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QUANTITATIVE ANALYSIS IN PROTEOMICS AND METABOLOMICS

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Summary

The quantification of differences between two or more states, physiological or pathological, in order to identify differentially expressed analytes in complex mixtures is among the most important objectives of proteomics and metabolomics.
Mass spectrometry (MS) is one of the key analytical technology on which the emerging ‘‘-omics’’ approaches are based. It may provide detection and quantization of thousands of proteins and biologically active metabolites from a tissue, body fluid or cell culture working in a ‘‘global’’ or ‘‘targeted’’ manner, down to ultra-trace levels.
Selected reaction monitoring (SRM) performed on triple quadrupole mass spectrometers has been actually the reference quantitative technique to analyze different molecules with high selectivity, sensitivity and a wide dynamic range. In SRM analysis, two stages of mass selection, i.e., selection of a precursor ion and monitoring of specific product ion(s) derived from the precursor, provide the highest analytical specificity applicable to complex mixture analysis.
The combination of m/z setting for the first and third quadrupole is referred to as ‘transition'.
New methodologies for selective quantitative analysis of targeted metabolites and proteins based on LC-MS/MS in Selected Reaction Monitoring scan mode. are developed. This approach has shown to be a valuable tool to identify altered pathways in pathological conditions and useful for quantitative measurements of a specific subset of known analytes such as same hormone and TDC, oxylipins, H2S sulfur metabolites, D, L Asp and NMDA and proteins as IDE and TSH.
The SRM strategies optimized in this thesis focused on 1) identification of a set of proteins and metabolites of interest capable of satisfying a specific biological or clinic request 2) selection of transitions maximizing sensitivity and selectivity 3) optimization of SRM transitions by tuning acquisition parameters of the mass spectrometer 4) validation of the transitions in biological matrix to account to unspecific contributions of fragment ions background 5) quantification by the external standard method with the realization of specific calibration curves.
The analysis in SRM mode (Selected Reaction Monitoring), has allowed us to achieve the specificity and selectivity to quantify the analytes of interest in relatively complex matrices, also at very low concentration with good reproducibility and accuracy.

1 Introduction

1.1 Omics and system biology

'Omic' technologies adopt a holistic view of the molecules that make up a cell, tissue or organism. They are aimed primarily at the universal detection of genes (genomics),mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in different biological compartments such as whole blood, plasma and urine. The integration of these techniques is called systems biology (Horgan RP, 2011).

Systems biology is based on the understanding that the whole is greater than the sum of the parts. It has been responsible for some of the most important developments in the science of human health and environmental sustainability (Kitano, 2002). It is a holistic approach to deciphering the complexity of biological systems integrating many scientific disciplines – biology, computer science, engineering, bioinformatics, physics and others – to predict how these systems change over time and under varying conditions, and to develop solutions to the world's most pressing health and environmental issues.

Components data yield information regarding the specific molecular content of the cell or system. Interactions data specify the connectivity that exists among the molecular species, thereby defining the network 'scaffold' within the cell or system. Finally, functional-states data reveal the overall behaviour, or phenotype, of the cell or system (Joyce A. R. 2006).

This ability to design predictive, multiscale models enables our scientists to discover new biomarkers for disease, stratify patients based on unique genetic profiles, and target drugs and other treatments. Systems biology, ultimately, creates the potential for entirely new kinds of exploration, and drives constant innovation in biology-based technology and computation. (Hartwell LH1, 1999)

1.1.1 Components data

Genomics. Genomics is defined as the study of the whole genome sequence and the information contained therein. It is clearly the most mature of the omics-sciences. Since 1995, nearly 300 genome-sequencing projects, with representative species from each of the three kingdoms of life, have been completed (Liolios 2006) and hundreds more are underway.

The genomic sequence is used to study the function of the numerous genes (functional genomics), to compare the genes in one organism with those of another (comparative genomics), or to generate the 3-D structure of one or more proteins from each protein family, thus offering clues to their function (structural genomics).

Genome annotation provide information about human and other major model organisms on complement of proteins and functional RNAs, transcription-factor-binding sites in genomic sequences and on complete set of translated open reading frames (ORFs) and the exon-intron structures from which they are assembled.

Transcriptomics. The transcriptome is the total mRNA in a cell or organism and the template for protein synthesis in a process called translation. The transcriptome reflects the genes that are actively expressed at any given moment (Horgan RP 2011). Microarrays and serial analysis of gene expression (SAGE) represent the most well-used approaches and have been applied to many model systems, as well as to the study of genes that are predominantly expressed in stem cells, to classifying the molecular subtypes of human cancers, and to monitoring the host-cell transcriptional response to pathogens (Joyce A. R. 2006).

Proteomics. The proteome is defined as the set of all expressed proteins in a cell, tissue or organism. Proteome analysis presents specialized analytical problems in two major areas: i) dynamic expression range and ii) diversity of protein expression (multiple protein forms). In order to clarify proteome–phenotype relationships,

several studies are dedicated to the development of new technology to explain the link between protein-expression profiles and distinct cellular processes or conditions (Kuster 2005). In particular, one strategy that is being developed involves overcoming the problem of detecting only the most highly represented proteins in biological samples by focusing on unique characteristic peptides for each protein or protein isoform (Joyce A. R. 2006).

Metabolomics. The metabolome refers to the complete set of low molecular weight compounds in a biological sample. These compounds are the substrates and byproducts of enzymatic reactions and have a direct effect on the phenotype of the cell. Metabolites are the final products of the genome, transcriptome and proteome integration. The metabolome consists of molecules that have high chemical and physical heterogeneity. Metabolomics is fast becoming a popular tool for studying the cellular state of many systems, including plants, the human red blood cell and microbes, as well as in metabolic-engineering applications, in pharmacology and toxicology and in human nutritional studies (Joyce A. R. 2006).

Lipidomics. Lipidomics is the systems-level analysis of lipids (fat molecules) and their interactions. It is a science still in its infancy but one that promises to revolutionize biochemistry. Lipids are grouped into eight categories that share common physical and chemical properties, and there are currently some 38,000 documented lipids (German, 2007).

Lipids that occur rarely or in small quantities are often the most effectual lipids in biological processes, meaning they are particularly important in disease diagnostics and in understanding pathology. Lipidomics can elucidate the pathology and treatment of many diseases such as cancer, diabetes, obesity, cardiovascular disease, arthritis, asthma, inflammatory bowel disease, Alzheimer's and others due to the associated disruption of lipid metabolic enzymes and pathways. A better understanding of lipidomics could significantly advance diagnostic medicine as well as provide novel treatment options (Rob Smith, 2014).

1.2 Mass spectrometry

Mass spectrometry is a reproducible methodology based on the determination of the molecular mass, which is not dependent on the experimental conditions. Most important features of mass spectrometry are: reproducibility, sensitivity, accuracy.

Mass spectrometry is based on the production of gas phase ions. These ions can interact with an electric field and can be resolved following their electro-dynamic attitude, which is dependent on their mass-to-charge ratio.

Today a wide variety of mass spectrometers is available, all sharing the capability to assign mass-to-charge values to ions, although the principles of operation and the types of experiments that can be done on these instruments differ greatly.

Mass spectrometers have four essential parts:

a) system for sample introduction

b) source that produces gas phase ions from the sample

c) one or more mass analyser to separate ions

d) ion detector.

Sample under investigation has to be introduced into the ion source of the instrument where the sample molecules are ionized. These ions are extracted into the analyser region of the mass spectrometer where they are separated according to their mass-to-charge ratios (m/z). The separated ions are detected and the generated signals sent to a data system where the m/z ratios are stored together with their relative abundance for presentation in the format of an m/z spectrum *(*Fig. 1.1*).*

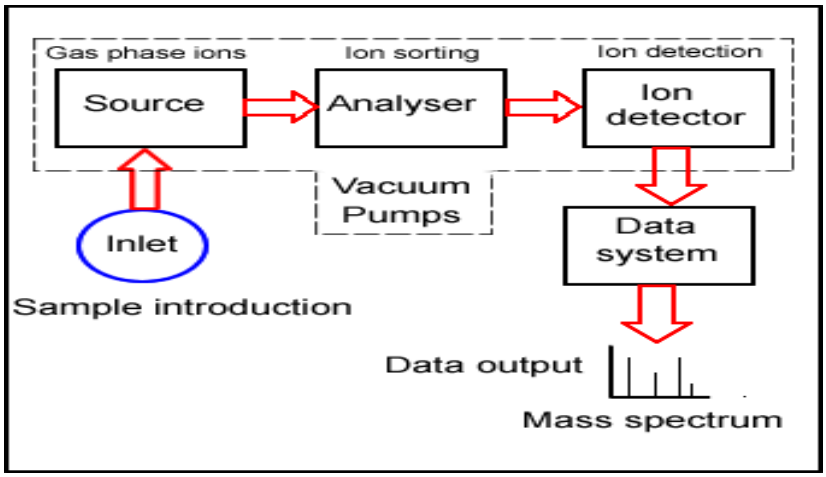


Figure 1.1 Process of measuring of a mass spectrometer

A class of mass spectrometers can be distinguished on its ionization system and on the type of analyser, that is an essential component to define the accessible mass range, sensitivity and resolution.

All mass spectrometers are operated at very low pressure in order to prevent collisions of ions with residual gas molecules in the analyser during the flight from the ion source to the detector.

Many ionization methods are available however the most widespread ionization methods in biochemical analyses are Electrospray Ionization (ESI) and Matrix Assisted Laser Desorption Ionization (MALDI). Mass analysers as quadrupoles (Q), ion traps (IT), time-of-flight (TOF), or combination of these in "hybrid instruments", are commonly used for their good resolution and sensitivity. TOF analysers are typically used in combination with MALDI sources (MALDI-TOF MS instruments); however both MALDI sources and TOF analysers can be used in different configuration.

The coupling of Liquid Chromatography (LC) and tandem Mass Spectrometry (MS/MS) is a widely used analytical technique for quantitative and qualitative analysis. Electrospray Ionization (ESI), Atmospheric Pressure Chemical Ionization (APCI), or Photo Ionization (APPI) allow the ionization of various semi-volatile, thermally labile, and polar to non polar compounds, such as pharmaceuticals, pesticides, personal care products, steroids, explosives, drugs of abuse etc., in trace levels. Generated ions will be transferred after ionization through a vacuum interface into the mass analyzer.

1.2.1 Electrospray (ESI)

Electrospray Ionization (ESI) has had a tremendous impact over the last few years on the use of mass spectrometry in biological research. ESI is well suited to the analysis of polar molecules ranging from less than 100 Da to more than 1,000,000 Da in molecular weight. In contrast to MALDI, in which the sample is a dried, crystalline admixture of protein/peptide sample and matrix, the peptides or proteins to be analyzed by ESI are in aqueous solution. Proteins and peptides exist as ions in

solution because they contain functional groups whose ionization is controlled by the pH of the solution.

During standard electrospray ionization sample is dissolved in a polar, volatile solvent and pumped through a narrow, stainless steel capillary (Fig. 1.2). A high voltage of 3 or 5 kV is applied to the tip of the capillary situated within the ionization source of the mass spectrometer and the sample emerging from the tip is dispersed into an a spray of highly charged droplets, a process that is aided by a co-axially introduced nebulising gas flowing around the outside of the capillary.

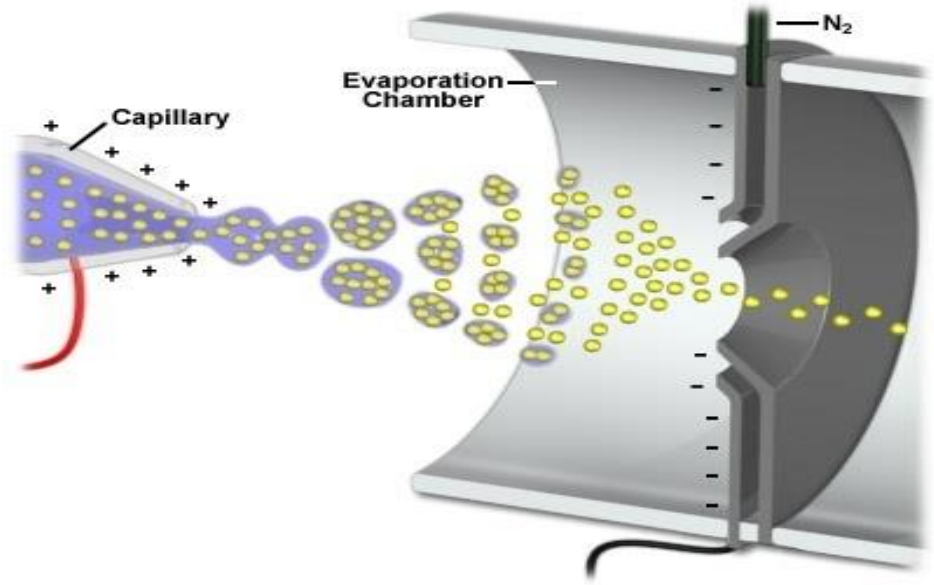


Figure 1.2 ESI source

This gas, usually nitrogen, helps to direct the spray emerging from the capillary tip towards the mass spectrometer. The charged droplets diminish in size by solvent evaporation, assisted by a warm flow of nitrogen known as the drying gas which passes across the front of the ionization source.

Eventually charged sample ions, free from solvent, are released from the droplets, some of which pass through a sampling cone or orifice into an intermediate vacuum region, and from there through a small orifice into the analyser of the mass spectrometer, which is held under high vacuum.

Electrospray is known as a “soft” ionization method (such as MALDI) as the sample is ionized by the addition or removal of a proton, with very little extra energy remaining to cause fragmentation of the sample ions.

The peculiar aspect of this technique is the fact that it gives rise to multiply charged molecular-related ions such as $(M+nH)^{n+}$ in positive ionization mode and $(M-nH)^{n-}$ in negative ionization mode. Because of the form assumed by the signal, this source is not useful to the analysis of complex mixtures. For this reason RP-HPLC system is generally coupled to ESI-source.
ESI source generally works with quadrupole and ion trap analyzers.

1.2.2 Quadrupole (Q) mass analyser

Quadrupole mass analyser (Fig. 1.3) has now become one of the most widely used types of mass analyser because of its ease of use, small size and relative low cost. It consists of four parallel rods that have fixed DC and alternating RF potentials applied to them. Ions produced in the source of the instrument are then focused and passed along the middle of the quadrupoles. Their motion will depend on the electric fields so that only ions of a particular *m/z* will be in resonance and thus pass through to the detector following a stable trajectory.

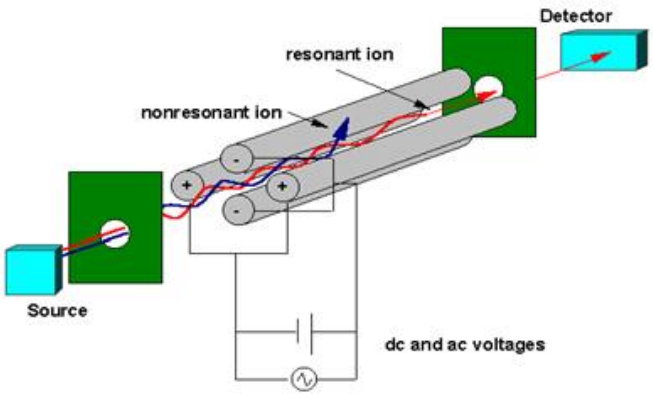


Figure 1.3 Quadrupole mass analyser

All other ions do not have a stable trajectory through the quadrupole mass analyzer and will collide with the quadrupole rods, never reaching the detector. The RF is

varied to bring ions of different *m/z* into focus on the detector and thus build up a mass spectrum.

1.2.3 Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) is used to produce structural information about a compound by fragmenting specific sample ions inside the mass spectrometer and identifying the fragment ions. Tandem mass spectrometry also enables specific compounds to be detected in complex mixtures on account of their specific and characteristic fragmentation patterns. Tandem mass spectrometry uses two stages of mass analysis, one to preselect an ion and the second to analyze fragments.
Generally in a tandem mass spectrometer the two analysers are separated by a collision cell into which an inert gas (e.g. argon, xenon) is admitted to collide with the selected sample ions and bring about their fragmentation.

1.2.4 Instruments for MS/MS analysis

Instruments for tandem mass spectrometry can be classified as *tandem in space* or *tandem in time* (Fig. 1.4). *Tandem in space* means that ion selection, ion fragmentation and fragments analysis, are events that occur in three different regions of the spectrometer; instruments of this type are the triple quadrupole (ESI-QqQ) and hybrid instruments such as ESI-Q-TOF, ESI-QqLIT or MALDI-TOF-TOF. In *tandem in time* instruments, those three steps of analysis occur in the same region of the spectrometer but in different times; three-dimensional ion trap are instruments classified as *tandem in time.*

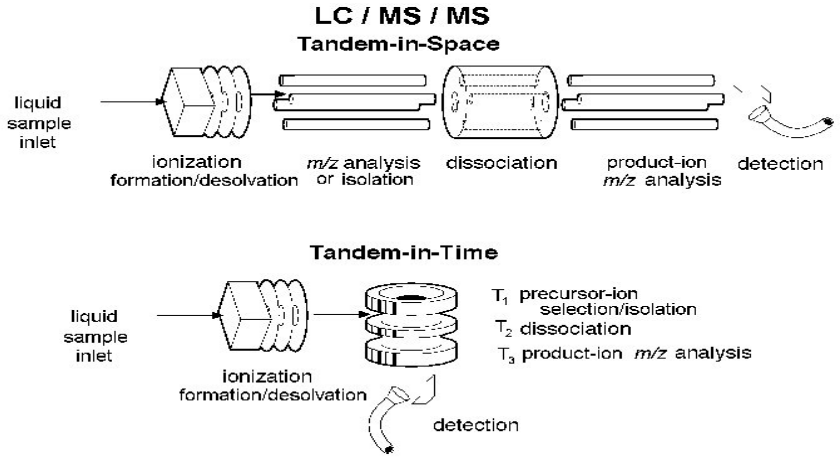


Figure 1.4 Schematic representation of tandem-in-space and tandem-in-time instruments

A commonly used instrument is the triple quadrupole, where two resolving quadrupoles mass filters are separated by a fragmentation cell. These are considered to be "beam-type" mass spectrometers because they are most often used with a continuous ion beam: when one of the mass filters is scanning, most of the ions are rejected by the instrument, resulting in poor sample use and low sensitivity, whereas when the mass filters are not scanning, but are transmitting a pre-selected ion, sensitivity and sample use are very high. MSMS mass spectrometer are Quadrupole ion Trap, Linear ion trap and triple quadrupole.

