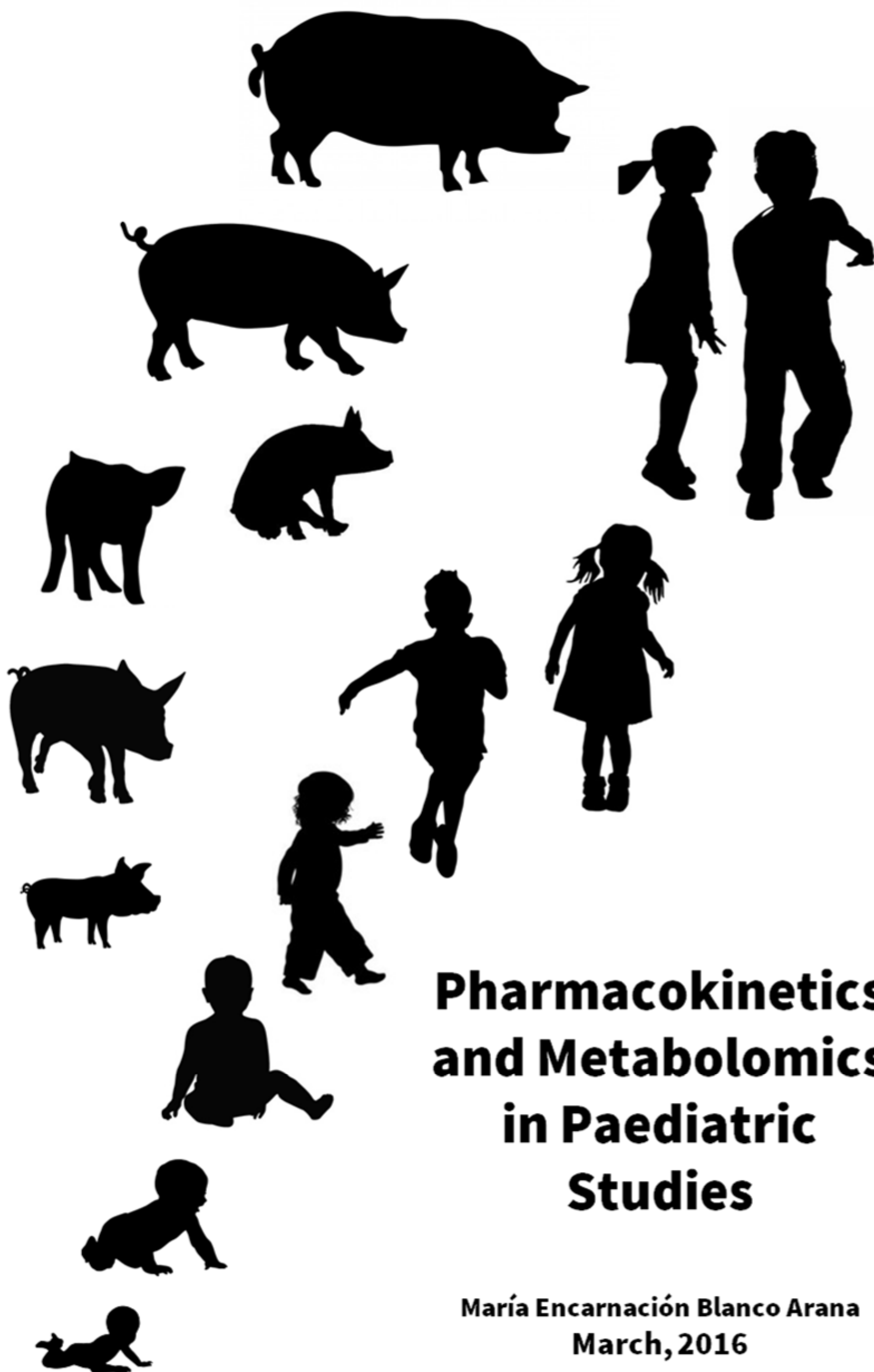

Pharmacokinetics and Metabolomics in Paediatric Studies

María Encarnación Blanco Arana

March, 2016

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UPV EHU

Universidad del País Vasco / Euskal Herriko Unibertsitatea

ZTF-FCT
Zientzia eta Teknologia Fakultatea
Facultad de Ciencia y Tecnología

UPV/EHU
Faculty of Science and Technology
Departament of Analytical Chemistry

Pharmacokinetics and Metabolomics in Paediatric Studies

Presented by
María Encarnación Blanco Arana

Supervisors
Rosa María Alonso Rojas
Oskar González Mendia

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Leioa, March 2016

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-Adiós–dijo el zorro. – Aquí está mi secreto. Es muy simple: sólo se ve bien con el corazón. Lo esencial es invisible a los ojos.

- Lo esencial es invisible a los ojos – repitió el principito a fin de recordarlo.

- Es el tiempo que has perdido en tu rosa lo que hace a tu rosa tan importante.

- Es el tiempo que he perdido en mi rosa… – dijo el principito a fin de recordarlo.

Antoine de Saint-Exupéry, El Principito

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ABBREVIATIONS AND ACRONYMS

%CV	Coefficient of variation
%RE	Relative error
%RSD	Relative standard deviation
^{1}H-MAS-NMR	Magic-angle spinning nuclear magnetic resonance
2PY	N1-methyl-2-pyridine-5-carboxamide
ACN	Acetonitrile
AC	Acylcarnitines
ADME	Absorption, distribution, metabolism and elimination processes
aEEG	Amplitude-integrated electroencephalography
AFMK	Acetyl-N-formyl-5-methoxykynurenamine
AGAT	L-arginine:glycine amidinotransferase
ASD	Autistic spectrum disorders
AUC	Area under curve
BBB	Blood brain barrier
BPCA	Best Pharmaceuticals for Children Act
Ce	Collision energy
CE	Capillary electrophoresis
CL	Clearance
C_{max}	Maximum concentration
CNS	Central nervous system
Cr	Creatine
CSF	Cerebrospinal fluid
CV	Cone voltage
CYP3A4	Cytochrome P450 isoform 3A4
DBS	Dried blood spot
DESI-MS	Desorption electrospray ionisation mass spectrometry
DI-MS	Direct infusion mass spectrometry
EBC	Exhaled breath condensate
EDTA	Ethylendiaminetetraacetic acid
EMA	European Medicine Agency
ESI	Electrospray ionization
F	Female
FA	Formic acid
FDA	Food and Drug Administration
FDAMA	FDA Modernization Act
FEN	Fentanyl
FTICR	Fourier transform ion cyclotron resonance
GAMT	Guanidinoacetate methyltransferase
GC-MS	Gas chromatography coupled to mass spectrometry

HCA	Hierarchical clustering analysis
HMP	Human Metabolome Project
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography coupled to tandem mass spectrometry
HPLC-UV	High performance liquid chromatography coupled to ultraviolet detection
HRMS	High resolution mass spectrometry
i.m.	Intramuscular
i.v.	Intravenous
IEM	Inborn errors of metabolism
inh.	Inhalatory
ISF	Brain interstitial fluid
IUGR	Intrauterine grow retardation
KEGG	Kyoto Encyclopedia of Genes and Genomics
LC-MS	Liquid chromatography coupled to mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lowest limit of quantification
M	Male
ME	Matrix effect
MF	Matrix factor
MMA	Methylmalonic acidemia
MRM	Multiple reaction monitoring mode
MS	Mass spectrometry
MVDA	Multivariate data analysis
NAD	Nicotinamide adenine dinucleotide
NMF	Normalised matrix factor
NMR	Nuclear magnetic resonance
OATP	Organic anion-transporting polypeptide
OPLS	Orthogonal partial least squares
OPLS-DA	Orthogonal partial least squares discriminant analysis
OSC	Orthogonal signal correction
PA	Propionic acidemia
PBPK	Physiologically Based Pharmacokinetic
PC	Principal component
PC1	First principal component
PC2	Second principal component
PC3	Third principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PD	Pharmacodynamic
PE	Process efficiency

Phe	Phenylalanine
PK	Pharmacokinetic
PK/PD	Pharmacokinetic/pharmacodynamic
PKU	Phenylketonuria
PLS	Partial least squares
PLS-DA	Partial least squares discriminant analysis
PREA	Pediatric Research Equity Act
QC	Quality control
QCs	Quality control samples
REC	Recovery
SOP	Standard operating procedure
SPE	Solid phase extraction
SPME	Solid phase microextraction
SUS	Similar and unique structures
$t_{1/2}$	Half life
TIC	Total ion current chromatogram
t_{max}	Time to reach maximum concentration
t_R	Retention time
Trp	Tryptophan
UHPLC-Q-TOF-MS	Ultra high performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometry system
ULOQ	Upper limit of quantification
UV	Ultraviolet detector
UVDA	Univariate data analysis
Vd	Volume of distribution
XIC	Extracted ion chromatogram

PREFACE

Therapeutic decision-making should always be guided by the best available evidence and the importance of the benefit for the individual patient. However, for the paediatric population, where sometimes little information is available for pharmaceutical drugs, practitioners must rely on other sources of knowledge as their previous experience in similar cases or the use of evidences from other population groups. One of the problems often encountered in paediatric clinical practice is the lack of information about the adequate dosage regimen, efficacy, and safety of drugs in this population. Ethical concerns as well as practical and commercial considerations have made pharmaceutical companies reluctant to consider the development of studies of medicines to be used in the treatment of children.

To stimulate the investigation in paediatric population and promote a greater involvement of children in clinical trials, the Food and Drug Administration (FDA) from the United States developed a series of regulatory strategies. In 1997, the FDA Modernization Act (FDAMA) offered an additional 6 months of market exclusivity for pharmaceutical companies to conduct paediatric studies. Laws as the Best Pharmaceuticals for Children Act (BPCA), enacted in 2002, or the Pediatric Research Equity Act (PREA), signed in 2003, have resulted in a significant increase in the knowledge about the efficacy and safety of drugs in paediatric population. However, despite these regulations, a large number of drugs are yet not authorised for its administration to children, and are commonly used '**off-label**'.

The off-label use does not imply that the drug is contraindicated or disapproved, but that insufficient data are available to obtain authorisation, and that the risk and benefits of using a drug in a particular situation have not been examined. In most of the cases, information about optimal dose is not available, and the administration is made based on previous experience of the paediatrician. There are also some cases where the drug is

manipulated by dissolving or splitting a tablet or by changing the indicated route of administration (e.g. to avoid intramuscular injections in young patients). The use of off-label drugs is especially common when treating rare or chronic diseases or sparse populations such as neonates. Studies performed in several countries provide different percentages of off-label prescription, reaching up to a 90% of patients prescribed with off-label drugs in neonatal intensive care units.[1]

The Spanish Royal-Decree Law 1015/2009—based on the European law CE 726/2004—that regulates the availability of drugs in special situations, defines off-label drugs as *«those used in different conditions than the included in the clinical data sheet»* and indicates that the use of drugs in these conditions should be limited to situations when no authorized therapeutic alternative is available for the patient. Also it is requested that parents must be informed and its use must be registered. However, as stated by the 2012-2013 paediatric national survey on off-label drug use in children in Spain (OL-PED study), many Spanish paediatricians do not comply with this rule, and commonly the use of these drugs is not properly registered or parents of the patient are not properly informed of the use of an off-label drug.[2]

Often, drugs administered without official authorisation for their use in children are dosed using empirical algorithms from allometric models optimised for adults, adjusting the dose by factors such as weight or body

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volume. However, this does not take into consideration the different grades of maturation of the organs involved in the absorption, distribution, metabolism and elimination (ADME) processes of drugs in children. *«Children are not small adults»* is a common statement—even almost a mantra—among paediatricians, based on the physiological and anatomical differences of children compared to adults that lead to pharmacokinetic (PK) and pharmacodynamic (PD) disparities between them. However, despite their differences, children and adults cannot be considered as different species. As many differences as there are between them, adult and paediatric populations also share similarities, and as children move from infancy through childhood and adolescence their developmental maturity approaches more closely that of adults. The transition from childhood to adulthood should be considered as a continuous process rather than a single migration, and children cannot be considered as a homogeneous unique group. In fact children are a highly fragmented population, where subjects rapidly change from one segment to another as they grow and develop. Paediatric population is generally divided into the following categories: neonates (birth up to 1 month), infants (1 month up to 2 years), children (2 to 12 years), and adolescents (from 12 up to 17 years).

Clinical practice in the paediatric field, especially in pharmacology, implies the understanding of the maturing process in a continuously changing subject, from preterm neonates to adolescents. Both safety and efficacy profiles of drugs may be significantly different for children due to differences in developmental physiology, disease pathophysiology, or developmental PK/PD. In many occasions, this practice leads to the necessity of a readjustment of the dosage based on the effect observed. This may be a dangerous practice with fatal consequences in cases of toxicity due to an excess of drug concentration. Alternative approaches to the extrapolation of dosing based in the actual development state of the children instead of on body weight, and that are able to predict the PK/PD behaviour of a compound in this individual paediatric patient are highly desirable.

To address the problem of the lack of information in paediatric pharmacotherapy and to increase the knowledge on PK and maturation and development in paediatrics in this manuscript are presented two different approaches, corresponding with the two parts in which it is divided. The first part, titled ***Pharmacokinetics in paediatrics***, will be focused on the development of analytical methods for the evaluation of PK predictive models in paediatric population, while in the second part, named ***Metabolomics in paediatrics*** metabolomics is used as a tool to differentiate the grades of maturation in paediatrics, both in animal models and humans. The use of animal subjects in this research has been kept to the minimum possible, and the experimental protocols met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005).

As discussed above, allometric scaling is insufficient to describe the ADME processes in paediatric population, as it does not take into consideration the different grades of maturation of the organs involved in these processes. Other approaches such as Physiologically Based Pharmacokinetic (PBPK) models or maturation based semi-physiologic approaches are considered more adequate for the prediction of the PK profile of drugs across paediatric population. In the research work presented in the first part of this thesis—developed in collaboration with the Pharmacology group of the Faculty of Medicine in the University of Basque Country/EHU—a simple analytical method based on high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) is developed, validated, and implemented for the evaluation of a maturation-based semi-physiologic approach of the opioid drug Fentanyl in animal models of newborns. Also, a brief introduction about PK modelling and prediction is included.

Metabolomics, the latest omics tool, can be a useful tool to gain knowledge about the metabolic changes observed in children along their maturation process. This information would be of great interest in order to improve the models used for the prediction of drugs behaviour in immature subjects. For

that reason, in the second part of this thesis, after studying the state of the art on metabolomics and a literature review of metabolomic studies in paediatric populations, two experimental studies are presented. The first one is a pilot study where the metabolic profile of plasma samples from pigs used as animal models of paediatrics are compared according to its classification into two groups based on their age: neonates and children. Based on the results obtained from this pilot study, a new experiment is designed for the search of biomarkers of maturation and development between piglets of different ages. In the second study—developed in collaboration with the Laboratory of Mass Spectrometry of the University of Padova—urine samples from children of different ages are analysed in order to search for metabolites able to be used as biomarkers of maturation. Along these chapters especial focus is made on the data handling, from pre-processing to statistical analysis using multivariate data analysis (MVDA).

Finally, in the Annex section, the scientific papers published during my period as a Ph.D. student are included. Although not all of them are directly related to the main theme of this thesis, they were an outcome of other research projects in which I have been involved, and so contributed to the skill development expected from a Ph.D. training period.

Part I: Pharmacokinetics in paediatrics

Chapter 1

Introduction to Pharmacokinetics

Dentro de nós há uma coisa que não tem nome, essa coisa somos nós.

(José Saramago, *Ensaio sobre a Cegueira*)

1 BASIC CONCEPTS OF PHARMACOKINETICS

Information on pharmacology in infants and children has increased considerably over the past 2 decades. However, there are still many aspects of the pharmacokinetic (PK) and pharmacodynamic (PD) behaviour of drugs that are not fully understood.[1]

Pharmacokinetics (PK) describes the time course of a drug in the different fluids and tissues of the body resulting from the administration of a certain drug dose. In simple words is «*what the body does to the drug*». Pharmacodynamics (PD) relates the effect observed with the concentration of the drug in the biophase, «*what the drug does to the body*».[2] Together PK and PD determine the relationship between dose and response.

1.1 ADME PROCESSES

The acronym ADME refers to the four fundamental processes affecting a drug while it is in the body: absorption, distribution, metabolism, and excretion (Figure 1.1).

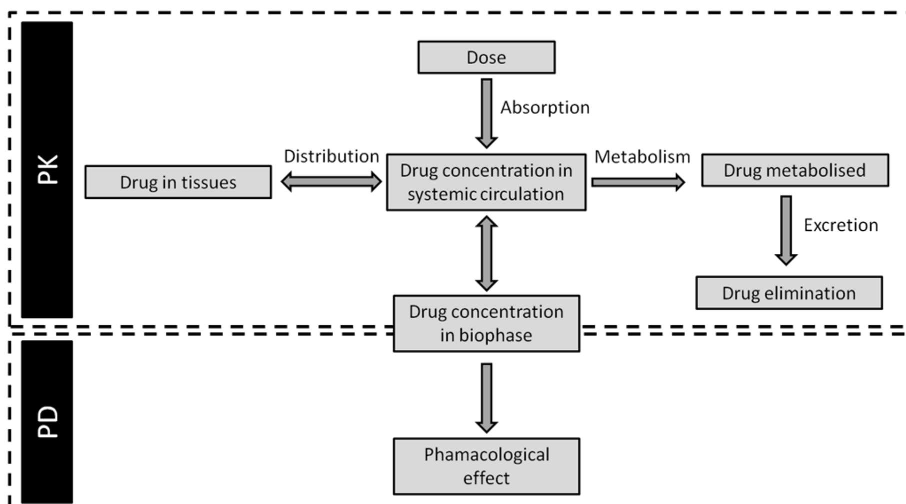

Figure 1.1 Interrelationship between PK and PD. Diagrammatic representation of the relationship between drug dosing, drug concentration, and pharmacological effect.

PK provides the mathematical basis to quantify the ADME processes. These processes determine the drug concentration and the time that it remains in the body. The understanding of these processes is required to prescribe the appropriate drug regiment for a patient.[3]

Absorption is the transfer of a drug from its site of administration into the bloodstream. The route of administration will affect drastically the rate and extent of absorption. When the drug is administered intravenously (i.v.) no absorption is required, as it immediately reaches the bloodstream. When the drug is administered by other routes—e.g. orally or intramuscular (i.m.)—the availability of the drug in the bloodstream is less straightforward, and also depends on other factors such as the formulation and chemical properties of the drug, or physiologic characteristics, as the drug must cross several biological membranes before reaching systemic circulation.

Once in the systemic circulation, drugs are distributed to organs and tissues, diffusing into interstitial fluid and cells. The lipid solubility of the drug greatly affects its **distribution**, as highly lipid soluble drugs diffuse easily through membranes into cells.[4] Other factors affecting distribution are organ blood flow—highly perfused tissues such as brain, heart, liver and kidney usually have rapid onset of action, while it is slower in less perfused tissues such as skeletal muscle, skin, or bone—, protein binding, or molecular size.

The **metabolism**—or biotransformation—and **excretion** of the drug are the processes responsible for the decline of blood drug concentration over time. Drugs are eliminated from the body through the kidneys and bile either unchanged or transformed into more easily excretable molecules. This process of transformation of drugs into more polar, water-soluble products, that takes place mainly on the liver and kidneys, is called metabolism. Drug metabolism is divided into two phases. Phase I metabolism includes oxidative, hydrolytic, and reductive reactions, while Phase II metabolism involves conjugation reactions with an endogenous

substance such as acetate, glucuronate, sulphate, or glycine. The main role of drug metabolism is to inactivate and detoxify drugs. However, in some cases the drug metabolite is pharmacologically active, and the biotransformation is necessary in order to obtain the effect. In either case, the formation of drug metabolites generates a decrease of the drug concentration. The excretion of the drugs from the body occurs primarily in the urine, although other routes of excretion include bile, sweat, saliva, tear, faeces, breast milk, and exhaled air.[5]

1.2 PK MODELS

PK models are mathematical approaches to reality that allow the description and prediction of the temporal evolution of drug levels in the body based on limited observations. PK parameter estimation is generally based on **noncompartmental**, **compartmental** or **physiologically based pharmacokinetic models (PBPK)**.

Noncompartmental models are simple and free of assumptions, based only on observations, but cannot be used to perform simulations or extrapolate the outcome upon drug administration in different situations.

Compartmental models are more complex, representing the body as a finite number of compartments arranged either in series or parallel to each other with specified interconnections, inputs and losses. These compartments do not represent a specific tissue or fluid, but simplifications of body structures. The most frequently used model is the simplest one, the one-compartment model, although two-compartments and multicompartment models are also used (Figure 1.2). Compartmental models are characterized by parameters (constant within a subject) and variables (dependent on the administered dosing regimen).

PBPK models include compartments for tissues involved in exposure, toxicity, biotransformation, and clearance processes connected by blood flow. Those compartments and blood flows are described using

physiologically meaningful parameters. PBPK can also be used to simulate different conditions, such as interspecies extrapolation—e.g. piglet to human—or simulation under various developmental stages—e.g. adult to child—or physiological conditions—e.g. healthy to not healthy—.[6]

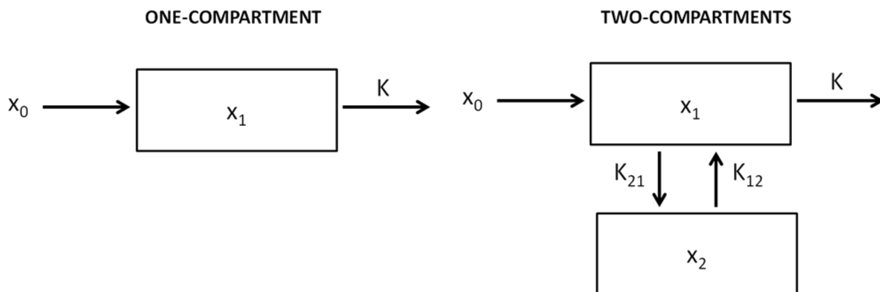

Figure 1.2 Compartmental models. One- and two-compartment models, where X_0=drug dose, X_1= amount of drug in central compartment, X_2= amount of drug in peripheral compartment, K = elimination rate constant from central compartment to outside the body, K_{12} = elimination rate constant from central compartment to peripheral compartment, K_{21} = elimination rate constant from peripheral compartment to central compartment.

1.3 DRUG CONCENTRATION PROFILE

The concentration–time profile of a drug is a graphical representation of the evolution of a drug concentration in the body, from the administration to its total excretion. It depends on the dose regimen and route of administration—e.g. i.v. administered drugs distribute rapidly without an absorption step, while for other routes of administration the absorption needs to be considered (Figure 1.3)—and the individual PK characteristics of both the drug and the subject. Ideally, the concentration of a drug should be measured at the site of action of the drug. However, receptor sites are usually inaccessible or widely distributed in the body, making unachievable the direct measure of drug concentration. In those cases drug levels in plasma are frequently measured, assuming that drug concentration in this fluid is in equilibrium with the drug concentration at the receptor. The predictable relationship between plasma concentration and concentration at the receptor site is known as kinetic homogeneity.[7] However, in some cases the ratio drug level in the site of action/drug level in plasma is not

directly correlated, and measures of the on-site concentration or fluids more proximate to the receptors—e.g. cerebrospinal fluid (CSF) for drugs acting in the central nervous system (CNS)—are necessary.[8]

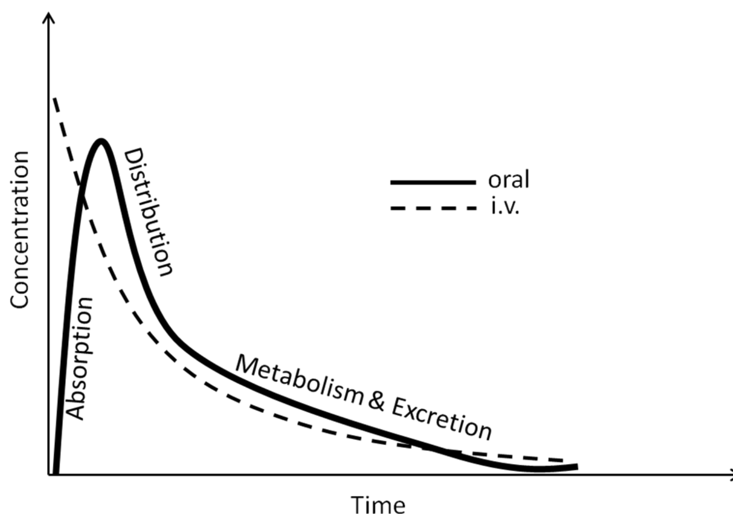

Figure 1.3 Plasma concentration–time profiles. Comparison of the typical plasma concentration–time profiles of an i.v. single dose and an oral single dose of a drug.

2 ANALYTICAL CHEMISTRY AND PK STUDIES

PK studies require repeated measurements over time in order to create complete concentration–time profiles, involving the use of multiple samples. A sufficient number of data points must be included to calculate pharmacokinetic parameters such as the area under the concentration–time curves (AUC), clearance (CL), maximum concentration (C_{max}), time to reach maximum concentration (t_{max}), half life ($t_{1/}2$) or volume of distribution (V_d) among other pharmacokinetic parameters.

In PK studies the minimisation of sample volume is of great importance, particularly when the volume of the fluids under study is limited as CSF, or in especial populations like neonates. The latest *in vivo* sampling techniques such as microdialysis[9] or solid phase microextraction (SPME) fibres[10] that

are being implemented with promising results also require analytical methods able to measure little amounts of sample. This is only possible thanks to the development of highly sensitive methods that allow to measure small concentrations of drugs or their metabolites in small volume samples.

Analytical methodology is also evolving in terms of comfort and ease of sampling procedure, methods based on salivary[11,12] or exhaled breath condensate (EBC)[13] allow less invasive procedures, which is of great importance when paediatric subjects are the object of study.

Once the sample is obtained, plasma concentrations of the drug—and, whenever it is necessary, its metabolites—must be quantified. Analytical methods used to quantify a drug in the biological fluid of interest in paediatric populations should be accurate, precise, sensitive, specific, and reproducible.[14] Also, methods used should be readily adaptable and, as stated, use a minimum volume of sample. Due to the great number of samples commonly measured in a PK study, simple and fast methods are broadly selected. The introduction of chromatographic techniques that permit the separation of complex mixtures and the quantification of their individual components in short time has greatly accelerated PK investigations. Nowadays, high performance liquid chromatography (HPLC) is the main technique used in most of the PK studies. Among the detectors employed, spectrophotometers—as ultraviolet (UV) detector—have been traditionally used. Notwithstanding, nowadays UV detection has been mainly replaced by mass spectrometers (MS) that offer much higher specificity and sensitivity.[15]

The analytical methods used in pharmacokinetic studies have to be validated to ensure that the quantitative determination of the concentration of a drug or metabolite in a particular biological matrix is reliable for the intended application.[16]

The different drug agencies—FDA, European Medicines Agency (EMA)—have published validation guides[17,18] collecting their requirements to declare a method validated. The parameters that require validation are selectivity and specificity, accuracy, precision, robustness, range and linearity of calibration curve, limit of quantification (LLOQ), recovery, stability, dilution integrity, carryover effect, incurred sample reanalysis, and system suitability in routine drug analysis. In addition, when MS is used matrix effect must be assessed.

However, there are still some ambiguous concepts in validation criteria and methodology. The methodology to calculate fundamental parameters such as the LLOQ has been defined in several ways without reaching a harmonised definition, which can lead to very different values depending on the applied criterion. Other parameters such as robustness or ruggedness are usually omitted and when defined there is not an established approach to evaluate them. Especially significant is the case of the matrix effect evaluation which is one of the most critical points to be studied in LC-MS methods but has been traditionally overlooked. To address this issue, I have written in collaboration with my colleagues González, Iriarte, Bartolomé, Maguregui and Alonso, a thorough critical review dealing with ambiguities in method validation with a special focus on these problematic parameters. The publication resulting from this research[16] is included in the Annex II: Published papers.

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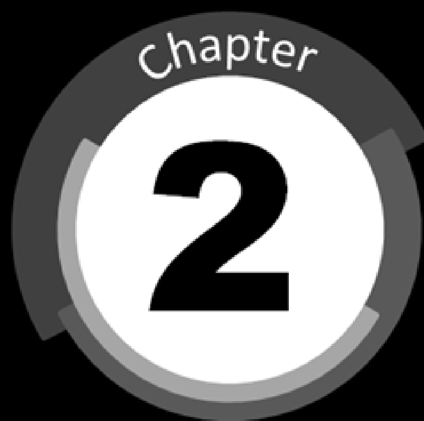
Part I: Pharmacokinetics in paediatrics



Determination of fentanyl in newborn pig plasma and CSF by HPLC-MS/MS

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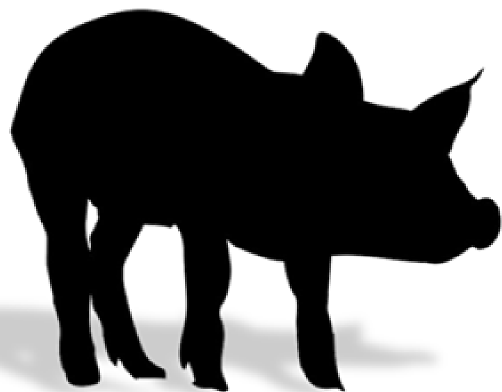
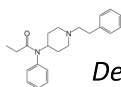


The creatures outside looked from pig to man, and from man to pig, and from pig to man again; but already it was impossible to say which was which.

(George Orwell, *Animal Farm*)



1 INTRODUCTION

Fentanyl (1-N-phenyl-N-(1-(2-phenylethyl)piperidyl)propanamide, FEN) (Figure 2.1) is a synthetic µ-opioid agonist used in neonatal and paediatric critical care units to provide analgesia and/or sedation when administered in continuous intravenous infusion during and after surgery[1] or in mechanically ventilated patients.[2,3]

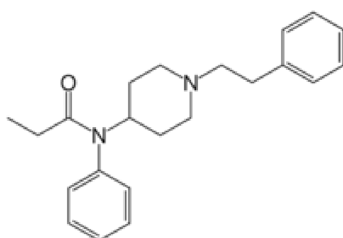


Figure 2.1 Molecular structure of Fentanyl. Systematic name: 1-N-phenyl-N-(1-(2-phenylethyl)piperidyl)propanamide

However, FEN administration is not indicated in infants (i.e., below 2 years of age) according to the manufacturer's product license, and the drug is therefore used off-label in this population. In order to increase the knowledge on the product within this context and try to reduce the degree of empiricism currently associated with the establishment of dosing regimens in this population, a maturation physiology based predictive pharmacokinetic/pharmacodynamic (PK/PD) model for fentanyl in neonatal care was built.[4] The performance of a PK/PD study in a suitable animal species was subsequently deemed convenient, as a complement and preliminary confirmation to the developed theoretical model. Concretely, the newborn piglet was considered a representative model of FEN behaviour in neonates because many of its anatomical and physiological characteristics more closely resemble those of humans than other non-primate species,[5,6] as supported by the frequent use of preterm and term neonate pigs in paediatric research.[7-9] In this respect, cytochrome P450 isoform 3A4 (CYP3A4), the enzyme responsible for hepatic fentanyl biotransformation in humans, is also present in pigs with comparable levels

and activity.[5,10,11] Moreover, the differences observed between juvenile and adult pig PK for some drugs were deemed as consistent with ontogenic changes reported for human PK.[12] Additionally, the swine cardiovascular system and its physiological development (related with the PD) are almost identical to those of humans.[6,13]

The general objective of this kind of experimental PK/PD studies is to characterise the systemic exposure of the drug after a given dose (assessed by plasma levels) as well as its relationship with the observed pharmacological effects. Nevertheless, FEN, a centrally acting drug that has to cross the blood-brain barrier to exert the majority of its analgesic and sedative effect, is known to exhibit certain degree of delay between its concentration–time profile in the blood and that observed in the central nervous system (CNS).[14] Under such circumstances, assessment of in vivo CNS availability may be of interest, as it is more likely to be directly correlated to the pharmacodynamic effects as compared to blood availability. Cerebrospinal fluid (CSF) is one of the biological matrices that can be sampled to provide an overall index of drug access to the CNS after systemic administration of a compound, thus being considered as a surrogate measure for drug concentrations at the target site within the brain.[15-17] Indeed, CSF penetration studies, often in combination with cerebral microdyalisis techniques measuring drug concentration in the brain interstitial fluid (ISF), are usually performed in preclinical species to investigate CNS drug distribution, as it is often a good reflection of the situation in humans.[17,18] Consequently, the development of a suitable, selective and sensitive analytical method capable of measuring FEN in both biological fluids is essential for the development of an experimental investigation where CSF and plasma samples are analysed.

Methods of high sensitivity and selectivity are specially required in the case of FEN, since due to its higher potency in comparison with morphine,[19-21] effective doses are much lower and, therefore, diminished concentrations (<10 ng/mL) are expected in biological fluids. In addition, the use of high sample volumes is impracticable for PK/PD studies in the newborn, where

several samples must be obtained periodically. Consequently, sensitive methods requiring low sample volumes must be used.

Some studies for the analysis of FEN in biological samples [22-24] using immunoassay methods have been reported, reaching in the best case a limit of detection of 0.0048 ng/mL[25] using 50 µL of plasma sample. However, these methods are prone to suffer from cross-interference of similar molecules such as structurally related compounds or metabolites.[26] Gas chromatography-mass spectrometry (GC-MS) methods for the analysis of FEN in plasma[27-29] have also been reported, obtaining values of lowest limit of quantification (LLOQ) ranging from 0.05 ng/mL up to 4 ng/mL when using a minimum of 500 µL of plasma. High performance liquid chromatography methods coupled to ultraviolet detection (HPLC-UV) found in literature[30,31] show the same problem, using 1 mL of plasma to reach a LLOQ of 0.2 ng/mL, in the best case. The only method using a suitable volume of plasma (100 µL)[32] is not sensitive enough for this PK/PD analysis (LLOQ equal to 3 ng/mL).

Several high performance liquid chromatography-mass spectrometry (HPLC-MS) methods are available for the determination of FEN and its derivatives in plasma. Methods reported by Koch et al.[33] and Huynh et al.[34] reached LLOQ values as low as 0.02 ng/mL and 0.025 ng/mL respectively; however, in order to reach those levels is required 1 mL of plasma sample and a liquid-liquid extraction (LLE) procedure was required. Lower sample volumes were used by Chang et al.[35] and Hisada et al.,[36] using an LLE procedure and a simple protein precipitation method respectively.

Studies using HPLC for the quantification of FEN in CSF or brain perfusate samples are scarce,[37,38] and to the best of our knowledge, HPLC-MS/MS has not been yet applied to the analysis of FEN in CSF samples from the newborn. The knowledge of drug concentrations in this biological matrix and their relationship to plasma or urine levels would add relevant information towards the establishment of PK/PD correlation for FEN.

The aim of this work was to develop an HPLC-MS/MS method with electrospray ionisation in positive mode that would allow rapid, sensitive and reproducible quantification of fentanyl in plasma and CSF, requiring small sample volume and quick sample processing, for its subsequent application on a PK/PD study of FEN in newborn pigs as an animal model of human neonates.

2 MATERIAL AND METHODS

2.1 INSTRUMENTATION

Chromatographic separation was carried out on an Alliance HPLC 2695 separation module (Waters, Milford, MA, USA). A Luna C18 (150 x 2 mm id, 3 µm) chromatographic column (Phenomenex, Torrance, CA, USA) was used as stationary phase. Mass spectrometric analysis was performed using a tandem mass spectrometer Quattro micro (Waters, Milford, MA, USA) equipped with an electrospray ionisation source operating in positive mode. Data acquisition was performed using MassLynx 4.0 software (Waters, Milford, MA, USA). Sample centrifugation was performed using an Eppendorf 5424 centrifuge (Eppendorf, Hamburg, Germany).

2.2 REAGENTS AND SOLUTIONS

FEN and $^{13}C_6$FEN, used as internal standard, were purchased from Alsachim (Illkirch Graffenstaden, France). HPLC quality formic acid and ammonium formate, from Sigma Aldrich (St. Louis, MO, USA), were used in the preparation of buffer solutions. LC-MS grade acetonitrile (ACN) (VWR, Radnor, PA, USA) was used as organic modifier. Purified water from a Milli-Q Element A10 System (Millipore, Billerica, MA, USA) was used in the preparation of buffer and reagent solutions.

Drug-free pig plasma samples were purchased from Seralab (West Sussex, United Kingdom) and collected in polypropylene tubes to be frozen at -20 °C. Due to the lack of drug-free pig CSF samples, artificial CSF was prepared

as an aqueous solution of NaCl (147mmol/L), KCl (2.7 mmol/L), $CaCl_2$ (1.2 mmol/L) and $MgCl_2$ (0.85 mmol/L).

2.3 PREPARATION OF STANDARD SOLUTIONS AND QUALITY CONTROL (QC) SAMPLES

FEN and $^{13}C_6$FEN were dissolved in dimethyl sulfoxide to give 1 mg/mL primary stock solutions. A 1000 fold dilution of the FEN primary stock solution was made in water to achieve a working solution with a concentration of 1 µg/mL. Aliquots of this working solution were added to drug-free plasma and artificial CSF to obtain Quality Control samples (QCs) at three concentration levels: Low, Mid and High QCs; being the Low QC three times the concentration at the LLOQ, the Mid QC the geometrical mean of the calibration range points, and the High QC the 85% of the upper limit of quantification (ULOQ). Calibration standards at seven levels ranging from 0.2 to 15 ng/mL for plasma and from 0.25 to 5 ng/mL for CSF were prepared also by dilution of the working solution with drug-free plasma or CSF. A dilution of the internal standard solution with ACN was made to give a 15 ng/mL solution. Primary stock solutions were stored at -20 °C and working solutions were stored at 4 °C until analysis. Calibration standards and QCs were freshly prepared immediately prior to analysis.

2.4 EXPERIMENTAL STUDY DESIGN

The analytical method developed was used for the quantification of FEN in pig plasma and CSF samples obtained in a prospective study that aimed to investigate the drug PK/PD behaviour when intravenously (i.v.) administered alone (in monotherapy) to mechanically ventilated newborn piglets (2-4 days, 1.7±0.2 kg, n=6) of each gender. The experimental protocol, which is explained in detail somewhere else,[39] met European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005) and was approved by the Ethical Committee for Animal Welfare of the Cruces University Hospital.

FEN dosage regimen (5 µg/kg bolus immediately followed by a 90 minute infusion of 3 µg/kg/h) was estimated as suitable for providing an adequate degree of sedation, measured by amplitude-integrated electroencephalography (aEEG), based on the results of a pilot study previously performed in two additional animals (data not shown).

Blood samples (n = 13-15 per animal) for the quantification of FEN were withdrawn at baseline, immediately after bolus administration, at t = 1, 10, 30, 90, 95, 120, 150 and 180 minutes after the start of the infusion and then every 30 minutes until experiment was stopped, which occurred at initial signs of awakening shown by each animal (i.e., t = 225-300 minutes). As restricted by the low volume of CSF in the study population as well as by the short evaluation period (maximum of 5 hours), the extraction of a single CSF sample in each animal was considered acceptable from an ethical perspective. CSF sample was drawn either at t = 10, 90 or 150 minutes (2 animals per time point), in order to allow comparison with the simultaneously extracted blood sample.

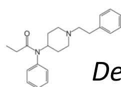
2.5 **SAMPLE COLLECTION**

Samples were collected by the Research Unit for Experimental Neonatal Respiratory Physiology at Cruces University Hospital (Barakaldo, Biscay, Basque Country).

Whole arterial blood samples were collected in EDTA tubes, and kept on ice until their immediate centrifugation at 3000 rpm at 4 °C in order to obtain the plasma. The supernatant was transferred to cryovials and stored at -80 °C until analysed. CSF samples were collected by lumbar puncture and stored in cryovials at -80 °C.

2.6 **SAMPLE PREPARATION**

Frozen samples from the studied animals were thawed until reaching room temperature. A volume of 150 µL of ACN with a concentration of $^{13}C_6$FEN of



15 ng/mL was added to 100 µL of plasma (final $^{13}C_6$FEN concentration 9 ng/mL) to promote protein precipitation and was vortex mixed for 5 min. Samples were then centrifuged at 10000 rpm during 5 min. The clean upper layer was transferred to a chromatographic vial to be injected in the HPLC-MS/MS system. CSF samples were injected without any sample preparation except the addition of 5 µL of the $^{13}C_6$FEN solution in ACN at a concentration of 15 ng/mL to 50 µL of sample (final $^{13}C_6$FEN concentration 1.36 ng/mL).

2.7 CHROMATOGRAPHIC AND MASS SPECTROMETRIC CONDITIONS

Chromatographic separation was achieved using an isocratic method, operating at a flow rate of 0.25 mL/min over a total run time of 3.5 min. The mobile phase was a mixture of ACN and water (40:60 v:v) containing 10 mM of formic acid/ammonium formate buffer, pH 3.5. A sample aliquot of 10 µL was injected into the column. The autosampler temperature was set at 10 °C and the column was kept at 30 °C.

Mass spectrometer source temperature was set at 120 °C. Nitrogen was used as desolvation gas at a temperature of 300 °C and at a flow of 450 L/h. Capillary voltage was set at 0.8 kV. FEN and $^{13}C_6$FEN were detected by multiple reaction monitoring mode (MRM) with a dwell time of 0.20 s. The following transitions were monitored in ESI +: *m/z* 337.0 → *m/z* 188.0—in accordance with the values reported in the literature [34-36]—using a cone voltage (CV) of 35 V and a collision energy (Ce) of 25 eV for FEN quantification, *m/z* 337.0 → *m/z* 105.0 using a CV of 25 V and a Ce of 45 eV for FEN confirmation and *m/z* 343.0 → *m/z* 188.0 using a CV of 45 V and a Ce of 25 eV for $^{13}C_6$FEN.

2.8 VALIDATION OF HPLC-MS/MS METHOD

The developed method was validated in terms of selectivity, linearity, sensitivity, accuracy, precision, carryover and matrix effect, following the FDA criteria established in the Bioanalytical Method Validation Guide. [40]

The selectivity of the method for plasma was evaluated by comparing the response of six individual drug-free plasma samples against a sample at the LLOQ, with reference to potential endogenous and environmental interferences. Due to the absence of blank real samples of CSF, the selectivity of the method in this matrix was evaluated analyzing aliquots of artificial CSF. The signal obtained in the blank matrices must be lower than 20% the response of FEN at the LLOQ and 5% the response of $^{13}C_6$FEN.

Calibration curves—consisting of a blank sample (blank matrix), a zero sample (blank matrix spiked with $^{13}C_6$FEN), and six non-zero calibration standards—were built plotting the corrected peak area of fentanyl (FEN/$^{13}C_6$FEN) against its nominal concentration. The acceptance criterion for the calibration curve was that at least four out of the six non-zero calibration standards had less than 15% deviation from the nominal concentration (20% for LLOQ standard). Sample concentration was calculated by interpolating the resulting corrected area in the regression equation of the calibration curve.

Sensitivity was examined by comparing blank samples with the response of calibration standards at the LLOQ, calculated using the Equation 2.1.

$$LLOQ = \frac{y_{blank} + 10 \cdot s}{b} \qquad \text{Equation 2.1}$$

Where y_{blank} is the average signal obtained from six different plasmas or six replicates of artificial CSF, s is its standard deviation and b is the slope of the calibration curve. The analyte response should be at least five times the response obtained from a blank sample.

In order to evaluate the intra-day accuracy, five replicate spiked samples were prepared in plasma and CSF at three concentration levels: Low, Mid and High QC; they were analysed the same day and their concentration value was obtained from interpolation of the resulting corrected area in the regression equation of the calibration curve. Accuracy was expressed as relative error (%RE). The acceptance criterion for accuracy was %RE <15%.

Inter-day accuracy was determined by calculating the %RE obtained when repeating intra-day accuracy experiments in three different days.

Intra and inter-day precision were evaluated as relative standard deviation (%RSD) of five replicates of the Low, Mid and High QCs in three different days, following the same procedure as for accuracy assay. The acceptance criterion for precision was %RSD <15%.

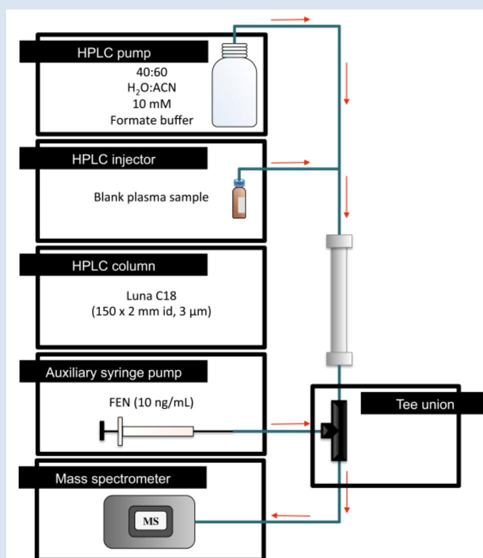
Carryover was tested by injection of a blank plasma sample directly after injection of the ULOQ standard. The response in the blank sample following the high concentration standard was then compared with the response at the LLOQ, and was considered acceptable if the signal obtained at the FEN and $^{13}C_6$FEN retention time was under 20% of the signal at the LLOQ and under 5% of the $^{13}C_6$FEN signal.

For the evaluation of matrix effect, five samples of each Low QC, Mid QC and High QC were prepared spiking five different blank plasmas with FEN and $^{13}C_6$FEN after protein precipitation. Normalised matrix factor (NMF) was determined as follows: NMF = (analyte peak area/IS area) in matrix/(analyte peak area/IS area) in pure solution. %RSD of the results in different plasma samples was calculated in order to demonstrate the absence of 'relative' matrix effect, referring to the variability of matrix effect among different sources of the same matrix. If %RSD was lower than 15%, the method was considered to be free of relative matrix effect.

Moreover, matrix effect was also qualitatively studied performing the post-column infusion experiments reported by Bonfiglio et al.[41] (as explained in Box 1). For this purpose, a solution of FEN (10 ng/mL) was infused post-column at a flow rate of 10 µL/min while the analysis of a blank plasma sample was carried out simultaneously. The eluent from the column and the flow from the infusion were combined using a zero-dead-volume Tee union and introduced into the source of the mass spectrometer.

Box 1 Matrix effect assessment

Matrix effect (ME) in mass spectrometry is defined as the effect of other compounds present in the matrix, different than the analyte, causing suppression or enhancement of the signal. ME could be considered one of the most important disadvantages of LC-MS methods in bioanalysis. It can diminish or enlarge the signal of the analyte and alter the S/N ratio modifying therefore the LLOQ. Furthermore, ME can vary dramatically among samples affecting the accuracy and precision of the method, and causing lack of linearity, which can lead to deceitful results. ME is mainly assessed by two strategies, a qualitative assessment based on post-column infusion and a quantitative evaluation as the absolute ME by post-extraction addition.



In the first protocol a constant flow of a solution of the analyte is added after the chromatographic column using a 'Tee' union while an injection of an extracted blank sample is carried out. Same procedure is followed with a blank solvent injection and afterwards both profiles are compared. Ideally, if there is no ME, the profiles of the analyte for the blank sample and the blank solvent should be the same. Ion suppression or enhancement is evidenced by variations in this profile. If more than one analyte is analysed with the same method each analyte must be infused individually to evaluate its ME in order to avoid self-induced ME.

For a quantitative assessment, is common to use a methodology based on the calculation of the matrix factor (MF) by comparing the signals from the analysis of three sets of samples: a matrix free standard (A), a sample spiked after the extraction of a blank matrix (B) and the same matrices spiked before extraction (C), all at different levels of concentration. MF is defined as the ratio of the response of B over the response of A. With these three sets the recovery (REC) and the process efficiency (PE) can be calculated as follows: REC = (C/B) and PE = (C/A) = (REC × MF). The advantage of this procedure is that recovery and ME are assessed together and can be combined with accuracy and precision studies.

According to this last definition, a MF value of one implies that the analysis is not affected by any ME, a MF lower than one indicates ion suppression, and a MF value higher than one indicates ion enhancement. The internal standard normalised MF (NMF) is defined as the MF of the analyte divided by the MF of the IS.

3 RESULTS AND DISCUSSION

3.1 CHROMATOGRAPHIC BEHAVIOUR OF FENTANYL

In the optimum chromatographic conditions the mean retention time of FEN was 2.05 min. As expected, the internal standard $^{13}C_6$FEN eluted at the same time as FEN, as can be seen in Figure 2.2.

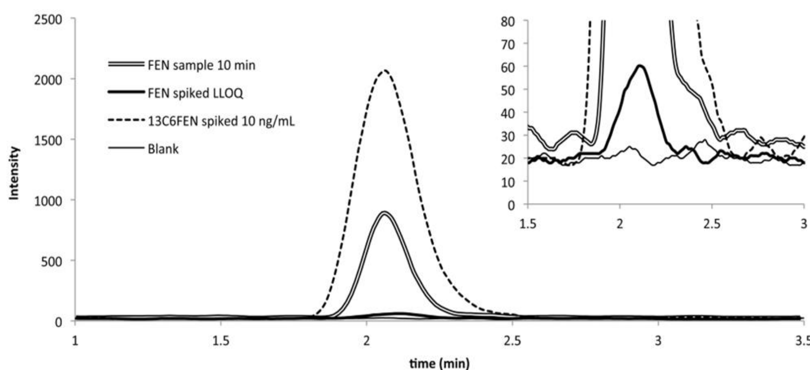


Figure 2.2 FEN chromatograms. Chromatograms of a blank pig plasma sample, the same sample spiked with 10 ng/mL of 13C6FEN and with 0.2 ng/mL of FEN at the LLOQ, and a pig plasma sample from the pharmacokinetic study taken 10 min after the fentanyl bolus dose.

3.2 METHOD VALIDATION

3.2.1 SELECTIVITY

No interfering peaks were observed at FEN retention time in any of the six individual pig plasma and CSF samples evaluated. For FEN and $^{13}C_6$FEN the response in blank plasma and CSF samples was lower than 20% and 5%, respectively, of the response at the LLOQ values (0.2 ng/mL for plasma and 0.25 ng/mL for CSF).

3.2.2 CALIBRATION CURVES AND SENSITIVITY

Calibration curves met the criteria established for linearity in the range of 0.2 ng/mL to 15 ng/mL for FEN in plasma and 0.25 ng/mL to 5 ng/mL in CSF

with values for R^2>0.999 in all cases. Moreover, the %RE value of all the non-zero standards was lower than 15%.

3.2.3 ACCURACY AND PRECISION

The results for accuracy and precision are presented in Table 2.1. Both, in plasma and CSF, the calculated %RE was lower than 15% at the Low, Mid and High QC for both the intra and inter-day assays, evidencing an adequate accuracy along the calibration range. Moreover, the %RSD was below 15% in all QC samples for both plasma and CSF samples, indicating that the precision of both methods was also suitable.

Table 2.1 Intra and inter-day accuracy and precision in terms of %RE and %RSD, respectively, for plasma and CSF samples at low, mid and high QC concentration values.

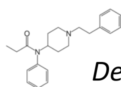
	Plasma					**CSF**			
	Intra-day			Inter-day		Intra-day			Inter-day
	Day 1	Day 2	Day 3	Days 1-3		Day 1	Day 2	Day 3	Days 1-3
Low QC (0.5 ng/mL)					**Low QC** (0.6 ng/mL)				
Mean	0.44	0.54	0.43	0.47	Mean	0.53	0.62	0.62	0.59
%RE	**8.17**	**10.61**	**2.40**	**5.67**	%RE	**12.04**	**2.58**	**3.47**	**2.00**
%RSD	**9.23**	**7.45**	**8.72**	**12.32**	%RSD	**14.23**	**12.69**	**9.06**	**8.89**
Mid QC (3 ng/mL)					**Mid QC** (1.2 ng/mL)				
Mean	2.80	2.96	3.33	3.03	Mean	1.12	1.29	1.18	1.20
%RE	**6.60**	**1.25**	**10.90**	**0.96**	%RE	**6.56**	**7.33**	**1.86**	**0.37**
%RSD	**12.53**	**3.75**	**9.48**	**8.88**	%RSD	**7.62**	**9.80**	**11.66**	**7.09**
High QC (12 ng/mL)					**High QC** (4.25 ng/mL)				
Mean	12.32	10.73	12.29	11.78	Mean	4.26	4.14	4.41	4.27
%RE	**2.67**	**7.71**	**13.35**	**1.85**	%RE	**0.25**	**2.53**	**3.68**	**3.09**
%RSD	**12.05**	**6.55**	**10.87**	**7.73**	%RSD	**4.26**	**6.43**	**7.85**	**0.47**

3.2.4 CARRYOVER

No quantifiable carryover effect was observed when injecting blank pig plasma or CSF solution immediately after the ULOQ.

3.2.5 MATRIX EFFECT

Post-column infusion experiments showed a substantial suppression of the ionisation of FEN due to matrix interferences as shown in Figure 2.3. Notwithstanding, this effect was compensated by the isotopically labelled



internal standard, with the average NMF among the different plasma sources being 93% and presenting a variability, in terms of %RSD, of 9% (n=5).

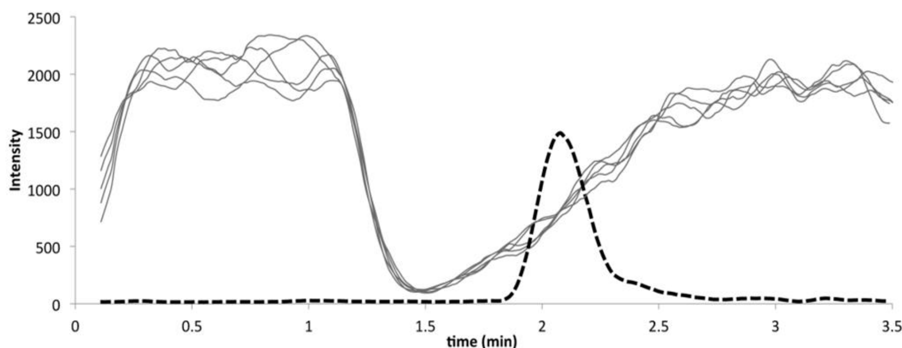


Figure 2.3 Post-column infusion experiment. Injection of 5 blank plasmas with post-column infusion of FEN (continuous lines) and injection of a blank plasma spiked with FEN at a concentration of 10 ng/mL (dashed line).

Notably, all parameters (i.e., selectivity, sensitivity, accuracy and precision, carryover and matrix effect) complied with the established acceptance criterion; therefore, the method was successfully validated.

3.3 Analysis of samples from PK/PD experimental study

The optimised HPLC-MS/MS method was implemented for the measurement of FEN concentration in pig plasma and CSF samples obtained from a PK/PD experimental study performed in newborn piglets.[39]

The developed method enabled the quantification of FEN concentrations from as low as 0.2 ng/mL, thus allowing the characterisation of the plasma profiles in piglets (Figure 2.4). The majority of the plasma concentrations calculated were above the LLOQ, except for the latest sampling points of two of the pigs in the study (no.3 and no.6), in which FEN has apparently already been nearly eliminated by that time.

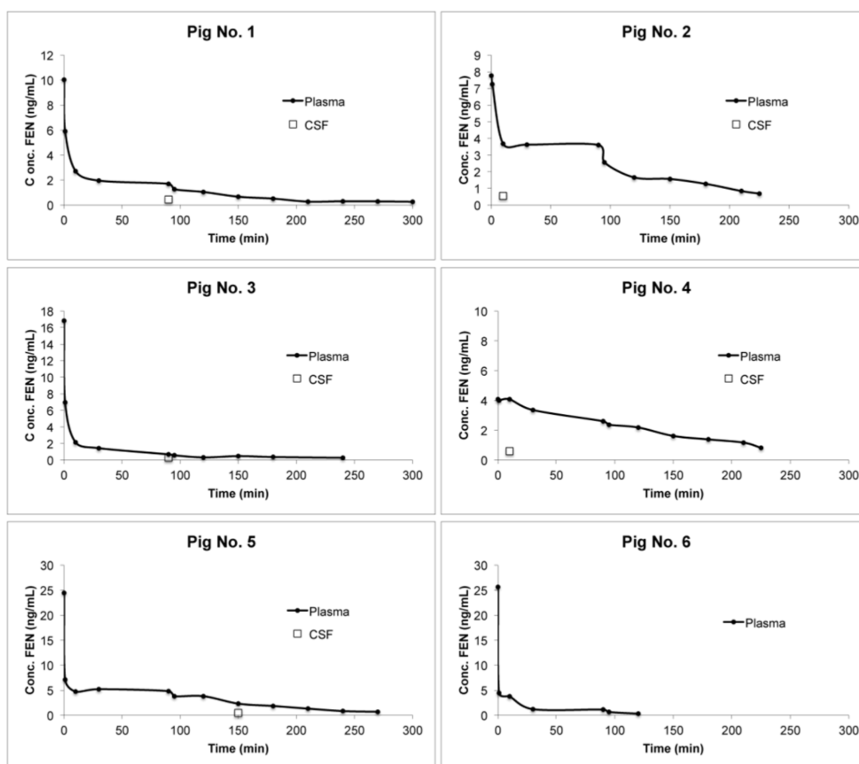
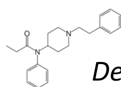


Figure 2.4 Fentanyl concentration profiles. Individual plasma profiles (black dots) and concentration measured in the available single CSF sample (white squares) of FEN piglets as quantified by the develop HPLC-MS/MS method. FEN level in CSF sample extracted from pig No. 6 was below the LLOQ and could therefore not be displayed.

The plasma concentration time curves obtained in all animals revealed multi-exponential disposition kinetics as expected, displaying a rapid initial distribution phase (compatible with high lipophilicity of FEN) followed by a slower decline. Although FEN plasma levels showed quite a large interindividual variability, the drug had, overall, been cleared up by the end of the experiments (225-300 min), which is consistent with animals showing initial signs of awakening at this point.

Fentanyl is primarily eliminated from the body by hepatic N-dealkylation via CYP3A4 to the inactive metabolite norfentanyl,[42-44] which is subsequently excreted in urine accounting for roughly 94% of the dose. The remaining



percentage of the dose is excreted unchanged in urine and stool,[45,46] so that quantification of the metabolites in study samples was deemed purposeless.

FEN was also determined in CSF samples and was detected from all of them, with the exception of the one taken at 150 min from pig no. 6, whose plasma FEN concentration was also below the LLOQ by that time. In the remaining CSF samples, FEN was detected even at the first time point (10 minutes post-dose) (Figure 2.4), thus confirming the rapid access of the compound to the CNS, in line with its high lipophilicity.

The CSF/plasma ratio provides insight into the CNS drug exposure or availability of centrally active compounds, thus serving as a reference for assessing the extent of delivery to the pharmacological targets within the CNS (biophase or effect site). This is especially true for those drugs crossing the blood brain barrier (BBB) mainly by diffusion via the transcellular route after systemic administration,[12,14] which seems to be the case for FEN in line with its high lipophilicity and the apparent lack of active transport at the level of BBB. Indeed, FEN has proved not to behave as a substrate of main transporters including efflux P-glycoprotein or influx organic anion-transporting polypeptide (OATP).[47,48]

The comparison of CSF and plasma concentrations is particularly applicable in elucidating the lag in the time course of a central pharmacologic effect relative to that of drug concentration in circulation, under the assumption that CSF is in equilibrium with the biophase.[14] Even if care should be taken when interpreting data with only a single time point CSF and plasma concentration available, this is, to the best of our knowledge, one of the first reports on the temporal inter-relationship of FEN plasma and CSF kinetics after i.v. administration of such low doses in preclinical species. Up to date, two single reports have been found in scientific literature describing this relationship in experimental animal models, but they refer to the administration of doses far higher than the ones concerned herein. The first one was performed in dogs injected tritium-labeled ^{3}H-FEN (10 or 100

µg/kg),[49] and the second one applied HPLC-UV to the quantification of FEN only at steady state conditions in piglets administered 30 µg/kg bolus followed by infusion at 10 µg/kg/h.[38]

In this sense, there also seems to be a paucity of published data on methods of analysis using HPLC-MS/MS for the quantification of FEN in CSF or cerebral microdialysis samples, despite the importance of determining the drug levels in the CNS with a sufficient degree of sensitivity. Even though in the present study only one CSF sample was obtained from each animal, the low volume of CSF needed (50 µL) allows the applicability of the method in future and more specific pharmacokinetic studies aimed to further evaluate the CSF distribution of FEN in larger preclinical populations and/or under different dosing protocols. The low volume of CSF needed (50 µL) eases the application of this method to the analysis of samples of the newborn. For instance, the performance of frequent serial CSF sampling over time would allow the calculation of the relative CSF exposure as compared to plasma, which is given by the ratio between the corresponding areas under the curve (AUC_{CSF}/ AUC_{plasma} ratio). Moreover, this HPLC-MS/MS method could also be applied to the quantification of FEN levels in brain ISF samples obtained via microdialysis techniques, thus providing the tool for the joint assessment of PK disposition in both matrices. This could help elucidating the existing PK inter-relationship of FEN concentrations in plasma, CSF and brain ISF, against the observed pharmacodynamic effects in suitable animal models [17,18,50]. This PK/PD correlation may then be extrapolated to humans based on the well-described predictive capacity of some preclinical species,[14,15] which is of great value in view of the extremely restricted access to sampling of these biophase surrogate markers (i.e., CSF and brain ISF as indicative of drug levels at the effect site) in humans.

4 CONCLUSION

A simple, selective and sensitive HPLC-MS/MS method was developed and validated for the quantitative determination of FEN in pig plasma and CSF

samples, which could be applied in future pharmacokinetic/pharmacodynamic assays.

This assay requires only a small volume of plasma (100 µL) and CSF (50 µL), which is of particular advantage in cases where sample volumes are limited (e.g., paediatric preclinical studies). The suitability of the method was assessed by its successful application to samples of both types of biological fluid from a pharmacokinetic study performed in newborn piglets.

