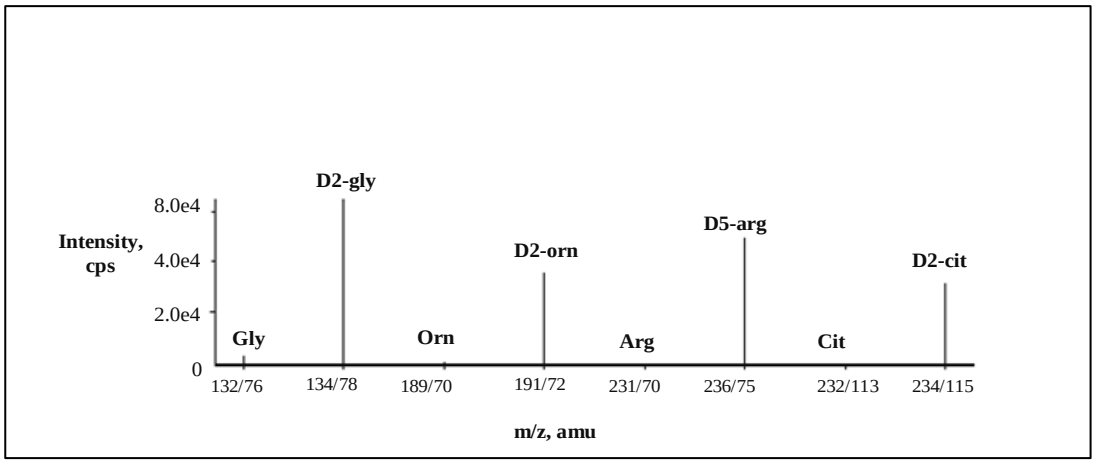


Figure 17. Basic Amino acids metabolic pathway analysed in our laboratory by MS/MS detection.

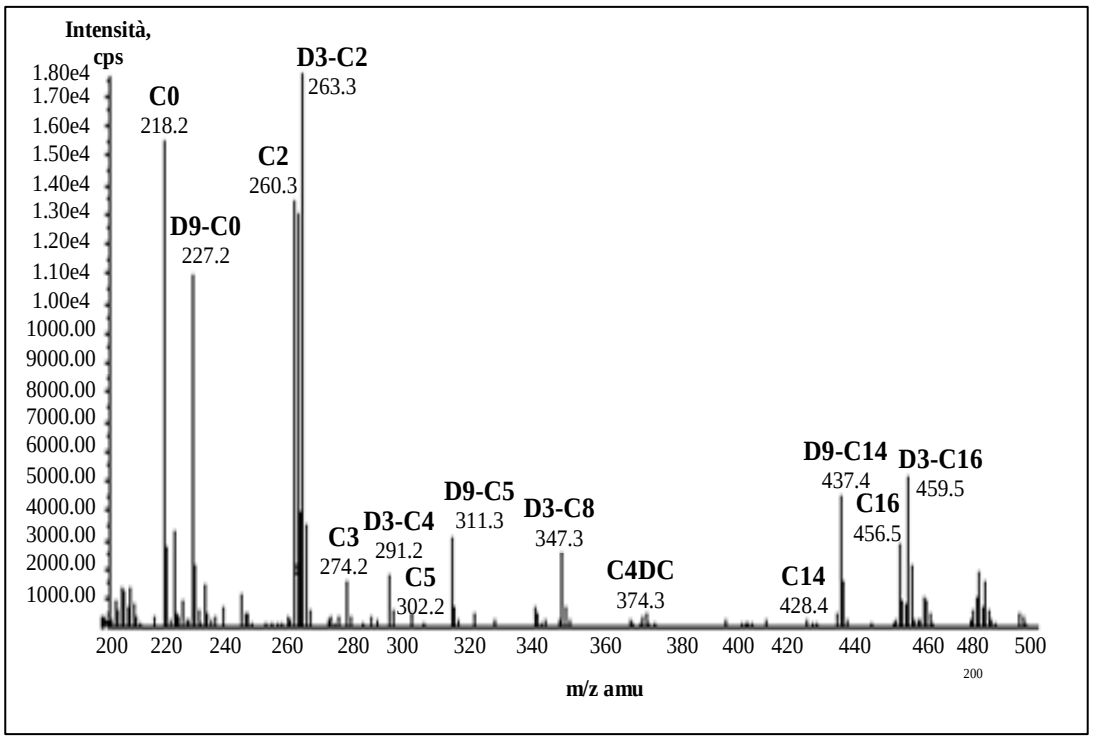


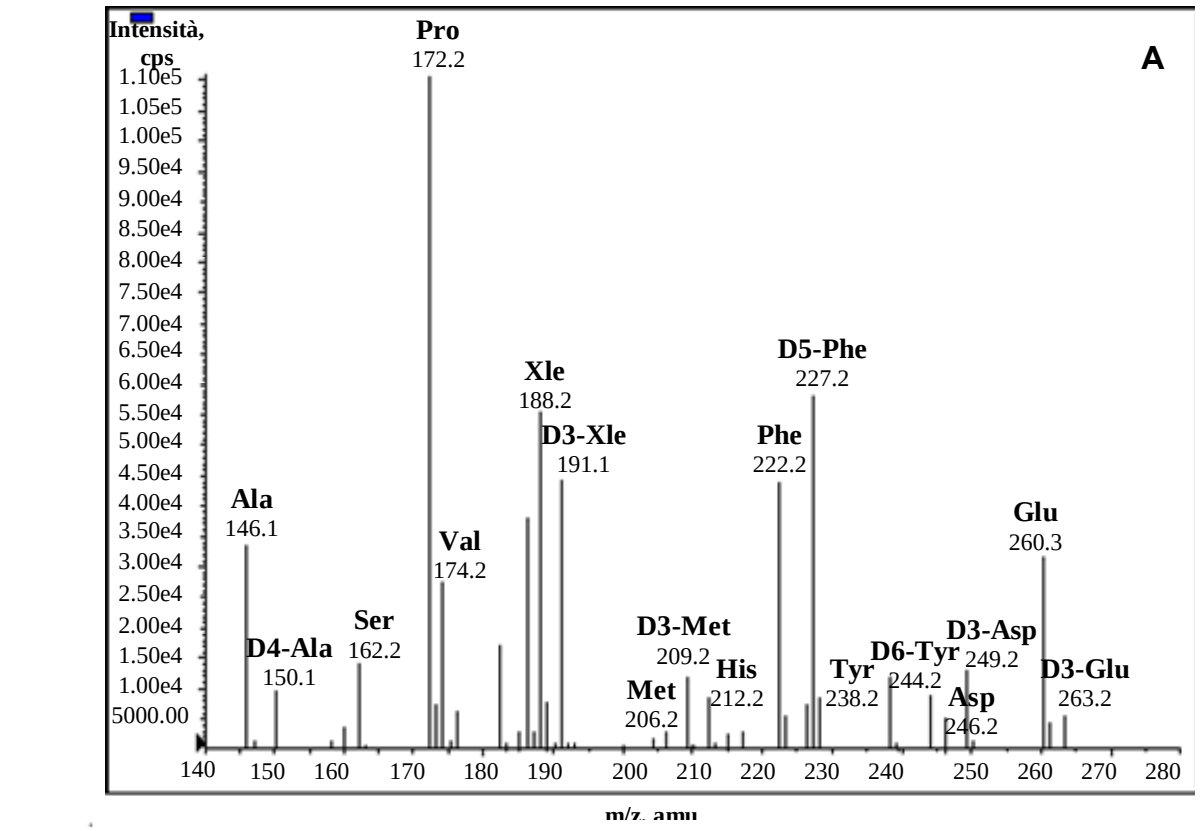
Figure 18. Acylcarnitines metabolic pathway analysed in our laboratory by MS/MS detection

Table 2. Amino acid and acylcarnitine cut-off values for healthy newborn and preterm neonates.

	Preterm (μmol/L)	**Newborn (0-1month) μmol/L**
Alanine	54-178	61-263
Valine	26-180	30-151
Leucine+Isoleucine+hydroxyproline	26-154	52-146
Methionine	8-24	6-30
Phenylalanine	10-67	21-63
Tyrosine	5-280	15-153
Aspartatic acid	2-44	5-42
Glutammic acid	70-306	57-359
Glycine	72-353	105-371
Ornithine	3-33	6-46
Citrulline	2-12	3-14
Arginine	0.1-10	0.6-10
C0 (Free carnitine)	3-49	4-44
C2 (Acetylcarnitine)	3-34	8-47
C3 (Propionylcarnitine)	0.01-2.7	0.04-3.18
C3DC (Malonylcarnitine)	0.01-0.15	0.01-0.15
C4 (Butyrylcarnitine)	0.01-0.61	0.02-0.65
C4DC (Methylmalonylcarnitine)	0.01-0.29	0.05-0.49
C5 (Isovalerylcarnitine)	0.01-1.00	0.02-0.29
C5DC (Glutarylcarnitine)	0.01-0.22	0.01-0.16
C5:1 (Tiglylcarnitine)	0-0.12	0.01-0.23
C5OH (3-OH-Isovalerylcarnitine)	0.04-0.30	0.03-0.28
C6 (Hesanoylcarnitine)	0-0.11	0.02-0.26
C6DC (Adipylcarnitine)	0.01-0.10	0.02-0.15
C8 (Octanoylcarnitine)	0.01-0.20	0.01-0.19
C8:1 (Octenoylcarnitine)	0.01-0.20	0.01-0.30
C10 (Decanoylcarnitine)	0-0.15	0.01-0.27
C10:1 (Decenoylcarnitine)	0.02-0.16	0.02-0.25
C12 (Dodecanoylcarnitine)	0.02-0.16	0.02-0.39
C12:1 (Dodecenoylcarnitine)	0-0.09	0.01-0.23
C14 (Myristoylcarnitine)	0.02-0.36	0.05-0.53
C14:1 (Tetradecenoylcarnitine)	0.01-0.17	0.02-0.28
C14:2 (Tetradecadienoylcarnitine)	0.02-0.07	0.01-0.19
C16 (Palmitoylcarnitine)	0.25-3.16	0.52-6.05
C16:1 (Hesadecenoylcarnitine)	0.01-0.30	0.01-0.45
C16OH (3-OH-Hesadecanoylcarnitine)	0-0.06	0.02-0.13
C18 (Stearoylcarnitine)	0.20-1.15	0.28-2.35
C18:1 (Oleylcarnitine)	0.29-1.95	0.41-3.51
C18:1OH (3-OH Oleylcarnitine)	0-0.06	0.02-0.14

Table 3. Amino acid and acylcarnitine cut-off values calculated on serum spot samples of healthy subjects.

	Infants (> 1 month) (µmol/L)
Alanine	89-328
Valine	63-221
Leucine+Isoleucine+hydroxyproline	63-198
Methionine	10-37
Phenylalanine	33-105
Tyrosine	32-112
Aspartatic acid	11-45
Glutammic acid	51-216
Glycine	86-320
Ornithine	13-70
Citrulline	7-45
Arginine	16-71
C0 (Free carnitine)	10.0-44.7
C2 (Acetylcarnitine)	3.5-15.4
C3 (Propionylcarnitine)	0.07-0.65
C3DC (Malonylcarnitine)	0.04-0.15
C4 (Butyrylcarnitine)	0.12-0.42
C4DC (Methylmalonylcarnitine)	0.04-0.15
C5 (Isovalerylcarnitine)	0.05-0.24
C5DC (Glutarylcarnitine)	0.03-0.17
C5:1 (Tiglylcarnitine)	0.03-0.12
C5OH (3-OH-Isovalerylcarnitine)	0.01-0.13
C6 (Hesanoylcarnitine)	0.04-0.18
C6DC (Adipylcarnitine)	0.02-0.11
C8 (Octanoylcarnitine)	0.07-0.25
C8:1 (Octenoylcarnitine)	0.03-0.40
C10 (Decanoylcarnitine)	0.09-0.43
C10:1 (Decenoylcarnitine)	0.08-0.32
C12 (Dodecanoylcarnitine)	0.04-0.21
C12:1 (Dodecenoylcarnitine)	0.04-0.20
C14 (Myristoylcarnitine)	0.03-0.15
C14:1 (Tetradecenoylcarnitine)	0.02-0.20
C14:2 (Tetradecadienoylcarnitine)	0.02-0.16
C16 (Palmitoylcarnitine)	0.01-0.23
C16:1 (Hesadecenoylcarnitine)	0.01-0.07
C16OH (3-OH-Hesadecanoylcarnitine)	0.01-0.05
C18 (Stearoylcarnitine)	0.01-0.18
C18:1 (Oleylcarnitine)	0.01-0.35
C18:1OH (3-OH Oleylcarnitine)	0.01-0.07



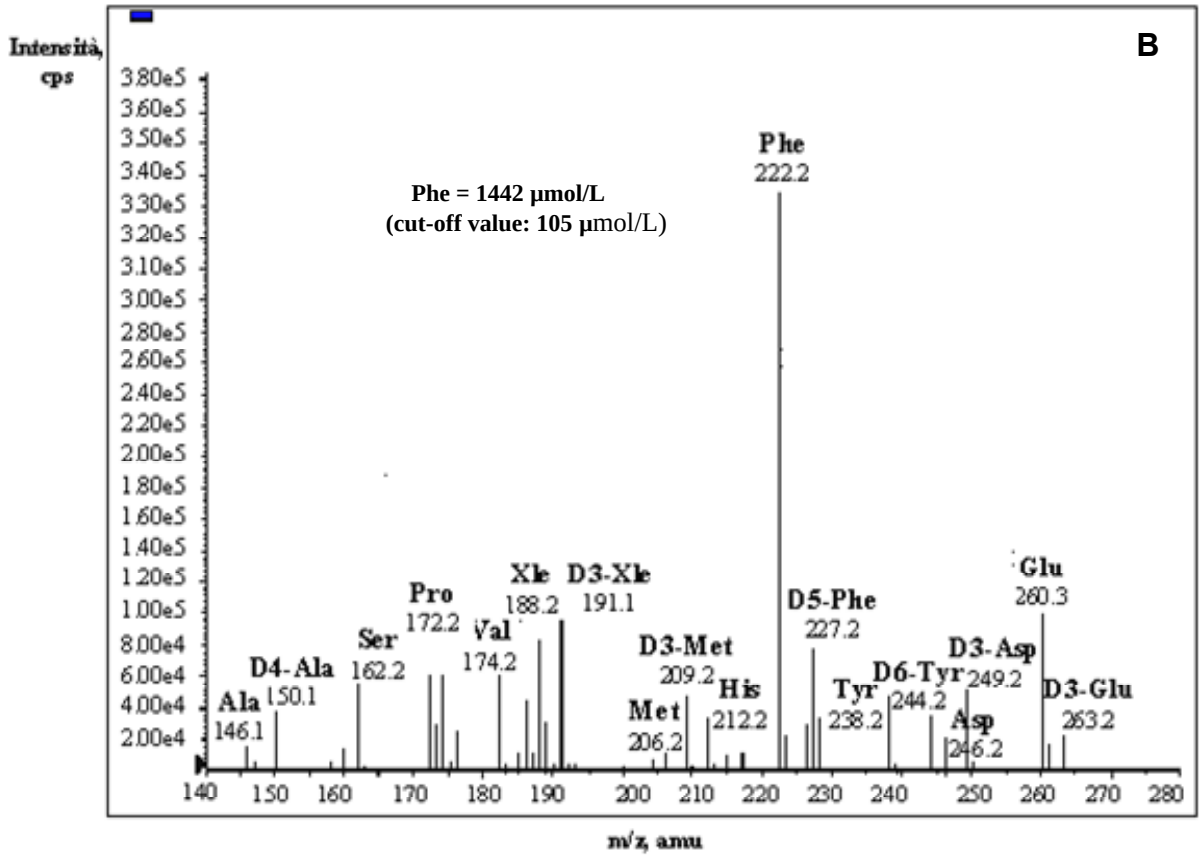


Figure 19. Blood Amino acid profiles obtained by ESI-MS/MS analysis, scanning for a constant NL of 102 Da. (A) Normal; (B) a case of PKU.