1.2.5 Quadrupole ion trap (IT)

The quadrupole ion trap is based on the same principle as the quadrupole mass filter, except that the quadrupole field is generated within a three-dimensional trap. The trap consist of three electrodes, a ring electrode and two hemispherical end caps electrodes. These electrodes allow to trap ion in a small volume (Fig. 1.5).

In quadrupole ion traps, ions are dynamically stored in a three-dimensional quadrupole ion storage device. The RF and DC potentials can be scanned to eject successive mass-to-charge ratios from the trap into the detector.

Ions are injected into an ion trap from an external source. Ions are dynamically trapped by the applied RF potentials. The trapped ions can be manipulated by RF events.

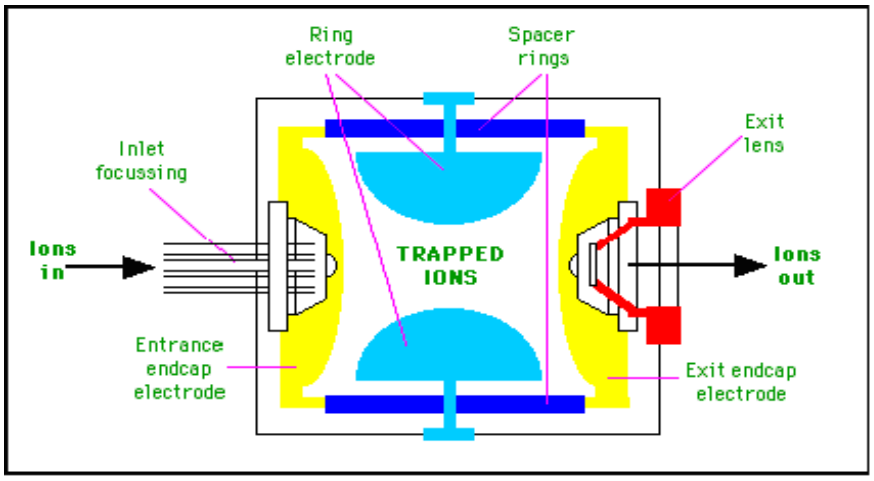


Figure 1.5 Quadrupole ion trap

The advantages of the ion-trap mass spectrometer include compact size, and the ability to trap and accumulate ions to increase the signal-to-noise ratio of a measurement. For these reasons ion traps are used in fragmentation experiments.
Conventional ion trap mass spectrometers operate with a three-dimensional (3D) quadrupole field, which confers to the analysis very high efficiency as regard the time to fill the ion trap and to generate a complete mass spectrum, but presents some problems as regard the trapping efficiencies, primarily due to their small volume. These problems have been overcome thanks to the introduction of linear ion traps, characterized by a larger ion storage capacity and a higher trapping efficiency.

1.2.6 Linear ion trap (LIT)

Trapping of ion can also be performed in linear 2D ion trap devices(LIT) (Le Blanc *et al*., 2003). The combination of triple quadrupole MS with LIT technology in a form of an instrument of configuration QqLIT (Fig. 1.6), is particularly interesting because this instrument retains the classical triple quadrupole scan functions such as selected reaction monitoring (SRM), product ion (PI), neutral loss (NL) and precursor ion (PI) while also providing access to sensitive ion trap experiments. In addition, for peptide analysis, the enhanced multiply charged (EMC) scan allows an increase in selectivity, while the time- delayed fragmentation (TDF) scan provides

additional structural information. The first commercially available instruments (Q-Trap ^{TM}) was manufactured by SCIEX and made available through the commercial area of Applied Biosystem.

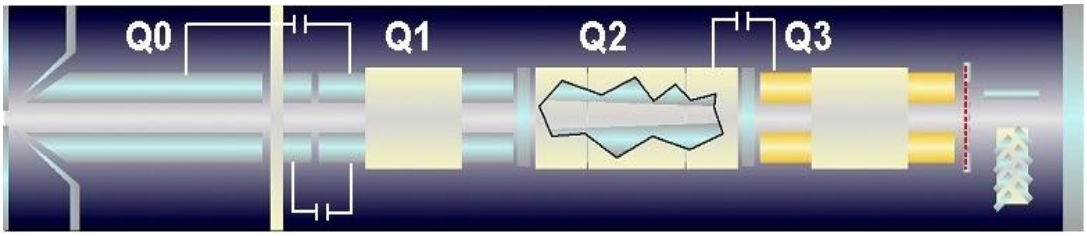


Figure 1.6 Schematic representation of QqLIT mass analyser

Ions generated by an ESI source, travel through an RF-only quadrupole (Q_0) (that is used to collisionally cool the incoming ion beam) before entering in the first quadrupole Q_1, that is a conventional RF/DC quadrupole mass filter and is tuned to transmit only the precursor ion of interest towards the second quadrupole (q_2), that is a collision cell. The final quadrupole , Q_3, can be operated as either another RF/DC mass filter or a linear ion trap with mass-selective axial ejection. Switching from triple-quadrupole to ion-trap modes is accomplished by changes in electrical voltages can be applied in < 1 ms. This means that analytical workflow can be devised that use both triple-quadrupole and ion-trap on a chromatographic timescale.

Linear ion trap combines the advantages of a triple quadrupole (very selective scanning of precursor ions) with that of three-dimensional ion trap, like high sensitivity. LITs have two major advantages over 3D-IT: a larger ion storage capacity and a higher trapping efficiency. Moreover there is no inherent low-mass cut-off in the product ion spectra because the fragmentation and the ion-trapping steps are spatially separated, while in conventional 3D ion traps the precursor and the lowest mass fragment must be stable simultaneously in the trap, which produces a low-mass cut-off in the resulting spectrum at about 25-30% of the precursor ion m/z.

Using Q_3 as a linear ion trap mass spectrometer it is also possible to obtain MS^n spectra, taking advantage of the characteristics of higher trapping efficiency of LIT upon 3D-IT and reduced space-charge interactions that limit the number of ions that can be stored. Moreover Q_3 is operated at a very low pressure ($3*10^{-5}$ Torr) that

insure that the ions entering the Q_3 trap are collisionally cooled thereby enhancing trapping efficiency and sensitivity of MS^n.

This instrument can operate in different scan modes (Fig1.7):

a) In the ***product or daughter ion scanning*** the Q_1 is used to select user-specified sample ions arising from a particular component; usually the molecular-related (i.e. $(M+H)^+$ or $(M-H)^-$) ions. These chosen ions pass into the q_2, are bombarded by the gas molecules which cause fragment ions to be formed, and these fragment ions are analysed (i.e. separated according to their mass to charge ratios) by the Q_3. All the fragment ions arise directly from the precursor ions specified in the experiment, and thus produce a fingerprint pattern specific to the compound under investigation.

This type of experiment is particularly useful for generating peptide sequence information.

b) In the ***precursor or parent ion scanning*** the Q_1 allows the transmission of all sample ions, while the Q_3 is set to monitor specific fragment ions, which are generated by bombardment of the sample ions with the collision gas in the q_2. This type of experiment is particularly useful for monitoring groups of compounds contained within a mixture which fragment to produce characteristic fragment ions.

c) In the ***constant neutral loss scanning*** both the Q_1 and Q_3 scan and collect data across the whole m/z range, but the two are off-set so that the second analyser allows only those ions which differ by a certain number of mass units (equivalent to a neutral fragment) from the ions transmitted through the first analyser. This scan mode is used, for example, to identify phosphorilated peptides in a peptide mixture. In fact a typical fragmentation of these peptides consists of the loose of a phosphoric acid residue, with a mass shift of -98 Da.

Other typical scan modes are the ***enhanced multiply charged scan*** and ***time-delayed fragmentation***. The *enhanced multiply charged* mode allows the removal of singly charged ions from the LIT (due to sample impurities), while doubly charged ions are enhanced in resolution and sensitivity. *Time-delayed fragmentation* mode is particularly interesting for spectra interpretation because it reduces multiple sequential fragmentations and so leads to simpler tandem mass spectra. This is obtained by eliminating ions with a kinetic energy over a threshold that don't fragment correctly.

The most common usage of MS/MS in proteomics is the product or daughter ion scanning experiment which is particular successful for peptide sequencing.

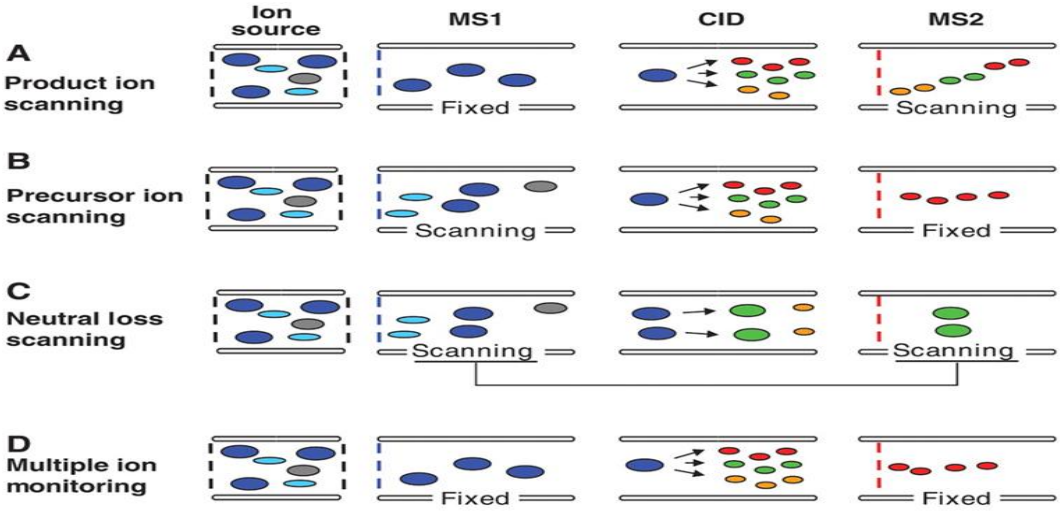


Fig 1.7 Different scan modes

1.2.7 Triple quadrupole

Single quadrupole systems contain only one mass filtering quadrupole while triple quadrupole systems consist of three quadrupoles. Q1 and Q3 are working as mass filters while Q2 is acting as collision cell.

Quadrupoles can be used in scanning or filtering mode. During a mass scan, DC and RF voltages are ramped resulting in the acquisition of full scan mass spectra. Such spectra are typically used for qualitative data analysis. However, scanning a quadrupole suffers from low sensitivity and slow scan speed. Thus, quantitative studies are performed with quadrupoles working in filtering mode. The most selective mode to use a single quadrupole MS is called Selected Ion Monitoring (SIM). Hereby, a fixed set of DC and RF voltages is applied to the quadrupole and thus only a single m/z can pass. Ions with different m/z are filtered out.

A fundamental understanding of the scan modes associated with the TQMS is essential for understanding the MS and MS/MS capabilities of the instrument. (March, 2009).

The triple quadrupole tandem mass spectrometer can readily be used to perform a single stage of mass spectrometry (MS) by setting two of the three quadrupoles into

RF-only (total ion) mode. Either Q1 or Q3 can be used as a mass filter, scanned to obtain a full spectrum, or set to pass a single fixed m/z (or a few fixed m/z values), a mode termed selected ion monitoring (SIM). When Q1 and Q3 are both used as mass analyzers, there are four possible MS/MS scan modes. In the daughter ion scan mode (also called product ion scan), Q3 is scanned to obtain a spectrum of all the daughter ions generated by CID in q2 of the parent or precursor ion mass-selected with Q1. In the parent ion scan mode (also called precursor ion scan), Q1 is scanned to obtain a spectrum of all the parent ions that upon CID in q2 produce the daughter or product ion mass-selected with Q3. In a neutral loss scan, both Q1 and Q3 are scanned at the same rate but with a fixed difference in mass to detect only those ions that undergo a specific neutral loss in q2 such as a loss of mass 18 (H2O). An important MS/MS scan mode for quantitating targeted compounds with maximum sensitivity is the selected reaction monitoring mode (SRM) in which one (or a few) selected parent-ion-to-daughter-ion transitions are monitored by setting Q1 and Q3 to pass specific m/z values. SRM is the MS/MS scan mode analogous to the MS selected ion monitoring scan mode (SIM).

Selected Reaction Monitoring (SRM) is the most common mode of using a triple quadrupole MS/MS for quantitative analysis, allowing enhanced sensitivity and selectivity. The first quadrupole filters a specific precursor ion of interest. Ions generated in the ion source having a different m/z can not pass Q1. The collision cell is optimized to produce a characteristic product ion by collision of the precursor ion with a neutral collision gas, such as nitrogen. This process is called Collision Induced Dissociation (CID). Generated product ions are transferred into the third quadrupole where only a specific m/z is allowed to pass. All other product ions are filtered out in Q3. In SRM mode, two stages of mass filtering are employed on a triple quadrupole mass spectrometer. In the first stage, an ions of interest (the precursor) is preselected in Q1 and induced to fragment by collisional excitation with a neutral gas in a pressurized collision cell (Q2). In the second stage, instead of obtaining full scan ms/ms where all the possible fragment ions derived from the precursor are mass analyzed in Q3, only a small number of sequence-specific fragment ions (transition ions) are mass analyzed in Q3.

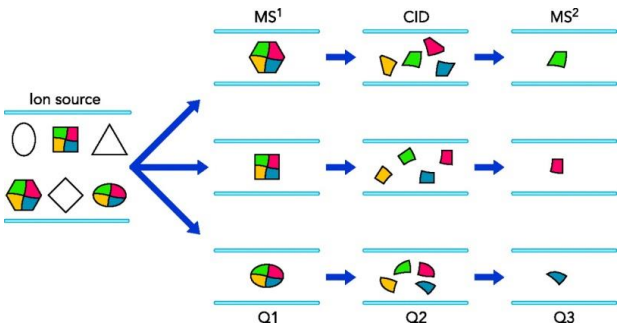


Fig 1.8 Selected ion mode

Thus SRM mode works like a double mass filter which drastically reduces noise and increases selectivity. Single quadrupole and triple quadrupole systems allow the detection of many SIM and SRM transitions, respectively. This enables quantitation of many targeted analytes in a single experiment. Typically, additional SIM and SRM transitions have to be detected to perform identification of quantified compounds. Hereby, the most intense ion is called the 'quantifier' and all additional ions are called 'qualifiers'. The EU Commission Decision 2002/657/EC defined performance criteria for confirmatory methods, such as MS and MS/MS, by introducing the concept of identification points. The required number of 4 identification points can be achieved by detecting 4 SIM on a single quadrupole MS or 2 SRM transitions on a triple quadrupole MS/MS. This targeted MS analysis using SRM enhances the lower detection limit for peptides by up to 100 fold (as compared to full scan ms/ms analysis) by allowing rapid and continuous monitoring of the specific ions of interest.

Selected reaction monitoring (SRM) coupled with stable isotope dilution mass spectrometry (SID-MS) using a triple quadrupole mass spectrometer is a powerful method for quantitative measurement of target molecules.. SRM methods, in principal, provide both absolute structural specificity for the analyte and relative or absolute measurement of analyte concentration when stable, isotopically-labeled standards are added to a sample in known quantities. When a synthetic, stable isotope labeled peptide is used as an internal standard, the concentration can be measured by comparing the signals from the exogenous labeled and endogenous unlabeled species. This can be done because they have the same physicochemical properties and differ only by mass. It has been a principal tool for quantification of small molecules in clinical chemistry for number of decades. MS-based quantitative assays have the necessary characteristics required for verification studies, namely: high specificity, sensitivity, multiplexing capability, and precision.

1.3 Mass spectrometry based omics

Dramatic technological advances in the biological sciences over the past few years have forged a new era of research including the emerging field of systems biology. Although the understanding of living organisms at the molecular system level is still in its infancy, it is evident that comprehensive investigations of the "omics cascade" with genomics, transcriptomics, proteomics, and metabolomics are important building blocks and will play a central role in this new science (Dettmer, 2007). The integrative analysis of an organism's response to a perturbation on the transcriptome, proteome, and metabolome levels will lead to a better understanding of the biochemical and biological mechanisms in complex systems.

Many "omics" techniques have been developed for one goal: identification of specific target compounds involved in diagnosis, prognosis, therapeutic response prediction and population screening of human disease. For example, recent ''omics'' technologies have opened a new road to biomarker discovery and early detection of cancer. A biomarker is a biologically derived molecule in the body, which is indicative of the progress or status of a disease.

Mass spectrometry (MS) is one of the key analytical technology on which the emerging ''-omics'' approaches are based. It may provide detection and quantization of thousands of proteins and biologically active metabolites from a tissue, body fluid or cell culture working in a ''global'' or ''targeted'' manner, down to ultra-trace levels. It can be expected that the high performance of MS technology, coupled to routine data handling, will soon bring fruit in the request for a better understanding of human diseases, leading to new molecular biomarkers, hence affecting drug targets and therapies.

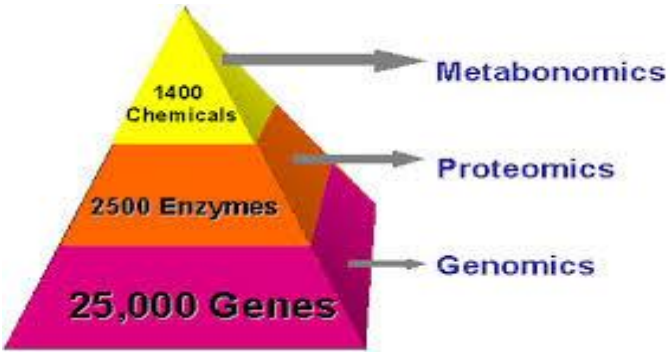
1.3.1 Mass spectrometry-based metabolomics

Mass spectrometry-based metabolomics aims at the comprehensive and quantitative analysis of wide arrays of metabolites in biological samples having very diverse physico-chemical properties and occurring at different abundance levels.