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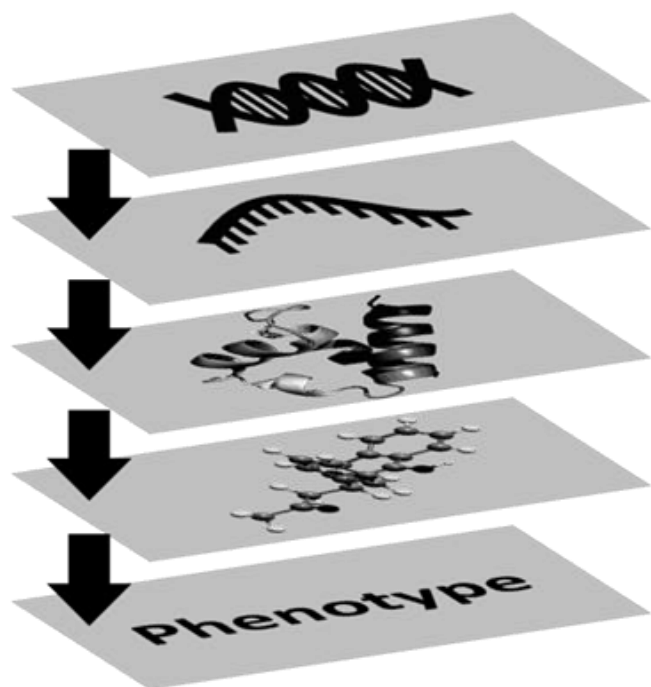
Part II: Metabolomics in paediatrics

Chapter 3

Introduction to metabolomics

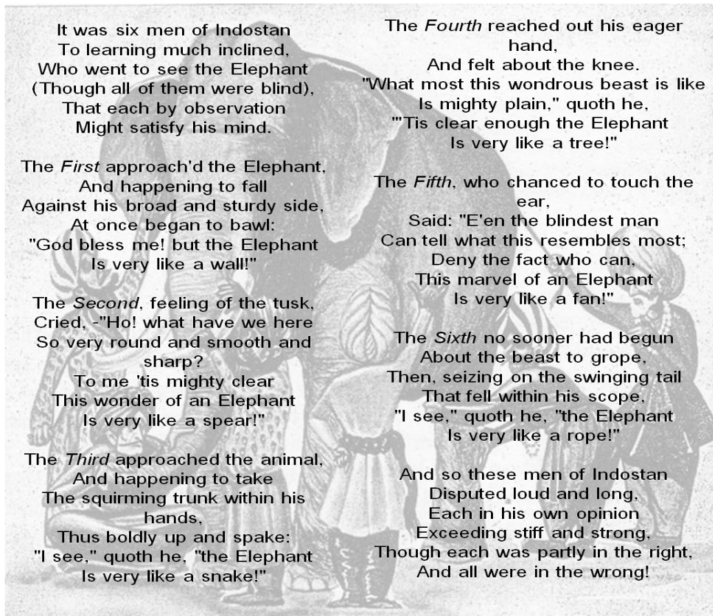


Did you ever feel, as though you had something inside you that was only waiting for you to give it a chance to come out? Some sort of extra power that you aren't using — you know, like all the water that goes down the falls instead of through the turbines?"

(Aldous Huxley, *Brave New World*)

1 FROM REDUCTIONISM TO SYSTEMS BIOLOGY

During the last half century reductionism has been the central issue in biology. Breakthrough discoveries and advances have been made in molecular biology—being the discovery of the double helix structure of the DNA undoubtedly the most popular—up to the point that it can be said that we have lived a 'molecular revolution'. However, in the last years, biology has experienced a change of direction in between the experimental and the philosophical. Rather than dissecting biological systems into their constituent parts to study them in isolation, the systems perspective takes a holistic approach, studying the whole organisation of individual components and their network behaviour. In words of Aristotle (B.C.E. 384 – 322) *«The whole is more than the sum of its parts»;* the differences between reductionism and systems biology can be compared with the Indian legend of the blind men and the elephant (Figure 3.1), where the limits of individual (or reduced) perspective are represented.



It was six men of Indostan
To learning much inclined,
Who went to see the Elephant
(Though all of them were blind),
That each by observation
Might satisfy his mind.

The *First* approach'd the Elephant,
And happening to fall
Against his broad and sturdy side,
At once began to bawl:
"God bless me! but the Elephant
Is very like a wall!"

The *Second*, feeling of the tusk,
Cried, -"Ho! what have we here
So very round and smooth and sharp?
To me 'tis mighty clear
This wonder of an Elephant
Is very like a spear!"

The *Third* approached the animal,
And happening to take
The squirming trunk within his hands,
Thus boldly up and spake:
"I see," quoth he, "the Elephant
Is very like a snake!"

The *Fourth* reached out his eager hand,
And felt about the knee.
"What most this wondrous beast is like
Is mighty plain," quoth he,
"'Tis clear enough the Elephant
Is very like a tree!"

The *Fifth*, who chanced to touch the ear,
Said: "E'en the blindest man
Can tell what this resembles most;
Deny the fact who can,
This marvel of an Elephant
Is very like a fan!"

The *Sixth* no sooner had begun
About the beast to grope,
Then, seizing on the swinging tail
That fell within his scope,
"I see," quoth he, "the Elephant
Is very like a rope!"

And so these men of Indostan
Disputed loud and long,
Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong!

Figure 3.1 Poem The Blind men and the elephant, by John Godfrey Saxe. The study of the parts in isolation is not erroneous, but a vision of the whole system is needed in order to understand it.

The systems biology approach provides a new perspective in medicine and pharmacological sciences, where physiology and disease are viewed as dynamic processes constrained by interrelationships among pathway and network components. Therefore, the detection, understanding, and treatment of a disease is starting to be approached through the identification and manipulation of global perturbed networks rather than focusing only on the individual components affected.[1]

2 OMIC TECHNOLOGIES

Omic technologies (e.g. genomics, transcriptomics, proteomics and metabolomics) have become key tools in the development of systems biology. Using a holistic view and studying the system as a whole, omic sciences are aimed at the universal detection of genes (**genomics**), mRNA (**transcriptomics**), proteins (**proteomics**) and metabolites (**metabolomics**), where no hypothesis is known or prescribed, but all data are acquired and analysed to define it.[2]

The development of the polymerase chain reaction (PCR) technique in 1983,[3] and therefore the significant falling of the cost of DNA sequencing, spurred a colossal revolution in biotechnology and genetic studies. In 1990 the Human Genome Project[4] was proposed, aimed to sequence the complete set of DNA in the human genome. The project was completed in 2003, more than two years ahead of schedule and under budget. However, the decoding of the genome is only the beginning of genomics: functional, comparative or structural genomics are some of the different areas of genomic studies. Nevertheless, genomics only gives us a glimpse of what may occur as dictated by the genetic code, and can be considered as an entry point for looking at other omic sciences.

With the aim of studying the level of expression of the genes, transcriptomics is focused on the analysis of messenger RNA (the transcriptome). A transcriptome forms the template for protein synthesis, resulting in a corresponding proteome, the final product of genome

expression. Proteomics is the discipline which studies the proteome and the expression, function and structure of the proteins that constitute it.

Although the genotype—the information in the genes of an organism—is largely responsible for the final state of an organism, the **phenotype**—the description of the total of physical characteristics of a biological system including its morphology, development, and metabolism—is also strongly influenced by environmental factors. It is metabolomics, the study of metabolites, the last discipline in the omics cascade (Figure 3.2), considered closer to this phenotype than the other techniques.

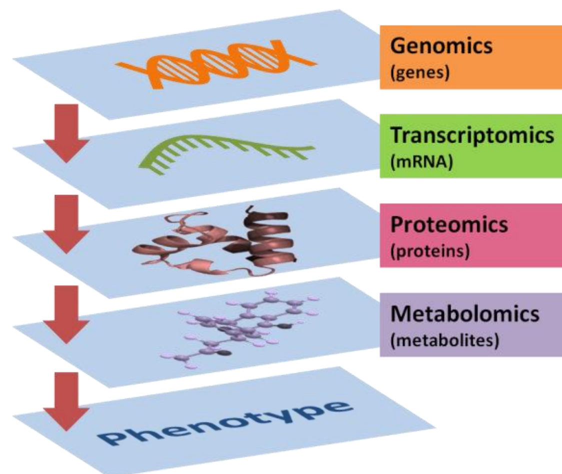


Figure 3.2 Omics cascade. From genes to metabolites, unravelling the phenotype.

3 WHAT IS A METABOLITE?

Metabolites are organic compounds of low molecular weight (<1,500 Da) that are substrates, intermediates or products of enzyme-catalysed metabolic reactions that naturally occur within cells and that do not directly come from gene expression. To be classified as a metabolite, a compound must be found inside cells, be recognised and acted upon by enzymes, its product must be able to enter into subsequent reactions, have a finite half-life and must serve some useful biological functions in the cell. Many metabolites are regulators that control the pace of the metabolism.

Two different kinds of metabolites can be distinguished based on their origin: endogenous and exogenous. **Endogenous metabolites** are synthesised by the enzymes encoded by the host's genome, whereas **exogenous metabolites** are generated from the degradative metabolism of xenobiotic compounds.

4 THE HUMAN METABOLOME

In contrast to the genome and proteome, the **metabolome**—term derived from the word metabolite and the Greek suffix –ωμα (–ōma), meaning collection or body—is not easily defined. Fiehn[5] defined the metabolome as the full suite of metabolites synthesised by a biological system, being this system defined by the level of biological organisation, such as organism, organ, tissue, cell, or cell compartment levels. However, the human metabolome is not only a result of the enzymes encoded by our genome, the endogenous metabolites, but is also composed of the products of what we ingest, what we are in contact with—the exogenous metabolites—and our microflora.[6]

The exact number of metabolites present in the human metabolomes remains imprecise, but it has been estimated to be several thousands of different metabolites in humans.[6] To identify and catalogue the metabolites in the human body is the main goal of the Human Metabolome Project (HMP).[7] Combining both dry-lab work—text mining and bioinformatics—and wet-lab work—by nuclear magnetic resonance (NMR), liquid chromatography coupled to mass spectrometry (LC-MS), or gas chromatography coupled to mass spectrometry (GC-MS) experiments—since its early beginnings in 2007[8] the HMP has identified thousands of compounds. Among them are drugs and drug metabolites, endogenous metabolites, and food additives or plant-derived products. The complexity of the human metabolome can be observed in the Kyoto Encyclopedia of Genes and Genomics (KEGG) Global Metabolism Map[9] (Figure 3.3), where the nodes represent metabolites and the lines a set of reactions.

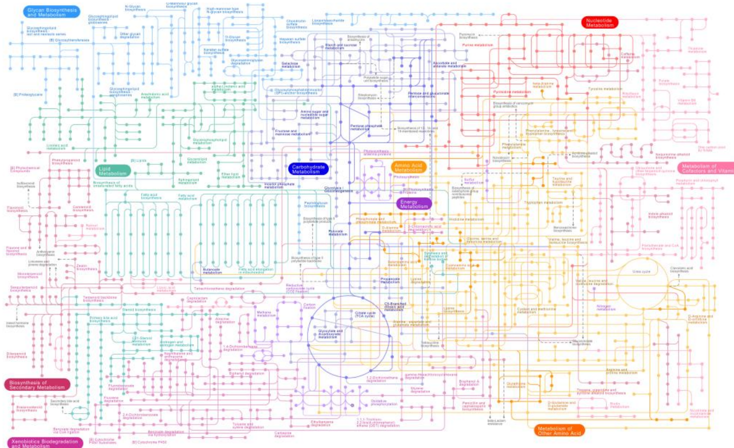


Figure 3.3 KEGG Global Metabolism Map (http://www.genome.jp/kegg/).

5 METABOLOMICS

Metabolomics is one of the newest omic sciences. It is complementary to the rest of omics, however, being at the end of the cascade (Figure 3.2), the metabolites are the final downstream product of the sum of gene transcription and environmental effects, and are strongly correlated with the host phenotype. Although the number of metabolites is lower than the number of genes or proteins, the metabolome domain is more complex, containing molecules of very different physical and chemical properties.

The importance of studying the metabolites in body fluids dates back to B.C.E. 1500-2000, when both traditional Chinese doctors and Hindus practicing Ayurveda used insects to detect high levels of glucose in the urine of patients.[10] The first metabolomics experiments could be considered those of Pauling et al.[11] in 1971. In this study they analysed about 250 metabolites in a sample of breath and 280 in a sample of urine vapour, conceiving the idea that from the quantitative and qualitative pattern of a high number of metabolites in body fluids, information that reflects the functional status of a complex biological system can be obtained. In the same year Horning and Horning[12] used the term **metabolic profiling** for the multicomponent analyses of urinary constituents.

At the end of the 1990s there was an avalanche of new acronyms related to omic technologies, but most of them were only used by the one who coined it. However, it was in 1999 when Nicholson[13] used the term **metabonomics** to describe *«the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification»*. There are several terms—metabonomics, metabolomics, metabolic profiling, metabolite fingerprinting, metabolite footprinting[6]—used for the holistic analysis of the metabolome. The main difference between metabolomics and metabonomics is that the objective of the first is the study of all the metabolites in a given biological system, e.g. the evaluation of normal levels of metabolites in urine, whereas the second aims to compare the metabolite levels of a system, e.g comparison of the metabolomes of healthy versus ill patients, in response to a particular stimulus or treatment.[14] However, in recent literature both terms are often used indistinctively to express the same discipline. Along this thesis, the term **metabolomics** will be used, defined as the comprehensive analysis of the metabolites present in a biological system, and will be used to refer to both types of studies.

The rapid evolution of the interest on this omic science since its beginnings is reflected in the exponential grow of the number of papers published on this discipline (Figure 3.4).

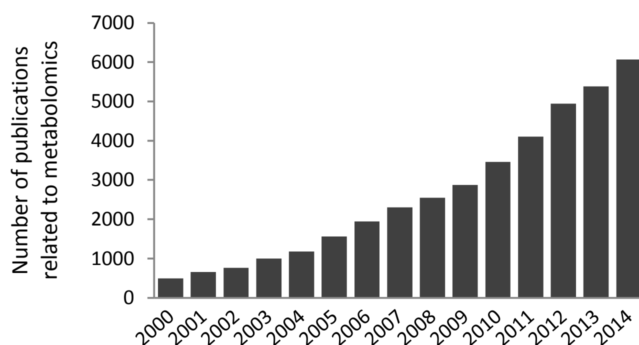


Figure 3.4 Metabolomics publications evolution. Number of publications in Scopus covering the search terms metabolomic*, metabonomic*, and/or metabolic profile.

6 METABOLOMICS WORKFLOW

Metabolomics is a complex discipline that requires from different steps to get from the biological question to biological interpretation of metabolomic results. The stages included in a typical procedure of the metabolomics pipeline are represented in the Figure 3.5.

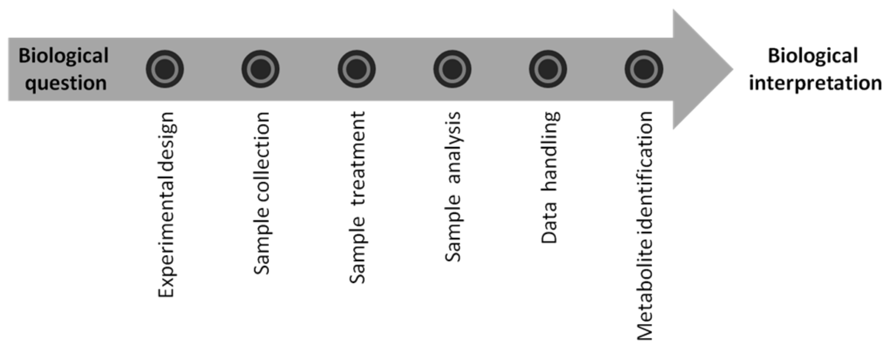


Figure 3.5 Metabolomics workflow. Steps followed in a metabolomics experiment.

6.1 EXPERIMENTAL DESIGN

The experimental design is one of the most important parts in the metabolomics workflow, notwithstanding it is often neglected. A careful design of the experiments is important[15,16] to avoid committing errors that would lead to lack of robustness or reproducibility of the study, to prevent spending a lot of unrequired time, money and effort, to use efficiently the samples—that is crucial when either animals or volunteers are studied—and to ensure that the results obtained are meaningful and answer the hypothesis or **biological question** of the study. The experimental design should be done collaboratively between all parts involved all along the metabolomics pipeline, from sample collection to statistical analysis and biological interpretation. In words of Sir Ronald Aylmer Fisher *«To call in the statistician after the experiment is done may be no more than asking him to*

perform a post-mortem examination: he may be able to say what the experiment died of».

The design must reflect the question that is being asked, the limitations of the experimental system, and the methods that will be used to analyse the data.

While designing a metabolomics experiment the subjects included in the study must be selected meticulously. Questions as the type of population—age, gender, race or species—, the selection of matching controls, or the number of subjects necessary to obtain enough statistical power are mandatory.[17] However, it is common that the number or type of samples to be used for the experiments are dictated by the reality of resources available in terms of money, time, availability, or other factors, rather than by scientific criteria. Although it is not an uncommon practice, it is unadvisable to perform a metabolomics study on samples leftover from another study and 'sitting in the back of the freezer'. Sampling should be design according to the study, and not the other way round.

The type of sample to be collected from the subjects must be selected taking into account the aim of the study, the availability, and the biological meaning. Typical samples used in metabolomics are biofluids—blood, plasma, urine, cerebrospinal fluid—, tissue, or cells.

6.2 SAMPLE COLLECTION

This step of the metabolomics workflow is mainly done outside the laboratory walls, often by physicians and ward nurses. Pre-analytical errors are more common than expected,[18] especially when samples are taken in the clinical field, where the daily clinical routine, lack of adequate equipments and time limitations may interfere with the accomplishment of a perfect pre-analytical process. It is important that the personnel in charge of sample collection is well trained, that the procedure for sample collection, transportation and storage is precisely defined in a standard

operating procedure (SOP) and that this is strictly followed to avoid pre-analytical problems. In particular, in multicenter studies it is still a major challenge to ensure that every hospital strictly follows the entire pre-analytical procedure defined in an SOP.

Important aspects to take into consideration when collecting the samples are the type of container used[19]—e.g. EDTA or heparin blood collection tubes—, temperature and time of storage before analysis or the effect of freeze-thaw cycles.[20,21] Other factors such as the time of day and circadian variations can be confounding variables,[20] and therefore should be taken into consideration.

6.3 SAMPLE TREATMENT

In all fields of analytical chemistry it is vital to use a repeatable sample preparation protocol in order to minimise the differences between samples due to the analytical process. A sloppy sample treatment can ruin the whole study, even if you are using the best analytical techniques. In metabolomics the choice of sample preparation method is a step extremely important, because it affects the results obtained and therefore the biological interpretation of the data. Nonetheless, it is often overlooked.

As in metabolomics the general aim is to study as many metabolites as possible, the best sample preparation protocol would be the one in which the sample is modified as little as possible to ensure the maximum metabolite coverage, simple and fast to prevent metabolite loss and degradation and reproducible to ensure that biological variation is higher than analytical variation. Furthermore, ideally it would incorporate a metabolism-quenching pre-treatment step to represent the true metabolome composition at the time of sampling.[14]

The most suitable sample treatment for a metabolomics study depends on the matrix being analysed, but also on the analytical technique to be used. For example, when gas chromatography is used in the analysis the sample

treatment generally requires a step of derivatisation, and for NMR, direct analysis is the most suitable option with minimum sample preparation (e.g. buffering with deuterated solutions). The sample treatment may also be different depending on the aim of the study.[22]

Plasma and urine are extensively used, as they are relatively easy to collect and provide information about the state of the organism at the time of collection and, in the case of urine, about the excreted metabolites discarded from the body.

The most common preparation protocols for plasma are **protein precipitation** with organic solvents or **solid phase extraction** (SPE).[22-25] Other selective methods as liquid-liquid extraction (LLE) are not so common,[26] and usually are avoided in metabolomics.

Common treatment procedures for urine are **dilution, centrifugation and/or filtration,** but using these procedures the high salt content can cause ionisation suppression, lead to adduct formation, and also negatively affect instrument performance by the presence of non-volatile residues when mass spectrometry (MS) is used. These aspects can be minimised by including, when possible, an effective extraction step as SPE.[27] However a SPE step will cause the loss of some metabolites.

6.4 SAMPLE ANALYSIS

Once the samples are transformed from their original form into a state compatible with the analytical instruments, they need to be analysed to acquire the metabolite information.

The final objective of metabolomics is to study the entire metabolome; however, metabolites form a very heterogeneous family of molecules with different structures, physicochemical properties and concentrations. This heterogeneity makes unfeasible to measure all the compounds using a single analytical technique. As a consequence, different analytical platforms are used in order to cover the maximum possible range of metabolites.

The main platforms used in metabolomics are **NMR** and **MS**, the latter often coupled to chromatographic separation techniques.[6]

Ideally both techniques should be used in order to obtain the maximum information as they are complementary, however, few are the studies that actually use both platforms, and if so, they are often used independently from each other, just comparing the metabolites obtained by both techniques. The selection of one technique or the other depends on diverse factors such as the access to the instrument, or the specific biological question. Nowadays new ways to combine NMR and MS are being explored.[28]

6.4.1 Nuclear magnetic resonance (NMR)

^{1}H-NMR spectroscopy possesses many general features that make it an excellent metabolic profiling technique. Due to the ubiquitous presence of the proton in the biochemical molecules, ^{1}H-NMR can operate in an untargeted fashion, generating many hundreds of simultaneous signals from metabolites without selection of specific analytes. ^{1}H-NMR also provides important structural information valuable for the identification of unknown metabolites. Furthermore, NMR is able to detect metabolites present in solution at concentrations larger than 1mM with an outstanding reproducibility between different NMR spectrometers and users.[28]

Both one-dimensional (1D) and two-dimensional (2D) ^{1}H-NMR experiments are used in metabolomics, being more common the formers. However the identification of metabolites in complex mixtures using only 1D ^{1}H-NMR is challenging because of overlapping peaks. 2D ^{1}H-NMR produces spectra with improved resolution, although it requires longer times of analysis and higher cost. On the other hand, for the analysis of intact tissues magic-angle spinning nuclear magnetic resonance (^{1}H-MAS-NMR) is used.[29]

Among the advantages of NMR over MS are the simple sample treatment required, that it is not destructive, its easy automatisation, and its reproducibility.

6.4.2 MASS SPECTROMETRY (MS)

MS based metabolomics has increased in popularity among metabolomics scientists. The developments in high resolution MS (HRMS) instruments such as Fourier transform ion cyclotron resonance (FTICR), Orbitrap and time-of-flight (TOF), have drastically increased ion resolution. The lower detection limits (pM – nM), its fast data acquisition, and the possibility of coupling to separation techniques make that nowadays MS can be considered as the preferred choice for most of the metabolomics studies.

Even though there are some metabolomics studies that use direct infusion MS (DI-MS),[30] the most common practice is to couple the spectrometer to a separation technique, in order to reduce the complexity of the spectra and diminish the ion suppression produced by the competition of the molecules for ionisation, especially when electrospray (ESI) is used as ionisation technique.

Gas chromatography was the first technique coupled successfully to MS,[31] and in its first years, GC-MS was the preferred choice for MS based metabolomics, especially applied to plants.[32,33] GC-MS is highly efficient, sensitive and reproducible, and the identification of compounds is easier than with other techniques because its reproducible mass spectra make possible to construct public databases. The main drawback of GC-MS is that only volatile compounds can be analysed, otherwise they have to be derivatised, which requires complicated and tedious sample treatment.

When the metabolites of interest are charged, the method of choice is capillary electrophoresis (CE). The information obtained from this technique is complementary to the chromatographic ones, because their mechanism of separation is different.

Nowadays GC-MS and CE-MS have been overshadowed by the popularity of liquid chromatography coupled to MS (LC-MS). Depending on the analytes of interest different types of chromatography should be used, being reverse phase used for non-polar metabolites, and normal phase or HILIC

chromatography for polar compounds. Usually the main aim of a metabolomic study is to analyse the maximum possible number of metabolites, therefore the use of complementary methods with different phases—i.e. reversed phase and HILIC—is highly recommendable.

The most common ionisation source used in LC-MS based metabolomics is ESI (Figure 3.6).

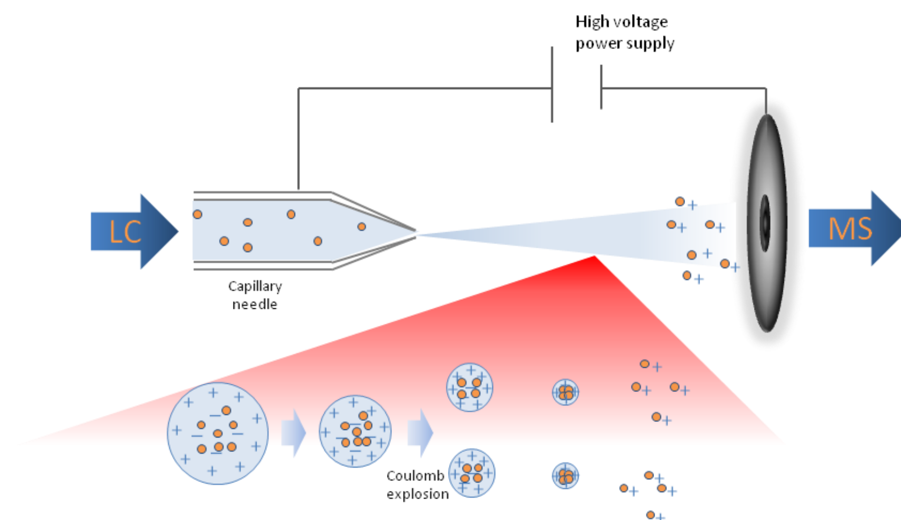


Figure 3.6 ESI ionisation. Transformation of the analytes from solution to charged ions.

ESI allows the ionisation of a wide range of compounds of many different species dissolved in a liquid phase. In the ESI source a process of charging and desolvation transforms the analytes in the liquid phase into gas ions that are introduced in MS analyser. The ESI source operates at atmospheric pressure spraying the sample through a thin needle into an electric field. An electrical potential is then applied to this needle, either positive (ESI+) or negative (ESI-), producing the formation of charged droplets in a process called nebulisation. These droplets are electronically driven and vaporised with a neutral gas (typically nitrogen). By solvent desolvation the size of the droplets is reduced, increasing the charge repulsion until it is higher than the superficial tension and the Coulomb explosion is produced, leaving bare ions that are transmitted into the optics of the mass spectrometer.

6.5 Data handling

Metabolomic analyses produce a huge amount of data that needs specific software and methodologies for its treatment. From the LC-MS system three-dimensional data set is obtained with information about intensity, m/z and retention time for each detected peak. After the data pre-processing the complexity of the data is reduced, obtaining a two-dimensional table with a list of **features** (Box 2) and their intensities. Then, these data are prepared for the statistical analysis by the pre-treatment steps that include normalisation, transformation, centring, and scaling in order to prepare the data for their statistical analysis. Depending on the software used for the data treatment the steps may change. In this section the general steps will be defined.

Box 2 Feature definition

In LC-MS based metabolomics a feature can be defined as a 3D signal induced by a molecular entity with a unique m/z and retention time. Features are the variables in the metabolomic data matrix. A feature does not necessarily correspond to a metabolite, and the number of features is always much higher than the number of metabolites.

6.5.1 Data pre-processing

The first step of data treatment is the extraction of features from the raw data obtained from the LC-MS system. This process involves data conversion, peak detection, peak alignment, zero filling, blank subtraction, and peak filtering.

Data conversion

The starting point is a set of raw data files, corresponding to each sample analysed. As different vendors use different data formats it may be necessary to convert the raw data into a common raw data format such as netCDF or mzXML.[34] Usually scripts to convert the data into these formats are included in the vendor software. In the case of Waters, the MassLynx

software is used to acquire the spectrometric data and DataBridge is the file conversion program included.

PEAK DETECTION

Peak detection, also called feature detection or peak picking, aims to separate real peaks generated by actual compounds from random electrical and chemical noise. Several programs are used for this purpose, both commercial as MarkerLynx (Waters), Mass Profiler Pro (Agilent), Metabolic Profiler (Bruker), or MarkerView (AB Sciex), and freely available as XCMS or MZmine.

There are three main strategies for peak detection:

- Vectorised peak detection method: search for data points with intensity above a threshold level in the *m/z* and the retention time direction.
- Slicing data in narrow sections in the *m/z* direction to obtain extracted ion chromatograms (XIC). Peaks are selected in each XIC using a second-order Gaussian filter or by applying a threshold filter.
- Model fitting the raw data to a three dimensional model of a generic isotope pattern.[35]

PEAK ALIGNMENT

The alignment consists of the assignment of peaks present in a defined percentage of samples and corresponding to the same feature, in order to eliminate retention time shifts between data sets. Each parameter such as noise tolerance and peak thresholds should be carefully adjusted based on the signal intensity and peak shape of the signals in the chromatogram to obtain an adequate alignment of peaks.[36]

As the retention time deviations in LC-MS are usually non-linear, sophisticated time correction methods have been developed, as Time Correlation Optimised Warping, Parametric Time Warping, Dynamic Time Warping or Calibration of Mass Values.[34]

ZERO FILLING

Missing intensity values in one or more samples is unfortunately a very common problem in omic sciences. Missing values can be originated biologically or technically. In LC-MS based metabolomics metabolites with low concentration, not-easily ionisable or with a strong suppression effect may not be detected due to sensitivity issues, giving a zero value in the matrix. Also peaks with bad quality shape can be missed in the peak picking step.

Missing values can have a strong influence in the results obtained, and can cause problems when statistical calculation is performed. To avoid this problem missing values have to be removed or filled with a number. Several statistical methods have been studied in order to fill these missing values.[37-39] In some cases, the raw data is revisited and the integration of the peak is 'forced', in other cases the zero is replaced by the minimum value found in the rest of the samples, by half of the minimum or by a random number smaller than the minimum.[40]

Software such as XCMS or MZmine include a step of gap filling in the data pre-treatment pipeline. In other cases this step has to be done using a statistical program.

BLANK SUBTRACTION AND PEAK FILTERING

A step to remove background signals helps to reduce the complexity of the data matrix. In this step the signals from the solvent are removed so that they are not identified as features.

In addition, peaks caused by adduct formation, fragments, multiple charge states or isotopologue ions—highly common in LC-MS metabolomic data—should be removed to reduce the number of observed features avoiding redundancies and therefore simplify biological interpretation. Some metabolomic data processing programs as Progenesis QI (Nonlinear Dynamics, Newcastle upon Tyne, UK) or MassHunter (Agilent Technologies,

Santa Clara, CA, USA) include a step to remove these artifacts. Also, free software as the CAMERA package for R[41] is available for this purpose.

6.5.2 Data pre-treatment

In metabolomics data it is usual to have differences in orders of magnitude between different metabolites and/or metabolites with large fluctuations in concentration, originated from unwanted experimental and biological variation. Experimental variation can arise from sampling, sample preparation or instrument variation, while biological variation is intrinsic to the sample. These factors may be confounded with the factors of interest and hinder the search for the differences in the metabolites that answer the biological question. Therefore, once the list of the detected features is obtained, and prior to the statistical analysis, the data is usually centred, normalised, scaled, and/or transformed in order to separate biological variations of interest from variations introduced in the experimental process.[42]

Normalisation

Normalisation aims to remove unwanted variation between data from different samples. The choice of an appropriate method of normalisation is data and experiment dependent, but should also rely on the aim of the analysis.

There are two main approaches for the normalisation of the data. The first is based in the use of a single or multiple internal or external standards added to the samples before or after the extraction, the second is based on statistical models.[43,44]

The use of standards to normalise the data exhibits high efficacy in increasing the homogeneity of replicate samples, but adds complexity to sample preparation.[45]

Among the statistical-based methods the total intensity and median fold change normalisations are widely used. Both methods assume that measured peak intensities are directly proportional to concentrations of metabolites in solution.

The **total intensity normalisation** forces all samples in a set of experiments to have equal total intensity, assuming that the total concentration for all features in a sample do not vary across samples.

The **median fold change normalisation** adjusts the median of log fold changes of peak intensities between samples in a set of experiments to be approximately zero.

Normalisation is fundamental to urine analysis, where large fluctuations in concentration can be found due to dilution factor.

TRANSFORMATION

Transformations are nonlinear conversions of the data, applied to correct heterocedasticity, convert multiplicative relations into additive, and make skewed distributions more symmetric.

A transformation often used is the log transformation.[42] Since it reduces large values in the data set more than the small ones, the transformation has a pseudo scaling effect. However, it may not be enough to correct for magnitude differences, and a scaling method can applied in addition to the transformation. A drawback of the log transformation is that it is unable to deal with the value zero.

An alternative to the log transformation is the power transformation, which uses the square root of the value instead of its logarithm.

CENTRING

Centring transfers all the feature variations to fluctuations around zero, instead of around the mean (Figure 3.7), leaving only relevant variation.

Centring is used in combination with normalisation, scaling, and transformation methods.

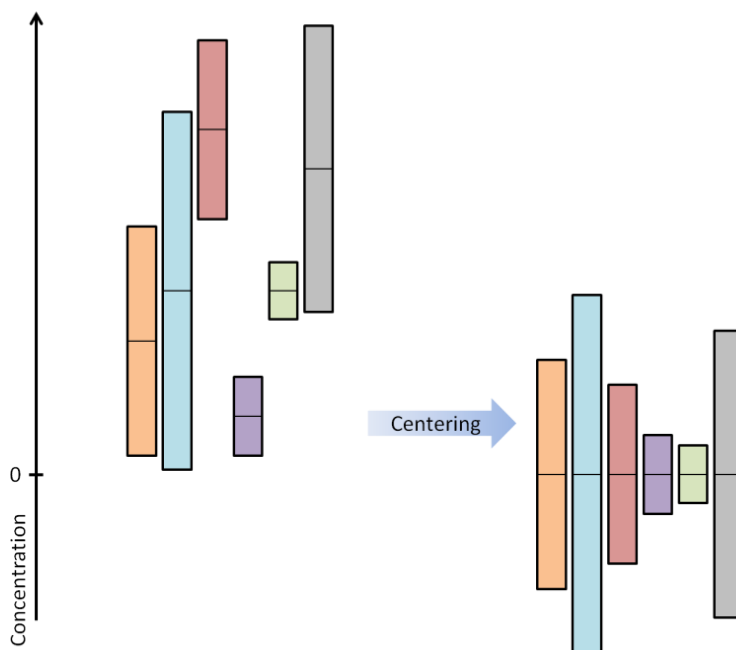


Figure 3.7 Centring of the data. Effect of centring in a set of compounds with different average values.

SCALING

The scaling methods divide each variable—i.e. feature—by a scaling factor, which is different for each variable, aiming to reduce the influence of the differences in fold changes between features. The scaling factor can be a measure of the dispersion or a size measure. Among the scaling methods that use the dispersion as a scaling factor are unit variance scaling, pareto scaling, range scaling, and vast scaling.[42] The methods of scaling more commonly used in metabolomics are unit variance and pareto scaling.

Unit variance scaling—also called autoscaling—uses the standard deviation as the scaling factor, equating the importance of the features. After autoscaling all variables have a standard deviation of one.

In **pareto scaling** the scaling factor is the square root of the standard deviation. This scaling method reduces the relative importance of large values keeping the data structure. After pareto scaling data remains closer to the original measurements than with autoscaling.

Although normalisation and scaling can seem similar they take care of different issues of the data. Normalisation removes variation between different samples, while scaling takes account of differences in concentration levels between features that are originated from differences in average abundance (Figure 3.8)

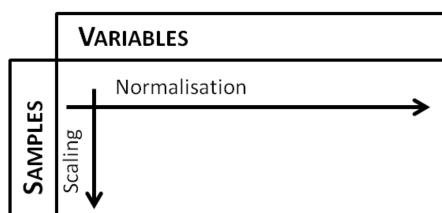


Figure 3.8 Normalisation vs Scaling. In a matrix with samples in different rows and variables on columns, normalisation is an operation on rows while scaling is an operation on columns.

6.5.3 DATA TREATMENT. STATISTICAL ANALYSIS

A metabolomics experiment generates a data set with high quantity of variables from a small number of observations. Also, these variables are usually correlated to each other. This multivariate data is opaque to simple interpretation, so data-reduction methods are needed to simplify its study. The purpose of the multivariate analysis is to decompose and model the data in order to find the latent information in it.

The starting point is an X-matrix, with *i* objects—the different samples, observations or subjects—in rows, and *p* variables—the features—in columns, that collectively characterise each and all of the n objects. After the data pre-processing and pre-treatment steps this matrix should be normalised, scaled, centred, and free of zero values.

This matrix can be studied in an unsupervised or supervised manner. In the unsupervised methods the classification of the samples is not taken into account when creating the model, while supervised methods rely on a known classification to design the model. The most common methods of multivariate analysis in metabolomics are principal component analysis (PCA) for the unsupervised methods and projection to latent structures by means of partial least squares (PLS) and orthogonal PLS (OPLS) for the supervised.

PRINCIPAL COMPONENT ANALYSIS (PCA)

PCA is the most commonly used method to explore relationships between samples in metabolic profiling studies.[46] It aims to extract a small number of latent components that summarises the measured data with minimal information loss by taking advantage of the correlation structure of peak intensities.

The X-matrix can be represented in a Cartesian system of dimension *p*, where the axis for the variables are orthogonal and have a common origin. This system is called the variable space. The representation of the data constitutes a swarm of points in this variable space where each object is characterised by its value in the axis. The first principal component (PC1) is located in the direction of maximum variance of this cloud of points representing the data. The second component (PC2) will be located in the direction of maximum variance orthogonal to PC1, and the third component (PC3) orthogonal to both PC1 and PC2, and so on. This new components can be considered as new variables that generate a new Cartesian coordinate system. Each new PC represents successively less variance, until they reach a point where the variance explained is only noise.

The projections of each observation in the new axis are named scores. The score plot (Figure 3.9) is called to the representation of a pair of score vectors plotted against each other. The score plot gives us an idea of the relationship among the observations.

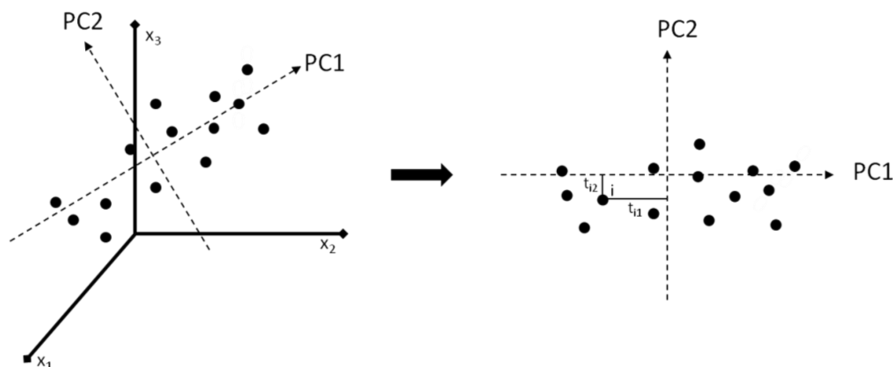


Figure 3.9 Score Plot. The score plot is built plotting the scores (ti1, ti2,…) of each object i in coordinates system generated by the principal components (PCs).

Each PC is a combination of the p unit vectors of the variable space, the original axis. The coefficients that compose the PC are called loadings. Loading vectors can be plotted against each other. The loading plot provides a view of the inter-variable relationship.

PCA can be considered as the basis of all multivariate modelling and is usually the first step in metabolomics data treatment. It is used to observe trends and grouping, and to detect outliers in the data. Notwithstanding, PCA should not be used for group discrimination, since it only detects gross variability and cannot distinguish 'among-groups' variability from 'within-groups' variability. For group discrimination supervised methods such as partial least squares discriminant analysis (PLS-DA) and orthogonal partial least squares discriminant analysis (OPLS-DA) should be used.

PROJECTION TO LATENT STRUCTURES BY MEANS OF PARTIAL LEAST SQUARES DISCRIMINANT ANALYSIS (PLS-DA)

PLS is a regression technique that relates the data (X) to the response (Y).[46] PLS finds information in the X-matrix related with another matrix Y, containing qualitative information.[47] A component in PLS is represented in the X space and the Y space, maximizing the correlation between the projections. PLS is especially necessary when within-groups variability

dominates the among-groups variability. In this case using PCA would not be possible to differentiate the groups.[48]

Partial Least Squares-Discriminant Analysis (PLS-DA) is a PLS regression method with a special binary 'dummy' Y-variable and it is commonly used for classification purposes and biomarker selection in metabolomics studies. In PLS-DA models, a relationship between the metabolomics data and the categorical variable Y is developed. The categorical variable Y is a vector which values indicate class membership of each sample—i.e. healthy vs sick—, and the separation of classes will be driven by this vector.

ORTHOGONAL PROJECTION TO LATENT STRUCTURES DISCRIMINANT ANALYSIS (OPLS-DA)

Variation not directly correlated with Y complicates the interpretation of PLS-DA results. To address this issue, OPLS-DA incorporates an orthogonal signal correction (OSC) filter into a PLS model, separating effectively the Y-predictive variation from Y-uncorrelated variation in X.[49] Therefore, OPLS can be considered as an extension of PLS.[46] OPLS-DA is of great utility to show which variables are responsible for class discrimination and can be considered as putative biomarkers.

6.6 METABOLITE IDENTIFICATION

Once a feature—or list of features—has been selected as putative biomarker to answer the stated hypothesis, the next step towards answering the biological question is the identification of this compound. Without metabolite identification, the results of any metabolomic analysis are biologically and chemically uninterpretable.

Given the chemical diversity of the metabolome and the large number of existent metabolites, metabolite identification remains a bottleneck in LC-MS based metabolomics.[50] Furthermore, reliable unambiguous assignments of observed features to a single metabolite are not always achievable. There is a large number of metabolites in human body that still remains

unidentified, despite the great efforts that have been made by the scientific community to make metabolite identification easier and more robust.

Several tools and strategies have been developed over the last years to identify subsets of metabolites detected in untargeted metabolomic studies.[51,52] The accurate measurement of the *m/z* is frequently the first process applied in the chemical identification of metabolic features acquired using LC-MS instruments. This *m/z* is compared with databases, and depending on the accuracy of this measure there will be more or less molecular formula matches, as a single molecular formula can correspond to multiple different metabolites. Nowadays there are numerous databases—METLIN,[53] Lipid Bank,[54] KEGG,[55] Lipids Maps,[56] or HMDB,[57] are some of them—that allow the search of metabolites based on mass matching. To reduce the number of possible matches a range of bioinformatics approaches can be used, as ionisation behaviour rules or isotope abundance ratios and peak area correlations.[58,59]

Experiments that offer fragmentation information of the biomarker such as MS/MS and MS^E are extremely useful for metabolite identification.[60,61] Using the fragmentation patterns the number of matching metabolites is significantly reduced.

Finally, to confirm the identity of the metabolite, the comparison with standards in terms of *m/z*, retention time and fragmentation pattern is necessary. Unfortunately not all the metabolites are commercially available, so this step is not always possible.

6.7 BIOLOGICAL INTERPRETATION AND VALIDATION

Finally, when the metabolites of interest have been selected and identified, its role as answers for the biological question stated as hypothesis must be studied. Extensive literature searches, pathway analysis and correlation studies are some of the strategies followed to determine the biological importance of the selected metabolite. Sometimes a targeted study derives

from a metabolomic one, where the metabolite selected as biomarker is analysed individually in order to gain more information about its role in the proposed biological question.

7 METABOLOMICS IN PAEDIATRICS

Over recent decades, the contributions of metabolomics in preclinical and clinical care have increasingly become more relevant. Since its beginnings as a promising tool limited to the academic field, metabolomics has become a relevant instrument towards personalised health care.[62] However, the implementation of omics-based tests in clinical laboratories has been difficult. The complexity of the acquisition and interpretation of metabolomics data in comparison with conventional tests has been an obstacle for its diffusion,[63] and despite the intense interest in biomarker development, few biomarker assays for diagnostic uses have been submitted to the US Food and Drug Administration (FDA). To facilitate the qualification of biomarkers to be used in drug development, regulation, and clinical practice, the FDA has encouraged several initiatives,[64] and as a consequence, it is foreseeable that the presence of biomarkers in clinical practice will increase in the next years.

Several studies have been conducted on humans showing that metabolomics can provide biomarkers for disease diagnosis and prognosis,[65-68] new insights into the mechanisms of disease pathogenesis,[69,70] relevant information about toxicology[71-74] or advance knowledge about drug metabolism.[75] However, the majority of these studies involved adult patients, and in comparison, fewer literature data can be found on paediatric metabolomics. Notwithstanding the number of paediatric and neonatal metabolomics papers is constantly increasing. Although the use of metabolomics in paediatrics, and especially in neonatology, is still in the pioneering phase, metabolomics appears to be a promising tool for this population, as information obtained from these studies would lead to a more personalised medicine, with applications in

both diagnosis and treatment of diseases. That is of special interest in paediatric population, usually neglected in clinical studies, and in which an early diagnosis can be crucial to avoid some life-threatening diseases.

The most commonly used biological samples in human metabolomics studies are urine, blood, plasma or serum. In paediatric studies urine present obvious advantages against the other matrices, because of its availability in large amounts, stability if well preserved, simple sample treatment, and uncomplicated and non-invasive method of collection even in small babies.[76] Blood is also a widely used matrix in neonatology, where dried blood spot samples (DBS) are being systematically collected from newborn babies for **neonatal screening**.

Metabolomic studies have been applied to paediatric and newborn research—in both human and animal paediatric models—in diverse areas such as the detection of inborn errors of metabolism (IEM), respiratory diseases, nutrition, nephrology, endocrine disorders, sepsis, among others.

7.1 INBORN ERRORS OF METABOLISM

In the study of inborn errors of metabolism (IEM) in neonatology, urine[77,78] and DBS[79] have traditionally been used. In the early 1960s Robert Guthrie developed a test for the analysis of DBS that could determine whether newborns had the metabolic disorder phenylketonuria (PKU).[80] Since then, the number of identified inherited metabolic diseases has been substantially increased. Nowadays millions of babies all around the world are routinely screened for certain genetic, endocrine, and metabolic disorders using targeted analysis by LC-MS/MS.[81-83] In this new omic era, the holistic approach of metabolomics using MS appears as an advantageous choice for the analysis of IEMs and it is destined to play a central role in the near future as a diagnostic tool for this kind of diseases,[84-86] including hyperammonemias, lactic acidemias, organic acidemias, and IEM of amino acids, pyrimidines, purines, carbohydrates, and other metabolites.[87]

Wikoff et al.[88] used untargeted metabolomics by LC-MS to differentiate plasma metabolomes of samples of methylmalonic acidemia (MMA) and propionic acidemia (PA) patients, from healthy individuals. In this study they were not only able to identify the propionyl carnitine—the compound tested for newborn screening by MS/MS in both MMA and PA—as a biomarker of disease, but also other metabolites such as unsaturated acylcarnitines (AC), isovaleryl carnitine, and butyrobetaine that were associated with these disorders and may be used as possible biomarkers.

More innovative techniques as nanospray ionisation and HRMS have been used as an alternative to the traditional targeted method by tandem mass spectrometry. These techniques were used by Denès et al.[85] for the detection of more than 400 components including AC, amino acids, organic acids, fatty acids, carbohydrates, bile acids, and complex lipids involved in IEMs in the analysis of DBS samples.

Other analytical platforms as desorption electrospray ionisation mass spectrometry (DESI-MS) and ^{1}H-NMR for the metabolomic analysis of IEM were explored by Pan et al.[89] In their investigation they demonstrated that both techniques can differentiate IEM patients from healthy controls based on the different peak patterns in their urine. Several other studies can be found using NMR[79,90] and GC-MS[91] based metabolomics for the analysis of IEMs.

7.2 Respiratory diseases

Respiratory infections are the leading cause of death among children under five years of age globally,[92,93] who experience from three to six acute respiratory illnesses per year.[94] The complex etiology and epidemiology of respiratory diseases complicates their diagnosis and treatment. This has spurred the search for methods to increase the understanding of these pathologies in the direction of a targeted approach and therapeutic monitoring. The usual diagnostic methods, such as spirometry, present great difficulty in their implementation in paediatric patients,[95] therefore

the analysis of a biomarker or set of biomarkers would represent a promising alternative as a diagnostic and monitoring tool for some respiratory diseases such as asthma, pneumonia or bronchiolitis, as well as being of great help to improve the disease models to make them more predictive and relevant to drug discovery.[96]

Given that the lung is the organ of interest in most of these diseases, airway fluid has been primarily used for monitoring biomarkers. Collection of exhaled breath condensate (EBC) is one of the least invasive methods for obtaining airway fluid. EBC is a dilute, complex solution of diverse biomarkers with various chemical properties. Although it is less complex than plasma or urine, EBC contains dozens of compounds—mainly water vapor, but also water-soluble volatile compounds and some non-volatile compounds[97]—which can be measured from the cooled and condensed exhalate (Figure 3.10). Some successful reports can be found using MS[98,99] in airway samples of children.

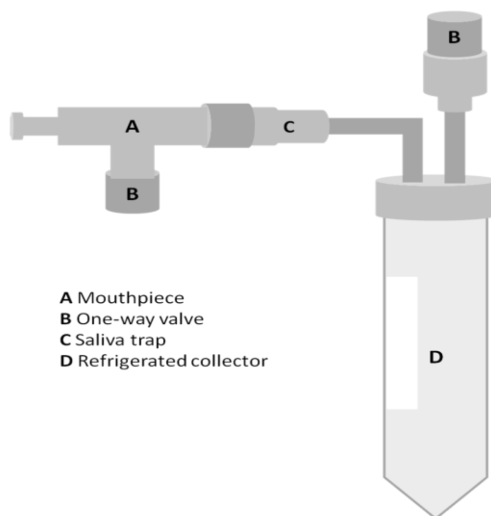


Figure 3.10 Collection of Exhale Breath Condensate (EBC). The collection system for the EBC is refrigerated for the condensation of exhaled breath.

Asthma is a heterogeneous disorder involving chronic airway inflammation, causing recurring periods of wheezing, chest tightness, shortness of breath, and coughing. Asthma is one of the most common chronic respiratory

illnesses, especially in children. Several metabolomics studies are reported using EBC,[100-104] urine,[105,106] and plasma[107] samples.

Other respiratory diseases that have been object of metabolomic studies are pneumonia,[108] bronchopulmonary dysplasia,[109,110] or cystic fibrosis.[111,112]

7.3 AUTISM

Autistic spectrum disorders (ASD) are a group of neurodevelopmental disorders characterised by the deficiency of social interaction and communication, restricted patterns of interest, and repetitive behaviours. Autism is diagnosed between the second and third years of life, based on behavioural criteria. However, several studies have shown metabolic alterations in individuals with ASD, being documented abnormal levels of amino acids in plasma, urine or cerebrospinal fluid in this population. In the last years various studies have been published reporting the use of metabolomic techniques in the study of these disorders.

Ming et al.[113] reported alterations in different pathways including amino acid metabolism and increased oxidative stress. De Angelis et al.[114] showed differences in the levels of free amino acids and volatile organic compounds of faecal samples in autistic children. In other studies[115-118] significant changes in the levels of several metabolites—aspartate, citrate, creatinine, dehydroepiandrosterone sulphate, hydroxyphenyllactate, indoleacetate, isoleucine, glutamate, glutarate β-alanine, glycine, taurine and succinate among others—between ASD and healthy individuals were identified.

7.4 NUTRITION

It is well known that diet and health are strongly associated, that nutrients affect metabolic regulation, and that nutrition can both prevent and promote disease. However it is not yet completely understood many of the links between diet and specific health outcomes. In the paediatric clinic, nutritional research can prevent the development of long-term diseases as

well as contribute to repair ongoing processes. Inappropriate diet in the first years of life may alter permanently the metabolic processes of the child and accelerate the development of chronic pathologies. Metabolomics can help track the interaction between nutrients and human metabolism, offering further insight into the connections of diet and health.[119-121] In recent years the literature has shown an increasing interest in paediatric nutrition and its relation with pathologies as obesity and the onset of diabetes.

Birth weight, either high or low, has found to be associated with increased risk of presenting a **metabolic syndrome** and **type 2 diabetes** in later life.[122,123] This association has also been studied by Dessì et al. using metabolomics. The authors were able to identify higher concentrations of myo-inositol,[124,125] associated with glucose intolerance and insulin resistance in adults,[126] in urine of neonates with intrauterine grow retardation (IUGR) compared with healthy controls, consistent results with those found in plasma of animal models.[127] In other metabolomic studies conducted by the same authors they have also studied the differences between the metabolomes of neonates with different birth weight.[128,129] Umbilical cord plasma has also demonstrated in several metabolomic studies to be a useful fluid to differentiate neonates with IUGR from children with normal birth weight.[130-134]

Infantile obesity is on the rise in most developed countries. In many European countries one child out of five suffers from obesity or is overweight. Childhood obesity influences the composition of the serum metabolome.[135] Rapid postnatal weight gain is considered as a potentially modifiable risk factor for obesity and metabolic syndrome. This rapid weight gain may be associated with maternal diet.[136] Branched-chain amino acids have been associated in various metabolomic studies in obese children with adiposity and cardiometabolic risk during mid-childhood.[137-139]

Type 1 diabetes is a major endocrine disorder, with serious long-term complications as cardiovascular diseases, chronic renal failure, or retinal damage. The incidence of type 1 diabetes among children and adolescents

has increased markedly in the Western countries during the recent decades. In consequence, there is an increasing interest in the study of this disease in order to effectively prevent it, and for this reason several metabolomic studies can be found in bibliography.[140-145]

Other pathologies in children related with nutrition have also been object of metabolomic analysis, as malnutrition,[146,147] nutritional rickets,[148] celiac disease,[149-151] or eating difficulties derived from prolonged parenteral feeding.[152]

7.5 NEPHROLOGY

Nephrology is an important area of paediatrics where metabolomics has an enormous potential, however, the number of reported clinical applications is scarce. The metabolome of the kidney is of great importance in cases of organ transplants,[153] nephrotoxicity,[154,155] and renal diseases,[156-158] but also to understand its function in drug kinetics.[159] Metabolic information on the role of this organ in the drug metabolism in paediatric subjects will allow improving dosing in this population. With this aim, urine metabolomics may be a great information source.

7.6 SEPSIS

Neonatal sepsis is defined as a complex clinical syndrome usually associated with several agents of disease, including bacteria, virus, and fungi. Sepsis is a complex multiorgan dysfunction resulting in large changes in the metabolites of the organism, and the success of its treatment is highly time-sensitive, where early diagnosis may be the difference between death and survival. Therefore, the rapid analysis of the whole metabolome may be an attractive methodology to improve the diagnosis of sepsis. However, very few researches have been developed and published using the metabolomics approach in the field of sepsis, mainly focused on the search of biomarkers for prediction and diagnosis.[160-163]

7.7 OTHER STUDIES

Paediatric oncology is an underexploited field for metabolomics compared to cases involving adults,[164] and only a few papers can be found in bibliography regarding metabolomics in brain and other tumors[165-167] and acute lymphoblastic leukemia.[168]

Other pathologies in children have been studied using metabolomics. Atopic dermatitis,[169-171] biliary atresia,[172,173] dental caries,[174,175] inflammatory bowel disease,[176] constipation,[177] or rabies[178] are some examples.

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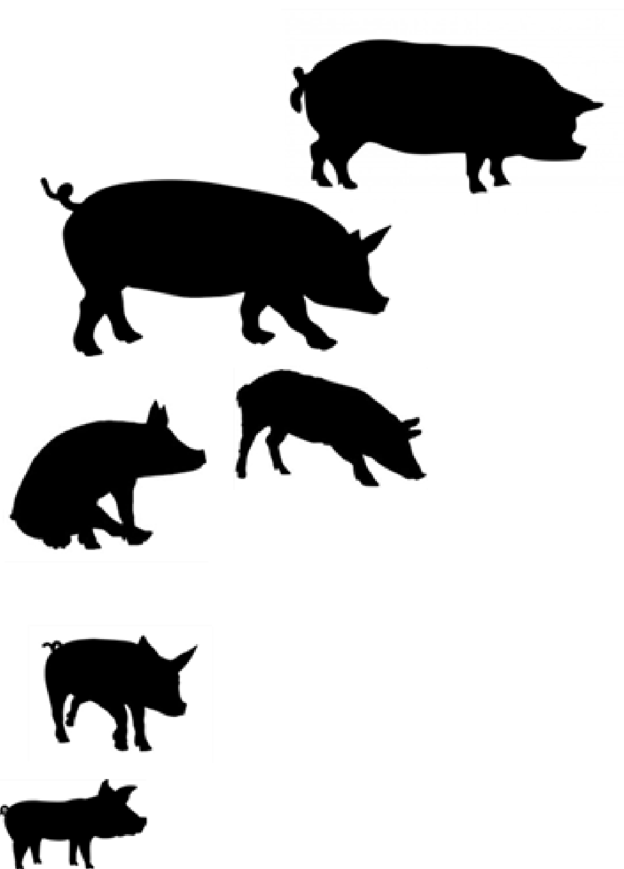
Part II: Metabolomics in paediatrics



Chapter 4

STUDY OF DIFFERENCES IN MATURATION GRADE OF PIGLETS

Sometimes, when you've a very long street ahead of you, you think how terribly long it is and feel sure you'll never get it swept. (...) You must never think of the whole street at once, understand? You must only concentrate on the next step, the next breath, the next stroke of the broom, and the next, and the next.

(Michael Ende, *Momo*)

1 INTRODUCTION

Aristotle (B.C.E. 384 – 322) called pigs *«the animals most like people»*. This belief has been proved right in the present, as pigs are commonly used as biomedical models of humans. The use of pigs as anatomic and physiologic model of humans dates back from the studies of ancient western medicine. As the Roman laws of its time did not allowed dissecting humans, Galen (C.E. 130-200), the prominent Greek physician and philosopher, used animals he believed to be similar, such as pigs, apes and goats as pedagogical and medical experimental subjects to prove or refute theories (Figure 4.1).[1] Since that time, the use of pigs has contributed remarkably to medicine as in the production of insulin[2] or the source of biologic heart valve replacements.[3]

Figure 4.1 Pig vivisection, from Opera Omnia, Galen. Galen performed public dissections of pigs to demonstrate that it is the brain that mediates sensation, cognition and movement, against the dominant theory affirming that those functions were served by the heart.

Animal models of humans are deemed relevant only if they reliably mimic the normal anatomy and physiology of human organs and tissues of interest. Among the different animals used in experimentation, pigs have demonstrated to be a suitable alternative to the dog or monkey as non-rodent species of choice.[4-6] Because of their similarity in size, physiology, organ development, and disease progression, swine are almost ideal biomedical models for humans.[7] Nowadays, the use of pigs as models is of special interest in paediatric research, where due to ethical issues and

difficulty of obtaining samples, the performance of experimental studies in suitable animal species is common practice.[8-10]

Pigs have been used as general surgical models of most organs and systems,[11-13] for pharmacokinetic (PK) and pharmacodynamic (PD) studies,[14-16] or for the study of several diseases.[17-19] However, the use of pigs in research is not only useful for the investigation of pathologies, as the examination of the metabolomes of healthy pigs may be convenient to obtain new insights into the different metabolic profiles inherent to a subject. In this regard, several metabolomics experiments using pigs as models can be found comparing metabolic profiles resulting from nutritional challenges, [20-22] weaning,[23] or cloned animals.[24] Nevertheless, no study was found using pigs as animal models to determine the intrinsic differences in the metabolome of children owing to development and organ maturation in the first stages of life.

Despite the advantageousness of using animal models instead of actual children it is important to bear in mind the importance of doing a responsible use of animal lives. A careful study design must be planed so that the minimum number of animals is used to obtain relevant answers to the biological question.

Therefore, this chapter describes a pilot study based on liquid chromatography coupled to high resolution mass spectrometry (LC-MS) metabolomics, using pig plasma samples collected from previous studies that were designed for other purposes—mainly blank samples for pharmacokinetic/pharmacodynamic (PK/PD) studies—, to investigate the differences between the plasma metabolomes of pigs with different ages as animal models of neonates and infant children. As this is a pilot study, aiming to obtain experimental evidences to design a more detailed study with a larger number of samples, the metabolic profile was also examined in relation to factors other than age such as gender, time of sample collection, sampling vein, or anaesthetic regimen in order to determine the variables

which need to be taken into account to obtain reliable results from the future study.

2 MATERIALS AND METHODS

2.1 REAGENTS AND MATERIALS

Gradient HPLC grade acetonitrile (ACN) (ROMIL, Cambridge, UK) was used as organic modifier for the chromatographic system. Purified water from a Milli-Q Element A10 System (Millipore, Billerica, MA, USA) was used in the preparation of mobile phases and reagent solutions. HPLC quality formic acid (FA) from Sigma Aldrich (St Louis, MO, USA) was employed as additive of the mobile phases.

2.2 INSTRUMENTATION

For the sample preparation step a Signature Digital Vortex Mixer 945303 (VWR, Radnor, PA, USA) and a centrifuge 5424 (Eppendorf, Hamburg, Germany) were used.

The metabolite profiling analysis was performed in an Acquity UPLC system, coupled to a high resolution Q-TOF mass spectrometer Synapt G-2 (Waters, Milford, MA, USA) equipped with an electrospray ionisation source (ESI).

2.3 SOFTWARE

MassLynx V4.1 and DataBridge 3.5 (Waters, Milford, MA, USA) were used for data acquisition and conversion, respectively. The statistical free software R 3.1.2 was used running the packages XCMS 1.42.0 (Metlin, La Jolla, CA, USA) and CAMERA 1.22.0 (Bioconductor Open Source Software for Bioinformatics) for peak detection, alignment, and isotopologues identification. Univariate statistical analysis was performed by Excel 2007 (Microsoft Office, Redmond, WA, USA) and SPSS Statistics 17.0 (IBM,

Armonk, NY, USA); and multivariate statistical analysis by SIMCA 13.0.1 (Umetrics, Umea, Sweden).

2.4 SUBJECTS

Plasma samples were obtained from mechanically ventilated newborn piglets (<5 days, n = 14) and infant piglets (8 weeks, n = 16) of Topig F-1 Large White x Landrace breed.

Subjects were divided into two main categories depending on the age: **Neonates** and **Children**. This classification as well as the recorded information about gender, time and vein of whole blood extraction, and the anaesthesia performed for each of the individuals is collected in Table 4.1. As the use of these animals was intended for different pharmacokinetic and pharmacodynamic studies, these factors were not specifically designed for this pilot study.