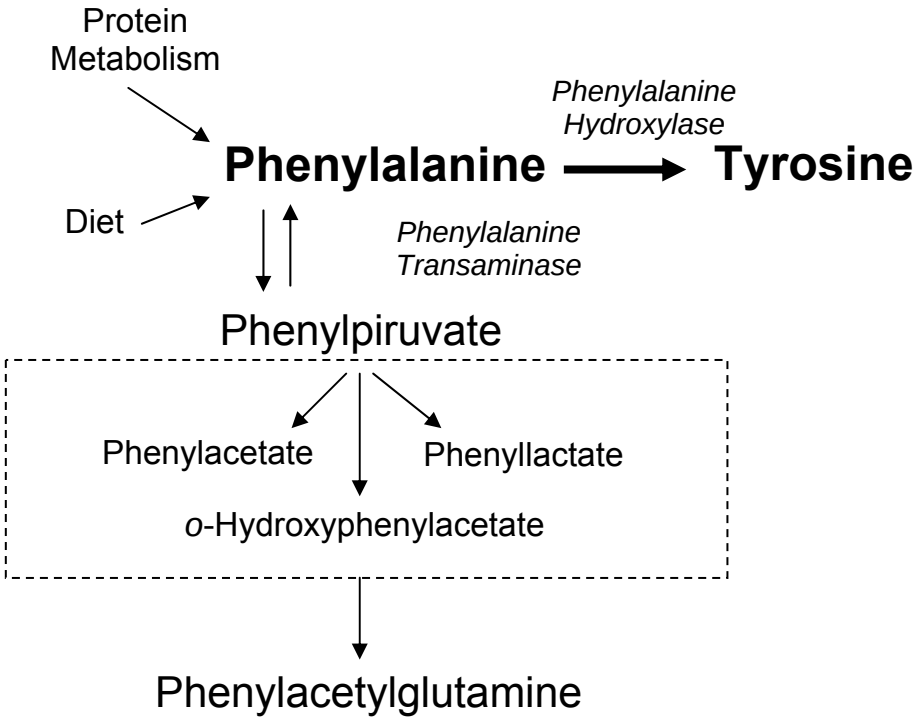
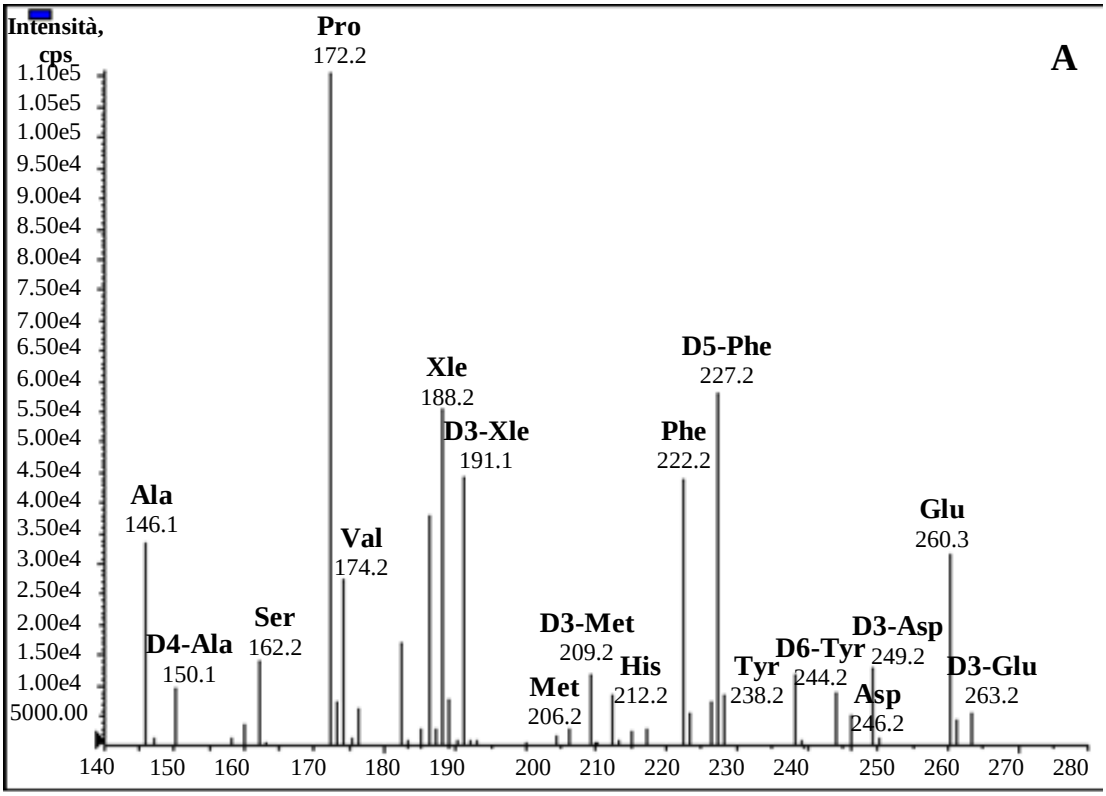


Figure 20. Overview of phenylalanine metabolism as it relates to PKU.



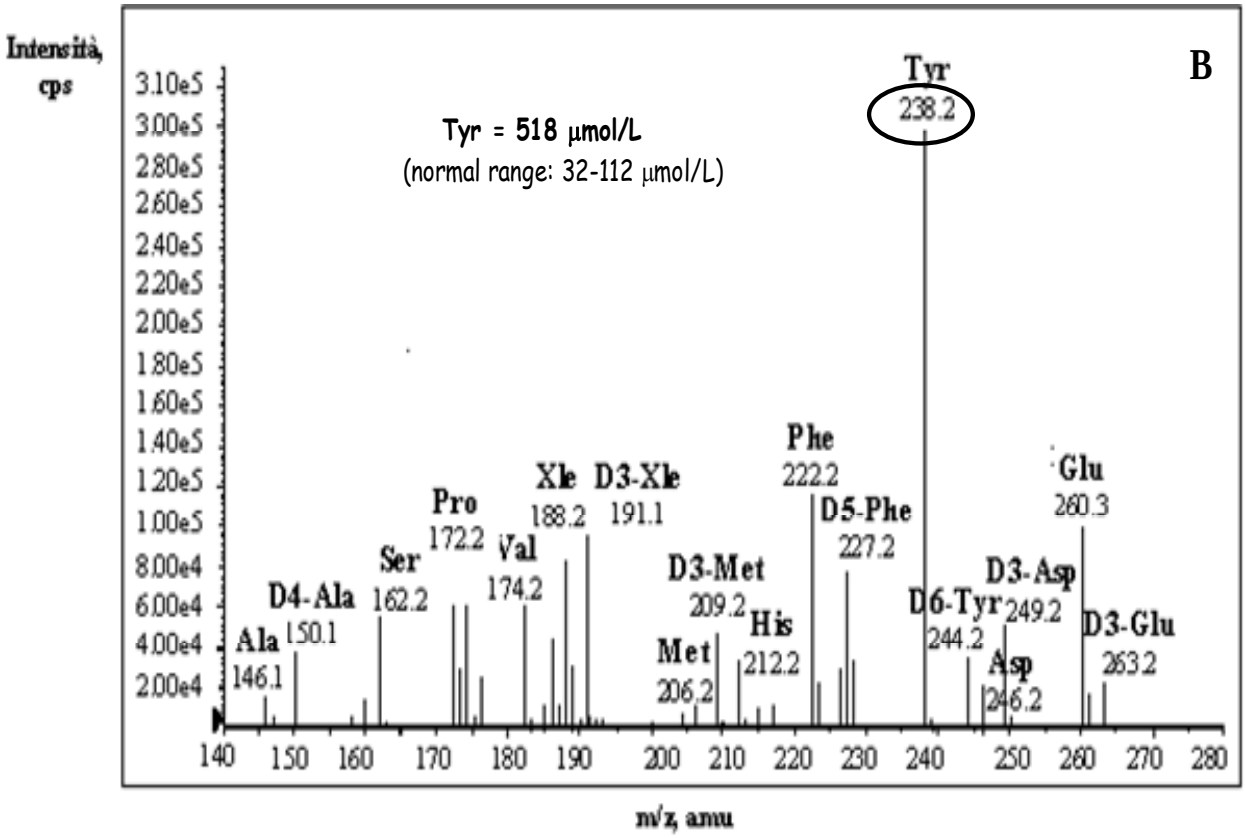


Figure 21. Blood Amino acid profiles obtained by ESI-MS/MS analysis, scanning for a constant NL of 102 Da. (A) Normal; (B) a case of Tyrosinemia type III.

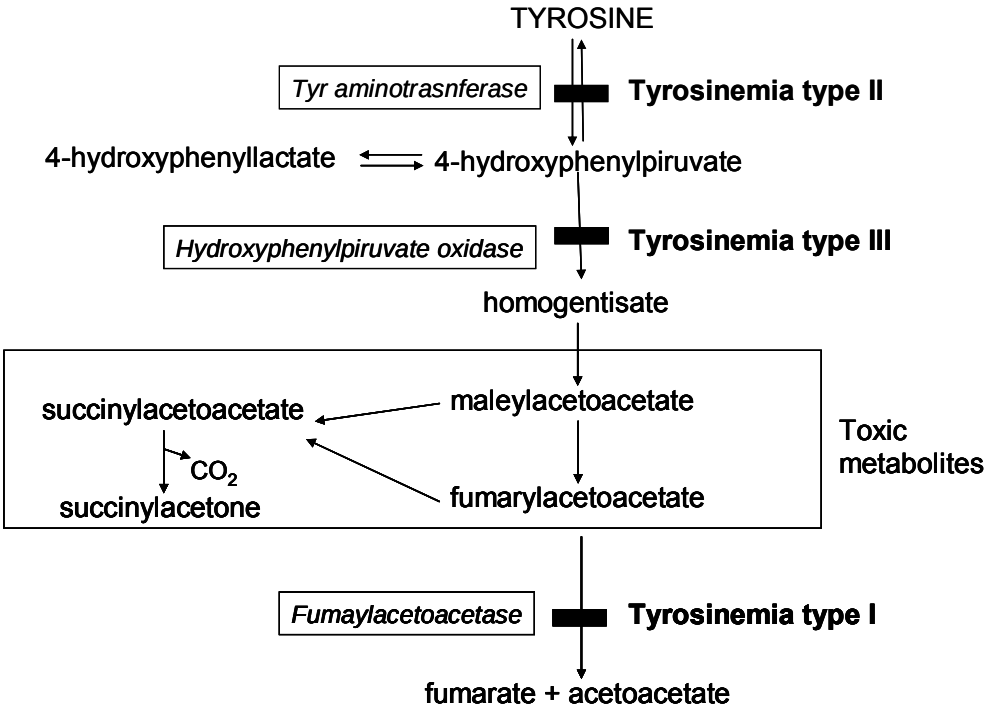


Figure 22. Metabolic pathway of Tyrosinemia type II

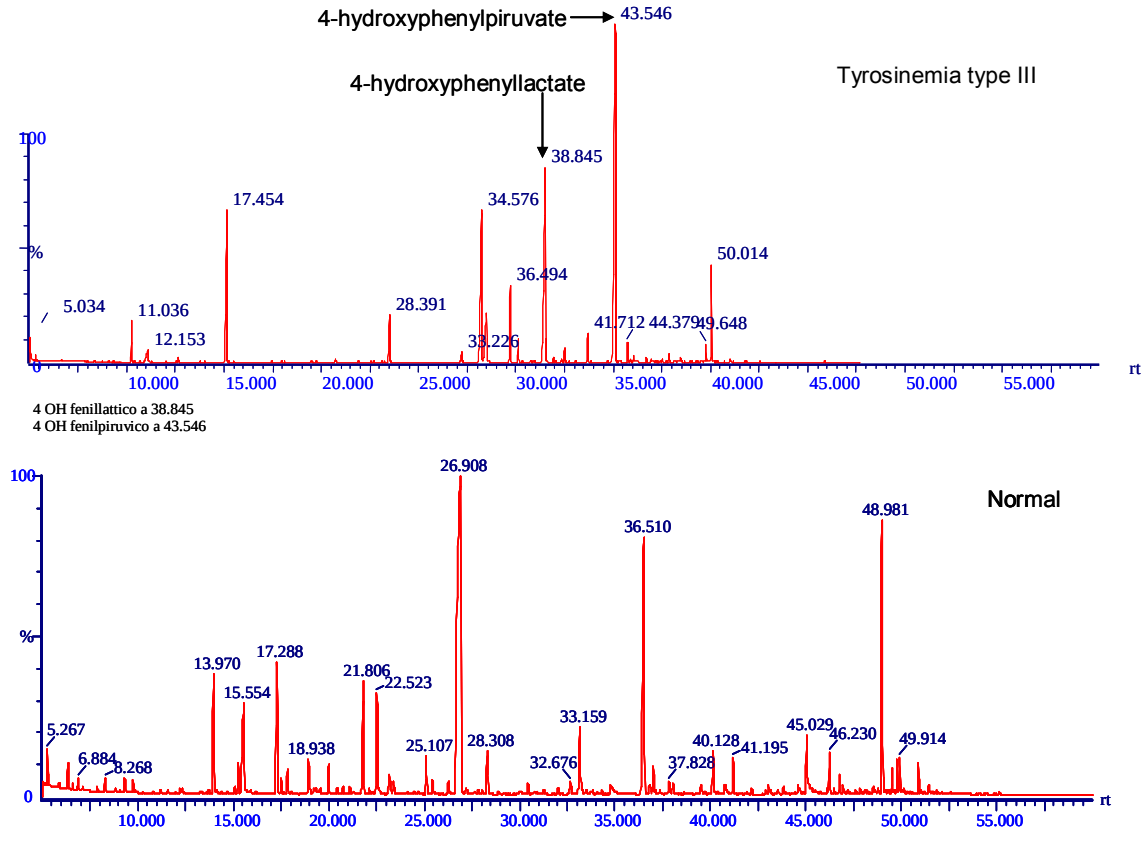
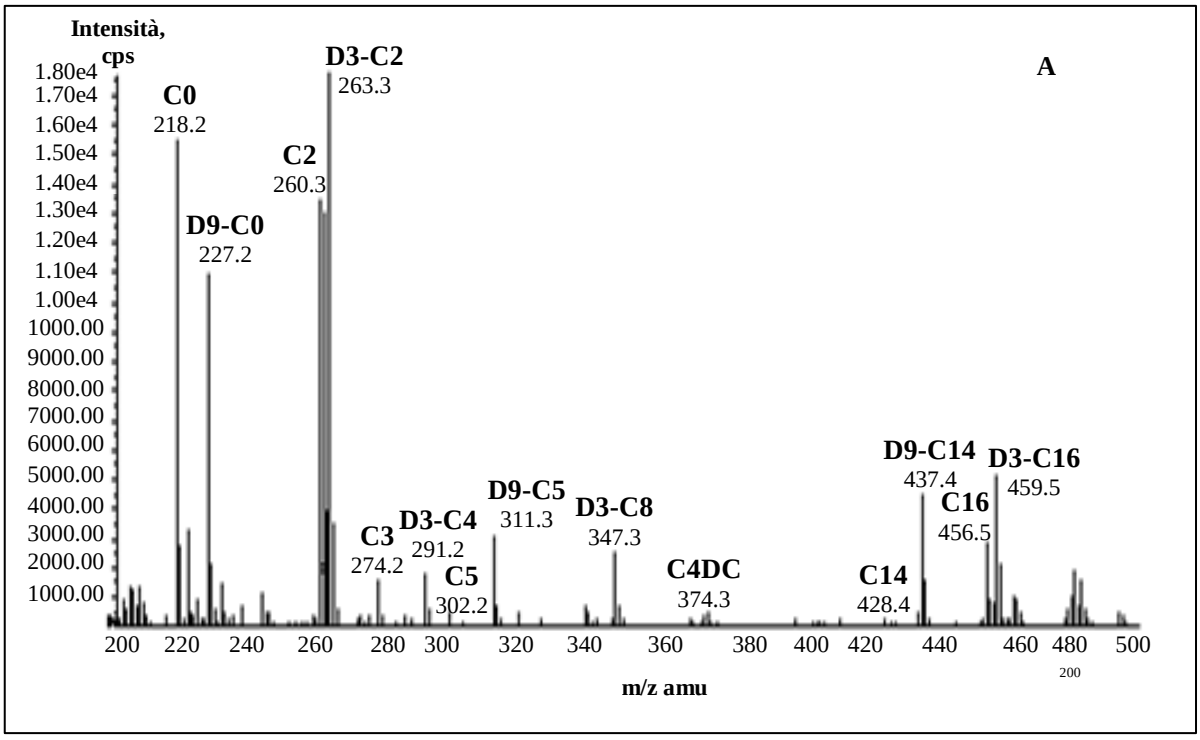


Figure 23. Organic acids profiles obtained by GC/MS analysis: Tyrosinemia type III Profile vs Normal Profile



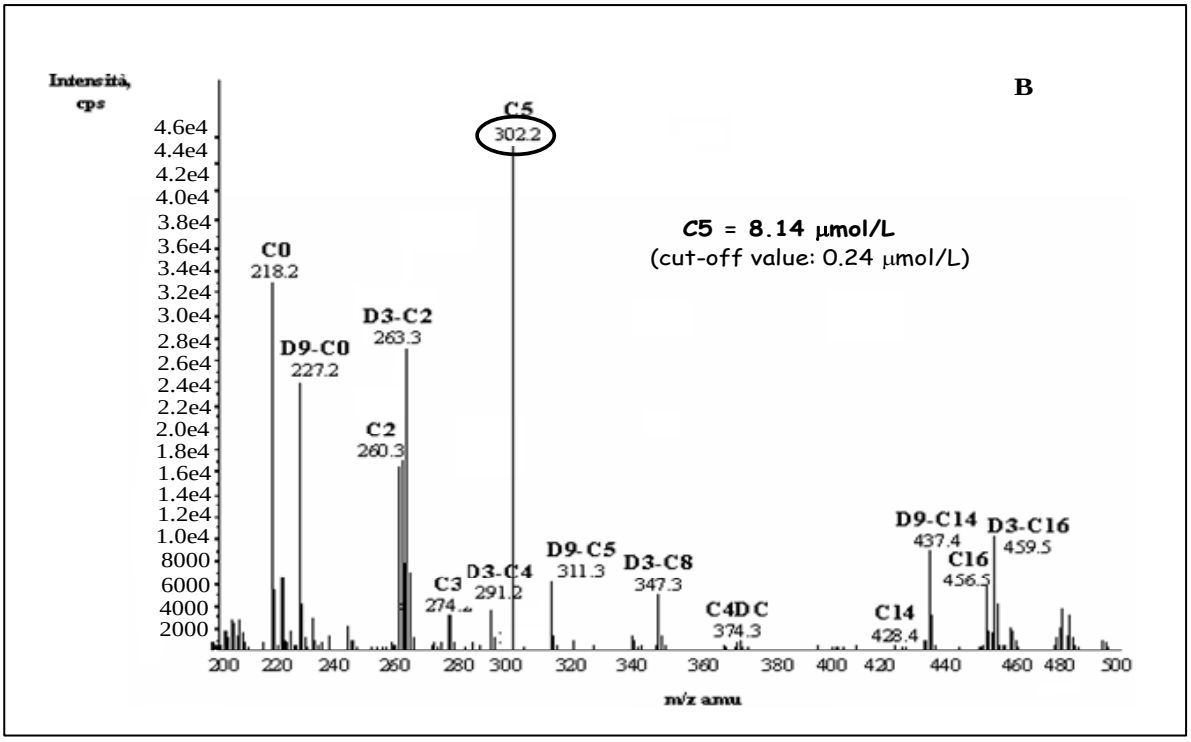


Figure 24. *Blood acylcarnitine profiles obtained by ESI-MS/MS analysis, scanning for a constant PI of 85 Da. (A) Normal; (B) a case of IVA.*