Consequently, comprehensive metabolomics investigations are primarily a challenge for analytical chemistry and specifically mass spectrometry has vast potential as a tool for this type of investigation. Metabolomics require special approaches for sample preparation, separation, and mass spectrometric analysis. It primarily focuses on metabolic fingerprinting, a technique that analyzes all detectable analytes in a given sample with subsequent classification of samples and identification of differentially expressed metabolites, which define the sample classes

Metabolome refers to the complete set of small-molecule metabolites (such as metabolic intermediates, hormones and other signalling molecules, and secondary metabolites) to be found within a biological sample, such as a single organism.

Metabolites usually represents small molecular species subjected to temporal, spatial and diet variability. Metabolites are produced during metabolism or at its end. The metabolome term (coined in the late 1990s by S.G. Oliver et al.,1998) is highly in homogeneous, because it represents a vast number of components that belong to a wide variety of compound classes, such as amino acids, lipids, organic acids, nucleotides, etc. in a high dynamic range of concentrations. According to Beecher, two thousand major metabolites seem to be a good estimate for humans and this number could greatly increase if we consider also the secondary metabolites (Di Girolamo F., 2013).



The word was coined in analogy with “transcriptomics” and “proteomics”; like the transcriptome and the proteome, the metabolome is dynamic, changing from second to second. Although the metabolome can be defined readily enough, it is not currently possible to analyze the entire range of metabolites by a single analytical method. Each type of cell and tissue has a unique metabolic ‘fingerprint’ that can elucidate organ or tissue-specific information, while the study of biofluids can give

more generalized though less specialized information. Commonly used biofluids are urine and plasma, as they can be obtained non-invasively or relatively non-invasively, respectively.

A metabolite is usually defined as any molecule less than 1 kDa in size. In plant-based metabolomics, it is common to refer to "primary" and "secondary" metabolites. A primary metabolite is directly involved in the normal growth, development, and reproduction. A secondary metabolite is not directly involved in those processes, but usually has important ecological function. Examples include antibiotics and pigments. By contrast, in human-based metabolomics, it is more common to describe metabolites as being either endogenous (produced by the host organism) or exogenous. Metabolites of foreign substances such as drugs are termed xeno metabolites. Global metabolic fingerprinting and quantitative metabolite profiling, represent two complementary strategies currently applied for metabolomic investigations (Dettmer K, 2004).

In most cases, the first step in a metabolite profiling experiment is to extract metabolites from the biological matrix. Extracted metabolites are often separated using gas chromatography/MS (GC/MS), liquid chromatography/MS (LC/MS), or capillary electrophoresis/MS (CE/MS). Critical to all MS-based approaches is the efficient desorption and ionization of metabolites, where the resulting gas phase ions can be separated by mass analyzers such as quadrupole, time-of-flight, and ion trap. Ions are typically detected using a microchannel plate and photomultiplier tube and identified through comparison of exact mass, retention time, and fragmentation information with genuine standards and spectral databases (Lee D.Y. 2010).

Nowadays, high throughput LC/MS/SRM allows simultaneous analysis of different class of small molecules, metabolites, or drugs in a short time, therefore enabling a quantitative profiling of hundreds to thousands of samples for target based metabolite profiling (Wei R., 2010).

Among different types of mass spectrometers, such as ion trap, time-of-flight, orbitrap, and quadrupole mass spectrometers, a triple quadruple mass spectrometer is optimal for targeted metabolomics, based on its high sensitivity, high specificity, and excellent quantitation ability. Two stages of mass selection reside in a triple quadrupole mass spectrometer: precursor ion (MS1) and a fragment of the precursor

ion (product ion, MS2) that produce a molecular weight and structure specific sensitive measurement for a given analyte. The potential power of utilizing a triple quadrupole mass spectrometry-based quantitation technique, namely, Selected Reaction Monitoring (SRM), for metabolomics has not been fully recognized until recently. A triple quadrupole mass spectrometry-based quantitation for small molecules has been heavily used by analytical chemists for analyzing drug metabolites, hormones, pesticides, and herbicides with great precision (CV < 10%). To further increase selectivity and sensitivity of the triple quadrupole mass spectrometry-based quantitation assay, a front-end separation technique, such as LC, GC, or CE, is often added as the third dimension of separation. While a variety of separation techniques can be used to couple a triple quadrupole mass spectrometer, the polarity-based liquid chromatography (LC) stands out for its speed, simple sample pre-treatment, and numerous choices of types of commercially available columns based on different separation mechanisms such as reverse phase, normal phase, or hydrophilic interaction, etc (Silas G. Villas-Boˆas 2005).

Mass spectrometry-based metabolomics offers quantitative analyses with high selectivity and sensitivity and the potential to identify metabolites. Combination with a separation technique reduces the complexity of the mass spectra due to metabolite separation in a time dimension, provides isobar separation, and delivers additional information on the physico-chemical properties of the metabolites. However, mass spectrometry-based techniques usually require a sample preparation step, which can cause metabolite losses, and based on the sample introduction system and the ionization technique used, specific metabolite classes may be discriminated. Therefore, parallel application of several techniques, for example, GC-MS and LC-MS is desired to study the metabolome comprehensively (Dettmer K, 2007).

1.3.2 Mass spectrometry-based proteomics

Recent successes illustrate the role of mass spectrometry-based proteomics as an indispensable tool for molecular and cellular biology and for the emerging field of systems biology. Proteomics was defined as the study of “the expressed protein complement of a genome at a specific time” by Wilkins in 1994. The terms

“proteomics” and proteome” mirror the terms “genomics” and “genome”. The drivers of genomic and proteomic analyses are the technological achievements of the past decade that enable the quantitative analysis of the DNA sequence, mRNA and protein expression inside cells and include tools such as DNA microarrays, two-dimensional gel electrophoresis (2D-GE) and mass spectrometry (MS).

Nowadays proteomics can rely on powerful analytical protein-separation technologies (chromatography, electrophoresis), that serve to simplify complex protein mixtures to compare apparent differences in protein levels between two samples. However the most powerful analytical tools for proteomic analysis is mass spectrometry (MS), whose instrumentation has undergone huge changes over the past years, culminating in the development of highly sensitive, robust instruments that can reliably analyze biomolecules, particularly proteins and peptides.

The introduction of ES and MALDI (1980’s), in combination with the accessibility of genome sequence information, has revolutionized MS, thus allowing routine MS analysis of protein molecules. Two main strategies for protein ID by MS are currently used in proteomics: top-down and bottom-up proteomics. In top-down proteomics, intact proteins are introduced into a mass spectrometer and then subjected to gas-phase fragmentation (Di Girolamo F., 2013).

However, the purpose to multiply charged product ions has always been a weak point of this approach, because it may prevent the determination of product ion masses. With the introduction of the modern mass spectrometers with high mass measurement accuracy, this obstacle has been overcome (e.g., modern MALDI TOF/TOF instruments). Conversely, in bottom-up proteomics, the proteins are firstly separated by gel electrophoresis or chromatography, subsequently digested by specific enzymes (e.g., trypsin to cut lysine and arginine) and then introduced into the mass spectrometer. Bottom-up proteomics approach is represented by peptide mass fingerprinting (PMF) and tandem MS analysis. PMF has largely characterized the early years of the proteomic era; it relies on the acquisition of mass spectra from a tryptic digest of a protein sample and on the measure of tryptic peptide masses searched against a protein database such as UniProt, employing different database search engines and performing, for each protein, an in silico tryptic digest, hence

generating a theoretical spectrum. The best overlap between the experimental and theoretical mass spectra then identifies the protein.

The 1D- or 2D-SDS-PAGE-LC-MS/MS based proteomics, often called shotgun proteomics, is now the main bottom-up proteomics technological approach. The workflow provides the protein separation by 1D-SDSPAGE according to MW, followed by in-gel tryptic digestion, peptide analysis by nanoLC-MS/MS and protein ID by database searching, as above described (Evans V.C, 2012). While the application of shotgun proteomics workflows to tissues, cells, and organelles usually results appropriate, the analysis of body fluids (e.g., serum, blood, plasma, intestinal fluids, urine samples) is particularly difficult because of the complexity and of the high dynamic range of contained analytes.

Recently, LC MS/MS in SRM ion mode has emerged as a promising technique for such precise quantification of targeted proteins. Originally applied to the measurement of small molecules (such as metabolites or drugs), where a capillary chromatography column is connected in-line to the electrospray ionization source of the mass spectrometer (Picotti P., 2012). SRM exploits the unique capability of triple quadrupole (QQQ) mass spectrometers to act as mass filters and to selectively monitor a specific analyte molecular ion and one or several fragment ions generated from the analyte by collisional dissociation. The number of such fragment ions that reach the detector is counted over time, resulting in a chromatographic trace with retention time and signal intensity as coordinates. Several such precursor–fragment ion pairs, termed SRM transitions, can be sequentially and repeatedly measured at a periodicity that is fast compared to the analyte's chromatographic elution, yielding chromatographic peaks for each transition that allow for the concurrent quantification of multiple analytes. This multiplexing capability has led to the term multiple reaction monitoring (MRM), which is frequently used as a synonym of SRM. When applied to proteomics, SRM measures peptides produced by the enzymatic digestion of a proteome as surrogates of the corresponding proteins. Molecular ions within a mass range centered around the mass of the targeted peptide are selected in the first mass analyzer (Q1), fragmented at the peptide bonds by collision-activated dissociation (in Q2) and one or several of the fragment ions uniquely derived from the targeted peptide are measured by the second analyzer

(Q3). A suitably chosen set of SRM transitions therefore constitutes a specific assay to detect and quantify a target peptide and, by inference, a target protein in complex samples (Lange V., 2008).

The application of SRM to proteomics has been slow and not without complications. Proteins are large molecules and by themselves at present are not compatible with the technique. The ensuing questions of how many and which of the many peptides generated by tryptic digestion of each target protein constitute optimal inputs for SRM assays are therefore of critical importance. In addition, peptides generally yield more complex fragment-ion patterns than metabolites or drugs, thus complicating the choice of appropriate SRM transitions (Picotti P, 2012)

1.3.3 SRM in clinical applications

The capability of SRM to quantify specific target compounds across a variety of samples appears particularly well suited for biomarker verification. Candidate biomarkers for their respective disease need to be verified across large sample sets to achieve sufficient statistical power, targeting easily accessible human specimens, such as serum or plasma (Rifai N, 2006). Classically, biomarker verification relied on antibody-based assays, but recently SRM has emerged as an alternative because of its superior multiplexing capabilities of 50–100 analytes, the shorter and cheaper assay development and the capability to discriminate between protein isoforms.

Numerous examples of the application of SRM for the analysis of low molecular weight chemicals with biological relevance exist in the literature and these span a broad range of analytes including endogenous compounds, therapeutic agents and their metabolites, environmental toxicants and compounds of abuse or malicious intent. Recent examples of the use of SRM for analysing endogenous compounds in humans include the measurement of vitamins, steroids and neurotransmitters, multipleanalyses of drugs and their metabolites are too numerous to list comprehensively, but include the measurement of therapeutic agents, such as warfarin, triazolam, nevirapine and antibiotics, the measurement of drugs of abuse, such as heroin, cocaine and cannabinoids and the assay of performance enhancers, such as androgen and other stimulants. In addition, SRM has been utilised for the

analysis of low molecular weight chemicals present in plants, fish and contaminated water courses, demonstrating the versatility and breadth of application of the technique for the routine quantification of low molecular weight products (Kitteringhama, 2009).

Over the last ten years, several studies that applied SRM to protein analysis increased exponentially and the subject of such studies is progressively shifting from technological advances to biological or biomedical applications, a development that attests to the increasing maturity of the technology.

A challenge in using SRM for candidate-biomarker verification is the required sensitivity for the quantification of low-abundance proteins, given a dynamic concentration range of plasma proteins over 12 orders of magnitude (Picotti P, 2012). SRM has also been applied to study signalling pathways, for example, Wnt/β-catenin signaling, a system of high biological and biomedical importance given its deregulation in different types of cancer. Similarly, SRM has been used to quantitatively monitor linked to mammalian stem cell renewal and pluripotency in nuclear extracts from mouse embryonic stem cell (Hewel J. A., 2010).

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2 SRM method

2.1 Analytical approach

Targeted metabolomics and proteomics focus on the quantitative measurements of a specific subset of known analytes representative of biologically relevant processes by LC-MS/MS in Selected Reaction Monitoring scan mode. This approach was shown to be a valuable tool to identify altered pathways in pathological conditions and/or to define therapeutic modes of action .

Aim of this project was the development of robust and versatile analytical procedures for the direct determination and quantification of proteins or metabolites in different biological samples.

The methodological approach developed here are based on different steps: 1) identification of a set of proteins and metabolites of interest capable of satisfying a specific biological or clinic request 2) selection of transitions maximizing sensitivity and selectivity 3) optimization of SRM transitions by tuning acquisition parameters of the mass spectrometer 4) validation of the transitions in biological matrix to account to unspecific contributions of fragment ions background 5) quantification by SRM.

2.1.1 Identification of targeted analytes

The first step of a targeted experiment is the selection of a set of compounds of interest. Depending on the sensitivity and accuracy required, hundreds and eventually up to 1000 analytes can be targeted in a single LC-MS analysis after the transitions have been optimized. The selection of the metabolites and proteins set might be on the basis of previous experiments or the scientific literature or in order to make possible in vitro and in vivo studies for specific disease.

In particular, for proteins analyses specific peptides (proteotypic peptides) (Bronstrup M. 2004). that are unique for target protein and easily detectable by mass

spectrometry should be choosen. The uniqueness of a peptide sequence in a proteome can be determined, in principle, from the genomic information, but the true complexity of proteomes is generally difficult to predict. The specific mass spectrometry signal response of different tryptic peptides from the same protein can differ by as much as 100-fold in intensity. The choice of peptides with favorable mass spectrometry properties is thus crucial, as it determines the sensitivity of the assay. Nowadays, information from prior experiments conducted on natural proteomes are available in different database as, for example, PeptideAtlas (Deutsch 2008)3, the Global Proteome Machine Database (GPMDB) (Craig 2004)4, that can be used for identification of peptides that can be reproducibly detected and are thus likely associated with the most intense signals. Proteotypic peptides can be easily identified from this database, and peptides can be selected that best fit the objectives of a scoring experiment, such as the identification of proteins, splice forms, SNPs, post-translational modifications and more.

Numerous software tools for evaluating the best SRM peptides have emerged such as Skyline (MacLean B. 2010, Geiger T 1987). These software use different algorithms that consider sequence features and predicted physico-chemical properties of peptides to classify them as “proteotypic” that is, found in only a single known protein and therefore serves to identify that protein (Fig.2.1).

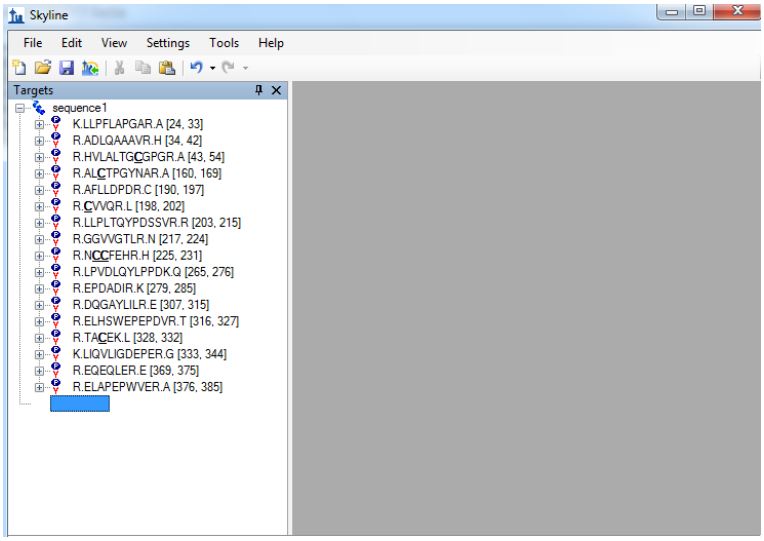

Figure1 Definition of proteotypic peptides by using Skyline software

Among the parameters critical for predicting peptides were hydrophobicity, charge, energetic and structural properties.

In general, short hydrophilic and long hydrophobic peptides should be avoided, whereas fully tryptic peptides with an average length of ~10 amino acids, devoid of residues prone to artifactual or post-translational modifications should be targeted.

The introduction of artefactual chemical modifications due to sample processing is a potential source of error in quantitative MS experiments because a fraction of the targeted peptide might be converted into the modified form in an irreproducible and unpredictable manner and can bias the quantification. Therefore, care should be taken to avoid targeting peptides with a high propensity for artefactual modifications such as peptides containing methionine or tryptophan residues should be avoided, as the side chains of these amino acids are prone to oxidation, peptides containing glutamine or asparagine residues may be chemically unstable and convert to glutamate or aspartate. The rate of conversion is dependent on the surrounding sequence, for example, asparagine followed by glycine or proline is particularly prone to deamidation. In addition, N-terminal glutamine residues are quickly transformed to pyro-glutamate under acidic conditions(Geiger T. 1987).

Similarly, peptide susceptible to undergo post-translational modifications, glycosylation, phosphorylation, etc. might to lead to bias as they can be present in various forms.

2.1.2 Selection of optimal transitions

The combination of m/z settings for the first and third quadrupole is referred as “transition”, it is important to select transition ions that maximize sensitivity and specificity of the SRM experiment. Several in silico methods are available, and continue to be refined, which facilitate the choice of the transitions. During selection of suitable transitions for a targeted compound one selects the fragment ions for each precursor-ion charge state that provide the highest signal intensity and lowest level of interfering signals. Transitions can be selected from (1) libraries of previously collected MS/MS spectra in online repositories, (2) computational tools that predict fragmentation, (3) analysis of synthetic peptide

standards. Just as criteria for selecting target peptides exist, characteristic peptide fragmentations empirically found to provide a high signal intensity have been described. Several software tools, for facilitating transition selection have been described. Because SRM assays are largely conducted with triple quadrupole instruments, optimization has been directed to this type of mass analyzer. These employ fairly sophisticated algorithms to deliver putative SRM transitions based on a combination of theoretical rules and empirical observation for optimal peptide MS/MS. The current practice is to select two transitions for metabolites analysis (the most abundant for screening said quantifier and a second for confirmation as qualifier) and at least two to five most intense transitions per peptides for proteins to build an SRM assay.