Samples collection followed the European and Spanish regulations for protection of experimental animals (86/609/EEC and RD 1201/2005), and was approved by the Ethical Committee for Animal Welfare of the Cruces University Hospital (Biscay, Basque Country, Spain).

2.5 SAMPLE COLLECTION

Samples collection was performed by the team of the Experimental Neonatal Physiology Unit (BioCruces Health Research Institute, Cruces University Hospital, Biscay, Basque Country, Spain).

Whole arterial blood samples were collected in EDTA tubes, and kept on ice until their immediate centrifugation at 3000 rpm at 4 °C in order to obtain the plasma. The supernatant was transferred to a cryovial and stored at -80 °C until analysis.

Table 4.1 Subject classification. Assignment of each of the 30 samples to the Children or Neonates group according to their age. Recorded information about gender, time and vein of extraction, and anaesthesia is included.

Name	Group	Age (days)	Gender	Time of extraction	Vein of Extraction	Anaesthesia
P41/12	Children	59	Female	Morning	Femoral	A1
P42/12		59	Female	Morning	Femoral	A1
P43/12		59	Male	Evening	Femoral	A1
P44/12		59	Male	Evening	Femoral	A1
P45/12		55	Female	Morning	Femoral	A1
P46/12		55	Female	Morning	Femoral	A1
P47/12		55	Male	Evening	Ear	A1
P48/12		55	Male	Evening	Ear	A1
P51/12		62	Female	Morning	Ear	A2
P52/12		62	Female	Morning	Ear	A2
P53/12		62	Female	Evening	Ear	A2
P54/12		62	Female	Evening	Ear	A2
P55/12		63	Female	Morning	Ear	A2
P56/12		63	Female	Morning	Ear	A2
P57/12		63	Female	Evening	Ear	A2
P58/12		63	Female	Evening	Ear	A2
P01/13	Neonates	1	Male	Morning	Femoral	A3
P03/13		1	Male	Morning	Femoral	A3
P04/13		2	Male	Morning	Femoral	A3
P05/13		1	Female	Morning	Femoral	A3
P06/13		2	Male	Morning	Femoral	A3
P07/13		1	Female	Morning	Femoral	A3
P09/13		1	Male	Morning	Femoral	A3
P11/13		1	Male	Morning	Femoral	A3
P12/13		2	Female	Morning	Femoral	A4
P13/13		3	Male	Morning	Femoral	A4
P15/13		5	Female	Morning	Femoral	A4
P66/13		1	Male	Morning	Femoral	A4
P67/13		2	Female	Morning	Femoral	A4
P69/13		2	Male	Morning	Femoral	A4

A1: Ketamine (15 mg/kg), Diazepam (2 mg/kg), Atropine (0.05 mg/kg), Fentanyl (0.005 mg/kg + 0.004 mg/kg/h), Propofol (1.2 mg/kg + 3 mg/kg/h), Lidocaine (3 mg/kg/h), Midazolam (0.5 mg/kg/h), Atracurium besylate (1mg/kg + 2 mg/kg/h);
A2: Ketamine (15 mg/kg), Diazepam (2 mg/kg), Atropine (0.05 mg/kg), Fentanyl (0.005 mg/kg + 0.004 mg/kg/h), Propofol (1.2 mg/kg + 3 mg/kg/h), Lidocaine (3 mg/kg/h), Midazolam (0.5 mg/kg/h);
A3: Ketamine (15 mg/kg), Diazepam (2 mg/kg), Atropine (0.05 mg/kg), Fentanyl (0.005 mg/kg + 0.004 mg/kg/h), Propofol (1.2 mg/kg + 3 mg/kg/h), Lidocaine (3 mg/kg/h), Midazolam (0.5 mg/kg/h), Vecuronium (1 mg/kg + 2 mg/kg/h);
A4: Ketamine (15 mg/kg), Diazepam (2 mg/kg), Sevoflurane (inh.)

2.6 SAMPLE TREATMENT

Frozen plasma samples from the studied animals were thawed until reaching room temperature. A **protein precipitation** protocol with ACN in a 2:1 v:v ACN:plasma proportion[25] was used as plasma sample treatment procedure. A volume of 50 µL of plasma was transferred to an Eppendorf tube and 100 µL of ACN were added. After vortex mixing for 2 min, samples were centrifuged at 10000 rpm for 5 min. The clean upper layer was transferred to a chromatographic vial to be injected into the ultra high performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) system.

2.7 QC SAMPLES PREPARATION

Quality control samples (QCs) are necessary for monitoring the performance of the analysis. In targeted analysis a QC is commonly a sample with the analytes of interest. In metabolomics this is not possible, as it is an untargeted analysis where samples contain hundreds to thousands of compounds, being most of them unknown. QCs in metabolomics are prepared as a pool sample obtained from aliquots of every sample[26]. By doing so, all the metabolites present in the sample will be contained in the QC. Then the QC is analysed at the beginning, end and randomly through the analytical run.[27] The same sample can also be used to equilibrate the system prior to analysis. After the analysis, QC data can be examined to assess the stability of the run. A selection of compounds of the sample can be chosen to evaluate peak shape, intensity, and mass and retention time precision. Also, using unsupervised analysis such as PCA, QC injections are expected to cluster together, and may be useful to discover drifts in the analysis.

Due to the small volume of plasma available, two types of QCs were prepared. On the one side, commercial pig plasma was used for system equilibration before the run and for the assessment of system stability, these were named **QCsys**. QCsys were treated following the same

procedure as study samples. On the other hand, proper **QCsamples** were prepared taking 5 µL from each treated study sample. Given that QCsamples closely represent the study samples, they were used for the estimation of analytical variability in the extracted features, as technical variation introduced during the analysis, data acquisition, and data pre-processing will be observable in their data.[28]

2.8 **UHPLC-Q-TOF-MS ANALYSIS**

Samples were randomised to avoid the effect of potential drifts during sample measurement, and analysed after the injection of 3 blank samples consisting of ACN, 15 QCsys for the conditioning of the system and a QCsample. The rest of QCsys and QCsamples were injected periodically through the run, and finally a QCsys, QCsample and a blank were injected at the end of the run (Figure 4.2).

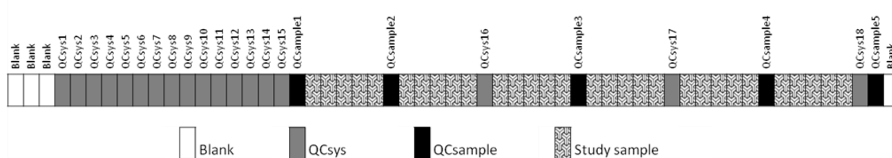


Figure 4.2 Scheme of the injection order. Study samples between QCs were randomly distributed. The same scheme with different randomisation for study samples was used both in ESI+ and ESI- modes.

The thermostated autosampler was set at 4 °C. 5 µL of the extracted plasma samples were injected into a BEH C18 (50 x 2.1mm, 1.7 µm) column (Waters, Milford, MA, USA), set at 40 °C. Water and ACN, both with 0.1 % FA, were used as mobile phases A and B respectively, at a flow rate of 500 µL/min. Gradient started from 0% to 100% B in 15 min, then was kept at 100 % B for 2 min, returned to starting conditions in 0.5 min, and kept for re-equilibration at 0% B for 1.5 min, for a total run time of 19 min.

Centroid data were collected in both positive (ESI+) and negative (ESI-) ionisation modes, in consecutive runs, at full scan range from 50 to 1200 m/z, using a scan and interscan time of 0.1 and 0.024 s, respectively. ESI parameters were: source temperature 120 °C, desolvation temperature 400

°C, cone gas flow 10 L/h, desolvation gas flow 800 L/h, cone voltage 30 V, and capillary voltage 0.7 kV for ESI+ and 0.5 kV for ESI-. A LockSpray interface was used to maintain mass accuracy during analysis. To this end, a solution of leucine enkephalin ($[M+H]^+$ = 556.2771 m/z, $[M-H]^-$ = 554.2615 m/z) at a concentration of 200 ng/L in 0.1% FA was continuously infused at 10 µL/min as lock mass. Mass spectrometer was externally calibrated previous to the analysis with a solution of sodium formate 0.5 mM.

2.9 DATA TREATMENT

Data were acquired with the MassLynx software as .raw and converted into CDF using the DataBridge converter, and the files were grouped into the different categories QCsample, Neonates, and Children.

Feature detection from the data was performed in R using the XCMS package. CentWave detection algorithm[29] was used for peak identification. Peak width was set to be between 3 and 20 s, and maximal tolerated m/z deviation in consecutive scans was set at 10 ppm. Peaks were grouped across samples and a retention time correction was performed. A peak filling step to force the integration of peaks where no signal was detected was included, in order to reduce the number of zero values in the resulting matrix. Using the CAMERA package, detected isotopologues of the molecular entities were excluded. The script used can be found in Annex I.

In order to eliminate missing values in the matrix, the few zero values remaining after the peak filling step were filled with random numbers between zero and the minimum value found in the feature, using the R statistical software.

To correct the intensity drop derived from the contamination or dirtying of the ion source components of the mass spectrometer a correction factor was applied, obtained from the regression curve of the signal of each feature in the QCsamples against the injection order. The corrected intensity was calculated according to the Equation 4.1.

$$x'_{i,j} = \frac{x_{i,j}}{f_{i,j}} \cdot x'_{i,j_1}$$ Equation 4.1

Where $x'_{i,j}$ is the corrected signal of the feature *i* in the sample *j* and $x_{i,j}$ is the signal without modifications. The correction factor $f_{i,j}$ is calculated as the theoretical value of the signal interpolating the order of injection in the regression curve of this feature in the QCsamples. The result is multiplied by $x'_{i,j1}$, the corrected signal for feature *i* in the first QCsample, j_1, in order to recover the original dimensions of the features. The effect of this correction was studied comparing the slope of the regression lines from the sum of the intensities against the number of injection, and by visual comparison of QCsamples position in the principal component analysis (PCA) score plot before and after correction.

The %CV of each feature in the QCsamples was calculated with Microsoft Office Excel software, and features with %CV>30%, which is considered an acceptable value of repeatability in biomarkers analysis,[30] were eliminated. %CV values higher than 30% are considered over the analytical variability tolerance limit and features with those values are considered to be noise.

As distribution normality, and homoscedasticity of the data are assumptions required to perform parametric tests—even if they are not always critical—, it is recommended to test those characteristics of the matrix to decide if a transformation of the data is necessary.[31] In this study normality of the data was examined applying a Shapiro-Wilk test[32] for each feature in each group. For homoscedasticity Levene test[33] was applied. Both tests were performed using SPSS software.

2.10 STATISTICAL ANALYSIS

Differences between the two age groups were studied using multivariate data analysis (MVDA) performed in SIMCA 13.0.1 software. Unsupervised analysis using PCA was used as an overview to look for trends and groupings, and to identify possible outliers. Partial least squares-discriminant analysis (PLS-DA) was used for supervised analysis, classifying

the study samples into two groups, Children for the 8 weeks old pigs and Neonates for the less than five days old pigs. PLS-DA models were validated to examine their suitability by means of permutation test and ANOVA. For the selection of relevant features in the separation of groups, S-plots from orthogonal partial least squares-discriminant analysis (OPLS-DA) models were used. The influence of gender, type of anaesthesia, time and vein of extraction was studied to determine their effect in the separation of the samples. Their distribution of the samples was inspected by PCA, and PLS-DA models were constructed classifying the samples by those characteristics (Table 4.2).

Table 4.2 Characteristics of the study subjects. Ratio of samples in each group with the different characteristics.

	Number of samples	Gender (♂:♀)	Time of extraction (M:E)	Vein of extraction (FV:EV)	Type of anaesthesia (A1:A2:A3:A4)
Neonates	14	9:5	14:0	14:0	0:0:8:6
Children	16	4:12	8:8	6:10	8:8:0:0

♂ = Male; ♀ = Female; FV = Femoral Vein; EV = Ear Vein; M = Morning; E = Evening; A1-A4 = types of anaesthesia (see Table 4.1).

3 RESULTS

3.1 SYSTEM STABILITY

The performance of the system in terms of retention time, mass precision, and intensity was tested using the QCsys. A total of 56 injections were analysed in a run—30 samples, 18 QCsys, 4 QCsamples, and 4 blank samples—in each ESI polarity, for a total time of approximately 36 h of analysis.

For each polarity mode three features—at the beginning, the middle, and the end of the chromatogram—with different m/z were selected to verify the stability of the system. Changes in m/z, retention time, and area of these features across the QCsys injections after conditioning were monitored for all of them. An example of this monitorisation for one of the selected features in ESI+ is shown in Figure 4.3.

Figure 4.3 System stability. Monitorisation of the stability of one of the selected features for ESI+ (m/z 414.3000, t_R 5.86 min). a) Extracted chromatogram of the selected feature in the four QCsys injections along the run, b) m/z monitorisation, c) t_R monitorisation, d) peak intensity monitorisation.

Values of m/z and t_R for the selected features remained almost unchanged for the whole run (Table 4.3). However, a drop of the intensity was observed for the three selected features in ESI+, while the intensity of the selected features in ESI- remained stable.

Table 4.3 System stability. Monitoring of mass and retention time precision, and intensity of selected features from QCsys along the run in both ESI+ and ESI- polarities.

		m/z		t_R		Intensity		
		Mean m/z	%CV	Mean t_R (min)	%CV	Intensity 1st QC	Intensity Last QC	% Intensity change
ESI+	Peak 1	120.0814	0.0004	1.21	0	15121	13812	–9
	Peak 2	414.3000	0.0001	5.86	0	4915	3421	–30
	Peak 3	637.3063	0.0003	11.87	0	82210	66290	–20
ESI-	Peak 1	121.0300	0.0004	3.24	0	2559	2419	–5
	Peak 2	391.2831	0.0004	7.84	0	2944	3188	8
	Peak 3	566.3500	0.0002	9.66	0	16399	17324	6

As a result, the intensities of each feature of the samples in ESI+ mode were corrected using Equation 4.1, while in ESI- mode, as no significant drop of intensity was observed, no correction was performed and raw data was used for the subsequent steps of data treatment.

3.2 DATA PRE-PROCESSING

In ESI+, a total of 1590 features were detected by XCMS. Among those features CAMERA package identified 368 carbon isotopologues, corresponding to the features with a pairwise m/z distance of 1.0033/z,[34] that were removed from the list. To eliminate missing values from the matrix 283 zero values were substituted by random numbers lower than the minimum value in each feature. On the other hand, in ESI- data XCMS software detected 318 features, 72 of them were removed from the list as they were identified as isotopologues by CAMERA. 65 missing values were substituted with random numbers lower than the minimum value recorded.

The frequency distributions for the %CV values were calculated for both ESI+ and ESI- modes (Figure 4.4).

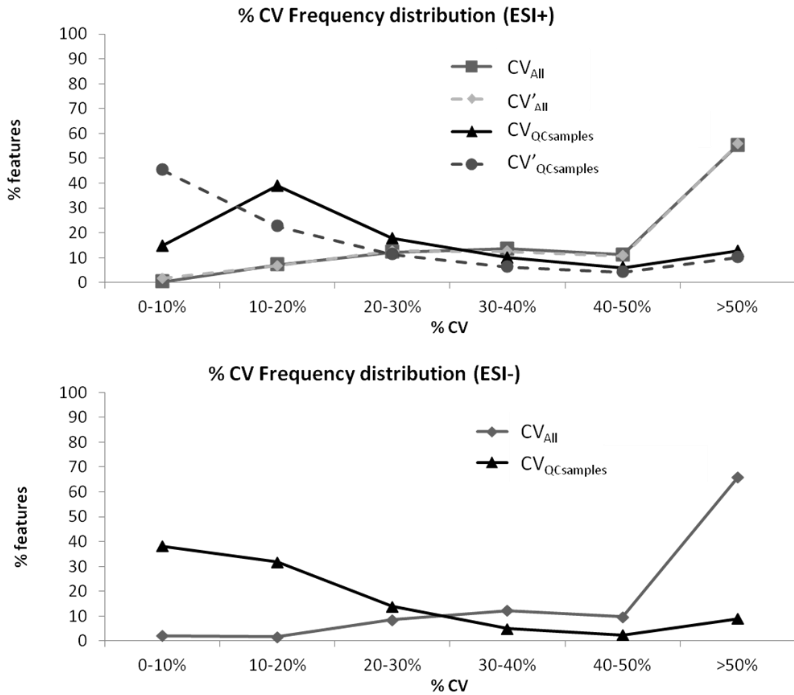


Figure 4.4 Frequency distribution of %CV values. Comparison of CV frequency distributions of raw data calculated either across all samples (CV_{All}) or QCsamples ($CV_{QCsamples}$) and, in ESI+ mode, also for corrected data (CV'_{All}, $CV'_{QCsamples}$).

The variation of QCsamples around their mean is expected to be low since they are replicates of the same pooled sample. Therefore, for QCsamples the majority of features had %CV values lower than 30%. Meanwhile, study samples are supposed to have higher variability. Accordingly, the highest percentage of the features detected on the study samples hold %CV values higher than 50%.

The correction of the intensity drop in ESI+ mode can be seen in the increase of the percentage of features with %CV values <10% in the Figure 4.4, as this drop of intensity through the run increased the variability of the features. This effect is also detectable plotting the sum of all intensities across QCsamples against the injection order (Figure 4.5). This trend was also observable in the sum of intensities of the study samples. Although these were randomly injected to minimise the influence of this kind of

analytical drift in the separation of the groups, the correction will reduce the possible confounding effect of the intensity drop. After correction the slope of the trend line of the data for the QCsamples is closer to zero.

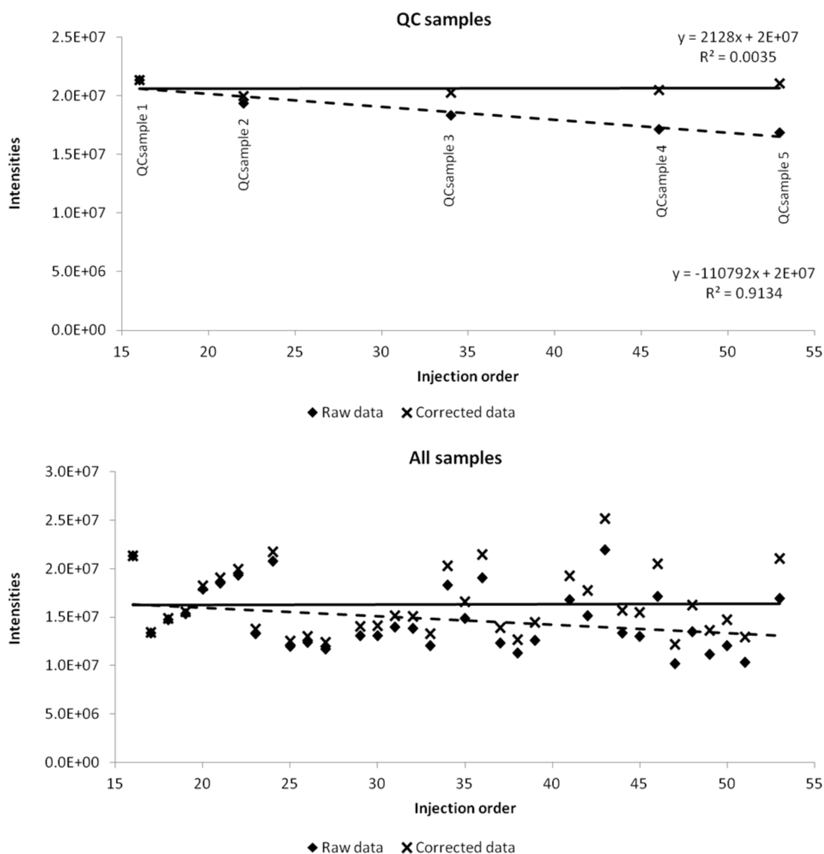


Figure 4.5 Intensity drop correction. Sum of intensities of all features against number of injection for a) QCsamples and b) All samples in ESI+ before and after correction of the intensity change across the run. Dashed and continuous lines represent the trend line of the data before and after correction, respectively.

A PCA model of the data before and after correction shows the improvement of QC samples clustering after data correction (Figure 4.6).

Figure 4.6 Effect of the intensity drop correction over the QCsamples. Score plot representing the first two components of the PCA a)before correction, b) after correction. Coloured in red the QCsamples (●) and in blue the study samples (●). Labels indicate the order of injection.

In the score plot of the PCA modelling the data before the intensity drop correction the tendency of QCsamples with the number of injection is clearly observed, while in the PCA obtained after correction of the data

QCsamples are perfectly clustered. Therefore, from the comparison of these score plots it could be deduced that using the correction of the intensity drop the correlation of the response with the order of injection was removed.

From the observation of these score plots a strong outlier, the sample numbered as 43 in Figure 4.6, corresponding to the pig P12/13, was detected. By visualisation of raw total ion current (TIC) chromatograms, this sample was completely different from the rest, both in ESI+ and ESI- modes (Figure 4.7), and was excluded from the data analysis. The subject from which the sample was taken, the piglet P12/13, showed higher levels of α1-acid glycoprotein than the rest of samples from its group as observed in a previous study.[35]

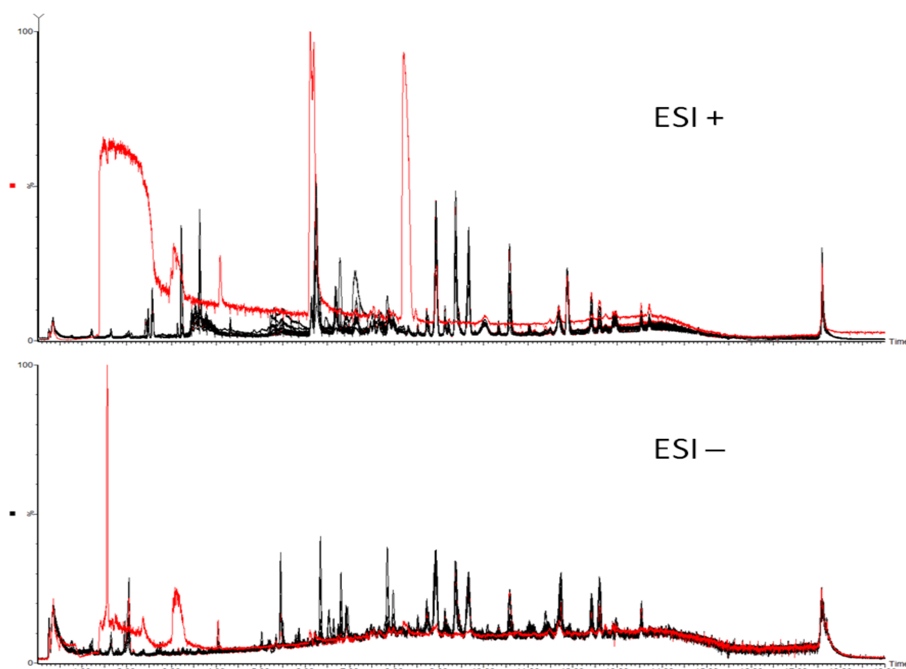


Figure 4.7 Confirmation of the outlier. Comparison of the TIC chromatograms of the outlier (coloured in red) against the rest of neonate samples (coloured in black).

In the final step of the pre-processing a total of 252 features in ESI+ and 40 in ESI- with %CV values higher than 30% in the QCsamples were eliminated, leaving two final lists of 970 and 206 features for ESI+ and ESI- modes, respectively.

Shapiro-Wilk test was applied to each feature in each group in order to determine if the matrix followed a normal distribution. Features with p-values lower than 0.05 for the Shapiro-Wilk test, refute the null hypothesis—H_0 = the data is normally distributed—and therefore are not considered to follow a normal distribution. To test homoscedasticity of the data Levene test was used, considering that features with p-values lower than 0.05 refute the null hypothesis—H_0 = both groups have equal variances— and therefore did not have equal variances. Shapiro-Wilk and Levene test were performed by SPSS software. In the Table 4.4 the normality and homoscedasticity of the features before and after a log transformation are compared.

Table 4.4 Normality and homoscedasticity test. Percentages of total number of metabolites that satisfied normality and homoscedasticity conditions (before log transformation ► after log transformation).

	Num. features	Group	Normality	Homoscedasticity	Normality & homoscedasticity
ESI+	970	Neonates	71% ► 76%	52 % ► 63%	39% ► 49%
		Children	67% ► 82%		37% ► 50%
ESI-	206	Neonates	58% ► 79%	46% ► 61%	29% ► 50%
		Children	67% ► 88%		30% ► 49%

It was verified that both normality and homoscedasticity of the data was improved using the logarithm. Therefore, data was log transformed in SIMCA software prior to multivariate statistical analysis.

3.3 Multivariate statistical analysis

3.3.1. Unsupervised analysis and detection of outliers

Data was centred using Mean Centring in SIMCA software and a PCA model was constructed with the study samples excluding the strong outlier P12/13 (Figure 4.8).

Figure 4.8 Data Visualisation using PCA. Score plot representing the first two components of the PCA in a) ESI+ (5 PC, $R2=0.780$, $Q2=0.596$) and b) ESI- (6 PC, $R2=0.878$, $Q2=0.701$). The sample marked in red was detected in the Hotelling's T2 plot as a moderate outlier.

Although by visualisation of the score plot using the two first components was not so obvious, sample P69/13 fell out of the 95% confidence level in the Hotelling's T2 Plot. Also visualisation of its TIC chromatogram in comparison with the rest of the samples showed a considerable difference, therefore this sample was excluded from further analysis.

3.3.2. Neonates versus Children supervised analysis

Two new PCA models were built excluding the outliers detected. A separation between the Children and Neonates groups can be clearly distinguished from these PCA models (Figure 4.9).

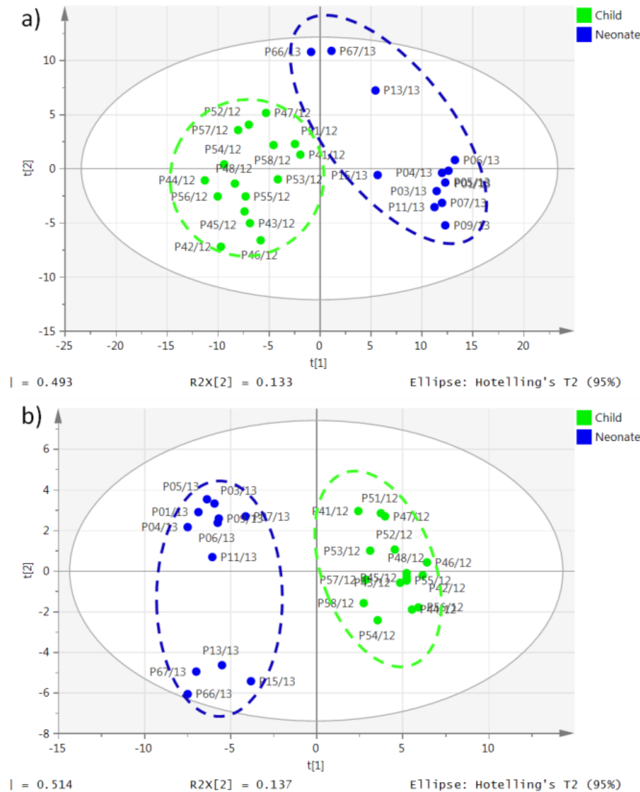


Figure 4.9 PCA model of study samples coloured by group. Score plots representing the first two components of the PCA models of study samples without the outlier samples in a) ESI+ (3 PC, R2=0.702, Q2=0.585) and b) ESI- (4 PC, R2=0.810, Q2=0.667) modes. Samples from group Children are coloured in green (●), samples from the Neonates group in blue (●).

For the supervised analysis a PLS-DA model was constructed to maximise the differences between the two classes (Figure 4.10).

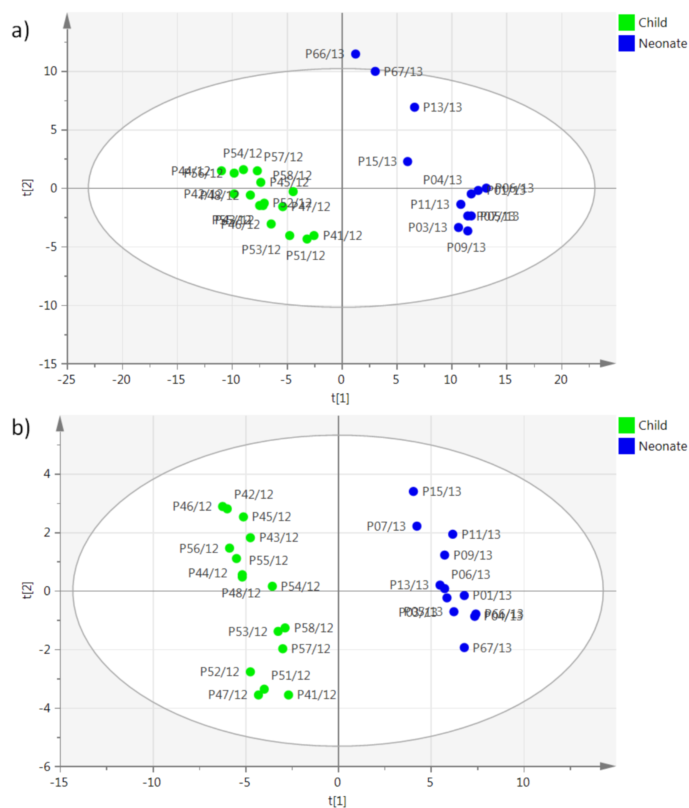


Figure 4.10 PLS-DA model of study samples classified by group (Neonates vs Children). Score plots representing the first two components of the of PLS-DA models in ESI+ (3 PC, R2=0.986, Q2=0.966) and ESI- (3 PC, R2=0.992, Q2=0.970).

These models were validated by permutation of Y variable corresponding to the two studied classes (999 permutations) (Figure 4.11). The negative intercept of the Q2Y trend line indicates that the model fit the data and the classification was not fortuitous. Validation plots for both models strongly indicate their validity.

Also an ANOVA test was used to assess the validity of the model, obtaining for both cases p-values lower than 0.05, pointing to a significant model.

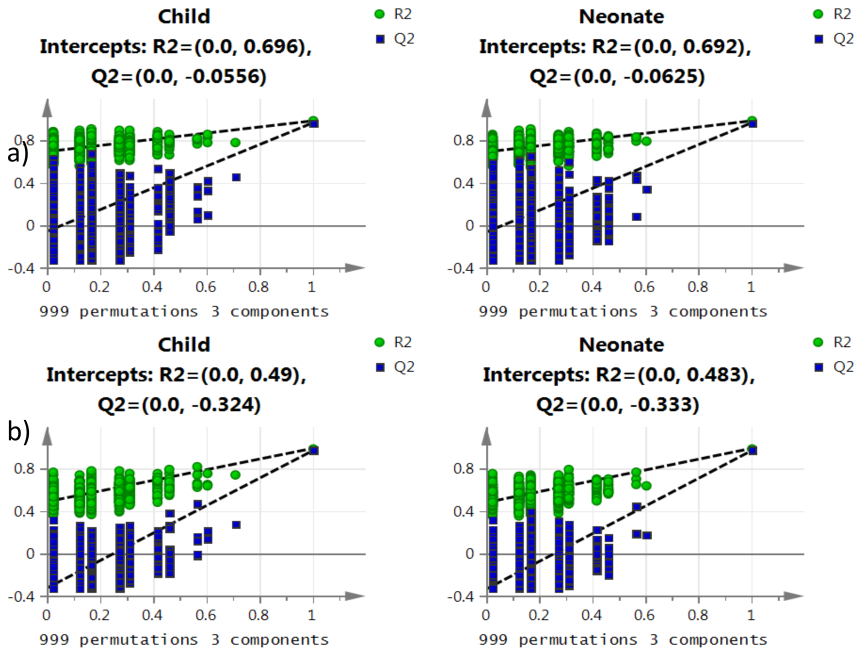


Figure 4.11 Validation of PLS-DA. Permutation plots for a) ESI+ and b) ESI- models.

Once the model was validated, OPLS-DA was used to find the features responsible for the maximum variation between the two classes (Figure 4.12), using the S-plot and VIP value of these variables (Figure 4.13).

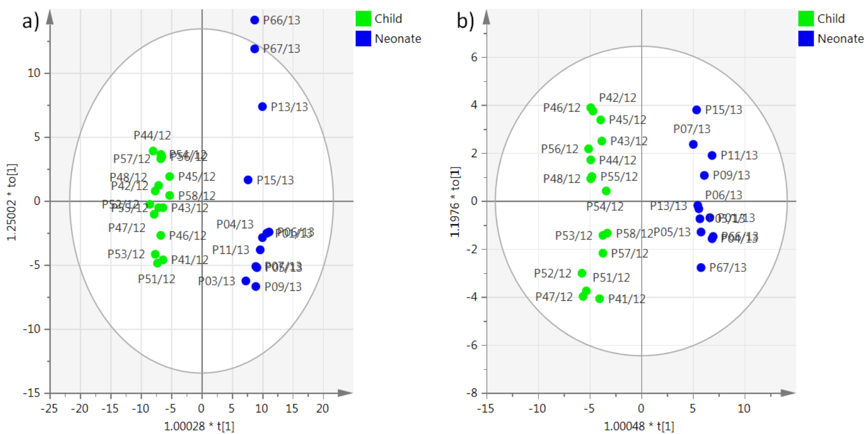


Figure 4.12 OPLS-DA models. Score plots for a) ESI+ (3 PC, R2=0.986, Q2=0.968), and b) ESI- (2 PC, R2=0.982, Q2=0.959) models.

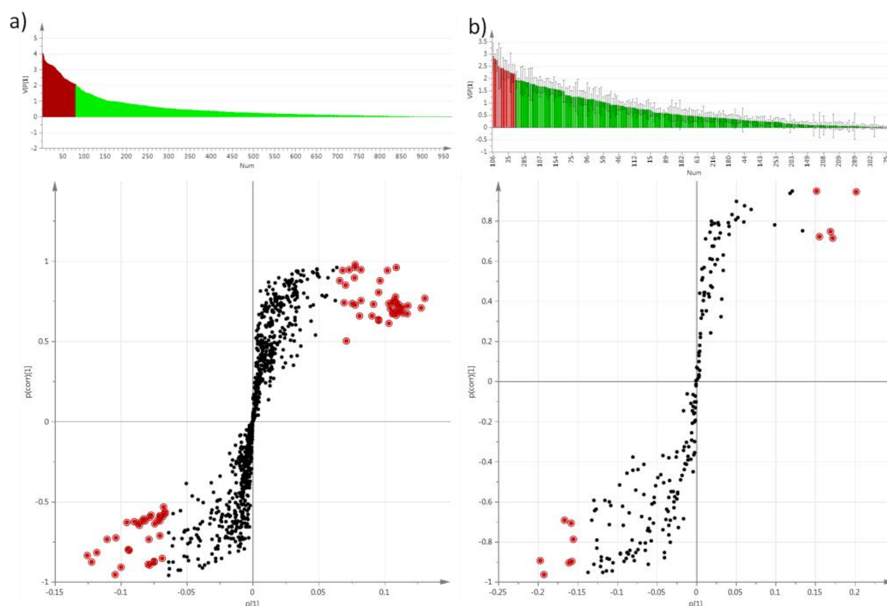


Figure 4.13 Selection of relevant feature. Both in a) ESI+ and b) ESI-, features with high absolute values of correlation (p(corr[1])) and covariance (p[1]) in the S-plot and VIP value higher than 2 (coloured in red) were selected as responsible for the differences between both groups for further investigation.

Features with high VIP value are situated far from the centre of the S-plot combining high model influence with high reliability on the correlation of the variables with the predictive component. Features laying on the right part of the plot are upregulated in the Neonate group, while features on the left are upregulated in the Children group.

The analysis of the features selected from the S-plot gave as a result the list of candidates shown in Table 4.5.

Mass-to-charge ratios with the same retention time and assigned to the same group by CAMERA may be different ions of the same chromatographic peak and are therefore suspicious of corresponding to the same compound (Box 3). Each feature from the group was studied individually and isotopes, adducts, and fragments were removed.

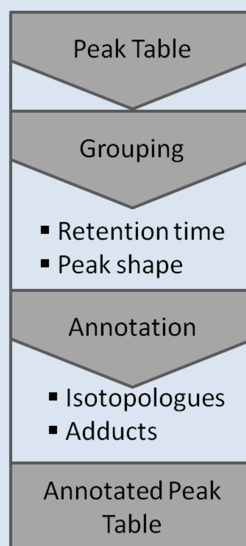
Table 4.5 Features selected from the S-plot. Classified as: Upper in Neonates = upregulated in Neonates; Upper in Children = upregulated in the Children group.

ESI+				ESI-			
Upper in Neonates		Upper in Children		Upper in Neonates		Upper in Children	
m/z	t_R (min)	m/z	t_R (min)	m/z	t_R (min)	m/z	t_R (min)
206.6644	3.65	146.0605	2.35	230.9966	2.68	226.0172	3.25
236.6750	3.65	206.1180	3.34	368.1933	2.70	384.2380	5.83
237.2092	3.14	256.0922	1.89	605.4169	3.23	415.1963	5.09
237.7105	3.14	359.2049	3.34	606.4197	3.23	437.1784	5.10
238.2123	3.14	360.2076	3.35	647.4279	3.65	766.3585	3.08
249.2093	3.64	368.2433	5.83			843.9076	2.64
258.2144	3.24	380.1836	3.34			897.6208	5.87
258.4017	3.23	408.2356	5.83				
258.7156	3.23	414.3005	5.87				
258.8080	3.23	430.1268	1.78				
259.2174	3.23	432.3110	5.87				
266.1396	2.22	445.2518	2.65				
276.1818	1.65	448.3060	5.96				
279.2199	3.65	470.2875	5.96				
279.3168	3.64	472.3037	5.87				
279.7217	3.65	558.2806	2.64				
279.8198	3.64	559.2809	2.67				
280.2230	3.65	564.2849	2.64				
314.2332	5.07	574.2955	2.65				
331.2275	4.87	585.2713	5.69				
356.2949	3.64	587.9639	2.68				
374.3057	3.23	594.7990	2.67				
375.3092	3.23	610.3682	10.98				
376.2732	11.91	645.3321	2.66				
386.2904	6.79	681.9797	2.48				
388.2962	6.79	682.3143	2.48				
392.1906	2.69	682.6482	2.48				
398.3055	3.64	767.8725	3.08				
405.2613	6.21	768.3742	3.08				
416.3161	3.23	768.8759	3.08				
416.3164	3.65	803.3914	3.09				
418.2741	13.79	803.8930	3.09				
430.3318	3.65	804.3940	3.09				
431.3348	3.65	845.9251	2.64				
433.2976	13.79	881.4435	2.67				
472.3425	3.65	881.9448	2.67				
474.4138	3.14	899.6365	5.86				
475.4165	3.14	1167.5203	12.98				
554.4199	11.90						
598.3865	10.28						
694.4226	11.90						

The remaining features, allegedly corresponding to the protonated molecule $[M+H]^+$, were searched in the online databases METLIN,[36] Lipid Bank,[37] KEGG,[38] Lipids Maps,[39] and HMDB.[40]

Box 3 CAMERA software

CAMERA (Collection of Algorithms for Metabolite pRofile Anotation) is an R-package designed to annotate isotope peaks, adducts, cluster ions, and fragments in peak lists. It clusters mass signals originated from a single metabolite, based on rules for mass differences and peak shape comparison.



In a first step CAMERA group peaks after their retention time. Then it verifies the grouping with a peakshape correlation step. Finally, CAMERA annotates isotopes and adducts. As default, CAMERA uses a dynamic rule set, created from the combination of lists of observable ions. Each rule describes a specific ion species with the mass difference to the molecular mass, ion charge and the number of molecules the ion species contains. All m/z differences within a compound spectrum are compared against this rule set. CAMERA searches for possible combinations from the standard ions H, Na, K, NH_4 and Cl, depending on the ionization mode. The detection of isotope patterns is done by calculation of m/z differences of 1.0033/z within a compound spectrum and by an intensity ratio check.

As a result an annotated peaklist is obtained, with information about the isotopologues and adducts. Also, pseudospectra of all the ions of the different species detected under a peak can be obtained.

Some of the features were identified as anaesthetic drugs or metabolites of anaesthetic drugs in the ESI+ model, indicating that the anaesthetic regimen was contributing to the separation of the groups. To study the influence of the different types of anaesthesia—A1 and A2 for Children, A3 and A4 for Neonates—in this separation, each group was studied individually by unsupervised analysis with PCA in both ionisation modes.

From the PCA score plots it can be distinguished a separation, clearer in Neonates than in the Children group, both in ESI+ and ESI- modes (Figure 4.14).

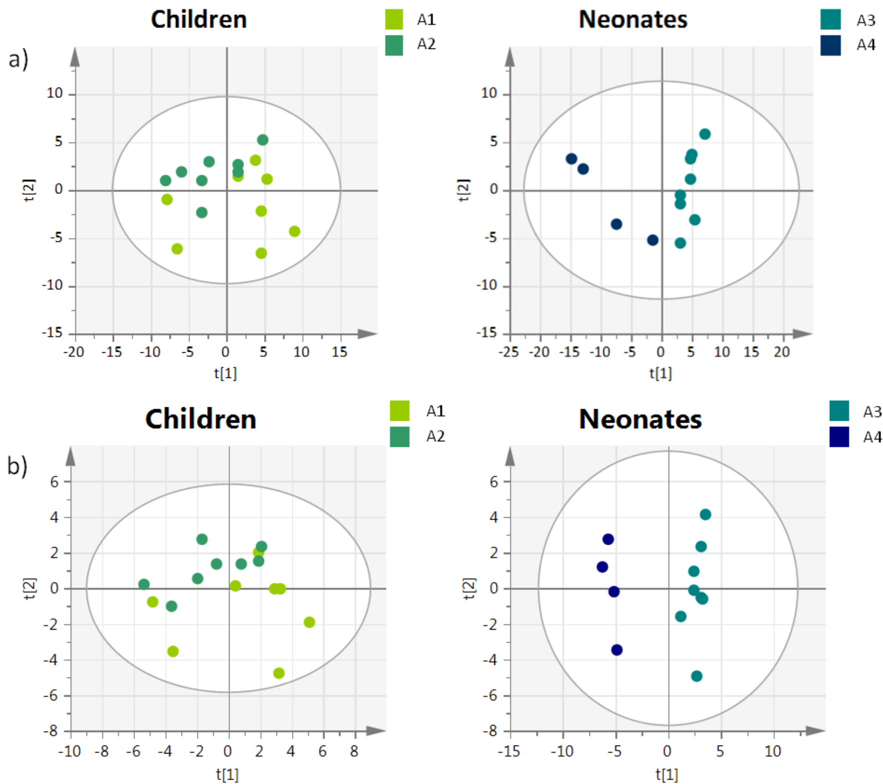


Figure 4.14 Anaesthesia PCA models. Score plots of Children and Neonates groups individually coloured according to the type of anaesthesia in a) ESI+ (Children: 3 PC, R2=0.593, Q2=0.240, Neonates: 3 PC, R2=0.712, Q2=0.378) and b) ESI- (Children: 3 PC, R2=0.723, Q2=0.397, Neonates: 5 PC, R2=0.886, Q2=0.527).

PLS-DA models of both groups classifying the samples according to the type of anaesthesia were built. From these models only the one built for the Neonates group in ESI- resulted significative according to the permutation test plot and the ANOVA p-value (Table 4.6). Notwithstanding, even though in ESI+ it does not pass the permutation test it is visible the trend to differentiate the two types anaesthesia.

Table 4.6 Validation of PLS-DA models using anaesthesia as class variable. The validity of the models was assessed according to the p-value obtained from the ANOVA and the Q2 intercept from the permutation test plots.

	Ionisation mode	PCs	R2	Q2	p	Q2 intercept	
						A1/A3	A2/A4
Children	ESI+	4	0.988	0.694	0.4306	0.315	0.334
	ESI-	6	0.998	0.911	0.4454	0.448	0.461
Neonates	ESI+	3	0.995	0.888	0.0164	0.188	0.194
	ESI-	2	0.987	0.927	0.0006	-0.338	-0.304

An OPLS-DA model was built for the Neonates group in ESI- (Figure 4.15), and the features responsible for the separation of the groups classified as the different types of anaesthesia were selected from its VIP value and position in the S-plot (Figure 4.16). From the S-plot it can be observed the higher amount of relevant features with higher concentration in the subjects treated with the anaesthesia A3, responsible for the separation.

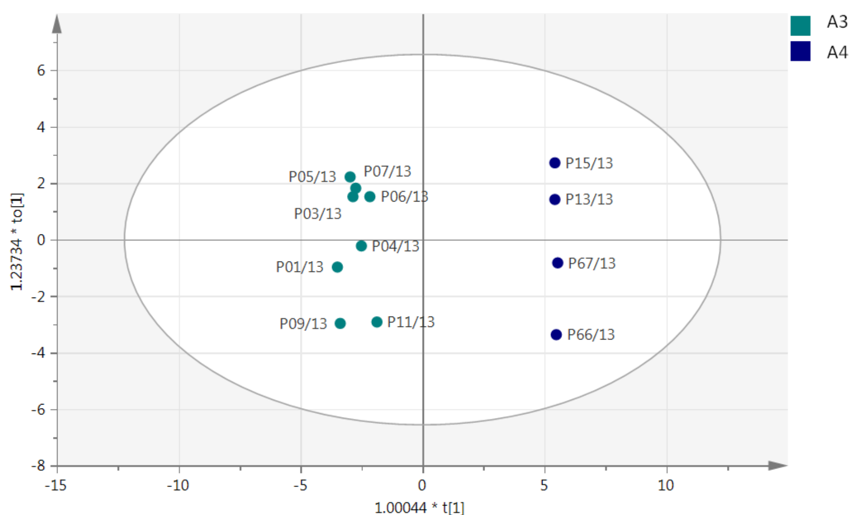


Figure 4.15 OPLS-DA model of the Neonates group in ESI- classified by the type of anaesthesia. Score plot (2PC, R2 = 0.987, Q2 = 0.942).

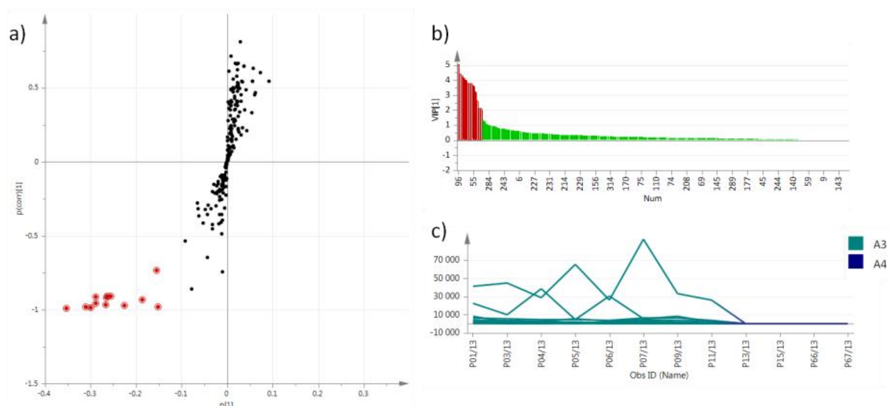


Figure 4.16 Selection of features responsible for the separation according to the type of anaesthesia. a) S-plot of the model. Coloured in red the features selected as differentiators of both groups of anaesthesia, b) VIP variables, c) Line plot of the selected features in the Neonate samples.

The features selected were extracted from the chromatograms to check that they corresponded to a real chromatographic peak and their exact masses were searched in the METLIN[36] online database in order to look for a possible identification. Several of the selected features were identified as compounds related with the anaesthetic drug propofol (Table 4.7), present in the A3 anaesthesia, but not in the A4.

Table 4.7 Feature discriminating the type of anaesthesia in ESI-. Results from the search of the features responsible for the separation between the two types of anaesthesia A3 and A4 in neonates.

m/z	t_R	Δppm	Name	Formula
177.1279	5.45	3	Propofol	$C_{12}H_{18}O$
273.0794	4.05	3	1,4-Benzenediol, 2,6-bis(1-methylethyl)-, 4-(hydrogen sulfate)	$C_{12}H_{18}O_5S$
353.1598	5.45	2	Propofol glucuronide	$C_{18}H_{26}O_7$
369.1548	3.72	1	4-hydroxy-3,5-bis(1-methylethyl)phenyl glucuronide 4-hydroxy-2,6-bis(1-methylethyl)phenyl glucuronide	$C_{18}H_{26}O_8$

The lack of validity of most of the models classified by type of anaesthesia indicates that the strong separation observed between Neonates and Children groups should not be only owed to the anaesthetic drugs. However, to reduce the effect of these drugs in the separation, new models were built, both for ESI+ and ESI-, removing the features responsible for the separation between the groups of anaesthesia—identified as anaesthetic

drugs, its metabolites and other features detected by CAMERA under the same chromatographic peak—from the features list.

Comparing the new PCA model with the model built with all the features it can be observed that the effect of the anaesthesia intra-group has been minimised and a better clustering of the Neonates group was obtained in both ESI+ and ESI- ionisation modes, while the strong separation between groups remains (Figure 4.17).

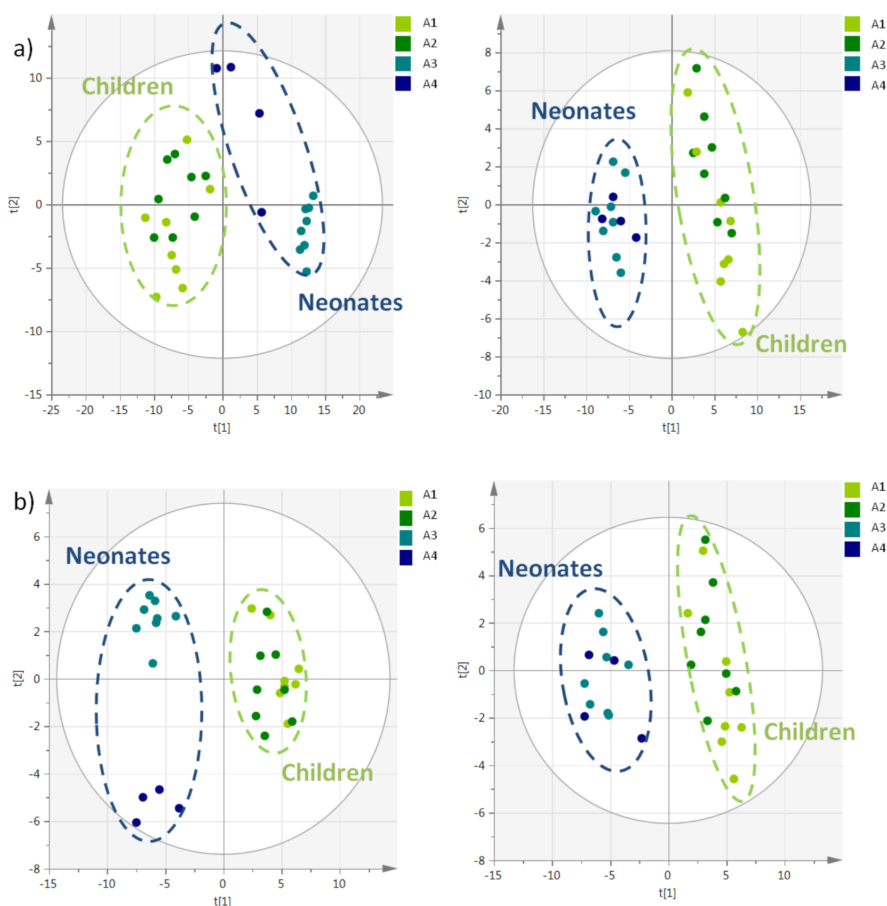


Figure 4.17 Comparison of PCA models before and after removing features corresponding to anaesthetics. Score plots of the PCA models before removing the features identified as anaesthetics (left), and after removing the features (right) coloured by type of anaesthesia in a) ESI+ and b) ESI- ionisation mode.

PLS-DA models were built with the new lists of features classifying the samples into the Neonates and Children group. These models were validated by means of permutation test and ANOVA. OPLS-DA was used to obtain the features responsible for the separation of groups.

Among the features with higher intensity values in ESI+ in the Neonates group several have been identified according to the exact mass as possible carnitines (Table 4.8).

Table 4.8 Features identified as possible carnitines. Features selected among those responsible for the separation between Neonates and Children identified as possible carnitines.

ID number	m/z	t_R	Possible metabolite
179	260.1853	3.17	Hexanoylcarnitine
45	162.1133	0.32	L-carnitine
402	370.2956	7.68	cis-5-Tetradecenoylcarnitine
244	288.2179	4.67	Octanoylcarnitine
115	232.1554	1.44	Butyril-L-carnitine
			Isobutyryl carnitine

3.3.3. Study of the influence of other variables

Other possible confounding factors as gender, and time and vein of sampling were individually studied.

Gender

PCA score plot was examined coloured according to gender in order to inspect possible trends by unsupervised analysis. To avoid the effect of the age each group was studied separately (Figure 4.18).

In ESI+ no separation can be seen by unsupervised analysis in any of the PCs of the model, neither for Children, nor for Neonates. Similar results were obtained in ESI- mode. PLS-DA models were built using gender as class variable for each group. None of these models could be validated by means of permutation test and ANOVA, and therefore it could not be said that the influence of gender was significative for the analysis.

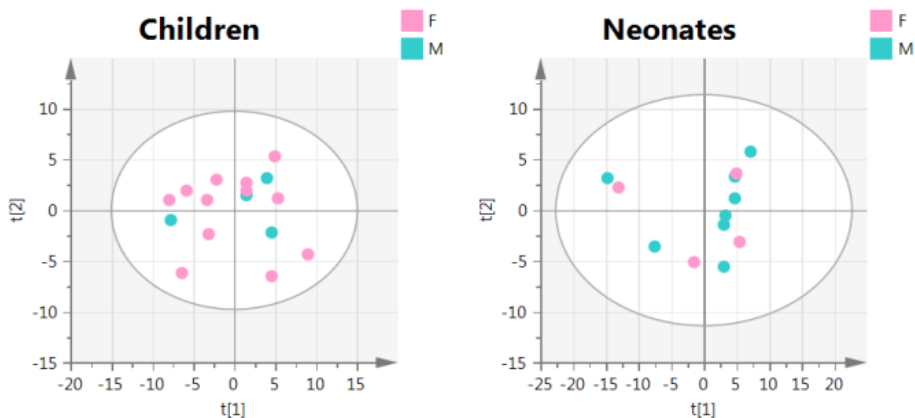


Figure 4.18 Gender PCA models (ESI+). Score plots of Children and Neonates individually coloured according to gender.

TIME OF SAMPLING

The visualisation of PCA score plots coloured by the time of sampling did not show any separation between the samples extracted in the morning and those extracted in the evening in ESI+ mode (Figure 4.19). As all neonate samples were extracted in the morning this group was not studied individually. Similar results were obtained in ESI-.

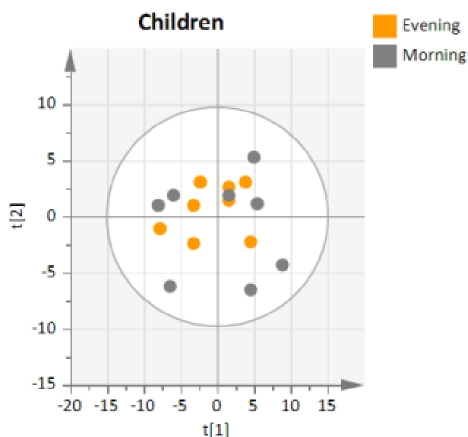


Figure 4.19 Time of sampling PCA model (ESI+). Score plots of the group Children individually coloured according to time of sampling.

The PLS-DA model using time of sampling as class variable could not be validated. Therefore, the influence of time of sampling in this analysis is not considered significative.

VEIN OF EXTRACTION

In the unsupervised analysis using PCA no clear separation was detected between samples taken from ear vein and from femoral vein, although it can be recognized a trend in the score plot (Figure 4.20). As all samples from the Neonates group were extracted from the femoral vein, this group was not studied individually. Similar results were obtained in ESI-.

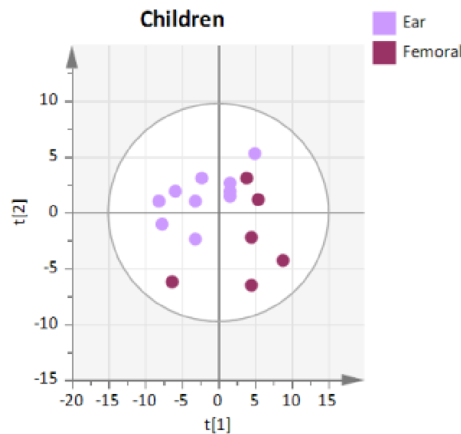


Figure 4.20 Vein of extraction PCA model coloured according to time of sampling (ESI+). Score plots of the Children group individually coloured according to vein of sampling.

Supervised analysis using PLS-DA with extraction vein as class variable resulted in no significative models for ESI+ or ESI-.

4 DISCUSSION

The possibility of reducing inter-individual biological variation by controlling environmental factors leads to more easily detectable intrinsic differences due to the different state of development. Due to the impracticability of doing so in human subjects, the use of pigs as animal models of paediatric

development may be a good alternative to the study in actual children. However, the use of animals to substitute children in research has to be justified, and the experiment must be carefully designed so that as few animals as possible are used to obtain sufficiently relevant results.

This pilot study was performed in order to obtain enough evidence about the feasibility of a future study involving the use of several animal subjects aiming to reveal the metabolites implied in the process of maturation. In the spirit of keeping the animal usage down to a minimum, blank samples from animals already involved in other studies were collected. As these animals were selected for different purposes they were not only differentiated by age, but also by other factors such as gender, time and vein of sampling, or anaesthesia. In metabolomics experiments this undesired variability caused by aspects other than the differences under study must be avoided. However, this primary study took advantage of these confounding factors in order to estimate their influence and effect in the data and determine their role in the future study.

From the MVDA models built a clear difference between the plasma metabolic profiles of piglets as models of neonates and children was observed. The separation between groups is appreciable by unsupervised analysis by PCA and by supervised analysis using validated PLS-DA models, indicating the potential of the proposed procedure to carry out a deeper study. However, this separation may be influenced by the different anaesthetic drugs used for the subjects, as several of the features found to be differentiators of the groups by age were identified as these drugs or their metabolites.

The study of the effect of anaesthesia in each group individually resulted in no statistical difference for most of the models built, with the exception of the Neonates group in ESI- mode, where a validated PLS-DA model was obtained classifying the samples according to the type of anaesthesia, and propofol and some of its metabolites were found to be responsible for the differences in this group of pigs. By removing the drugs and their

metabolites from the features list the effect of the type of anaesthesia was minimised—as observed by the better intra-group clustering obtained in the new PCA model—while the strong separation between groups was maintained. This effect was also observed in ESI+ mode, where even though the separation based on anaesthetics was not statistically significant, the removal of the features identified as drugs or its metabolites also resulted in a better intra-group clustering. This possibility of re-interrogating the data is one of the advantages of untargeted analysis, since based on the results obtained from the MVDA data can be re-examined to search for specific metabolites or family of metabolites, or as in our case to minimise the influence of undesired compounds.

The comparison of piglets of 8 weeks based of their gender did not result in any significant difference. This may be due to several reasons, as the fact that these piglets have not reached puberty—expected around the 14 to 26 weeks of life—that is the period when the differences between male and female subjects become more evident. In addition, the limited number of samples may not be enough to unmask subtle differences between genders. Compounds as steroid hormones, well known gender differentiators, are not detected among the relevant features by the method here presented. However, it is unclear if this may be a consequence of the lack of difference between genders or that they are not detected by the applied method due to their low concentration. A further investigation will be required in order to search for different levels of concentration in this family of compounds depending on the gender of the subject.

Time and vein of sampling resulted in no statistically significant effect, indicating that possible differences derived from the circadian cycle of the metabolites or the extraction vein, if any, are not detected by the method developed.

Although some metabolites as several carnitines appeared to be relevant for the differentiation of piglets according to age, no further identification was carried out, as the main aim of this study was not the identification of

biomarkers, but the confirmation of the usefulness of metabolomics for the differentiation of the different grades of development of piglets. Nevertheless, evidence was obtained as outcome of this study that features which exact masses were identified in databases as carnitines may be upregulated in the first stages of life does not go in accordance with other studies in humans found in bibliography,[41,42] where the levels of L-carnitine and several acetyl carnitines are found to increase with age. Identification of these metabolites should be confirmed by comparison with standards. If the identification is confirmed, a targeted analysis aiming to study differences in plasma levels of this family of metabolites in correlation with age will be proposed in order to determine the evolution of plasma levels of these compounds in correlation with age. Also, further experiments of MS/MS or MS^E would be of great interest to facilitate the identification of these and other metabolites.

Valuable insights were obtained into the feasibility of the implementation of LC-MS-based metabolomics in the study of age-related differences in the plasma metabolome of piglets as paediatric models. This work shows evidences that by using metabolomics with LC-Q-TOF-MS it is possible to detect changes in the metabolic profile of piglets according to their grade of maturation.

5 DESIGN OF FUTURE STUDY

Considering the promising results provided by the preliminary research here presented, a larger-scale study was designed in collaboration with the team of the Experimental Neonatal Physiology Unit of the BioCruces Health Research Institute (Cruces University Hospital, Biscay, Basque Country, Spain).

Information about the effects of different times and veins of sampling, gender of the subjects, or confounding factors such as anaesthesia obtained from this pilot study was applied to avoid in the future some of the

problems encountered in this research. Also, based on these preliminary results, an estimation of the sample size was performed.

In the pilot study, noticeable differences between the plasma metabolome of piglets of less than 5 days—models of neonates—and of 8 weeks—models of children—were detected. For the future study it is proposed the collection of four age groups: less than a week, two weeks, four weeks, and twelve weeks, coincident with the different stages of childhood, namely newborns and neonates, toddlers, and children (Figure 4.21).