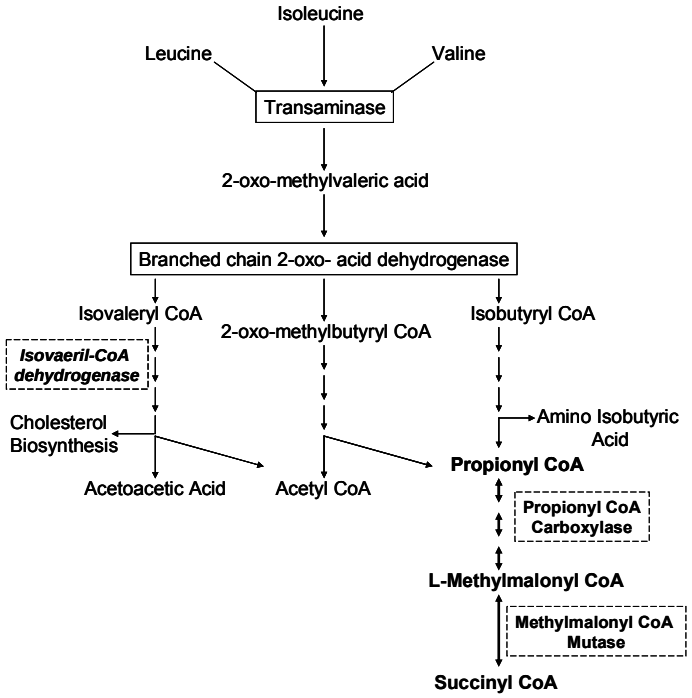


Figure 25. *Illustration of the metabolism of selected amino acids, their production of ketogenic substrates, and the subsequent formation of organic acyl CoAs from these substrates. Overview of isovaleric aciduria, Propionic aciduria and Methymalonic aciduria.*

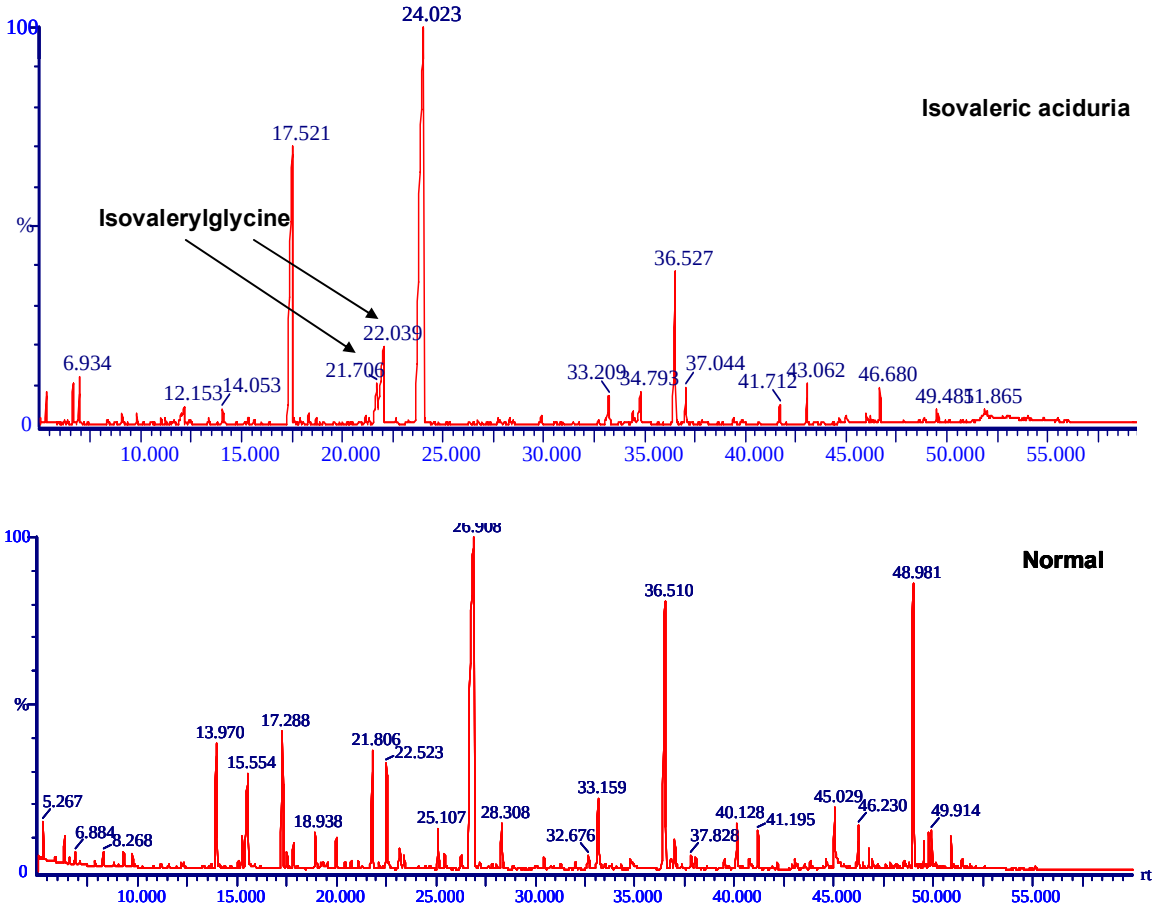
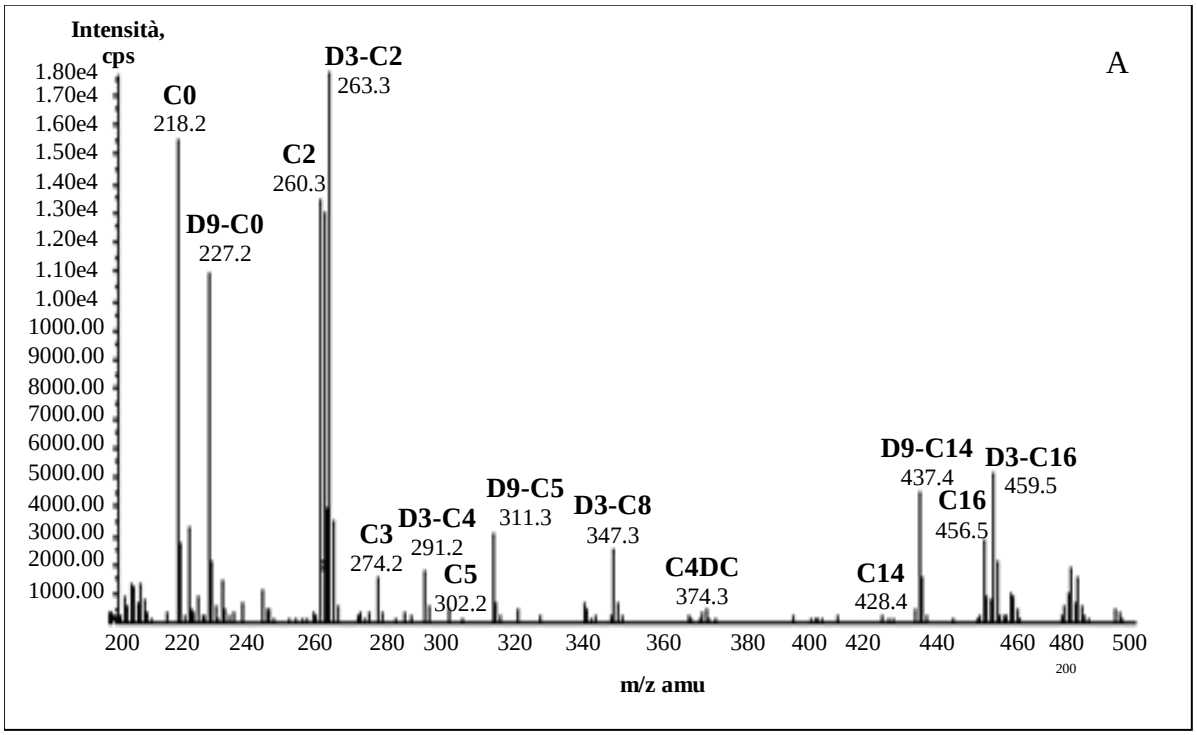


Figure 26. Organic acids profiles obtained by GC/MS analysis: Isovaleric aciduria Profile vs Normal Profile



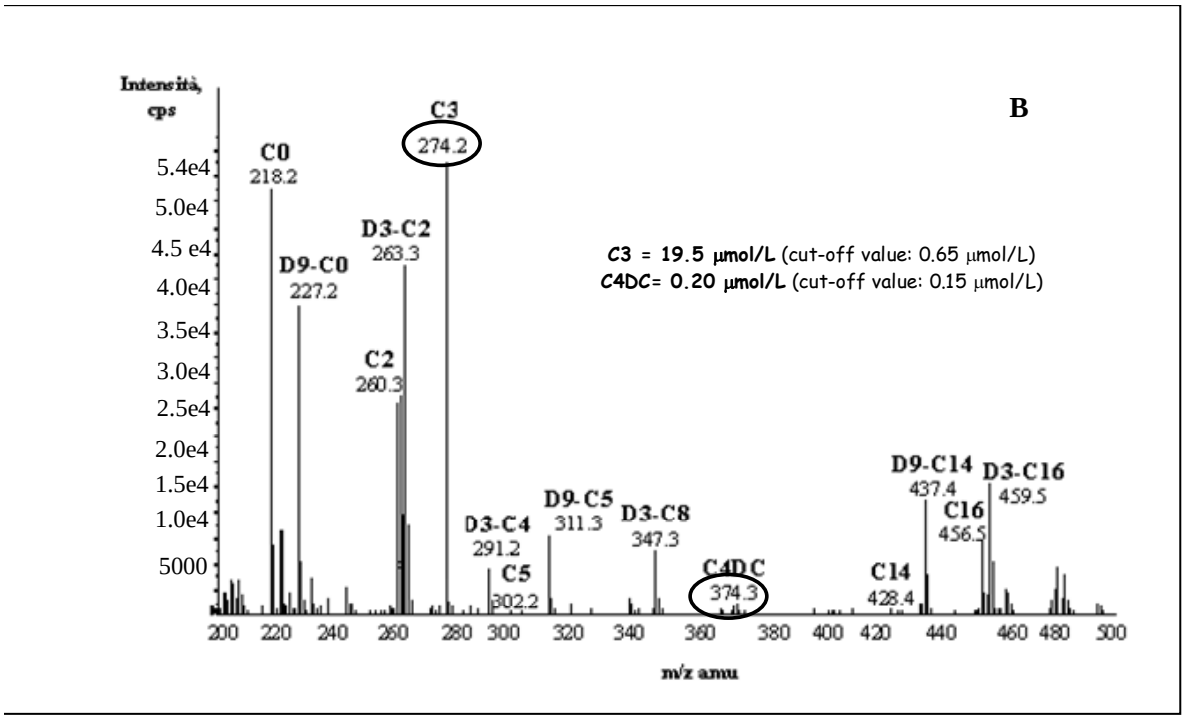
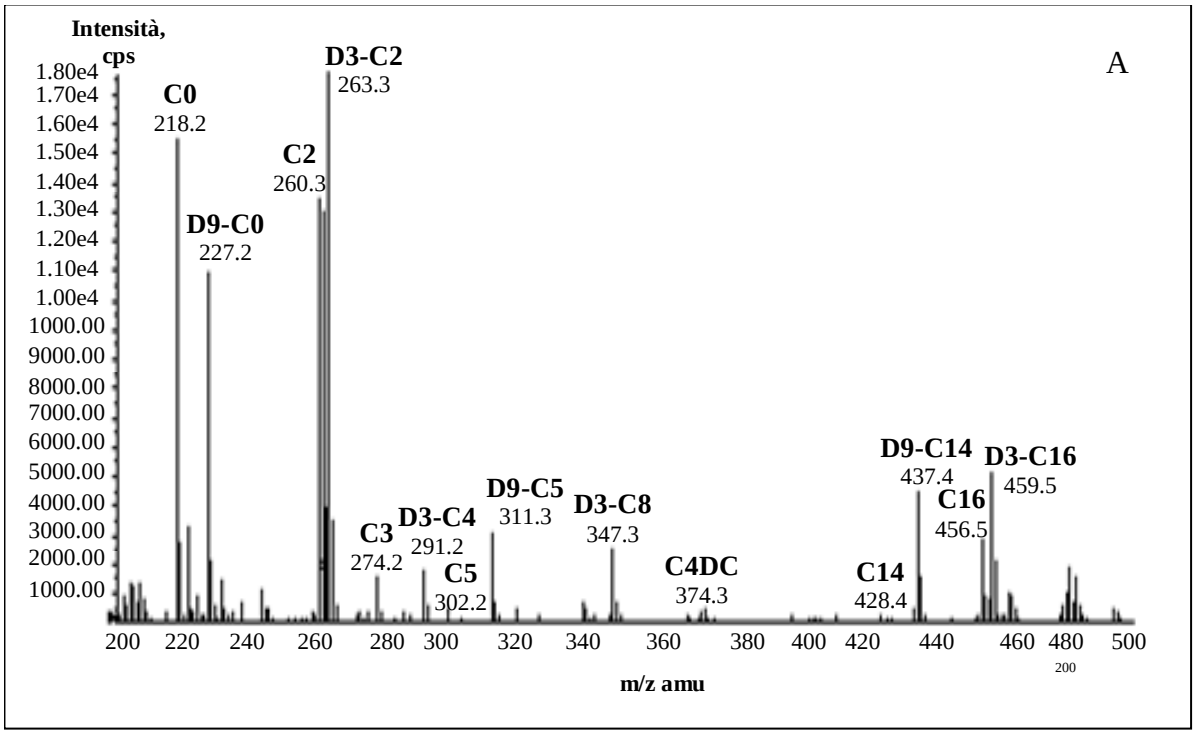


Figure 27. Blood acylcarnitine profiles obtained by ESI-MS/MS analysis, scanning for a constant PI of 85 Da. (A) Normal; (B) A case of Propionic aciduria (PA).



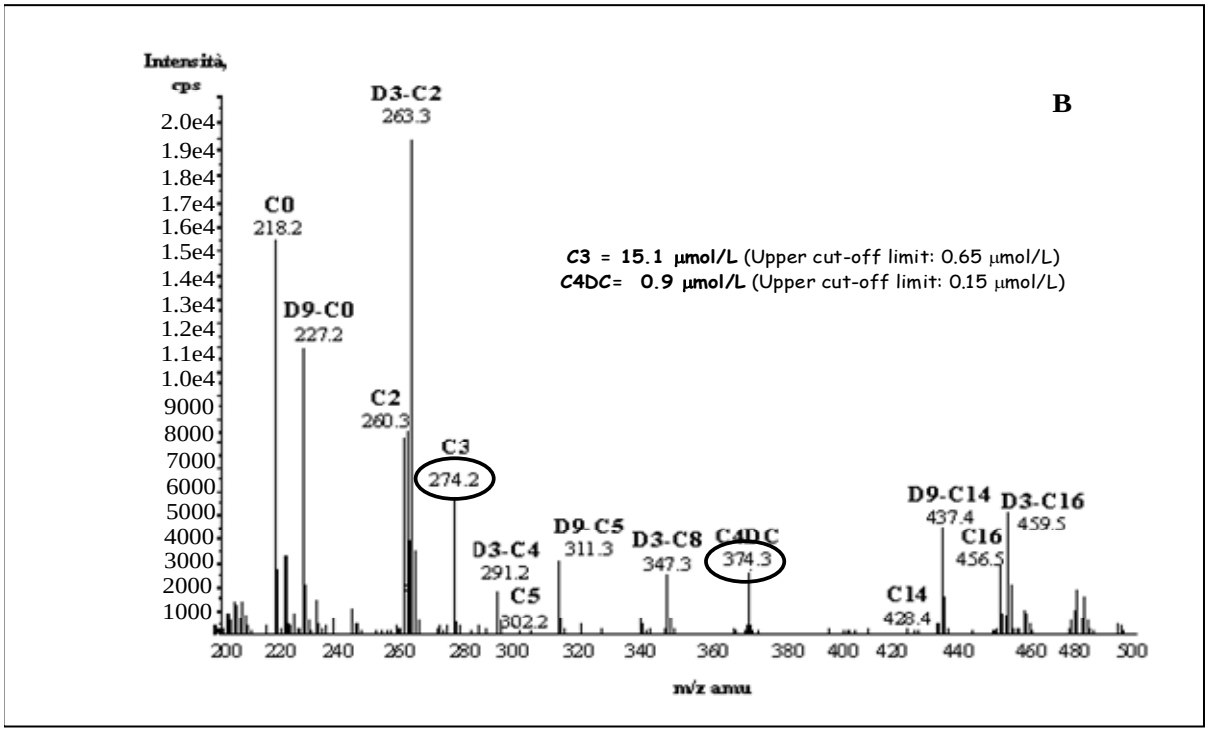


Figure 28. Blood acylcarnitine profiles obtained by ESI-MS/MS analysis, scanning for a constant PI of 85 Da. (A) Normal; (B) A case of Methylmalonic aciduria (MMA).