For metabolomics analysis, it has been used MassHunter Optimizer, part of the MassHunter Workstation, a commercial software package from Agilent for triple quadrupole LC-MS. It allows fast, sensitive compound identification and confirmation that helps design SRM acquisition. MassHunter also enables the optimization collision energy as well as select the best transitions from empirical data generated on the Agilent platforms using Optimizer-designed methods.

For peptides and proteins analysis, it has been used Skyline, an open-source software platform that provides support not only for experimental design (selecting peptides and optimization of transitions) but also for downstream data analysis (T. D. MacLean B. 2010).

Skyline utilizes the ProteoWizard libraries (Kessner D. 2008) to allow analysis of data from all MS instrument platforms, thus providing a vendor-neutral resource for sharing and creation of both methods and results across instrument platforms. Skyline facilitates the generation and refinement of proteomic peptide lists from protein sequences or database entries, both by utilizing online MS/MS spectral repositories and by supporting the generation of custom-built libraries based upon sets of locally acquired tandem spectral data.

Skyline creates transition lists and vendor-specific instrument methods that can be imported directly into instrument control software for MS instruments from several vendors. Skyline also provides a platform for standardized analysis of SRM result files for peak integration and visualization and data quality assessment across

multiple analyses. Finally, Skyline allows the export of processed data in custom report formats compatible with subsequent statistical analyses, publication, and database deposition (Fig. 2.1).

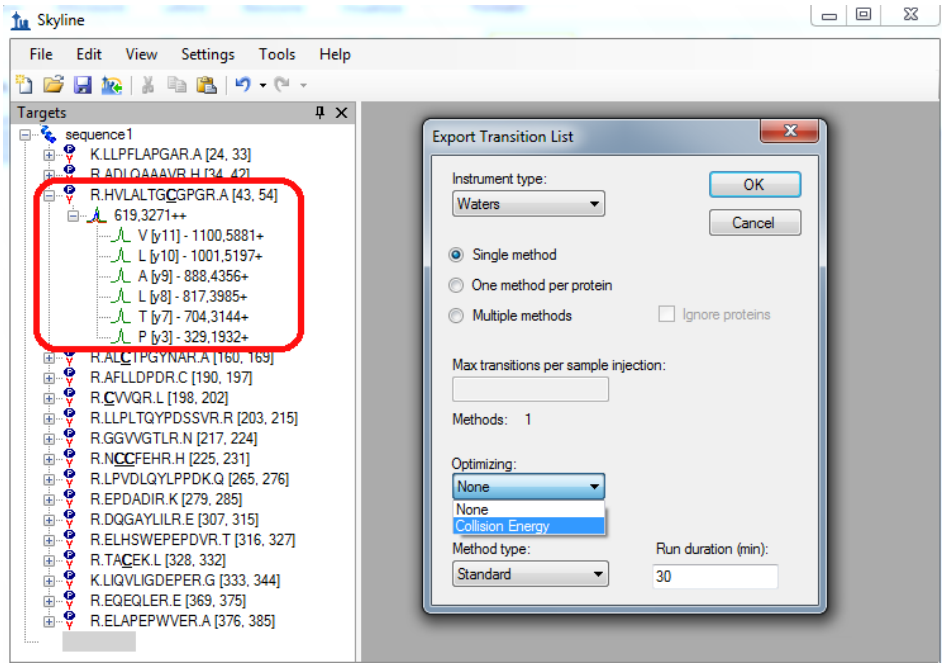

Figure 2.2: Skyline, definition of the transitions for a peptide: is the doubly charged precursor m/z , while the others are y-fragment m/z . The near window is the panel control to export transition list by optimizing the collision energy.

However, there is a physical limit to the number of transitions that can be measured in the same analysis. The SRM cycle time is the product of the number of transitions recorded in the cycle and the time spent on acquiring each transition signal (dwell time). Monitoring too many transitions in an SRM run results in too long a cycle time and hence an insufficient number of data points to reconstruct the chromatographic elution profile of the targeted peptide, compromising accurate quantification . Alternatively, this results in a low dwell time and hence a reduced signal-to-noise ratio, compromising the detection of low-abundance components. This generates the well-known trade-off between the number of transitions and the limit of detection of an SRM experiment **(**Picotti P 2012**)**.

2.1.3 Optimization of SRM transitions

In order to increase the limit of detection and quantification of the SRM assays mass spectrometry parameters that are molecule-dependent (such as declustering potential or cone voltage) or transition-dependent (for example, collision energy) optima. can be optimazed. To this end, instrument vendors often provide functions that linearly correlate collision energy or declustering potential with the precursor mass-to-charge ratio (m/z) (MacLean B. 2010, Kuzyk 2009).

In particular, ionization conditions have been optimized. Precursor charge state is essential for a sensitive detection. Charge states can be inferred from previous experiments on other ESI instruments. However, ionization devices and experimental conditions (flow rate, solvents, background) can influence charge state distributions. Moreover, during the ionization process, single ions need to be generated. The process of dissolvation and dissociation of ion clusters is supported by a voltage potential referred to as ‘declustering potential' (DP), ‘fragmentor voltage' or ‘ion transfer capillary offset voltage' depending on the manufacturer. At too high DP, compounds are fragmented already in the source. From plotting many experimentally determined DP optima, a positive linear correlation of precursor m/z value and DP optimum was determined (unpublished results). As the DP displays a broad optimum, individual optimization usually does not result in a significant signal increase. Individual transitions of a peptide have the same DP optimum if they are derived from the same precursor charge state.

During fragmentation singly charged y ions are the predominant type of fragments generated by CID in a linear collision cell. Only small b ions are usually observed. Fragments with *m/z* values close to the precursor should be avoided as such transitions are usually noisy. Fragments with *m/z* values above the precursor generally display the highest selectivity, as the singly charged chemical background cannot result in fragments with higher *m/z* than the precursor. In contrast, tryptic peptide ions are predominantly doubly or triply charged with one charge at each terminus. Upon fragmentation, one charge is lost and therefore a part of the fragments has an *m/z* value bigger than the precursor value. A parameter that is of

considerable importance is the collision energy, that is tuned to optimize the intensity of the fragment ions of interest: with increasing collision energy, a larger part of the precursor ions is fragmented and fragment ion intensity increases until this increase is overcompensated by the losses due to secondary fragmentation events. The optimal collision energy is approximately linearly correlated with the precursor mass for a given charge state. However, particular peptides or fragments deviate considerably from the predicted value.

The easiest and most systematic way of optimizing ionization and fragmentation conditions is to test possible transitions in direct infusion mode and ramp the parameters . This process is partly automated by add-ons for the acquisition software.

2.1.4 Validation of the transitions

Transitions extracted for an SRM assay should be validated by addressing the likelihood that the chosen transitions and their intensity distribution are associated with the target peptide or metabolite. Each transition selected for a specific target should be evaluated in the context of the actual biological matrix to account for unspecific contributions of the fragment ions deriving from co-eluting species with similar properties. Typically, one measures 3–5 intense transitions for each endogenous peptide. When one applies an assay to a biological sample, the first question is whether it could be used to unambiguously identify the target peptide in a complex sample.

Plasma is probably the most complex biological matrix, so it is important to evaluate the matrix effect that can affect the goodness of the analysis and the quality of the result obtained. Whilst this is readily achievable for low molecular weight markers, it provides a major challenge when looking for proteins or peptides. It has been estimated that human plasma contains proteins that span 10 orders of magnitude of concentration, and includes resident plasma proteins (high and medium abundance) and signaling proteins such as hormones and cytokines (low abundance).

High abundance species comprise a small population of approximately 20 proteins, including albumin, haptoglobin, hemopexin and the immunoglobulins, which between them account for 99% of the total protein content.

Specifically, the matrix affects not only ion suppression, a major pitfall for the analyst, but also detection capability, repeatability and accuracy.

The mechanism of the matrix effect in LC–MS, well described by Trufelli et al.2011, is not fully understood. One of the most important aspects is competition between an analyte and a co-eluting matrix component during ionization. As a result, there is a decrease in analyte ionization (ion suppression) or an increase in this ionization (ion enhancement). The matrix effect depends strongly on the type of ionization source. In ESI droplets are produced and a greater amount of additives (from eluents or sample matrices) may lower evaporation efficiency and the ability of analytes to reach the gas phase.

Reactions in the electrospray source depend on the properties of solvents and additives (like volatility, viscosity, pH or electrolyte concentration), the physicochemical properties of analytes (pKa, hydrophobicity, proton affinity or ion solvation energy) and the operating parameters of LC–MS instruments (flow rate, temperature and voltage). Hence, all these variables make ionization a highly complex and changeable process. Furthermore, co-eluting components may produce similar ions in MS or MS/MS experiments, which leads to the erroneous interpretation of results, especially when these components are present at high concentrations in the extract and eluted in the same retention window as the target compounds.

In summary, the consequence of the matrix effect is primarily a reduction or increase in the detector signal of the analyte; there may also be problems with repeatability, ion ratio (modification of typical fragmentation patterns, and hence difficulties with database searching), linearity and quantification (false negative diagnostics or overestimation of analyte concentration) (Trufelli H. 2011).

Several strategies have been put forward to eliminate the problems resulting from matrix (Frenich A.G. 2009, Kostiainen R. 2009). The most effective of them is exhaustive sample clean-up used immunoaffinity depletion of highly abundant plasma proteins in combination with peptide fractionation by strong- or mixed-mode cation exchange chromatography, which can help to remove interferents, and improves the LOQs to 1–10 ng ml− but it does run the risk of analyte loss. The second strategy is to improve chromatographic separation. This allows the analytes

to be eluted in an appropriate period of time, in order to avoid co-elution with matrix components. A third approach is to dilute the final extract several times so that fewer matrix components will be injected into the analytical system

The matrix effect can be compensated for with appropriate calibration methods, firstly, by using calibration standards in pure solvents, and secondly, by using standards in the matrix (Frenich A.G. 2009).

Finally, the standard-addition method is a much cheaper and useful approach when no blank matrix is available for the calibration. Here, a known amount of analyte (spike) is introduced into aliquots of sample extracts containing the target compound, so that any co-extracted impurity is accounted for in the calibration; one has to bear in mind, however, that the volume of standard added must be small enough to prevent sample extract dilution. By using this methodology, the unknown concentration initially present in the sample can be calculated by extrapolation.

At this point, for each analyte the recovery value (%) was estimated by measuring the concentration of analyte added to real samples (spiked samples).

$$Recovery(\%) = \frac{C_1 - C_2}{C_3} * 100$$

where is that:

C_1: analyte concentration measured after the addition

C_2: analyte concentration measured before the addition

C_3: added concentration

2.1.5 Quantification by SRM

SRM-based quantification can be coupled to different strategies for relative or absolute protein quantification.

The aim of relative quantification is to express the amount of a targeted compound in one state or sample relative to that of a second state or sample. Approaches to relative quantification that can be coupled to SRM include label-free or stable isotope–labeling methods. Isotope-labeling methods introduce stable isotope tags to proteins or peptides and rely on the concomitant mass spectrometry measurement of

heavy and light or differently labeled peptides as an internal standardization, thus correcting for experimental and instrumental variability, downstream of the label-mixing step (Picotti P 2012). Label-free approaches (Rinner O. 2007)) rely on the direct evaluation of mass spectrometry signal intensities of naturally occurring peptides contained in a sample. Although in principle each of these quantification methods can be coupled to SRM analysis to be noted that methods based on chemically added tags normally change peptide-fragmentation patterns as well as the precursor-ion mass. In such cases, new SRM assays should be developed that are specific for the labeled peptides. In contrast, labels that conserve the chemical structure of peptides, such as metabolic labeling, also conserve their fragmentation patterns and thus the relative transition signal intensities. One can thus easily derive the assays for the heavy form of a peptide from the fragment-ion spectrum of the light peptide and the knowledge of the site and type of incorporation of the heavy label. A drawback of label-based methods is the introduction of additional sample-processing steps. An intermediate solution between the high precision of label-based approaches and the simplicity of label-free methods is the use of a single labeled peptide as a reference standard for all other measured endogenous peptides (Zhang 2011).

The gold standard for absolute quantification is stable-isotope dilution. The most commonly used approach relies on isotopically labeled reference peptides that are chemically identical to the light native peptides (AQUA peptides) (Gerber 2003). The analytical precision of this method is high and can result in up to 5% errors in the estimation of the amount of peptide originally loaded onto the liquid chromaography system. However, issues such as incomplete digestion of the target protein, partial artifactual modification of the target peptide or partial loss of the synthetic peptide before addition can affect the accuracy of this approach. For such reasons, the absolute quantification method is sometimes referred to as 'precise relative quantification (Picotti P 2012).

In our SRM experiment, quantification was carried out by the external standard method with the realization of calibration curves, considering that areas subtended to peaks, for each transition, are proportional to concentration. The area under this curve is the extracted ion current (XIC) of a specific transition. XICs of peptides of

interest are extracted from the original data file. Filters are used to correct baselines or to remove artefactual spikes. XICs are then smoothed with an average filter before performing a closing and an opening mathematical morphology operation with a small flat structuring element. The closing operation eliminates thin valleys and conserves the intensity of local maxima, while the opening operation eliminates thin peaks (i.e. remaining spikes) and conserves the intensity of local minima. Hence, detection of peak positions is performed on the closed profile, and the opened profile is used to eliminate remaining spikes. The peak boundaries are searched on the closed profile, and the peak area (i.e. the quantification value) is computed.

Another important and critical procedure in protein quantification is data normalization. Linearity determines the highest measurable concentration within the specified conditions. The lower limit of detection (LLOD, often referred to as LOD) and the LOQ are defined, respectively, as the concentration level at which the analyte can be reliably detected in the sample under consideration and as the level at which the analyte can be detected and measured with sufficient precision. Several methods are used to determine LOD and LOQ (Armbruster D. A. 1994) (Zorn M. E. 1997). The simplest one consists in calculating LOD and LOQ for a given analyte as the amount of this analyte providing a signal corresponding to the mean value of repeated blank sample measurements +3 and +10 standard deviations, respectively.

These strategies are simple and cost-effective and have demonstrated high reproducibility and linearity.

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3 Metabolomics and Xenobiotics

3.1 Introduction

In recent years, metabolomics has been widely used in toxicology, mainly because it has the ability to provide information to better understand the mechanisms of toxicity, while also providing the ability to identify biomarkers.

In this context, metabolomics become a valuable tool for toxicology. Like the other "omics" sciences, metabolomics uses high-throughput performance analysis and the information that it provides are closer to the phenotype and therefore more easily referable to the classic toxicological endpoint. The metabolomic analysis may be performed on biological fluids, as well as on tissues and cellular cultures; unlike traditional histopathological methods, it requires small volume of sample, allowing a non-invasive sampling, constitute an important advantage for toxicological studies that are based on the use of small rodents as animal models (Ebbels ,2007). Moreover , the low cost allows a greater number of replicas and evaluations of concentrations at different time for monitoring of physiological states up to overt stages of intoxication (Craig , 2006; Schnackenberg et al., 2009).

Exposure to chemicals (xenobiotics) by feeding, environment or drugs is almost inevitable for all living beings, including humans. Interactions between xenobiotics and biological systems are bi-directional; in fact although the xenobiotics can be processed by the biological system through absorption, dislocation, metabolism and excretion, the entire biological system, or part of it, it may be severely compromised, particularly when subjected to high doses or repeated exposures.

The metabolic activities, which include both the xenobiotic to endogenous metabolism, play a central role in the damage caused by exposure to xenobiotics, especially for xenobiotics that induce toxicity (XIT); numerous toxicological studies have shown that the formation of reactive metabolites and disruption of endogenous metabolites, constitute the key events of the beginning and progression of toxicity caused by the XIT (Nicholson, 2002). Chemical and biochemical analysis of genes,

proteins, and metabolites are commonly conducted for acquiring information on toxic activity of any XIT in order to understand the mechanisms of toxicity that involve changes at the gene, protein and metabolic level, respectively. However, while changes at the gene and protein level, reflecting potential physiological changes, alterations of the metabolome are directly related to the real consequences of exposure to toxic xenobiotics; this is due to the fact that metabolites are the end products of biological processes. Therefore the study of the metabolome, resulting from exposure to XIT, is fundamental to understanding the mechanisms that regulate the toxicity.

“Endocrine Disrupting Chemicals" (EDC), constitute a special class of xenobiotics that can alter the normal hormonal homeostasis. According to the definition adopted by the European Union "an interferent endocrine is an exogenous substance or a mixture , that alters the endocrine system function, causing adverse effects on the health of an organism, or its progeny, or (sub) population”. The assessment of risks associated with exposure to EDCs concerns various areas, as this class of compounds, characterized by high chemical and physical heterogeneity, including environmental contaminants, herbicides, pesticides and compounds commonly used in industrial manufacturing, including food grade (De Coster, 2012)

The thyroid, endocrine gland strongly influenced by the external environment, is the target of a large number of these interfering, currently classified as Thyroid-Disrupting Chemicals (TDC) (Crofton, 2005). Among these, ethylene bis-dithiocarbamate fungicides, of which ethylene thiourea (ETU) is the major metabolite, and organophosphorus pesticides, chlorpyrifos (CPF) are widely used in agriculture for the poor tendency to bio-accumulation. The massive use of these substances has clearly led to higher exposure often unconscious, not to high doses for several organisms, including humans (Nicholson 1999).

Although the thyroid alteration linked to Etu and CPF exposure at has been studied both in vivo models massively exposed as well as in workers exposed to high amounts (Bouhifd 2013), in both cases it was found reduced serum levels of thyroid hormones T3 and T4 (Watkins 2002). Recently it has been shown that chronic exposure, even at low doses, is associated with hormonal and homeostatic alterations in sex hormones, with possible impact on male and female fertility.

The research activity, in collaboration with Prof. De Felice and prof. Concetta Ambrosino at Biogem , Ariano Irpino (AV), have been focused on some hormone of hypothalamic-pituitary-thyroid axis and two TDC which alter the endocrine activity, in order to assess the direct effects of exposure to pesticides in humans through the use of model organisms.