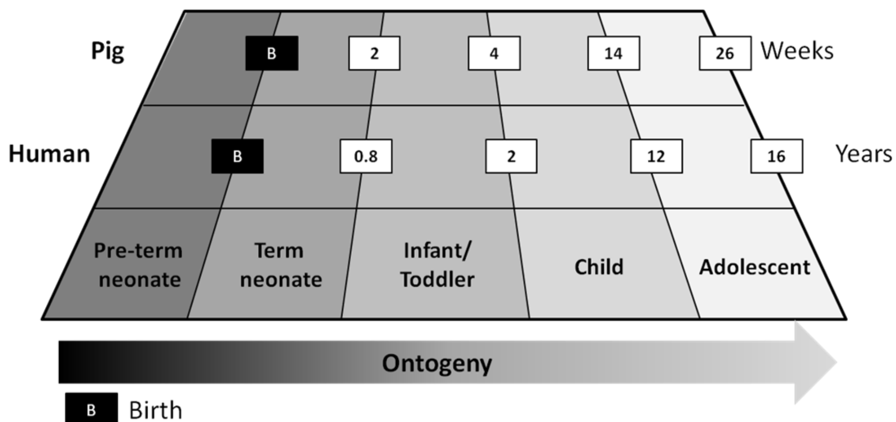


Figure 4.21 Comparative age categories based on overall central nervous system (CNS) and reproductive development. Adapted from Buelke-Sam 2001.

Assessment of the sample sizes—i.e. number of subjects per group—is of great importance in the study design. Enough subjects have to be enrolled in the study so that sufficient statistical power is achieved to find differences among groups. Statistical power depends on effect size—magnitude of the difference between groups—and sample size. If the effect size of the intervention is large, it is possible to detect such an effect in smaller sample numbers, whereas a smaller effect size would require larger sample sizes.[43] However, the common saying *«the more the better»* does not apply here, as an unnecessarily high number of subjects will lead to a waste of animal lives and resources for a minimum information gain.

In metabolomics experiments the assessment of sample size is a complex exercise, and there is not a standard method established. Several methods

can be found in bibliography.[31,44-46] Here, the method proposed by Ferreira et al.[47] was used through the adaptation as the SSPA package for R[48] (Figure 4.22).

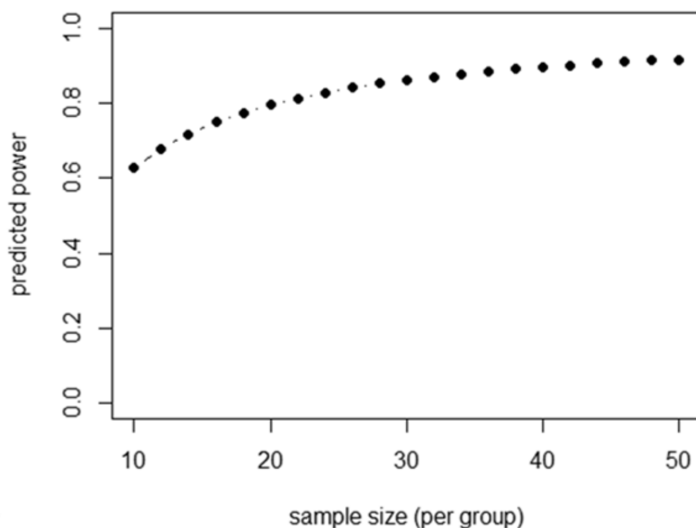


Figure 4.22 Sample size calculation.. For sample sizes from 10 to 50 the power curve is predicted based on the information obtained from the pilot data.

With ten samples per group it is estimated 65% power of the analysis, reaching 80% for twenty samples. As a compromise between the predicted power of the analysis and ethical and economical reasons, it is proposed the use of 12 subjects per group, leading to a 70% predicted power. As no differences were detected in the previous results based on the subject gender both male and female piglets will be used.

Ideally, the same 12 subjects would be kept for 12 weeks and plasma samples would be collected at the different times estimated. In this way, not only the number of animals used would be drastically reduced, but also the inter-group differences due to the intrinsic diverse metabolomes of the subjects will be minimised. However, due to the impossibility of keeping the animals for such a long time in the facilities of the Experimental Neonatal Physiology Unit, 12 different subjects for each time point are needed. In order to make the most of this animals and gain the maximum information

possible, not only plasma samples will be collected, but also other biological fluids as urine and CSF, and tissues from the liver, kidney, heart, brain and lungs.

As it has been seen in the pilot study the administered anaesthesia can be a confounding factor, therefore, to minimise its effects, the same anaesthetic drugs will be administered to all the subjects: ketamine and diazepam (i.m) and sevoflurane (inh.), keeping them to a minimum to avert as much as possible the modification of the metabolome, but enough to prevent causing suffering to the animals, and avoiding the use of the drugs with bigger influence in the separation of the samples based on the type of anaesthesia in this pilot study.

As vein extraction of blood is not always possible, for the future study it is proposed that all blood samples will be collected from femoral vein in EDTA tubes, and kept on ice until the extraction of plasma by centrifugation at 3000 rpm at 4 °C. The supernatants will be transferred to cryovials and stored at -80 °C until analysis. Urine and CSF samples will be collected by bladder and lumbar puncture, respectively, and stored with no further preparation. All samples will be stored at -80 °C until analysis.

All samples will be collected at the approximate same time in the morning, because despite no differences have been detected in the pilot study here presented depending on the circadian cycle, it is well known its effect on the levels of several metabolites.[49]

Same sample treatment used in the pilot study will be applied to the plasma samples in the new experiments. For the analysis only ESI+ ionisation mode will be performed, as little extra information was obtained from the ESI- analyses, but in addition MS^E experiments will be added to facilitate the identification of biomarkers. For the rest of biofluids and tissues, sample treatment and analysis will be optimized.

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Part II: Metabolomics in paediatrics

Chapter 5

Study of differences in maturation grade of Children

It's no use going back to yesterday, because I was a different person then.

(Lewis Carrol, *Alice in Wonderland*)

1 INTRODUCTION

Human maturation in early life is a complex multifactorial process, influenced by genetic and environmental factors.[1] Little is known about human intra- and inter-individual metabolic profile changes during childhood. Studies of biological variation during development provide insight into the physiological changes that characterise the process of maturation in the first stages of life. Values obtained from such investigations are important for the determination of the **biological age** of a patient.

While chronological age is defined by the time the subject has lived, biological age estimates the functional status of an individual in comparison to his chronological equals.[2] The American Food and Drug Administration (FDA) describes the age ranges for paediatric population based on their chronological age as shown in Figure 5.1.[3]

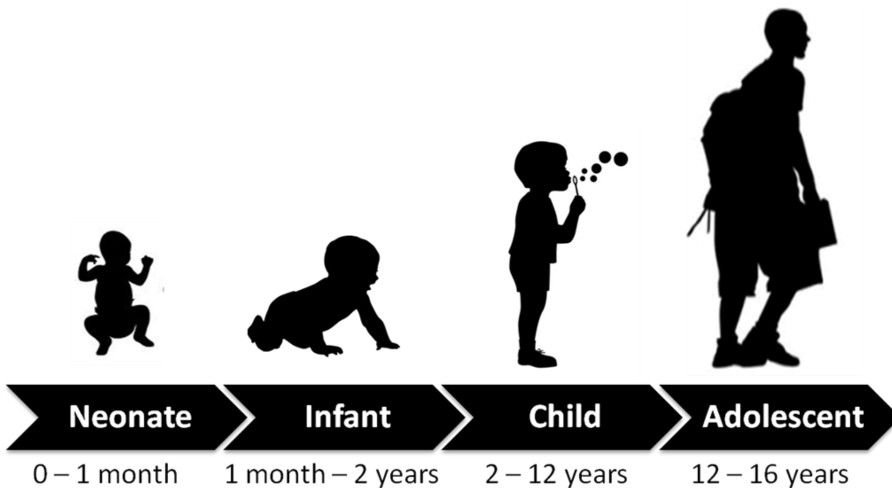


Figure 5.1 Paediatric age ranges. FDA classifies paediatric population into 4 groups depending on the age: neonates, infants, children, and adolescents.

Although the concept of biological age has been primarily applied to gerontology and life expectancy,[4] its determination is of great importance in

paediatrics, where big differences may be found between chronological and biological age due to slow or fast maturation of the individual.

The assessment of biological age in a patient—and therefore its maturation state—offers an interesting step towards personalised medicine. Chronological age alone does not provide enough information for the prediction of the effect of drugs in the children, as in their developing system, enzymes involved in drug metabolism and conjugation usually do not mature at the same rate. The process of maturation is continuous throughout all the stages of life of paediatric population, from birth through childhood and adolescence until reaching the adulthood and therefore complete maturation. From this point ahead they can also be found changes in the metabolism, however, does would not be related with organ maturation but with ageing.

Metabolomic techniques may offer new insights into the changes that occur during growth and development, and may offer an interesting approach to studying the metabolic changes that characterise the process of maturation and ageing. The untargeted study of metabolic profiles in children through a hypothesis-free approach may lead to the discovery of new biomarkers of ageing and development in this population.

The study presented in this chapter is consequence of a previous study designed and developed for the comparison of urine samples collected from children younger than one year of age and affected with bronchiolitis—an infectious disease that affects the bronchioles of infants and young children—with follow-up samples of those children taken at 6 and at 12 months of age, and with age-matched healthy children (control groups). While treating the data obtained from the analysis of these samples by ultra high performance liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) a clear effect of the age was observed in the construction of models by multivariate data analysis (MVDA). To investigate this effect independently from the bronchiolitis, data obtained from the control groups were separately processed and

differences among samples based on the age were studied. Also, the influence of gender in the data was studied.

Therefore, the main aim of this study is to investigate the effect of the age in the metabolomic data obtained from a control group of children of less than approximately a year of age.

2 MATERIALS AND METHODS

2.1 REAGENTS AND MATERIALS

LC-MS-Ultra CHROMASOLV® grade acetonitrile (ACN) and methanol (MeOH) (Sigma-Aldrich; St Louis, MO, USA) were used as organic modifiers for the chromatographic system. Purified water from a Milli-Q® Integral System (Millipore; Merck, Darmstadt, Germany) was used in the preparation of mobile phases and reagent solutions. Mass spectrometry grade formic acid (FA) from Fluka (Sigma-Aldrich; St Louis, MO, USA) was employed as additive of the mobile phases.

2.2 INSTRUMENTATION

For the sample preparation step a Super Mixer Vortex (Cecchinato; Mestre, VE, Italy) and a Centrifuge 5415D (Eppendorf, Hamburg, Germany) were used.

The metabolite profiling analysis was performed in an Acquity UPLC system, coupled to a high resolution Q-TOF mass spectrometer Synapt G-2 (Waters, Milford, MA, USA) equipped with an electrospray ionisation source (ESI).

2.3 SOFTWARE

MassLynx V4.1 and DataBridge 3.5 (Waters, Milford, MA, USA) were used for data acquisition and conversion, respectively. The statistical free software R 3.1.2 was used running the packages XCMS 1.42.0 (Metlin, La

Jolla, CA, USA) and CAMERA 1.22.0 (Bioconductor) for peak detection, alignment, and isotopologues identification. Univariate statistical analysis was performed by Excel 2007 (Microsoft Office, Redmond, WA, USA) and SPSS Statistics 17.0 (IBM, Armonk, NY, USA); and multivariate statistical analysis by SIMCA 13.0.1 (Umetrics, Umea, Sweden).

2.4 SUBJECTS

Urine samples were obtained from children of both genders of less than a year of age admitted to the emergency room (ER) of the University Hospital of Padua (Padua, PD, Italy) between September 2013 and March 2014 with a diagnosis of bronchiolitis (n=55). Follow up samples were collected, when available, for those children when they reached the age of 6 months (n=38) and 12 months (n=27) approximately. Samples were divided therefore in three groups named **BRO**, **BRO 6M**, and **BRO 12M**. Control samples were collected from healthy children of different age and were separated into three groups: **CTRL** for children up to 4 months of age (n=19), **CTRL 6M** for children between 4 and 10 months (n=33), and **CTRL 12M** for children older than 10 months (n=32). Controls were considered healthy if they met no chronic medical condition, no acute infection or antibiotic assumption over the last four weeks, and if they had no history of atopy. Age and gender data for each group are compiled in the Table 5.1.

Table 5.1 Characteristics of the study participants. Number of subjects enrolled for each group according to the pathology, age, and gender.

	n	Age (months)			Gender	
		Average	min	max	Female	Male
BRO	55	2.7	0.4	11.9	20	35
BRO 6M	38	7.4	5.9	10.0	13	25
BRO 12M	27	13.0	11.9	15.1	10	16
Total Bronchiolitis	**120**	**6.5**	**0.4**	**15.1**	**43**	**77**
CTRL	19	1.7	0.7	4.0	11	8
CTRL 6M	33	6.3	4.5	8.0	11	22
CTRL 12M	32	12.4	11.1	13.7	12	20
Total Controls	**84**	**7.6**	**0.7**	**13.7**	**34**	**50**
Total	**204**	**6.9**	**0.4**	**15.1**	**77**	**127**

Written informed consent signed by a parent was required for inclusion in the study and for the treatment of their personal data according to the

Italian law. This study was approved by the University Hospital of Padua Ethical Committee (n° 3112).

2.5 SAMPLE COLLECTION

Urine samples were collected at the University Hospital of Padua (Padua, PD, Italy). A urine sample of at least 5 mL was collected from each patient within 24 h of enrolment (day 0 samples) and at 6 and 12 months of age (when available). For urine collection, sterile screw-capped polypropylene containers were used. Urine samples were immediately placed in the refrigerator at 4 °C and transferred within 12 h of collection at -20 °C, to minimise bacterial growth.

Urine samples collected were shipped on dry ice to the Laboratory of Mass Spectrometry of the Department of Women and Children Health of the University of Padua for their storage and analysis.

1 or 2 aliquots—depending on the volume of urine collected—of 3 mL of each sample were transferred into 5 mL polypropylene tubes and stored at -80 °C. All collection and storage elements were previously washed with MeOH to eliminate possible contaminants from plastic additives.

2.6 SAMPLE TREATMENT

Frozen urine samples, stored at -80 °C were transferred first to -30 °C freezer and then thawed at 4 °C. Once thawed, samples were vortex mixed and centrifuged for 10 min at 6000 rpm at 10 °C to remove insoluble particles.

Samples were **diluted** with a solution of 0.1% FA in a 1:5 v:v sample:solution ratio. A volume of 100 µL of urine was transferred to an Eppendorf microtube and 400 µL of 0.1% FA were added. Samples were then vortex mixed and 200 µL were transferred to the correspondent well of a 384 wells plate to be injected by the UHPLC-Q-TOF-MS system.

2.7 QC, BLANK AND TEST MIX SAMPLES PREPARATION

A QC sample (**QCsys**) for system equilibration before the run and for the assessment of system stability was prepared by mixing 20 µL of each urine sample. Also QC samples were prepared for each of the groups (**QCG**) by mixing another 20 µL of each sample within groups. QC samples were then diluted with a solution of 0.1% FA in a proportion 1:5 v:v sample:solution, taking the adequate solvent volume corresponding to the volume of the QC prepared.

A vial with a solution of 0.1% FA was used for blank samples to check absence of carryover and to examine possible changes in the baseline.

A **test mix** sample was prepared for the assessment of mass accuracy, retention time deviation, and intensity along the run. For the preparation of the test mix nine compounds (sulfaguanidine, acetaminophen, hippuric acid, caffeine, leu-enkephalin, sulfadimethoxine, verapamil, terfenadine and cholic acid) of known t_R and m/z in the conditions of analysis (Table 5.2) were dissolved in 0.1% FA and transferred to a vial to be injected.

Table 5.2 Test Mix. Compounds of the test mix sample, with their t_R and m/z both in ESI+ and ESI- ionization modes.

Compound	t_R (min)	m/z	
		ESI+	ESI-
Sulfaguanidine	1.00	215.0603	213.0446
Acetaminophen	2.65	152.0712	150.0555
Hippuric Acid	3.68	180.0661	178.0504
Caffeine	3.94	195.0882	-
Leu-enkephalin	4.98	556.2771	554.2615
Sulfadimethoxine	5.05	311.0814	309.0658
±Verapamil	5.41	455.2910	-
Terfenadine	5.99	472.3216	-
Cholic Acid	6.88	-	407.2797

2.8 UHPLC-Q-TOF-MS ANALYSIS

In the pre-run several injections of blank samples, test mix and QCsys were done. Then, study samples (SS) from the six groups and QCGs were injected randomised to avoid the effect of potential bias and drifts during sample

measurement. Every ten injections and at the end of the run a QCsys, test mix, and a blank sample were injected (Figure 5.2).

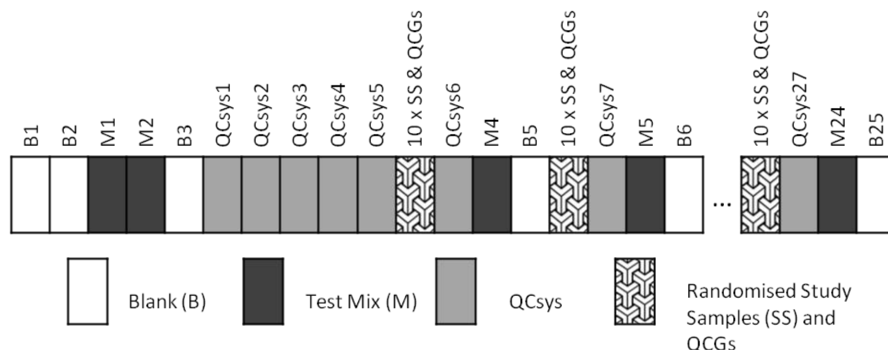


Figure 5.2 Scheme of the injection order. Study samples were randomly distributed. The same scheme with different randomisation for study samples was used both in ESI+ and ESI- modes.

The thermostated autosampler was set at 6 °C. 5 µL of the samples were injected into an Acquity UPLC HSS T3 (100 x 2.1mm, 1.8 µm) column (Waters, Milford, MA, USA), set at 50 °C. Water acidified with 0.1% FA was used as aqueous mobile phase (A). As organic modifier (B) a mixture of MeOH:ACN in a 90:10 ratio acidified with 0.1% FA was used. The gradient started after an isocratic step of 1 min at 5% B. Then the % B reached 95% in two linear gradient steps: up to 30% in 2.5 min and to 95% in 3 min. The 95% B was maintained for 1.5 min, and after that, the system returned to initial conditions, which were kept for 3 min, being the total run time of 11 min. The mobile phase flow was 0.5 mL/min. This flow was splitted after the column so that only one part out of ten entered into the detector.

The samples were analysed operating the Q-TOF-MS in both **MS Scan** and **MS^E** modes of data acquisition (Box 4), both in ESI+ and ESI- ionisation mode, in different runs.

For the MS Scan experiments centroid data were collected in both positive (ESI+) and negative (ESI-) ESI ionisation modes at full scan range from 20 to 1200 m/z, with a scan and interscan time of 0.3 and 0.024 s, respectively.

ESI parameters were: source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 20 L/h for ESI+ and 50 L/h for ESI-, desolvation gas flow 600 L/h, cone voltage 30 V, and capillary voltage 3 kV for ESI+ and 1.5 kV for ESI-.

Box 4 Data acquisition methods: MS Scan vs MS^E

The TOF mass spectrometers are high mass accuracy and high resolution instruments widely used in metabolomics. TOF spectrometers are ideal for untargeted analysis since they provide the ability to collect both high-resolution precursor and fragmentation data through their different data acquisition methods, facilitating the characterisation of metabolites.[5]

MS Scan. Usually it is the first step on a metabolomics analysis. In this method a full scan of all the *m/z* range, typically wide, is collected. Every ion within this *m/z* range is counted.

MS^E. MS^E method records exact mass precursor and fragment ion information without quadrupole selection. To do so, MS^E rapidly alternates between two functions: the first one acquires low collision energy spectra that provides precursor exact mass information; the second acquires elevated collision energy spectra providing exact fragment ion exact mass data. The acquired data are aligned based on the retention time for each component, so that the fragmentation spectra obtained can be linked to its precursor ion. Softwares as the MS^E Data Viewer (Waters, Milford, MA, USA) facilitate the interpretation of these data.

For the MS^E experiments, two acquisition functions with different collision energies were created, one acquiring low energy spectra (LE) and the other high energy spectra (HE). In the LE function the transfer collision energy was set to 4 eV while in the HE function a ramp from 20 to 30 eV was used.

MS Scan data were used for the feature extraction and statistical analysis, while MS^E experiments were used as a useful complement for the structural identification of potential biomarkers.

To ensure mass accuracy and reproducibility, the mass spectrometer was calibrated over a range of 20–1200 Da with a solution of sodium formate. A LockSpray interface was used to maintain mass accuracy during analysis. To this end, a solution of leucine enkephalin ($[M+H]^+$ = 556.2771 m/z, $[M-H]^-$ = 554.2615) was continuously infused at 5 µL/min as lock mass.

2.9 DATA TREATMENT

MassLynx software was used for data acquisition. MS Scan files were converted into CDF with the DataBridge converter software.

Firstly, MS Scan data was processed for all samples—QCsys, QCGs, and the six groups of BRO and CTRL—for each ESI+ and ESI- ionization modes.

The XCMS package in R was used for peak detection, using the CentWave algorithm[6] and setting 3 to 20 as peak width range and 10 ppm as maximal m/z deviation in consecutive scans. Grouping, retention time correction, and peak filling steps were performed. The script used can be found in Annex I.

To minimise the confounding effect of the antibiotic presence in the children treated for bronchiolitis over the multivariate analysis, only features present in a minimum of 30% of the samples at least at four out of the six groups were kept. On the other hand, when control children were studied separately, only features present in at least more than 30% samples in all groups were kept to reduce the effect of xenobiotics in the analysis.

Features appearing after the 6.5 min of the chromatogram, corresponding to the step of column cleaning and re-equilibration were removed.

The CAMERA package was used for the detection of isotopologues and adducts, and remaining zero values in the matrix were replaced by random numbers between 0 and the minimum value found in the feature, using R.

Urine specimens present higher inherent variability than other biological fluids as plasma, serum, or cerebrospinal fluid. Different physiological and pathophysiological factors, water consumption, circadian rhythms or diet among many other factors are responsible for differences in urine volume, so that up to 15-fold variations are commonly observed among volumes of normal urine samples.[7] This volume variability—together with differences in the plasmatic concentration and the excretion rate of the compounds—

leads to disparities in metabolite concentration and to heteroscedasticity of the data. As a consequence, the achievement of relevant results by MVDA may be jeopardized. To compensate this variation several strategies have been used. Flow-rate correction, electrical conductivity, specific gravity, osmolarity, or creatinine-based methods are commonly used approaches in urinalysis.[8] However, for the holistic approach of metabolomic analysis, statistical strategies may be more adequate. In this chapter a **median fold change normalization** method was used for the normalization procedure to reduce variance caused by urine dilution[9,10] following the Equation 5.1.

$$x'_{i,j} = \frac{x_{i,j}}{f_{i,j}} \qquad \text{Equation 5.1}$$

Where $x'_{i,j}$ is the corrected signal of the feature i in the sample j and $x_{i,j}$ is the signal without modifications. The correction factor $f_{i,j}$ is calculated as the median fold change of the intensity between each feature in the sample $x_{i,j}$ and a target profile, here chosen as the median of the samples $M_e(x_i)$, as shown in the Equation 5.2.

$$f_{i,j} = M_e\left(\frac{x_{i,j}}{M_e(x_i)}\right) \qquad \text{Equation 5.2}$$

The effect of this correction was studied comparing the plot of the sum of intensities against the number of injection, the number of features with %CV lower than 30% in the QCsys samples, and by visual comparison of QCsys samples repeatability in the PCA before and after correction.

The %CV of each feature in the QCsys samples was calculated with the Microsoft Office Excel software, and features with %CV>30%, which is considered an acceptable value of repeatability in biomarkers analysis,[11] were eliminated. %CV values higher than 30% are considered over the analytical variability tolerance limit and features with those values are considered to be noise.

A power transformation was applied to the data matrix. Normality of the data was examined applying a Shapiro-Wilk test for each feature in each

group. Homoscedasticity was checked by means of Levene test. Both statistical tests were performed by SPSS software.

2.10 STATISTICAL ANALYSIS

Differences between the groups were studied by MVDA using SIMCA 13 software.

Unsupervised analysis by means of PCA was used as an overview to look for trends, groupings, and outliers. Hierarchical cluster analysis (HCA) was used to study the clustering observed in PCA. Unsupervised analysis by PCA was also applied to the study of the effect of the gender.

PLS models were built using the age as the Y-variable. Pairwise comparison by PLS-DA was used, classifying the samples into the different groups according to the age. PLS-DA models were validated by means of permutation test and ANOVA.

Also soft independent modelling of class analogy (SIMCA) was used as a supervised method to study the validity of the groupings. To do so, five samples from each group were randomly selected and assigned to a test set. The remaining samples constituted the training set, and were used for building the model. The test set samples classes were then predicted according to the built model. Cooman's plots were used to study the classification and prediction of the model.

For the selection of putative biomarkers of maturation, S-plot and similar and unique structures (SUS) plot from OPLS-DA models were used.

Selected features were studied by univariate data analysis (UVDA), using Kendall and Spearman's tests in order to examine the correlation of the concentrations of these features observed in the samples with the age.

2.11 METABOLITE IDENTIFICATION

For metabolite identification the scheme represented in Figure 5.3 has been followed.

First, it was checked that the feature selected as possible biomarker corresponded to a real chromatographic peak by extracting the m/z from the chromatogram of the sample where the intensity is the highest.

The feature was then searched in the list obtained by CAMERA in order to examine if it was identified as the protonated in ESI+ or deprotonated in ESI- molecule or an adduct or fragment. When the feature is identified as an adduct or fragment, the m/z of the protonated/deprotonated molecule is calculated.

The accurate masses from the protonated or deprotonated molecules were searched in the online databases (METLIN,[12] Lipid Bank,[13] KEGG,[14] Lipids Maps,[15] and HMDB[16]). Among the hits obtained from these databases the possibility of the feature being the proposed metabolite was checked. Some of the reasons for exclusion of metabolites in this step may be due to the physicochemical properties of the molecules—as discrepancies in polarity or type of ionisation with the selected feature—but they may also be biochemical—as the unfeasibility of some metabolites appearing in the fluid or the system under study—.

When available, the MS spectrum from the databases or bibliography was compared with the spectra obtained experimentally. For MS^E experiments, both low and high collision energy functions were compared.

If the MS spectra found in bibliography corresponds to the spectra obtained experimentally the next step would be the confirmation of the metabolite identity by comparison with standards. In this step MS/MS experiments are of great interest, as comparison of retention time and fragmentation pattern leads to an almost decisive identification. In the work here presented, due to the lack of standards, this step was not performed.

Metabolite identification

Search m/z in chromatogram
It is a real peak?

Yes

No

Discard Feature

Search in CAMERA list

$[M+H]^+$

Adduct/Fragment

Calculate $[M+H]^+$

Search m/z in online databases
Are there any hits?

Yes

No

Discard Feature

Check viability of the ionization mode, t_R, biofluid location…
Is the metabolite feasible?

No

Check another compound

Yes

Is the MS spectra available (databases+bibliography)?

Yes

Compare Spectra
Does it match?

No

Yes

No

Compare with a standard
Does it correspond?

Yes

No

Identification confirmed

Figure 5.3 Scheme of the metabolite identification process. Steps followed from the feature selection to the metabolite identification.

3 RESULTS

3.1 SYSTEM STABILITY

A total of 299 injections were analysed in each run—204 samples, 28 QCsys, 18 QCGs, 25 blank samples and 24 test mix samples—for approximately 60 h of analysis for each polarity and acquisition mode, resulting in a total of 240 hours. Between runs the system was stopped and the ion source was cleaned.

The performance of the system along the MS Scan runs in terms of mass accuracy, retention time, and intensity both in ESI+ and ESI- ionisation modes was tested with the test mix samples (Table 5.3).

Table 5.3 System stability. Monitoring of retention time %CV, mass accuracy as the average ppm deviation from the accurate mass, and intensity drop from the first to the last injection in a run of the compounds in the test mix along the MS Scan runs in both ESI+ and ESI- polarities.

	ESI+			ESI -		
	t_R %CV	m/z ppm	Intensity drop (%)	t_R %CV	m/z ppm	Intensity drop (%)
Sulfaguanidine	0.28	3.20	10.5	0.00	0.63	19.8
Acetaminophen	0.13	7.32	18.7	0.19	0.90	20.8
Hippuric Acid	0.07	6.15	12.1	0.14	0.24	17.8
Caffeine	0.13	4.68	18.6	-	-	-
Leu-enkephaline	0.00	0.31	27.5	0.08	0.12	29.5
Sulfadimethoxine	0.04	1.38	15.0	0.00	0.27	22.2
±Verapamil	0.04	0.90	18.2	-	-	-
Terfenadine	0.00	0.77	11.9	-	-	-
Cholic Acid		-		0.00	0.76	26

Values of t_R and m/z for the selected peaks remained almost unchanged for the whole run and mass accuracy remained below 10 ppm in all cases.

On the other hand, a drop of the intensity was observed for the compounds in both ESI+ and ESI- ionisation modes. This drop of intensity is not homogeneous, but compound and polarity mode dependant (Figure 5.4), being for instance almost three times more pronounced for leu-enkephaline than for sulfaguanidine in positive mode.

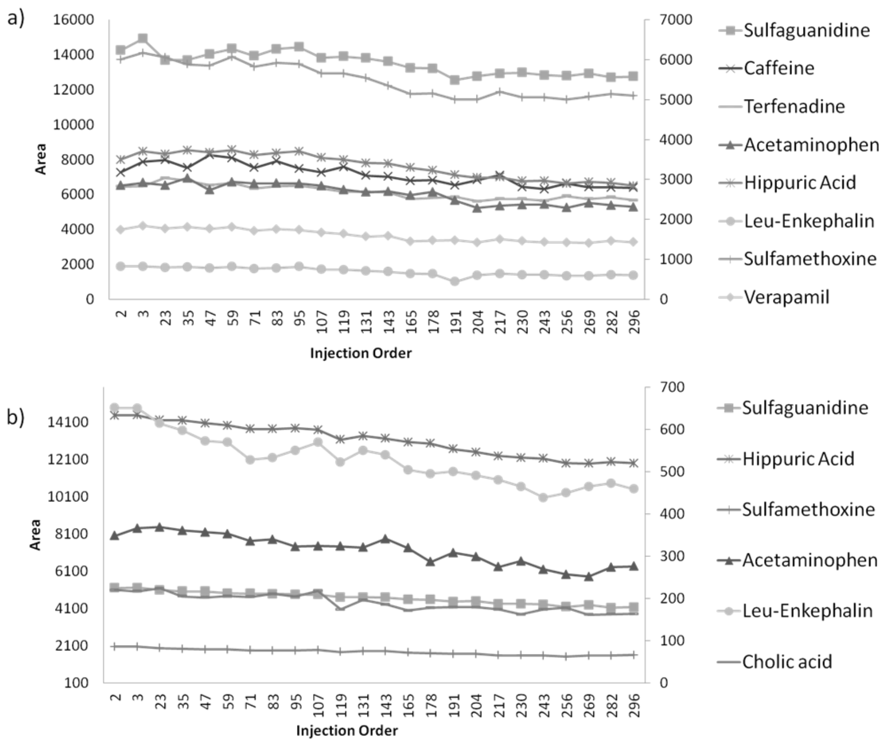


Figure 5.4 Intensity monitoring. Areas of the compounds of the test mix along the run in a) ESI+ and b) ESI-.

3.2 DATA PRE-PROCESSING

3.2.1 PRELIMINARY STUDY: BRONCHIOLITIS VS CONTROLS

In ESI+, a total of 5042 features were detected by XCMS. 2486 features were removed as they were present in three or less groups and could probably be xenobiotics resulting from medication. 416 variables appearing after 6.5 min in the chromatogram were also removed. Among the remaining features, 218 were removed as they were identified as carbon isotopologues by the CAMERA package.[17] To eliminate missing values from the matrix 139 zero values were substituted by random numbers lower than the minimum value in each feature. In ESI- data the XCMS software detected 5923 features, 3336 of them removed as only present in three or

less groups and 344 appearing after 6.5 min. 281 features were identified as isotopologues by CAMERA and 198 missing values were substituted with random numbers lower than the minimum value recorded for each feature.

Based on their visual and volumetric diversity, differences in the chromatographic profiles of the samples were expected. In the TIC chromatograms different elution patterns were observed, even between children within the same group. Those differences were especially intense in the BRO group, due to the presence of pharmacological drugs (Figure 5.5).

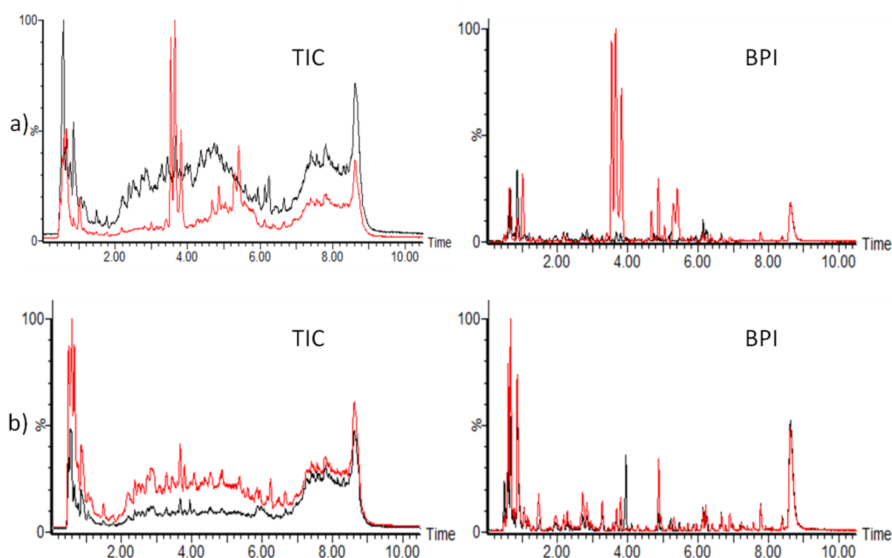


Figure 5.5 Differences in the chromatographic profile. TIC and BPI chromatograms in MS Scan analysis and ESI+ ionisation mode of a) two children from the BRO group and b) two children from the CTRL group, all of the same approximate age (~0.7 months).

The effect of the pharmaceutical drugs was mainly eliminated by the previous removal of the features appearing in only three or less groups of samples. For the compensation of differences based on the diverse volumes and the drop of intensity along the run, median fold change normalization was applied using the Equations 5.1 and 5.2 (Figure 5.6).

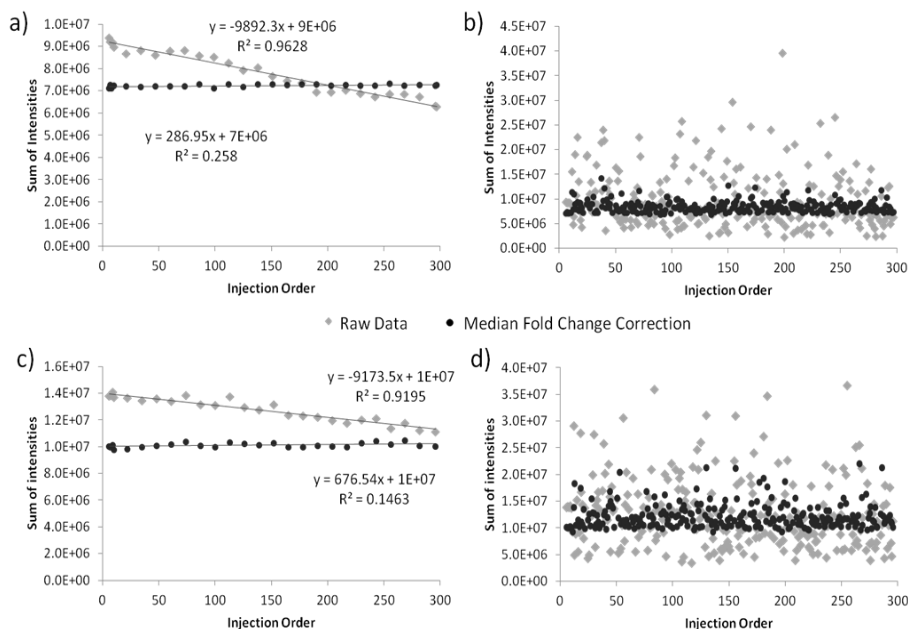


Figure 5.6 Median fold change normalisation. Sum of intensities of all features in ESI+ of a) QCsys samples and b) all samples, and in ESI- also for c) QCsys samples and d) all samples.

As it can be seen in the Figure 5.6, the normalisation by median fold change reduced the variability of the samples and compensates the intensity drop due to the detector sensitivity drift.

The correction of the intensity drop is also reflected in the percentage of features with %CV values lower than 30% in the QCsys samples that increased from a 75% to 87% in ESI+ and from 84% to 87% in ESI- and by the position of the QCsys samples in the PCA (Figure 5.7).

In the PCA score plot of the raw data a clear trend of the QCsys samples with the order of injection can be seen, while this trend disappears in the normalised data, where QCsys samples are perfectly clustered.

Figure 5.7 Effect of the median fold change normalization in the QCsys samples in ESI+. Score plot representing the first two components of the PCA in a) ESI+ and b) ESI- before the normalisation (left) and after normalisation (right). Coloured in red (●) the QCsys and in grey (●) the study samples. Labels indicate the order of injection.

289 features in ESI+ and 348 in ESI- had %CV>30% in the QCsys samples and were therefore removed from the list, leaving two final lists of 1994 and 1929 features for ESI+ and ESI-, respectively.

These data were studied by MVDA building a PCA model using SIMCA 13.0.1 software. When analysing these data it was seen a clear trend of the

samples distribution with the age, that veiled the effect of the pathology (Figure 5.8).

Figure 5.8 Impact of the age in the PCA. Score plots of the PCA models in a) ESI+ and b) ESI-. Samples sized by age.

This observation gave rise to the study presented in this chapter, where the changes in the metabolome as a consequence of the maturation process and change of diet in the first year of age are studied.

3.2.2 Study of the effect of age in the metabolome

To avoid the effect of the pathology and the pharmacological treatment, the groups of children affected with bronchiolitis were excluded, and the samples corresponding to the CTRL, CTRL 6M, and CTRL 12M groups were individually processed and studied. The results of the preprocessing steps are resumed in the Figure 5.9.

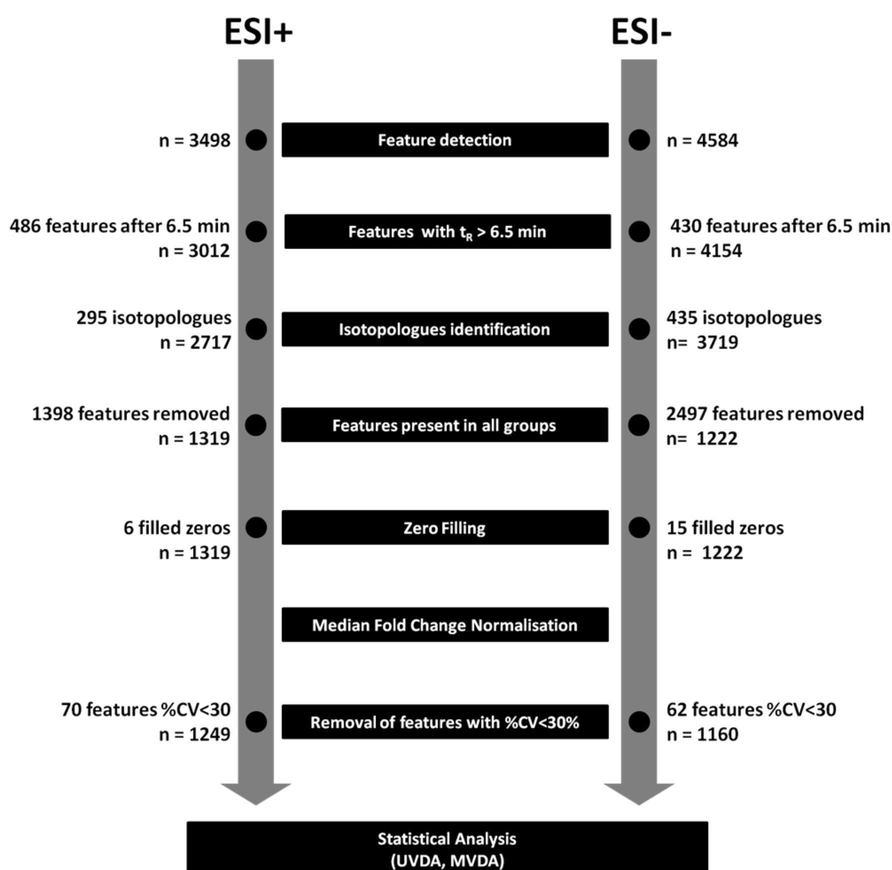


Figure 5.9 Preprocessing of CTRL samples. Workflow followed for the preprocessing of the samples of healthy children. n represents the number of features of the list in each step.

QCsys samples used in the preliminary study were prepared with all the samples from both children diagnosed with bronchiolitis and healthy, and are therefore not representative of this subset of samples. In these cases, were no QC sample is available, Vinaixa et al.[18] recommend to discard the features with %CV<20% in the study samples, as they are supposed to be below the analytical variation threshold. As 30%, a common threshold for analytical variation in bioanalysis, has been used along this manuscript, features with %CV below this value were removed in this study.

A power transformation was applied to the data. Normality and heteroscedasticity before and after the transformation were assessed by Shapiro-Wilk and Levene tests using the SPSS software. As it can be seen in Table 5.4 both normality and heteroscedasticity of the data was improved with the transformation.

Table 5.4 Normality and homoscedasticity test. Percentages of total number of metabolites that satisfies normality and homoscedasticity conditions (before transformation ► after transformation).

ESI mode	Num. features	Group	Normality	Homoscedasticity	Normality & Homoscedasticity
ESI+	3101	CTRL 0	61% ►75%	53% ►67%	31% ►48 %
		CTRL 6M	37% ►60%		19% ►41 %
		CTRL 12M	53% ►66%		20% ►44%
ESI-	4084	CTRL 0	48% ►70%	52% ►68%	26% ►48%
		CTRL 6M	26% ►54%		16% ►40%
		CTRL 12M	25% ►56%		16% ►41%

3.3 MULTIVARIATE STATISTICAL ANALYSIS

3.3.1. STUDY OF THE EFFECT OF AGE AND THE INFLUENCE OF GENDER

Data was centred by Mean Centring in SIMCA software and a PCA model was constructed with the study samples and the QCG samples (Figure 5.10).

From the score plot representation a distribution of the three groups along the first PC according to the age can be observed. This distribution can be more easily seen in the representation of the first component of the PCA against the age of the subjects (Figure 5.11).

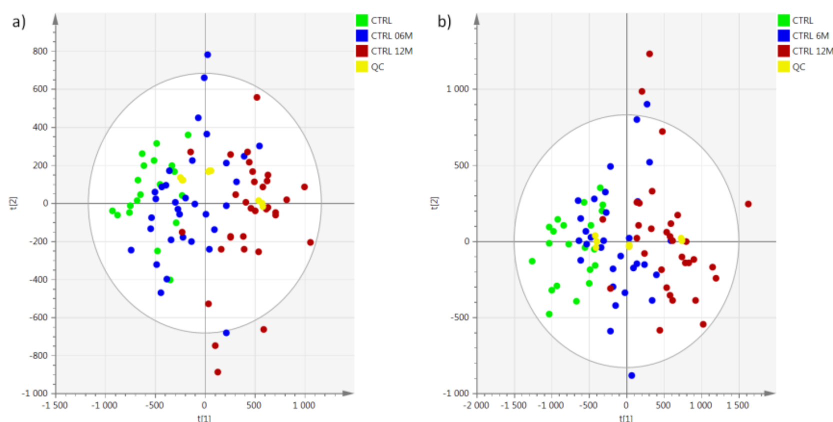


Figure 5.10 Data visualisation by PCA. Score plot representing the first two components of the PCA in a) ESI+ (12 PC, R2=0.745, Q2=0.408) and b) ESI- (9 PC, R2=0.678, Q2=0.304), coloured by group. In yellow are coloured the QCG samples from the three groups.

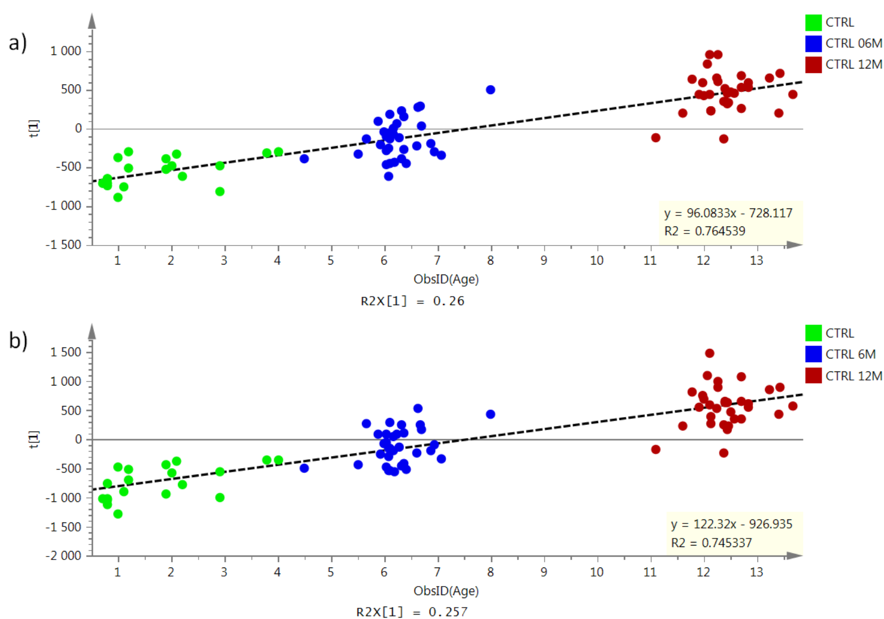


Figure 5.11 Distribution of age along the first component. Score plot of the first component of the PCA model built for the three groups of the study against the age of the subject in a) ESI+ and b) ESI-. Dashed black line represents the regression line.

PCA score plots were coloured according to the groups Male and Female to look for possible trends in the data owed to the gender of the subject (Figure 5.12).

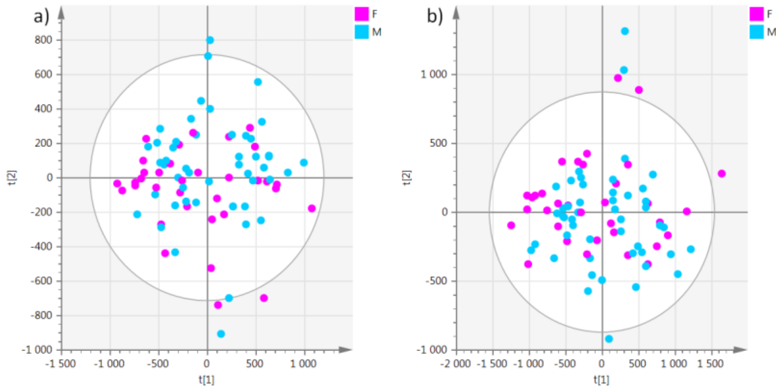


Figure 5.12 Effect of gender in the PCA. Score plot of the first two components of the PCA model built in a) ESI+ and b) ESI-. Coloured according to the gender of the subjects: in magenta are female (F) subjects, in light blue male (M) subjects.

Also PCA models were built for each group of age individually and the influence of the gender in the distribution of the score plot was studied (Figure 5.13).

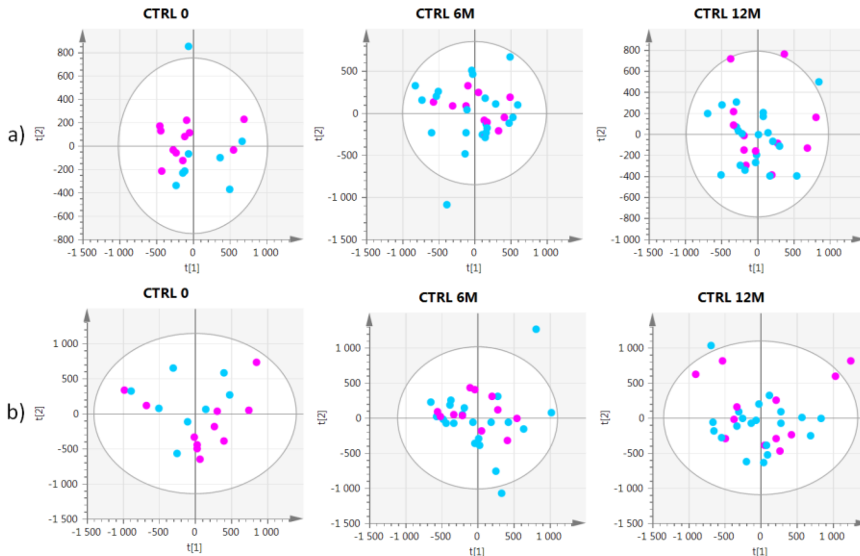


Figure 5.13 PCA per group. PCA score plot in a) ESI+ and b) ESI- coloured according to the gender. Coloured in magenta female subjects (F) and in light blue male subjects (M).

Neither on the PCA score plots with all the subjects, nor on the groups individually was detected a separation between male and female subjects. The lack of effect of the gender on the data was confirmed by PLS-DA models. None of the PLS-DA models classifying samples into Male and Female groups passed the validation tests (data not shown).

A PLS model was built using the age as Y variable (Figure 5.14). From the score plot it can be seen the distribution of the three groups, where the CTRL and CTRL 12M groups are perfectly separated while the CTRL 6M group is situated between them.

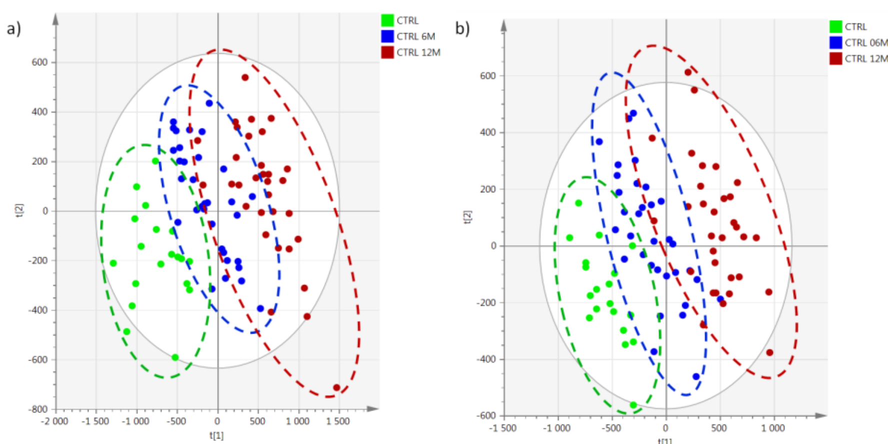


Figure 5.14 Grouping in the PLS model. Score plot of the first two components of the PLS model built in a) ESI+ and b) ESI-. Samples coloured by group. Dashed lines encircle all samples belonging to each group.

Also, PLS models were built for each group individually, to study if the trend observed for the three groups is maintained when they were analysed individually. From the Figure 5.15 it can be observed that, even if the individual models are weaker than the model built with all the samples, score values of the samples in the first component of the PLS models increase with age.

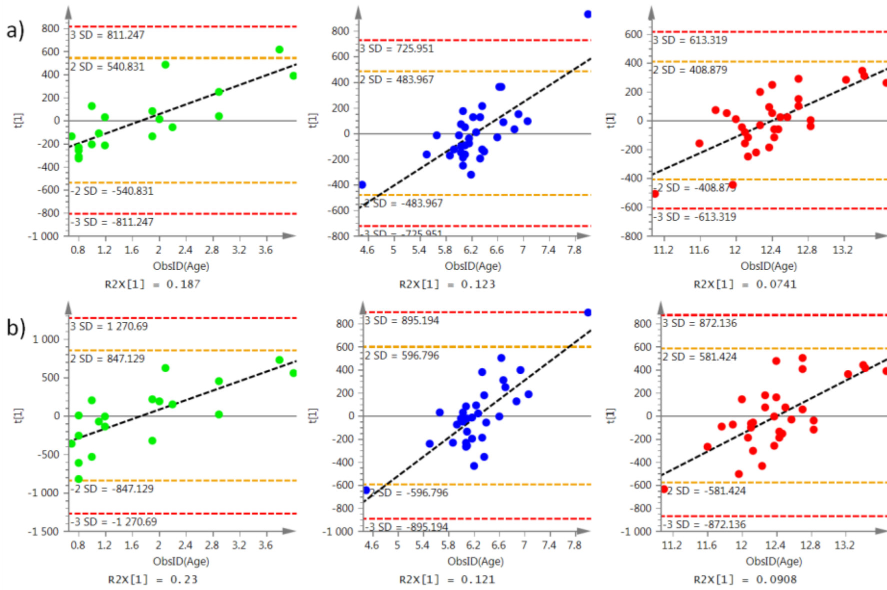


Figure 5.15 Individual PLS models. Score plots vaslues of the first components of the PLS models of each group individually against the age in a) ESI+ and b) ESI-. Group CTRL is coloured in green, CTRL 6M in blue, and CTRL 12M in red.

To study the differences among the groups a pairwise comparison was performed comparing CTRL vs CTRL 12M, CTRL vs CTRL 6M, and CTRL 6M vs CTRL 12M.

3.3.2 Pairwise comparison

Unsupervised analysis

Data was first studied by unsupervised analysis by PCA and Hotelling's T2 plot for outlier detection (Figure 5.16). Outliers were removed and new PCA models were built, checking that no other sample was detected as an outlier and that the separation observed remained.

CTRL vs CTRL 12M | CTRL vs CTRL 6M | CTRL 6M vs CTRL 12M

ESI+: 7 PC, R2=0.694, Q2=0.421 | 9 PC, R2=0.712, Q2=0.221 | 10 PC, R2=0.717, Q2=0.306

ESI-: 7 PC, R2=0.676, Q2=0.316 | 5 PC, R2=0.560, Q2=0.194 | 10 PC, R2=0.707, Q2=0.197

Figure 5.16 Unsupervised analysis of pairwise comparisons by PCA. Score plots of the PCA models and respective Hotelling's T2 plots. Samples coloured by group. Outliers marked in black.

Dendrograms obtained from the HCA of the paired groups are represented in the Figure 5.17.

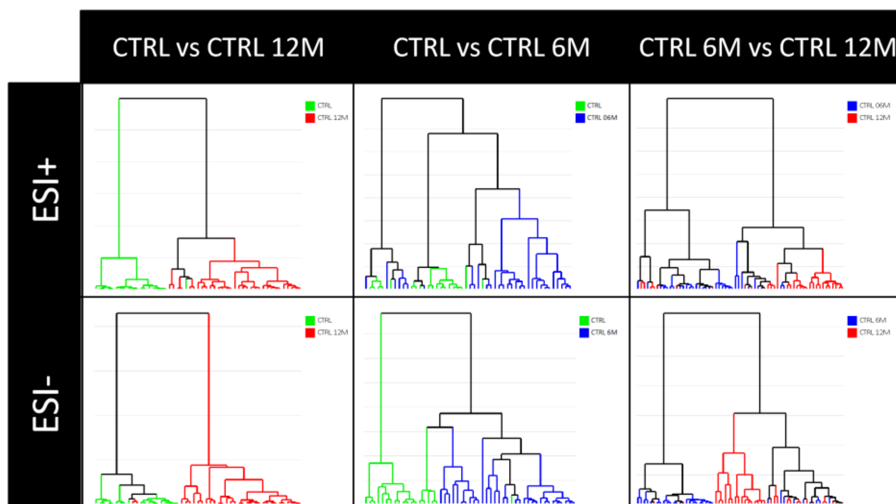


Figure 5.17 Unsupervised analysis of pairwise comparisons by HCA. Dendrograms obtained from the HCA using Ward linkage. Samples coloured by group.

The HCA dendrograms show similar results to those of PCA. In the CTRL vs CTRL 12M comparison the samples are almost perfectly organized in two clusters coincident with the assigned classes. For the other two comparisons the clustering is in general less definitive, however, the trend with the age can be observed.

SUPERVISED ANALYSIS

Outliers detected in the unsupervised analysis were excluded from the list of observations and PLS-DA models were built to differentiate the groups. These models were validated by permutation of Y variable corresponding to the two studied classes (999 permutations) (Figure 5.18). The negative intercept of the Q2Y trend line of the permutation plots indicates that the models fit the data and the classifications are not fortuitous. Also ANOVA was performed obtaining p-values lower than 0.05.

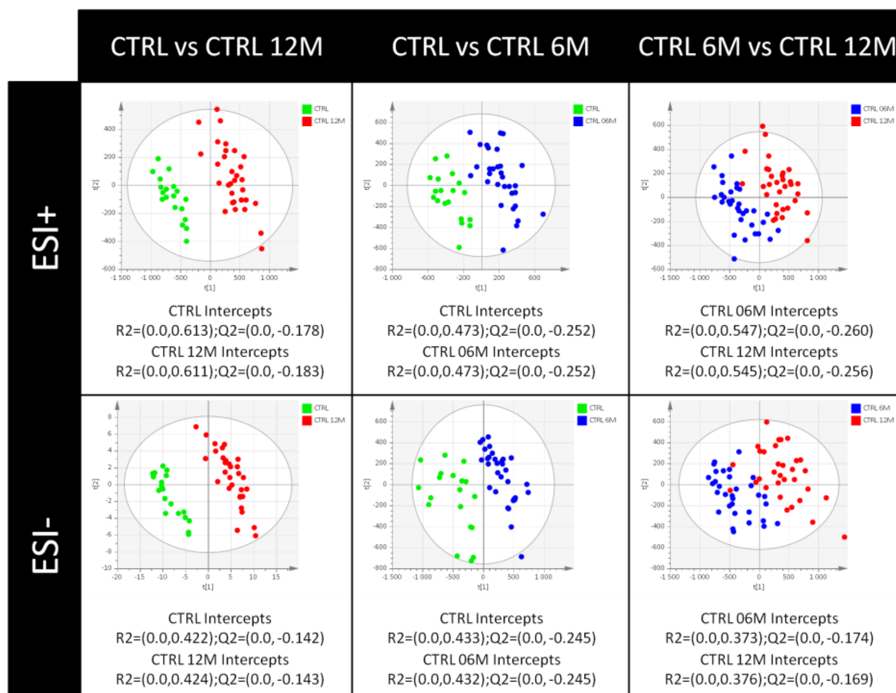
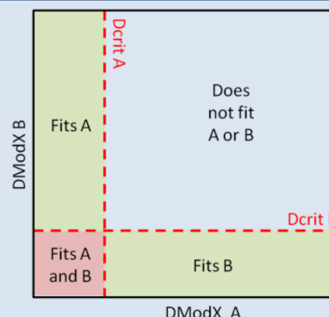


Figure 5.18 Supervised analysis of pairwise comparisons by PLS-DA. Score plots of the PLS-DA models and respective permutation test plots intercept values.

For the SIMCA analysis first PCA-Class models were built for the samples in the training set. A Cooman's plot (Box 5) was built plotting the class distances (DModX) for the CTRL and CTRL 12M groups against each other and representing samples from both training and test sets (Figure 5.19).

Box 5 Cooman's plot of SIMCA

In a Cooman's plot[19] class distances (DModX's) for two classes are plotted against each other in a scatter plot. Critical distance to the model (DCrit) lines delimitate four areas of diagnostic interest. Samples lying in the top-left or bottom-right areas fit one of the models but not the other. Samples in the bottom-left corner fit both models. In the top-right corner are found samples that do not fit any of the models.



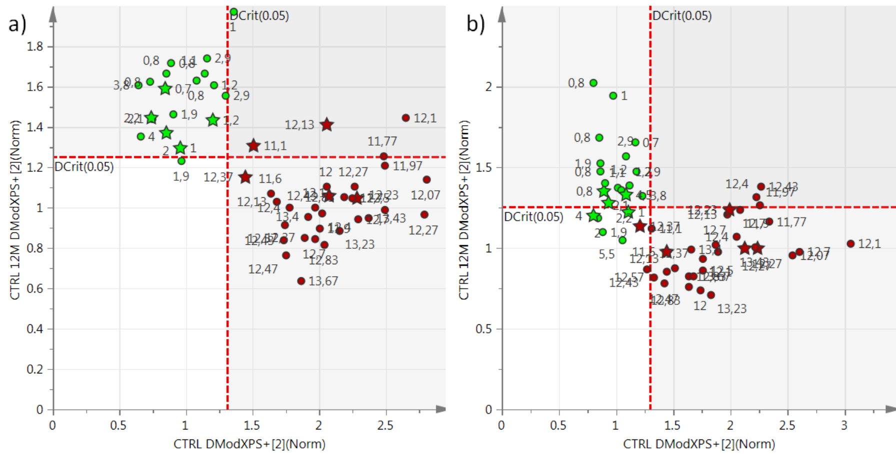


Figure 5.19 Cooman's plot of the CTRL and CTRL 12M groups in the SIMCA classification. In a) are represented the results from ESI+ and in b) the results from ESI-. Coloured in green are the samples from the CTRL group, and in red are the samples from CTRL 12M group. Samples belonging to the training set are represented as circles, while samples from the test set—whose class is predicted—are shaped as stars. Labels indicate the age, in months, of the subject. Dashed red lines indicate critical sample residual thresholds.

In ESI+ the class of all samples from the CTRL group was correctly predicted, while some of the samples from the CTRL 12M group was not classified in neither of the groups. In ESI- two samples from the CTRL group and one from the CTRL 12M group appeared in the zone shared by both groups.

SELECTION OF FEATURES OF INTEREST

OPLS-DA was used for the search of putative biomarkers. Features of interest were selected by visualization of S-plots, jack-knifed confidence interval from the loading plot and VIP value from the three pairwise comparisons. The results from the CTRL vs CTRL 12M comparison are shown in the Figure 5.20.