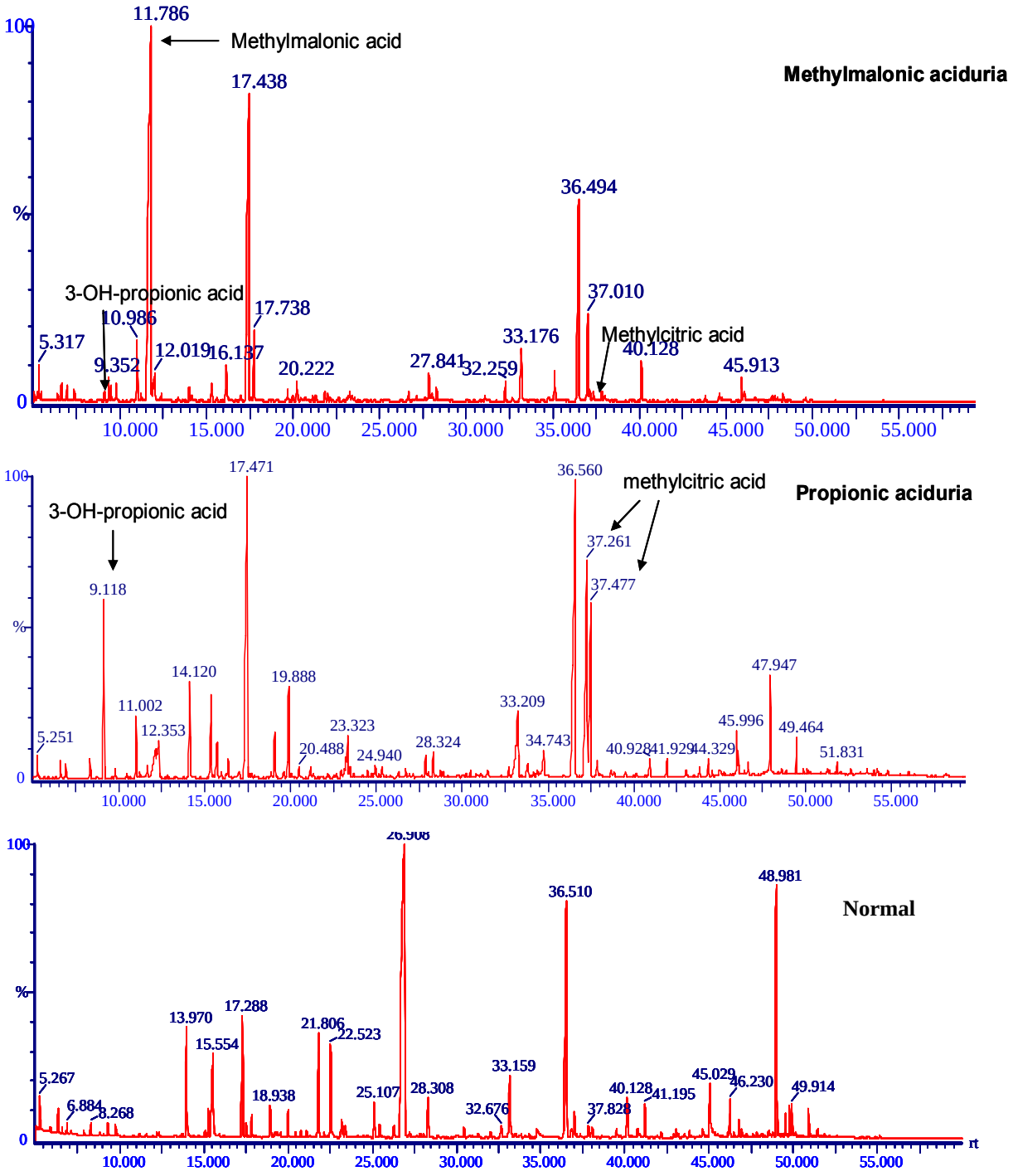


Figure 29. Organic acids profiles obtained by GC/MS analysis: Propionic and Methylmalonic aciduria Profiles vs Normal Profile

2.5.1 Accuracy and Precision

For all samples, the method reproducibility was evaluated for each analysis by repeating three times the amino acids and acylcarnitines extraction and derivatization of the same sample. The results was very good because the average precision (area CV) for n=3 samples is in the range between 4 and 8. The accuracy was assessed by standard deviation (SD) evaluation for n=3 samples. Data were demonstrated a good correlation between theoretical and experimental values for all amino acids and acylcarnitines.

2.6 Newborn Screening Quality Assurance Program

Many factors contribute to the success of a newborn metabolic screening program. In actuality, blood sample collection after birth and before hospital discharge marks the beginning of the newborn-screening process. Proper analyses rely heavily on blood-spot quality. Consequently, an improperly collected blood spot may disrupt the entire screening process for a newborn. A properly collected newborn blood specimen requires several elements, including suitable collection time, appropriate application of heel-stick blood to filter paper, sufficient drying before packaging, and timely shipment to the screening laboratory. A disturbance in any part of this sequence can lead to poor or insufficient sample analysis (Figure 30). Proper collection time avoids the possibility of dubious results affected by age. Careful spotting of the blood sample on filter paper avoids inaccurate analysis results caused by undersaturation, oversaturation and abraded specimens. Ample drying of a sample and its prompt receipt by a laboratory can prevent sample degradation (*Khoury MJ et al., 2003*).

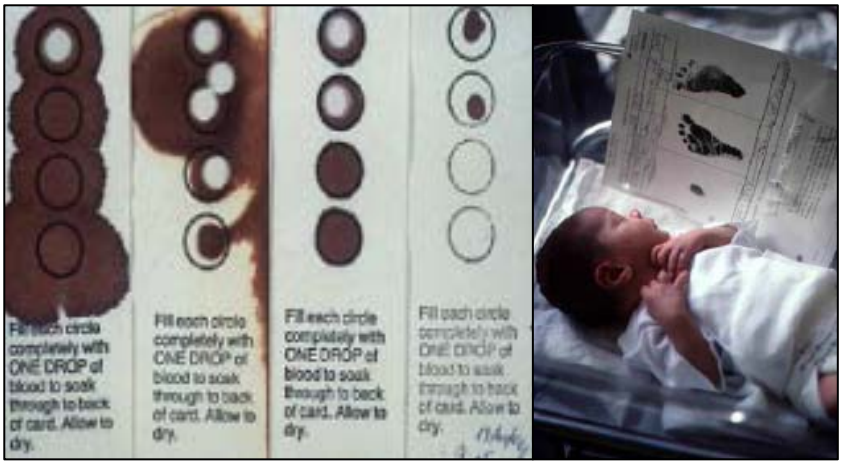


Figure 30. Examples of blood spot sample collections.

Blood-spot materials have been developed by several organizations (e.g. "*Center for Disease Control and Prevention*" CDC) to be used in newborn-screening laboratories for Quality Assurance (QA) / Quality Control (QC) (*Adam BW et al., 2000; Hannon WH et al., 1997 & Webster D et al., 1999*). One of the challenges of developing

QA/QC materials for MS/MS arises from the complexity of multianalyte systems that measure not one, but many metabolites for which a positive screening result may not be based solely on increases in a single metabolite. Therefore, were performed a newborn screening quality assurance program of “Center for Disease Control and Prevention” (CDC), an interlaboratory quality control program for amino acids and acylcarnitines that artificially creates the disease state by enrichment levels of some amino acids or acylcarnitines in the blood spot samples. The CDC is a program to help state health departments and their laboratories maintain and enhance the quality of test results.

2.7 Conclusions

The recent and continuing impact of tandem mass spectrometry (MS/MS) in newborn screening is considerable. Unquestionably, the use of MS/MS to detect metabolic disorders in newborns is one of the most important advancements in neonatal screening since the introduction of the Guthrie test for PKU because represents an effective tool in screening for multiple metabolic disorders in a single analysis (*Wilcken B et al., 2003*). MS/MS technology offers a new vision to newborn-screening programs that now have the ability to screen for 30 or more metabolic disorders in a single analysis from one small disk of dried blood or serum and in a few minutes.
Our study was designed to introduce in our laboratory the MS/MS technology for expanded newborn screening program. This approach was revealed a reliable method for the early diagnosis of different inborn errors of metabolism: amino acidemias, fatty-acid organic disorders (FAO) and organic acidurias. Now it is possible to screen rapidly, simultaneously, and inexpensively many very rare disorders. It is very important that once the MS/MS has been set-up in the laboratory with an experienced team, it is much easier to add another disorder for screening without the need for additional sample or analysis time. Due to the robustness of this technology, a number of metabolic disorders with unclear natural history have been added to the screening programs. The number of disorders is based on experience with detection of increased metabolites in blood from children affected with these disorders, although not all have been demonstrated to be detectable in the neonate. The large increase in the number of inherited metabolic disorders detectable in the newborn period because of tandem MS screening greatly extends the possibilities of early, generally presymptomatic, diagnosis and treatment to minimize morbidity and mortality for many affected children. Therefore, whether detected presymptomatically (ie, by screening) or after symptoms manifest, IEM in infants and children is expensive to manage. In the future, the goal of our expanded newborn screening program will be the establishing appropriate reference intervals for subpopulations of newborns, the update of limits to minimize false positives and false negatives, the development protocols for the differential diagnosis of suspected disorders and the compilation of incidences of disorders. Nevertheless, is very important to consider that the semi-quantitative method developed is based on the analysis of metabolites extracted from spot samples. The extraction from spot samples, because of the analytical limitations that will have indicated in the next chapter, has not sufficient statistical power to generate high confidence in the precision of the result; therefore, this analytical method can be used in rapid metabolite screening where the desired results do not need to be highly precise but rather to answer the question of whether or not a metabolite falls within a certain range. In fact, in the presence of an inborn error of metabolism, for example the classic PKU disease, the metabolite concentrations exceed unequivocally their respective cut-off levels; therefore, the positive result is very manifest. Instead, in the follow-up of patients where is evaluated the efficacy therapy by phenylalanine measurement it would be better to use a quantitative analytical method that provides the more precise result. In addition, for monitoring of phenylketonuric patients is necessary to measure only phenylalanine and tyrosine concentrations; therefore it would be time spending and expensive to detect a complete metabolic profile by the classic analytical method for newborn screening. Development of “quantitative” analytical method for the follow-up of patients Phenylketonuria affected was an other aim of this research project that will have discussed in the next chapter.

Chapter 3

3.1 Phenylketonuria (PKU)

PKU (*OMIM 261600*) and its milder variant HPA are genetic disorders characterised by a deficiency in PAH (*EC 1.14.16.1*), an enzyme that is required to metabolise L-Phenylalanine (L-Phe) to L-Tyrosine (L-Tyr). On the basis of blood Phe concentrations, PAH deficiency can be classified into classic PKU (Phe>1200 µmol/L), mild PKU (Phe=600-1200 µmol/L) and mild HPA, where blood Phe is elevated above upper reference limit, but <600 µmol/L (*Hanley WB, 2004*). The decreased PAH activity found in most forms of PKU and HPA are caused by mutations In the *PAH* gene, resulting in a non-functional PAH enzyme.
Untreated PKU is associated with an abnormal phenotype including growth failure, microcephaly, seizures and intellectual impairment caused by the accumulation of toxic by-products of Phe metabolism. The incidence of PKU or HPA is highest amongst Caucasian, occurring in approximately 1 in 10,000 births. PKU can be detected in newborn screening as performed in most Western countries, and early dietary treatment consisting of a low protein diet with Phe restriction can prevent the development of metabolic and pathological sequelae, including intellectual impairment.

3.2 History of PKU

PKU was first described by Asbjørn Følling, one of the first Norwegian physicians to apply chemical methods to the study of medicine (*Centerwall SA., 2000*). In 1934, the mother of two intellectually impaired children approached Følling to ascertain whether the strange musty odour of her children's urine might be related to their intellectual impairment (*Følling I., 1994*). The urine samples were tested for a number of substances including ketones. When ketones are present, urine usually develops a red-brown colour upon the addition of ferric chloride, but in this instance the urine yielded a dark-green colour (*Følling I., 1994*). After confirming that the unusual result was not due to any medications and repeating the test every other day for two months, Følling proceeded with a more detailed chemical analysis involving organic extraction and purification of the responsible compound, and determination of its melting point (*Følling I., 1994*). The basic elements were quantitated by combustion, and an empiric formula of $C_9H_8O_3$ derived (*Følling I., 1994*). Mild oxidation of the purified substance produced a compound which smelled of benzoic acid, leading Følling to postulate that the compound was phenylpyruvic acid (*Følling I., 1994*). There was no change in the melting point upon mixing of the unknown compound with phenylpyruvic acid thus confirming the mystery compound was indeed phenylpyruvic acid. Følling subsequently requested urine sample from 430 intellectually impaired patients from a number of local institutions and observed a similar result upon addition of ferric chloride, in a further eight individuals (*Følling I., 1994*). These eight individuals all presented with a mild complexion (often with eczema), stooping figure with broad shoulders, a spastic gait, and severe intellectual impairment (*Følling I., 1994*). Family studies of the affected individuals led to the suggestion of an inherited recessive autosomal trait. Dr. Følling suggested the name "imbecillitas phenylpyruvica" relating the intellectual impairment to the excreted

substance (Følling A., 1934), thereafter renamed “phenylketonuria” (*Penrose L., Quastel JH., 1937*). The discovery of PKU by Dr Asbjørn Følling was an important milestone in medicine. The PKU model was used to illustrate how metabolic abnormalities could have neurological effects and how treatment could dramatically affect the clinical manifestations of the disorder. The development of Guthrie’s screening test and dietary treatment led to the prevention of intellectual impairment in affected children throughout the world. Furthermore, the PKU model has since been used as a template to shed light on over 200 other inborn errors of metabolism (*Applegarth DA. et al., 2000 & Raughuveer TS. et al., 2006*).

3.3 Biochemistry of PKU

Phe exists as D and L enantiomers and L-Phe is an essential amino acid required for protein synthesis in humans (*Young VR., Pellet PL., 1987*) (Figure 1).

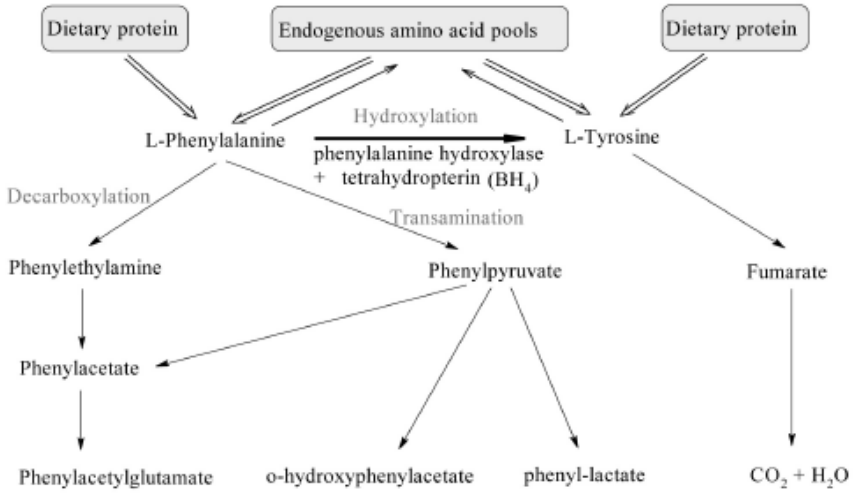


Figure 1. Phe metabolism in humans. Intake of L-Phe is via the diet and it is recycled through amino acid pools. Hydroxylation by PAH with its cofactor BH4, in the presence of molecular O2, produces L-Tyr. Alternative metabolism of L-Phe by decarboxylation or transamination produces various metabolites which are excreted in urine. Based on Scriver CR and Kaufman S (2001).15

As with many other metabolites, Phe concentrations are regulated to a steady-state level with dynamic input and run out flux. Persistent disturbance to the flux will eventually result in alteration of the steady-state concentrations. Dietary intake of Phe along with endogenous recycling of amino acid stores are the major sources of Phe, whereas, utilisation or run out of Phe occurs via integration into proteins, oxidation to Tyr or conversion to other metabolites (*Scriver CR., Kaufman S., 2001*).
The conversion of Phe to Tyr occurs by a hydroxylating system consisting of: 1) PAH, 2) the unconjugated pterin cofactor, tetrahydrobiopterin (BH_4), 3) enzymes which serve to regenerate BH_4, namely dihydropteridine reductase and 4α-carbinolamine dehydratase. While the *para*-hydroxylation of Phe is essential for the rupture of the benzene ring, it is not required for further metabolism of the alanine side chain (*Kaufman S., 1999*). This alternative pathway of transamination and decarboxylation leads to the formation of metabolites such as phenylpiruvate, phenyllactate and *o*-

hydroxyphenylacetate which are excreted in urine. Conversion of Phe to Tyr (Figure 2) has two outcomes. First, it drives the endogenous production of the non-essential amino acid Tyr (*Kaufman S., 1999*). Second, the hydroxylation reaction is the rate limiting step for complete oxidation of Phe to CO_2 and H_2O and contributes to the pool of glucose and 2-carbon metabolites (*Scriver CR., Kaufman S., 2001 & Hufton SE et al., 1995*).

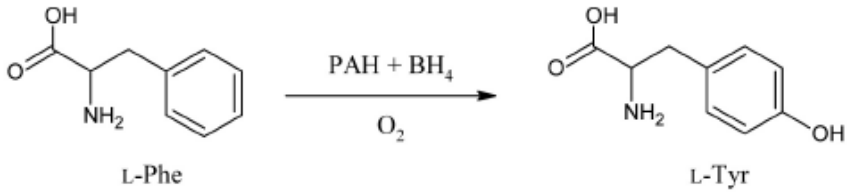


Figure 2. Conversion of Phe to Tyr is via a pathway involving the para-hydroxylation of the benzene by PAH, the cofactor BH4 and molecular oxygen.

A number of rare, related disorders due to defects in the BH_4 regeneration system can also affect Phe homeostasis and catecholamine and serotonin biosynthesis, as this cofactor is common to the Phe, Tyr and tryptophan (Trp) hydroxylating enzymes (*Blau N. et al., 2001*). PAH catalyses the stereospecific hydroxylation of L-Phe, the committed step in the degradation of this amino acid. Phe catabolism and PAH activity is mainly associated with the liver, although minor activity has been demonstrated in rat kidney (*Richardson SC. et al., 1993*). In humans, the PAH enzyme exists as a mixture of tetramers and dimers; the monomer is about 50 KDa in size and is comprised of 452 amino acids (*Hufton SE et al., 1995*). The enzyme PAH requires BH_4 as a cofactor, as well as molecular oxygen for its activity.
PAH can be divided into a number of functional domains (Figure 3).