3.2 Material and methods

3.2.1 Chemicals and reagents

Triiodothyronine (T3), thyroxine (T4), 17β-estradiol (17βe), testosterone (T), chlorpyrifos (CPF) and ethylenethiourea (ETU) were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis and purchased from J. T. Baker (Phillipsburg, NJ). All stock solutions were stored at −20 °C.

3.2.2 Preparation of standard solutions

Standard solutions were prepared by dissolving the equivalent of 1 mg of the respective metabolites in 1mL of methanol. Testosterone standard solution of 1000 ng\uL was dissolved in a solution of dichloromethane and acetonitrile (9:1).
A standard solution of 500 pg\uL of each metabolite was used for optimization of the SRM transition. Standard solutions at different concentrations were prepared by serious dilution and were used for calibration curves. Standard mixtures for each analyte were prepared as follows: 0.5, 1,0, 5, 10, 25, 50, 100 pg/µl for T3, T4, ETU; 1, 5, 10, 25, 50, 100, pg/µl for CPF and 17β-estradiol; 5, 10, 25, 50, 100, 150 for T. All standards were kept at -20ºC before LC MS/MS analysis.

3.2.3 Spike and sample preparation

In order to validate the optimized method, serum samples were spiked, prior to the extraction, with appropriate concentrations of the standard mixture of target analytes. Finally, for each of 155 samples of mouse serum, an aliquot of 100 µl was treated with 300 µl of cold ethanol. The mixtures were stored at -20°C for about 30 minutes followed by centrifugation at 10000 rpm for 10 min at room temperature. The clear supernatant, subsequently subjected to analysis, was transferred to a 1.5-mL tube and stored at -20°C. 1 ul of supernatant was used for analysis.

3.2.4 LC–MS/MS instrumentation and condition

SRM analysis was performed using a Agilent 1200 series LC coupled to a 6420 triple quadrupole MS. Analytes were separated on a Phenomenex Kinetex reverse-phase C-18 column (100mm x 2, 1 mm; 5 µm, 100 Å) at a flow rate of 200 µL/min with a 5% to 95% linear gradient in 12 min (A solvent 0.1% formic acid, 2% ACN in water; B solvent 0.1% formic acid, 5% water in ACN).
For each compound, data acquisition parameters for SRM in positive ion mode, were automatically optimized by MassHunter Optimizer software, using a standard solution of 500ng\ml (500ppb) Based on the analytical parameters optimized in the previous stage, the acquisition methods were processed through software MassHunter Data Acquisition.

3.2.5 Data Processing

Extracted mass chromatogram peaks of metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00). Peak areas of corresponding metabolites are then used, as quantitative measurements, for assay performance assessments such as assay variation, linearity etc.

3.3 Results

3.3.1 Mass spectrometer parameters optimization

The setup of the SRM analytical method has required the identification of the specific acquisition parameters for each molecules For this purpose, standard solutions 500 ng \ ml of each analyte were introduced for DI (direct injection), and analyzed by the Mass Hunter Optimizer software. The optimization has proceeded by a suitable definition of the following parameters:

- Molecular weight of the analyte
- Polarity of ionization
- Minimum m / z ratio detectable for product ions "low mass cut-off"
- Collision energy (CE)
- Fragmentor Voltage.

The low mass cut-off refers to the m / z value below with ions has unstable trajectories and for this reason is excluded from fragmentation.

The optimization of collision energy and fragmentor values guarantees for each analyte the best result in terms of signal intensity. Finally, for each analyte the two product ion with the major abundance are selected.

The transition corresponding to the highest signal has defined as quantifier, useful for quantification. However, by acquiring additional signal corresponding to the next highest product ion (qualifier), enough information may be considered available for confirmation, particularly if the ratio of signal between the two product ions is consistent between the calibration standards and the unknown samples.

Based on the analytical parameters optimized, a SRM methods has been developed using the software MassHunter Data Acquisition, It includes transitions, specific parameters related to the ionization and fragmentation of each analyte and the dwell time that indicates time spent to monitor single transition. The results are showed in the table 3.1.

Compound Name	Precursor Ion m/z	Product Ion m/z	Dwell (ms)	Fragmentor (V)	Collision Energy (V)	Polarity
Thyroxine (T4)	777,9	731,6	200	135	28	Positiva
		322,8	200		92	
Triiodothyronine (T3)	651,9	605,7	200	135	20	Positiva
		197,1	200		84	
Testosteron	289,4	108,9	200	135	24	Positiva
		97,1	200		20	
17βEstradiol	255,4	144,1	200	135	44	Positiva
		115,1	200		72	
Ethilenthiourea	103,5	60,1	200	135	8	Positiva
		44,1	200		8	
Chlorpiriphos	350,5	199,1	200	135	16	Positiva
		108,9	200		76	

Table. 3.1 Precursor ion, product ions and mass spectral parameters optimized for all analytes.

3.3.2 Quantification and normalization

1 ul of a mixture of targeted metabolites standard solution at different concentrations were separated in a single run by a LC-system. The total ion current (TIC) chromatogram as a function of retention time was registered from detector, showing a good peak shape for each analyte.

Identification and quantification is based on extracted ion chromatogram (XIC) signals of different transitions (Fig 3.1).

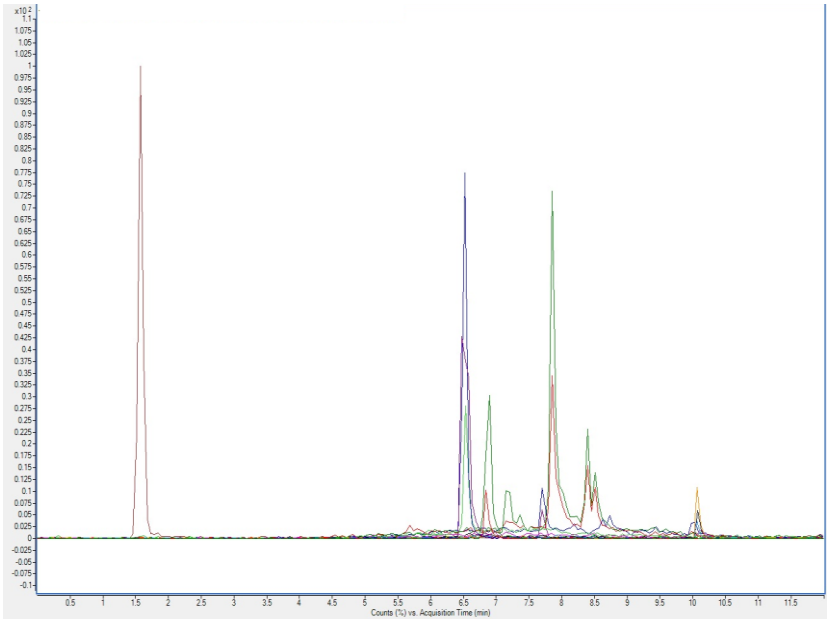
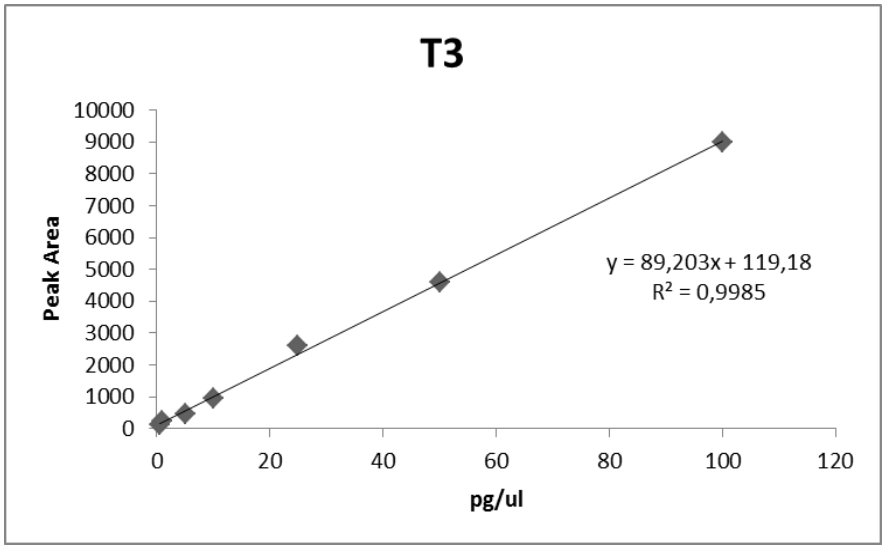
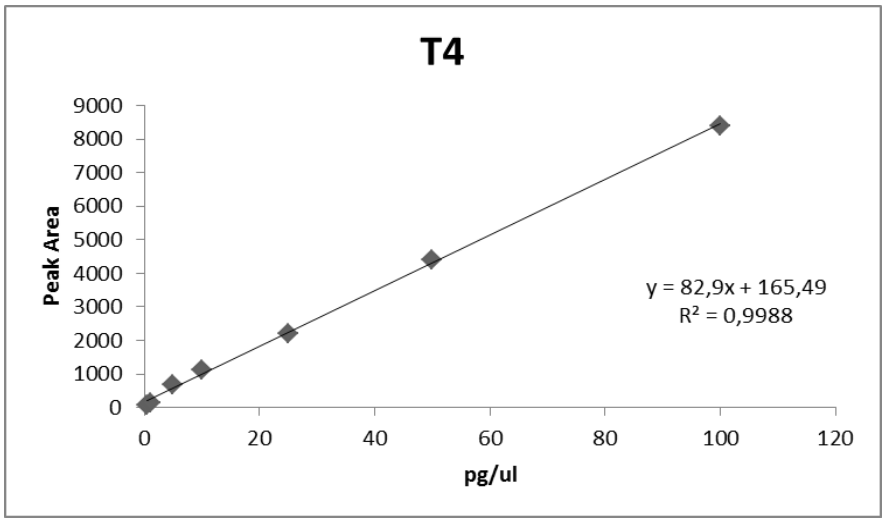
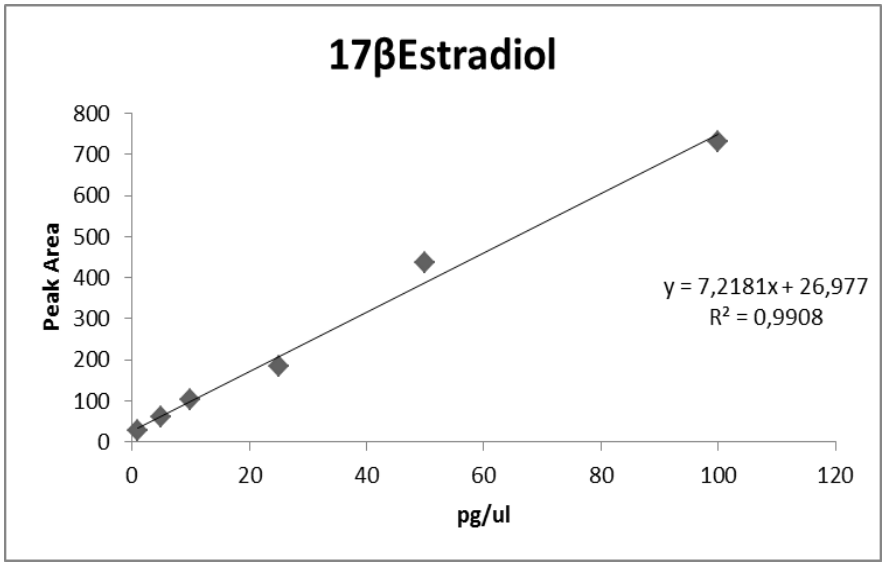


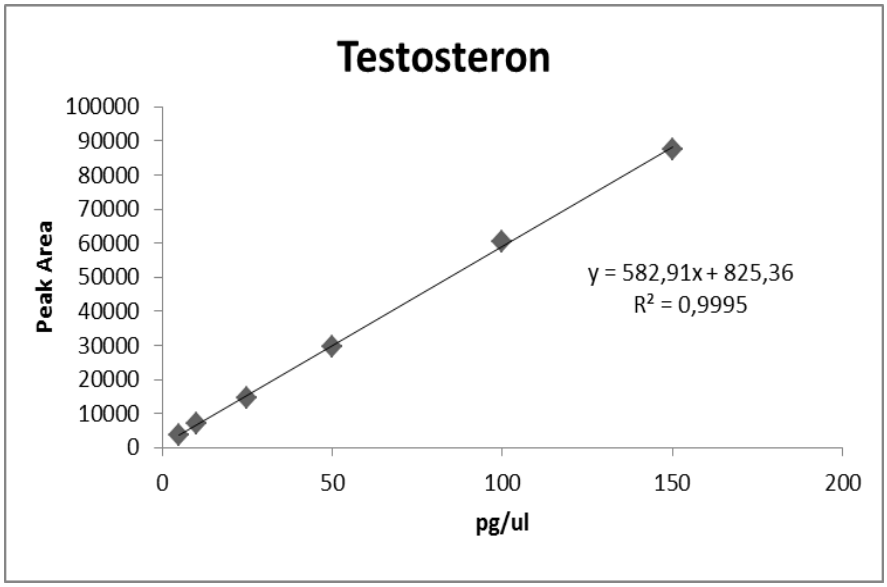
Figure 3.1- Extracted ion chromatogram (XIC) chromatogram for a standard mixture solution of six metabolites (T3, T4, CPF; ETU; T; 17β).

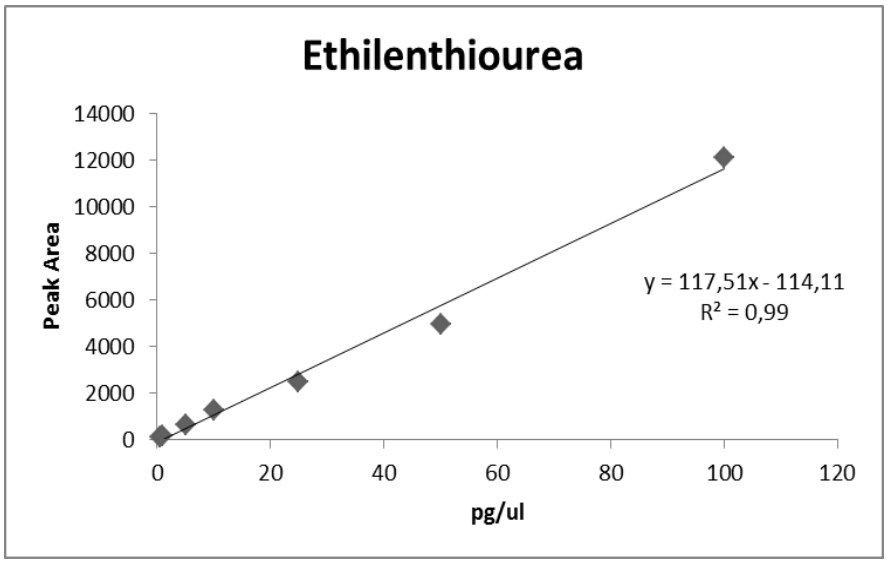
The calibration curves were obtained by analyzing standard solutions at different concentrations. Good linearity (R^2 > 0.99) is obtained for all compounds over two orders magnitude in concentration range (Fig 3.2).




T4
Peak Area
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
20
40
60
80
100
120
pg/ul
y = 82,9x + 165,49
R² = 0,9988


17βEstradiol
Peak Area
800
700
600
500
400
300
200
100
0
0
20
40
60
80
100
120
pg/ul
y = 7,2181x + 26,977
R² = 0,9908


Testosteron
Peak Area
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
50
100
150
200
pg/ul
y = 582,91x + 825,36
R² = 0,9995



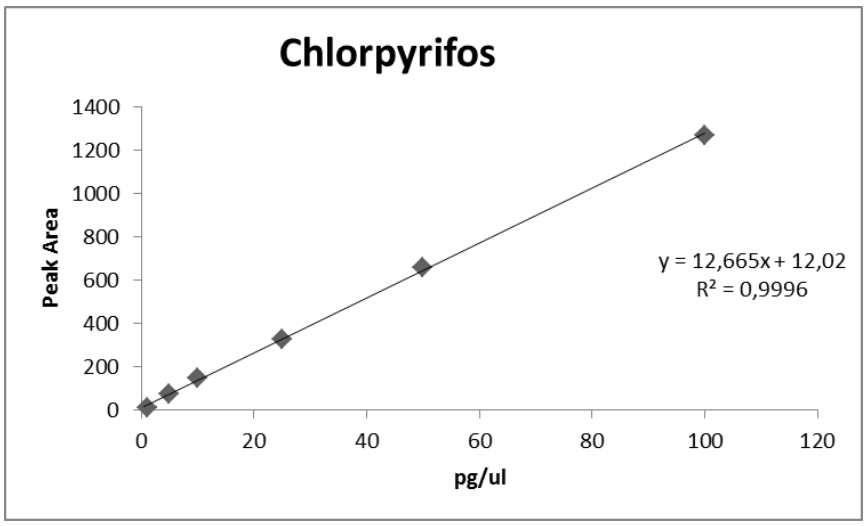


Figure 3.2 : Standard calibration curves obtained for the six analytes.

Limit of detection (LOD) and limit of quantification (LOQ) were determined as the lowest spiked concentration of each compound which, upon addition to the serum, was experimentally determined with acceptable precision (better than 10%) and accuracy (100 ± 20%). The matrix effect and relative recovery was determined interpolating from respective calibration curve the value "abundance" obtained by spike analysis of each analyte (Tab.3.2).

Compound Name	Linear range (pg/ul)	Recovery %	LOD (pg/ul)	LOQ (pg/ul)
Thyroxine (T4)	0,5-100	90	0,34	1,02
Triiodothyronine (T3)	0,5-100	85	0,27	0,80
Testosteron	5-150	89	2,58	7,81
17βEstradiol	1-100	88	1,99	6,04
Ethilenthiourea	0,5-100	75	0,53	1,61
Chlorpiriphos	1-100	80	0,92	2,78

Table 3.2 Normalization parameters for all compounds.

3.3.3 Validation of the method

According to the conditions of the method developed during optimization step, 155 murine serum samples from model organisms, felt with different quantitative of the two pesticides via food and water, were subjected to quantitative analysis. Target analytes were extracted from murine serum by protein precipitation prior to LC-MS/MS analysis. Quantification was achieved by the external standard method by interpolation on calibration lines using Agilent MassHunter Quantitative Analysis software (B.05.00).