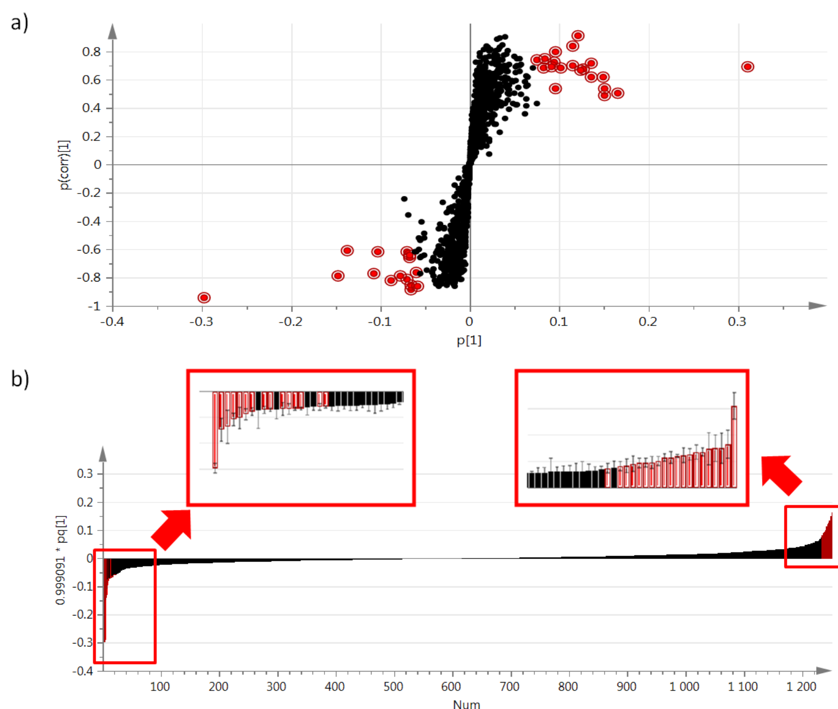


Figure 5.20 Selection of putative biomarkers. a) S-plot of the OPLS-DA models for the CTRL vs CTRL 12M comparison in ESI+, b) loading plot with jack-knifed confidence intervals. Coloured in red features selected for further identification.

Features of interest were selected based on their correlation and covariance values for further identification. The putative biomarkers obtained in each of the three pairwise comparisons in ESI+ are collected in the Table 5.5, indicating in which comparison they showed a significant effect in the separation.

Table 5.5 Selected features in ESI+. Features selected from the S-plots of the three pairwise comparisons as putative biomarkers and the comparison where they had significant effect in the separation.

ID number	m/z	t_R (min)	CTRL vs CTRL 12M	CTRL vs CTRL 6M	CTRL 6M vs CTRL 12M
39	84.9593	0.49	X	X	
104	105.0329	3.68	X	X	X
123	110.0080	0.49	X	X	
149	114.0655	0.59	X		X
177	118.0855	0.61	X	X	X
193	120.0801	2.18	X	X	X
197	121.0276	2.64	X	X	

Table 5.6 (Cont.) Selected features in ESI+. Features selected from the S-plots of the three pairwise comparisons as putative biomarkers and the comparison where they had significant effect in the separation.

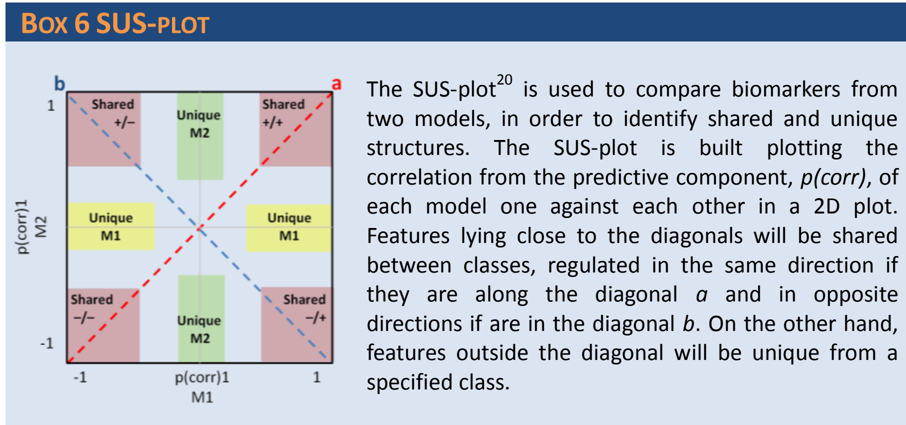
ID number	m/z	t_R (min)	CTRL vs CTRL 12M	CTRL vs CTRL 6M	CTRL 6M vs CTRL 12M
211	130.0491	5.40			X
228	125.9856	0.49	X	X	
253	129.0176	0.92	X	X	
266	130.0492	0.99	X	X	
267	130.0491	3.80	X	X	X
292	132.0756	0.62	X	X	X
322	137.0450	9.30		X	
328	137.0702	0.58	X		
340	139.0019	0.92	X		
420	146.9959	0.49	X	X	
423	147.0465	1.22		X	
427	147.0756	0.60	X		X
463	151.0383	3.18	X	X	X
491	153.0399	1.03		X	
497	153.0651	1.49	X	X	X
584	162.1118	5.86		X	
588	163.0594	0.62	X		
611	166.0844	2.18	X	X	X
635	169.0349	0.86	X	X	X
693	166.0844	3.59		X	
702	176.0698	4.32			X
734	180.0649	3.68	X	X	X
743	181.0601	3.27	X		X
757	182.0805	1.05	X		X
764	182.9618	0.49	X	X	X
793	185.1278	2.99	X	X	
805	188.0699	2.94	X	X	
817	190.0492	3.67			X
841	192.1047	3.59		X	
940	203.1496	5.95			X
960	205.0966	2.94	X	X	X
973	206.8853	5.41			X
1035	214.9170	0.49	X	X	
1139	226.1071	4.11		X	
1204	232.1538	2.30	X		X
1237	237.1228	2.82	X		X
1297	243.1334	4.11		X	X
1308	244.1541	3.05			X
1326	245.1492	4.53		X	
1344	246.1694	3.24	X		X
1350	247.1072	3.80	X		X
1351	247.1284	2.94	X		
1354	247.1285	2.13			X
1364	248.0915	3.80	X	X	X
1437	257.1490	4.82	X	X	
1472	261.1439	3.43		X	

Table 5.7 Selected features in ESI+. Features selected from the S-plots of the three pairwise comparisons as putative biomarkers and the comparison where they had significant effect in the separation.

ID number	m/z	t_R (min)	CTRL vs CTRL 12M	CTRL vs CTRL 6M	CTRL 6M vs CTRL 12M
1487	262.1645	1.47			X
1492	263.1023	4.06	X		
1514	264.1628	5.20		X	
1521	265.1182	3.80	X	X	X
1529	265.4730	3.80	X	X	X
1568	271.1646	5.30		X	
1746	288.2894	6.22	X		X
1747	288.2895	6.14	X	X	X
1760	290.1344	2.16		X	X
1766	290.8472	5.41			X
1837	300.2166	5.23		X	
1853	302.1595	2.43		X	
1858	302.2324	5.47	X	X	X
1877	304.1289	4.04	X		X
1881	304.1752	2.88		X	
1919	310.2010	5.27		X	
1920	310.2009	5.16		X	
1951	314.2193	5.62		X	
1969	316.3208	6.41	X		X
1981	318.1910	3.67		X	X
1990	319.1651	3.98			X
2018	325.1126	0.62	X		
2066	330.2273	5.17		X	
2099	334.1858	2.64		X	
2146	342.2273	4.70		X	
2166	344.8752	0.49	X	X	
2334	374.2532	5.38		X	
2363	381.0793	0.59	X		X
2728	474.1841	6.13		X	
2729	474.8333	4.93		X	
3137	638.0255	4.99		X	
3138	638.3598	4.99		X	
3145	642.2949	2.23		X	

From the total of 85 features selected, 17 of them were common in the three comparisons, 12 in the comparisons of CTRL with CTRL 6M and CTRL 12M, but not in the comparison of CTRL 6M and CTRL 12M, 12 only in the comparison of CTRL 12M with CTRL and CTRL 6M; and 3 only in the comparison of CTRL with CTRL 6M and CTRL 12M. Six features were exclusive from the CTRL vs CTRL 12M comparison, 25 from the CTRL vs CTRL 6M and 10 from the CTRL 6M vs CTRL 12M.

To study the common features between the different comparisons SUS-plots were also used (Box 6).



BOX 6 SUS-PLOT

The SUS-plot[20] is used to compare biomarkers from two models, in order to identify shared and unique structures. The SUS-plot is built plotting the correlation from the predictive component, *p(corr)*, of each model one against each other in a 2D plot. Features lying close to the diagonals will be shared between classes, regulated in the same direction if they are along the diagonal *a* and in opposite directions if are in the diagonal *b*. On the other hand, features outside the diagonal will be unique from a specified class.

These plots are commonly used in cases where a unique single group is compared with two other groups independent from each other. As an example, in a study where a control group is compared to two different treatments, biomarkers obtained from the comparison of each treatment with the controls may be shared between both treatments and therefore would appear in the diagonal of the SUS-plot, or may be unique for each treatment and appear outside the diagonals.

Although in the study here presented the groups are not independent, SUS-plots have demonstrated to be suitable to search for differences and similarities in the relevant features found in the ranges of age compared (Figure 5.21).

As an example, the feature with ID number 1521—with m/z = 265.1182 and t_R = 3.80—appears as a significant feature in all three comparisons, with high correlation and covariance in the S-plots, increasing its value with age, being a candidate of special interest. In the SUS-plots this feature appeared in the diagonal, in the area of shared structures.

ESI+ ESI-

Figure 5.21 Shared and unique structures. SUS-plots confronting the different pairwise comparisons against each other in both ESI+ and ESI-. Only the features selected from the S-plots are represented.

For ESI- relevant features were selected from the S-plots of the pairwise comparisons, and the obtained lists of features were compared (Table 5.6) to search for shared and particular features among comparisons.

Table 5.8 Selected features in ESI-. Features selected from the S-plot of the three pairwise comparisons as putative biomarkers and the groups were they appeared in.

ID number	m/z	t_R (min)	CTRL vs CTRL 12M	CTRL vs CTRL 6M	CTRL 6M vs CTRL 12M
100	124.0070	0.59	X		
343	167.0206	0.86	X		
391	172.9910	2.97	X	X	X
413	174.9556	0.49	X	X	
427	175.0607	3.69	X	X	
447	178.0504	3.68	X		X
450	178.3409	3.68	X		
526	187.0065	4.30	X		X
550	188.9857	2.57	X	X	X
581	191.0193	0.93	X	X	
612	194.0453	2.64	X	X	
738	204.9806	1.55	X		
761	206.9963	2.45	X		X
803	212.0017	3.18	X	X	X
939	222.9912	1.16	X		X
1049	232.0582	5.37	X	X	
1136	241.1188	4.11		X	
1217	246.0742	5.91	X		
1229	246.9912	2.91	X	X	
1329	255.1344	4.82		X	
1439	263.1032	3.80	X	X	X
1445	263.4559	3.80	X		X
1897	304.9133	0.49	X	X	
2302	343.0665	2.90	X	X	
2625	377.0852	0.62	X		X
2682	383.1526	5.58	X	X	
2683	383.1528	5.92	X	X	
2887	405.1761	5.43	X		X
2956	411.2379	6.14	X	X	
3078	427.1812	6.22		X	
3108	429.1964	5.91		X	
3148	434.8707	0.49	X	X	
3173	439.0792	0.62			X
3635	523.1430	0.65	X		X
3658	527.2146	3.80	X	X	X
3676	530.2788	5.82		X	
3744	541.2647	5.81		X	
3829	564.8295	0.48	X	X	
3892	585.1357	0.66	X		X
4078	632.2038	0.69	X		

A total of 40 features were selected in ESI-. Five of them were common for the three comparisons, 13 were selected from the comparisons of CTRL group vs CTRL 6M and CTRL 12M, and 9 from the comparisons of CTRL 12M vs CTRL and CTRL 6M. 6 features appeared only in the comparison of CTRL vs CTRL 12M, other 6 in the comparison of CTRL vs CTRL 6M, while only 1 feature was exclusive from the comparison of CTRL 6M vs CTRL 12M. To compare graphically the outcome of the different OPLS-DA models SUS-plots were used (Figure 5.21).

3.4 UNIVARIATE DATA ANALYSIS OF THE SELECTED FEATURES

Features selected as putative biomarkers were studied by univariate statistical analysis. First, those appearing in the three pairwise comparisons were examined.

Normalised concentrations of these features in the different samples were plotted against the age of the subjects excluding the samples detected as outliers by MVDA. Most of the selected features showed a positive correlation with age, although some of the features from ESI+ decreased with the age. One example for each situation is represented in the Figure 5.22.

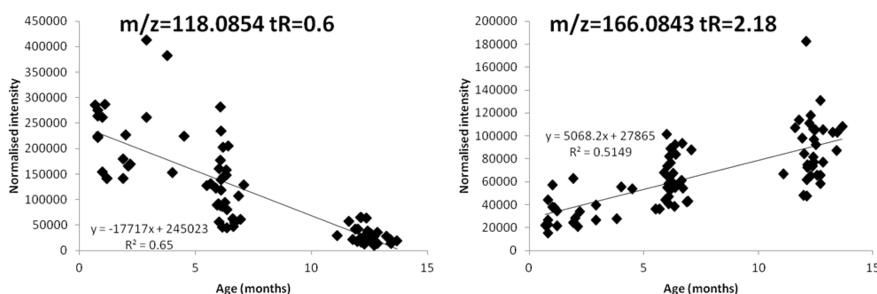


Figure 5.22 Correlation of the intensity with the age. Correlation plot of the normalised intensity of two of the features selected in ESI+ with the age.

To examine the correlation of each feature with the age Spearman and Kendall tests were performed with SPSS. The results are collected in the Table 5.9.

Table 5.9 Kendall and Spearman tests. Features with a significant correlation with the age according to the Kendall and Spearman test of correlation.

	ID number	m/z	t_R	Kendall's τ	Significance (2-tailed)	Spearman's ρ	Significance (2-tailed)
ESI+	104	105.0329	3.68	0.379**	0.000	0.538**	0.000
	177	118.0855	0.61	-0.673**	0.000	-0.870**	0.000
	193	120.0801	2.18	0.564**	0.000	0.755**	0.000
	267	130.0491	3.80	0.469**	0.000	0.656**	0.000
	292	132.0756	0.62	0.334**	0.000	0.508**	0.000
	463	151.0383	3.18	0.358**	0.000	0.556**	0.000
	497	153.0651	1.49	0.402**	0.000	0.594**	0.000
	611	166.0844	2.18	0.552**	0.000	0.747**	0.000
	635	169.0349	0.86	-0.119	0.114	-0.178	0.110
	734	180.0649	3.68	0.388**	0.000	0.553**	0.000
	764	182.9618	0.49	0.404**	0.000	0.562**	0.000
	960	205.0966	2.94	0.472**	0.000	0.644**	0.000
	1364	248.0915	3.80	0.470**	0.000	0.660**	0.000
	1521	265.1182	3.80	0.469**	0.000	0.660**	0.000
	1529	265.4730	3.80	0.473**	0.000	0.667**	0.000
	1747	288.2895	6.14	-0.377**	0.000	-0.608**	0.000
	1858	302.2324	5.47	0.360**	0.000	0.518**	0.000
ESI-	391	172.9910	2.97	0.566**	0.000	0.755**	0.000
	550	188.9857	2.57	0.494**	0.000	0.717**	0.000
	803	212.0017	3.18	0.344**	0.000	0.513**	0.000
	1439	263.1032	3.80	0.444**	0.000	0.625**	0.000
	3658	527.2146	3.80	0.451**	0.000	0.642**	0.000

** Correlation significant at the 0.01 level (2-tailed)

Only the feature with ID number 635 exhibited a non significant correlation with the age in neither the Kendall not the Spearman test. This feature was identified as uric acid.

3.5 METABOLITE IDENTIFICATION

From the list of features collected in the Table 5.9 and following the scheme represented in Figure 5.3 nine metabolites were identified. As an example the identification of the compound detected in ESI+ with ID number 1521 is explained.

3.5.1 IDENTIFICATION OF FEATURE WITH ID NUMBER 1521 IN ESI+

The feature with ID number 1521 appeared at 3.80 minutes, with an m/z of 265.1182. The chromatogram of the extracted peak is represented in Figure 5.23.

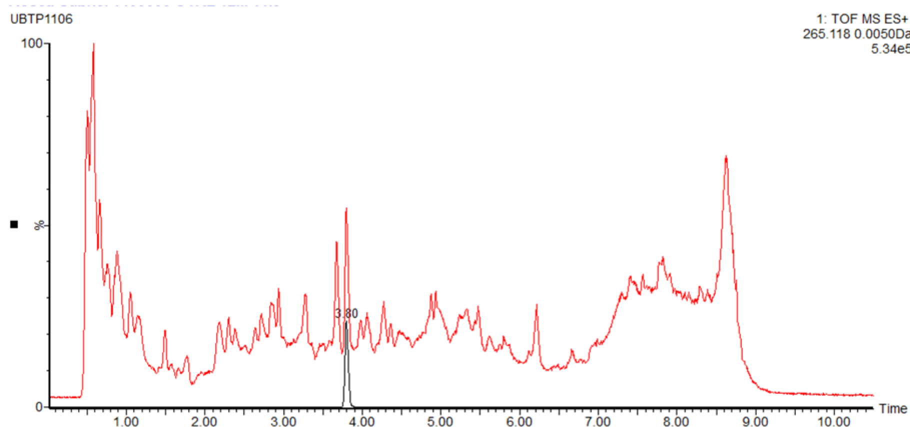


Figure 5.23 Chromatogram of the feature 1521. Line in red represents the TIC chromatogram, in black the chromatogram extracted for the m/z 265.1182.

From the list resulting from CAMERA, 40 features were grouped under the same peak as the selected feature, being the selected feature the most intense (Figure 5.24). Among them, fourteen features were identified as isotopologues and fifteen as different adducts, fragments, dimers or trimers, while the feature 1521 was identified as the $[M+H]^+$ ion of a 264.1110 mass.

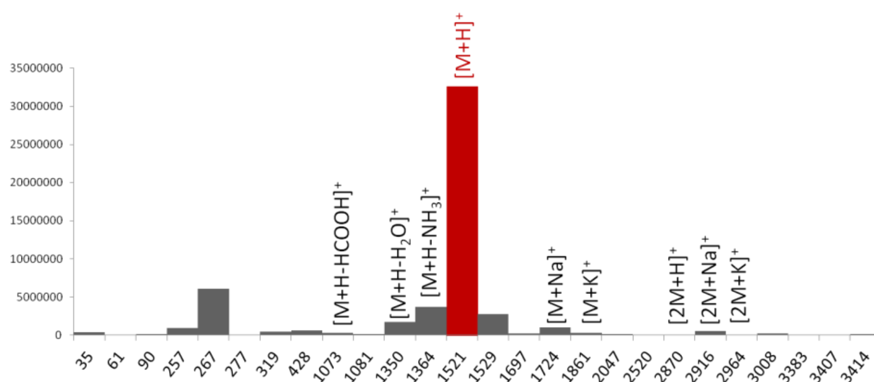


Figure 5.24 Features under the same CAMERA group. Sum of intensities of each feature in all the samples. Over the bars are indicated the type of ion identified by CAMERA. Marked in red is the feature 1521.

The search of this feature in METLIN database resulted in two hits (Table 5.10).

Table 5.10 METLIN search result. Molecules proposed by the METLIN database as possible identifications of the mass 265.1183.

METLIN ID	Mass	Δppm	Name	Structure
44788	[M+H]+ m/z 265.1183 M 264.1110	0	**Acetyl-N-formyl-5-methoxykynurenamine (AFMK)** Formula: $C_{13}H_{16}N_2O_4$ CAS: 52450-38-1	
58397	[M+H]+ m/z 265.1183 M 264.1110	0	**Alpha-N-Phenylacetyl-L-glutamine** Formula: $C_{13}H_{16}N_2O_4$ CAS: 28047-15-6	

The acetyl-N-formyl-5-methoxykynurenamine (AFMK), is a metabolite resulting from the oxydation of melatonin by myeloperoxidase, mainly found in cerebrospinal fluid[21] and plasma[22], but not in urine[23]. In addition, the MS fragmentation spectrum of this compound found in bibliography, presenting a main fragment of m/z 178.0857, did not correspond to the MS spectra obtained from our feature in the MS^E experiments. Therefore, this metabolite was rejected.

The alpha-N-phenylacetyl-L-glutamine is a common constituent of human urine, formed by the conjugation of phenylacetate and glutamine. Because of its structure, with groups amino and hydroxyl, both ESI+ and ESI- ionisations are feasible. The mass correspondent to a negative ionisation, 263.1037, was also found among the relevant features in ESI-, with the approximate same t_R, as the feature with ID number 1439.

As the spectra were not found in the online databases, those reported by Fukui *et al.*[24] were used to compare with the fragments obtained experimentally by MS^E (Figure 5.25). The spectra obtained experimentally were coincident with bibliographical results, obtaining correspondence in the main fragments, with m/z = 265, 248, 147, 130, and 84.

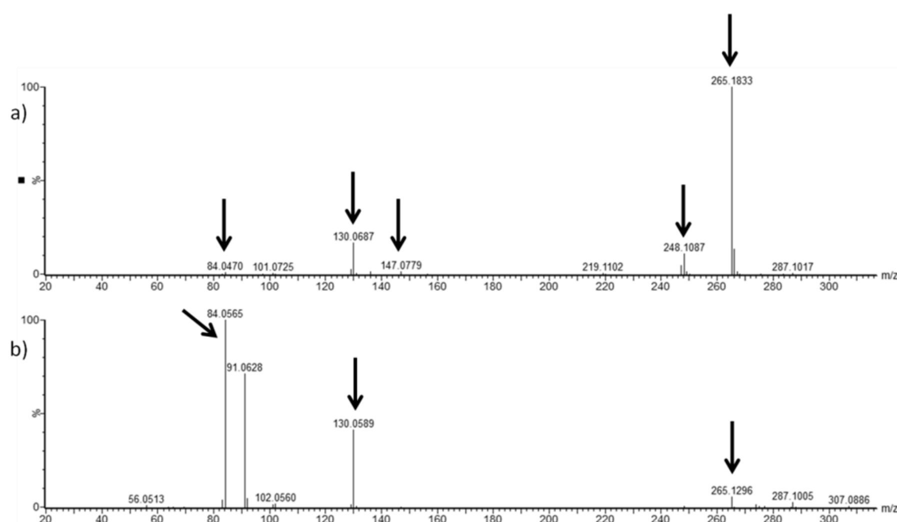


Figure 5.25 MS^E spectra in ESI+. Spectra obtained from the feature with ID number 1521, in a) low energy and b) high energy functions. Arrows indicate the fragments coincident with bibliographic results.

The m/z = 243 and 130 fragments were also detected as relevant features—ID numbers 267 and 1364—in the list of putative biomarkers, and were associated—along with the unidentified feature with ID number 1529—under the same group by CAMERA.

Also the spectra in ESI- coincide with the bibliography (Figure 5.26). Fragments with m/z 263, 145, and 127 obtained in the experiments in ESI- were in agreement with the results obtained by Fukui et al.[24] for the spectra of the N-phenylacetyl-L-glutamine.

The fragmentation proposed for this molecule in ESI+ and ESI- is represented in Figure 5.27.

Although no standard was available for the last step of the identification, the closeness of the experimental and bibliographical spectra allows to confidently assure the identification of the feature 1521 in ESI+ and 1439 in ESI- as N-phenylacetyl-L-glutamine, and features 267, 1364, and 1529 in ESI+ as fragments from this metabolite.

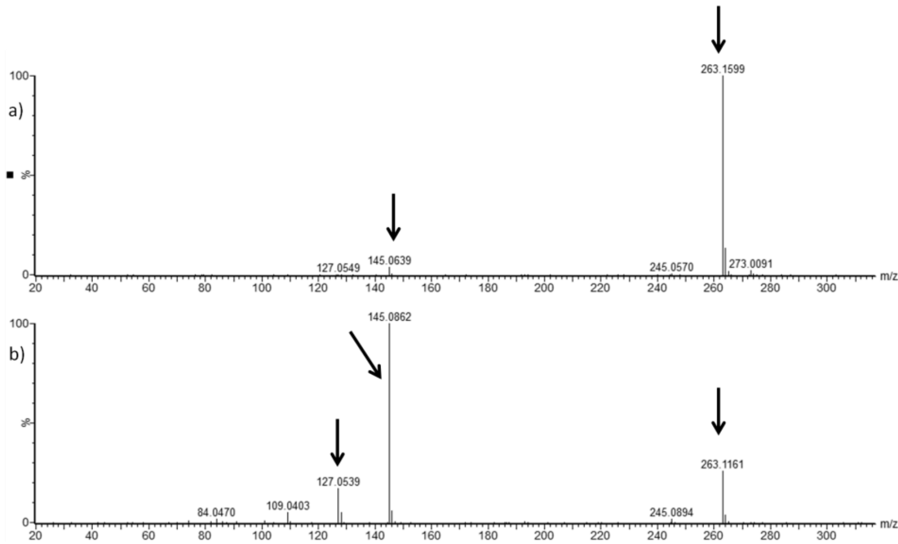


Figure 5.26 MS^E spectra in ESI-. Spectra obtained from the feature with ID number 1439 in a) low intensity and b) high intensity functions. Arrows indicate the fragments coincident with bibliographic results.

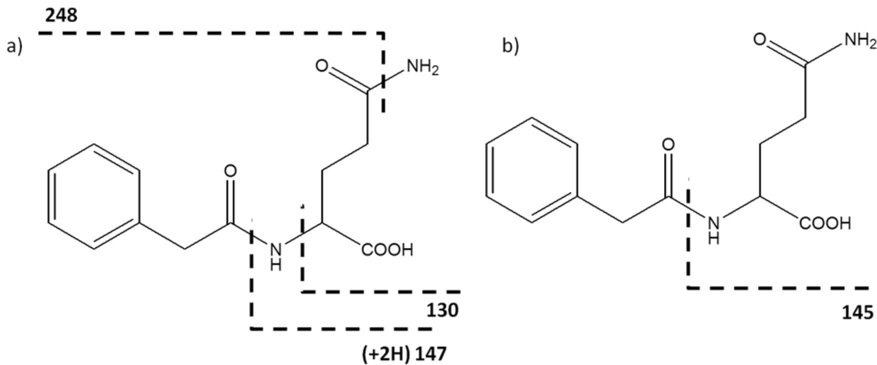


Figure 5.27 N-phenylacetyl-L-glutamine fragmentation. Proposed fragmentation of the molecule in a) ESI+ and b) ESI-.

3.5.2 Putative biomarkers

Similar process was followed for the identification of the rest of metabolites, and 9 metabolites were identified. More information regarding these metabolites is gathered in the Table 5.11.

Table 5.11 Metabolite identification. Relevant compounds identified as possible biomarkers of age.

Name	Formula	Monoisotopic Mass (Da)	t_R (min)	Adduct ion	Molecular weight (Da)	Error (ppm)	Correlation With age	Confirmed by spectra
Creatine	$C_4H_9N_3O_2$	131.0694	0.62	$[M+H]^+$	131.1332	8	↑	Y
N1-Methyl-2-Pyridone-5-carboxamide	$C_7H_8N_2O_2$	152.0586	1.49	$[M+H]^+$	152.1506	5	↑	Y
Phenylalanine	$C_9H_9NO_3$	179.0582	2.18	$[M+H]^+$	165.0790	11	↑	Y
Hippuric acid	$C_9H_9NO_3$	179.0582	3.68	$[M+H]^+$	179.1727	3	↑	Y
L-Tryptophan	$C_{11}H_{12}N_2O_2$	204.0899	2.94	$[M+H]^+$	204.2252	2	↑	Y
α-N-Phenylacetyl-L-glutamine	$C_{13}H_{16}N_2O_4$	264.1110	3.80	$[M+H]^+$ $[M+H]^-$	264.2771	0	↑	Y
C17-Sphinganine	$C_{17}H_{37}NO_2$	287.2824	6.14	$[M+H]^+$	287.4812	0	↑↓	N
2,6-dimethyl-heptanoyl carnitine	$C_{16}H_{31}NO_4$	301.2253	5.47	$[M+H]^+$	301.4216	0	↑	N
Indoxylsulfuric acid	$C_8H_7NO_4S$	213.0095	3.18	$[M+H]^-$	213.2100	0	↑	Y

Among those metabolites, only the hippuric acid was confirmed by comparison of MS^E experiments with a standard, as it was included in the Test Mix sample used for system stability monitorisation.

4. DISCUSSION

The results of this study have shown significant differences in the metabolic composition of urine between children with different chronological ages, younger than a year. MVDA of urinary LC-MS data indicated that levels of creatine (Cr), N1-methyl-2-pyridine-5-carboxamide (2PY), phenylalanine (Phe), hippuric acid, L-tryptophan (Trp), α-N-phenylacetyl-L-glutamine, 2,6-dimethyl-heptanoyl carnitine, and indoxylsulfuric acid increase with age, while C17-sphinganine levels increase from 0 to 6 months followed by a decrease when reaching the year of life.

Cr in mammals can be obtained from diet or synthesized *de novo* endogenously by a two-step mechanism involving L-arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT).[25] The first step of the synthesis, the formation of guanidinoacetate, greatly depends on the level of renal function.[26] The higher levels of Cr found in older children in this study is consistent with the results obtained by Gu et al. [27] In their research they have found a first increase of the Cr levels in the three first years of life of the subjects, followed by a decrease correlated with age. They attribute this decrease to the rise of conversion of creatine to creatinine in the muscle. Creatinine was also among the parameters most influencing the OPLS-DA models built for pairwise comparison of group CTRL vs CTRL 12M and CTRL 6M vs CTRL 12M, were a decrease in concentration has been seen with age.

Three of the identified metabolites belong to the tryptophan metabolic pathway. Trp is one of the essential amino acids absorbed exclusively from meals, and the precursor of nicotinamide. 2PY is major product of nicotinamide adenine dinucleotide (NAD) degradation, which is involved in numerous cellular redox reactions. It has been demonstrated that levels of

2PY in urine are affected in some disorders, being increased in burned or scalded children and cirrhotic patients, and decreased in patients with Parkinson. Changes in levels of 2PY have been also observed under varying physiological status, as diurnal variation, physical stress or cold exposure. Also, the indoxylsulfuric acid is a metabolite of Trp. It may be presumed that the increase in 2PY and indoxylsulfuric acid is a consequence of the more elevated levels of Trp in older children. This higher concentration of Trp in older children may be derived from changes in diet, as it is commonly at the age of 4-6 months that solid food is introduced on the diet.

Phe is another essential amino acid, precursor of Tyrosine, and the neurotransmitters catecholamines in the body, and one of its metabolites is the α-N-phenylacetyl-L-glutamine. A positive correlation has been observed between plasma levels of Phe and age in patients with phenylketonuria (PKU),[28] and levels of Phe have been linked with aging in urine of subjects ranging from 23 to 74 years old by Psihogios *et al.*;[29] however, no information has been found about the effect of age in urine levels of healthy children.

Similarly, Psihogios et al. reported a variation of hippurate with age for adults. However, Gu et al. concluded that this variation is less pronounced among the paediatric population. Levels of hippuric acid are increased with consumption of phenolic compounds—such as fruits—so the implication of the change of diet in the elevated concentration with age must be taken into account.

The acyl esters of L-carnitine, the acylcarnitines (AC), function as intermediates in fatty acid transport across mitochondrial membranes, facilitating the entry of long-chain fatty acid into the mitochondria. Cavedon et al.[30] claimed a decrease with age in the levels of AC in dried blood spot samples, however, this decrease was in many cases non significant. In the study here presented an increment of the levels of 2,6-dimethyl-heptanoyl carnitine with age has been observed.

Urinary research is especially useful for the evaluation of renal maturation process during the first year of life, as it is well known that several aspects of renal function vary considerably during this period, differing markedly from the equivalent values in the adult.[31] A few studies can be found associating renal development with physical parameters as glomerular filtration rate or urine flow rate. However, when studies are being performed about organ development in the first stages of life there are several factors to consider other than the maturation, as it has been said, development is a complex multifactorial process involving not only organ maturation but also other factors as e.g. physical growth or changes on diet. Hayton[32] developed a model to discriminate and characterize the influence of physical growth and maturation on several renal function parameters as glomerular filtration rate, active tubular secretion and renal plasma flow. They observed that when plotting these parameters against body weight the relationship appears linear for older children, but not for children below 2 years of age, in which renal maturation has a great influence on these parameters. Charlton et al. [33] found differences in protein urinary biomarkers between preterm infants and full-term infants, establishing the importance of taking into account gestational age when performing this kind of studies.

In the research here presented the correlation of the urine metabolic profile with age has been observed. However most of the metabolites selected as potential biomarkers from the pairwise group comparisons may be influenced by the change in diet characteristic at 4 to 6 months of age. Whether the metabolic differences are related to the development associated with age or are a consequence of the change of diet remains to be determined in further studies. Nevertheless, investigation of the metabolic pathways giving rise to these substances—e.g. by targeted analysis of the metabolites involved—could provide new clues to the metabolism changes involved in organ development, in order to examine differences between chronological and biological age of individual children.

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CONCLUSIONS

Pharmacokinetics in paediatrics

- High resolution liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) has demonstrated to be an efficient technique for the development of analytical methods applicable to pharmacokinetic (PK) studies in animal models of paediatric population.
- It was accomplished the determination of the analgesic drug Fentanyl in both cerebrospinal fluid (CSF) and plasma samples using small sample volumes—50 µL and 100 µL, respectively—with a simple and fast method, capable of detecting concentrations as low as 0.2 ng/mL, and therefore, allowing the monitorisation of the whole plasma profile of the drug in the subjects.
- The determination of Fentanyl in CSF and its relation with the plasma concentration provides insight into the CNS drug exposure or availability of centrally active compounds, thus serving as a reference for assessing the extent of delivery to the pharmacological targets within the CNS.
- The validation of the developed analytical method in bioanalysis is crucial to ensure the reliability of the obtained results. Whenever mass spectrometry is used, it is of special importance the assessment of the matrix effect.

Metabolomics in paediatrics

- Metabolomics has confirmed to be an excellent tool for the study of different grades of maturation in paediatric population, either animal or human.
- It is vital to take into consideration possible confounding factors as the regimen of anaesthesia, gender, or time of sampling among others when designing a metabolomic study.

- One of the great advantages of metabolomics is the possibility of reinterrogation of the data. This characteristic is especially useful to minimise the effect of the mentioned confounding factors.
- The use of animals as subjects of experimentation, if it cannot be avoided, should always be kept to the minimum possible. To do so, it is useful to establish a first pilot study with few subjects in order to determine the feasibility of a larger study and to improve its design by identifying the problems and confounding factors that affect the results.
- In the research here presented the correlation of the urine metabolic profile with age has been observed. MVDA of urinary LC-MS data indicated that levels of creatine (Cr), N1-methyl-2-pyridine-5-carboxamide (2PY), phenylalanine (Phe), hippuric acid, L-tryptophan (Trp), α-N-phenylacetyl-L-glutamine, 2,6-dimethyl-heptanoyl carnitine, and indoxylsulfuric acid increase with age, while C17-sphinganine levels increase from 0 to 6 months followed by a decrease when reaching the year of life.

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XCMS SCRIPTS

Your eyes can deceive you; don't trust them.

(Obi-Wan Kenobi, Star Wars IV: A New Hope)

CHAPTER 4. STUDY OF DIFFERENCES IN MATURATION GRADE OF PIGLETS

ESI+

```
> x<-xcmsSet(method="centWave",peakwidth=c(3,20),ppm=10 )

> g1x <-group(x)

> rtc1x <-retcor(g1x,family="symmetric",plottype="mdevden")

> g2x <-group(rtc1x,bw=10)

> fpx<-fillPeaks(g2x)

>diffreport(fpx ,"children","neonates","all_POS",metlin=0.15,100)

>xsax<-xsAnnotate(fpx)

>xsaFx<-groupFWHM(xsax)

>xsacx<-groupCorr(xsaFx)

>xsaFIx<-findIsotopes(xsacx)

>xsaFAx<-findAdducts(xsaFIx,polarity="positive")

>write.csv(getPeaklist(xsaFAx),file="POS_CAMERA.csv")
```

ESI-

```
>x<-xcmsSet(method="centWave",peakwidth=c(3,20),ppm=10)

>g1x<-group(x)

>rtc1x<-retcor(g1x,family="s",plottype="m")

>g2x<-group(rtc1x,bw=30)
```

```
>fpx<-fillPeaks(g2x)

>diffreport(fpx,"Neonates","Children","all_NEG",metlin=0.15,100)

>xsa<-xsAnnotate(fpx)

>xsaF<-groupFWHM(xsa)

>xsac<-groupCorr(xsaF)

>xsaFI-findIsotopes(xsac)

>xsaFA<-findAdducts(xsaFI, polarity="negative")

>write.csv(getPeaklist(xsaFA),file="NEG_CAMERA.csv")
```

CHAPTER 5. STUDY OF DIFFERENCES IN MATURATION GRADE OF CHILDREN

- **All samples (BRO+CTRL)**

ESI+

```
>x<-xcmsSet(method="centWave",ppm=10,peakwidth=c(3,20))

>g1x<-group(x)

>rtcx<-retcor(g1x,family="s",plottype="m",missing=10,extra=10)

>g2x<-group(rtcx,bw=2,mzwid=0.025,minfrac=0.5)

>fpx<-fillPeaks(g1x)

>diffreport(fpx,"CTRL 0","CTRL 06","POS_All",metlin=0.15,100)
```

```
>xsax<-xsAnnotate(fpx)

>xsaFx<-groupFWHM(xsax)

>xsacx<-groupCorr(xsaFx)

>xsaFIx<-findIsotopes(xsacx)

>xsaFAx<-findAdducts(xsaFIx,polarity="positive")

>write.csv(getPeaklist(xsaFAx),file="POS_AllBRO_CAMERA.csv")
```

ESI-

```
>x<-xcmsSet(method="centWave",ppm=10,peakwidth=c(3,20))

>g1x<-group(x)

>rtcx<-retcor(g1x,family="s",plottype="m",missing=10,extra=10)

>g2x<-group(rtcx,bw=2,mzwid=0.025,minfrac=0.5)

>fpx<-fillPeaks(g1x)

>diffreport(fpx,"CTRL 0","CTRL 06","NEG_All",metlin=0.15,100)

>xsax<-xsAnnotate(fpx)

>xsaFx<-groupFWHM(xsax)

>xsacx<-groupCorr(xsaFx)

>xsaFIx<-findIsotopes(xsacx)

>xsaFAx<-findAdducts(xsaFIx,polarity="negative")

>write.csv(getPeaklist(xsaFAx),file="NEG_AllBRO_CAMERA.csv")
```

- **CTRL Samples**

ESI+

```
>x<-xcmsSet(method="centWave",ppm=10,peakwidth=c(3,20))

>g1x<-group(x,mzwid=0.025)

>rtcx<-retcor(g1x,family="s",plottype="m",missing=5,extra=5)

>g2x<-group(rtcx,bw=2,mzwid=0.025)

>fpx<-fillPeaks(g2x)

>diffreport(fpx,"CTRL","CTRL 6M","POS_CTRL",metlin=0.15,100)

>xsax<-xsAnnotate(fpx)

>xsaFx<-groupFWHM(xsax)

>xsacx<-groupCorr(xsaFx)

>xsaFIx<-findIsotopes(xsacx)

>xsaFAx<-findAdducts(xsaFIx,polarity="positive")

>write.csv(getPeaklist(xsaFAx),file="POS_CTRL_CAMERA.csv")
```

ESI-

```
>x<-xcmsSet(method="centWave",ppm=10,peakwidth=c(3,20))

>g1x<-group(x,mzwid=0.025)

>rtcx<-retcor(g1x,family="s",plottype="m",missing=5,extra=5)

>g2x<-group(rtcx,bw=1,mzwid=0.025)

>fpx<-fillPeaks(g2x)
```

```
>diffreport(fpx,"CTRL 0","CTRL 06","NEG_CTRL",metlin=0.15,100)

>xsax<-xsAnnotate(fpx)

>xsaFx<-groupFWHM(xsax)

>xsacx<-groupCorr(xsaFx)

>xsaFIx<-findIsotopes(xsacx)

>xsaFAx<-findAdducts(xsaFIx,polarity="negative")

>write.csv(getPeaklist(xsaFAx),file="NEG_CTRL_CAMERA.csv")
```

PUBLISHED ARTICLES

En resolución, él se enfrascó tanto en su lectura, que se le pasaban las noches leyendo de claro en claro, y los días de turbio en turbio, y así, del poco dormir y del mucho leer, se le secó el cerebro, de manera que vino a perder el juicio.

(**Miguel de Cervantes Saavedra**, *Don Quijote*)

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RESEARCH PAPER

Evaluation of human plasma sample preparation protocols for untargeted metabolic profiles analyzed by UHPLC-ESI-TOF-MS

Estitxu Rico · Oskar González · María Encarnación Blanco · Rosa María Alonso

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Abstract Eight human plasma preparation protocols were evaluated for their suitability for metabolomic studies by ultra-high-performance liquid chromatography coupled with electrospray ionization time-of-flight mass spectrometry: organic solvent protein precipitation (PPT) with either methanol or acetonitrile in 2:1 and 3:1 (v/v) ratios with plasma; solid-phase extraction (SPE) using C_{18} or HybridSPE cartridges; and a combination of PPT and SPE C_{18} cartridges and microextraction by packed sorbent. A study design in which the order of injection of the samples was not randomized is presented. The analyses were conducted in a BEH C_{18} column (1.7 μm, 2.1 mm×100 mm) using a linear gradient from 100 % water to 100 % methanol, both with 0.1 % formic acid, in 21 min. The most reproducible protocol considering both the univariate and the multivariate analysis results was PPT with acetonitrile in a 2:1 (v/v) ratio with plasma, offering a mean coefficient of variation of the area of all the detected features of 0.15 and one of the best clusterings in the principal component analysis plots. On the other hand, the highest number of extracted features was achieved using methanol in a 2:1 (v/v) ratio with plasma as the PPT solvent, closely followed by the same protocol with acetonitrile in a 2:1 (v/v) ratio with plasma, which offered only 1.2 % fewer repeatable features. In terms of concentration of remaining protein, protocols based on PPT with acetonitrile provided cleaner extracts than protocols based on PPT with methanol. Finally, pairwise comparison showed that the use of PPT- and SPE-based protocols offers a different coverage of the metabolome.

Keywords Metabolomics · Plasma · Sample treatment · Reproducibility · Liquid chromatography–mass spectrometry · HybridSPE

E. Rico (✉) · O. González · M. E. Blanco · R. M. Alonso
Analytical Chemistry Department, Faculty of Science and Technology, University of Basque Country/EHU, P.O. Box 644, 48080 Bilbao, Spain
e-mail: estitxu.rico@ehu.es

O. González
Analytical Bioscience Division, LACDR, Leiden University, Einsteinsweg 55, Leiden 2333 CC, Netherlands

Introduction

Metabolomics consists of the untargeted analysis of low molecular weight metabolites (less than 1 kDa) in biological samples [1–4]. The principal aim of this omic science is to provide insight into the metabolic status of complex living systems. Comparison of the metabolic profiles from different phenotypes can be used to identify specific metabolic changes, leading to the understanding of physiology, toxicology, and disease progression [5, 6].

The more widespread analytical platforms in metabolomics are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with liquid chromatography (LC) or gas chromatography (GC) [7]. Although NMR spectroscopy is more robust, repeatable, and needs a simpler sample treatment, which is ideal for high-throughput analysis, this technique has an important drawback that is overcome by MS: its low sensitivity. Historically, most metabolomic studies were done using GC coupled with MS, but the limitations related to the molecular weight and type of metabolites that can be analyzed and the extensive preparation required led to the emergence of LC coupled with MS as a metabolite profiling tool. When MS is coupled with ultra-high performance LC (UHPLC), it is able to analyze simultaneously thousands of metabolites, requiring a small sample volume for analysis. Nonetheless, MS does not provide as much structural information as NMR spectroscopy, and with MS it is more difficult to accomplish the identification of biomarkers. As the

aforementioned techniques have different strengths and drawbacks, the ideal case would be to analyze the same sample set with a combination of them [7, 8].

In all fields of analytical chemistry it is vital to use a repeatable sample preparation protocol in order to minimize the differences between samples due to the analytical process. This is especially important when human samples are analyzed because the influence of diet, environmental effects, and genetics-related factors causes high interindividual variability itself. In a metabolomic study, two different approaches can be used for the comparison of different sample treatment protocols: univariate and multivariate analysis. With use of a traditional statistical approach, the median and mean coefficient of variation (CV) of the intensities of all detected features [mass-to-charge ratio (m/z) and retention time (RT) pairs] can be calculated among the replicates of each of the different protocols. It is also possible to know the percentage of features that have a CV lower than a certain value among the replicates—for example, 0.3, which is considered the maximum acceptable value for the total error for targeted LC–MS analysis [9]—and the percentage of features present in all replicates or in a certain number of replicates. The comparison of these values between protocols gives an overview of the differences in repeatability. However, it is important to bear in mind that metabolomic data sets are complex matrices composed of thousands of features that should be analyzed also using a multivariate approach to combine multiple features and enhance the statistical comparison of methods. Principal component analysis (PCA) is the most suitable analysis for this purpose. One way of analyzing the repeatability in PCA is by calculating the intrareplicate distance within the model—the Euclidean distance (ED)—a value that gives a comparison of relative repeatability among the replicates.

As in metabolomics the general aim is to study as many metabolites as possible, the best sample preparation would be the one in which the sample is modified as little as possible (e.g., a simple dilution is the usual protocol for urine metabolomic studies [10]). Previous works have reported the study of sample treatment of matrices such as liver [11, 12], plasma [13–18], cells or microbes [19–24], and feces [25] by different analytical techniques. In the case of plasma samples, direct injection is not possible without the rapid degradation of the columns and loss of sensitivity caused by the gradual buildup of nonvolatile compounds in the cone of the ionization source. For these reasons and because of the important ion suppression caused by endogenous compounds in LC–MS, it is crucial to apply a cleanup step before the analysis. This is usually achieved by protein precipitation (PPT) using an organic solvent, methanol (MeOH) or acetonitrile (ACN) being most often used. An alternative approach that does not involve necessarily PPT and thus allows the study of only non-strongly protein bound metabolites is solid-phase extraction (SPE). A combination of organic solvent PPT and SPE can be used with the aims of releasing metabolites bound to proteins and prolonging column life. These SPE approaches remove substances that are prone to being strongly retained on the column if the same stationary phase is used. Because one of the major challenges in metabolomics is the analysis of a great number of plasma samples, the removal of these substances could be very helpful in order to keep the ionization source clean and lengthen the column life. However, to our knowledge, SPE has been applied in only one metabolomic study of plasma samples analyzed by LC–MS [17]. Finally, another cleanup option is offered by phospholipid-removal cartridges such as HybridSPE cartridges. The packed-bed filter/frit assembly of these cartridges acts as a depth filter for the concurrent physical removal of precipitated proteins and chemical removal of phospholipids, whereas small molecules pass through unretained, giving, as a result, a cleaner sample in which ion suppression caused by phospholipids is eliminated [26].

It is important to bear in mind the final purpose of a study. This means that the protocol that provides the highest number of metabolites is not always the most suitable. For instance, if the interest resides in nonpolar compounds that are not phospholipids, the use of HybridSPE cartridges could help to reduce the matrix effect in that chromatographic area. In contrast, if we are interested in the lipid region, the use of a solvent such as 2-propanol or dichloromethane during the extraction would be a better option.

In this study, eight human plasma preparation protocols were evaluated for their suitability for UHPLC–electrospray ionization (ESI) time-of-flight (TOF) MS based untargeted metabolomics: four of them based on organic solvent PPT with either ACN or MeOH in 2:1 and 3:1 (v/v) ratios with plasma; two approaches in which SPE C_{18} cartridges were used, with and without a previous PPT step; and HybridSPE cartridges and microextraction by packed sorbent (MEPS). To our knowledge, this is the first time that the effectiveness and repeatability of HybridSPE and MEPS protocols have been evaluated in human plasma metabolomics. Other plasma sample preparation protocols, as lowering pH with acid or protein denaturation using heat, were not considered from the beginning because according to Want et al. [15] the number of extracted features obtained with these protocols is considerably low. The aim of this work was, therefore, to study these different human plasma preparation protocols by means of univariate and multivariate analyses in order to select a reliable and repeatable sample preparation protocol for the untargeted metabolic analysis of human plasma that offers a high number of extracted features. Other parameters, as the number of extracted features, the reproducibility of a series of selected features, and the amount of protein remaining in each reconstituted plasma extract, were also studied.

Materials and methods

Reagents and materials

The solvents for LC–MS and sample preparation (MeOH and ACN) were OPTIMA® LC–MS grade and were obtained from Fisher Scientific (Los Angeles, CA, USA). Formic acid (FA) and sodium formate, both LC–MS grade, leucine enkephalin (high-performance LC grade), bovine serum albumin solution, and Bradford reagent were from Sigma-Aldrich (St Louis, MO, USA). Purified water from a Millipore (Milford, MA, USA) Milli-Q Element A10 water system was used. For UHPLC–ESI-TOF-MS analysis, 96-well plates were from Waters (Milford, MA, USA), well plate cap mats were purchased from VWR International (Leicestershire, UK), HybridSPE precipitation cartridges were purchased from Sigma-Aldrich (St Louis, MO, USA), and Strata-X SPE polymeric cartridges were from Phenomenex (Torrance, CA, USA). For MEPS, an eVol® XR handheld automated analytical syringe coupled to a 500 μL MEPS syringe that contains an MEPS C_{18} cartridge, all purchased from SGE Analytical Science (Melbourne, Australia), was used.

Plasma samples

Blood samples from ten healthy volunteers were collected in EDTA tubes. Written informed consent was obtained from all the volunteers. Plasma samples were prepared by centrifugation in a 5804 R centrifuge (Eppendorf, Hamburg, Germany) at 1,200*g* at 4 °C for 10 min. Pooled samples were prepared by mixing equal amounts of the ten plasma samples in one vessel. This plasma pool was divided into 10 mL fractions, which were used to evaluate the different sample preparation protocols. All the samples were stored at -20 °C until analysis.

Plasma sample preparation

Frozen plasma was thawed on ice. Eight sample preparation protocols were compared (Fig. 1), each using six preparation replicates and three instrumental replicates. All the sample extracts at the end of the treatment contained the same proportion of plasma to reconstitution solvent (50:50 v/v MeOH:H_2O).

Organic solvent PPT

This type of sample preparation consisted of PPT with either cold MeOH or cold ACN. The starting volume of plasma was 250 μL. Two different ratios of organic solvent to plasma were tested: 2:1 and 3:1 (v/v). After brief vortexing, plasma samples were centrifuged at 13,000*g* for 5 min. Then 600 μL and 800 μL of the supernatant were taken for the 2:1 and 3:1 (v/v) ratios, respectively, and they were dried under a stream of N_2 at 40 °C in a TurboVap evaporator (Zymark, Barcelona, Spain). Finally, the dried extracts were reconstituted in 200 μL of 50:50 (v/v) MeOH:H_2O.

SPE procedures

Two SPE approaches were studied: one included a PPT step prior to the extraction and the other included a simple dilution of the plasma sample with H_2O. The PPT was done with MeOH in a 3:1 (v/v) ratio with plasma, as detailed in the previous section, but instead of evaporating the supernatant to dryness, when the volume was less than 200 μL, we added 800 μL of H_2O. In the case of the dilution with H_2O, 800 μL of H_2O was added to 200 μL of plasma. SPE cartridges were previously activated with 1 mL of MeOH and then further conditioned with 1 mL of H_2O. Diluted samples were loaded into the cartridge and cleaned with 1 mL of H_2O. Subsequently, 1 mL of MeOH was used as the elution solvent. Finally, the eluate was dried under a N_2 stream at 40 °C and reconstituted in 200 μL of 50:50 (v/v) MeOH:H_2O.

Microextraction by packed sorbent

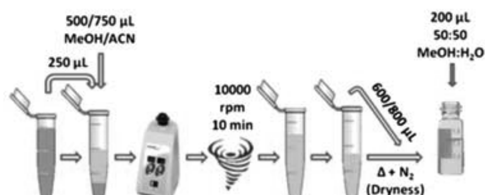
First, an MEPS C_{18} cartridge was activated with 100 μL of MeOH and then further conditioned with 1 mL of H_2O. Then, 100 μL of plasma diluted with 400 μL of H_2O was loaded into the cartridge. After the sample had been cleaned with 100 μL of H_2O, 500 μL of MeOH was used for elution. The guide provided by the manufacturer (SGE Analytical Science, Melbourne, Australia) was followed for this protocol. Finally, the eluate was dried under a N_2 stream at 40 °C and reconstituted in 100 μL of 50:50 (v/v) MeOH:H_2O.

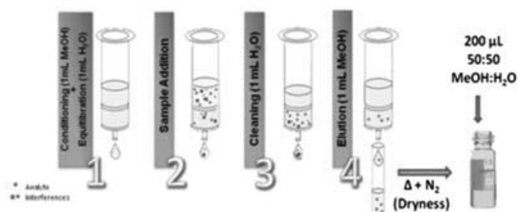
HybridSPE procedure

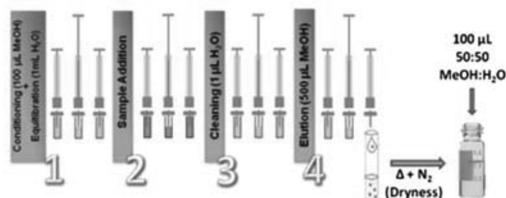
The protocol performed was the one recommended by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). Firstly, a PPT step was performed by the addition of 750 μL of cold 1 % FA in ACN to 250 μL of plasma. After brief vortexing, the mixture was centrifuged at 1,600*g* for 3 min. Then, 800 μL of the supernatant was loaded into the HybridSPE cartridge and a vacuum was applied. At this point the eluate is ready for immediate LC–MS/MS analysis, but in order to make this method comparable with the other protocols, it was dried under a N_2 stream at 40 °C and reconstituted in 200 μL of 50:50 (v/v) MeOH:H_2O.

UHPLC–ESI-TOF-MS analysis

Analysis was performed using an Acquity UPLC system coupled to an ESI quadrupole TOF Synapt-G2 mass







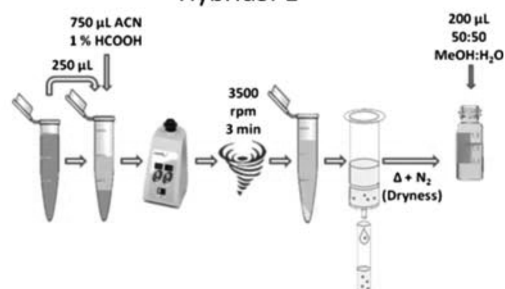


Fig. 1 Sample treatment protocols evaluated. *ACN* acetonitrile, *MeOH* methanol

spectrometer (Waters, Milford, MA, USA) operated in positive ESI mode. Chromatography was done at 40 °C on a Waters Acquity BEH C_{18} column (1.7 μm, 2.1 mm× 100 mm) with the following solvent system: solvent A 0.1 % FA in H_2O, and solvent B 0.1 % FA in MeOH. A linear gradient was used at a flow rate of 0.5 mL/min from 100 % solvent A to 100 % solvent B in 21 min. Then 100 % solvent B was held for 2 min, after this time the initial conditions were reached, and finally, a 3-min reequilibration step was included. The injection volume was 5 μL. The ESI conditions were a source temperature of 120 °C, a desolvation temperature of 450 °C, a cone gas flow rate of 10 L/h, a desolvation gas flow rate of 900 L/h, a capillary voltage of 0.7 kV, and a cone voltage of 30 V. The TOF detector worked in resolution mode (approximately 2,000 full width at half maximum), and all mass spectral data were acquired in centroid mode by scanning an *m*/*z* range of 50–1,200 with a scan time of 0.1 s and an interscan delay time of 0.02 s. The quadrupole was not used in this study. Leucine enkephalin ($[M + H]^+$=556.2771 *m*/*z*) (2 ng/μL in 0.1 % FA in 50:50 v/v ACN–H_2O) was used as a lock mass, and was introduced straight into the mass spectrometer at a flow rate of 10 μL/min. The instrument was calibrated before the analysis using a 0.5 mM sodium formate solution (calibration error less than 1 ppm). All UHPLC–ESI-TOF-MS operations were run under the control of MassLynx 4.1 (Waters, Milford, MA, USA).

Design of the analysis

A blank, consisting of 50:50 (v/v) MeOH:H_2O solution, was included at the beginning and at the end of the run in order to test for any possible contamination or carryover effect. A quality control (QC) sample, prepared by combining equal aliquots of the replicates from each method, was injected regularly every eight injections throughout the run to monitor the sensitivity and the stability of the UHPLC–ESI-TOF-MS platform. This QC sample was also used to condition the system at the beginning of the analysis. It was observed in a previous test that at least 15 injections of a sample containing the matrix studied were necessary to stabilize the system, and that even after the analysis of a low number of plasma samples the sensitivity fell (data not shown). For this reason and because of the purpose of the study (evaluating the repeatability of each of the sample preparation protocols), the order of injection of the samples was not randomized to minimize the effect of the instrumental drift arising from column degradation or contamination of the MS source on the evaluation of repeatability within each protocol. The analysis order is shown in Table 1. The preparation replicates of each protocol were injected one after the other. Instrumental replicates of one of the preparation replicates were injected between them (replicates 1_1, 1_2, and 1_3). Although this way of analyzing the samples is the most convenient to study the repeatability, it is not the optimum to set the number of extracted features,

Table 1 Order of sample injection

Order of injection of the different protocols	Injection schedule within each protocol
15 QC conditioning	QC
MEPS	Replicate 1_1
SPE	Replicate 2
HybridSPE	Replicate 3
SPE + PPT	Replicate 1_2
MeOH 2:1	Replicate 4
ACN 2:1	Replicate 5
MeOH 3:1	Replicate 6
ACN 3:1	Replicate 1_3
	QC

ACN 2:1 acetonitrile in a 2:1 (v/v) ratio with plasma, *ACN 3:1* acetonitrile in a 3:1 (v/v) ratio with plasma, *MEPS* microextraction by packed sorbent, *MeOH 2:1* methanol in a 2:1 (v/v) ratio with plasma, *MeOH 3:1* methanol in a 3:1 (v/v) ratio with plasma, *PPT* protein precipitation, *QC* quality control, *SPE* solid-phase extraction

taking into consideration the drop of sensitivity throughout the run. This problem was overcome by analyzing in a different batch three replicates of each protocol randomly (data not shown).

Data processing

The raw spectrometric data acquired were processed using XCMS [27] (version 1.30.3) in order to convert the three-dimensional LC–MS raw data (RT, *m*/*z*, intensity) into a table of time-aligned detected features, with their RT, *m*/*z*, and intensity in each sample. XCMS is written in the R statistical programming language and is freely available under an open-source license. The version of R used was 2.14.1. The samples were grouped according to the different sample preparation protocols. Blanks and QC samples were treated as separate groups. The CentWave algorithm [28] was used for peak picking with a peak width window of 3-20 s—peakwidth= c(3,20)—and a maximum tolerated *m*/*z* deviation in consecutive scans for a peak of 15 ppm (ppm=15). The *m*/*z* width for the grouping was set to 0.015 Da (mzwid=0.015), and the bandwidth parameter chosen was 5 s (bw=5) for the first grouping and was then determined from the time deviation profile after RT correction. These values are the ones commonly used in UHPLC systems coupled to a high-resolution mass spectrometer, and after studying the raw data and the results obtained, we decided that they were appropriate for the analysis performed. The rest of the parameters were set to the default values. Finally, the missed peaks during the peak picking algorithm were integrated automatically with the fillPeak function. This step is essential for reducing the observed CV values, as many zero values are replaced with real peak intensities. To avoid LC–MS artifacts (those peaks that do not represent molecular ions of metabolites such as isotopes, common adducts and fragments, multiple charge states, etc.), R-package CAMERA was used to filter the detected peaks. The peak marker tables (comprising *m*/*z*–RT pairs and their corresponding intensities for each sample) generated were exported into Microsoft Office Excel 2007 (Microsoft, Redmond, WA, USA) for univariate analysis and into SIMCA-P+ 11.5 (Umetrics, Umeå, Sweden) for multivariate analysis. Data outside the interval 0.5–21 min—that is, up to the elution peak and from the point at which the column washing step of the analysis started—were discarded.

Evaluation of method repeatability

To obtain the most in-depth information on sample preparation repeatability, the data were analyzed using two different approaches: univariate and multivariate statistics. Furthermore, other parameters calculated directly from the resulting table from XCMS, as the total number of features and the CV of the area of some selected features for each protocol, can be used to study the repeatability of each protocol.

Multivariate statistics

PCA was performed on all data after logarithmic transformation—10log(peak area)—and mean centering. Scores plots were examined to assess the degree of similarity between the different protocols and to identify outliers or trends in the data. The ED within the PCA data was calculated for the intrareplicate distance within the model [29] for each protocol taking into account the first three principal components. These EDs give a comparison of relative repeatability among the replicates, which can be difficult to observe in the three-dimensional PCA scores plots obtained. The lower the value, the better is the repeatability it indicates.

Univariate statistics

Univariate statistics were used to compare the CV distribution of feature intensity among the replicates for each protocol. Another way of comparing the CV distribution is by calculating the percentage (or number) of features which have a CV lower than a certain value. In this work, the number of features with a CV lower than 0.15, 0.20, and 0.30 were calculated. The two first values are the CV values considered to represent an acceptable degree of repeatability according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use [30] for bioanalytical methods for targeted analysis in any concentration (0.15), except for the one corresponding to the limit of quantitation (0.20), and 0.30 is the acceptable CV in biomarkers analysis [17].

Other parameters

To select the most suitable sample preparation protocol for metabolic profiling, there are some other parameters that should also be considered: the total number of features obtained and the area deviation of some selected features for each protocol. As the behavior of each detected feature depends on its own chemical structure and the RT at which it is eluted from the chromatographic column, no internal labeled standards are commonly used in metabolomics. For this reason the area deviation offered by each protocol was assessed by studying six features with different RTs and *m/z* present in all the replicates of all the protocols.

Protein concentration estimation

The Bradford assay was used to estimate the amount of protein remaining in three replicates of each reconstituted plasma preparation protocol. Samples were first diluted 1:2 (v/v) with distilled H_2O and then mixed with Bradford reagent in a 1:20 (v/v) ratio of sample to Bradford reagent. A calibration curve was generated using known concentrations (from 0.78 to 100 mg/L) of a standard protein (bovine serum albumin).