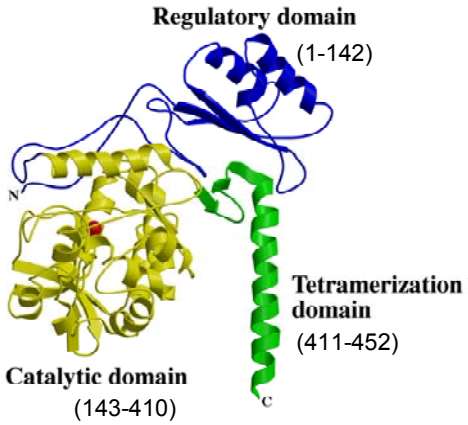


Figure 3. The domain structure of PAH. Each PAH subunit is classified into three functional domains which are involved with regulation, catalytic activity, and subunit binding.

The regulatory domain contains a serine residue which is thought to be involved in activation by phosphorylation. The catalytic domain contains a motif of 26 or 27

amino acids responsible for cofactor and ferric iron binding. The C-terminal domain is thought to be associated with inter-subunit binding (*Hufton SE et al., 1995*) (Figure 4).

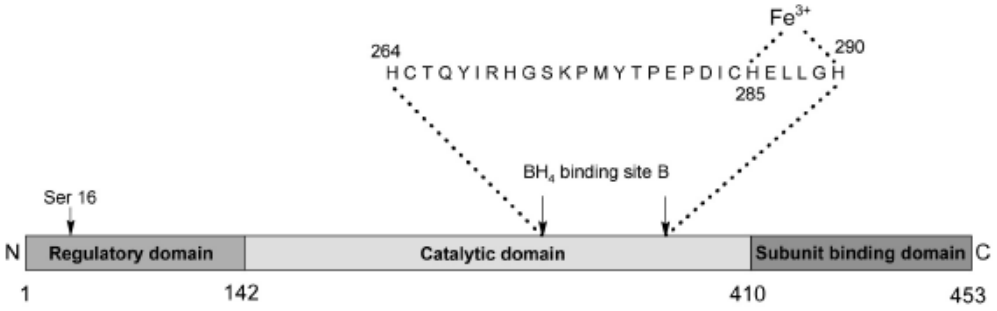


Figure 4. Structural components of PAH. The catalytic domain of PAH contains a motif of 26 or 27 amino acids which are responsible for ferric iron and cofactor (BH4) binding. Adapted from Huften IG et al (1995).17

PAH is regulated by a number of possible mechanisms. After a protein meal, it is postulated that the increased Phe in the amino acid pool causes a release of glucagons from the pancreas (*Kaufman S., 1986*).
Hepatic PAH is subject to control by cAMP-dependent protein kinase and α-adrenergic agent stimulated Ca^{2+}/calmodulin-dependent protein kinase phosphorylation–dephosphorylation processes (*Richardson SC. et al., 1993*). It has been further reported that these control mechanisms influence BH_4 co-factor interaction with PAH (*Richardson SC. et al., 1993*). In addition, there is evidence that Phe may also be able to cause a conformational change in PAH, as well as up-regulate cAMP activity (*Hufton SE et al., 1995*). X-ray crystallographic studies are consistent with these mechanisms (*Andersen OA et al., 2002*). Taken together, these mechanisms enable fine regulation of Phe concentrations by balancing levels sufficient for maintenance of protein biosynthesis while minimising tissue exposure to high concentrations of Phe.

3.4 Genetics of PKU

The human *PAH* gene (Figure 5) is located on chromosome 12q23.2, spans about 171 kb and contains 13 exons (*Konecki DS et al., 1992 & Lidsky AS et al., 1985*).
The total cDNA length is about 2.4 kb; it encodes for a polypeptide of 452 amino acids of near identical sequence to the human PAH protein, indicating little post translational modification (*Lidsky AS et al., 1985*).
HPA can be caused by either mutations at the *PAH* locus, which results in PKU, or from mutations in a number of loci which effect BH_4 synthesis and regeneration resulting in non-PKU HPA (*Scriver CR., Kaufman S., 2001).*

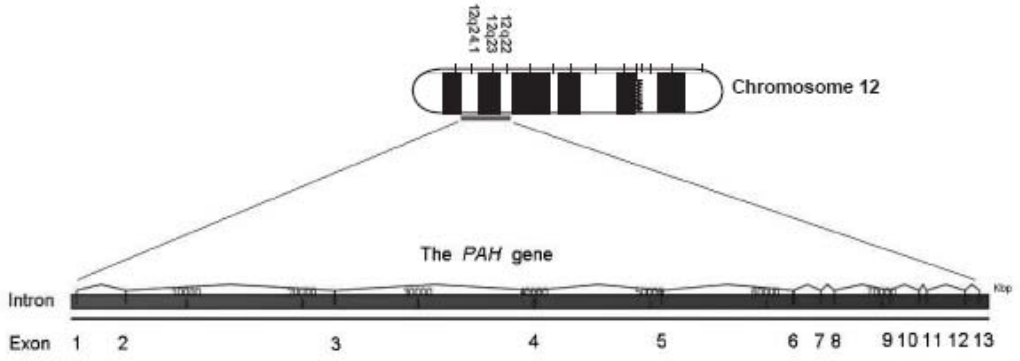


Figure 5. The basic structure of the human PAH gene. Found on the long arm of chromosome 12 (12q23.2), the human PAH gene contains 13 exons which encode a polypeptide of 452 amino acids.

Mutations can either be neutral with respect to phenotype, or pathogenic due to their disruption to enzyme structure and function. More than 500 disease-causing mutations have been identified in patients with PKU or HPA and recorded on the mutation database for *PAH (PAH: Phenylalanine hydroxylase locus knowledgebase. http://www.pahdb.mcgill.ca/ Accessed 12 April 2007*).

The human *PAH* gene shows great allelic variation and pathogenic mutations have been described in all 13 exons of the *PAH* gene and its flanking region. The mutations can be of various types: 1) missense mutations: 62% of *PAH* alleles; 2) small or large deletions: 13%; 3) splicing defects: 11%; 4) silent polymorphisms: 6%; 5) nonsense mutations: 5%; 6) insertions: 2%.

Since the *PAH* gene is biallelic, and there are many disease causing mutations, most patients are compound heterozygotes (*Michals-Matalon K., 2001*). Some *PAH* mutations are more severe than others depending upon their effect on enzyme structure and function. However, the effect of *PAH* mutations on the clinical phenotype is variable (*Gizewska M et al., 2003 & Kayaalp E et al., 1997*). Compliance of dietary treatment is the primary factor which dictates blood Phe concentration and so plays a pivotal role in the severity of the clinical manifestations of PKU in an individual. The effect of a specific mutation on enzyme function can be assessed through a number of approaches including enzyme assays, in vivo isotopic studies or by analysis of in vitro gene expression (*Kayaalp E et al., 1997*). Given that the crystal structures of the catalytic and regulatory domains have now been resolved to 2 Å, a fourth approach by "virtual" molecular modelling techniques (*in silico*) is also possible (*Andersen OA et al., 2002*).

Large studies have shown a good correlation between the severity of mutation and hydroxylation rates in most individuals (*Treacy EP et al., 1997*). However, exceptions do occur and this is to be expected since hydroxylation by PAH is also dependent on availability of its cofactor BH_4. in this regard, some *PAH* genotypes are known to be more BH_4-responsive than others (*BH4 – Tetrahydrobiopterin. http://www.bh4.org Accessed 12 April 2007*). Although intelligence quotient (IQ) score is one of the most complex of human traits, some correlation between the severity of *PAH* mutations and IQ has been observed (*Ramus SJ et al., 1993*).

The incidence of PKU in Caucasian populations is between 1 in 10,000 and 1 in 15,000 people (Table 1).

Table 1. Incidence of PKU by population. Adapted from Scriver and Kaufman (2001).[15]

Region / Country		Incidence of PKU
Asian Populations	China	1 : 17,000
	Japan	1 : 125,000
	Turkey	1 : 2,600
	Yemenite Jews (in Israel)	1 : 5,300
European Populations	Scotland	1 : 5,300
	Czechoslovakia	1 : 7,000
	Hungary	1 : 11,000
	Denmark	1 : 12,000
	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
Arabic Populations		Up to 1 : 6,000
Oceania	Australia	1: 10,000

It has been suggested that the high incidence of PKU in Turkey is due to the high prevalence of consanguinity and the low incidence seen in Finland and Japan is due to a pronounced negative founder effect in Finland and genetic drift in the founding of the Japanese island population (*Guldberg P et al., 1995 & Okano Y et al., 1992*).
Untreated PKU is associated with an abnormal phenotype including growth failure, microcephaly, seizures and intellectual impairment caused by the accumulation of toxic by-products of Phe. Moreover, decreased or absent PAH activity can lead to a deficiency of Tyr and its downstream products, including melanin, L-thyroxine and the catecholamine neurotransmitters (*Scriver CR., Kaufman S., 2001*).
The effect of PAH mutations on hepatic enzyme function and the resultant disruption to Phe homeostasis has been well described, but the major clinical effect in PKU relates to brain development and cognitive function (*Kayaalp E et al., 1997*).
The mechanism by which high Phe concentrations result in intellectual impairment is yet to be clarified, but the hallmark of the neuropathology seen in both treated and untreated PKU seems to involve hypomyelination and demyelination (*Dyer CA, 1995*).
A number of factors have been as contributing to the neurotoxicity in PKU, including: Tyr deficiency, the effect of elevated Phe concentrations on transport of other metabolites across the blood brain barrier, and the effects of a potential and relative Tyr deficiency on neurochemistry and metabolism.
Tyr is converted to L-DOPA (3,4-dihydroxy-L-Phe), a precursor of dopamine and other catecholamine neurochemicals. It has been suggested that the lack of PAH activity results in relative Tyr deficiency for the foetus, with mothers that are carriers for PKU possibly having limited supply of Tyr (*Scriver CR., Kaufman S., 2001*). However, there are observations which do not support this suggestion: a) restriction of Phe alone should not prevent the development of the phenotype, and b) supplementation of Tyr alone in the post-natal period does not prevent developmental defects nor improve neuropsychological functions. The L-type amino acid carrier is the sole transporter of large neutral amino acids (LNAA), which includes Phe, across the blood brain barrier. The competition for this carrier when elevated concentrations of Phe exist may have the effect of blocking transport of the serotonin and catecholamine precursors Tyr and Trp (*Pietz J et al., 1999*). Whilst some have proposed a strong causal link between these disrupted transport

mechanisms and PKU, others dispute this claim (*Pietz J et al., 1999 & Hommes FA, 1989*). Magnetic resonance imaging (MRI) has shown white matter lesions in the brain of adult PKU patients, the size and number of which directly relate with blood Phe concentrations (*Thompson AJ et al., 1993*). Such changes have been reversed by lowering blood Phe levels (*Cleary MA et al., 1995 & Walter JH et al., 1997*).
This has led to the establishment of Phe treatment targets (birth to 8 years, Phe 100-350 µmol/L; older children and adults, Phe <700 µmol/L) (*Australian Society for Inborn Errors of Metabolism. PKU handbook. Dennison B, editor. Alexandra, Australia: Human Genetics Society of Australasia; 2005 & Blau N, Burgard P, 2006*).
A recent meta-analysis of the cognitive profile of adolescents and adults with PKU compared with control subjects showed significantly reduced Full Scale IQ, processing speed, motor control and inhibitory abilities, and reduced performance on tests of attention in the PKU groups (*Moyle JJ et al., 2007*).
The link between MRI changes and cognition in PKU remains elusive, although the use of electroencephalogram and event-related potentials has been shown to be a useful tool in identifying the loci of neurophysiological deficiencies during cognitive tasks in PKU (*Moyle JJ et al., 2006*).

3.5 Screening and Diagnosis of PKU

Since the late 1960s, and following the development of Guthrie's biochemical assay for diagnosis of PKU and a number of other diseases, Australian health services have been conducting newborn screening programs as part of a worldwide initiative (*Muchamore I et al., 2006*).
Collection of newborn blood by heel prick onto filter paper cards has become an accepted facet of newborn care throughtout the modern world (*AAP Newborn Screening Task Force. Newborn screening: a blueprint for the future. A call for a national agenda on state newborn screening programs. Pediatrics 2000; 106 (Suppl): 389-422*). These blood spots can be analysed by a variety of methods to (in the case of PKU) measure the Phe concentration in the blood (*Scriver CR., Kaufman S., 2001*).
More recently, tandem mass spectrometry has been developed as a routine method for newborn screening tests.
The cost effectiveness of this new approach has enabled an increase in the number of disorders screened in these programs form four (PKU, congenital hypothyroidism, galactosemia and cystic fibrosis) to over 20 (*WA newborn Screening Program. Expanded newborn screening: information for health professionals. Perth, Australia: Departement of Health, Government of Western Australia; 2004*) (*Scriver CR., Kaufman S., 2001*).
A positive screening result identifies an infant with HPA, but it is possible that the increased Phe concentrations may be transient due to other non-PKU disease (e.g. transient 4α-carbinolamine dehydratase deficiency) or be a result of maternal HPA. Of the children with persistent HPA, over 98% have the condition because of mutations at the *PAH* locus (*Scriver CR., Kaufman S., 2001*).
Diagnosis of HPA is made on the basis of an elevated blood Phe concentration on a repeat blood sample. The upper reference limit for Phe in whole blood or plasma in neonates is <150 µmol/L and slightly lower (<120 µmol/L) in older children (*Scriver CR., Kaufman S., 2001*).

The measurement of Phe metabolites in urine is not an accepted PKU screening method as excretion depends upon transaminase activity (which can be low in neonates) and great variation between blood Phe and urine metabolite concentrations have been demonstrated (*Knox WE, 1970*).
Differential diagnosis of PKU from the disorders of synthesis or recycling of BH_4 may involve various testing regimes including BH_4 loading tests, measurement of urine and plasma pterin metabolites and neurotransmitter metabolites as well as blood spot dihydropteridine reductase measurement (*Scriver CR., Kaufman S., 2001*). Tests targeting direct enzyme measurement (PAH, 4α-carbinolamine dehydratase) would require tissue biopsy.
Conventional PKU diagnosis is based on the aberrant metabolic phenotype, disease causing mutations and associated polymorphic haplotypes can be analysed at the *PAH* locus. PKU mutation analysis is particularly useful in the detection of carriers, for prenatal diagnosis.
A wide variety of molecular genetic techniques have been utilised including Southern blotting, restriction enzyme digestion, detection of mutations by sequencing and multiplex ligation probe amplification (*Desviat LR et al., 2006 & Kozak L et al., 2006*).

3.6 Treatment of PKU

The foundation of PKU treatment is a low Phe diet which, by reducing or normalising Phe concentrations, prevents the development of the neurological and psychological changes. Since neurological changes have been demonstrated within one month of birth, it is recommended that dietary restriction should be started early and be continued through childhood when neural development is maximal (*Konecki DS et al., 1992*).
Clinical neurological abnormalities, affected neuropsychological performance and brain imaging in adults with PKU has led to a consensus opinion that the PKU diet should be followed for life (*Phenylketonuria (PKU): screening and management. NIH Consensus statement 2000; 17:1-33 & Management of PKU: A consensus document for the diagnosis and management of children, adolescents and adults with phenylketonuria, The National Society for Phenylketonuria (United Kingdom) Ltd; 2004*). An ever more stringent regime of Phe restriction is required for women with PKU contemplating starting a family, particularly during pregnancy, as elevated blood Phe concentrations are teratogenic towards the developing foetus.
A low Phe diet is used for treatment with, initially, the small amounts of Phe coming from breast milk or commercial infant formula considered sufficient intake in babies. In older children, protein intake is calculated each day, whereby a child is allocated a certain number of grams or units of daily protein, depending upon longitudinal plasma Phe concentrations. Food such as eggs, milk, cheese, meat, poultry, fish, dried beans and legumes which are high in protein are excluded from the diet (*Michals-Matalon K, 2001*). However, this regime would not normally provide enough protein for growth requirements and therefore, commercially available supplements of essential amino acids, lacking Phe, need to be taken on a daily basis. Children undergo regular blood Phe testing which, in concert with complete food diaries, is used by dieticians to make adjustments to the recommended diets (*Michals-Matalon K, 2001*). A special problem persists in the PKU diet with aspartame (L-aspartil-L-Phe