The results which take into account the recovery values for each analyte and are summarized in the table 3.3.

Analyte	♀ Concentration range (pg/µL) in 74 samples (*)	♂ Concentration range (pg/µL) in 81 samples (*)
Testosterone	8,32 - 53,34	47,90 - 159,99
Estradiol	6,49 - 173,44	6,06 - 81,50
Triiodothyronine	0,87 - 18,27	0,94 - 4,60
Thyroxine	14,88 - 74,60	17,58 - 101,32
Chlorpyrifos	2,98 - 11,54	3,17 - 12,31
Ethylenethiourea	1,96 - 255,26	1,87 - 1028,82

Table 3.3. Murine serum concentratio ranges determined for both male and female.

3.4 Discussion

Mass spectrometry-based metabolomics is a valuable tool for the study of various biological processes and is highly diffused in many scientific fields, including the toxicological, for its efficiency and reliability.

The mass spectrometry applied to targeted metabolomics has been shown a number of important advantages respect to classical method as immunoassays that are typically used for quantitative analysis,. In fact, the proposed analytical methods have allowed to the simultaneous and high sensitivity quantification, of specific molecules in complex mixtures. The SRM mode have a strong selective nature that can significantly reduce the risk of false positives. In addition, the reduced required volume (100ul for extraction), associated to a relatively non-invasive sampling, demonstrate the absolute compatibility with the toxicological in vivo studies, limiting the sacrifice of the animal models. The high analytical productivity, combined with a wide versatility of the techniques developed, had led to the evaluation of several samples and monitoring of treatments and physiological states emphasizing the effects of these pesticides on hormonal activity and fertility.

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4 H_2S SULFUR METABOLITES

4.1 Introduction

H_2S is an important biological signalling molecule involved in vascular and nervous system functions, whose biogenesis and regulation are still poorly understood. Excessive H_2S production in hyperhomocysteinemia may contribute to the associated cardiovascular pathology (Chiku T, 2009). However, despite the growing interest in H_2S biology and the therapeutic potential of H_2S-releasing compounds (Kashfia K., 2013), surprisingly little is known about the enzymatic production of this gas and how it may be influenced by changes in sulfur amino acid levels in disease states (Fig. 4.1).

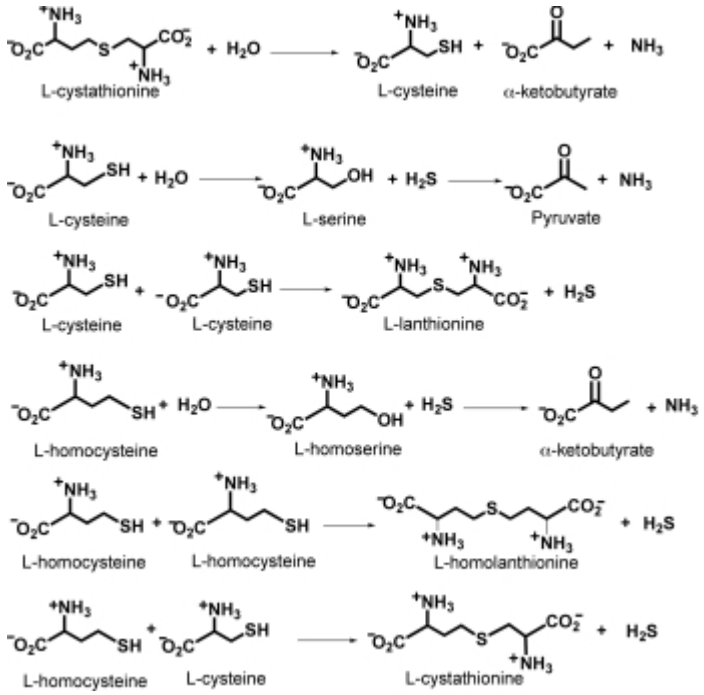

Figure 4.1 Cystathionine cleavage and H_2S-generating reactions

Several studies demonstrated that H_2S occurrence can be inferred by a novel biomarker, homolanthionine, which has been reported in urine of homocystinuric

patients, and by lanthionine generated by condensation reaction of two molecules of cysteine (Chiku T, 2009) .

It has been shown that early detection and/or pharmacological treatment can prevent many clinical complications associated with these diseases and can substantially improve the quality of life of patients. The determination of these molecules require screening methods with an high degree of specificity and sensitivity, avoiding high cost and lengthy analysis.. Currently, there is increasing interest in developing accurate methods for the quantitative analysis of metabolites in biological samples. Liquid chromatography-tandem mass spectrometry (LC-MSMS) methods have been successfully used in screening protocols to identify and determinate a wide range of metabolites in different matrices, as well as to study their metabolisms (Dettmer K., 2007; Kitteringhama N. R., 2009). Nowadays, automatic rapid high performance liquid chromatography (HPLC) tandem mass spectrometry has become the technique of choice for several metabolite analysis thanks to the capacity to simultaneously separate and determinate multi component mixtures. Modern multiplex instruments can analyze thousands of samples per month so that, the generally high instrumental costs, the cost of the individual assay is affordable (Gosetti F., 2013). In addition, the improved specificity and resolution offered by triple quadrupole mass spectrometry allow their application in profiling (namely identification and determination) of metabolites in human biological matrices. The selectivity is achieved by the selected reaction monitoring characteristic of triple quadrupole mass spec instrument.

A number of methods for measuring cystathionine and homocysteine have been reported (Bártl J, 2014), although no analysis of H2S diagnostic metabolites has yet been published.

Here, a method utilizing liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring ion mode has been developed and evaluated for the determination of keys H2S metabolites in plasma, in collaboration with First Division of Nephrology, Department of Cardio-thoracic and Respiratory Sciences, Second University of Naples. The simultaneous, sensitive, fast, and reproducible measurement of homocysteine, homolanthionine, homoserine, lanthionine, and cystathionine concentrations might be useful in the highlighting the mechanism responsible for hyperhomo-cysteinaemia.

4.2 Materials and methods

4.2.1 Chemicals and reagents

Homocysteine, homoserine, lanthionine, and cystathionine were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis. All stock solutions were stored at −20 °C.

4.2.2 Preparation of standards solutions

Standard solutions were prepared at 1 mg/mL concentration in 50:50 methanol/water with 0.1% formic acid. A stock solution of 500 pg\uL of each metabolite was used for optimization of the SRM transition.

Standard solutions at different concentrations were prepared by serious dilution and were used for calibration curves. All standards were kept at -20ºC before LC MS/MS analysis.

4.2.3 Spiked and samples preparation

To prepare spikes, blood samples from healthy anonymous donors without any hematological diseases were collected in EDTA tubes and enriched with different amount of lanthionine. These spiked blood samples were stored at −20 °C until the analysis.

A 200 µL of plasma sample was submitted to protein precipitation with 600 ul di Ethanol and vortexed thoroughly. The mixture was stored to – 20°C for about 30 minutes to complete protein precipitation. After centrifuged at 13,000×g for 10 minutes, the supernatant was then directly transferred into HPLC auto sampler and 5 µl analyzed using LC–MS/MS.

4.2.4 LC–MS/MS instrumentation and conditions

The LC–MS/MS analyses were carried out by using a 6420 triple Q system with a HPLC 1100 series binary pump (Agilent, Waldbronn, Germany). The chromatographic separation was achieved on reverse-phase C-18 column (Kinetex - Phenomenex 100mm x 2,1 mm; 5 µm, 100 Å) with eluent A (0.1% formic acid, 2% MeOH in water) and eluent B (methanol) and the flow rate was 0.200 mL/min. Starting condition was 5% to 95% A in 8 minutes than to 100% for 2 minutes. Tandem mass spectrometry was performed using a turbo ion spray source operated in positive mode, and the selected reaction monitoring (SRM) mode was used for the specific analytes.

4.2.5 Data processing

The ideal conditions for transitions detection were determinated via Agilent MassHunter Optimizer software. Extracted mass chromatogram peaks of metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00).

4.3 Results

4.3.1 Mass spectrometer parameters optimization

The transitions for each metabolite were properly selected by MassHunter Optimizer software in post-column infusion experiments by directly infusing analytes dissolved in the mobile phase. A SRM methods has been developed using the software MassHunter Data Acquisition and all analytical parameters optimized are showed in the table 4.1.

Compound Name	Precursor Ion m/z	Product Ion m/z	Fragmentor (V)	Collision Energy (V)	Polarity
Cystathionine	223,3	134,1	81	5	Positive
	223,3	88	81	25	Positive
Lanthionine	209,3	120	81	9	Positive
	209,3	74	81	25	Positive
Homocysteine	136,2	90	81	5	Positive
	136,2	56,1	81	17	Positive
Homoserine	120,1	74	81	5	Positive
	120,1	56,1	81	17	Positive
Homolanthionine	237,1	148	81	5	Positive
	237,1	102	81	30	Positive

Table 4.1 Precursor ion, product ions and mass spectral parameters optimized for all analytes.

4.3.1 Quantification and normalization

Calibration curves were obtained by external standard method by plotting peak areas against concentration (pg/µl), and linear functions were applied to the calibration curves. Data were integrated by Mass Hunter quantitative software showing a linear trend in the calibration range for all molecules (Fig 4.2).

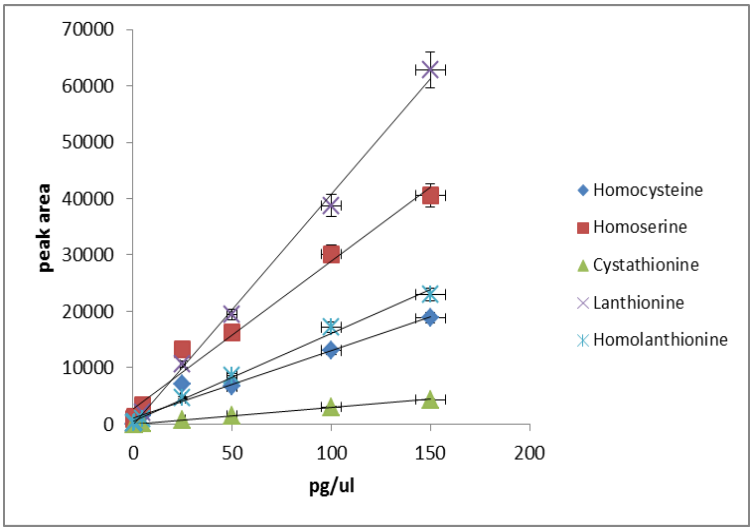

Figure 4.2 : Standard calibration curves obtained for the five analytes.

The limits of detection (LODs) were determined by evaluating the noise signal in plasma and employing a signal-to-noise ratio of 3:1. The lower limits of quantification (LLOQs) were determined as the lowest spiked concentration of each analyte which, upon addition to the blood sample, was determined. Recovery was calculated by comparing the extraction yield before and after the standard addition of the different analytes into the control blood samples The results of LOD, LLOQ and recovery are reported in Table 4.2.

Compound Name	Calibration range (pg/µl)	Recovery %	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification
Cystathionine	0,5-150	90	0,54	1,62
Lanthionine	0,5-150	94	0,38	1,15
Homocysteine	0,5-150	92	0,31	0,93
Homoserine	0,5-150	91	0,14	0,43
Homolanthionine	0,5-150	94	0,42	1,07

Table 4.2 Normalization parameters for all compounds.

4.3.1 Validation of the method

To evaluate the applicability of this method, a control group of 22 healthy subjects and 67 uremic subjects on chronic hemodialysis were analyzed: plasma sample was submitted to protein precipitation and the supernatant was then directly analysed in a LC-MS/MS assay.

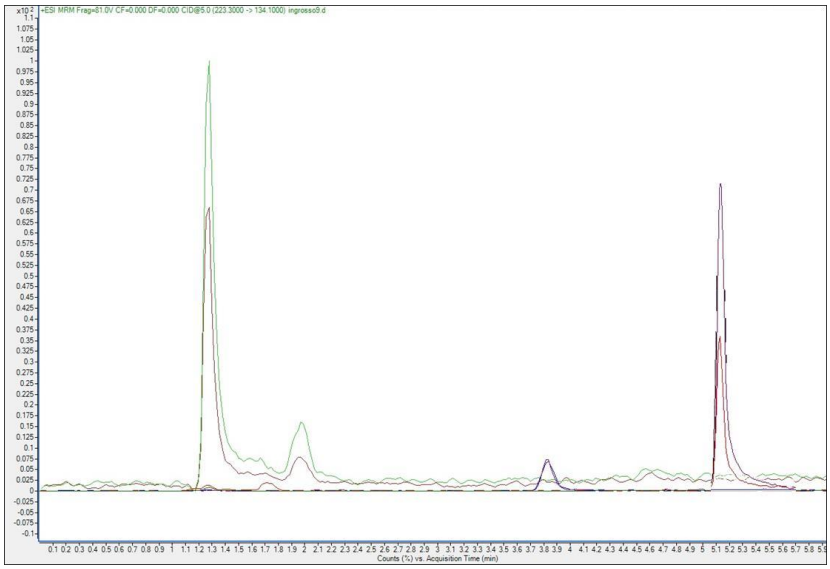

Figure 4.2 XIC chromatograms of all targeted analytes in a serum sample.

The concentrations were calculated by integration on calibration curves using Agilent MassHunter Quantitative Analysis software (B.05.00) Table 4.3.

Metabolites	ctr	patients
Cystathionine (pg/µL)	148,4	1096,1
Homoserine (pg/µL)	330,7	637,4
Lanthionine (pg/µL)	-	64,8
Homolanthionine (pg/µL)	70,5	112,2
Omocysteine	92,7	177,5

Table 4.3 Sulfur metabolites levels in healthy subjects and uremic subjects (mean value).

The experiments were performed in duplicate and averaged. To evaluate the applicability of this method to routine clinical practice, we evaluated the stability of the metabolites of interest in serum over a 2-week period, which represents sufficient time for transporting a sample from the patient to the laboratory. The stability was tested by analyzing serum samples spiked with target molecules at final concentrations of 50 pg/ul. All the samples were stored at -20°C, and the SRM analyses showed a decreases in concentrations of about 10 % after 14 days of storage. Because these decreases in the analyte concentrations were lower than the inter-assay variability, they did not appear to be significant.

4.4 Discussion

A simple and rapid method for the simultaneous measurement of five clinically relevant sulfur metabolites in plasma has been set up. The analytical performance of our method, i.e., its linearity, recovery and reproducibility, is analogous to the performance previously described in other studies based on the detection of metabolites in plasma matrix, indicating the suitability of our method for routine use (Perna A. F., manuscript submitted). The novelty of our method based on targeted mass spectrometry in multiple reaction monitoring ion mode, lies in the determination of two important markers of the H2S pathway, lanthionine and homolanthionine while simultaneously analyzing homocysteine, homoserine, and cystathionine. The diagnostic utility of this method is concerned its application at clinical samples as a rapid screening procedure for detecting patients with severe hyperhomocysteinemia.

Low reagent costs together with the relative simplicity of sample preparation make the LC-MS/MS method well suited, not only for research work but also in those laboratories with a tandem mass spectrometer, for the measurement of routine clinical samples. Once set up, this SRM method was successfully applied as a rapid screening procedure for detecting patients with severe hyperhomocysteinemia (Perna A. F., Manuscript submitted).

4.5 References

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5 Oxylipins

5.1 Introduction

Recently, a new class of oxygenated fatty acid metabolites, previously strongly studied in mammals and higher plants, was also found in marine diatoms, amongst the most abundant forms of photosynthetic plankton in the ocean (d'Ippolito et al., 2005, Fontana et al., 2007) and referred to as oxylipins (Fig.5.1), oxidized compounds formed from fatty acids by a reaction involving at least one step of mono- or dioxygenase-dependent oxidation (Gerwick et al., 1991).

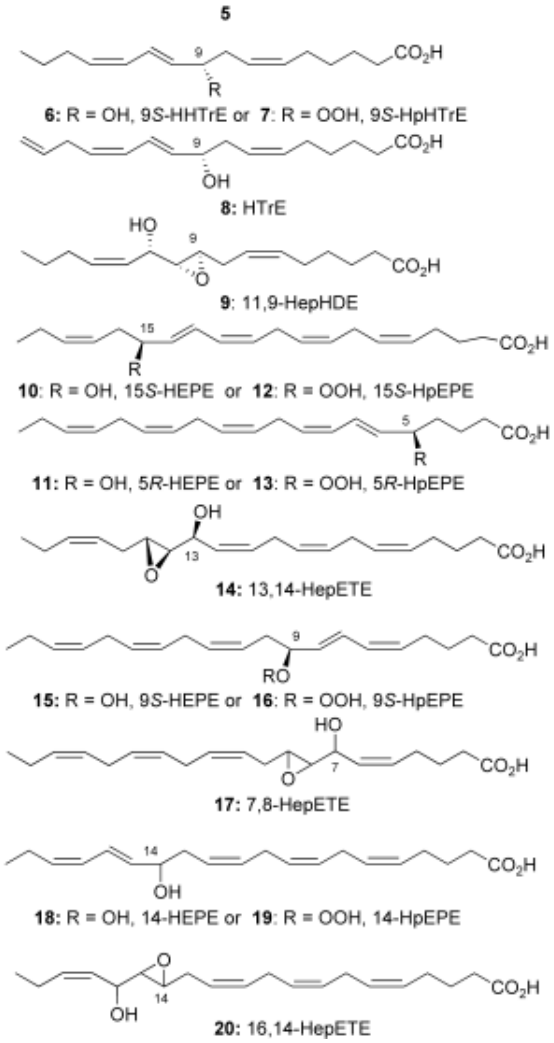

Figure 5.1 Oxylipins from lysed diatom cells.

The presence of a series of these products ranging from 16 to 20 carbon atoms were reported (d'Ippolito et al.,2005). Oxylipins have been recognized as important chemical mediators in ecological and physiological processes of marine and freshwater diatoms leading to the production of a great multiplicity of species, such as hydroperoxy-hydroxy-, keto-, oxo-acids,epoxy alcohols and aldehydes (Wendel&Jüttner, 1996; Pohnert& Boland, 2002; Wichard et al., 2005a; d'Ippolito et al., 2005).