Results

The eight sample preparation protocols (each performed on six preparation replicates and three instrumental replicates) were compared, resulting in a set of 90 injections, 64 plasma samples plus 24 QC samples (QC and QC conditioning), and two blanks. Different criteria were used to evaluate the protocols: repeatability by multivariate and univariate analyses, number of extracted features, repeatability of a series of selected features, and the amount of protein remaining in each reconstituted plasma extract.

Feature distribution

The base peak intensity chromatograms obtained with all the sample preparation protocols show the feature distribution with chromatographic time (Fig. 2). In general, until 16 min, all of them, except for the HybridSPE and MEPS protocols, had similar profiles with different intensities and different number of visible features, which were especially higher for an ACN-to-plasma ratio of 2:1 and an MeOH-to-plasma ratio of 2:1. From this time on, in the cases of the HybridSPE, MEPS, direct SPE, and SPE plus PPT protocols, fewer chromatographic peaks were detected in comparison with the other protocols, and again an ACN-to-plasma ratio of 2:1 and an MeOH-to-plasma ratio of 2:1 provided the highest peak intensities and the greatest number of features. Taking into account that the last few minutes correspond to the lipophilic region, we conclude that solvent precipitation extracts had more lipophilic material than those of other protocols and that the removal of a great part of compounds of this kind was achieved when any cartridge was used for sample preparation.

Evaluation of method repeatability using multivariate statistics

The repeatability of the chromatographic method used was assessed in the PCA. A clear tendency of the QC samples was observed as the instrument sensitivity falls (44 % of signal drop from the first QC sample to the last one, data not shown). The explained variation (R^2X) was 70.5, 79.9, and 86.7 for the first, second, and third principal components, respectively, and the predicted variation (Q^2X) for these components was 70.2, 79.5, and 86.1, respectively. When the model was built without including the QC samples in the data processing step (Fig. 3), in order to avoid their effect in the PCA, a large increase in R^2X and Q^2X was observed for the first three components (91.6, 96.0, and 97.9 and 88.5, 92.6, and 95.8, respectively). All the protocols were well separated from each other, and the results agreed with the differences observed in the base peak intensity chromatograms. This can be observed in the distribution of the sample treatment protocols along the first principal component.

To give a number to the relative repeatability of the protocols shown by the PCA scores plot, the EDs generated from the first three components were calculated. In Fig. 4, the EDs represent the mean of the distances among the replicates of a particular protocol.

The protocol involving an ACN-to-plasma ratio of 2:1 yielded the lowest mean ED. When this value was statistically compared (95 % confidence level) with the EDs of the other sample preparation protocols, significant differences were obtained in all cases, except for SPE plus PPT.

Evaluation of method repeatability using univariate statistics

Box plots were used to visualize the distribution of feature intensity CV values among the replicates (Fig. 5). Each box shows the degree of dispersion of CV values of features for one condition by displaying the 25th percentile (bottom of the box), the median, the mean, and the 75th percentile (top of the box). According to this plot, the protocol based on solvent precipitation with ACN in a 2:1 ratio with plasma, showing a median and a mean CV of 0.12 and 0.15, respectively, was the most repeatable protocol. An analysis of variance confirmed that the observed differences between this protocol and the other protocols were significant.

Another way of visualizing the distribution of feature intensity CV values is by calculating the number of extracted

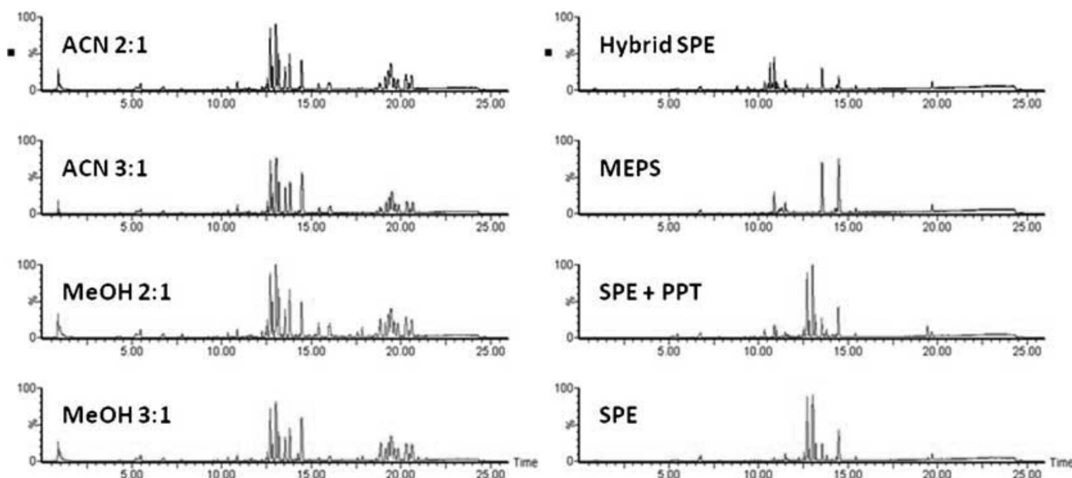


Fig. 2 Raw base peak intensity ultra-high-performance liquid chromatography (UHPLC)–electrospray ionization-time-of-flight mass spectrometry chromatograms of a pooled plasma sample treated with the eight preparation protocols—ACN in a 2:1 (v/v) ratio with plasma (*ACN 2:1*), ACN in a 3:1 (v/v) ratio with plasma (*ACN 3:1*), MeOH in a 2:1 (v/v) ratio with plasma (*MeOH 2:1*), MeOH in a 3:1 (v/v) ratio with plasma (*MeOH 3:1*), HybridSPE, microextraction by packed sorbent (*MEPS*), solid phase extraction (*SPE*) and protein precipitation (*PPT*), and direct SPE—injected through a BEH C_{18} UHPLC column (2.1 mm×100 mm)

features for each method with an intensity CV lower than 0.15, 0.20, and 0.30. These data are shown in Fig. 6. In terms of the number of consistently detected features, again the protocol with a 2:1 ACN-to-plasma ratio seemed to be the most repeatable protocol, with 93 % of the features having a CV below 0.30. It is important to bear in mind that the values of the total number of features indicated in this section are not real because they are affected by the fall of sensitivity during the analysis.

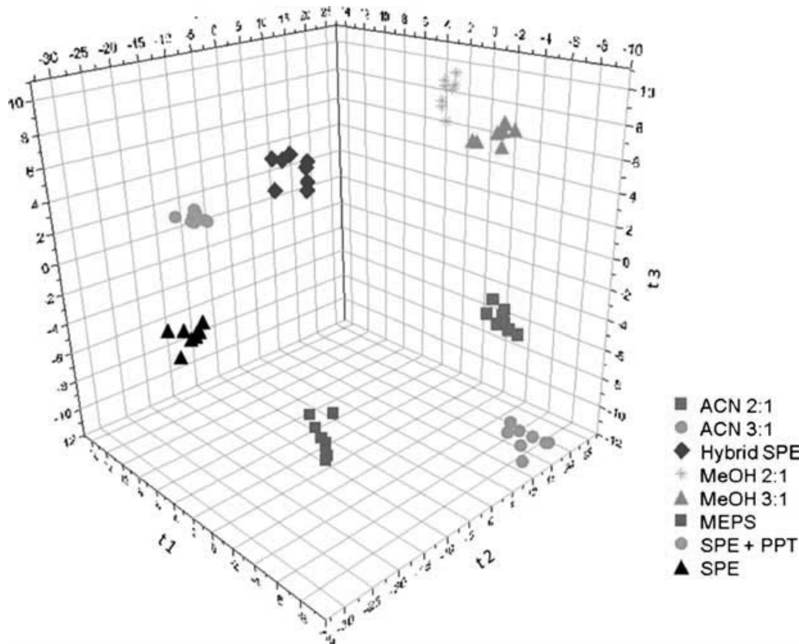


Fig. 3 Three-dimensional principal component analysis scores plot for all the plasma preparation protocols after removal of liquid chromatography–mass spectrometry artifacts and quality control samples

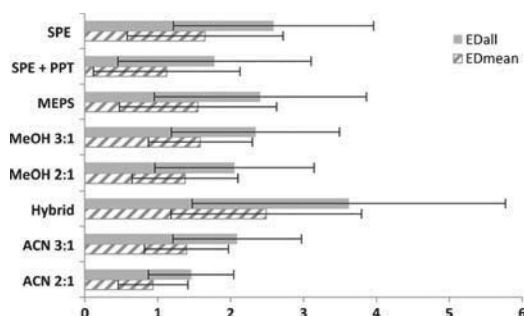


Fig. 4 Calculated Euclidean distances (*ED*) (expressed as mean±confidence interval (95 % confidence level)

Number of extracted features

The number of extracted features from XCMS for each protocol was also considered in the selection of the optimal conditions. As mentioned in "Design of the analysis," this value was calculated by injecting in a different batch only three replicates from each protocol randomly, so the drop in sensitivity during a long run does not lead to an underestimation of the number of features of the samples analyzed at the end of the batch (data not shown). The order obtained, in terms of the number of extracted features, did not differ from the one observed in the designed run. As Table 2 shows, the maximum number of detected features was achieved with PPT with MeOH in a 2:1 ratio with plasma as the protocol, closely followed by PPT with ACN in a 2:1 ratio with plasma. The best feature extraction efficiency of MeOH in serum samples was reported by Want et al. [15], and the best feature extraction efficiency of MeOH in plasma samples was reported by Bruce et al. [16]. A large decrease in the number of extracted features was observed for the HybridSPE, MEPS, and direct SPE protocols, which makes sense if one takes into account that the former includes a mechanism for the removal of phospholipids and in protocols based on C_{18} cartridges without a previous PPT step protein-bound compounds are not studied, whereas protocols including a PPT step result in a drastic alteration of the three-dimensional structure of proteins that allows the release of metabolites bound to them.

The number of common repeatable features between methods was calculated. Pairwise comparisons of the different plasma preparation protocols showing the number of common features and the percentage, taking as 100 % the total detected features in the protocol, and the total detected features in each case are indicated in Table 2. A high percentage of common features (more than 76 %) can be seen for the different proportions of organic solvent to plasma used, with the percentage being highest in the case of ACN in a 3:1 ratio with plasma (87 %). On the other hand, it is observed that with use of SPE protocols it is possible to detect features that are not observed in PPT protocols, meaning that both types of protocol are complementary.

Repeatability of selected metabolites

Analytical and sample preparation repeatability was further investigated using selected features. For this purpose six features with different RTs and *m*/*z* values present in all the replicates of all the protocols were chosen. Table 3 lists these features and their measured *m*/*z* value, RT, and intensity CV among the replicates for each protocol.

All the protocols provided a mean CV below 0.2, with the SPE plus PPT protocol yielding the best results in terms of repeatability, offering a mean CV of 0.12 among preparation replicates and 0.04 when only instrumental replicates are taken into account.

Protein concentration

The concentration of residual protein was estimated for each plasma preparation protocol using the Bradford assay. According to the results obtained by Bruce et al. [16], ACN has better protein removal efficiency than MeOH in plasma samples. As can be observed in Table 4, our results agree with these observations, with both PPT protocols based on PPT with MeOH being the ones that resulted in the largest amount

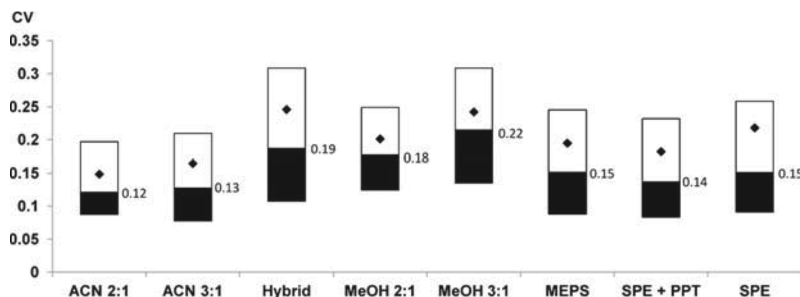


Fig. 5 Box plots comparing the distribution of feature intensity coefficients of variation (*CV*) among replicates for the different sample preparation protocols. For each *box*, the *bottom* corresponds to the 25th percentile, the *middle band* corresponds to the median (numerical value indicated), the *diamond* corresponds to the mean, and the *top* corresponds to the 75th percentile

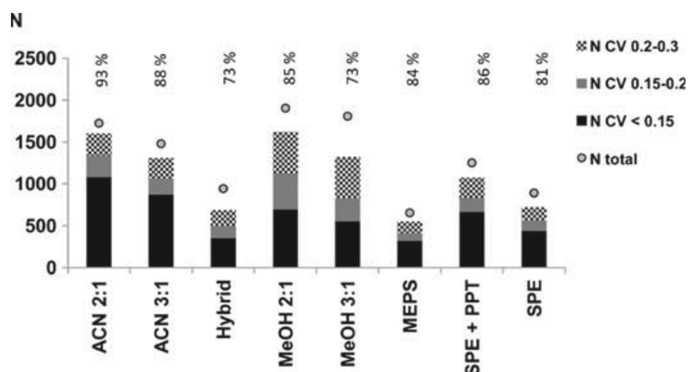


Fig. 6 Distribution of repeatable features for each protocol. The *numbers above the columns* express the percentage of features with a CV<0.30 of the total number of detected features

of residual protein. The efficiency of SPE cartridges in terms of protein removal was not reported in any of the aforementioned studies, but from these results, the use of these cartridges helps to get a cleaner extract compared with a simple PPT with MeOH, as was expected. It is worthwhile remarking that in the case of the SPE plus PPT protocol, MeOH was used as the organic solvent for PPT and, therefore, maybe if ACN had been used as the PPT solvent, this combination would have resulted in the lowest remaining protein concentration.

Discussion

Many reported approaches for plasma sample preparation have focused on a small subset of metabolites [14, 16–18]. As our aim was to perform untargeted metabolic profiling of plasma samples encompassing a wide range of chemical structures, it was also essential to study the repeatability of the different plasma preparation protocols used for all the detected features, as reported by Want et al. [15]. For this reason the efficiency and repeatability of the selected plasma preparation protocols were investigated using univariate and multivariate analyses, and other parameters, as feature distribution, number of extracted features, the repeatability of some selected features, and the amount of protein remaining in the reconstituted samples, were studied. All of these criteria should be taken into account when selecting a sample preparation protocol for metabolic profiling studies.

To our knowledge, the use of SPE cartridges in metabolomics has been evaluated in only one work [17], but the authors did not study sample preparation repeatability. Our work is the first in which the efficiency of the SPE plus PPT, MEPS, and HybridSPE protocols in metabolomics has been evaluated.

Before starting to analyze the different plasma preparation protocols studied, we point out that an appropriate study design is essential to fulfill the desired aim. The LC–MS method applied entails a drop of sensitivity during a single run (44 %), and this needs to be taken into consideration, otherwise the results would not be reliable at all. The proposed design to solve this problem is to inject the samples, instead of randomly, classified by protocols. In this way, the highest drop of sensitivity within a protocol is only 7 % (calculated from the instrumental replicates), and the distance from the first

Table 2 Pairwise comparison showing the number of common features and the percentage, taking as 100 % the total detected features in the protocol, and the total detected features in each case

	ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE	Total
SPE	592 (67)	533 (60)	494 (56)	566 (64)	496 (58)	423 (64)	730 (82)		890
SPE + PPT	796 (64)	719 (58)	545 (58)	846 (68)	732 (59)	436 (66)			1,250
MEPS	351 (54)	324 (49)	479 (73)	353 (54)	298 (45)				656
MeOH 3:1	1201 (70)	1109 (75)	347 (37)	1366 (76)					1,808
MeOH 2:1	1289 (75)	1158 (78)	429 (45)						1,905
HybridSPE	420 (45)	363 (38)							943
ACN 3:1	1287 (87)								1,480
Total	1,725	1,480	943	1,905	1,808	656	1,250	890	

Table 3 Repeatability of six features with different *m/z* and retention times (*RT*) present in all the replicates of each sample preparation protocol

Feature	*m/z*	RT (s)	CV							
			ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE
M227T46	226.952	45.8	0.06	0.15	0.09	0.11	0.10	0.27	0.04	0.22
M195T406	195.088	405.6	0.15	0.23	0.23	0.20	0.03	0.10	0.21	0.11
M314T587	314.233	587.0	0.11	0.06	0.29	0.16	0.13	0.14	0.08	0.19
M460T652	460.270	652.3	0.21	0.18	0.12	0.22	0.57	0.10	0.18	0.48
M637T772	637.306	772.3	0.27	0.24	0.25	0.18	0.20	0.11	0.15	0.10
M804T862	803.543	862.3	0.05	0.07	0.03	0.09	0.13	0.06	0.06	0.12
Mean CV			0.14	0.15	0.17	0.16	0.19	0.13	0.12	0.20
Mean CV of instrumental replicates			0.11	0.16	0.05	0.18	0.11	0.07	0.04	0.17

CV coefficient of variation

replicate to the last one is constant for all the protocols. Another point to bear in mind related to the drop of sensitivity is its effect on the number of detected features. To overcome this, a different analysis in which only three preparation replicates of each protocol are analyzed randomly is performed.

After the evaluation of the different criteria applied, we found that PPT with ACN in a 2:1 (v/v) ratio with plasma produced the best results for most of the parameters studied. This protocol offered one of the lowest EDs between replicates in multivariate analysis, the lowest CV values in univariate analysis, good repeatability of the selected features, and one of the lowest concentrations of remaining protein in the final extract. Furthermore, although PPT with ACN in a 2:1 ratio with plasma offered 9.5 % fewer total detected features than PPT with MeOH in a 2:1 ratio with plasma, the difference in terms of repeatable features (CV<0.30) was only 1.2 %. The satisfactory results in terms of repeatability could be explained by the combination of the simplicity of the sample preparation protocol itself (the same for all the PPT protocols) and the high efficiency of ACN as a PPT agent (the HybridSPE protocol also included a PPT with ACN step). The better results achieved by PPT with ACN when compared with MeOH in terms of repeatability could be explained by the fact that the extracts obtained with PPT using ACN as an organic solvent exhibited lower ionization suppression than the ones obtained with MeOH [31]. On the other hand, in the comparison of PPT with ACN with the HybridSPE protocol it must be taken into account that the HybridSPE protocol includes an additional step that could be the cause of the observed differences in repeatability. The lower number of extracted features of the protocols that include retention mechanisms was probably due, on the one hand, in the case of direct SPE and MEPS, to the fact that metabolites bound to proteins are not released and, on the other hand, when HybridSPE cartridges were used, to the removal of phospholipids. Finally, according to the Bradford assay, the extracts obtained using ACN as an agent for PPT were cleaner than the ones obtained with MeOH, which were the extracts that contained the highest concentration of remaining proteins. This agrees with the results reported by Bruce et al. [16], and thus, MeOH should not be an option when a long column life is desired.

Although Michopoulos et al. [17] observed that SPE offers good repeatability, our results do not totally agree when SPE is compared with PPT protocols. A reason for this difference could be that they did not include preparation replicates and that they focused on only a small subset of metabolites. Another difference between their study and ours is that all our protocols were performed manually, but they used an automatized SPE station, which could significantly improve

Table 4 Absorbance measurements and concentrations of residual protein (expressed as mean±confidence interval; 95 % confidence level) estimated for each plasma preparation protocol using the Bradford assay

Bradford assay	ACN 2:1	ACN 3:1	HybridSPE	MeOH 2:1	MeOH 3:1	MEPS	SPE + PPT	SPE
Measure 1	0.625	0.598	0.586	1.619	1.559	0.635	0.680	0.735
Measure 2	0.603	0.599	0.605	1.614	1.49	0.590	0.734	0.777
Measure 3	0.622	0.589	0.596	1.664	1.499	0.622	0.695	0.658
Mean	0.617	0.595	0.596	1.632	1.516	0.635	0.703	0.723
Concentration (mg/L)	2.5±0.4	1.9±0.2	2.0±0.4	26±1	24±2	3±1	4±1	5±3

repeatability. Want et al. [15] stated that ACN was the organic solvent which provided less repeatability in terms of univariate analysis in serum samples. The reason for the difference between their results and ours cannot be easily established, but could be because serum and plasma are similar, but different matrices. Another explanation could be that they injected the sample preparation replicates randomly, and therefore, sample treatments with replicates further from each other show a higher CV owing to the decay in sensitivity.

The MEPS and HybridSPE protocols did not result in the expected good repeatability, but maybe the automatization of the processes and/or the optimization of the parameters that affect the extractions could significantly improve the results.

In terms of complementarity it is important to mention that pairwise comparison showed that many of the detected features in protocols that included an SPE mechanism were not detected when only PPT protocols were used. These findings mean that if both types of protocols are combined, a higher coverage of the metabolome could be obtained.

Finally, this work shows an approach to compare the results obtained from different sample preparation protocols for untargeted metabolomics that takes into consideration many different parameters that affect directly the results. This strategy could be applied to any other matrix and/or sample preparation protocols.

Conclusions

We have reported an appropriate design of a study for the evaluation of different human plasma sample preparation protocols for use in a metabolomic study. After the evaluation of the eight plasma preparation protocols studied (based on organic solvent precipitation or three different cartridges or a combination of both), we can conclude that a simple PPT with ACN in a 2:1 (v/v) ratio with plasma is the protocol that globally better satisfies all the requirements established: the best results in terms of univariate and multivariate repeatability, a high number of extracted features, and good removal of proteins from the sample, which is very relevant when a great number of samples is to be analyzed. On the other hand, the use of PPT- and cartridge-based extraction protocols offers a different coverage of the metabolome.

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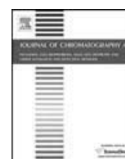

Review

Bioanalytical chromatographic method validation according to current regulations, with a special focus on the non-well defined parameters limit of quantification, robustness and matrix effect

Oskar González[a,b], María Encarnación Blanco[a], Gorka Iriarte[c], Luis Bartolomé[d], Miren Itxaso Maguregui[a], Rosa M. Alonso[a,*]

[a] Analytical Chemistry Department, Science and Technology Faculty, the Basque Country University/EHU, P.O. Box 644, Bilbao, Basque Country 48080, Spain
[b] Analytical Bioscience Division, LACDR, Leiden University Einsteinsweg 55, CC Leiden 2333, Netherlands
[c] Laboratory of Public Health of Alava, (Public Health and Addictions Directorate, Basque Government), Santiago 11, Vitoria-Gasteiz 01002, Basque Country
[d] Central Analytical Service (SGIker), Science and Technology Faculty, the Basque Country University/EHU, P.O. Box 644, Bilbao, Basque Country 48080 Spain

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ABSTRACT

Method validation is a mandatory step in bioanalysis, to evaluate the ability of developed methods in providing reliable results for their routine application. Even if some organisations have developed guidelines to define the different parameters to be included in method validation (FDA, EMA); there are still some ambiguous concepts in validation criteria and methodology that need to be clarified. The methodology to calculate fundamental parameters such as the limit of quantification has been defined in several ways without reaching a harmonised definition, which can lead to very different values depending on the applied criterion. Other parameters such as robustness or ruggedness are usually omitted and when defined there is not an established approach to evaluate them. Especially significant is the case of the matrix effect evaluation which is one of the most critical points to be studied in LC-MS methods but has been traditionally overlooked. Due to the increasing importance of bioanalysis this scenario is no longer acceptable and harmonised criteria involving all the concerned parties should be arisen. The objective of this review is thus to discuss and highlight several essential aspects of method validation, focused in bioanalysis. The overall validation process including common validation parameters (selectivity, linearity range, precision, accuracy, stability. . .) will be reviewed. Furthermore, the most controversial parameters (limit of quantification, robustness and matrix effect) will be carefully studied and the definitions and methodology proposed by the different regulatory bodies will be compared. This review aims to clarify the methodology to be followed in bioanalytical method validation, facilitating this time consuming step.

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* Corresponding author. Tel.: +34 94 601 2686; fax: +34 94 601 35 00.
E-mail address: rosamaria.alonso@ehu.es (R.M. Alonso).

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1. Introduction

Method validation is a necessary process to demonstrate that an analytical method is suitable for its intended use, thus, that it can offer accurate, precise and reproducible results. These reliable results are essential for bioavailability, bioequivalence, pharmacokinetic, pharmacodynamics or toxicological studies where analytes must be quantified in biological matrices such as urine or plasma. Consequently, method validation is a crucial step in bioanalysis and essential for laboratories to adhere to current Good Manufacturing Processes (GMP), Good Laboratory Practices (GLP) or International Organisation for Standardisation (ISO) regulations, such as ISO17025 [1] and ISO15189 [2]. Nowadays several regulatory bodies deal with bioanalytical validation and even though there are still some divergences, a relative consensus has been reached by the scientific community. Nevertheless, the continuous advances in instrumentation and the emergence of more demanding analytical challenges make bioanalysis a field in permanent evolution. Therefore, method validation guidelines should keep up with this progression in order to cope with real requirements, and to this end the participation of all the concerned parties (pharmaceutical industry, statisticians, analytical chemists, academicians. . .) is imperative.

The first attempt to harmonise bioanalytical validation dates back to 1990, when the United States Food and Drug Administration (FDA) and the American Association of Pharmaceutical Scientists (AAPS) sponsored the first bioanalytical method validation workshop in Crystal City (Arlington, VA). The aim of this workshop was to reach a consensus on the requirements in validation for analytical methods focused on bioavailability, bioequivalence and pharmacokinetic studies. Parameters considered essential were set (stability, accuracy, precision, sensitivity, specificity, response and reproducibility) and the outcome of this workshop was well received by the scientific community eager of a harmonised policy to work with. In consequence, the report of this workshop published in 1992 [3] became the basis of nowadays bioanalytical validation and the starting point for a stimulating discussion. Soon, due to the importance of method validation and the great advances made in this field during the following years, the need for an official document became apparent, and finally, in 1999 the FDA issued the *"Draft Guidance for Industry: Bioanalytical Method Validation"* [4]. Shortly after, the FDA and AAPS organised the second Crystal City workshop in order to discuss the draft and give the scientific community a chance of sharing the experience accumulated over the 10 years elapse since the first workshop took place. New topics such as partial, full and cross-validation were discussed; stability experiments were studied more in depth and flourishing hyphenated mass spectrometry techniques (MS) were addressed. The summary of this workshop was published in 2000 [5] and it became the backbone for the official *"Guidance for Industry: Bioanalytical method validation"* issued in 2001 [6].

This document can be considered the cornerstone of bioanalytical validation since many laboratories standardised their validation procedures following this guideline and the later proposals for new guidelines used it as reference. Nevertheless, analytical chemistry and drug analysis are in an unceasing development and therefore new worries and problems are continuously emerging. This is so that after the publication of the guidance two new Crystal City meetings were organised in 2006 and 2008 and their respective white papers published in order to complete the initial guideline [7,8]. The first one dealt with some questions that remained unresolved such as metabolite and stability studies, carryover and especially, matrix effect (ME) [9]. In this workshop incurred sample reanalysis (ISR) was discussed for the first time [10] but it was not until 2008 workshop that this topic was carefully studied. The white papers arising from these workshops are widely accepted by the scientific community despite not being official documents. Nevertheless and after more than 10 years the FDA needed to release an update of the validation guideline. Therefore, the draft of the guideline update was published in September 2013 [11]. The AAPS called the scientific community for a new workshop held in Baltimore in December 2013 where the FDA draft was carefully studied. As expected, a vivid discussion about hot topics such as validation of biomarkers, endogenous compounds or anticoagulant change took place. Once the outcome of this conference is considered, the final guide will be released.

Besides FDA other regulatory agencies and organisations have dealt with bioanalytical method validation. In Europe, this field was to some extent covered by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) "*Validation of Analytical Procedures Q2 (R1)*" [12], developed between 1994 and 1996 by experts on method validation from European Union, Japan and USA. Nevertheless, this guideline is addressed to identification tests, control of impurities or active ingredient quantification. Obviously the European Union needed an official document to establish the regulation for bioanalytical validation and consequently, in December 2008 European Medicines Agency (EMA) released the "*Concept paper/recommendations on the need for a guideline on the validation of bioanalytical methods*" [13]. Immediately the European Bioanalysis Forum (EBF), an organisation comprised of bioanalytical scientists working within the pharmaceutical industry, expressed their concern about the possibility of contradiction between this new guideline and the world-widely recognised FDA guidance [14].

When the draft guidance was released in November 2009 [15] a lively discussion arose again and several meetings were organised in order to achieve a more harmonised document [16–20]. In this framework the Global Bioanalysis Consortium (GBC) was founded with the idea of merging all the existing and emerging bioanalytical guidances in a unified consensus document [19]. The GBC is a worldwide organisation of representatives of scientific associations with a well-coordinated organisation involving different harmonisation teams that are connected to the Steering Committee via the Scientific Leadership team [21]. Besides all the aforementioned there are other organisations which deal with bioanalytical validation, either organising frequent workshops and conferences such as the Canadian Calibration and Validation group (CVG) [22–27] and the Global CRO Council (GCC) [28] or developing domestic guidelines such as Brazilian ANVISA (*Agência Nacional de Vigilância Sanitária*) [29], Japanese MHLW (*Ministry of Health, Labour and Welfare*) [30] or Spanish AEFI (*Spanish Association of the Industry Pharmaceutics*) [31].

The numerous meetings organised yearly around bioanalytical validation evidence the importance of the topic as well as the lack of consensus in some important issues which need to be further discussed and agreed. This review aims to summarise the different approaches around validation parameters in a clarifying way, providing clear information about the whole validation process but with special focus in the most controversial validation parameters: robustness, lower limit of quantification (LLOQ) and matrix effect, since according to our experience, these are the most ambiguous or overlooked parameters.

To our knowledge this is the first work that includes relevant aspects from the worldwide recognised FDA, EMA and ICH official guidelines plus ANVISA, MHLW and AEFI domestic guides. In addition it includes first comments on FDA guideline update draft.

2. Bioanalytical method validation

As aforementioned, method validation is the process through which the reliability of a method to quantify an analyte in a certain matrix is demonstrated. Depending on the previous degree of development of the method to be validated this process is more or less exhaustive leading to three different types of method validation: full, partial and cross-validation.

When a method is implemented for the first time or a new drug or metabolite is included a *full validation* is mandatory. According to FDA, a full validation should study all the fundamental parameters including accuracy, precision, selectivity, calibration curve, sensitivity, reproducibility and stability. In addition, EMA adds matrix effect to these fundamental parameters. In general, this kind of validation is necessary for each matrix and species under study and it should be carried out in the same matrix and using the same anticoagulant (if the matrix should be blood/plasma) as the study samples. Only an alternative matrix can be used if justified, as is the case of the rare matrices.

Usually, before a full validation is carried out a pre-validation process is necessary in order to characterise some parameters. The most important step during pre-validation is the definition of the lower and upper limits of quantification (quantification range) and the response function to be used during the validation. Additionally the number of calibration standards needed to build the calibration curve, recovery (REC) and selectivity can be evaluated [32,33].

Sometimes minor changes are made to a previously validated method such as transfer between laboratories, change in concentration range, change in sample processing procedure, change in storage conditions.. .. In these cases a full validation may not be necessary and a *partial validation* may be enough. According to the guidelines, the validation process to be carried out during partial validation can range from as little as the determination of the within-run precision and accuracy, to an almost full validation. The absence of an established protocol means that the validation process will rely on the criterion of the analyst.

Some of the minor changes that lead to a partial validation can be controversial. For example, even if EMA states that *“generally a full validation should be performed for each species and matrix concerned”* partial validation may be enough for a change in animal species or matrix. Anyway, when an extrapolation between animal species is carried out species-specific metabolism should be taken into consideration and when a change in matrix within species is performed it must not affect the outcome of the study [20]. There is still a lack of consensus regarding the anticoagulant change (for example from heparin to EDTA), even if it is clear that at least a partial validation must be carried out there is not full agreement regarding the use of the same anticoagulant with different counter-ion (for example sodium heparin and lithium heparin) [7,24]. A survey carried out among 15 EBF companies showed that a change in counter-ion has not impact on assay performance and therefore they concluded that plasma samples containing same anticoagulant with different counter-ion should be regarded as equal matrices [34,35]. This point was also a matter of discussion during the 5th Workshop on Recent Issues in Bioanalysis organised by the CVG [26] where they recommended a benchtop stability study on matrix despite the fact that it has been demonstrated that the change in the counter-ion does not affect the sample beyond a slight change in the pH.

Finally, the last type of validation is *cross-validation* [36]. It consists of a comparison of the validation parameters of two or more different analytical procedures that are used in the same study. It can consist of a comparison between methods using different analysis techniques (LC-MS/MS vs ELISA) or between laboratories using the same method. According to EMA for the cross-validation the same quality control (QC) samples should be analysed by the different methods (laboratories) and the results cannot differ more than a 15%.

3. Validation parameters

Along this section the different parameters that should be evaluated in bioanalytical method validation for chromatographic assays will be studied. This review will be mainly focused on the guidelines published by the two main regulatory agencies in bioanalysis: *“Guideline on bioanalytical method validation”* by EMA [37] and “*Guidance for Industry: Bioanalytical Method Validation”* by FDA. For the latter, the document presented in 2001 [6] and the white papers issued in 2007 [7] and 2009 [8] will be discussed. The guideline update, which draft has been recently published [11], will be also taken into account but always bearing in mind that it is still in the review phase. Other documents regarding method validation will also be taken into consideration such as *“Validation of analytical procedures: text and methodology Q2(R1)”* [12] by ICH, *“Guide for validation of analytical and bioanalytical methods (RE 899/2003)”* [38] and “*Dispõe sobre os requisitos mínimos para a validação de métodos bioanalíticos empregados em estudos com fins de registro e pós-registro de medicamentos (RDC 27/2012)”* by Brazilian ANVISA [29], *“Draft Guideline on Bioanalytical Method Validation in Pharmaceutical Development”* [30] by Japanese MHLW, *“Validation of Analytical Methods”* [31] by Spanish AEFI and *“The Fitness for Purpose for Analytical Methods. Laboratory Guide to Method Validation and Related Topics”* by EURACHEM [39].

One of the main drawbacks of all these guidelines is that they are rather conceptual and even if they define validation criteria the methodology to carry out the validation procedure is not precise enough. In order to fill this gap, and help the professionals from the pharmaceutical industry the *“Société Française des Sciences et*

Techniques Pharmaceutiques" (SFSTP) elaborated a validation procedure proposal with a smart and careful experimental design which has also be taken into consideration in this work [33,40–42].

3.1. Selectivity and specificity

These two parameters that are closely related are usually inappropriately interchanged [43]. Both FDA and EMA agree in the definition of selectivity as the ability of a bioanalytical method to measure and differentiate the analyte(s) of interest and internal standard (IS) in the presence of components which may be expected to be present in the sample. On the other hand, EMA defines specificity as the ability to measure the analyte unequivocally in the presence of other compounds, either exogenous or endogenous, in the matrix. Even if the difference between both terms is apparently faint, a clear distinction should be made: selectivity is a parameter that can be graded whereas specificity is absolute [44–48]. Selectivity is the measure of the extent to which an analytical method can determine an analyte without interference from other compounds. Only when a method is perfectly selective for the analyte it is considered specific. Very few methods are specific and therefore selectivity is considered to be the best term to be used in validation.

Main guidelines agree in the methodology for selectivity studies. At least six independent sources of the same matrix should be used and the response of the interferences compared to the signal of the analyte at the LLOQ. ANVISA RDC 27/2012 is more exhaustive and specifies that from the 6 different sources one should be hyperlipidaemic if the matrix is whole blood and one hyperlipidaemic and another one hemolysed if the matrix is plasma. The response of the interference should be lower than 20% the response of the analyte at the LLOQ and 5% the response of the IS. EMA allows the use of a smaller number of different sources if the matrix is considered rare, and FDA does it if LC-MS methods are used. In this case, checking the matrix effect is encouraged in order to demonstrate that precision and sensitivity are not affected. Regardless the detection method used, the authors recommend checking the selectivity in at least 6 different sources especially when low molecular mass analytes or complex matrices are to be analysed.

The regulatory agencies also propose a second approach for the study of selectivity based on the investigation of potential interfering substances, such as metabolites, degradation compounds or concomitant medication. This is a common practice in industry, where effect of co-medication is widely studied according to a survey carried out by EBF [49]. On the other hand, very few scientific papers follow this approach [50–53]. Ansermot et al. studied the selectivity of a method for the quantification of selective serotonin reuptake inhibitors by spiking plasma samples with 63 common medications in the psychiatric population [52]. They found out a potential interference with the same retention time and m/z as the analyte and optimised the fragmentation of the confirmation ion in order to avoid false positives in routine analysis. Hu et al. found out that the hydroxylated metabolite of the brominated hypoglycemic agent G004 interfered with the parent drug after in-source dehydration by analysing incurred samples [53]. It is evident that this infrequent methodology offers complementary information about method selectivity and therefore it should be considered especially when metabolites or degradation products are available and common co-prescribed drugs known.

3.2. Accuracy

According to the Joint Committee for Guides in Metrology [54] accuracy of an analytical method is defined as the closeness of agreement between a measured quantity value and a true quantity value of a measurand. In the FDA guideline text accuracy is defined as the closeness of *mean* test results obtained by the method to the true value (concentration) of the analyte. This definition, which involves the mean of some replicates, is misleading and corresponds to trueness, as it has been discussed in depth by Rozet et al. [43,55]. According to ISO definition, accuracy involves both random error and bias while trueness only reflects the bias of the analytical method [56]. Therefore, by using the methodology described in the guidelines, trueness of the analytical method is calculated instead of the accuracy. This inadequate definition of accuracy is thoroughly accepted in bioanalysis as it can be observed in different guidelines and publications. Nevertheless, in order to achieve a harmonised definition in the frame of the analytical validation this term should be reviewed in the close future.

The regulatory organisations recommend to measure accuracy using a minimum of 5 replicates at least at three different concentrations (QC samples) covering the whole calibration range: within 3 times the LLOQ, near the centre, and close to the upper calibration point. Taking into account the allowed error, the authors suggest setting the upper QC sample concentration between 75% and 85% of the upper calibration point in order to avoid extrapolation. Besides this three concentration levels, EMA and ANVISA ask to measure accuracy at LLOQ. Within-run (intra-batch) accuracy and between-run (inter-batch) accuracy should be evaluated and expressed as Relative Error (%RE). The deviation (in percentage) of the mean from the true value should be less than 15% except for LLOQ where up to 20% of error is accepted. For between-run accuracy at least 3 runs analysed on at least two days should be used.

3.3. Precision

Precision is defined as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions and it is expressed as relative standard deviation or absolute coefficient of variation (%CV) (ratio between standard deviation and mean, multiplied by 100). The %CV should not exceed 20% for the LLOQ or 15% for the rest of the QC samples. Precision can be evaluated with the same experiments used for the determination of the accuracy. In this way, within-run (intra-batch) precision assesses precision during a single analytical run and between-run (inter-batch) precision measures the precision with time. Despite FDA suggesting that the between-run precision may involve different analysts, equipment, reagents and laboratories, the inter-laboratory study of the precision fits better with the term reproducibility used in the glossary of the guideline.

In the guidelines for analytical method validation, ICH and ANVISA classify precision in a more unambiguous way by defining repeatability, intermediate precision and reproducibility. In this way, repeatability assesses precision under the same operating conditions within a short period of time and would be equivalent to within-run precision. Intermediate precision expresses within laboratory variations (different days, analysts or instrumentation) and would be equivalent to between-run precision without considering different laboratories. Finally, reproducibility is the precision inter-laboratory and involves collaborative studies. These organisations are less demanding in terms of number of replicates and request a minimum of three determinations per concentration level. This classification is unequivocal and avoids the confusion in inter-batch precision that can derive from FDA and EMA definitions. Reproducibility and intermediate precision are closely related to robustness and ruggedness, in fact ICH defines reproducibility as ruggedness, but this point will be thoroughly discussed in the following section.

3.4. Robustness and ruggedness

Robustness and ruggedness are terms related to the ability of a given method to provide reliable results despite variations in the analysis conditions. Nevertheless, there is considerable confusion in the scientific literature with regard to the use of the terms robust and rugged and the associated characteristics robustness and ruggedness as applied to the description of analytical methods. This confusion does not only take place in the pharmaceutical or bioanalytical field but also in others such as environmental or food analysis. Differences between these terms have not been properly defined by IUPAC, ISO or similar bodies. Indeed, within IUPAC documents, confusion exists within the use of ruggedness as implying an inter-laboratory use, or a single-laboratory situation [57]. The most accepted and used definitions for robustness and ruggedness are given by ICH and the U.S. Pharmacopeial Convention (USP). ICH defines robustness as follows: *"The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage"* [12]. Therefore a study of robustness will involve an investigation into the effect of these small, but deliberate variations. The USP has accepted the ICH definition for robustness and defines ruggedness as: *"The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions such as different laboratories, different analysts, different instruments, different lots of reagents, different elapsed assay times, different assay temperatures, different days, etc. Ruggedness is normally expressed as the lack of influence of operational and environmental factors of the analytical method. Ruggedness is a measure of reproducibility of test results under the variation in conditions normally expected from laboratory to laboratory and analyst to analyst"* [58]. As can be seen in the definition, the ruggedness test could be performed without deliberately changing the method parameters but only under different test conditions. Afterwards, their influences can be evaluated using a nested design or nested Analysis of Variance (ANOVA). In fact, this definition is equivalent to that for intermediate precision or reproducibility already mentioned in Section 3.3. In this sense, is moderately considered by some authors [59] that ruggedness is a property hierarchically above robustness as put forward by the Canadian Drugs Directorate in their three level testing system. In this system, Level I refers to the ICH definition of robustness and should include verification of repeatability by using a second analyst. In Level II testing, the effects of more severe changes in conditions are examined when the method is intended to be applied in a different laboratory with different equipment. Level III considers "a full collaborative testing", which is rarely done. On the other hand, other researchers such as Youden and Steiner [60] used the term ruggedness test for an experimental set-up that examines influences of minor but deliberate and controlled changes in the method parameters on the response, in order to detect those factors with a large influence (non-rugged factors). In fact, Youden and Steiner's ruggedness test application could be more related with robustness as described by ICH. As can be seen, several definitions for robustness or ruggedness exist which are, however, closely related and, in some cases, exchanged.

As it has been already mentioned the main objective of validation of bioanalytical methods is to demonstrate that the procedure is suitable for its intended purpose. For this reason, if a bioanalytical method is to be used routinely it needs to be robust so that the results obtained are reliable regardless small methodological variations. Although the robustness/ruggedness could be a significant parameter to be studied during the optimisation or validation step in bioanalysis, neither of these parameters is taken into account in the most important bioanalytical validation guidelines. According to FDA, bioanalytical method validation should include all the procedures/parameters that could make it reliable and reproducible for the intended use [6]. Nevertheless, neither robustness nor ruggedness is mentioned along the guide and no recommendation has been included regarding the study of the influence of small variations or different conditions. EMA guideline also omits robustness and ruggedness. On the other hand, both guidelines ask for a partial validation when methods are transferred between laboratories, which could be an unstated alternative to check the ruggedness (as defined by USP) of an initial bioanalytical method.

In contrast, ANVISA guideline [38] provides detailed information about robustness, which is defined according to ICH and is supposed to be evaluated before the validation step by studying the main parameters susceptibility to variations. Depending on the analytical methodology used (spectrometry, liquid chromatography or gas chromatography) the guide recommends checking different parameters. For example, in the case of LC, parameters that could affect to robustness are the pH variation of the mobile phase, variation in the composition of the mobile phase, different batches or column manufacturers, temperature and flow of mobile phase. It should be noted that these recommendations do not apply to identification or performance purpose methods.

Although the literature cited above refers almost exclusively to drug analysis and bioanalysis, the clear distinction between the descriptors robust and rugged is of wider application to other fields of analysis under regulation, such as those of human foods, animal feed, environmental samples and of articles subject to tariff/customs control. In this sense, the council directive 96/23/CE [61] lays down measures to monitor drugs and pharmaceuticals, among others, in live animals and animal products. Although strictly speaking it is not a guide in the bioanalytical field, it could be a great example of discussion about how robustness should be considered during the validation of the analytical procedures. This directive defines ruggedness (in the same way as ICH robustness) as *"the susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and/or sample preparation conditions under which the method can be applied as presented or with specified minor modifications. For all experimental conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH, temperature) any variations which could affect the analytical result should be indicated"*. Furthermore, not only is given the definition but also some recommendations about how the analytical method should be tested. This directive suggests the inclusion of relevant changes in the robustness study such as stability of reagents, sample composition, pH or temperature. Besides that, unlike with all previous guides, according to this directive responses of these changes should be analysed by means of, for example, Youden focus [60]. Briefly, according to this methodology, the average differences between measurements under normal conditions and measurements under slight metrological changes are compared. The standard deviation of the sum of the quadratic differences should be less than the reproducibility standard deviation of the validated method. If this assumption is satisfied, the robustness of the method is assured [61]. In summary, in this particular case there are not only some suggestions to check the parameters relative to robustness but also, as well as in the case of other parameters such as reproducibility or accuracy, minimum tabulated levels to assure the applicability of analytical methods.

3.4.1. Methodological approach

If a partial literature revision of the last 5 years articles related to bioanalytical validation methods is carried out, only in approximately 20% of the total cases, a robustness study was performed by the authors [62–67]. Some of the authors explain that the lack

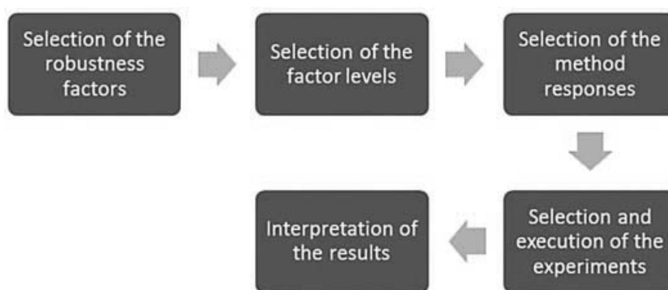

Fig. 1. Summary of the procedure for robustness testing.

of these studies is due to the fact that according to the guides they followed, robustness test was not required. In other works, robustness (following USP ruggedness concept) has been studied as a part of a reproducibility study but not deeply. In the articles in which a proper robustness study is performed it is carried out by means of One Variable At a Time (OVAT) procedures [68–70]. In this cases method overall precision, chromatographic resolution or peak symmetry are evaluated for changes in factors such as flow rate, wavelength, column temperature, pH of buffer and organic content in mobile phase as a measure of robustness. Briefly, levels of a given factor are varied while keeping the other factors at method working (nominal) levels to evaluate the effect of the former factor on the method response(s). This classical approach can be fast (only if few factors are studied), easy and applicable for bioanalytical methods [71,72], but is not recommended for robustness testing [73] since interactions among factors are not taken into account. Moreover, when robustness tests are performed following this procedure, they are usually carried out at a late stage in the method validation probably due to the relationship between robustness/ruggedness concepts and reproducibility of the method, for which inter-laboratory studies are commonly performed in the final stage of the validation process. However, performing a robustness test at this point involves the risk of finding out a lack of robustness of the method too late, which involves that it should be redeveloped and reoptimised. At this stage much effort and money have already been spent in the optimisation and validation, and therefore this is an incident to be avoided.

A different approach to study robustness which is spreading lately and overcomes OVAT methodology drawbacks is based on the use of experimental designs. This option has large advantages compared to univariate treatment. When applying an experimental design, the effect of a given factor is calculated at several level combinations of the other factors, while with the OVAT approach it is performed only at one level. Thus, in an experimental design, a reported factor effect is an average value for the whole domain, and it represents more globally what is happening around the nominal situation. Moreover, the univariate approach requires more experiments, especially when the number of examined factors becomes larger, and the importance of factor interactions cannot be taken into account. Design of Experiments (DoE) with predictive probability is a very innovative framework to simultaneously optimise the separation and estimate the method robustness over the experimental domain. Vander Heyden and co-workers explain thoroughly the use of DoE for robustness testing during validation step in a brilliant guidance [73]. In Fig. 1, the steps they propose for a robustness/ruggedness test can be observed.

According to this author, regardless robustness is investigated as part of the development of the method, at the end of method development during optimisation, or after method development during validation, the method parameters which will be investigated remain the same, e.g., % organic modifier in the mobile phase, pH of the mobile phase. . .[73,74]. These are referred to as the robustness factors and the first step of robustness testing is to choose them. The number of factors selected determines how much information is gathered about how the changes in method parameters affect the results, so, the higher number of factors studied the more information but also the higher number of experiments to be performed. Once the factors have been chosen, the levels at which they will be studied need to be defined. The amount of variation is referred to as the factor level, typically, limits around a nominal value are investigated and the magnitude of these limits has to be defined. It must be kept in mind that the aim is to understand the effect of changing a method parameter, whereas when determining the robustness of a method during validation the aim is to examine the variation which might be expected in routine use of the method. Thus the limits for the former may be expected to be wider than in the latter scenario. The factors and factor levels define the way in which the method will be set up for robustness experiments, but a way of measuring the effect of the method variations is also required. This needs to take into account quantitative aspects of the method such as an assay result (e.g., %w/w for main components or impurities), but also chromatographic criteria such as resolution, retention factor, asymmetry factor or number of theoretical plates. These are referred to as the method responses. Once factors, levels and responses have been selected, an experimental design is chosen and executed. The recommended designs are fractional factorial and Plackett–Burman [73,74] although two-level full fractional designs also can be used, provided that the number of examined factors does not exceed four, since otherwise too many experiments are required. Statistical and graphical methods may be used to aid interpretation [74]. Although not addressed to bioanalysis, some international guides such as the FDA and ICH request a Quality-by-Design (QbD) approach based on DoE. QbD is defined as *"a systematic approach to development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management"*. Although the ICH guideline Q8 (R2) either does not explicitly discuss analytical method development, the QbD concept can be extended and results in a systematic approach that includes defining methods goal, risk assessment, developing of a Design Space, implementing a control strategy and continual improvement to increase method robustness and knowledge.

The emergence of the use of QbD principles in pharmaceutical manufacturing has led to the application of QbD to analytical methods in other fields such as bioanalysis. This in turn has highlighted the importance of the study of robustness during method development as the design space concept of QbD translates into knowledge about the effect of each method parameter on the final analytical result. A very smart and computer-assisted way of developing a chromatographic method is by using different

kind of software modelling packages (R 2.13 free-ware software, DryLab®4 chromatography modelling) [75,76]. Based on a small number of experiments, these software applications can predict the movement of peaks in reversed-phase liquid chromatography separations when changing the mobile phase composition or pH, temperature, flow rate and the column dimensions and particle size [77]. The DoE is not usually used as a test of robustness in bioanalytical methods but it is possible to find it in some validations of drugs in other matrices than bulks or pharmaceutical formulations [50,78–80].

3.4.2. Discussion

Robustness and ruggedness are overlooked in the main bioanalysis validation guides taking into account the importance of these parameters to guarantee the good performance of the analytical method during routine analysis. Despite the intensive method validation procedures required in bioanalysis in order to meet the strict regulations set by the regulatory authorities, only ANVISA, in the RE 899/2003 requires the study of robustness as a method validation parameter.

In order to cover these parameters, non bioanalysis specific guidelines have to be consulted and in these documents there is also significant confusion in defining both terms (robustness/ruggedness) and making a difference between them. For that reason, it would be highly recommended that organisations such as IUPAC would define these terms unambiguously. Ideally, robustness should be investigated as part of method validation because a method is not complete without an evaluation of its reliability in routine use, but unfortunately, as has been observed, it is often deferred, or completely overlooked, because of the time-consuming nature of the study. In the ICH validation guidelines, robustness is not included in the tabular summary of required characteristics to be tested during validation, which could lead to the mistaken belief that a study of robustness is not required. Robustness should be included in the guides in which is not currently present as one of the parameters for the validation and optimisation process such as accuracy, precision, selectivity, sensitivity, reproducibility or stability. Given the different ways to ensure robustness found in the literature in recent years, also would be advisable to include methodological recommendations. The use of design of experiments (DoE) seems to be a good alternative as a tool to evaluate robustness better than OVAT procedure. Moreover, the performance of a robustness test has been shifting to earlier stages in method development. Some guidelines, including ICH, as well as some authors working in bioanalysis consider robustness a method validation topic performed during the development and optimisation phase of a method, while others consider it as belonging to the development of the analytical procedure. Therefore, the robustness test could be viewed as a part of method validation that is performed at the end of method development or at the beginning of the validation procedure. In any case, the exact position has relatively little influence on how it is performed.

3.5. Calibration curve: range and linearity

The calibration curve is the mathematical equation that relates the instrument response to the calibration standards for a defined calibration range. After the pre-validation step this range should be fixed between the LLOQ and the upper limit of quantification (ULOQ). The former depends highly on the sensitivity of the analysis technique but it does not need to be the limit value which fulfils the specifications of the regulatory authorities. In fact, the LLOQ should be adapted to expected concentrations and to the aim of the study (fit-for-purpose). For example, EMA establishes the limit for the LLOQ for bioequivalence studies to be 5% of the C_{max} of the analyte. This means that even if the analysis method is able to quantify 0.01% of the C_{max} such a low LLOQ may not be necessary since it will not provide any additional information to the study. Regarding the ULOQ, it should be high enough to ensure that all the study samples will fall within the calibration range. If a sample concentration is higher than the ULOQ the concentration should not be calculated by extrapolation but by means of a reassay after dilution with the matrix as will be explained later (point 3.10).

According to the guidelines a calibration curve should consist of a blank sample (blank matrix), a zero standard (blank matrix spiked with the IS) and at least six calibration standards covering the whole calibration range. The use of replicates is not necessary but it can be helpful when labile compounds are to be analysed. The simplest mathematical model that can explain adequately the relationship between concentration and signal should be used. It is important to mention that forcing the calibration curve to have zero as intercept can introduce a significant bias, so this approach should not be followed. Ideally a non-weighted univariate equation would be utilised, but taking into account the wide concentration ranges of bioanalytical methods, weighted linear regression or complex quadratic/logarithmic equations may be necessary [81–83]. Even if the guidelines do not mention it, homoscedasticity along the calibration curve should be studied and if the distribution of the variance is not homogeneous, statistical weights should be applied [84,85]. When this step is not performed, the influence of the low concentration standards is usually underestimated due to the overestimation of the high concentration standards. That is the reason why many bioanalytical methods use $1/X$ or $1/X^2$ weights for the regression models [86–91].

Regarding the acceptance criteria for the calibration curve a slight incongruity can be found in the FDA official guideline. In the Section 3.5 the requirement for acceptance is that 4 out of six non-zero standard have less than 15% of deviation from the nominal concentration (20% for LLOQ standard) whereas in the *Acceptance Criteria for the Run* section same cut-off is required for 75% of the standards. The latter criteria seem to be more adequate and fits with EMA's one. Furthermore, both organisations agree that at least 6 of the non-zero standards must satisfy the accuracy criteria including LLOQ and ULOQ standards. When replicate standards are used at least 50% of the calibration standards tested per concentration level should fulfill the accuracy criteria. In addition to standards accuracy, FDA also requires a goodness of fit test for the calibration curve and in the new update they ask to perform at least six calibration curves over the whole validation process with at least 4 concentrations analysed in duplicate (LLOQ, low, medium and high). This last requirement was only applied to microbiological and ligand-binding assays in previous guidelines and if it is finally accepted for chromatographic methods it would lengthen the validation procedure considerably.

3.6. Limit of quantification

The LLOQ is the lowest concentration of analyte which can be determined with acceptable precision and accuracy. The bioanalytical validation guidelines establish well harmonised acceptance criteria. %RE and %CV values of five replicates should be less than 20% and the analyte response at the LLOQ should be at least 5 times the response of the blank. In other words, the signal of the blank at the retention time of the analyte must have an area no greater than 20% of the area corresponding to the LLOQ [31].

3.6.1. Methodological approach

Even if all guidelines agree in the definition of the LLOQ, there is not such agreement in the calculation of this parameter. In this way, different approaches can be found in the literature and the guidelines to determine the LLOQ in the bioanalytical field. Taking into account the importance and ambiguity on the calculation

of this parameter many other approaches have been proposed in other analytical fields which could be applied to bioanalysis [92]. Nevertheless due to the scope of this review only the approaches dealing with LLOQ have been considered.

3.6.1.1. Based on the practical examination. Based on a trial and error concept, the LLOQ can be determined by using at least five QC samples spiked at a concentration close to the estimated LLOQ, from a single pool of matrix different from the calibration standards [3,93]. The mean values should be within pre-defined acceptance criteria [94]. The advantage of this approach is the fact that the estimation of LLOQ is based on the same quantification procedure used for real samples [95]. On the other hand it can be time consuming since many different QC samples may be needed until reaching the concentration that meets the criteria.

3.6.1.2. Based on the "standard deviation at the LLOQ" (IUPAC approach). In practice, the quantification limit can be expressed as the concentration that can be determined with a specified relative standard deviation [96]. Thus:

$$LLOQ = k \cdot \sigma \quad (1)$$

where k is the multiplier whose reciprocal equals the selected quantifying %CV and σ is the standard deviation of the concentration at the level of the LLOQ [97]. In bioanalysis, the required precision at the LLOQ is of 20%, thus, $k = 5$. To estimate σ a number of independent determinations ($n > 10$) must be carried out using samples spiked at a concentration close to LLOQ. Since LLOQ is not known, some general analytical validation guides recommend to analyse a sample with a concentration between 2 and 5 times the estimated detection limit (LOD) [57]. However it is important to point out that in contrast to analytical method validation, LOD is not a term used in regulatory bioanalysis [16], thus, the analyst may need to perform some extra experiments to estimate it [12,98–101]. Other guidelines suggest to perform the determinations on a blank sample [39]. Such a procedure, however, is discouraged, unless there is a strong evidence that the precision is constant between $C = 0$ and $C = \text{LLOQ}$.

3.6.1.3. Based on the well-known signal-to-noise (S/N) ratio approach. This approach is only applicable to instrumental analysis procedures providing a blank signal, background noise or baseline signal, such us UV–visible spectrophotometry or chromatographic methods [12,31,38,102].

The value of the LLOQ is given by the concentration of analyte providing a signal at least 5 times higher than that of background noise, since a 5:1 S/N ratio is considered to be sufficient to discriminate the analyte from the background noise. Nevertheless, the required ratio can vary between 5 and 20 depending on the guideline [31] and the procedure followed to measure the noise. In this aspect, S/N can be defined as the ratio between the height of the analyte peak (signal) and the amplitude between the highest and lowest point of the baseline (noise) in a certain area around the analyte peak [103]. However, very often the noise is defined as half of the amplitude and consequently the limit for S/N becomes twice bigger.

Even if this is one of the most used approaches in chromatographic methods, this procedure presents some disadvantages, starting with the fact that the calculation of the signal of the blank is not straight forward which turns the estimation of the S/N ratio into a difficult task. Nevertheless some rules have been detailed to facilitate it [104]. According to Hartmann et al. [93], in order to calculate the signal of the blank, it is advisable to perform from 6 to 10 consecutive analyses of a blank sample recording the maximum fluctuation of the background noise measured over a certain distance, in an area corresponding to 20 times the half peak width. Another inconvenience of this approach is that it is dependent on the manner the noise is measured; thus, the S/N ratio will vary from one instrument to another depending on the internal operational set-up such as signal data acquisition rate or the detector time constant. Furthermore, thresholding and smoothing can have a dramatic effect on the apparent S/N without necessarily reflecting an instrument improvement in terms of precision or accuracy of the results, making the S/N ratio measure an extremely subjective estimation [55,105,106]. In addition, the estimation of baseline noise can become quite difficult in bioanalysis, if matrix peaks elute close to the analyte peak [95]. These peaks can interfere with the background noise and lead to an overestimation of the noise amplitude and consequently also of the LLOQ. From the point of view of experimental verification, it has been observed in many cases, that it is possible to obtain equally precise and accurate results at concentration levels below the theoretical LLOQ [31]. Another problem of this approach is associated to the magnitude used to measure the S/N ratio and to build the calibration curve. For example, in chromatography with photometric detection, the signal height is measured in absorption units, but calibration curve is generally built using areas. Therefore, the quantification limit is not expressing the lowest level of the analyte, but the lowest quantified absorbance [55].

Despite all the drawbacks, this is an interesting approach since the LLOQ is related to the ability of the method to distinguish analyte signal from the background signal or noise. However, some authors [107] identify LLOQ directly with sensitivity when these terms are conceptually different, being the sensitivity the capability of the method to discriminate small differences in concentration of the analyte [108]. Therefore in practical terms, sensitivity is the slope of the calibration curve obtained when representing the response versus concentration. In this regard, a high sensitivity of the analytical method does not always imply lower LLOQ and it could be preferable a method with lower background noise at the expense of sensitivity [31,109].

3.6.1.4. Based on the "standard deviation and the slope of the calibration curve". LLOQ can be calculated using the calibration curve and the signal of the blank using Equation 2 [31]:

$$LLOQ = \frac{y_{blank} + 10 \cdot \sigma}{b} \quad (2)$$

Where y_{blank} is the background signal or the signal of the blank, b is the slope estimated from a calibration curve built close to the concentrations levels of the LLOQ and σ is the standard deviation of the response. Depending on the approximation used to obtain σ this function will have different ways.

Using the standard deviation of the blank: $\sigma = s_{blank}$

This approach is only applicable for methods where s_{blank} can be estimated from replicate analyses of blank samples. In this case σ is the standard deviation of the response obtained from the analysis of the blank (i.e., the background noise of the system, y_{blank}). The application of Eq. (2) is only valid if the major source of variation is due to the standard deviation of the blank (thus, s of the blank is much bigger than the s of the y-intercept and the s of the slope). Otherwise, the term corresponding to the standard deviation of the blank s_{blank} would be more complex to calculate [31].

As happens with the S/N approach the estimation of the y_{blank} and the s_{blank} is certainly the weak point of this approach. [110]. In order to avoid the sometimes challenging calculation of these parameters, Eq. (2) can be modified using some approximations. On the one hand, y_{blank} is replaced by the y-intercept with the calibration curve (extrapolating the equation to zero concentration). On the other hand, s_{blank} is replaced by the standard deviation, obtained from the calibration curve, of this hypothetical blank [31].