metyl ester) an artificial sweetener which, when metabolised, releases Phe, L-aspartic acid and methanol.
The benefits of the Phe-restricted, low protein diet, are clear and include: avoidance of the biochemical abnormality (increased Phe concentrations), improved neurological and psychological performance, and prevention of neurological damage. However, dietary treatment does not come without challenges such as: compliance with the diet, the requirement of social support, and risk of imbalances in essential dietary nutrients (*Hanley WB, 2004*). Blood Phe concentrations are determined by both the severity of the *PAH* mutation and by intake of Phe through dietary protein. In milder forms of PKU (non-PKU HPA), where there is residual enzyme activity, blood Phe concentrations may be only mildly elevated even under conditions of poor treatment diet compliance. It is accepted that the best indicator of dietary compliance in classical PKU is regular monitoring of blood Phe concentrations.
The Phe-restricted diet with semi-synthetic supplementation is not without risk. PKU patients under dietary treatment can have low concentrations of trace elements and cholesterol, and some disturbance to folate metabolism as well as distortion of their fatty acid profile (*Lucock M et al., 2002, Moseley K et al., 2002 & Schulpis KH et al., 2004*). However, it remains controversial as to whether all of these dietary imbalances occur as a result of insufficient intake and supplementation, or are due to endogenous disturbances in the biosynthesis of these essential constituents.
The finding of low serum cholesterol concentrations in PKU patients, whilst having been clearly established by various groups (presumably across a wide genotypic spectrum) remains unexplained (*Acosta PB et al., 1973, Colome C et al., 2001, DeClue TJ et al., 1991, Galluzzo CR et al., 1985 & Verduci E et al., 2004*).This association could be due to dietary influences, variation in lipoprotein metabolisms between individuals due to their lipid controlling mechanisms, or perhaps due to influence of increased concentrations of Phe or its metabolites. While much work remains to be done, the low cholesterol concentrations in PKU may have broader implications to both the pathophysiology of PKU and the development of cardiovascular disease (CVD). Animal models have been used to demonstrate inhibition of sterol synthesis by various phenolic compounds (*Ranganathan S, Ramasarma T, 1973, & Shama Bhat C, Ramasarma T, 1979*). This led to work that investigated inhibition of a number of important enzymes involved in cholesterol biosynthesis; again in animal models under conditions of induced HPA (*Castillo M et al., 1988*). Dietary supplementation of L-Phe has been shown to be associated with inhibition of the rate determining enzyme for cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A reductase (*EC 1.1.1.88*), in the liver and brain and decreased concentrations of mevalonic acid.
Decreased coenzyme Q10 (ubiquinone-10; CoQ10) concentrations have been found in both plasma and in lymphocytes from patients with PKU (*Colome C et al., 2002 & Artuch R et al., 1999*). CoQ10 shares a common biosynthetic pathway (the mevalonate pathway) to cholesterol. Apart from being a cofactor in the mitochondrial electron transport chain, the reduced form of CoQ10 has an important role as an antioxidant in both the mitochondria and lipid membranes as well as in preventing LDL oxidation (*Colome C et al., 2002*). The effect of decreased CoQ10 concentrations and, presumably increased oxidative stress in PKU would support an inhibitory effect of Phe upon the cholesterol, and CoQ10 pathways. There is a paucity of studies investigating CVD risk and cardiovascular events in PKU. Vascular risk factors and homocysteine were assessed in children with PKU under dietary treatment and they were found to have low total cholesterol, vitamin B6, B12 and

folate concentrations resulting in moderate hyperhomocysteinaemia (*Schulpis KH et al., 2002*). It is possible, that the hypocholesterolaemia in adult PKU patients may confer a protective effect against CVD. However, an assessment of adult PKU patients using biochemical markers along with non-invasive imaging techniques is needed to test this hypothesis. Highly elevated concentrations of Phe are teratogenic and are a cause of increased risk of miscarriage (*American Accademy of Pediatrics: Committee on Genetics. Maternal phenylketonuria*). Specifically, the foetus can be affected by elevated Phe concentrations which lead to intrauterine growth retardation, facial dysmorphism, microcephaly, congenital heart disease and developmental delay and has led to families consisting of multiple children with birth defects and intellectual impairment (*Knerr I et al., 2005; Lee PJ et al., 2005 & Shaw-Smith C et al., 2004*).
Foetal exposure to HPA in PKU affected pregnancies is exacerbated by the transplacental gradient for Phe; an average foetal/maternal ratio of 1.5 is suggested although ratios up to 2.9 can be seen in early pregnancy when foetal development is maximal (*Scriver CR et al., 2001*). Because of the foetal morbidities associated with HPA, a more stringent control of blood Phe concentrations is required in mothers with PKU. A preconception diet is required with a Phe target interval of between 100 and 360 µmol/L in affected mothers (*Lee PJ et al., 2005*). In addition, weekly monitoring of the Phe concentrations is advised to aid in achieving low baseline levels (*Australian Society for Inborn Errors of Metabolism. PKU handbook. Dennison B, editor. Alexandra, Australia: Human Genetics Society of Australasia; 2005*).

3.7 Emerging PKU Therapies

Although dietary restriction of Phe is the cornerstone of treatment for PKU, the practicalities of following the strict diet have led to trials of additional therapies.

3.7.1 BH4 Therapy

Recent clinical trials have shown that a subset of ‘classical’ PKU children respond to BH_4 therapy, dependent upon their PAH gene mutation(s) (*Hennermann JB et al., 2005*). Sapropterin dihydrochloride (*Kuvan, Biomarin Pharma*) is an orally active synthetic form of BH_4 that has received Orphan Drug status and Fast Track designation for the treatment of PKU. Phase II and III clinical trials have shown that Kuvan is a safe and effective therapy in selected patients with HPA and mild-to-moderate PKU who responded to a BH_4 loading test (*Burnett JR et al., 2007*).

3.7.2 Enzyme Replacement Therapy

Unfortunately, patients with more severe forms of classic PKU and some non-PKU HPA do not respond to BH_4 treatment, presumably because these individuals lack sufficient residual PAH activity for stimulation by BH_4. Such nonresponders could benefit most from enzyme replacement therapy. Unlike BH_4 treatment, enzyme replacement is not dependent on the *PAH* genotype.
Replacement of the enzyme could be facilitated by partial liver or normal hepatocyte transplantation. Although liver transplantation would correct the metabolic phenotype

in PKU, the high risk of major surgery and lifelong immunosuppression precludes its routine use (*Vajro P et al., 1993*).
An alternative enzyme therapy for PKU has been trialled which involves the substitution of PAH with Phe ammonialyase (*PAL, EC 4.3.1.5*), a non-cofactor dependent plant protein involved in Phe degradation.
This treatment has been shown to be effective in mouse models causing modest but short-lived falls in Phe concentrations (*Sarkissian CN et al., 1999*).
Indeed, PhenylaseTM (*PAL*), Biomarin Pharma is currently under investigation for the potential treatment of patients with PKU who do not respond to BH_4.

3.7.3 Large Neutral Amino Acid Therapy

As discussed previously, it is hypothesised that competition for the L-type amino acid carrier by Phe with other LNAAs may occur in PKU. This hypothesis has led to LNAA supplementation trials (*Pietz J et al., 1999*).
Increasing the blood concentrations of various LNAAs has led to reduced brain concentrations of Phe (*Pietz J et al., 1999*). Furthermore, the increased Tyr and Trp intake may be of benefit in disorders of BH_4 regeneration.
A new LNAA formulation (*NeoPhe, Solace Nutrition*) has been effective in reducing blood Phe concentrations (*Matalon R et al., 2006*).

3.7.4 Gene Therapy

Complete and persistent correction of HPA has been reported in the *Pahenu2* mouse using somatic gene therapy, site specific genome integration of *Pah* cDNA and by liver directed recombinant adeno-associated virus vectors (*Ching Z et al., 2004 & Harding C et al., 2006*).
Early work on gene therapy for children with PKU was considered inappropriate as the therapy involved administration of immunosuppressant agents to block the immune response to the vector so as to prolong the therapeutic effect (*Ding Z et al., 2004*). These trials involved the use of recombinant adenoviral vectors. Other trials involving the use of recombinant retroviral vectors have been abandoned following the observation that these vectors may induce leukaemia-like disorders (*Ding Z et al., 2004*).
PAH-deficient (*Pahenu*2) mouse models have been used to trial gene transfer via a recombinant adeno-associated virus vector encoding the human *PAH* gene with promising results (*Oh HJ et al., 2004*). This vector appears to be a safer mode of transfer as it possesses minimal antigenicity and showed no signs of inducing liver damage (*Oh HJ et al., 2004*). However, administration of the vector would ideally be facilitated by a less intrusive means than via the hepatic portal vein, which was used in the aforementioned study.

3.8 Materials and Methods

Patients diagnosed with phenylketonuria (PKU) following newborn screening are treated with a phenylalanine-restricted diet and monitored by plasma phenylalanine concentrations. A quantitative plasma phenylalanine method suitable for babies and infants requires a small sample volume, good precision at critical levels, a rapid turnaround time to confirm presumptive screening results, and timely management of the diet. Such a method must be simple and robust enough for routine use. The ability to measure tyrosine by the same method is an advantage, particularly for the management of PKU during pregnancy, and also offers a means of monitoring treatment of patients with tyrosinaemia. Several methods are currently available for the quantification of plasma phenylalanine and tyrosine, for example high-performance liquid chromatography (HPLC). However, many of these methods involve relatively large sample volumes or time-consuming analysis.
Tandem mass spectrometry has been widely advocated in screening for various metabolic disorders using dried blood samples. Although dried blood spots can be used for quantification of phenylalanine, they are not ideal for the precise quantification required for dietary management because of the imprecision of the sample volume (*Holub M et al., 2006*).
Quantification in dried blood spot of metabolites is based on the fact that a disk of 3 mm contains approximately 3.1 µl of whole blood with a hematocrit of 50.1% or approximately 3.2 µl with a hematocrit of 53%, but the concentrations of metabolites may vary between plasma and cellular compartment within a blood spot and are therefore amenable to changes of hematocrit. In fact, the blood volume within a given 3 mm disk may increase significantly with increasing hematocrit levels (*Holub M et al., 2006*). Hematocrit levels in neonates vary significantly from each other and frequently differ from the assumed value of 50.1% or 53%. On the other hand premature infants especially those who are critically ill may have significantly lower hematocrit levels at the time of screening (*Holub M et al., 2006*).
Proper analyses rely heavily on spot quality; consequently, an improperly blood spot preparation can lead to poor or insufficient sample analysis.
In addition, the metabolites may be distributed according to chromatographic effects within the spot and therefore location of the punch may have a significant effect. In particular the effect of the punch position on levels of some amino acids was more pronounced in samples with low hematocrit, in which higher metabolite concentrations were found in peripheral compared to central punches due to chromatographic effects that take place on paper (*Holub M et al., 2006*).
Because of analytical limitations, the analysis of metabolites extracted from spot samples has not sufficient statistical power to generate high confidence in the precision of the result; therefore, this assay have to consider a semi-quantitative analysis (*Chace et al., 2001*).
This technique can be used in rapid metabolite screening where the desired results do not need to be highly precise but rather to answer the question of whether or not a metabolite falls within a certain range. In fact, in the presence of an inborn error of metabolism, for example the classic PKU disease, the metabolite concentrations exceed unequivocally their respective cut-off levels (i.e. classic PKU); therefore, the positive result is very manifest. In addition, when the metabolite concentrations is very high in comparison with cut-off values, the hematocrit and/or punch location on the disk do not affect decision making (*Chace et al., 2001*).

Instead, in the follow-up of patients where is evaluated the efficacy therapy by phenylalanine measurement it would be better to use a quantitative analytical method that provides the more precise result. In addition, for monitoring of phenylketonuric patients is necessary to measure only phenylalanine and tyrosine concentrations; therefore it would be time spending and expensive to detect a complete metabolic profile by the classic analytical method for newborn screening.
The aim of the present study was to develop an alternative analytical method respect to the commonly method for neonatal screening involving butylation; it was decided to develop a method rely on a rapid chromatographic separation and MS/MS detection of underivatized phenylalanine and tyrosine.

3.8.1 Subjects

The studies were performed in 24 controls and 6 PKU patients. The patients were young with an age range between 4 and 10 years old. Diagnosis of Phenylketonuria based on the results obtained by newborn screening was confirmed by tandem mass spectrometry analysis using serum spot samples.

3.8.2 Instrumentation

All experiments were performed on an Agilent Series 1100 LC system (Agilent Technologies, Waldbronn, Germany) binary pump equipped with an HTC PAL autosampler. The LC system was coupled to an API 4000 triple-quadrupole mass spectrometer (Applied Biosystem/Sciex, Toronto, Canada) that operated in the ion evaporation mode with ionspray source.
The ionspray probe was operated at a sprayer voltage of 5500 volts. The ESI source temperature was set to 400°C. Curtain gas back pressure 10 psi, nebulizer gas 55 psi and desolvation gas 40 psi were applied.
All data, acquired in the positive ion mode, were collected and processed using Analyst 1.4.1 software (Applied Biosystem/Sciex, Toronto, Canada).
The multiple reaction monitoring (MRM) acquisition mode was applied for tandem mass spectrometric measurements of amino acids. Diagnostic fragments were determined for phenylalanine and tyrosine from product ion scans obtained using continuous flow sample infusion with a flow rate of 10 µL/min. All the source parameters, gas flow and collision energy values were optimized for each component in the MRM experiments. Applied parameters and characteristic CID fragment ions of the amino acids are shown in Table 2.
Chromatographic separations were performed on a normal phase (Thermo Hypersil silica 50x4.6 mm I.D) chromatographic column using water/formic acid 0.1% (A) acetonitrile/formic acid 0.1% (B) as eluent. The chromatographic gradient profile is shown in the Table 3.

Table 2. The optimum LC-MSMS parameters for each amino acid.

Amino acids	Declustering potential (eV)	Collision Energy (eV)	MRM channel (Q1/Q3 m/z)	Retention time (min)
Phenylalanine	45	25	166.1/120.0	1.93
Tyrosine	50	45	182.1/91.0	1.95
D5-Phenylalanine	40	25	171.3/125.2	1.94
D4-Tyrosine	50	22	186.3/140.1	1.95

Table 3. Chromatographic gradient profile

Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	2000	5	95
1	1.50	2000	70	30
2	2.00	2000	70	30
3	2.10	2000	5	95
4	3.00	2000	5	95

3.8.3 Chemicals

D4-alanine as internal standard, D5-phenylalanine, D4-tyrosine and unlabeled phenylalanine and tyrosine, were obtained from Sigma-Aldrich (Budapest, Hungary). Stock solutions at 1mg/mL were made in water containing 5% of NH_3 except for D4-tyrosine where the NH_3 was 10% while successive dilutions were made in methanol.

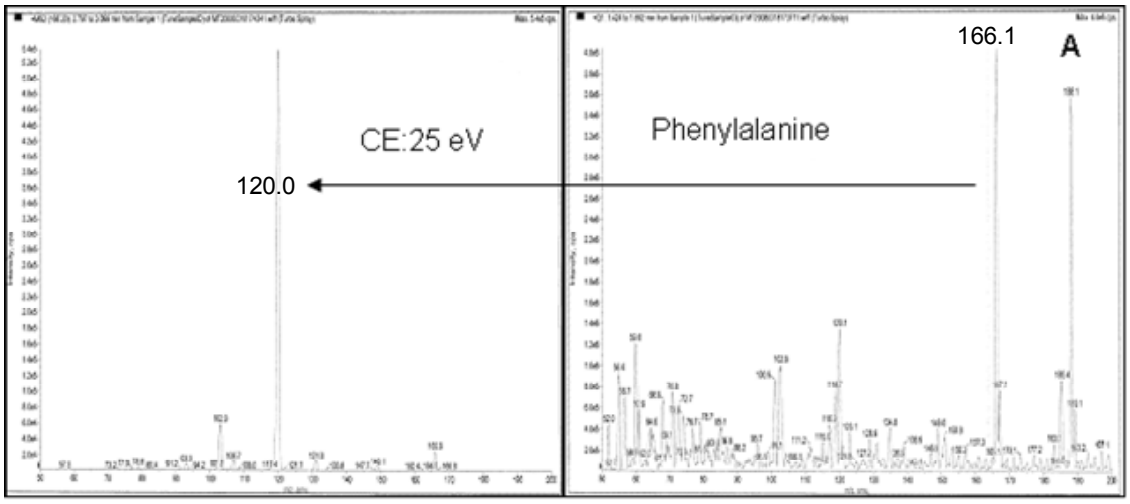
3.8.4 Serum samples

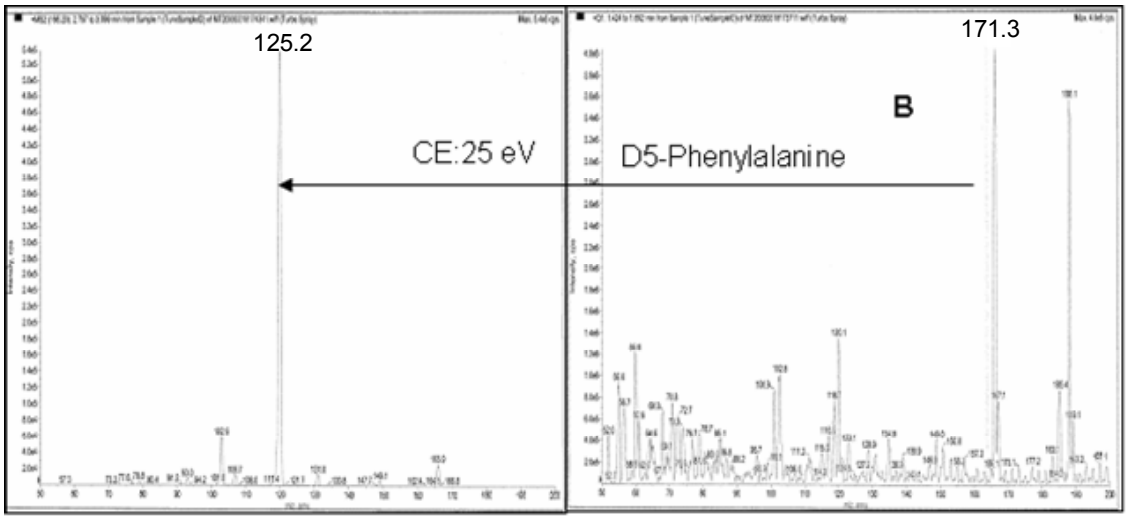
Samples were prepared by adding to 50µL of human serum, 200µL of methanol containing D5-phenylalanine and D4-tyrosine labelled internal standards. After that, they were vortexed and centrifuged at 14000 x g x 10 minutes. Finally 5µL of supernatant were loaded onto the LC-MS/MS system.