Oxylipin analysis is a powerful but under-utilized approach, which may give important information on the cell physiology. In plants, animals and fungi the sequence of lipoxygenase pathway leading to the oxylipins formation shows striking similarities and evidence on their role in the pathological and physiological processes in these organisms was accumulated in the last decades. Thus the presence of the complex network of oxygenated products in marine diatoms opens intriguing questions about the role of fatty acid derivatives for example in the regulation of phytoplankton communities.

Oxylipin distribution is relatively wide within different diatoms and these compounds characterize an evident species-dependent specificity of the metabolic signature that arises from the variability of both the fatty acids recognized as lipoxygenase (LOX) substrates and the enzymatic activities downstream of the LOX processing (d'Ippolito et al., 2005). Methods generally used for their analysis are all based on GC/MS and take advantage of the two basic types of detection: electron ionization and chemical ionization. However, GC is not the best analytical method for all oxylipins as some, although stable in the fridge, degrade or rearrange when analysed at high temperatures, causing losses and/or the artifactual generation of other oxylipins. However, compounds such as fatty acid (FA) hydroperoxides and some other low-stability oxylipins are often better analysed by liquid chromatography (LC). In addition, many large or polar oxylipins (e.g. oxylipin glycosides) are difficult to fractionate with GC and are better separated by LC (Schmelz et al. 2003; Muller 2006).

A method utilizing liquid chromatography-electrospray tandem mass spectrometry (LC-MS/MS) in selected reaction monitoring in positive ion mode has been developed and for the first time, the developed SRM method was applied to the

determination of oxylipins pattern in extracts of marine diatoms Skeletonema marinoi from laboratory cultures (Pane F. et al, manuscript submitted) .
The research activity was developed in collaboration with Dr. Adrianna Ianora and Dr. Giovanna Romano from the Zoological Station ' A. Dohrn ' Naples.

5.2 Material and methods

5.2.1 Chemicals and reagents

5-HEPE (5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid) and 15-HEPE ((15R)-15-hydroxyeicosa-5Z,8Z,11Z,13E,17Z-pentaenoic acid were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis and purchased from J. T. Baker (Phillipsburg, NJ. All standards and solutions were stored at −20°C.

5.2.2 Preparation of Standard solutions

The stock solutions were prepared by adding 1.00 mL aliquots of each analyte to a 10-mL volumetric flask and bringing the standard to volume with 20/80 methanol/water (v/v) to yield a standard solution with 1000 μg/mL of each analyte. The stock solutions were stored at −20 C until the analysis. Final 2 μg/mL individual analyte standard solutions were prepared, by serial dilution from stock solutions, and were used for mass spectrometric tuning and for calibration curves. Standard mixtures for each analyte were prepared as follows: 5.0, 25.0, 50.0, 125.0, 250.0 pg/ul for each analyte. All standards were kept at -20ºC before LC MS/MS analysis.

5.2.3 Cell culturing and experimental conditions

Batch cultures of *Skeletonema marinoi* (CCMP 2092) were grown in silicate-enriched Guillard's f/2 medium (Guillard, 1975) prepared with 0.2-μm-filtered and autoclaved seawater collected in the gulf of Naples. Cultures were growth in two-litre polycarbonate bottles constantly bubbled with air filtered through 0.2 μm

membrane filters (Sartorius, Goettingen, Germany). Cultures were kept in a climate chamber (RefCon, Napoli, Italy) at 20°C on a 12 h : 12 h light: dark cycle at 100 µmol photons m^{-2} s^{-1}.Cells were harvested at the stationary phase by centrifugation at 3,900 g for 30 minutes at 4°C using a refrigerated centrifuge with a swing-out rotor (DR 15P, Braun Biotechnology International, Allentown, PA, USA). Samples were frozen in liquid nitrogen and kept at -80°C until analysis.

5.2.4 Spiked and samples preparation

A cell pellet (about 5 g wet weight) was suspended in distilled water (1 ml/g of wet pellet), sonicated for 1 min at 4° C and left at room temperature in order to allow wound induced reactions to take place. After 30 min, acetone in equal volume with water was added. The standards, 5HEPE and 15-HEPE (30 µg/g of pellet) was added to a saline buffer used as control matrix, to correct the recoveries of the analytes. The resulting suspension was centrifuged at 2000 g for 5 min at 5° C and successively extracted with dichloromethane (equal volume with aqueous phase). Using a Pasteur pipette, the upper organic layer was transferred into a centrifuge tube. 0.2 ml of upper organic layer was filtered through a 0.2-µm PTFE syringe filter (Pall Acrodisc 13mm) into an LC vial for analysis.

5.2.5 LC–MS/MS instrumentation and conditions

1 µl of supernantant were analysed by using a 6420 triple Q system with a HPLC 1100 series binary pump (Agilent, Waldbronn, Germany). The oxylipins were separated by using as analytical column anC18column 10 cm x 4,6mm, 5u. The mobile phase was generated by mixing eluent A (2% ACN 0.1 % Formic Acid) and eluent B (methanol 0.009 % Formic Acid) and the flow rate was 0.200 mL/min . Starting condition was 98 % to 95% B in 9 minutes .Tandem mass spectrometry was performed using a turbo ion spray source operated in positive ions mode, and the selected reaction monitoring (SRM) mode was used for the selected analytes. A standard solution of 500 pg\uL of each metabolite was used for optimization of the

SRM transition. Standards were automatically (flow injection) tuned for ionization polarity, optimal declustering potential (DP), product ion, and collision energy (CE) using metabolite standard solutions via Agilent MassHunter Optimizer software.

5.2.6 Data Processing

Extracted mass chromatogram peaks of metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00). Peak areas of corresponding metabolites are then used, as quantitative measurements, for assay performance assessments such as assay variation, linearity etc

5.3 Results

5.3.1 Mass spectral parameters selection

The first step consisted in the selection of the best mass spectral parameters for the SRM detection of the analytes. Method sensitivity, limits of detection, and dynamic range were assessed using a mixture of two standards, 5-HEPE and 15-HEPE. Thus, the two standard molecules were analysed to establish the optimal instrument settings for each compound. The SRM transitions for standard metabolites were properly selected by MassHunter Optimizer software by directly infusing each standard dissolved in the mobile phase to select the best product ion (Q3 ion) and optimize collision energy (CE), as well as declustering potential (DP). The most intense fragment ions were selected as the quantification ion for each molecule. The results are summarised in the Table 5.1.

Compound Name	Precursor Ion m/z	Product Ion m/z	Dwell (ms)	Fragmentor (V)	Collision Energy (V)	Polarity
5 hepe	317,1	255	200	81	12	Positive
	317,1	59,1	200	81	7	Positive
15 hepe	317,1	219,1	200	81	9	Positive

	317,1	255	200	81	12	Positive

Table 5.1 Precursor ion, product ions and mass spectral parameters optimized for all analytes.

Figure5.2 shows the SRM chromatograms obtained for each target analyte with all transitions correctly occurring at the same retention time for the different species. Mass spectral parameters were optimised to obtain the best signal/noise ratio. The transition were selected in order to yield the best sensitivity and selectivity in the subsequent SRM analysis.

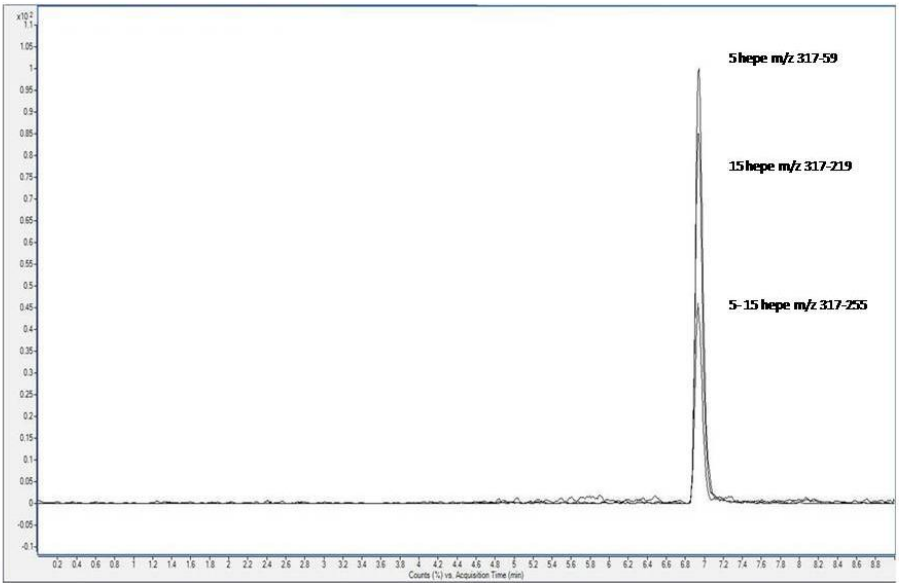

Figure 5.2 XIC chromatograms obtained for the two target analytes

5.3.2 Quantification and normalization

Linearity and matrix effect were studied using standard solutions and matrix matched calibrations. Matrix matched calibration curves were built by spiking a diatom extract with 15 and 5 HEPE. Both standard and matrix matched calibration curves were constructed by plotting peak areas against concentration (pg/µl), and linear functions were applied to the calibration curves. Data were integrated by Mass Hunter quantitative software showing a linear trend in the calibration range for the standard molecules. The coefficients of determination (R2) were greater than 0.99.

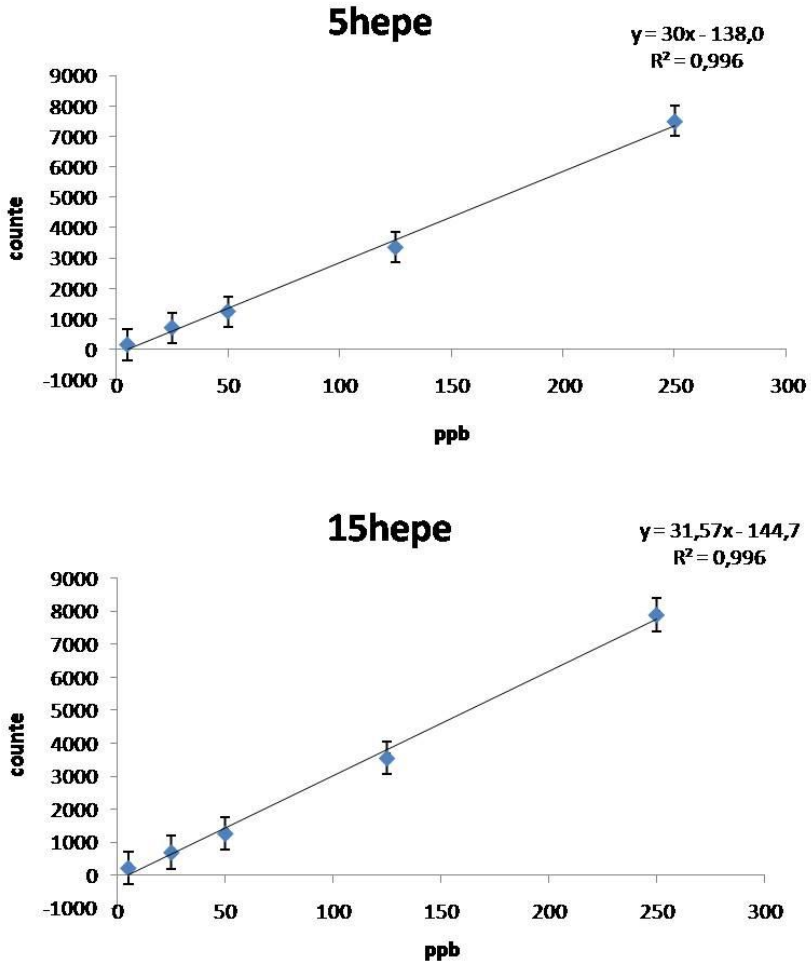

Figure 5.3 : Standard calibration curves obtained for the two analytes

The limits of detection (LODs), were defined as the lower limit of concentration below which the sample could not be revealed and were determined by making 10 replies of blank samples spiked with low concentrations of each analyte. Calculations were made according to the following formula: LOD = 3*STD (STD=standard deviation).The LODs of 5-HEPE, and 15-HEPE resulted to be 0.61 pg/µl, 0.64 pg/µl, respectively (Tab 5.2).

In order to define the recovery in matrix and the LOQ, standard solutions were then prepared by spiking known amount of standards in the control sample. LOQ was calculated as s/n=10, resulting to be 1,85 pg/µl for 5-HEPE and , 1,90 pg/µl for 15-HEPE respectively. Recovery was calculated by comparing the extraction yield

before and after the addition of the different standard analytes into the control samples. Quantitation was achieved by using the calibration curves.

Compound Name	Linear range (pg/ul)	Recovery %	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification
5 hepe	5 - 250	91	0,612	1,853
15 hepe	5 - 250	93	0,640	1,9

Table 5.2 Normalization parameters for all compounds.

The lower LOD determined by SRM method in comparison to reported methods (Cutignano et al 2011) indicates that this method is highly sensitive for determination of the level of this class of molecules without any derivatization step. In particular, the LOD determined by SRM measurements resulted to be at least 10 times lower than the values reported by other methods for the determination of these metabolite thus underlining the higher sensitivity of the proposed procedure and making this procedure well suited for *in vivo* determination.

5.3.3 Oxylipins SRM method

Our intent was to develop a rapid screening and quantifying method of targeted oxylipins in diatoms. Thus, to set up the SRM method we selected known oxylipins structures from literature data (Jang. 2009, Fontana 2007). In order to define the SRM parameters, precursor ions and fragment ions were selected taking advantage by characteristic oxylipins fragmentation pattern extensively studied by Fontana and co-workers in important papers (Fontana 2007). It was reported that, in MS/MS mode, synthetic fatty acid epoxydes, such as 8-hydroxy-9,10-epoxy-octadecanoic acid methylester (*m/z* 351 $M+Na^+$) typically give fragments arising by the heterolytic

break of the carbon-carbon bond of the epoxyde ring. This cleavage is very specific and allows the positioning of the functional group

Analogously, cleavages of natural epoxy alcohol derivatives of fatty acids provide information for the location of both the epoxy and the alcohol groups present on the fatty acid alkyl chain. Because the charge resides always on the daughter ion containing the ester function, the cleavage mechanism of both epoxides and epoxy alcohol derivatives of polyunsaturated fatty acid is very unambiguous and allows the positioning of the functional group. Fragmentation typically generates daughter ions arising from losses of oxygen (M-16), rupture of C-C bond of the oxyrane ring and between alcoholic and epoxide functions,

The fragmentation mechanism arising by α-cleavage is common to other keto acids and is sufficiently diagnostic to assign the position of the functional group (Fontana 2007a).

We firstly focused on known and already studied diatoms oxylipins, then we introduced in SRM method other oxylipins determined in matrices different from diatoms in order to obtain a metabolite panel as complete as possible. In general, these transitions were selected to yield the greatest sensitivity and selectivity for SRM quantification and are summarised in Table 5.3.

precursor	**product**	**name**
261	152	**6-KHTrE**
261	113	**6-KHTE**
263	154	**6-HHTE**
263	107	**9-HHTE**
265	149	**6-HHTrE**
269	177	**9-HHME**
279	179	**12-Htre**
281	83	**11,9-hephed**
295	195	**13-HODE**
295	171	**9-HODE**
300	152	**6-KHTE**
301	257	**EPA**
303	259	**ARA**
313	201	**9,10-DHOME con 12,13-DHOME**
317	174	**8-hepe**
317	94	**14-hepe**

317	179	**12-HEPE**
319	275	**20-HETE**
319	219,2	**14,15-EET**
319	219,1	**15-HETE**
319	203,1	**5-HETE**
319	191	**5,6-EET**
319	179,2	**12-HETE**
319	167	**11,12-EET CON 11-HETE**
327	283	**DHA**
329	211	**9,12,13-TRIHOME**
333	190	**7,5-hepete**
333	147	**10,8-hepete**
333	126	**16,14-hepete**
337	207	**14,15-DiHETrE**
337	166,9	**11,12-DIHETRE**
337	144,8	**5,6-DIHETRE**
337	127	**8,9-DIHETRE**
343	281,4	**17-HDOHE**
361	272	**19,20-DiHDoPe**
317	115	**5-hepe**
317	255	**5-hepe_15hepe**
317	219	**15-hepe**

Table 5.3 The whole the SRM panel for the detection and quantification of 40 oxylipins.

5.3.4 Validation method

A reliable sample extraction procedure was optimised keeping sample manipulation to a minimum. First, extraction and cleanup steps were developed in a control matrix containing no endogenous analytes and consisting in 100 mM phosphate-buffed saline (pH 7.4)

Possible mass spectral interferences from biological specimens were investigated by adding a known amount of standards to the control. The absence of any signal except from 5 and 15-HEPE was verified by LC-MS/MS analysis using the SRM method containing the transitions selected for the two analytes. Moreover,

neither interferences nor carryover peaks were observed in the blank (matrix) samples by SRM analysis thus indicating the selectivity of the method.

5.3.5 Oxylipins determination in Skeletonema marinoi extracts

The reliability of the developed procedure and its applicability to routine practice were evaluated by determining the concentration of oxylipins in *Skeletonema marinoi* extracts. Diatoms samples were prepared by cells sonication and simple protein precipitation as described before; so they were directly analysed by tandem mass spectrometry in SRM positive ion scan mode. The experiments were performed in duplicate and averaged. Figure 5.4 showed the transition for each molecules.

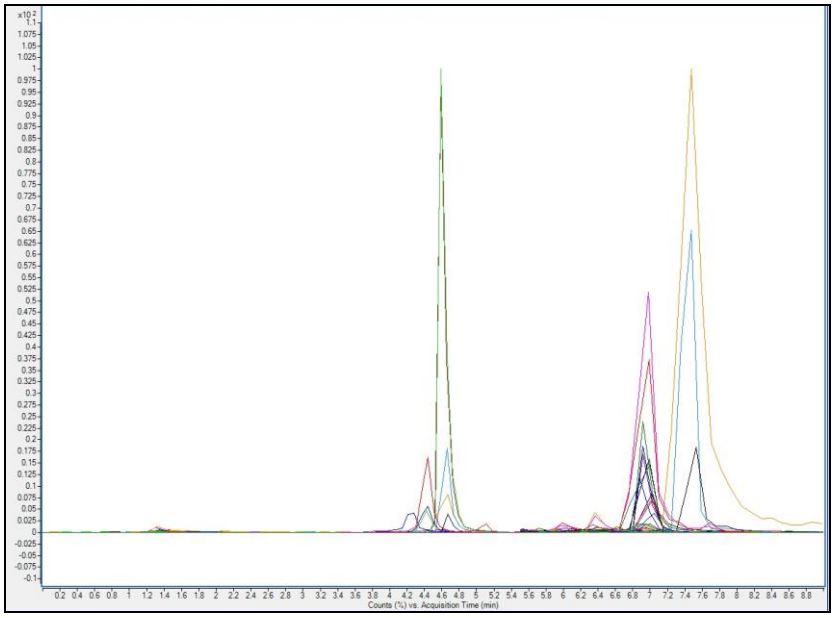

Figure 5.4 Xic chromatograms reported all transition monitored in a diatoms sample

The panel of 40 oxylipins could be easily quantitated by SRM analysis by using the calibration curves (Table 5.4).