Residual Standard Deviation of the Calibration Curve: $\sigma = s_{x/y}$

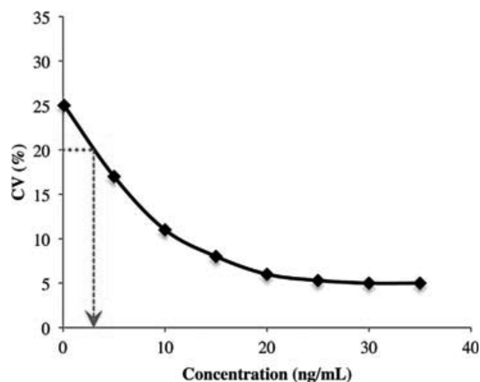

Fig. 2. Example of precision profile, showing the evolution of the bioanalytical method precision with respect to analyte concentration. The LLOQ corresponds to the smallest concentration with a %CV not exceeding 20%. Based on the "Graphical Examination of the Relative Error versus Concentration Level" (Accuracy Profile approach).

In this case σ is obtained from the residual standard deviation of the calibration curve ($s_{x/y}$) [12,38,102]. It is advisable to remember that this approach can only be used under homoscedasticity conditions. If the precision along the calibration curve is significantly different a weighted calibration curve should be used and the LLOQ calculated using the corrected $s_{x/y}$ [105].

Standard Deviation of the Intercept with the y-Axis of the Calibration Curve: $\sigma = s_a$

In this case σ is the standard deviation of y-intercept of the calibration curve (s_a). As explained in the first option for this approach, it can be obtained directly from the standard deviation of the zero concentration, but ANVISA states that s_a should be calculated as the standard deviation of the y-intercept of at least 3 curves [38].

This approach has some problems that are worth mentioning. On the one hand, ICH Q2(R1) Guide assumes that the calibration is linear, what is not always true [55]. On the other hand no guidance has established any requirement related to calibration curves at low concentration ranges (number of standards, correlation coefficient, number of calibration curves...). Obviously, the obtained LLOQ will depend on all these parameters. Finally, it is important to take into account that all these approaches based on the calibration curve should be used under homoscedasticity conditions, but the use of a weighting procedure should be considered [110].

3.6.1.5. Based on the "graphical examination of the %CV versus concentrations close to the expected LLOQ" (EURACHEM approach) [39]. The LLOQ can be calculated based on the %CV following the EURACHEM approach (also known as "Target %CV approach"). This approach is different in essence from those related to the estimation of the standard deviations since it is based on the direct measure of precision, in other words, it is based on the establishment of the minimum concentration of analyte that can be analysed with a precision equal to a %CV of 20%. For this aim, six replicates of a series of samples with decreasing concentration levels are measured. Then, the obtained %CV for each level is plotted against analyte concentration, as shown in Fig. 2. Using this chart the LLOQ is calculated as the concentration for which the %CV is equal to 20% [39].

The strong point of the EURACHEM approach is that the precision of the measurement, i.e. one of the defining criteria for the LLOQ, is directly measured. This approach overcomes some of the problems stressed in the previous approaches, since it is no longer equipment and operator dependant [105]. However, only the precision of the analytical procedure is assessed without taking into account the trueness or the accuracy.

3.6.1.6. Graphical examination of relative error versus concentration level" (Accuracy Profile approach). Another procedure which allows the estimation of the LLOQ is the Accuracy Profile approach (also named Total Error Profile approach) which is part of a complete systematic validation strategy, based on the construction of the so called accuracy profiles that reflect the total error of the method [32,33,40–42,111–114].

This approach re-focuses some validation criteria and proposes harmonised protocols by distinguishing, in particular, diagnosis rules and decision rules. These decision rules are based on the use of the Accuracy Profile, which integrates essential elements for the validation in a single graph (or table), i.e. the bias, the precision, and the quantification limits [41].

This powerful tool is based on tolerance intervals for the measurement's error [40]. As it can be seen in Equations 3 and 4, two terms are contained in the tolerance interval: one is trueness (bias or systematic error) and the other one, is the intermediate precision coefficient variation (random error). In this way the tolerance interval expresses the result's accuracy (understood as total error). Furthermore, the tolerance interval integrates an additional dimension, the chance (or risk), for future results, conditionally to past results, to fall within (outside) the acceptance limits [41].

For this approach two parameters must be set by the analyst: the acceptance limits (established by the regulatory Guidelines) and the risk of having future measurements falling outside those acceptance limits ($1-\beta$) [115]. If the analyst is ready to assume, for example a risk of a 5% (i.e. a ±5% acceptance limit or 95% of tolerance level, β = 95%), (s)he will be able to guarantee that 95 times out of 100 the future measures given by his procedure will be included within the acceptance limits fixed according to the regulatory requirements [40].

In practice, the tolerance interval at each concentration level for the validation standards is computed as follows [33] (in order to achieve a better understanding of this approach the reader is addressed to Hubert et al.'s work [41]):

$$\textit{Low Tolerance Interval}_j\,(LTI_j) = Bias_j(\%) - Q_t\left(\upsilon; \frac{1+\beta}{2}\right)\sqrt{1+\frac{1}{p\cdot n\cdot B_j^2}}\cdot CV_{IP,j} \quad (3)$$

$$\textit{Upper Tolerance Interval}_j\,(UTI_j) = Bias_j(\%) + Q_t\left(\upsilon; \frac{1+\beta}{2}\right)\sqrt{1+\frac{1}{p\cdot n\cdot B_j^2}}\cdot CV_{IP,j} \quad (4)$$

Where:

- $\text{Bias}_j(\%)$ is the trueness of the back-calculated concentration of the j-concentration level $RE\% = \frac{x-\mu_T}{\mu_T} \times 100$
- Q_t (υ; $(1+\beta)/2$) is the β-quantile of the Student t distribution with υ degrees of freedom $\upsilon = \frac{(R+1)^2}{\frac{(R+(1/n))^2}{p-1}+\frac{1-(1/n)}{p\cdot n}}$
- (where β is the chance level, p is the number of series analysed and n is the number of independent replicates per series).
- $B_j = \sqrt{\frac{R_j+1}{n\cdot R_j+1}}$
- $R_j = \frac{\hat{\sigma}^2_{B,j}}{\hat{\sigma}^2_{W,j}}$.

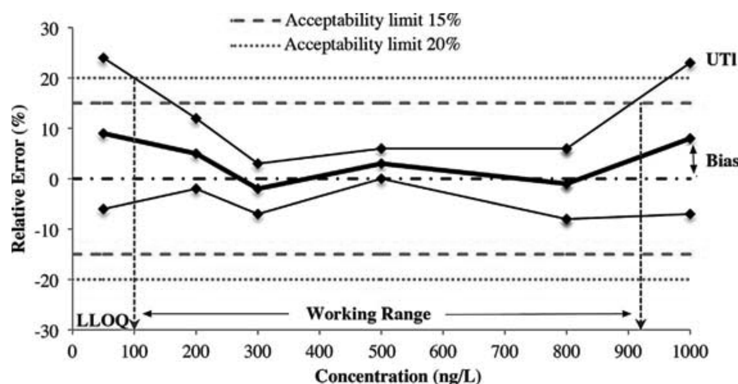

Fig. 3. Schematic representation of an Accuracy Profile based on a 6 concentration levels. Two acceptance limits have been represented: in red, upper and lower acceptance limits (ER = 15%) for the calibration range, except the LLOQ. In blue, upper and lower acceptance limits for the LLOQ (ER = 20%). β-expectation tolerance limits (intervals) for each concentration level are represented in fine black. The observed relative bias (trueness) is reproduced in bold-black. While the intersection between the tolerance limit and the acceptance limit of 20% defines the LLOQ, the intersection between the tolerance limit and the acceptance limit of 15% defines the ULOQ.

- $CV_{IP,j} = \frac{\hat{\sigma}^2_{IP,j}}{\hat{\mu}_j} \times 100$ is the intermediate precision (in terms of coefficient of variation%) for a *j*-concentration level, ($\hat{\sigma}^2_{IP,j} = \hat{\sigma}^2_{W,j} + \hat{\sigma}^2_{B,j}$ is the intermediate-precision variance for a j-concentration level, $\hat{\sigma}^2_{W,j}$ the intra-series variance and $\hat{\sigma}^2_{B,j}$ the inter-series variance; and $\hat{\mu}_j$ is mean of the back calculated concentrations of the j-concentration level).

To obtain an Accuracy Profile, the relative error is plotted versus the validation standards concentration levels and then, the lower tolerance limits LTl_j are joined together ($LTl_1 \rightarrow LTl_2 \rightarrow \ldots \rightarrow LTl_m$), as well as the upper tolerance limits UTl_j ($UTl_1 \rightarrow UTl_2 \rightarrow \ldots \rightarrow UTl_m$). Finally, the previously specified acceptance limits are reported in the graph as represented in Fig. 3. If the whole Accuracy Profile is within in the acceptance limits, the analytical method is expected to provide accurate results for its intended purpose and therefore declared valid over the whole range studied. If some point of the profile steps outside these limits then the method cannot be considered valid for that concentration level. In order to define the LLOQ the concentration value in which the 20% acceptability limit line crosses either the lower or higher tolerance limit should be graphically found.

Summarising, this approach not only simplifies the validation process of an analytical procedure, but, also constitutes a visual tool allowing the analyst to evaluate the capability of its method and even its future performance [41]. The analytical interpretation is easy and all the useful required statistics, such as trueness, precision, quantification limits, risk, linearity, are integrated. The use of the Accuracy Profile as single decision tool allows not only to reconcile the objectives of the procedure with those of the validation, but also to visually asses the capacity of the analytical procedure to fit its purpose [40,111].

3.6.2. Discussion

Despite the consensus existing around the definition of the LLOQ concept, there is a lack of agreement regarding the way this parameter should be calculated. This is highly problematic since it can mean that the LLOQ values given by different laboratories using the same analytical procedure are not necessarily comparable. Consequently, when reporting the quantification limit for a given analytical method, it should be necessary to specify exactly how the limits have been determined.

The S/N approach is one of the most widely used, despite the difficulties in calculating the noise and the significant equipment dependence. Thus, all the approaches based on the measure of the background noise or its standard deviation do not appear to be robust. In fact, Mermet et al. confirmed that the estimation of the standard deviation of the blank signal is certainly the weak point of these procedures [110]. The approaches based on the deviations of the slope or the Y-intercept could be an alternative but none of the guides set specific requirements in relation to the calibration curves that should be used in this approach.

The EURACHEM approach could provide a good compromise for evaluating the LLOQ. However, it is only able to discriminate based on the obtained precision values. It does not reflect any information about accuracy (trueness), which obviously must be also considered. A possible improvement would include the graphical presentation of %RE obtained from the same experiments, but in that case a calibration curve would be necessary, making the procedure more time-consuming.

The Accuracy Profile approach seems to be the best way to take into consideration both the bias and the precision. In this case, the LLOQ is the concentration that fulfils the acceptability limits established for the total error. This approach turns out to be revolutionary, since it involves a substantial change in the concept of validation itself. The resources and efforts are optimised in an obvious way, while the analysis of the results, in addition to be simple and complete, is visual and easy to interpret.

3.7. Matrix effect

Matrix effect (ME) is a parameter specifically related to mass spectrometry (MS) methods and is defined as the effect of other compounds in the matrix, different than the analyte, on the quantification of the analyte: signal suppression or enhancement, elevated baseline, impact on the extraction or the retention time. . .[116]. With the spread of MS methods in bioanalysis this phenomenon has gained importance in the last years due to the impact that coeluting substances can have in the ionisation efficiency, to the extent of becoming a fundamental parameter in method validation. The high sensitivity and selectivity of the MS technique, especially when coupled to liquid chromatography and used in tandem (LC-MS/MS), has led to a growing trend in methods with little to no sample preparation and minimal chromatographic separation [117]. However, the high selectivity of

LC-MS/MS methods does not eliminate completely the problems caused by coeluting compounds. With less selective techniques, such as photometric detection, these compounds can usually be visually detected as they appear as peaks causing an interference, whereas in LC-MS they may not be visible in the m/z ratio or transition monitorised but still modify the signal of the analyte of interest by suppressing or enhancing it.

Selectivity and ME are terms closely related although they name different events. As it has been said previously, selectivity deals with interferences whose signal overlap with the signal of the analyte of interest. On the contrary, compounds producing matrix effect may not be visible, so that method appears to be selective and yet be affected by signal suppression or enhancement. The use of MS detectors instead of other less selective techniques such as photometric or electrochemical detection, especially when used in multiple reaction monitoring (MRM) mode, reduces the selectivity problem, but it is susceptible to ME complications. Even though ME is characteristic of the MS detection independently of the technique used for separation, it is largely more common in LC than in other techniques such as gas chromatography (GC) analysis, being usually neglected when GC is used. Some studies explore the effect of matrix in the signal intensity when GC/MS is used, but are mainly from environmental or alimentary fields [118–120]. For this reason this section will be focused on the observed ME when LC-MS is used in bioanalytical studies.

Although many theories have been proposed to explain ion suppression [121–123], the exact mechanism of the competition between analyte and coeluting components remains yet unknown. It is well known that ME is generated in the ion source, the interphase between LC and MS, where analytes are desolvated and charged. The most common ion sources used in bioanalysis are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Since ionisation of molecules takes place differently in the different ion sources [124], the mechanisms by which ME is generated are specific for each type of source. Also, ion suppression has been demonstrated to be affected by the ionisation source design [125].

In the ESI source a process of charging and desolvation transforms the analytes in the liquid phase into gas ions that are introduced in MS analyser. Different theories have been proposed to explain this complicated and yet not fully understood process [126–128], but it seems clear that the coeluting compounds interfering with either the desolvation or the charging step alter the ionisation of the analyte. Although they are generally the principal cause, not only endogenous components in the biological matrix (salts, amines, triglycerides...) cause ME, also some exogenous compounds (plasticizers from sample containers or anticoagulants in case of plasma) are susceptible to alter the ionisation process [123]. Furthermore, there are other substances that can be present in the mobile phase and can alter the signal of the analyte by causing ion suppression or enhancement. Nevertheless this is not considered a ME source since it is not sample specific. Usual cases are some mobile phase additives such as non-volatile phosphate or sulphate salts which provoke poor desolvation of droplets [129–131] and components such as surfactants or ion-pairing reagents which make difficult the migration of an analyte to the surface of the droplet.

APCI source is less prone to suffer from ME. Since the analytes are not charged in the liquid phase but in the gas phase [132], droplet generation or desolvation problems are eliminated. Nonetheless, it is not completely free of ME [133]. This ionisation source is also vulnerable to non-volatile compounds and to competition between analyte and coeluting compounds for available charge.

Over the years, several researchers have proposed diverse methods to control and reduce matrix effects in bioanalysis [134–138]. Commonly the attempts to reduce matrix effect go through extensive clean-up, sample dilution, improved chromatography separation or the use of isotope labelled standards. When complex biological matrixes are studied it is recommendable a sample treatment step before the analysis [139,140]. Therefore, in the choice of the most adequate sample treatment method, its impact in ME should be taken into account. For example, when plasma samples are studied, a major source of suppression comes from the residual phospholipids from the matrix that can be minimised by an extraction step previous to the analysis [141–143]. ME could be considered one of the most important disadvantages of LC-MS methods in bioanalysis. It can diminish or enlarge the signal of the analyte and alter the S/N ratio modifying therefore the LLOQ. Furthermore, ME can vary dramatically among samples affecting the accuracy and precision of the method, and causing lack of linearity, which can lead to deceitful results [144]. In spite of its importance, ME has been traditionally overlooked during method validation and even though current guidelines include the quantification of this parameter as a validation requirement, there are still important voids in this topic especially regarding methodology.

The matrix effect phenomenon was first reported by Kebarle and Tang [145] in 1993, who showed that the electrospray response of an analyte decreased as the concentration of other electrolytes was increased. Notwithstanding, it was not until the 2nd Crystal City meeting held in 2000 that matrix effect was considered by the FDA [5], later defined in its guideline as *"the direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample"* [6]. However, no methodology was suggested by the FDA to evaluate the existence and magnitude of ME until the white paper published in 2007, product of Crystal City III meeting. In this paper, the matrix factor (MF) is defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions [7]. According to this last definition, a MF value of one implies that the analysis is not affected by any ME, a MF lower than one indicates ion suppression, and a MF value higher than one indicates ion enhancement. The IS normalised MF (NMF) was also defined as the MF of the analyte divided by the MF of the IS.

EMA gave the same definition for ME and NMF on the *Guideline on bioanalytical method validation* of 2011 [37]. The definition of MF is expanded saying that the peak area in presence of matrix should be measured by analysing blank matrix spiked after extraction and in absence of matrix by analysing a pure solution of analyte. Both guides agree that the variability in the MF, which would cause lack of reproducibility in the method, must be studied using six lots of blank matrix from individual donors. In the case of matrices that are difficult to obtain, FDA as well as EMA accept the use of less than six individual blank matrices. It is also admissible according to the FDA guideline to use less than six matrices when the NMF with stable isotope IS is used. EMA emphasises that pooled matrices should not be used. The variability of MF should be determined in terms of %CV. A %CV of 15% is considered by both agencies as the maximum acceptable value.

Although FDA does not include any indication about the concentration at which ME should be studied, EMA suggests that the determination of the MF should be done at two levels of concentration, a lower level of maximum 3 times the LLOQ and a higher level close to the ULOQ, but no indication is given to compare the consistency of the matrix effect at the different levels.

ANVISA did not consider matrix effect among the validation parameters in RE 899/2003 document [38], however, a section focused on matrix effect assessment was included in RDC 27/2012 [29] recommending similar procedure as EMA.

In the case of plasma and serum, EMA suggests that hemolysed and hyperlipidaemic samples should be also studied. ANVISA RDC 27/2012 is more specific and states that for plasma eight samples

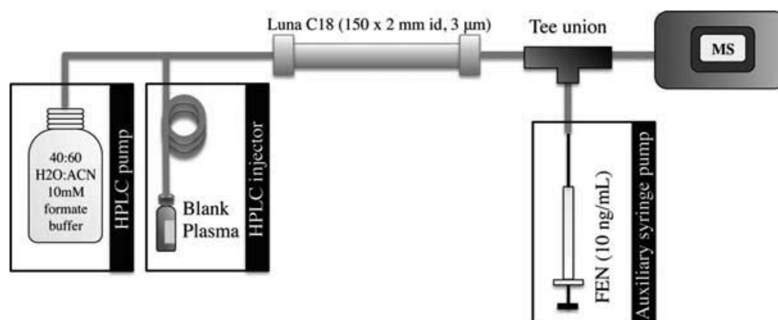

Fig. 4. Scheme of the post column infusion system.

must be studied, being two of them hemolysed, two hyperlipidaemic and four normal; for total blood six samples are necessary, being two of them hyperlipidemic and four normal. These kinds of samples are rather common and are considered to be particularly affected by ME. Even though ideally all type of populations should be included in a validation, this is unfeasible on a routine basis, but should be considered when those populations are targeted, for example in clinical trials of renally or hepatically impaired populations.

Sometimes, excipients used in formulations remain unaltered in-vivo and can be a source of ME. In these cases, EMA proposes that ME should be determined not only for blank matrix but also for matrix containing these excipients by spiking them directly into the blank matrix. In the case that those excipients are metabolised or somehow altered, matrix should be collected from subjects administered only with the excipients.

Finally, Japanese MHLW [30] also briefly considers matrix effect as a parameter of validation when mass spectrometric methods are used. This guideline, similar to the FDA, recommends to determine the MF by comparing the analyte response in presence and in absence of matrix from 6 different sources and that it may be normalised with IS so that the precision should not exceed 15%.

3.7.1. Methodological approach

Matrix effect is mainly assessed by two strategies, a qualitative assessment based on post column infusion described by Bonfiglio et al. [129] and a quantitative evaluation as the absolute matrix effect by post extraction addition firstly defined by Matuszewski et al. [146].

In the first protocol a constant flow of a solution of the analyte is added after the chromatographic column using a "Tee" union while an injection of an extracted blank sample is carried out (Fig. 4). Same procedure is followed with a blank solvent injection and afterwards both profiles are compared. Ideally, if there is no ME, the profiles of the analyte for the blank sample and the blank solvent should be the same. Ion suppression or enhancement is evidenced by variations in this profile as shown in Fig. 5. If more than one analyte is analysed with the same method each analyte must be infused individually to evaluate its ME in order to avoid self-induced ME.

This methodology has been vastly used to study ME in bioanalysis [88,134,147] and provides a qualitative way to examine the areas of the chromatogram affected by ME. Estimations of ME in terms of percentage of signal intensity must be considered as approximations [136]. Kaufmann and Butcher [148] developed a smart variation of this strategy in order to correct the ME. Segments of analyte solution and analyte-free solvent are alternatively infused post column so that a pulsating signal is registered. In this way the ME is quantitatively evaluated in real time and after data-processing the signal of the analyte can be corrected. Tudela et al. [149] proposed the use of a ME marker to identify samples whose ME differ from the accepted values. They found that isotopically labelled budesonide may be a suitable marker to indicate samples with severe ME in the analysis of diuretics in urine.

For a quantitative assessment, Matuszewski et al. [146] proposed a methodology based on the calculation of ME by comparing the signals from the analysis of three sets of samples: a matrix free standard (A), a sample spiked after the extraction of a blank matrix (B) and the same matrices spiked before extraction (C), all at seven levels of concentration. They define ME as the ratio of the response

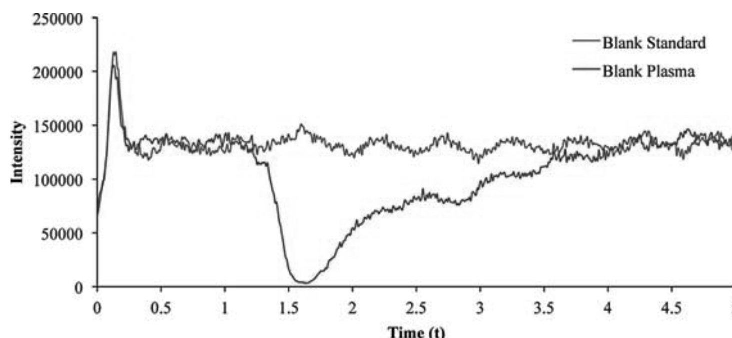

Fig. 5. Post column infusion of fentanyl while injecting through the chromatographic system a blank standard (blue) or a blank plasma treated with protein precipitation using acetonitrile (red). The ion suppression between 1.5 and 3.5 min can be clearly observed.

of B over the response of A, which is equivalent to the MF defined in the guidelines.

With these three sets the recovery (REC) and the process efficiency (PE) can be calculated as follows: REC(%) = (C/B) × 100 and PE(%) = (C/A) × 100 = (REC × ME)/100.

The advantage of this procedure is that recovery and matrix effect are assessed together [150–153] and can be combined with accuracy and precision studies. Nevertheless it is not a common practice to study them at seven levels. Usually recovery and ME are studied at three levels of concentration (low, medium and high QC) or at two levels as it is recommended by EMA and ANVISA guidelines (close to the LLOQ and the ULOQ). The authors of this approach also proposed assessing ME using five different lots of matrices in order to demonstrate the absence of "relative" ME, referring to the variability of the ME among different sources of the same matrix. However, currently it is more common to use six different sources as recommended by the guidelines.

An alternative approach to determine the presence or absence of ME was also proposed by Matuszewski [154]. By this methodology the slope of a calibration curve prepared in matrix is compared to the slope of the same calibration curve prepared in clean solvent. Superposed lines indicate absence of ME whereas differences in the slopes reveal the presence of ion suppression or enhancement. Thus, the relative matrix effect would be assessed by comparison of the slopes obtained in different matrix sources.

3.7.2. Discussion

ME could be considered as one of the major drawbacks of the LC-MS technique, especially when such complex matrices as the bioanalytical ones are studied. As it is not visible unless it is specifically addressed it could go unnoticed and appear as lack of reproducibility, poor precision or deficient accuracy among other problems. Despite its potential deleterious effects, ME was initially overlooked in the international guidelines of validation where it is vaguely defined or not even mentioned. Due to the wide expansion of LC-MS use in bioanalysis laboratories, the assessment of ME has become imperative for the favourable outcome of many bioanalytical methods. Consequently, it is included in the most recent versions of almost all the guidelines. However, the methodology to be followed remains sometimes unclear. In our opinion clear and concise instructions about the methodology to evaluate ME should be given.

The quantitative methodology proposed by Matuszewski et al. [146] has proven to be adequate to determine the presence or absence of ME interfering with the analyte of interest, and determining the recovery of the method. Contrary to bioanalytical validation guidelines, the authors recommend applying this methodology at three levels of concentration (low, medium and high QC) simultaneously with the accuracy and precision studies. In this way the number of experiments to perform is minimised and at the same time information of ME along the whole calibration range is obtained.

As it happens with the recovery, none of the guidelines gives a value admissible for the MF. According to their indications, as long as ME is reproducible it does not necessarily need to be eliminated, but identified and quantified. Nevertheless, Kollipara et al. [107] indicate that most of the bioanalytical laboratories accept 0.80-1.20 as the limit for acceptable IS normalised MF value, and consider ME negligible if it is in ±15% of nominal value with a %CV no more than 15%.

3.8. Recovery

Recovery measures the ability of a method to extract an analyte from a biological matrix and it is expressed as the percentage of the known amount of the analyte carried through the sample extraction and processing steps of the method. It is important to point out that there is not a minimum established value for recovery since a bioanalytical method with a low recovery could be suitable for a certain analyte if the sensitivity of the detection technique is high enough. Nevertheless, recovery of the analyte and the IS should be precise, reproducible and consistent over the calibration range. Indeed, as happens with ME, the guidelines do not focus on the recovery value (even if 100% is desirable) but in demonstrating that the obtained values are consistent.

According to FDA and ANVISA, recovery is calculated by comparing the analytical response for extracted samples at three concentrations with unextracted standards. Even if it is not explicitly indicated, by this approach the absolute recovery or PE is obtained (see Section 3.7), which includes relative recovery and matrix effect. In our opinion both terms should be separately calculated and therefore the methodology for the calculation of recovery defined in the MHLW would be more suitable, where they compare the analyte response in a biological sample spiked before the extraction and processed with the response in a biological blank processed and then spiked with the analyte. This guideline recommends performing the analysis at each concentration at least in triplicate while FDA and ANVISA do not set the number of replicates. To our knowledge, the most efficient way to determine recovery is described in *matrix effect* section, where matrix effect, recovery and process efficiency are calculated simultaneously. The three required concentration levels can be the same ones used for accuracy and precision study.

It is noteworthy that recovery term is mentioned not even once in EMA guideline. This fact did not go unnoticed by the scientific community as it can be observed in the large amount of comments received by EMA during the consultation period regarding this topic [155]. The organisation claims that recovery is an issue to be investigated during the analytical method development and as such is not considered to be included in a validation guideline. The authors agree with EMA in this point and more taking into consideration that extraction recovery reproducibility is implicitly demonstrated when the accuracy, precision and linearity of the method fulfil the acceptance criteria. Nevertheless, from a practical point of view the authors encourage to include relative recovery assay during method validation since the number of experiments to be added is minimal if the procedure is properly designed, and it can provide very useful information to understand the whole analytical procedure.

3.9. Stability

Studying the stability of the analyte in stock solutions and matrix is vital to ensure the reliability of the results provided by the analytical method [156]. After the first Crystal City meeting only stability during the collection process, sample storage period and two freeze-thaw cycles was demanded. Nowadays instead, the different guidelines ask for more exhaustive studies. These include assesses that cover all the situations that can be encountered during the whole analytical procedure such as freeze-thaw stability, short and long term stability, stock stability and post preparative stability. Even if there is not a complete consensus among the guidelines regarding the last point, it has been already thoroughly discussed by Kollipara et al. who distinguished four assays for processed sample stability: wet state stability, processed sample integrity, autosampler stability and reinjection reproducibility [107]. It is noteworthy that FDA guideline does not specify acceptance criteria for the stability exercises whereas the rest of the regulation bodies deemed that the deviation between the calculated and the nominal values should be within ±15%.

Additionally to the aforementioned stability assays, during the last bioanalysis meetings, several points regarding stability

have been widely discussed such as the stability of the drug in blood before plasma has been separated, stability in hemolysed or hyperlipidaemic samples, influence of coanalysed compounds in analyte stability, the assumption of the stability at −70 °C if it has been demonstrated at 20 °C [27,28,157]. All these aspects are still matter of debate and should be eventually integrated in the official guidelines.

3.10. Dilution integrity

Sometimes, study samples' concentration may exceed the ULOQ, which means that a dilution and a reassay of the sample are necessary. During the validation procedure it should be demonstrated that this step do not affect the quality of the final results. It is important to emphasise that the dilution should be carried out before the sample treatment using the same matrix than the study samples (e.g., human to human urine). Otherwise, dilution of the processed sample would alter the amount of matrix compared to the calibration standards (unless they are diluted too) leading to unreliable results.

Both FDA and EMA include the demonstration of dilution integrity but only the latter proposes a methodology to study it. At least five replicate samples should be spiked at the highest expected concentration above the ULOQ and properly diluted to fit in the calibration range. The precision and accuracy should be within the set criteria (%CV, %RE 15%) and it should be assessed on at least one of the validation days [158,159]. ANVISA RDC 27/2012 includes dilution integrity test with the study of precision and accuracy by adding one more QC level named CQD (Dilution Quality Control) in each batch.

3.11. Carryover effect

Carryover is a phenomenon caused by the presence of a residual amount of the analyte in the analytical instrument after an injection, which can affect the accuracy and precision of the results. Carryover can be a great obstacle when developing LC-MS/MS methods in bioanalysis due to its intrinsic high sensitivity and the broad calibration range necessary for some studies. This effect affects to a greater extent the low concentration samples due to the higher percentage influence of the remaining analyte in the measured response. This is especially troubling when the analysis of a low concentration sample is preceded by the analysis of a high concentration one, which can often happen when analysing study samples. But carryover does not affect only to subsequent sample(s), it can be random too, for example, if late eluting compounds are accumulated and eluted several injections later.

Despite the importance of carryover, FDA does not mention it in its official guideline and it is not until the White Paper published in 2007 that this effect is addressed [7]. They recommend injecting one or more blank samples after a high concentration sample or standard and compare the response of the analyte in the blanks with the response at limit of quantification. Even if there is not a cut-off value for the carryover (it should be minimised as much as possible), taking into account that the response at the LLOQ should be at least 5 times higher than the blank response, the carry-over cannot be higher than the 20% the response of the analyte at the LLOQ or 5% the response of the IS. This is indeed the procedure recommended by EMA, MHLW and ANVISA.

In some cases carryover cannot be avoided, hence special measures should be taken such as the use of a fast cleaning gradient or the injection of blank samples between study samples and after high concentration standards or QC samples [160,161]. In these cases randomisation of the standards or study samples is not recommended in order to minimise the impact of the carryover effect in the accuracy and precision

3.12. Incurred sample reanalysis

Incurred Sample Reanalysis (ISR) has been a hot topic in bioanalysis during last years [162]. This topic was brought out in Crystal City III workshop when it was shown that even if the inter-day precision and accuracy was usually less than 6% when analysing incurred samples repeatedly the obtained concentration values could differ by 30-80% [7,10]. It was obvious that in some cases the QC samples were not able to mimic the study samples mainly due to metabolites conversion to the precursor drug, differences in protein binding, recovery issues, sample inhomogeneity or matrix effects. Due to the importance of this topic a new workshop was organised in 2008 in order to set some recommendations to be followed by bioanalysis laboratories [8].

According to this workshop the number of samples to reanalyse depends on the size of the study, with a minimum of 5% of the samples for the large studies (5-10% for the rest). These samples should be representative of the study and therefore it is recommended to choose samples around the t_{max} and around the elimination time. The results obtained for the reanalysed samples are then compared with the original values, so the number of replicates used in the analysis should be the same. In order to pass the acceptance criteria for the ISR the concentration value of at least 4 out of 6 samples should agree within the 20%. When EMA released the guideline ISR was included following the same criteria with the only exception that they set the number of reanalysed samples to 10% if the total sample number in the study is less than 1000 and 5% if the number of samples exceeds this number, which is the same procedure that MHLW draft guideline recommends. In the last FDA draft the number of ISR samples is set in the 7% of the study sample size, regardless the size. Both guidelines agree that ISR is at least expected for in vivo human bioequivalence studies, all pivotal pharmacokinetic/pharmacodynamic studies and toxicokinetic studies.

3.13. System suitability in routine drug analysis

Although this review is focused on method validation, system suitability and acceptance criteria for the run when applied to routine analysis will be shortly discussed due to the connection with the topic and the fact that the guidelines also address these points. System suitability is a test to confirm that the instrument performance is adequate and is carried out before running the batch, usually using a standard solution [108]. The parameters to be studied during system suitability test are selected before the validation procedure and depend on the analytical instrument to be used: retention time or peak width for chromatographic methods; analyte response or fragmentation ratios for MS detectors. Once the proper instrument performance has been demonstrated by verifying that the values of those parameters are inside the tolerance range the analytical run can be carried out.

An analytical run consists of a calibration curve (blank sample, zero sample and calibration standards), QC samples at least at three different concentrations in duplicate (low, medium and high concentrations) and the study samples. The QC samples and the calibration standards should be spiked using different stock solutions and the whole batch should be processed together without interruption. In the case of large studies the number of QC samples should be at least the 5% of the number of studied samples.

During method validation the criteria explained in point 3.5 should be applied to the calibration standards and the regression model should be the one used during method validation. According to EMA, even if the calibration standards defined as LLOQ or

ULOQ do not fulfil the accuracy criteria the calibration curve can be used, as long as six calibration standards meet the criteria and all the QC samples remain inside the calibration range. In those cases, the LLOQ for that run is the next lowest concentration standard and the ULOQ the next highest concentration standard. FDA does not give specific information about this fact but states that the values falling out the accuracy limits can be discarded, provided they do not change the model, so the same approach can be followed. In case the sample concentration falls below the LLOQ, it should not be reported as zero but as "BQL" (below the quantification limit) and it should not be used for any pharmacokinetic calculations [163].

Concerning the QC samples at least 4 out of 6 samples should meet the accuracy criteria, always taking into account that at least one of the replicates for each concentration level should be within the 15% of the nominal values (4-6-15 rule). The authors recommend designing the calibration curve and the QC samples in such a way that the concentration of the lowest QC sample is slightly higher than the second calibration standard and the concentration of the highest QC sample is lower than the second to last calibration standard. This allows the rejection of calibration curves when one of the extreme points does not fulfil the acceptance criteria. Another approach to minimise the risk of discarding a whole analytical run due to a single point out of the accepted range is to run duplicate calibration standards only for the LLOQ and the ULOQ. In that way, even if only one of the replicates would meet the acceptance criteria the run could be accepted.

4. Discussion

Current bioanalytical validation guidelines have demonstrated to be essential documents to orientate bioanalytical laboratories in method validation and to set the criteria that the developed methods should fulfil. Nevertheless, as it has been thoroughly discussed along this review, these guidelines are too ambiguous when defining some parameters, the methodology to be followed or when setting some of the acceptance criteria.

Among the established criteria for validation all guidelines agree that the accuracy and precision, expressed as %RE and %CV values respectively, should be lower than 15% (20% for LLOQ). This acceptance criterion is easy to implement but it can lead to unreliable results. On the one hand, % RE is calculated using the mean of the measures and therefore it is possible to arrive to the paradoxical situation where the accuracy and precision are acceptable but the bias of more than half of the measures is higher than 15% (e.g. a sample with a reference value of 100 a.u. for which the measure of 5 replicates are 84, 86, 84, 100 and 116). On the other hand both acceptance criteria are independent from each other and consequently there is not a combined measure of the bias and the precision. This means that a method could be validated with both %CV and %RE values close to 15%. Once again in this extreme situation the bias of most of the measures could be unacceptable. Hubert et al. [40] already stated that for a normal distribution with a %CV of 15% and no relative error only 66% of the measures would have a bias less than 15%. Obviously when increasing the relative error until being close to 15% this number would decrease (to around only 45% of the measures with a bias lower than 15%). It means that this hypothetical method that could be successfully validated according to current regulations, would hardly meet the 4-6-15 rule set for routine analysis. This fact is really contradictory taking into account that the main aim of validation is to demonstrate that the validated method offers reliable results during routine analysis. Obviously this scenario should be avoided in bioanalysis and an approach that implies the total error (accuracy + precision) should be considered. An interesting option is to use the Accuracy Profile approach explained in *limit of quantification* section in which the construction of the accuracy profiles and the use of the acceptability limits lead to more reliable results besides providing more complete information regarding the regression mode and the working range.

This approach does not only offer a different way to process the data obtained from validation experiments but a new methodology to face the whole validation procedure about which some other authors have discussed earlier [32,40,43,55].

While bioanalytical validation guidelines define the majority of the validation parameters' acceptance criteria (with the exception of robustness), they leave a wide margin for interpretation in terms of methodology. The required content is clear, but not the overall process for obtaining it, i.e., a validation program is not defined and the consecution of steps which the analyst must accomplish is not clearly expressed. Obviously the validation guidelines cannot just consist of a rigorous schedule detailing all the steps to follow because there should be room for modifications depending on each particular analysis but nevertheless, reaching some standardisation degree in the methodology would also help to achieve a more harmonised validation procedure in bioanalysis.

The lack of agreement in methodological approach to calculate the LLOQ and the little attention that is paid on ME have been already carefully discussed together with the omission of robustness assays. Another issue not covered by the current regulations relates to the quantification of biomarkers. In the last years the advances in the field of personalised medicine and metabolomics have led to the need of quantifying these endogenous compounds that can indicate a certain biological condition such as an illness. Unfortunately, the bioanalytical guidelines are more addressed to exogenous compounds such as drugs and their metabolites and therefore no indications can be found related to the quantification of endogenous compounds. Aware of this situation, FDA included a section in the last draft of the guideline update called *"special issues"* where information about biomarkers and endogenous compound analysis is shown. They propose the validation procedure to be carried out using analyte free matrix, what is usually complicated in these cases. The use of alternative matrices (e.g. use a buffer instead of urine) is justified provided there is not matrix effect difference whereas the possibility of using alternative methods such standard additions is not yet considered. The growing interest in quantification of biomarkers will probably result in a rise of the relevance of this topic in the bioanalysis field.

5. Conclusions

Bioanalytical method validation is a fundamental process to confirm that the results obtained for a certain analyte in a biological matrix are reliable. Despite the need of trustworthy results in all the fields involving drug or metabolite analysis (toxicology, pharmacokinetic studies, bioavailability studies...) there are still some vague aspects that have not been properly addressed by the different regulatory bodies.

After carefully reviewing the regulatory guidances and scientific works dealing with bioanalytical method validation, the authors concluded that the most controversial validation parameters are robustness, lower limit of quantification (LLOQ) and matrix effect.

Robustness assay is not yet mandatory according to the regulatory bodies, taking into consideration the importance of this parameter when routine analysis is carried out, a deep discussion about the different approaches to evaluate it was included. Experimental design emerges to be a very powerful tool for this aim, allowing a fast, systematic and easy to implement methodology [73].

Regarding to LLOQ, main bioanalytical validation guidelines agree in the definition of the LLOQ concept, but do not give

information about the procedure to follow for its calculation, probably because it is considered to be evaluated in a pre-validation step. This turns problematic since the LLOQ values given by different laboratories using the same analytical procedure are not necessarily comparable. The Accuracy Profile approach seems to be the best way to estimate LLOQ, integrating trueness, precision, risk and linearity. The use of the Accuracy Profile provides a tool to assess the capacity of the analytical procedure to fit its purpose.

Even though in the last versions of the main validation guidelines ME has been included as an essential validation parameter, the specific methodology to be used remains imprecise. As ME could be considered one of the major drawbacks of the LC-MS technique, especially when complex matrices are studied, more precise directions should be given in order to avoid mismanagement of such an important phenomenon. The methodology proposed by Matuszewski appears to be adequate to determine the presence or absence of ME.

2001 FDA guideline is still followed in many laboratories although taking into account nowadays technology and requirements some of its aspects are out of date. The appearance of EMA guideline in 2011 solved some of the issues that were not covered by its predecessor such as ME or ISR, but there are still some aspects that should be included or improved in the following updates: robustness studies, validation of biomarkers and endogenous compounds, stability issues, acceptance criteria for IS repeatability, conditions under which partial validation is allowed... FDA 2013 guideline will deal with some of these points but due to the unceasing development in bioanalysis it is necessary that all the concerned parties keep involved in order to reach harmonised criteria for bioanalytical method validation.

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Review

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Bioanalysis

Cardiovascular drug determination in bioanalysis: an update

The great impact of cardiovascular diseases in human health has led to the development of a huge number of drugs and therapies to improve the treatment of these diseases. Cardiovascular drug analysis in biological fluids constitutes an important challenge for analytical scientists. There is a clear need for reliable methods to carry out both qualitative and quantitative analysis in a short time of analysis. Different problems such as drug monitoring, analysis of metabolites, study of drugs interactions, drugs residues or degradation products, chiral separation, and screening and confirmation of drugs of abuse in doping control must be solved. New trends in sample preparation, instrumental and column technology advances in LC and innovations in MS are described in this work.

Oskar González[1], Nerea Ferreirós[2], María Encarnación Blanco[1] & Rosa M Alonso*,[1]
[1]Department of Analytical Chemistry, Faculty of Science & Technology, University of the Basque Country (UPV/EHU), 48080 Bilbao, Basque Country, Spain
[2]Pharmazentrum Frankfurt/ZAFES, Institute of Clinical Pharmacology, Goethe-University Frankfurt, D-60590 Frankfurt am Main, Germany
*Author for correspondence:
Tel.: +34 946012686
Fax: +34 946013500
rosamaria.alonso@ehu.eus

Cardiovascular diseases & combined cardiovascular therapy

Cardiovascular diseases (CVDs) are a group of diseases including several pathologies related to the heart and blood vessels, such as angina, heart attack, stroke and heart failure, among others [1,2], which remain the leading cause of mortality over the world. The etiology of CVDs is complex and multifactorial. There are a group of risk factors, which plays an important role contributing sinergically to the increase of CVD. Among them, we can find factors such as hyperlipemia, arterial hypertension, obesity and diabetes mellitus. These factors constitute the so-called metabolic syndrome.

Treatment of CVDs may include lifestyle changes as well as combined cardiovascular therapy with drugs that have different targets and mechanisms of action [3]. In Table 1, the families of drugs commonly prescribed in combined cardiovascular therapy are collected.

The development of new and improved drugs belonging to the different families of combined cardiovascular therapy drugs is an important goal of the pharmaceutical industry. However, in the development of cardiovascular drugs several problems are encountered. Adverse effects may occur at all stages of drug development, in both nonclinical animal studies and in human patients [4]. There is also a potential risk of drug-induced toxicity that must be identified, quantified and reduced at the clinical level [5]. The success of the therapeutic treatment depends not only on selecting the proper medication but also on the appropriate dosage of the drugs, since drug effectiveness is related to drug exposure and plasma concentrations of drug. Individual variation in drug metabolism and drug–drug interactions affect the concentration and, therefore, the effectiveness of cardiovascular medications. One of the ultimate goals for the treatment of CVDs is to personalize the combined therapy at an accurate dosage that is optimal for an individual patient, with the potential benefits of increasing the efficacy and safety of medications. Medication adherence also affects patient outcomes, but the development of effective interventions to control and improve it is limited by the lack of objective analysis procedures such as therapeutic drug screening [6,7]. Therapeutic drug monitoring in biological fluids is crucial for PK and PD studies. Therefore, the use of bioanalytical methods for cardiovascular drug analysis is crucial in pharmacological and clinical fields.

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Table 1. Families of drugs used in combined cardiovascular therapy.

Drug family	Group of drug
Antihypertensive drugs	• Diuretic – Loop diuretics – Thiazides – K^+-sparing diuretics – Type I receptor antagonists – Carbonic anhydrase inhibitors • Vasodilators – Angiotensin-converting enzyme inhibitors (ACEI) – Angiotensin receptor antagonists (ARA II) – Direct vasodilators – Calcium channel blockers – Nitrates – Ganglionar blockers – Renine inhibitors – Calcium channel openers • Cardioinhibitors – β-blockers – Calcium channel blockers • Total action sympaticolytic
Hypolipemic drugs	• HMG CoA inhibitors (statins) • Nicotinic acid • Fibrates • Bile acid-binding resins
Anticoagulant drugs	• Heparin • Oral anticoagulants
Antiplatelet drugs	• Glycoprotein platelet inhibitors • Platelet aggregation inhibitors
Hypoglucemic drugs	• Insulin • Sulfonylureas • Biguanidines • Meglitinides analogues • Thiazolidinediones • Inhibitors of alfa-glucosidase

Other aspect to be considered in cardiovascular drug bioanalysis is doping analysis. Some of the cardiovascular drugs, such as diuretics or β-blockers, are considered forbidden substances in some sports, they are included in the current list published by the Word Anti-Doping Agency (WADA) in 2015 [8] and their use can be punished. Therefore, these drugs must be accurately and selectively determined in urine. For this purpose, validated screening and confirmation methods for these substances need to be applied.

The existence of cardiovascular drugs with chiral center(s) in their molecules, and the different biological activity of their enantiomers is another important challenge to take into consideration. Pharmaceutical research calls for efficient and selective analytical methods for chiral analysis in order to determine pharmaceutical activity and safety of any enantiomer of a racemic mixture drug [9].

The great number of commercialized cardiovascular drugs and the frequent use of combined cardiovascular therapy make that one of the challenges of cardiovascular drug analysis is the need of the simultaneous determination of several drugs from different families with different physicochemical properties (molecular weights, acid–base properties, polarities) in complex matrices as biological fluids (plasma, urine, saliva). On the other hand, the great number of samples included in clinical studies gives rise to a considerable increase in the use of high-throughput separations in bioanalytical applications in the recent years. Therefore, the main objective of any bioanalytical laboratory is to develop reliable and efficient methods to carry out both qualitative and quantitative analysis with short analysis time.

Pharmaceutical and clinical laboratories need to solve the problems previously described in cardiovascular analysis: drug monitoring, analysis of metabolites, study of drugs interactions, drugs residues or degradation products, chiral separation and screening and confirmation of drugs of abuse in doping control [10]. Therefore, new progresses are continuously being developed in cardiovascular drug analysis.

In the case of sample preparation, this is an important step of analytical process to remove potential interferences while recovering the highest proportion of the analytes. The choice of the extraction procedure depends not only on the analytes but also on the objective of the assay, laboratory resources and matrix composition. An ineffective sample preparation can easily become a bottleneck for the quality of the whole analytical method [10]. Traditional procedures such as protein precipitation (PPT), LLE and SPE have been commonly used, but other procedures such as liquid–liquid microextraction (LLME), SPME and microextraction by packed sorbent (MEPS), among others, are being increasingly used [11]. Regarding separation, chromatographic methods have been long used as reference methods [12]. HPLC is a powerful separation technique due to its accuracy, precision, versatility and robustness, which has been employed extensively in cardiovascular drug analysis. However, nowadays to solve new bioanalytical applications satisfying the need of reducing the total analysis time in a field with a great variety of analytes, complex sample matrices and an increased demand on fast analytical results, UHPLC with sub-2-μm particles columns, monolithic and core–shell columns and 2D-LC have been introduced [12]. Environmentally friendly alternative methodologies such as supercritical fluid chromatography (SFC) and miniaturization of chromatography have also emerged.

However, challenges in bioanalytical laboratories include the development of LC–MS methods [13–15], which provide the sensitivity and **selectivity** required for quantitative analysis at very low concentration levels and also allows structural elucidation of analytes. Triple quadrupole (QqQ) coupled with LC is nowadays the reference methodology for quantitative purposes in bioanalysis. Nevertheless, HRMS, which offers an excellent **mass accuracy** and mass **resolving power**, emerges as a feasible alternative to LC–QqQ. The application of this technique for the screening, characterization and quantitation of cardiovascular drugs is described in this work.

This article is not intended to be an exhaustive examination of the literature but to show the current LC–MS methods used and new trends emerging in the field of cardiovascular drug analysis in biological fluids. This review covers the works reported in the last 10 years, describing modern sample treatment procedures, trends in instrumental and column technology in LC and innovative applications in MS.

Sample preparation for cardiovascular drug determination

Nonselective approaches, such as PPT, are preferred for screening and multitargeting purposes [7,16–18] and advantaged by factors of cost, throughput and minimal method development, while more selective extraction procedures as LLE or SPE can be favored for quantitative determination of small groups of analytes, often structurally related and analytes expected in low concentrations, requiring preconcentration steps [19–21]. These traditional approaches have already been reviewed for cardiovascular drugs and metabolites [12] and will not be discussed here.

It is very important to assure the stability of the analytes during the sample preparation conditions and that the extracted amounts of the analytes are sufficient for their analytical quantitation. International validation recommendations do not describe a minimal recovery percentage, but usually higher recoveries as possible as desired (typically higher than 80%). In the case of stability, variations of the total amount of the analytes of less than 15% are recommended.

> **Key terms**
>
> **Selectivity:** Ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants or matrix components.
>
> **Mass accuracy:** Ability of a mass spectrometer to offer *m/z* values close to the exact mass of the ion. It is reported as the deviation in parts per million from the measured mass of an ion to its exact mass. It strongly depends on the calibration of the instrument.
>
> **Resolving power:** Ability of a mass spectrometer to distinguish between two peaks at *m/z* values differing by a small amount. It is usually calculated as $m/\Delta m$, where m is the *m/z* of the ion and Δm is the full width at half height maximum of the peak. Not to be confused with resolution term.

We describe new trends in extraction procedures applied to cardiovascular drugs in bioanalysis. In this way, one of the novelties in extraction techniques is the use of molecularly imprinted polymers (MIPs). These are synthetic materials obtained via the copolymerization of a functional monomer with a cross-linker in the presence of a template molecule. After polymerization, the polymer is washed to eliminate the template molecules, and the obtained binding sites are able to recognize the template in terms of size, shape and chemical functionality, which increases enormously the selectivity of the extraction procedure (Figure 1) [22,23]. MIPs can be used to construct extraction columns for online [22] or offline [24] SPE procedures and SPME procedures [25,26]. The main application in cardiovascular analysis deals with β-blockers determination, although it has also been applied to salicylate [25] and the angiotensin II receptor antagonist telmisartan [27], in this case combined with LLME. MIPs can also be packed in pipette tips to facilitate automation.

Column switching is also an example of automation during extraction processes, which use has decreased in the last years in bioanalysis. Nowadays, sample-

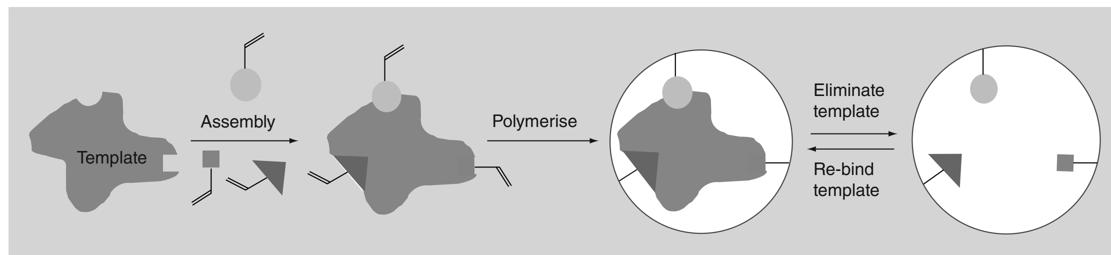

Figure 1. Molecular imprinted polymers.

Key term

Desorption electrospray ionization: Ionization procedure in which ions are generated from the sample surface by way of bombardment with high velocity, charged microdroplets through the atmosphere. The spray impact causes the formation of microscopic liquid layers on the sample surface in which the condensed-phase analyte dissolves. This process is followed by desorption via momentum transfer when additional droplets collide with the liquid layer forcing the dissolved analyte into the gas (atmospheric air) phase in the form of micron-sized droplets.

pretreatment methods that are miniaturized, simpler (preferably one-step), with minimum use of solvents and amenable to online coupling with analytical instruments, for high-throughput performance and semiautomated or automated operation are favored. SPME integrates sampling, extraction, concentration and sample introduction into a single almost solvent-free and simple step [28]. This procedure has been used for antidiabetics in human plasma [29], lidocaine in saliva [30] or captopril in human plasma using in this last case, headspace and gas chromatography (HS-SPME-GC) [31]. The so-called dispersive liquid microextraction (DLLME) is an approach of the previously mentioned microextraction. It is a rapid, easy to operate and low-cost technique based on a ternary solvent system in which the extraction and disperser solvents are rapidly injected into the aqueous sample to form a cloudy solution. Extraction equilibrium is quickly achieved due to the large amount of surface contact between the droplets of the extraction solvent and the aqueous sample. Therefore, the extraction time is very short. After the centrifugation of the cloudy solution, the extraction solvent generally settles at the bottom of the tube and is aspirated with a microsyringe for instrumental analysis [32]. This procedure has been applied to the determination of digoxin in urine samples [33], antihypertensive drugs in rat serum [34] and β-blockers and losartan [35–37]. This technique has two main drawbacks: on the one hand, the number of extraction solvents for DLLME is limited due to the need to use solvents with a higher density than water, which are collected at the bottom of centrifuge tube and, on the other hand, the high toxicity of the used extraction solvents, such as chlorobenzene, chloroform and carbon tetrachloride. To overcome these drawbacks, a DLLME technique based on solidification of floating organic droplets (DLLME-SFO), developed in 2008 by Leong and Huang [38], using low-density and low melting point organic compounds as extraction solvents has been applied to the determination of carvedilol in plasma [36].

Sometimes, the liquid-phase microextraction is based on the use of hollow fiber, as shown for isradipine in microsomal fractions of rat liver [39]. Hollow fiber stabilizes and protects the organic extractant phase. When the fiber is immersed in or suspended over the donor phase, the desired analytes can be extracted into the acceptor phase and into the lumen of the hollow fiber through the pores of the fiber wall. The acceptor solution can be directly analyzed by any further processing, providing high analyte preconcentration and excellent sample cleanup.

Analytes can also be extracted from the biological matrices using electromembrane extraction. This is a three-phase miniaturized extraction system where analytes are extracted from an aqueous sample solution, across an organic-supported liquid membrane and into an aqueous-acceptor solution by the use of an electric field, which greatly reduces the extraction time [40] (Figure 2). This procedure has been applied to the simultaneous determination of atenolol and betaxolol from urine and plasma samples [41].

A novel approach is described for the quantitative analysis of metoprolol and propranolol in blood samples by ionization of the analytes collected on SPME fibers by inserting the fiber directly into the MS [42]. Nielsen *et al.* developed a fast drug-screening method for metoprolol, propranolol and verapamil in urine including a sample treatment with MEPS (Figure 3) and analysis using a circular microchip with 60 micropillar electrospray ionization (μPESI) tips [43] without further chromatographic separation. Raterink *et al.* optimized a quantitative method for verapamil and propranolol in plasma by direct-infusion nano-ESI coupled to an Orbitrap instrument [44], with a previous gas pressure-assisted micro-LLE (GPA-μLLE) step. Manicke *et al.* developed a quantitative method with desorption electrospray ionization (DESI) in 96-sample plates for propranolol (and carbamazepine) in urine and brain extract. The method allows the analysis in 2.5 s per sample with precision (RSD) values of around 10% and accuracy (RE) values of less than 3% [45].

Another approach for sample miniaturization is the use of DBS for analysis of the drugs. After collecting about 20–30 μl whole blood and extracting the punched disc with an adequate solvent, bisoprolol, ramiprilat, propranolol and midazolam in rats [46], bisoprolol, ramipril and simvastatin in humans [47] and metformin and sitagliptin in humans [48] could be determined.

The influence of the different extraction procedures in the matrix effects during the analysis of cardiovascular drugs using nontraditional extraction approaches has unfortunately not always been studied. In some cases, the authors mention the complete elimination of matrix effects without deeply evaluation [36],

but in most cases, only the selectivity is evaluated. It is well known that enhancing selectivity reduces matrix effects, but the quantitation of this parameter should be included in the analysis of any biological sample. Isotopically labeled standards undergo similar matrix effects and therefore the quantitation of the analytes is typically not adversely affected. A reliable LC–MS method can only be built on the availability of appropriate standards and internal standards.

LC in the cardiovascular drug analysis

The analysis of cardiovascular drugs by LC has traditionally involved the use of HPLC with reversed phase columns (RP-HPLC) [20,49], and even nowadays this technique is still widely used. The introduction of UHPLC and stationary phases based on sub-2-µm particles constituted a watershed in bioanalysis. The shorter analysis times, less consumption of solvents and better resolution and sensitivity of UHPLC have caused an increase in the use of this technique. Nowadays, the UHPLC technique has been introduced in analytical laboratories of all specializations. PK studies [50], drug monitoring [51], doping control [52] or toxicity analyses [53] are some of the fields where UHPLC is established among the first choice approaches. The advantages of the use of UHPLC for the analysis of cardiovascular drugs have been already thoroughly discussed by Baranowska *et al.* [12] as well as other advances in chromatography as high-temperature HPLC (HT-HPLC), HILIC and monolithic and superficially porous stationary phases. Therefore, those aspects will be only briefly covered in this article.

Advances in columns: monolithic, core–shell & chiral columns

Since the introduction of UHPLC, great efforts have been made in order to develop new and improved stationary phases. Monolithic and core–shell columns were initially developed as a low-pressure alternative to the sub-2-µm packing materials, but the upgraded characteristics of UHPLC in comparison with HPLC, in terms of operation pressure as well as dwell and system volume [54], allowed the exploitation of the improvements in efficiency reached with these type of columns.

Monolithic columns consist of a skeleton of a continuous porous rod of silica or polymeric material. The large through-pores, that could be comparable to the interstitial voids of a particle packed column, give the material a higher permeability, which allows the use of higher flow rates, speeding the separation process without exceeding the pressure limits of HPLC. Silica-based monolithic columns, with a bimodal pore-size distribution, are preferred for the analysis of small molecules, while polymer-based monolithic columns have been proven advantageous for the rapid separation of large molecules as proteins, nucleic acids or synthetic polymers [55]. Great efforts have been made in order to optimize the polymerization process, so that mesopores are created in the organic monolithic structure, improving its application for the separation of small molecules [56,57]. The improvement of silica-based monolithic columns is also a field in constant development. For the separation of cardiovascular drugs, mainly silica-based columns have been used [58–61].

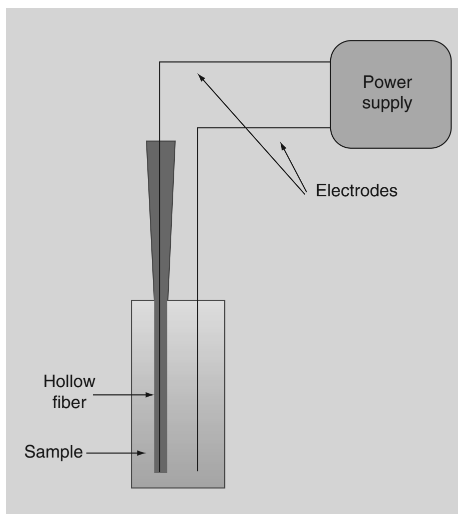

Figure 2. Equipment used in hollow fiber electromembrane extraction.

Core–shell columns are constituted of partially porous particles, with a solid core of solid-fused silica covered by a porous layer. This type of packed particles offers lower eddy dispersion and a less longitudinal diffusion compared with fully porous particles. These columns are a valid alternative to UHPLC for the laboratories equipped with HPLC systems to improve their performance, when a limited number of analytes are studied in a single run, without the higher economical investment in new equipment. Nowadays, several manufacturers commercialize a wide range of stationary phases based on the core–shell technology, being C18 the most used for cardiovascular drug analysis. Several publications compare the performance of fully porous particle columns with new core–shell columns. Ruta *et al.* [62] evaluated in 2012 different commercially available columns in terms of kinetic performance, retention capability, loading capability, selectivity and peak shape, exposing the advantages and disadvantages of each of those columns. Since then, core–shell particle columns have been continu-

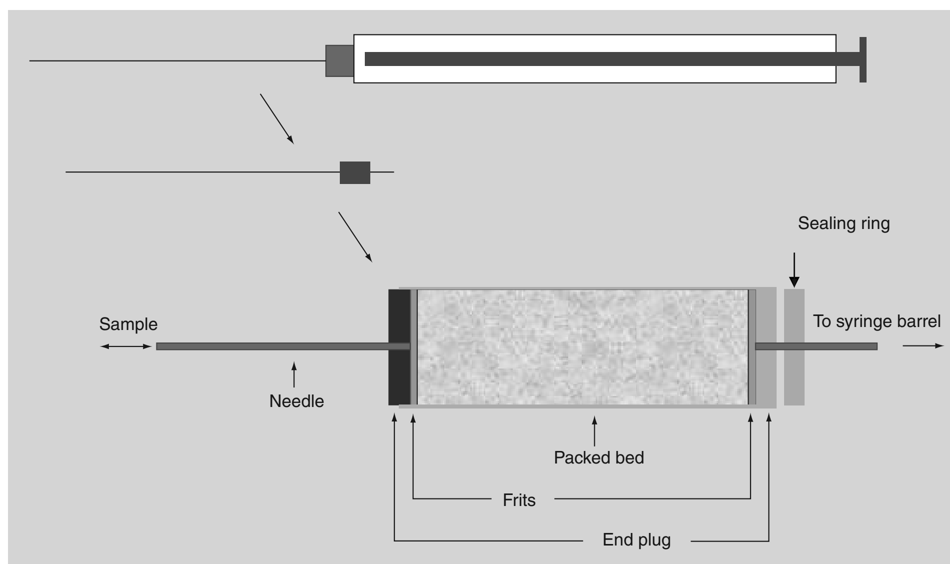

Figure 3. Microextraction by packed sorbent product syringe (250 µl).

ously evolving [63], including a wider range of stationary phases and column sizes. Dubbelman *et al.* [64] compared the Acquity UPLC BEH column, one of the most common fully porous columns for drug analysis, with six commercially available core–shell columns for the analysis of various drugs representing a large range in molecular mass, hydrophobicity and pKa values. Among the different columns studied, they found that the Cortecs UPLC C18 1.6 µm was the most suitable column for their analysis. Nowadays, it is easy to see those columns used for the analysis of different drugs, including cardiovascular ones [65,66].