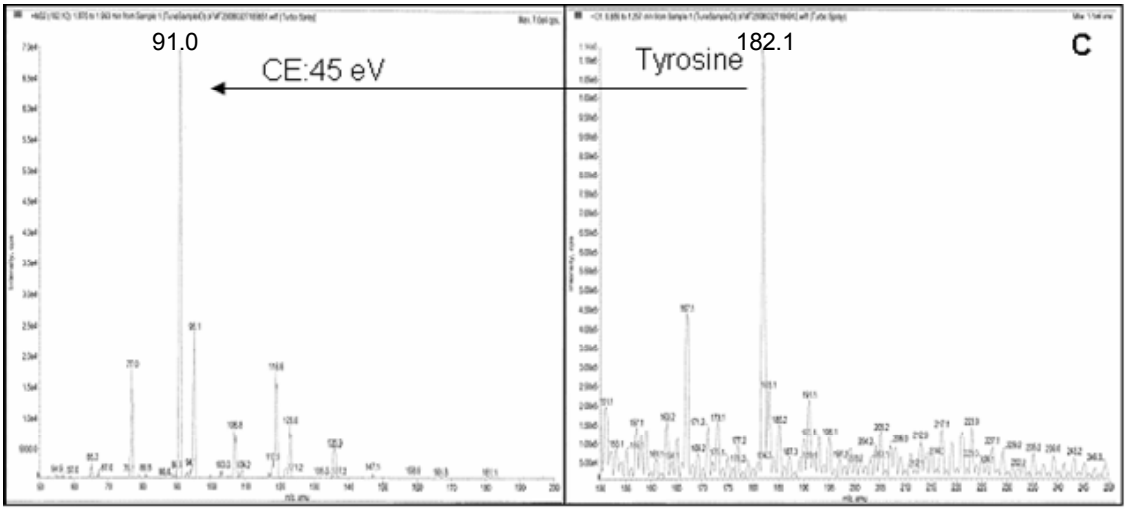
3.9 Results

3.9.1 Tandem Mass Spectrometry optimization

The fragmentation pattern of the protonated molecular ions ($[M+H]^+$) of each amino acid under collision-induced dissociation in the tandem mass spectrometer is shown in Figure 6. Figure 7 shows the LC-MRM profile from each amino acid spiked in human serum samples







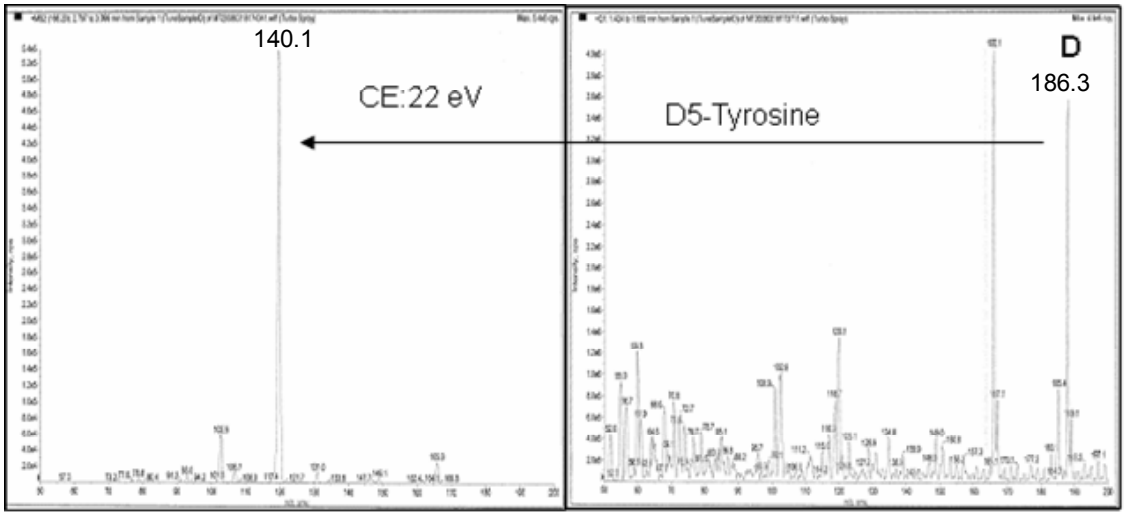
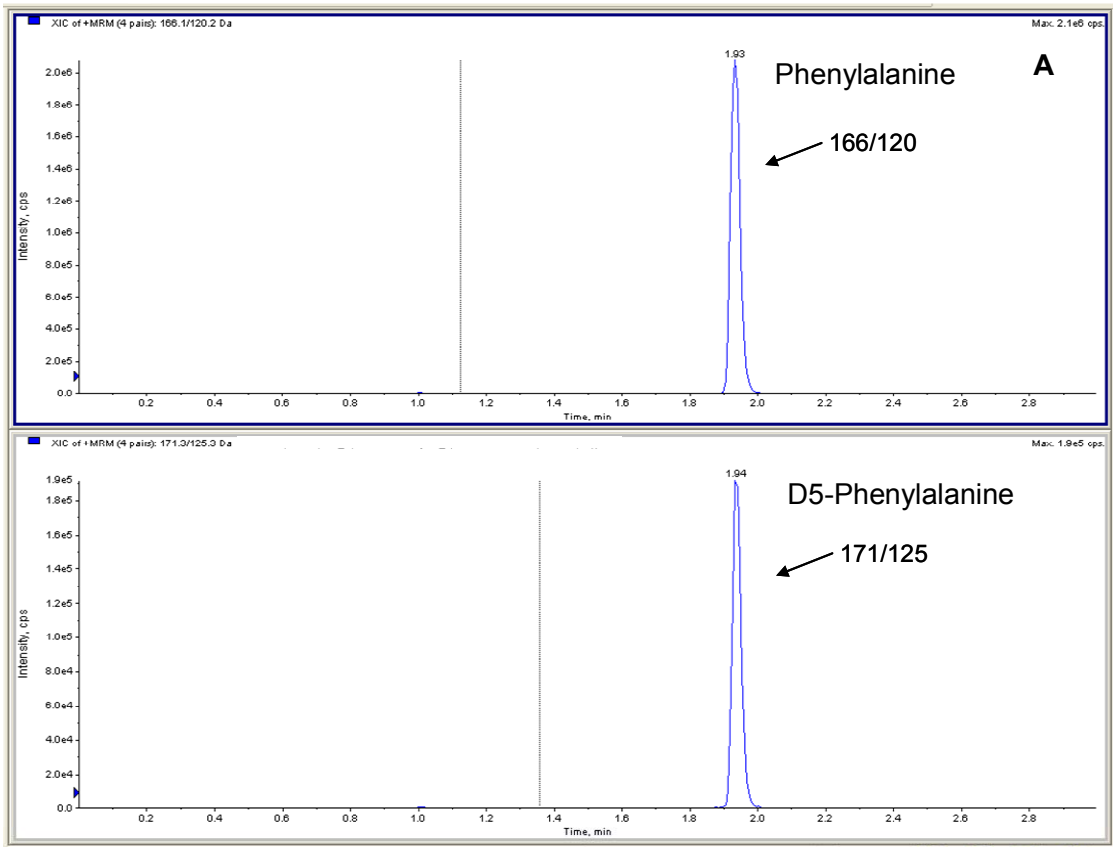


Figure 6. *Full scans and MS/MS of phenylalanine (A), D5-phenylalanine (B), tyrosine (C), D4-tyrosine (D).*



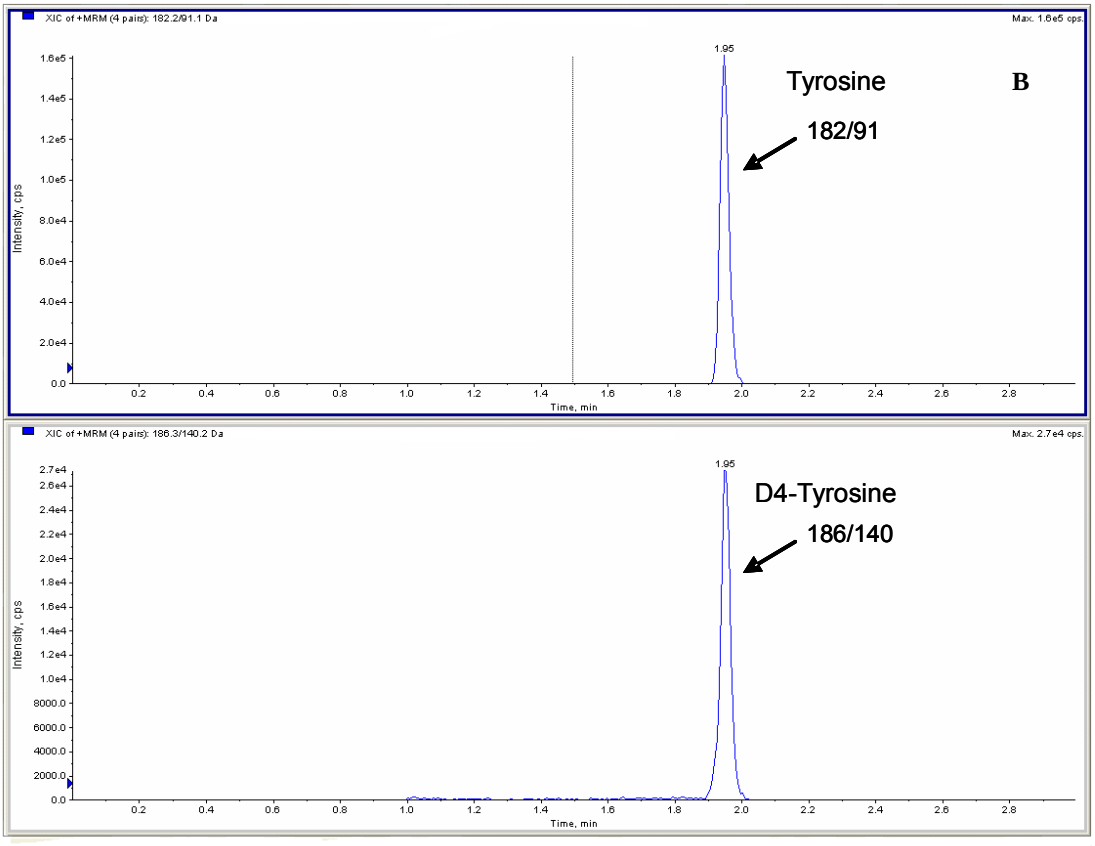


Figure 7. *LC-MS/MS analysis of labelled and unlabeled (A) phenylalanine and (B) tyrosine. Identities and MRM channels are indicated above the LC peaks.*

3.9.2 Assay linearity

Calibration plots of analyte/internal standard peak area ratio versus the nominal concentration of D5-phenylalanine and D4-tyrosine in serum were constructed and weighted $1/x^2$ linear regression applied to the data.
The dynamic ranges of calibration curves for D5-Phe and D4-Tyr were established on the basis of the phenylalanine and tyrosine values results from neonatal screening (Table 4 and Table 5).
D5-Phe show a linear response that ranged from 25000 to 400000 ng/mL (R^2= 1) (Figure 8); the curve for added D4-Tyr showed good linearity over the concentration range 250-64000 ng/mL (R^2= 0.9999) (Figure 8).
The accuracy of linearity assay was determined by comparing the nominal concentration values with experimental concentration values for D5-Phe and D4-Tyr. Results show a good correlation between the two values of concentration (Table 6 and Table 7).

Table 4. *Dynamic r*ange of calibration curve for D5-*phenylalanine*

Area ratio (D5-Phe/D4-Ala)	Experimental Phe concentration (ng/mL)
18	23890
34	52117
68	110293
122	203064
208	351084

Table 5. *Dynamic r*ange of calibration curve for D4-*tyrosine*

Area ratio (D4-Tyr/D4-Ala)	Experimental Tyr concentration (ng/mL)
0,085	234
0,117	557
0,173	1107
0,254	1915
0,428	3635
0,832	7800
1,639	15487
3,321	31506
6,001	57029

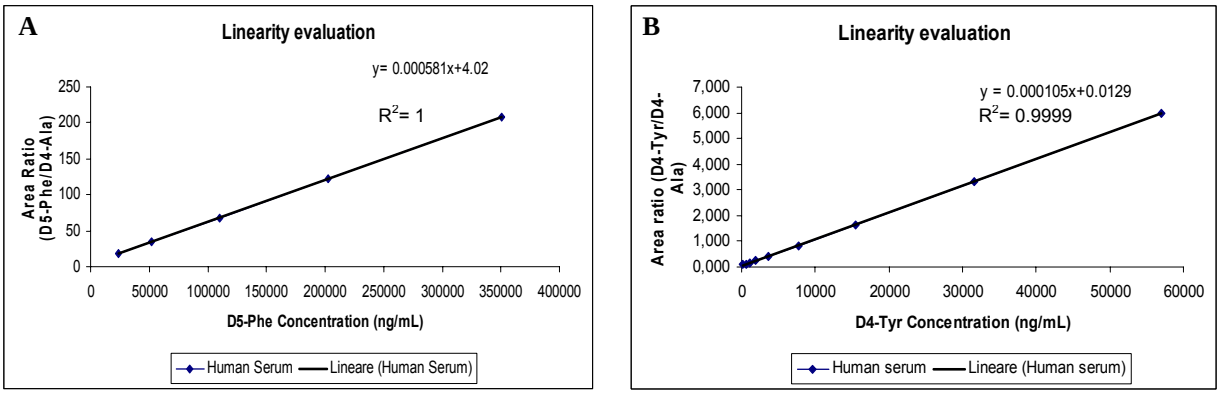


Figure 8. Human serum calibration curve of D5-Phe and D4-Tyr

Table 6. Accuracy of linearity assay for D5-phenyalanine

D5-Phe Nominal Concentration (ng/mL)	D5-Phe Experimental Concentration (ng/mL)	Accuracy (%)
25000	23120	92
50000	55014	110
100000	111921	112
200000	197982	99
400000	341794	85

Table 7. Accuracy of linearity assay for D4-tyrosine

D4-Tyr Nominal Concentration (ng/mL)	D4-Tyr Experimental Concentration (ng/mL)	Accuracy (%)
250	234	94
500	557	111
1000	1107	111
2000	1915	96
4000	3635	91
8000	7800	98
16000	15487	97
32000	31506	98
64000	57029	89

3.9.3 Recovery and Matrix Effect

The Recovery was assessed by comparison of peak areas of analyte standards in matrix sample extracted with peak areas of analyte standards spiked in blank matrix extracted. It was determined by the following calculate:

$$\textbf{Recovery= A/B*100}$$

where A is peak area of analyte standard spiked extracted and B is peak area of analyte standard spiked in blank matrix post extraction.
Data reported in Table 8 demonstrated good recovery (99.7 ± 1.0% for D5-Phe and 98.8 ± 1.3% for D4-Tyr) (Figure 9).
The Matrix effect was evaluated by comparison of peak areas of analyte standards in absence or presence of the matrix by the following calculate:

$$\textbf{ME=(B-A)/A*100}$$

where A is peak area without matrix and B is peak area with matrix.
Data reported in Table 8 demonstrated a minimal matrix effect (17.0 ± 0.4% for D5-Phe and 15.9 ± 0.3% for D4-Tyr) (Figure 9).

Table 8. Recovery (A) and Matrix Effect (B) extimation of D5-phenylalanine and D4-tyrosine

AA	*Recovery (%)*
D5-Phe	99.7
D4-Tyr	98.8

AA	*Matrix Effect (%)*
D5-Phe	17.0
D4-Tyr	15.9

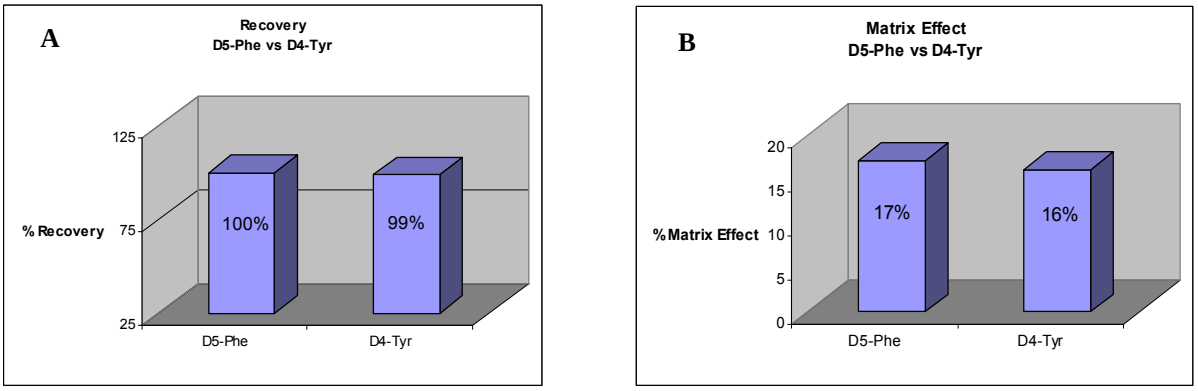


Figure 9. Recovery (A) and Matrix Effect (B) estimation for D5-phenylaline and D4-tyrosine

3.9.4 Reproducibility

Extraction method reproducibility was evaluated by repeating the protein precipitation of the same spiked serum sample. Samples were run in six replicates and six different serum samples were used.
The precision calculated for D5-phenylalanine and D4-tyrosine was very good (CV of D5-Phe 4.8% and D4-Tyr 6.5% for n=6).

3.9.5 Quantitative analysis by LC-MRM

For controls, were used 24 serum samples that had already been analyzed by tandem mass spectrometry analysis using spot sample and that were considered normal. In this control group, the mean ± SD of Phe and Tyr were 404 ± 113 µmol/L and 270 ± 45 µmol/L, respectively; the Phe/Tyr molar ratio in each sample was 1.50 ± 0.36.
Figure 10 shows an example of LC-MS/MS spectrum of a serum sample that had been categorized as PKU. The peak area of Phe and Tyr of PKU patient is very high in comparison with peak area reference in the control sample.
Results are summarized in Table 9 including results of 24 control samples.
The Phe/Tyr ratio was calculated for each sample (Table 9).
For all cases of PKU, the main ratio was 12 ± 9.

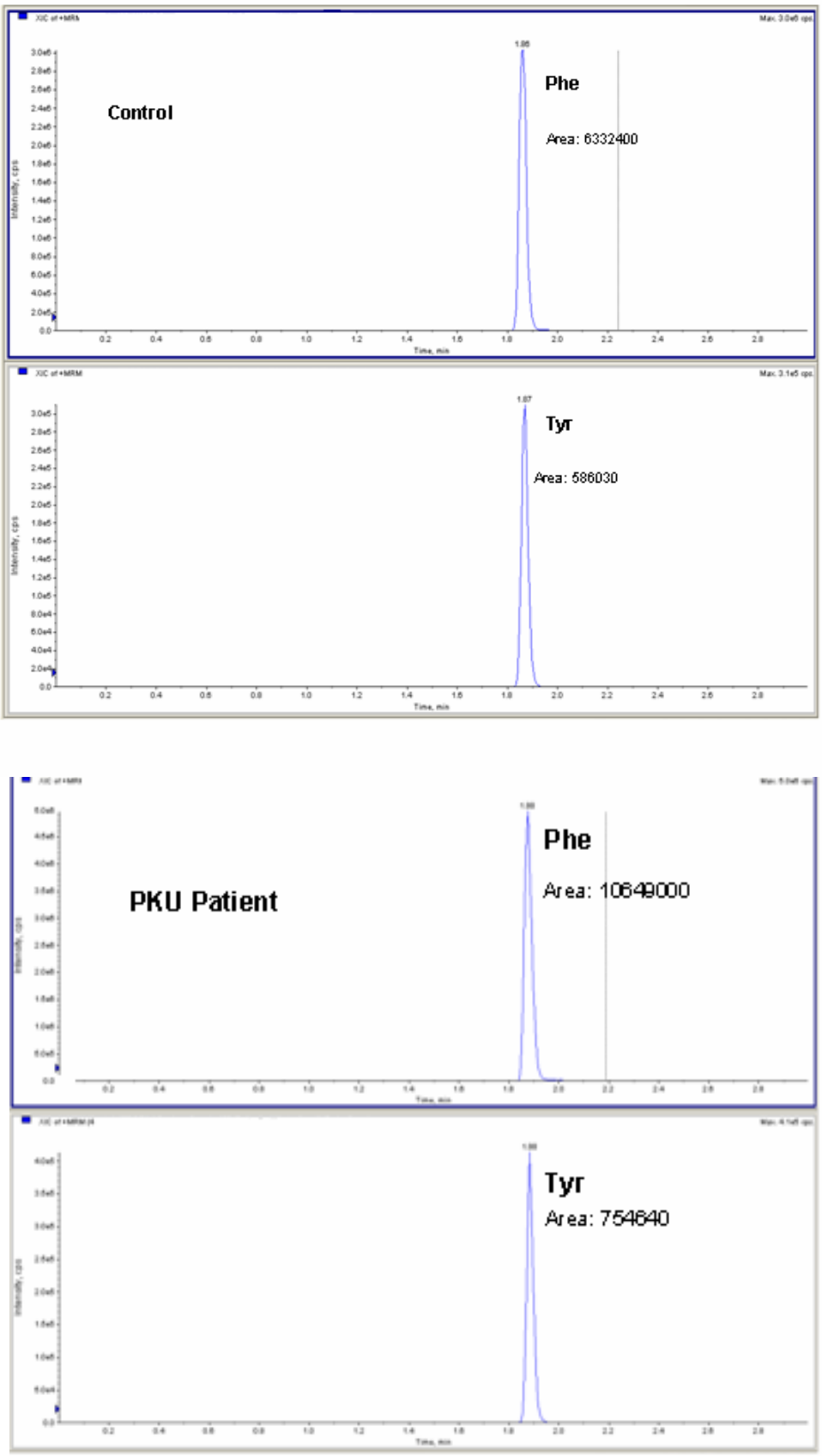


Figure 10. LC-MRM profile of phenylalanine and tyrosine: control vs PKU patient

Table 9. Quantitative analysis for phenylalanine and tyrosine in serum samples

	Phenylalanine (ng/mL)	Tyrosine (ng/mL)	Phe/Tyr Ratio	Phenylalanine (µmol/L)	Tyrosine (µmol/L)	Phe/Tyr Ratio
Control 1	99278	69652	1,43	602	383	1,57
Control 2	97721	64063	1,53	592	352	1,68
Control 3	94303	63987	1,47	572	352	1,63
Control 4	90488	58273	1,55	548	320	1,71
Control 5	89045	52281	1,70	540	287	1,88
Control 6	85597	51476	1,66	519	283	1,83
Control 7	84766	44042	1,92	514	242	2,12
Control 8	76403	41270	1,85	463	227	2,04
Control 9	73332	39949	1,84	444	219	2,02
Control 10	68700	39168	1,75	416	215	1,93
Control 11	66067	40820	1,62	400	224	1,79
Control 12	64905	46593	1,39	393	256	1,54
Control 13	59576	50176	1,19	361	276	1,31
Control 14	57896	46218	1,25	351	254	1,38
Control 15	54819	44508	1,23	332	245	1,36
Control 16	51720	46928	1,10	313	258	1,22
Control 17	51782	46590	1,11	314	256	1,23
Control 18	49523	49038	1,01	300	269	1,11
Control 19	48420	44871	1,08	293	247	1,19
Control 20	49243	37739	1,30	298	207	1,44
Control 21	47770	46656	1,02	290	256	1,13
Control 22	47986	50316	0,95	291	276	1,05
Control 23	44727	50316	0,89	271	276	0,98
Control 24	45955	53124	0,87	279	292	0,95
AV	**66668**	**49086**	**1,36**	**404**	**270**	**1.50**
SD	18594	8144	0,33	113	45	0.36
Patient 1	468221	19792	24	2838	109	26,09
Patient 2	436776	40900	11	2647	225	11,78
Patient 3	306567	23959	13	1858	132	14,11
Patient 4	102400	30231	3	621	166	3,74
Patient 5	63551	41003	2	385	225	1,71
Patient 6	210456	20004	11	1275	110	11,60
AV	**264662**	**29315**	**16**	**1604**	**161**	**12**
SD	168836	9775	8	1023	54	9

The calibration curves and samples analysis results show a good quantitative response that confirms the presence of a phenylalanine hydroxylase defect identified by newborn screening test.

As shown in Table 9, concentration of phenylalanine is very high in PKU patients in comparison with phenylalanine concentration of the controls while concentration of

tyrosine in the PKU patients is lower than or equal to that of control groups (Figure 11).

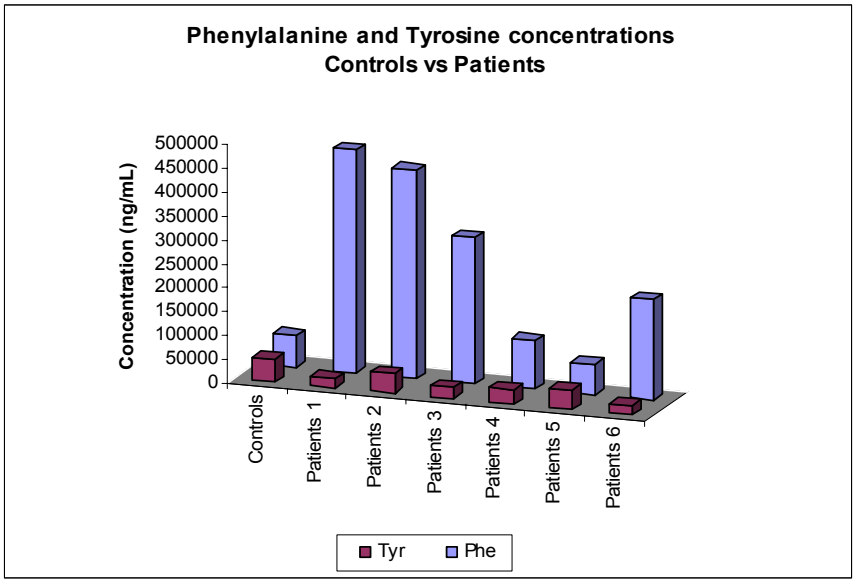


Figure 11. Phenylalanine and tyrosine concentration in control groups and PKU patients

Phe/Tyr ratio of PKU patients was calculated because was suggested that this ratio allows detection of affected infants under 24 hours of age without increasing the rate of false positives (*Chace et al., 1998*); further, the calculation of the ratio of phenylaline to tyrosine was shown that to be appropriate for the discrimination of most of the different forms of HPA (*Schulze A et al., 1999*).
As shown in Figure 13, Phe/Tyr ratio of PKU patients is very high in comparison with Phe/Tyr ratio of control groups (Figure 12).

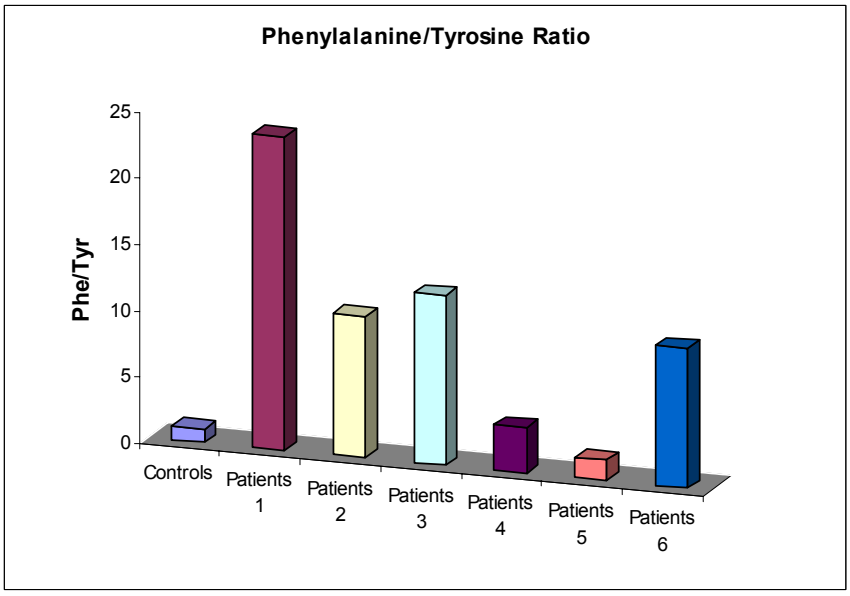


Figure 12. Phenylalanine/tyrosine ratio of control groups and PKU patients

So the results show that the new analytical method presented here is able to distinguish pathological profiles from normal profiles.

3.9.6 Method Accuracy

The accuracy of present analytical method was determined by comparing the individual values of phenyalanine and tyrosine in liquid serum samples with those obtained from the same samples by standard method for newborn screening involving tandem mass spectrometry analysis on dried filter paper.
Data are reported in Table 10 and demonstrate that the concentrations of phenylalanine and tyrosine extracted by protein precipitation and quantified by LC-MRM method are higher than the concentrations of phenylalanine and tyrosine extracted from dried serum spot and quantified by ESI-MSMS analysis in newborn screening.

Table 10. Comparison of concentrations of phenyalanine and tyrosine in liquid serum samples (A) and in dried serum spot samples (B) by different extraction procedures and different analytical methods.

A	Phe (ng/mL)	Tyr (ng/mL)	Phe/Tyr Ratio	Phe (µmol/L)	Tyr (µmol/L)	Phe/Tyr Ratio
Patient 1	468221	19792	24	2838	109	26
Patient 2	436776	40900	11	2647	225	12
Patient 3	306567	23959	13	1858	132	14
Patient 4	102400	30231	3	621	166	4
Patient 5	63551	41003	2	385	225	2
Patient 6	210456	20004	11	1275	110	12
AV	**264662**	**29315**	**16**	**2448**	**155**	**17**
SD	168836	9775	8	1023	54	9

B	Phe (ng/mL)	Tyr (ng/mL)	Phe/Tyr Ratio	Phe (µmol/L)	Tyr (µmol/L)	Phe/Tyr Ratio
Patient 1	260205	7826	33	1577	43	37
Patient 2	211035	16198	13	1279	89	14
Patient 3	146685	8918	16	889	49	18
Patient 4	50820	12922	4	308	71	4
Patient 5	30690	16380	2	186	90	2
Patient 6	112530	8372	13	682	46	15
AV	**135328**	**11769**	**14**	**820**	**65**	**15**
SD	89516	3935	11	543	22	12

As shown in Table 10, the concentrations of phenylalanine and tyrosine extracted from dried serum filter paper and measured by "semi-quantitative" MS/MS method are much lower than those of phenylalanine and tyrosine extracted from liquid serum by protein precipitation and determined by "quantitative" LC-MRM analytical method.

But an important thing to note is that the trend of phenylalanine and tyrosine of PKU patients is the same in both serum samples types (Figure 13).

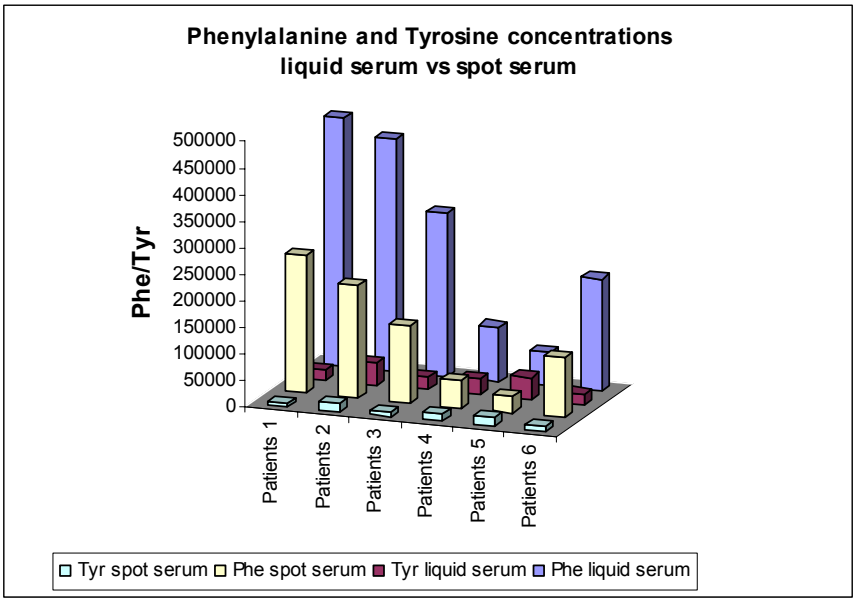


Figure 13. Phenylalanine and tyrosine measurements in liquid serum and spot serum samples of PKU patients

In addition, as shown in Table 9, the Phe/Tyr ratio values of each patient are very similar between the two serum sample types (Figure 14).

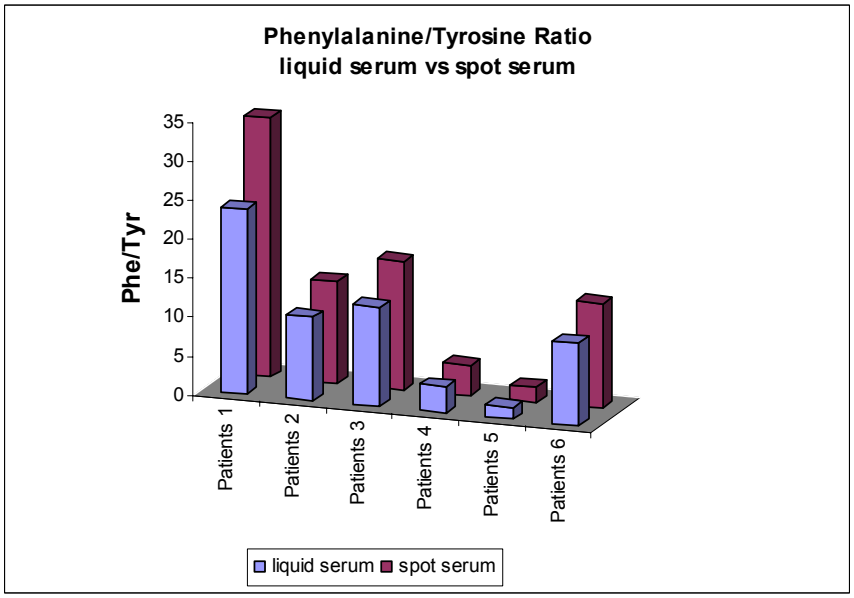


Figure 14. Phenylalanine/tyrosine ratio in liquid serum and spot serum samples of PKU patients

3.10 Conclusions

Comparing the concentrations of phenylalanine and tyrosine of the same PKU patients measured with the two different analytical methods, were found significant differences in the concentration of each single analyte. In particular, concentrations of both phenylalanine and tyrosine analyzed with new analytical method are always about twice compared with those analyzed with the classic procedure used for neonatal screening.
It is interesting to note however that the phe/tyr ratio of the same patient calculated with the two different methods is very similar. Therefore, both analytical methods are able to identify the presence of Phenylketonuria disease.
But is probably that the semi-quantitative analytical method, using paper card, is less accurate and precise respect to quantitative method in liquid serum; therefore it underestimates the single concentrations of phenylalanine and tyrosine and so normalizes the analytical error through the ratio of amino acids.
The new quantitative LC-MS/MS analysis developed is simple to execute, precise and reliable, with rapid analysis times. The method requires small sample volumes and ensures a more rapid turnaround of analysis.
Specimen preparation is simpler and rapid (10 min) than preparation of phenylalanine and tyrosine in the common analytical method for newborn screening that, over the extraction phase, includes a butylation step (1 hour).
This method potentially allows the analysis of screening, diagnostic and routine monitoring of specimens from PKU patients; therefore it represents a valid alternative to the common analytical method using for the diagnosis and follow-up of PKU patients.

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