Chiral separation is an issue of great concern in pharmaceutical analysis, since the different enantiomers of a racemic mixture can be responsible for different pharmacological and toxicological effects in the body. In this way, it is important to characterize the biological activity of each enantiomer and its metabolites, which could derive of the administration of a drug. The cardiovascular drugs β-blockers constitute an example of this enantiomeric-related activity. The S (-) enantiomeric form of β-blockers is a stronger antagonist of the β-receptor than the R (+) one [67]. There are numerous different possible chiral stationary phases (CSPs) used in HPLC columns, both polymeric (synthetic as methacrylate or natural as cellulose, amylose or proteins) or based in small-molecule ligands (as cyclodextrins or copper complex). The research on new CSPs is an active field, however, only some of them are commercialized, while the rest remain in the research stage. For the analysis of β-blockers, different CSPs may be used. Ali *et al.* developed a chiral method to separate the R (+) and S (-) isomers of four β-blockers in plasma using the chiral column CelluCoat and different combinations of *n*-heptane–ethanol–diethylamine [68], Wang *et al.* used an amylose CSP for the analysis of four β-blockers [69], and some studies using cyclodextrins can be found in the literature [70]. The transition from chiral HPLC to enantiomeric UHPLC (eUHPLC) faces some practical and fundamental problems, as mechanical resistance and long-term stability of particles functionalized with chiral selectors or the incompatibility of some of the commercially available UHPLC equipments with many apolar solvents used in normal-phase chromatography, commonly used in chiral chromatography that are currently object of investigation [71].

Analysis of polar compounds: HILIC

Despite its wide use in bioanalysis, there are some challenges that RP-UHPLC is not able to solve yet. The limitations of this technique with respect to retaining highly hydrophilic compounds opened the door to the popularization of HILIC for both endogenous and pharmaceutical compounds [72]. HILIC uses a polar stationary phase, such as bare silica, amino or sulfobetaine-type zwitterionic columns, and a mobile phase with a high content of aprotic organic solvent, where water is the strong eluent. The retention mechanism is a complex system involving partition, polar and ion exchange interactions, therefore the optimization of a separation is not always as predictable as it can be with a C18 column.

HILIC is especially useful for the simultaneous determination of parent drugs and their commonly more polar metabolites. Kovaříková *et al.* [73] compared different HILIC columns to separate the cardioprotectant dexrazoxane from three of its highly polar metabolites. Using a zwitterionic HILIC stationary phase and studying systematically different variables, column temperature, mobile phase strength and pH, they were able to separate all compounds, including two position isomers which differed each other only by the position of a methyl group. HILIC chromatography was also used by Kasagić-Vujanovića *et al.* [74] to determine amlopidine besylate and bisoprolol fumarate in pharmaceuticals using chemometrically-assisted optimization. The authors applied a central composite design for the optimization of the mobile phase–acetonitrile content, pH of the aqueous phase and concentration of ammonium acetate. Mazzarino *et al.* [75] used HILIC for the screening and confirmation analysis of different drugs, including several β-adrenergic agents in human urine.

2D-LC

Despite the progress in instrument technology and the development of new and improved stationary phases, 1D chromatography either using RP or HILIC may not have enough resolution power to separate a large number of compounds of very different polarities in a reasonable time of analysis. An approach for the analysis of this complex samples is given by 2D-LC, where columns of independent (orthogonal) retention mechanisms are used. Under ideal conditions, where the coupled columns are completely orthogonal, the peak capacity of the system will be equal to the product of the peak capacity of each dimension [76]. Even though the first mentions to multidimensional chromatography date back to the late 1970s [77], it is still nowadays object of discussion and development [78,79], and there is an increased interest for its use in pharmaceutical analysis in recent years.

Two commonly used 2D-LC separation modes are heart-cutting (LC–LC) and comprehensive (LC × LC) (Figure 4) [80] . In the LC–LC approach, fractions of interest are collected from the first column and transferred to the second. This process can be done offline, reinjecting collected fractions into a second LC system. However, this offline approach requires handling the sample to transfer it from one dimension to the other, which may lead to some disadvantages like sample contamination or losses, and also increases the time of analysis while diminishing the repeatability of the method. To perform this approach online, a switching valve is used, so that fractions from the first dimension are collected in a loop and introduced in the second column without flow interruption. This technique is commonly used when the number of fractions of interest is limited, and it is useful to resolve overlapping peaks from the first chromatographic step, to be used with sample-cleaning purposes or to preconcentrate some analytes of interest.

In LC × LC, the entire eluate is transferred from the first into the second dimension, so that the whole sample is subjected to two different separations. There are three conditions, so that a 2D-LC separation could be called comprehensive [81]: every part of the sample must be subjected to two independent separations, equal percentages of all sample components must pass through both columns and reach the detector, and the separation from the first dimension have to be maintained. One of the drawbacks of LC × LC is the possible incompatibility of mobile phases used in each dimension. Therefore, the interface technique used should be carefully studied. Some of the techniques used are dual loop, stop flow and vacuum evaporation interfaces. Also, the data obtained from LC × LC are more complex than the data obtained from monodimensional chromatography. Several articles found in bibliography deal with the treatment of these complex data [82,83] for 2D-chromatographic systems.

Applications of 2D-LC have been reported mainly for protein analysis [84] and also for the separation of polymers [85] and some small molecules [86]. Despite its high potentiality of separation in pharmaceutical samples, the applications of these methods to the analysis of cardiovascular drugs are scarce. Sheldon applied in 2003 LC–LC to the analysis of a synthetic mixture of ten pharmaceutical compounds, including atenolol [87]. Rainville *et al.* [88] combined the use of DBS and multidimensional chromatography technology for the analysis of five drugs, one of them rosuvastatin. By using a 2D-LC system with at column dilution, they were able to inject directly into a reversed-phase system the highly organic extract from DBS, reaching a limit of detection for rosuvastatin of 0.5 ng/ml.

Supercritical fluid chromatography

SFC has emerged in recent years as an alternative of other LC techniques in the pharmaceutical industry [89]. The introduction of modern SFC instruments with improved back pressure regulation, pumping and injection systems, as well as the coupling with MS detectors made SFC suitable for analytical, semipreparative and preparative separation of small molecules.

SFC utilizes carbon dioxide in a supercritical state in combination with a limited proportion of polar organic solvent, commonly alcohols, as mobile phase. The low viscosity and high diffusivity inherent to supercritical fluids allow the use of higher flow rates without a high

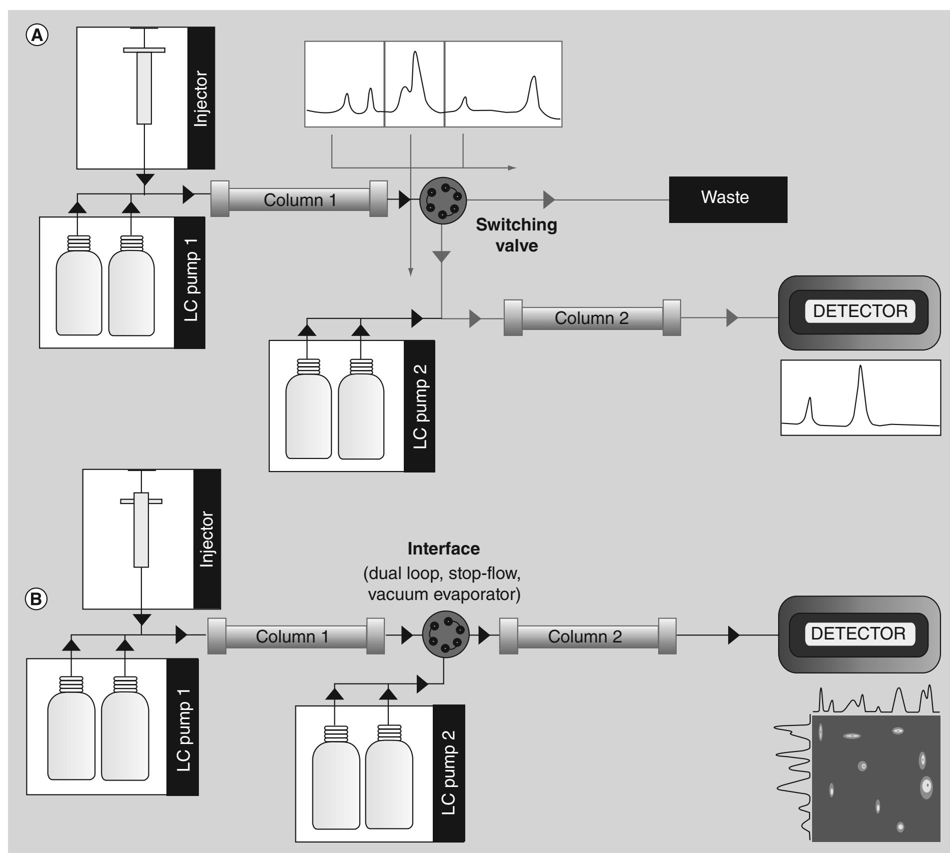

Figure 4. 2D-LC. Schematic representation of **(A)** heart-cutting 2D-LC (LC–LC), and **(B)** comprehensive 2D-LC (LC × LC).

increase in the backpressure. The retention mechanism of SCF is multimodal, combining partitioning, adsorption through H-bonding and electrostatic and ionic interactions [90]. Compared with conventional LC, SFC offers lower mobile-phase consumption, an option for an alternative selectivity and shorter time of analysis, and it is considered environmentally friendly as it reduces the consumption of contaminant solvents [91].

SCF has emerged as the preferred choice for the separation of enantiomeric mixtures. Many pharmaceutical companies use SFC at analytical and preparative scale for chiral separations [92,93]. Methods for the separation of enantiomers using SFC have been published for some cardiovascular drugs as clopidogrel [94], warfarin [95], propranolol and pindolol [96,97] or timolol [98], among others.

Even though the enantiomeric separation has been the main application of SFC in the pharmaceutical field the growing interest for its use in achiral separations is noticeable. Highly remarkable is the work of Novákovác *et al.* [14], using ultrahigh performance SFC (UHPSFC) for the screening of doping agents in urine samples.

Innovation in MS

QqQ as a gold standard

Since the first commercial LC–MS devices appeared in the late 1980s and early 1990s, QqQ coupled to LC gained an exponential importance in bioanalysis until they became the gold standard for quantitation in only a few years [99]. QqQ (and also ion-trap instruments) still plays this prominent role especially thanks to the possibility of working in SRM, which offers a high selectivity, sensitivity and analysis speed.

SRM has been widely used in cardiovascular drug analysis both for screening and quantitative purposes. Since the aim of this review is to focus in the most innovative trends in MS and the use of SRM for cardiovascular drugs has already been reviewed somewhere else [12,100,101], this topic will not be thoroughly

discussed. Initially, QqQ multicomponent analysis was seriously hampered by the dwell time [102] and consequently only a few analytes could be simultaneously analyzed. In the course of time, the new instruments overcame this problem by increasing the acquisition rate and splitting the SRM in time windows, allowing in this way the simultaneous analysis of multiple compounds [17]. Another inconvenient for fast analysis is the time needed to switch polarity when both positive and negative polarity modes need to be used in one analysis. In these cases, two different runs are usually necessary [103,104], but there are also instruments which offer a fast switching time allowing the analysis in positive and negative modes in a single run [105,106]. Due to all these improvements, cardiovascular drugs (especially diuretics and β-blockers) can be analyzed together with other drugs of doping or toxicological interest.

SRM is a powerful tool for screening and usually two transitions are used per analyte for confirmation purposes. However, sometimes, this is not enough and leads to false-positive results. The lack of selectivity can be overcome using data-dependant acquisition methods (DDA) in QqQs or ion traps. These methods use different survey scan functions to trigger the collection of fragmentation spectra in MS/MS mode that are afterward compared with a spectral library to identify the compound or confirm the positive match. By using a MS scan function as survey function, general unknown screening methods (GUS) can be developed. For instance, Sauvage *et al.* [107] used a MS scan function to trigger an enhanced product ion (EPI) spectrum for a maximum of three ions using collision energy spread. The EPI spectra are then compared with a spectral library (1000 spectra in positive mode and 250 in negative mode) in order to identify the compounds. Sturm *et al.* followed the same approach for a method including online SPE with atmospheric pressure chemical ionization (APCI), but selecting only the most intense ion for MS/MS analysis [108]. They successfully applied the method for the analysis of several drugs and metabolites in urine and plasma samples using a spectral library of more than 365 compounds. Mueller *et al.* developed an online extraction with turbulent flow chromatography method with a MS scan survey function that only triggers the DDA function when an ion included in a candidate list exceeds the intensity threshold [109]. The linear ion trap switches polarity within the same run and performs both MS^2 and MS^3 fragmentation spectra.

Dresen *et al.* developed a targeted method for the screening of 700 compounds in biofluids including many cardiovascular drugs using a 'scheduled' SRM survey scan [110], where the DDA function is used to confirm the positive match. Above a certain threshold for the SRM, an EPI spectrum is performed for a maximum of two parent ions at a time point and the obtained information is compared with a spectral database with more than 5600 spectra of 1253 compounds [111]. Same approach was used by Politi *et al.* for the screening of 24 diuretics in urine after sample dilution [112].

Drift toward HRMS

Even if QqQ instruments working in SRM are the most common analyzers used in bioanalysis [13], they have some limitations. On the one hand, the number of transitions that can be measured depends on the duty cycle, which can be a problem when dealing with hundreds of mass transitions and narrow chromatographic peaks. On the other hand, only targeted compounds are detected, which means that valuable information about metabolites or degradation products is lost. Some of these problems can be overcome with the DDA, but these analysis rarely allow quantification and show some limitations when several analytes coelute [113]. Due to all these facts, HRMS is gaining importance in bioanalysis. Some authors have compared the shift from SRM to HRMS with the paradigm shift from UV detection to MS detection [114]. HRMS solves problems associated to duty cycle, allows untargeted analysis and data reinterrogation, does not require extensive optimization and provides excellent mass accuracy and mass resolving power while keeping quantification performance. The negative side is that the instruments are obviously more expensive, require frequent calibration and the sensitivity cannot be compared with QqQ instruments yet. Therefore, for analytical purposes for which sensitive quantification of a reasonable number of compounds is necessary without additional untargeted information, QqQ will remain the gold standard. Despite these drawbacks and considering all the advantages, HRMS has been lately applied to bioanalysis [115,116] and consequently to cardiovascular drug analysis for screening, characterization and quantification purposes.

Screening of cardiovascular drugs using HRMS

While low-resolution MS screening methods rely on mass transitions (or spectral libraries with DDA), HRMS screening methods are based on accurate mass and isotopic pattern. This is only possible thanks to the ability of these instruments to acquire information over a whole *m/z* range. The two most important parameters in terms of identification are mass accuracy and resolving power [117]. Even with a sub-1-ppm mass accuracy, several elemental compositions are possible; thus, additional information such as isotopic pattern (mass, abundance and spacing) or precursor fragmentation is necessary for a more reliable identification [118]. In order to get

fragmentation information, hybrid mass spectrometers can be used including a quadrupole prior to the HRMS to select the precursor ion. Depending on the approach used for analyte identification, different screening methods can be found for cardiovascular analysis.

Using a single MS scan

Analysis methods using a single MS scan function are very common for the screening of cardiovascular drugs in biofluids, especially in toxicological and doping fields, where these drugs (mainly diuretics and β-blockers) are analyzed together with many other banned or toxic substances. The most usual platforms include an HPLC or UHPLC separation coupled to ToF [119–121] or Orbitrap [122] spectrometers, with fast polarity switching in the same injection if the instrument allows it or one injection for each polarity if it does not. Screening methods usually include a database with the retention time and the accurate mass of the analyte (sometimes also the isotopic pattern), so that when a compound falls inside the retention time and mass tolerance window, a positive match is reported. In some cases, hybrid MS (Q-TOF) instruments are used performing a first injection in wide-pass quadrupole mode and a second injection with quadrupole selection for a more reliable identification [119,121,123].

Using DDA & library search

As is feasible with low-resolution instruments, DDA can be employed using a quadrupole prior to the HRMS detector. In this way, in addition to obtain cleaner spectra high-accuracy fragmentation information is available for a more reliable identification. Oberacher's group contributed remarkably to this field with a wide spectral library for small-molecule identification with more than 10,000 spectra for over 1200 compounds [124]. In the last few years, they have made important advances in spectral search algorithm development and in the transferability between laboratories and instruments. The library includes all type of cardiovascular drugs and has been successfully integrated in LC–MS methods [125]. Broecker *et al.* also developed a LC–Q-ToF platform including 234 cardiovascular drugs for toxicological analysis that can also be applied to metabolite identification [126]. Thomas *et al.* developed a DDA method for DBS analysis using Orbitrap in which a survey function switches between polarities with 75,000 full width at half height maximum (FWHM) resolving power and the DDA of a maximum of three ions is acquired at 17,500 FWHM [127]. They successfully applied method for the identification of 28 model drugs (including bisoprolol, hydrochlorothiazide, metoprolol and propranolol).

Using MS^{All}

All ion fragmentations, also known as MS^{All} or MS^{E}, use a data independent acquisition (DIA) approach with a collision energy switch between low-energy and high-energy status. In the low-energy mode, mass spectra of the intact molecules are obtained and in the high-energy mode mass spectra of the fragments. In this way, low-energy mode provides information comparable to a MS scan, while high-energy mode gives fragmentation information. Since the quadrupole operates in wide-pass mode, all the fragments are obtained and they are assigned to the corresponding precursor using advanced algorithms. This approach is extremely useful to obtain complete information in a single run but offers dirtier fragmentation spectra than DDA due to the fact that quadrupole is not used as a filter. Pedersen *et al.* used a low-energy function (4 eV) and a high-energy function with a collision energy ramp (10–40 eV) for the screening of 256 compounds in whole blood including several cardiovascular drugs (e.g., β-blockers, diuretics, statins and calcium channel agonists), which was applied to more than 1000 toxicological samples. Similarly, Chindarkar *et al.* [128] applied MS^{All} for drug screening in urine samples previously analyzed by other techniques. The method was able to confirm 92% of the true positives and additionally identified other drugs missed by the reference methods. De Castro *et al.* proposed a different screening approach where precursor and fragmentation information is included in a single function [129]. The so-called in-source collision-induced dissociation (ISCID) requires a careful optimization of the fragmentation parameters, so precursor and fragment ions of different analytes can be simultaneously detected. Yet, it will be possible only depending on their physicochemical properties. They compared the method with a traditional LC–ToF method using only precursor ion information and ISCID provided more reliable identification due to the additional information provided by the fragment ions.

Using SWATH

Sequential window acquisition of all theoretical fragment ion mass spectra (SWATH) is a novel DIA acquisition technique based on the fragmentation of all the precursor ions entering the mass spectrometer in *m/z* windows isolated by the quadrupole (usually in 20–35 *m/z* range). This technique keeps the benefits of MS^{All} and also increases the selectivity and the quality of the fragmentation spectra thanks to the quadrupole selection. Obviously, depending on the width of the isolation windows, the quality of the fragmentation spectra will vary and will not be as clean as for DDA where a single mass is selected. Therefore, compounds with a similar *m/z* will pass the quadrupole simulta-

neously and interfere in their corresponding MS/MS spectra, although this problem can be overcome with deconvolution algorithms similarly to those for MS^{All}.

Oberacher and colleagues already compared the SWATH and DDA performance for their toxicological screening library and found that SWATH was much more sensitive with only a false-positive rate of 0.06% [130]. Roemmelt *et al.* also obtained satisfactory identification results using their spectral library with SWATH and with a forced coelution experiment of 20 analytes (including amiodarone) proved that SWATH can detect more analytes than DDA [113]. Zhu *et al.* additionally compared MS^{All} with SWATH and DDA for the analysis of microsomal incubations with eight test compounds (including propranolol) and urine samples [131]. They found DDA to offer the best fragmentation spectra but to miss some parent ions, while MS^{All} and SWATH offered a 100% hit rate. Among the DIA methods, SWATH outperformed MS^{All} considering the quality of the fragmentation spectra.

HRMS for untargeted analysis & characterization

HRMS is a powerful technique for drug screening thanks to the fast acquisition time in a wide *m/z* range. This ability makes it also extremely suitable for other fields related to drug discovery such as metabolite and degradation product identification [115,116]. These compounds could also be identified by low-resolution mass spectrometers using neutral loss or precursor ion scan, but still some biotransformations can be overlooked and isobaric compounds could lead to misunderstandings. Consequently, there has been a shift in the last years toward HRMS for metabolite/degradation product identification, supported by different data mining methods (e.g., mass defect filter, dealkylation tools and isotopic pattern filter).

One of the works that best explains this shift was performed by Rousu *et al.* [132]. They carried out metabolism experiments for verapamil in human liver microsomes and compare the results obtained by TOF, Orbitrap, QqQ and hybrid linear ion-trap QqQ coupled to LC. ToF proved to be the best choice identifying 69 metabolites without requiring any metabolite prediction. Although Orbitrap offered the best mass accuracy, its lower acquisition rate required slower chromatography and the lower sensitivity led to false negatives. Nevertheless, this technique is extremely useful when more accurate structural elucidation is necessary. Regarding the low-resolution MS detectors, the required metabolism prediction made them time-consuming and laborious. And yet, with a careful design of the acquisition experiments, several metabolites were missed. Another applicability of HRMS in bioanalysis was developed by Erve *et al.* for the fast and complete metabolism study of prazosin in urine, bile, feces, plasma and brain homogenate using Orbitrap with direct nanospray infusion including a ZipTip cleanup procedure [133]. The use of direct infusion (no finite peaks as in LC) allowed the use of the highest resolving power of the instrument (100,000 FWHM) with a mass accuracy below 1 ppm, thus, metabolites in the different matrices could be detected with a high sensitivity and accurately elucidated with the exception of isomeric compounds. Hopfgartner *et al.* compared the use of DDA, SWATH and MS^{All} for the elucidation of talinolol metabolites in liver microsomes [134]. They found SWATH to be the best approach for metabolite discovery, identifying six hydroxylated metabolites and offering high-quality spectra. DDA offered even cleaner spectra, but some relevant precursor ions were not selected for fragmentation experiments due to their low response. On the other hand, MS^{All} was not able to provide high-quality spectra for low abundant metabolites due to the interference of coeluting compounds.

Besides using HRMS for metabolite identification, it has also been applied in other fields of drug discovery especially in the study of degradation products. Using ToF detection in combination with MS^n (up to MS^7) and hydrogen/deuterium exchange studies, Singh and colleagues found 11 stress degradation products for rosuvastatin [135], 7 for fosinopril [136] and 3 for irbesartan [137]. Same research group identified the degradation/interaction products of a combination of atenolol, lisinopril and aspirin [138] and of atenolol with different excipients [139] by comparing the fragmentation patterns. Indeed, HRMS is very useful to elucidate the fragmentation pathways and better understand the molecule of interest, as Chytil *et al.* showed for rilmenidine using an Orbitrap instrument [140].

HRMS for quantitative analysis

Traditionally, HRMS instruments have not been used for quantitative analysis mainly due to their limitations in dynamic range (detector saturation) and sensitivity, but thanks to the latest advanced in HRMS technology (e.g., improved detectors, fast electronics and tube temperature stabilization) more sensitive instruments have been developed with dynamic ranges of more than three orders of magnitudes and an excellent mass accuracy (<5 ppm) and resolving power (>20,000 FWHM) (Table 2).

Jiang *et al.* showed an interesting applicability of HRMS by quantifying rosiglitazone and its metabolite 5-OH-rosiglitazone in rat plasma without authentic metabolite standard [141]. Initially, a calibration curve for rosiglitazone was built and the samples of interest analyzed by using the calibration curve of the parent

Table 2. Quantification methods for cardiovascular drug analysis using high-resolution mass spectrometry.

	Analyte	Matrix	Sample treatment	Calibration range	Ref.
1	Rosiglitazone 5-OH-rosiglitazone	Rat plasma	PPT	2.5–5000 ng/ml	[141]
2	Atenolol	Human blood	DBS and solvent extraction	25–1500 ng/ml	[142]
3	Bisoprolol Ramipril Simvastatin	Human blood	DBS and solvent extraction	0.1–100 ng/ml 0.5–100 ng/ml 1–100 ng/ml	[47]
4	Ten thiazide diuretics	Human urine	Extraction, hydrolysis and SPE	25–400 ng/ml	[143]
5	Amiloride Atenolol Propranolol Warfarin	Human hair	Methanolic sonication extraction	50–2500 pg/mg	[144]
6	Verapamil	Mouse brain and plasma	PPT	2–500 ng/ml (no Q selection) 0.1–100 ng/ml (Q selection)	[145]

PPT: Protein precipitation.

compound to quantify also 5-OH-rosiglitazone. Once the metabolite standard is obtained, the difference in response for both analytes is calculated and a correction ratio estimated. In this way, previous results were recalculated. These results were compared with the direct quantification of the metabolite and showed to be highly comparable. This approach opens new perspectives in PK/PD since data obtained in early stages by HRMS can be reanalyzed once a metabolite is found to be of interest.

Lawson *et al.* applied ToF detection for the analysis of atenolol, bisoprolol, ramipril and simvastatin in DBS [47,142], obtaining LLOQ values as low as 0.5 ng/ml and a dynamic range of 4 orders of magnitude. Furthermore, they successfully applied the method for assessing medication adherence in control volunteers.

Peters *et al.* developed an ambitious LC–ToF method with a generic sample preparation for the quantitative screening of 56 doping agents in urine [143]. Among these compounds, ten thiazide diuretics were analyzed in ESI mode. All of them showed an accuracy within 70–140%, which was considered to be acceptable. Dominguez-Romero *et al.* also developed a LC–ToF quantitative screening method for several drugs in hair (including amiloride, atenolol, propranolol and warfarin) obtaining a good precision, linearity and LLOQ as low as 15 pg/mg [144]. Additionally, they optimized the fragmentor voltage in order to get in-source fragmentation and increase the identification reliability.

HRMS quantification can also be performed in tandem mode by using hydride instruments such as Q-TOF. Ding *et al.* compared the quantitative performance of a SRM method with a QqQ, a ToF scan method and a Q-ToF method for the analysis of verapamil (and another eight compounds) in a rat plasma/brain homogenate-mixed matrix [145]. The ToF scan offered the lowest sensitivity but for SRM and Q-ToF analyses LLOQ were similar. In terms of selectivity, no significant matrix interferences were observed in any of the analysis methods. It is important to highlight that while the selectivity of a QqQ is predefined by the acquisition settings (quadrupole mass window), the selectivity of a ToF method depends on the mass extraction window chosen in postacquisition data analysis. The authors also proved that there was a good correlation between verapamil concentrations obtained using QqQ and Q-TOF. In this way, ToF analysis is advised for general quantitative screening considering the time saved in method optimization turning to the Q-ToF approach when the sensitivity is not enough.

Conclusion & future perspective

The most important innovations in cardiovascular drug analysis deal with low sample volume (using miniaturized sample preparation procedures), short analysis time (increasing the applications of UHPLC and chromatographic column miniaturization) and fast and selective MS techniques (accurate mass and isotopic patterns) for quantitative approaches. Other trend is the use of screening procedures for a large number of analytes, to avoid analyzing one sample in several runs.

Miniaturization is not only emerging in cardiovascular drug analysis but also in modern bioanalytical appli-

cations. The need to maximize the amount of information obtained while minimizing sample volume spurred the development of miniaturized techniques. Other advantages of miniaturized LC systems are lower solvents consumption, reduction of system dead volume, faster analysis, reduction of matrix effect for some applications and portability of the analytical system. The use of packed silica capillary columns and the advances made in the production of polymeric and silica monoliths led to the development of several miniaturized LC systems as nano-LC instruments and chip formats.

Nano-LC/MS applications have mainly been focused on proteomic analysis, however, its advantages compared with conventional LC/MS make it attractive for the analysis of small molecules as metabolomics or pharmaceutical analysis [146,147], even though the application of the nanoscale LC to the pharmaceutical field is still limited. Fanalli *et al.* [148] applied nano-LC with UV detection for the separation of several β-blockers, using two capillary columns packed with C18 silica particles. Also, a nano-LC system coupled to MS/MS using a nano-ESI source was employed for the analysis of valsartan in plasma using only 10 µl of sample [149]. Nowadays, nano-ESI–MS without further separation technique is applied for single-cell analysis in lipidomics and proteomics and could be very interesting for pharmaceutical analysis, especially for targeted therapeutics.

Another emerging technique in drug analysis is ion mobility spectrometry (IMS). This separation technique based on the size, shape and charge of the molecule is known since the beginning of 20th century, but it was not until the last decade that it began to be used for small-molecule analysis [150]. The hyphenation of IMS to LC–MS adds an orthogonal separation dimension, which can be very useful to separate chiral compounds, to increase the selectivity of a method or to better understand the shape of the molecule. Considering all these advantages and the latest improvement in instrumentation, IMS will probably have a significant impact in cardiovascular drug analysis during the following years. So far, its applicability has already been proved by some authors: Dwivedi *et al.* used chiral IMS to separate (R) and (S) atenolol by using the chiral modifier (S)-(+)-2-butanol in the drift gas [151]; Kapron *et al.* used FAIMS (high-field asymmetric waveform IMS) to improve the selectivity of norverapamil detection in urine by removing matrix interferences [152] and Shimizu *et al.* proposed a smart method to determine the position of glucuronidation of ezetimibe metabolites with IMS-ToF [153].

In this way, the authors propose for the next years automated, faster and more sensitive applications for cardiovascular drug determination in bioanalysis, in which extraction procedures and separations techniques are going to be gradually replaced by more selective innovations in MS, such as IMS, allowing sample analysis times of about 1 s.

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Executive summary

Sample preparation for cardiovascular drug determination

- Extraction procedures tend to miniaturization of sample using techniques that also are suitable for automation.

LC in the cardiovascular drug analysis

- UHPLC equipped with new and improved columns is replacing reversed phase-HPLC in high-throughput pharmaceutical and biological analysis.
- 2D-LC is a powerful technique to enhance resolution in cardiovascular drug analysis.

Innovation in MS

- Although triple quadrupole remains as the first choice detector for cardiovascular drug analysis, HRMS is gaining importance not only in qualitative but also in quantitative analysis.
- Detection approaches such as MS^{All}, SWATH or DDA have emerged due to the need of a more reliable identification of multiple compounds in doping and toxicological analysis.

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- **Innovative approach to elucidate the position of the glucuronidation using ion mobility and calibration cross-section regression lines.**

Sindrome metabolikoa: XXI. mendearen madarikazioa

Oskar González[*1,2], *María Encarnación Blanco*[1], *Gorka Iriarte*[3], *Estitxu Rico*[1], *Itxaso Maguregui*[4], *Rosa M. Alonso*[1]

[1] Kimika Analitikoa Saila, Zientzia eta Teknologia Fakultatea (UPV/EHU),

[2] Analytical Bioscience Division, LACDR (Leiden University)

[3] Arabako Osasun Publikoko Laborategia (Osasun Publikoaren eta Adikzioen Zuzendaritza, Eusko Jaurlaritza),

[4] Pintura Saila, Arte Ederretako Fakultatea (UPV/EHU),

* oskar.gonzalezm@ehu.es

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Laburpena: Bizi-baldintzen etengabeko bilakaeran gaur egungo gizarteak bereganatu dituen hainbat ohituren ondorioz (dieta desorekatua eta ariketa fisiko eskasa besteak beste), esan daiteke eritasun kardiobaskularren intzidentzia goraka doala. Eritasun kardiobaskularren izenpean hainbat patologia topa ditzakegu (bihotzekoa, istripu zerebrobaskularra...) eta hauen atzean dauden arrisku-faktoreak desberdinak dira: hipertentsioa, kolesterola, diabetesa, obesitatea… Arrisku-faktore hauek ez dira normalean banaka agertzen, eta elkarrekin konbinatzen dira *sindrome metabolikoa* delakoaren barruan. Sindrome metabolikoa kontrolatzeko bizimoduan aldaketa txikiak baina garrantzitsuak ezarri behar dira eta neurri horiek nahikoak ez direnean bakarrik hasi behar gara medikamentuak erabiltzen. Arrisku-faktore bakoitzak tratamendu ezberdina behar duenez, beharrezkoa gertatzen da farmako ezberdinez osaturiko *terapia kardiobaskular konbinatua* delakoa. Orokorrean, terapia honetan, hipertentsioaren aurkako botikak erabiltzen dira presio arteriala jaisteko, hipolipemianteak gorputzeko gantz kontzentrazioak kontrolatzeko, eta bestetik intsulina edo diabetesaren aurkako botikak glukosa mailak murrizteko. Horretaz gain, koagulazioa eta agregazio plaketarioa saihesteko botikak erabiltzen dira, sarritan gertatzen den arterien buxadurak ekiditeko.

Hitz gakoak: eritasun kardiobaskularrak, sindrome metabolikoa, terapia kardiobaskular konbinatua.

Abstract: The evolution in life-style the contemporary society has undergone, such as a movement towards unbalanced diet and scarce physical exercise, leads to an increase in cardiovascular diseases. Among these diseases we can find different pathologies

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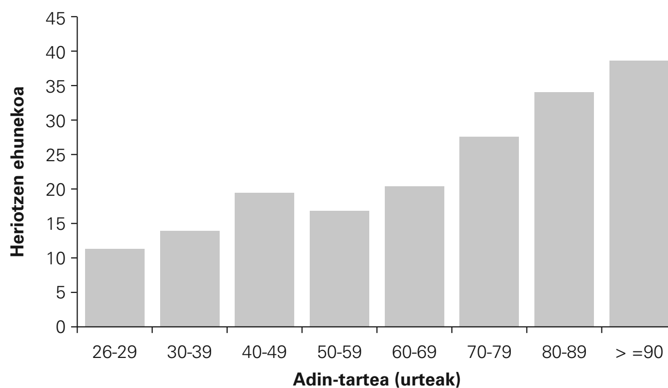
(strokes, heart attacks...) with incidence dependent on some risk factors such as hypertension, cholesterol level, diabetes, obesity... These risk factors often appear together which is known as *metabolic syndrome*. In order to control this syndrome some minor but important changes in life-style must be carried out. When this is not enough, a medical treatment is necessary to combat the different risk factors. These require different treatments and therefore a *combined cardiovascular therapy* involving different drugs is necessary. Usually, in this therapy, antihypertensives are used to decrease blood pressure, hypolipidemic agents to control fat levels and insulin or antidiabetics to reduce glucose levels. Furthermore anticoagulants and antiplatelets are frequently prescribed in order to avoid arterial clotting.

Keywords: cardiovascular diseases, metabolic syndrome, combined cardiovascular therapy.

1. SINDROME METABOLIKOA ETA ERITASUN KARDIOBASKULARRAK

Urteen poderioz, gizarteak bizi-baldintzen etengabeko bilakaera jasan du: ehiztari eta nomada-ohiturei bizitza sedentario eta erosoak gailendu zaizkie; esku-lanak lan mekanikoari utzi dio bidea eta aisialdiak aktibitate fisikoak baztertu ditu. Gainera, kontuan izanda gure dieta gantz saturatuetan eta azukreetan aberastu dela, ulertzekoa da azken urteetan eritasun kardiobaskularrak lurralde garatuetan lehenengo heriotza-kausa bihurtu izana.

Hala ere, pentsa daitekeenaren kontra, gaitz kardiobaskularrek eragindako heriotzen %80a garapen bidean dauden lurraldeetan gertatzen da. Izan



1. irudia. 2012 urtean eritasun kardiobaskularrek EAEn eragindako heriotzak, adinaren arabera.

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ere, zailtasunak daude herrialde hauetan prebentzio eta tratamendu osasun-zerbitzu egokiak eskaintzeko, eta egoera ekonomikoak dieta desorekatuak ekarri ditu [1].

Euskal Autonomi Erkidegoan (EAE) 2012ko datuen arabera, zirkulazio-arazoak direla kausa, 6210 biztanle hil ziren, hildako guztien %31. Eritasun hauek pairatzeko arriskua adinarekin estuki lotuta dago; izan ere, hildakoen %87ak 69 urtetik gorakoak ziren (1. irudia). Era berean, sexuaren arabera ezberdintasun nabarmenak daude, eta gizonezkoen artean heriotzatasa %28koa da baina emakumeen artean %33koa [2].

1.1. **Eritasun kardiobaskularrak**

Eritasun kardiobaskularren izenpean hainbat patologia topa ditzakegu: besteak beste, kardiopatia iskemikoa, garuneko istripu baskularrak, arteriopatia periferikoa, tronbosi benoso sakona, sukar erreumatiko akutua edo bihotzaren sortzetiko malformazioa.

Eritasun kadiobaskularrak pairatzeko posibilitatea hainbat arrisku-faktoreren menpe dago: hipertentsioa, kolesterola, diabetesa, obesitatea… Arrisku-faktore hauek ez dira normalean banaka agertzen, eta elkarrekin konbinatzen direlarik, gaixotasun kardiobaskularrak azaleratzen dira [3]. Arrisku-faktoreen konbinaketa honi, hain zuzen ere, *sindrome metabolikoa* deritzo.

1.2. **Sindrome metabolikoa**

Azaldu den bezala, sindrome metabolikoa eritasun kardiobaskularrak pairatzeko probabilitatea areagotzen duten arrisku-faktoreen konbinaketa da. Hala ere, komunitate zientifikoan ez dago adostasun handirik gaixotasun hau diagnostikatzerako orduan, ikuspuntu ezberdinak baitaude arrisku-faktoreak zehaztean. Berri samarra den eritasun honen lehenengo aipamena Reaven doktoreak [4] egin zuen 1988. urtean eta hortik aurrera, definizio ezberdinak agertu dira baita izen ezberdinak ere: X-sindromea, intsulina-erresistzentziaren sindromea, sindrome plurimetabolikoa, laukote hiltzailea… Lehenengo definizio bateratua ez zen 1998. urtera arte plazaratu Osasunaren Munduko Erakundearen (OME) eskutik [5]. Dena den, sindrome metabolikoa irizpide honen arabera detektatzea neketsua eta garestia izateagatik gehien erabiltzen den definizioa NCEP-ATPIII (*National Cholesterol Education Program's Adult TreatmentPanel III*) txostenean agertzen dena da. 2001. urtean egindako biltzarrean AEBetako *National Heart, Lung, and Blood Institute* eta *American Heart Association (*AHA) erakundeek sindrome metabolikoaren definizioa berrikusi zuten. Ordutik hainbat aldaketa egin dira azken urteetan [6]. 1. taulan ikus daitekeen definizioak 5 aldagai hartzen ditu kontuan: obesitate abdominala [7], hipertentsioa, tri-

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glizeridoak, dentsitate altuko lipoproteinak (HDL kolesterola) eta glukosa mailak. Parametro hauetatik hiru mugaz kanpo daudenean sindrome metabolikoa diagnostikatzen da [8].

1. taula. NCEP-ATPIII-ko irizpideak sindrome metabolikoa identifikatzeko.

Parametroak	Mugak
Hipertentsioa	>135/85 mmHg (Sistolikoa/diastolikoa)
Loditasun abdominala*	>102 cm (Gizonezkoak) >88 cm (Emakumeak)
Triglizeridoak	>150 mg/dL
HDL kolesterola	<40 mg/dL (Gizonezkoak) <50 mg/dL (Emakumeak)
Glukosa baraurik	>100 mg/dL

* Etniaren arabera balioak alda daitezke.

Ikerketa ezberdinen arabera, uste da munduan populazio helduaren %20-30ak jasaten duela gaitz hau, talde horretakoek eritasun kardiobaskularrak pairatzeko aukera bikoitza daukatelarik [9]. Mundu mailan aurrera eraman diren ikerketa ezberdinen artean, Massachusetts-eko Framingham herrian 1948. urtean hasi zen ospetsuena eta sakonena izan dena [10]. Lortutako emaitzei esker gaixotasun kardiobaskularrak pairatzeko aurresateko egitasmoa garatu zuten [11].

1.3. **Sindrome metabolikoaren arrisku-faktoreak**

1.3.1. *Hipertentsioa*

Odolak zirkulatzean gorputzeko odol-hodietan eragiten duen indarrari, presio arteriala deritzo. Indar hori gehiegizkoa denean, hipertentsioa gertatzen da, zeharkatzen dituen organoak erasatean.

Presio arterialaren (PA) kuantifikazioa bi parametroren neurketaren bidez egiten da:

— Presio sistolikoa (PAS) edo presio altua: Bihotzak odola bultzatzen duenean, eragiten den presioa maximoa da.
— Presio diastolikoa (PAD) edo presio minimoa: Bihotza atsedenaldian dagoenean presio minimoa da.

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OMEk 1999an Hipertentsiorako Tratamenduaren Gidan plazaraturikoaren arabera, presio arterial normala hurrengo era honetan definitzen da: gizaki heldu batentzat 140 mmHg baino baxuagoko PAS eta 90 mmHg baino baxuagoko PAD. 140/90 mmHg balioetatik gorako kasuetan (muga hauek barne), hipertentsioa dugu. Balio antzekoak agertzen dira «Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure» (JNC) batzordearen azken txostenean [12]. Bigarren definizioan aldiz, OMEren Gidan ez bezala, pre-hipertentsio tarte bat definitzen da. Hipertentsioa berez ez da gaixotasun modura, gaixotasun bezala kontuan hartzen, baina hipertentsioa bera garatzeko arrisku handia duten pertsonak identifikatzeko erabiltzen da. 2. taulan jasotzen dira JNCk presio arteriala ebaluatzeko ezarritako irizpideak.

2. taula. JNCren presio arterialaren sailkapena

Sailkapena	PAS (mm Hg)	PAD (mm Hg)
Normala	< 120	<80
Pre-hipertentsioa	120-139	80-89
Hipertentsioa I	140-159	90-100
Hipertentsioa II	>160	>100

Hala ere, JNCk 2014an argitaratutako txostenean 60 urtetik gorako gizakientzat aurretik ezarritako mugak malgutzen ditu; izan ere, tentsioa kontrolatzeko farmakoen erabilera presio sistolikoa 150 mm Hg-tik gorako kasuetan edo presio diastolikoan 90 mm Hg-tik gorako mailak ematen direnean aholkatzen da [13].

Erraza izan daiteke hipertentsioa kontrolatzea, baina gaitz honek badarama bere baitan arrisku bat: nekez azaleratzen du sintomarik. Hori dela eta, kezka nagusia ez da hipertentsio arterial nabarmena duten gizakien kopuru erlatiboki baxua, tentsio arteriala arinki altua duen gizartearen espektro zabala baizik.

1.3.2. *Dislipemia*

Dislipemia (kolesteremia edo hiperlipidemia bezala ere ezaguna) edozein lipido plasmatikoren kontzentrazioa bere balio normaletik urruntzen denean gertatzen da. Eritasun kardiobaskularren ikuspuntutik, kolesterolean edo triglizeridoetan gertatzen diren aldaketak dira interesgarriak, haiek baitira gaitz askoren erantzule.

Dislipemia, triglizieridoak (kolesterol txarra) edota dentsitate baxuko lipoproteinek (LDL), 3. taulan ikus daitezkeen muga-balioak gainditzen di-

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3. taula. Dislipemiarekin zerikusia duten, lotutako lipidoen kontzentrazioen sailkapena.

Sailkapena	Kolesterol totala (mg/dL)	HDL kolesterola (mg/dL)	Triglizeridoak (mg/dL)
Egokia	< 200	>60	<150
Muga	200-239	40-60	150-200
Ezegokia	>240	<40 (gizonak) <50 (emakumeak)	>200

tuztenean gertatzen da [14]. Gainera, kolesterol onaren (HDL) kontzentrazioa txikiegia denean eta ondorioz kolesterolaren lan onuragarria txikitzen denean ere dislipemia gertatzen da. Bi baldintza hauek lotuta daude sarritan eta horrela, eritasun kardiobaskularra jasateko probabilitateak areagotzen dira.

1.3.3. *Diabetesa*

Sindrome metabolikoaren arrisku-faktoreren artean, diabetesa da eztabaida gehien sortu duena. OMEk emandako definizioaren arabera diabetes mellitusa edo intsulinari erresistentzia izatea beharrezko baldintza zen sindrome metabolikoa diagnostikatzeko. Azkeneko urteetan ordez, beste arrisku-faktoreen parean aztertu da.

4. taulan jasotzen da OMEk odoleko glukosa mailaren arabera egiten duen diabetesaren sailkapena. Sindrome metabolikoaren diagnostikoan, glukosa maila 100-110 mg/dL-tik gora egotea nahikoa da arrisku-faktore bat dagoela pentsatzeko.

4. taula. OMEren odoleko glukosa mailen araberako sailkapena.

Sailkapena	Glukosa maila baraurik (mg/dL)
Normala	< 110
Glukosa maila eraldatua	110-126
Diabetes Mellitus	>126

Diabetesa eta sindrome metabolikoen arteko loturari dagokionez desadostasunak egon diren arren, argi dago [15] zerikusia badutela. Izan ere, sindrome metabolikoa duten eta diabetikoak ez diren gaixoen artean, dia-

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betesa garatzeko probabilitatea bost aldiz handiagoa da. Gainera, bi gaixotasun hauek aldi berean jasaten dituzten eriek gaitz kardiobaskularrak pairatzeko arrisku handiagoa daukate [9].

2. SINDROME METABOLIKOAREN TRATAMENDUA

Sindrome metabolikoaren aurkako lehenengo neurria bizimoduaren aldaketa izan beharko litzateke eta tratamendu farmakologikoa, soilik lortutako emaitzak behar bezalakoak ez direnerako utzi beharko litzateke.

2.1. Farmakorik gabeko tratamendua

Sindrome metabolikoa kontrolatzeko bizimoduan aldaketa txikiak baina garrantzitsuak egin behar dira. Ikerketa batzuen arabera pisu-galtze txikiak (%5-10) sindromearen hainbat arrisku-faktoreren hobekuntza dakar [16]. Beraz, gaixotasunari aurre egiteko dieta eta ariketa fisikoa ezinbestekoak direla esan daiteke.

NCPE-ak proposatutako dieta 5. taulan ikus daiteke [17]; dieta horretan funtsezkoa da azukre eta gantz saturatuen kontsumoa txikitu eta fruituena eta barazkiena handitzea. Orokorrean komenigarria da egunero 500-1.000 kaloria gutxiago kontsumitzea.

5. taula Sindrome metabolikoari aurre egiteko NCEP erakundeak proposatutako dieta.

Elikagaia	Gomendatutako dosia
Gantz saturatuak	< %7 Kcal-eko osoko kopurua
Gantz poliinsaturatuak	≈ %10 Kcal-eko osoko kopurua
Gantz monoinsaturatuak	≈ %20 Kcal-eko osoko kopurua
Gantz kopuru osoa	%25-35 Kcal-eko osoko kopurua
Karbohidratoak	%50-60 Kcal-eko osoko kopurua
Proteinak	≈ %15 Kcal-eko osoko kopurua
Zuntza	20-30 g/egun
Kolesterola	200 mg/egun
Osoko Kaloria kopurua	«prebentzio» pisuari eusteko

Ariketa fisikoak, pisua galtzen laguntzeaz gain, sindrome metabolikoaren arrisku-faktore guztiak kontrolatzen laguntzen du; horregatik gomendatzen da egunero gutxienez 30 minutuz ariketa fisiko aerobikoa egitea (hobe ariketa hau ordu bat baino gehiagokoa bada). Kontrola beharrekoak dira era berean tabakoa, alkohola, kafeina eta gatzaren kontsumoa (2. irudia) zeren

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2. irudia. Sindrome metabolikoa kontrolatzeko terapia ez-farmakologikoa.

azken hauek presio arteriala igotzen baitute. Izan ere, tabakoa da kontrola daitekeen heriotza goiztiarraren arrazoi garrantzitsuena ~~da~~ herrialde garatuetan [18]. Sinetsi daitekeenaren aurka, heriotza gehiago eragiten dituzte arazo kardiobaskularrekin zerikusia duten gaitzek, biriketako minbiziarekin loturikoek baino. Droga honen kaltea agerikoa da: Europa mailan 2004. urtetik hona tabakoaren aurka ezarri diren legeen ondorioz bihotzekoen kopurua %15 murriztu da [19].

Dena den, terapia farmakologikoa erabiltzeak ez du esan nahi dietarik eta ariketarik egin behar ez denik. Izan ere, dietak eta ariketa fisikoak nahitaezkoak dira tratamendu farmakologikoa arrakastatsua izateko.

2.2. **Terapia farmakologikoa**

Esan bezala, sindrome metabolikoa arrisku-faktore ezberdinez osatuta dago. Hauek tratamendu ezberdinak behar dituzte eta ondorioz ezinezkoa da medikamentu bakarrarekin tratatzea; hori dela eta, beharrezkoa izaten

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da hainbat farmako biltzen dituen *terapia kardiobaskular konbinatua*. Orokorrean, terapia honetan, anithipertentsiboak erabiltzen dira presio arteriala jaisteko, hipolipemianteak gorputzeko gantz kontzentrazioak kontrolatzeko, eta intsulina edo antidiabetikoak glukosa mailak murrizteko. Gainera, inongo arrisku-faktoreri aurre egin ez arren, antikoagulatzaileak eta antiagregatzaile plaketarioak erabiltzen dira sarritan gertatzen den arterien buxadura ekiditeko [20].

2.2.1. *Antihipertentsiboak*

Farmako antihipertentsiboak aspaldi hasi ziren terapia kardiobaskularrean erabiltzen eta ondorioz, ugariak eta ezagunak dira. Haien bidez, lortu nahi da presio arteriala egunean zehar arrisku tartetik kanpo egonkor mantentzea. Izan ere, presio arteriala ziklikoki aldatzen da (lotan gaudenean minimoa da eta esnatzean igo egiten da) [21]. Presio arteriala kontrolatzeko terapia bizitza osorako denez, ezin da bertan behera utzi, eta hori gerta ez dadin pazienteari kontua errazten dioten tratamenduak erabili behar dira.

Hipertentsioaren aurkako farmakoak beren eragite-mekanismoaren arabera sailka daitezke [22]:

— Diuretikoak: Eragite-mekanismoa odol-bolumenaren murrizketan oinarritzen da. Nahiz eta erabilitako dosi handiek eragindako efektu kaltegarriek kolokan ipini zituzten, gaur egun terapia konbinatuan oso erabiliak dira haien efektu hipotentsorea gehigarria baita [23]. Familia honetan hainbat azpitalde daude: tiazidak (*hidroklorotiazida*), potasio aurreztaileak (*amilorida*)...
— Angiotentsina-II Hartzailearen Antagonistak (AHA-II) edo «sartanak»: AT_1-hartzailearen blokatze espezifiko-selektiboaren bidez angiotentsina-II-ren ekintza inhibitzen dute. Angiotentsina da presio arterialaren igoeraren eragile nagusia. AHA-II gehienek itzulezina edo ez lehiakorra den blokatzea eragiten dute, Angiotentsina-II maila igo arren blokatze hau itzulgarri bihurtzen ez delarik [24]. Farmako hauen artean, badaude *valsartan, telmisartan, candesartan…*
— Angiotentsinaren Entzima Bihurtzailearen Inhibitzaileek (AEBI) edo «priloek»: Angiotentsina-I Angiotentsina-II-ra bihurtzen duen entzima lehiakorki blokeatzen dute, eta horrela, sintesia oztopatzen da [24]. Droga hauen artean badaude *enalapril, ramipril…* daude.
— Kaltzioaren antagonistak: Zelula-mintzean zehar Ca^{2+} iragatea galarazten dute. Efektu antihipertentsibo nagusia bultzatzen duen efektu natriuretikoa eragiteko ahalmena dute. Merkatuan salgai daudenen artean, badaude *amlodipino, lercandipino, lacidipino…*
— Beta blokeatzaileak: Bihotzaren uzkurdura moteltzen dute eta horrela presio arteriala murrizten dute. Beta blokeatzaileak efektu

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antihipertentsiboa ere duen renina-angiotentsina-aldosterona ardatzaren inhibizio partzialean eragiteaz gain, kolesterolaren metabolismoan efektu positibo bat eragiten dute. *Bisoprolol* eta *atenolol* dira erabilienak.

Espainar estatuko lurralde mailan paziente hipertentsoen artean egindako ikerketa batek, erakutsi du, agerian utzi du gehien erabiltzen diren antihipertentsiboak AEBI-ak direla. Jarraian, AHA-II, kaltzioaren antagonistak, tiazidak eta azkenik beta blokeatzaileak daude [25].

Askotan terapia eraginkorra lortzeko, beharrezkoa gertatzen da hainbat farmako konbinatzea, eta horretan diuretikoek berebiziko garrantzia dute, beste antihipertentsiboekin erabilita efektu sinergikoa lortzen baita (osoko efektua banakako efektuen batuera baino handiagoa da). Beraz, terapia antihipertentsibo konbinatuan diuretiko bat erabili ohi da beste antihipertentsibo batekin, konbinazio arruntena tiazidek eta AHA-II-ek edo AEBI-ek osatutakoa izanda [26].

2.2.2. *Hipolipemianteak*

Nahiz eta eritasun kardiobaskularren eta kolesterolaren arteko harremana aspalditik ezagutu, farmako hipolipemianteen erabilera ez zen zabaldu lehen mailako prebentzio neurri bezala hartu ez ziren arte. Hipolimemianteen kontsumoa %442 igo da 2000. urtetik 2012. urtera. Plazaratutako lehenbiziko medikamentu hipolipemianteak fibratoak izan ziren 60.eko hamarkadan eta beste batzuk agertu baziren ere (azido nikotinikoaren deribatuak, behazun-azidoen bahitzaileak...), haiek izan ziren gehien erabili zirenak, estatinak agertu arte. Hauek tratamendu hipolipemiatearen merkatua goitik behera aldatu zuten eta egun, erabiltzen diren farmako hipolipemianteen %89 dira [27].

2003. urtean kolesterolaren absortzioa galarazten zuen ezetimiba izeneko farmakoa agertu zen. Oso harrera ona izan zelarik, gantzen aurkako terapia irauliko zuela zirudien. Alabaina, lortutako emaitzen eztabaidagarritasuna dela eta, ez zuen lortu estatinak ordezkatzea. Gaur egun ezetimiba beste estatina batekin konbinatuta hartzen da gehien bat.

Jarraian azalduko ditugu Hipolipemianteen ezaugarri ezberdinak:

— Fibratoak: PPARα hartzaile nuklearraren transkripzioa aktibatzen dute; honek eragina du gantz azidoak oxidatzen eta garraiatzen dituen proteinetan eta lipasa lipoproteikoan. Honi esker, dentsitate oso baxuko lipoproteinak (VLDL) eta LDLen katabolismoa handitzen da, HDLen ekoizpenarekin batera. Triglizeridoen maila murrizteko oso egokiak dira baina miopatiak (min muskularrak) sor ditzakete. Farmako hauen artean *gemfibroziloa* da gehien erabiltzen dena [28].

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— Behazun azidoen bahitzaileak: behazun azidoen iraizketa igoaraziz kolesterolaren degradazioa eragiten dute eta horrela, LDLena. Triglizeridoen maila ez da aldatzen eta HDLena apur bat igo daiteke. Hala ere, gorputzak duen tolerantzia motelagatik oso gutxi erabiltzen dira, baina *colestiramina* eta *colestipol* dira ohikoenak [29].
— Azido nikotinikoaren eratorriak: VLDLen sintesia inhibitzen dute eta triglizeridoen gibelerako garraioa oztopatzen dute. Horrela, triglizerido eta LDL mailak jaitsi eta HDLenak, igo egiten dira. Emaitza oso onak lortu arren, erakutsitako efektu sekundarioak direla eta, estatu mailan bere erabilera kasu jakin batzuetara mugatuta dago. Familia honetako farmako erabiliena *niceritrol* da [30].
— Estatinak: Hidroxi-metil-glutaril koentzima A (HMGCoA) erreduktasa inhibitzen dute; erreduktasa hau, funtsezkoa da kolesterolaren sintesian. Honen ondorioz LDLak eta VLDLak murriztu eta HDLak igo egiten dira. Gaur egun, haiek dira dislipemiari aurre egiteko farmakorik egokienak dira, batez ere LDLa txikitu behar denean. Gainera efektu antihipertentsiboa dutenez, terapia konbinaturako oso interesgarriak dira. Honez gain, medikuntzaren beste arlo garrantzitsu askotan ikertzen hasiak dira efektu terapeutiko asko eransten zaizkielako, batez ere HIESA, Alzheimer eta minbiziaren aurkako tratamenduetan. Gaur egun merkatuan dauden estatinak hauek dira: *Atorvastatina, Fluvastatina, Lovastatina, Pravastatina*, *Rosuvastatina* eta *Simvastatina*. 2001. urtean *Cerivastatina* merkatutik kendu zuten albo-ondorio arriskutsuak eragiten zituelako. Hala ere, ez dirudi beste estatinek inongo arazo larririk eragiten dutenik kasu batzuetan miopatiak eragin eta transaminasak igo ditzaketen arren.
— Ezetimiba: Enterozitoetan dagoen Niemann Pick C1L1 proteina inhibitzen du hesteen kolesterol xurgaketa oztopatuz. Ondorioz LDL gutxiago sortzen da. VLDL mailak ere arinki txikitu eta HDLenak igo egiten dira. Gehien bat estatinak nekez toleratzen direnean edo eta hauekin batera hartzen dira. Medikamentu nahiko berria denez, bere albo-ondorioei buruzko informazio gutxi dago [31]. Ezetimibaren kontsumoa 2012. urtean estatinak edo fibratoak ez diren hipolipemianteen kontsumoaren %70a izan zen 2012. urtean [27].
— Beste hipolipemiante batzuk: Badaude oso gutxi erabiltzen edo frogetan dauden beste hipolipeminate batzuk, adibidez arritmiak eragiten zituen eta merkatutik at dagoen probukol antioxidatzailea, arrainen gantz azido poliinsaturatuak, heparinak...

Gogoratu behar da komenigarria dela gaixotasun kardiobaskularrak saihesteko «kolesterol txarra» (LDL) txikitu eta eta «kolesterol ona» (HDL) igotzea. Triglizeridoekin (TG) harremanetan dagoen VLDL ere murriztu behar da. Hainbat ikerketak, frogatu dute estatinak direla hilkortasuna gehien murrizten duten hipolipemianteak, seguru asko LDLa gehiago murrizten dutelako eta presio arterialean eragin handiagoa dutelako [32].

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2.2.3. *Antidiabetikoak*

Orokorrean, diabetesaren aurka gehien erabiltzen den tratamendua intsulinan oinarrituta dago. Hala ere, Diabetes Mellitusaren kasuan bakarrik erabiltzen da, hau intsulinaren menpekoa denean edo ahozko antibiabetikoak eraginkorrak ez direnean. Azken farmako hauen artean bi multzo bereiz daitezke: alde batetik intsulinaren jariatzea areagotzen dutenak (sulfonilureak eta metiglinidak); bestetik, intsulinaren aurreko erantzuna hobetzen dutenak [33] (biguanidak eta tiazolidindionak).

— Sulfonilureak: Langerhasen irletako β-zelulen intsulina jariatzea eragiten dute. Gainera, dosi altuetan glukosaren ekoizpen hepatikoa murrizten dute. Eriak obesitate arazorik ez duenean lehen aukerako tratamendu bezala erabiltzen dira. Hauen artean erabilienak *glibenclamida*, *gliquidona* eta *glimepirida* dira.
— Metiglinidak: Sulfonilureen portaera berbera dute, baina beste zelula-hartzaileen gune bati lotzen zaizkio. Haien efektua azkarragoa da baina laburragoa ere. Bestalde, erantzuna glukosa kontzentrazioaren menpekoa denez, baraurik hipogluzemia jasateko probabilitateak txikiagoak dira. Epe luzeko ikasketa gutxi daudenez eta garestiagoak direnez, ez dira gehiegi erabiltzen. Gainera ezin dira sulfonilureekin batera hartu, onura gehigarririk lortzen ez delako. *Repaglidina* da familia honen farmakorik ezagunena.
— Biguanidak: Intsulinaren jariatzea piztu ordez honekiko sentikortasuna areagotzen dute, baita glukosaren metabolismoa ere. Gainera lipido mailetan efektu lagungarria dute. Biguaniden artean gaur egun onartutako bakarra *metformina* da, farmako antidiabetikoen artean erabilienetariko bat. Egokia da obesitate arazoak dituzten pazienteentzat, pisua handitzen ez duen antidiabetiko bakarra izateagatik.
— Tiazolidindionak: Gantz arrean, muskulu eskeletikoan eta gibelean intsulinaren aurkako erresistentzia txikitzen dute PPARγ hartzaile nuklearrari lotuz.
— Gibelaren glukosa-ekoizpena murrizten dute eta bere erabilera areagotzen dute periferian. Familia honetako farmakoen artean *Rosiglitazona* zen ezagunena baina estatuan 2010eko abenduaren 29an bere merkaturatzea eten egin zen infartu-arriskua igotzen zuen susmoagatik [34].

2.2.4. *Antikoagulatzaileak eta antiagregatzaile plaketarioak*

Farmako hauek ez dute, berez, inolako efektu onuragarririk sindrome metabolikoa osatzen duten arrisku-faktoreen aurka. Hala ere, oso erabilgarriak dira gaitz honen tronboak bezalako ondorio hilgarriak ekidin ditzaketelako. Nahiz eta antikoagulatzaileek eta antiagregatzaileek efektu berdina

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izan, haien mekanismoak guztiz ezberdinak dira eta bestelako kasu batzuetan erabiltzen dira.

Antikoagulanteak bihotzekoak, garun-infartuak, tronbosi benoso sakonak eta biriketako tronboenbolismoak saihesteko erabiltzen dira. Alde batetik, ahoz hartzen diren K bitaminaren antagonistak daude. Izenak dioen bezala, K bitaminaren menpekoak diren koagulazio-faktoreen aktibazioa galarazten dute. *Acenocoumarol* da erabiliena Europan eta *Warfarina* AEBetan. Beste alde batetik, injektagarriak diren *heparina* bezalako antikoagulatzaileak daude eta hauek gehiago erabiltzen dira ospitale esparruetan.

Antiagregatzaile plaketarioak tronbosi arteriala eta arteroeskleriosia saihesteko erabiltzen dira. Farmako hauek plaketen elkarketa galarazten dute eta ondorioz tronboen sorkuntza ekiditen da. Antikoagulatzaileek ezin dute lan hau egin, zirkulazio arterialean efektu gutxi baitute. Zalantzarik gabe kostu-eraginkortasun onena eskaintzen duen farmakoa *aspirina* da. Hau, efektu analgesikoa izateaz gain dosi txikiagoetan terapia kardiobaskularrean erabil daiteke. Izan ere, lehenengo aukerako farmakoa da eta beste medikamentuak (*clopidogrel, ticlopidine*…) aspirinak arazoak eragiten dituenean baino ez dira erabiltzen.

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