Compound Name	Abbreviation	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6
10-hydroxy-8-epoxy-5Z,11Z,14Z,17Z-eicosatetraenoic acid	10,8-hepete	0,48	1,21	0,58	0,12	0,25	36,87
(±)11,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid	11,12-DIHETRE	nd	nd	nd	nd	nd	nd
11(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid / (±)11-hydroxy-5Z,8Z,12E,14Z-eicosatetraenoic acid	11,12-EET / 11-HETE	nd	nd	nd	0,06	1,46	nd

11-hydroxy-9- epoxy-8Z,11Z,14Z,17Z-eicosatetraenoic acid	11,9-hepete	0,04	0,06	0,01	0,31	0,63	nd
(±) 12-hydroxy-5Z,8Z,10E,14Z,17Z-eicosapentaenoic acid	12-HEPE	nd	nd	nd	0,13	1,82	nd
12-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid	12-HETE	nd	nd	nd	nd	0,23	0,38
12-hydroxy-5Z,8E,10E-heptadecatrienoic acid	12-Hhtre	nd	nd	nd	0,10	0,59	nd
(5Z,8Z,11Z)-14,15-Dihydroxy-5,8,11-eicosatrienoic acid	14,15-DiHETrE	nd	nd	nd	nd	nd	2,73
(±) 14(15)-epoxy-5Z,8Z,11Z-eicosatrienoic-16,16,17,17, 18,18,19,19,20,20,20-d11 acid	14,15-EET	0,01	nd	nd	nd	nd	nd
(±) 14-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid	14-hepe	nd	0,005	nd	nd	nd	nd
(±) 15-hydroxy-5Z,8Z,11Z,13E,17Z-eicosapentaenoic acid	15-hepe	17,58	1,09	66,43	nd	nd	7,48
15-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid	15-HETE	nd	nd	nd	0,06	nd	nd
16-hydroxy-14- epoxy-8Z,11Z,14Z,17Z-eicosatetraenoic acid	16,14-hepete	1,09	2,30	1,25	7,23	51,10	184,74
(±) 17-hydroxy-4Z,7Z,10Z,13Z,15E,19Z-docosahexaenoic acid	17-HDOHE	0,005	0,02	0,02	0,54	7,07	216,13
(+/-)19,20-DIHYDROXY-4Z,7Z,10Z,13Z,16Z DOCOSAPENTAENOIC ACID	19,20-DiHDoPe	nd	nd	nd	nd	nd	nd
20-hydroxy-eicosatrienoic acid	20-HETE	nd	nd	nd	0,02	nd	1,86
(±) 5,6-dihydroxy-8Z,11Z,14Z-eicosatrienoic acid	5,6-DIHETRE	nd	nd	nd	nd	0,05	nd
(±) 5(6)-epoxy-8Z,11Z,14Z-eicosatrienoic acid	5,6-EET	nd	nd	nd	nd	nd	nd
(±) 5-hydroxy-6E,8Z,11Z,14Z,17Z-eicosapentaenoic acid	5-hepe	3,50	2,59	4,28	39,99	234,65	1389,13
5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid	5-HETE	0,01	nd	0,01	0,17	0,63	5,62
(7E,9Z,12Z)-6-hydroxyhexadeca-7,9,12,15-tetraenoic acid	6-HHTE	nd	nd	0,00	nd	nd	1,10
(7E,9Z,12Z)-6-hydroxyhexadeca-7,9,12-trienoic acid	6-HHTrE	0,16	0,37	0,06	nd	nd	8,02
(7E,9Z,12Z)-6-ketohexadeca-7,9,12,15- tetraenoic acid	6-KHTE	nd	0,003	nd	nd	3,03	1,10
6-ketohexadeca-7,9,12-trienoic acid	6-KHTrE	nd	0,003	nd	nd	nd	nd
7-hydroxy-5- epoxy-8Z,11Z,14Z,17Z-eicosatetraenoic acid	7,5-hepete	nd	0,02	nd	0,07	nd	nd
8,9-dihydroxy-5Z,11Z,14Z-eicosatrienoic acid	8,9-DIHETRE	nd	nd	nd	nd	nd	nd
8-hydroxy-5Z,9E,11Z,14Z,17Z-eicosapentaenoic acid	8-hepe	nd	nd	0,03	0,01	nd	nd
9,10,13-Trihydroxyoctadec-11-enoic acid	9,10,13-TRIHOME	nd	nd	nd	0,15	1,84	nd
(Z)-9,10-dihydroxyoctadec-12-enoic acid/ 12,13-dihydroxyoctadec-12-enoic acid	9,10-DHOME con 12,13-DHOME	nd	nd	nd	0,03	nd	1,48
9,12,13-Trihydroxyoctadec-11-enoic acid	9,12,13-TRIHOME	0,01	nd	0,02	0,05	0,20	4,81
9-hydroxy-7E-hexadecenoic acid	9-HHME	nd	0,07	0,01	nd	nd	nd
(6Z,10E,12Z,15)-9-hydroxyhexadeca-6,10,12,15-tetraenoic acid	9-HHTE	nd	0,25	0,07	0,86	2,23	21,09
(6Z,9E,12Z)-hexadeca-6,9,12-trienoic acid	9-HHTrE	0,02	0,05	0,03	nd	nd	1,40
(±)-9-hydroxy-10E,12Z-octadecadienoic acid	9-HODE	nd	nd	nd	0,37	63,74	nd
(±)-13-hydroxy-9Z,11E-octadecadienoic acid	3-HODE	nd	0,01	0,01	0,25	0,94	41,79
docosahexaenoic acid	DHA	0,04	0,02	0,01	1,45	nd	834,54
arachidonic acid	ARA	0,01	0,003	0,01	0,09	0,95	23,80
eicosapentaenoic acid	EPA	0,27	0,12	0,17	11,41	14,29	556,36

Table5.4 Oxylipins identified in the microalgal pellets collected at each sampling site. Quantities for each compound are reported in ng/mg of proteins.

5.4 Discussion

This method allowed simultaneous 40 oxylipins profiling thus expanding the knowledge of this class of molecules in diatoms. Once set up this SRM procedure led to a successful definition of quantitatively changes in oxylipins profiles in different single strain cultures of diatoms (Lauritano - manuscript submitted). However, this SRM method, even detailed is not exhaustive and can be easily extended to other oxylipins detection taking advantage by increasing interest in this field. Therefore enabling a quantitative profiling of hundreds to thousands of samples in a short time.
Eicosanoids, derived from metabolism of Arachidonic acid and related polyunsaturated fatty acids (Dennis, 2011), are bioactive lipids derived from polyunsaturated essential fatty acids involved in a wide variety of important physiological processes (Funk,2001). This entire and complex enzymatic system gives rise to hundreds of eicosanoids showing very similar structures, chemistries, and physical. properties . Up today more than 100 biologically active oxylipins have been identified (Dennis, 2011) and this number is constantly in progress thus indicating the ever increasing interest in this field. In this respect, new sensitive and easily handling analytical methods for their characterization are needed.
It was set up a simple and rapid method for the simultaneous direct measurement of oxylipins in diatoms extract based on HPLC fractionation and tandem mass spectrometry in multiple reaction monitoring mode avoiding any derivatization step . Because of the complexities of biological samples, a hyphenated technology is a common choice for metabolomics, among which, LC/MS has been widely used in recent years. Mass spectrometry (MS) has been the best selection because of its high sensitivity, specificity (molecular weight detection), low cost, and coupling capability to separation technologies. Among different types of mass spectrometers, a triple quadruple mass spectrometric optimal for targeted metabolomics, based on its high sensitivity, high specificity, and excellent quantitation ability. MS spectral integrated approach had provided sufficient data for a qualitative and quantitative metabolite analysis in marine diatoms, demonstrating that , also at the end of a natural diatom bloom, diatoms release a series of secondary metabolites, most represented by oxylipins, that are deterrent for their predators (i.e. inducing a

reduction in the egg production and hatching success), but at the same time copepods activate a series of defense systems (e.g. the activation of the chaperonine HSPs, detoxification enzymes and inhibitor of apoptosis IAP) that may indicate a copepod stress response (Lauritano, .manuscrip submitted).

The simultaneous, sensitive, fast and reproducible measurement of these metabolites might be useful in the highlighting the cell physiological status. The final goal of this work was to propose a general protocol for diatoms characterization avoiding methods that are time consuming and labour intensive and do not allow the processing of large numbers of samples for metabolic profiling.

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6 Metabolomics and degenerative diseases

6.1 Introduction

Clinical diagnosis of major neurological disorders, for example Alzheimer's disease and Parkinson's disease, on the basis of current clinical criteria is unsatisfactory. Emerging metabolomics is a powerful technique for discovering novel biomarkers and biochemical pathways to improve diagnosis, and for determination of prognosis and therapy. Identifying multiple novel biomarkers for neurological diseases has been greatly enhanced with recent advances in metabolomics that are more accurate than routine clinical practice. Cerebrospinal fluid (CSF), which is known to be a rich source of small-molecule biomarkers for neurological and neurodegenerative diseases, and is in close contact with diseased areas in neurological disorders, could potentially be used for disease diagnosis (Zhang AH1 2013) .

Metabolomics analytical platforms and informatics tools are being used to map potential biomarkers for a multitude of disorders including those of the central nervous system (CNS) and are enabling us to describe detail perturbations in many biochemical pathways and links among these pathways this information is key for development of biomarkers that are disease-specific (Quinones MP 2009).

Chemical synthesis of amino acids produces a 1:1 ratio of D and L-amino acids. About 50 years ago, scientists believed that D-AAs do not exist in living organisms. D-amino acids are believed to have been excluded in the process of evolution, as all proteins in living things on earth are composed of L-amino acids. Studies in the last decades have shown that D-AAs are widely present in the tissues of higher organisms, including humans. Recent studies have determined the involvement of D-amino acid in diseases closely related to aging process and age-related disorders, such as cataracts and Alzheimer’s disease.

D-serine plays an important role in neuroplasticity, memory, and learning; D-aspartate is involved in developmental and endocrine functions. The main pathways for the appearance and metabolism of D-AAs have been described. The pathogenicity of D-AAs is associated with excess activation of NMDA receptors and incorporation into normal protein molecules (conformational changes), which results in functional inactivation of the protein or its toxicity, and an increase in the

concentration of reactive oxygen species (oxidative stress) during disintegration of D-AAs by D-AA oxidase (DAAO). The level of D-AAs in biological fluids, the activity of enzymes, and mutations in genes that encode these enzymes may be used as a diagnostic marker in some diseases (Chervyakov A. V. 2011).

Temporal regulation of D- aspartate content depends on the post-natal onset of D-aspartate oxidase expression, the only known enzyme able to catabolize this D-amino acid. Pharmacological evidence indicates that D-aspartate binds and activates NMDA receptors (NMDARs). To decipher the physiological function of D-aspartate in mammals, in the last years, genetic and pharmacological mouse models with abnormally higher levels of this D-amino acid have been generated. Overall, these animal models have pointed out a significant neuromodulatory role for D-aspartate in the regulation of NMDAR-dependent functions (Errico F 2013).

The involvement of D-Asp in the regulation of the synthesis and/or release of different hormones has been clearly demonstrated. However, its biological significance in the brain is still obscure. D-Asp appears with a peculiar temporal pattern of localization, being abundant during embryonic development and strongly decreasing after birth. This phenomenon is the result of the postnatal onset of D-Asp oxidase (DDO) expression, the only known enzyme that strictly controls the endogenous levels of D-Asp. The pharmacological affinity of D-Asp for the glutamate site of NMDARs has raised the intriguing question whether this D-amino acid may have some in vivo influence on responses mediated by this subclass of glutamate receptors. In order to unveil the physiological function of D-Asp and of its metabolizing enzyme, genetic and pharmacological approaches have been recently developed. It has now become possible to generate animal models with abnormally elevated levels of D-Asp in adulthood based on the targeted deletion of the Ddo gene and on the oral administration of D-Asp. These animal models have thus highlighted that D-Asp has a neuro modulatory role at NMDARs in brain areas where they regulate crucial nervous functions. Indeed, abnormally high D-Asp levels in the hippocampus are able to strongly enhance NMDAR-dependent LTP and, in turn, to facilitate spatial memory of mice. Moreover, in both mutant and treated animals, this deregulated D-Asp content completely suppresses striatal LTD, most likely via over activation of NMDARs. The later synaptic plasticity alteration resembles that

produced by chronic administration of haloperidol and is probably the neurobiological substrate responsible for the attenuation of prepulse inhibition deficits induced by amphetamine and MK-801 in Ddo knockout and D-Asp-treated mice. These in vitro and in vivo findings, together with others reported in this review, support a neuromodulatory action for D-Asp at glutamatergic synapses. In addition, they suggest that this D-amino acid may play a potential beneficial role in conditions related to a pathological hypo functioning of NMDARs in the mammalian brain (F. N. Errico 2013).

Due to its physiological relevance, a number of different methods have been proposed to detected d-Asp, including gas chromatography, high-performance liquid chromatography, high-performance capillary electrophoresis and enzymatic procedures (Hamase 2002).

The most difficult part of this analysis is the enantio separation of d-Asp from l-Asp, since their chemical and physical properties, except configuration, are similar. Chiral HPLC sensitive separation methods for the determination of d-Asp levels in tissue samples have been developed (Maier 2001 Nguyen, 2012).

Nowadays, automatic rapid HPLC tandem mass spectrometry (LC-MS/MS) has become the technique of choice for metabolite analyses due to its capability to simultaneously separate and quantitative many specific molecules in multi component mixtures. Petritis and co-workers successfully performed simultaneous chiral separations of underivatized amino acids have by using a teicoplanin-based chiral stationary phase and used ion spray tandem mass spectrometry for their ionisation and detection (Teleki A 2015)**.**

6.2 Material and methods

6.2.1 Chemicals and reagents

N-methyl D-Asp (NMDA), D-Asp, L-Asp, were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis and purchased from J. T. Baker (Phillipsburg, NJ). All stock solutions were stored at −20 °C. Tissue samples derive from Ddo knockout mice (*Ddo*$^{-/-}$) brains.

6.2.2 Preparation of Standard solutions

The stock solutions were prepared by adding 1.00 mL aliquots of each analyte to a 10 mL volumetric flask and bringing the standard to volume with methanol to yield a standard solution with 1000 ng/mL of each analyte. The stock solutions were stored at −20 C until the analysis..

6.2.3 Individual Standard Solution

Final 2000 pg/µl individual analyte standard solutions were prepared, by serious dilution from stock solutions, and were used for calibration curves. Standard mixtures for each analyte were prepared as follows: 0.5, 1,0, , 25, 50, 125, 200, 300, pg/µl for D-Asp; 1, 5, 25, 50, 80, 125 pg/µl for L-Asp; 0,25, 2,5, 5, 10, 25, 50 for NMDA having higher, medium, and lower measuring sensitivities, respectively, All standards were kept at -20ºC before LC MS/MS analysis.

6.2.4 Sample preparation

All tissues were homogenized by sonication in Tris buffer (Tris-HCl 50mM pH 8) until a clear solution was obtained, and stored at −20 C until the time of analysis. For an initial screening extraction batch run, the control tissue was fortified in triplicate at three different target levels (50, 100 and 200 pg/µl)by adding the s standard solution containing same concentration of the three analytes to a 200 µl of homogenized control tissues (wt) corresponding to 0.5-g portion of control tissue. Aliquot of 0.6 ml of methanol was added (0.2ml) to each sample and the tube was mechanically shaken vigorously for 5min. The tube was centrifuged at 10000rpm (7600 rcf) at 10 C for 10min. Using a Pasteur pipette, the upper organic layer was transferred into a centrifuge tube. 0.2 ml of upper organic layer was filtered through a 0.2-µm PTFE syringe filter (Pall Acrodisc 13mm) into an LC vial for analysis.

6.2.5 LC–MS/MS instrumentation and condition

2µl of supernantant were analysed by using a 6420 triple Q system with a HPLC 1100 series binary pump (Agilent, Waldbronn, Germany). The enantiomers were separated by using as analytical column an Astec chirobiotic T column 10 cm x 4,6mm, 5µm. The mobile phase was generated by mixing eluent A (0.1 % Formic Acid in 2% ACN) and eluent B (0.009 % Formic Acid, methanol) and the flow rate was 0.200 mL/min. Starting condition was 50% to 95% B in 6 min.

Tandem mass spectrometry was performed using a turbo ion spray source operated in positive mode, and the selected reaction monitoring (SRM) mode was used for the selected analytes.

A standard solution of 500 pg/µl of each metabolite was used for optimization of the SRM transition. Metabolites were automatically (flow injection) tuned for ionization polarity, optimal declustering potential (DP), product ion, and collision energy (CE) using metabolite standard solutions via Agilent MassHunter Optimizer software.

6.2.6 Data Processing

Extracted mass chromatogram peaks of metabolites were integrated using Agilent MassHunter Quantitative Analysis software (B.05.00). Peak areas of corresponding metabolites are then used, as quantitative measurements, for assay performance assessments such as assay variation, linearity etc.

6.3 Results

The entire analytical procedure including the assessment of sensitivity, limits of detection, and dynamic range was developed using standard metabolite solutions.

6.3.1 Enantiomeric separation

An equimolar mixture of D- and L-Asp was resolved on a HPLC Chirobiotic chiral phase column consisting of the amphoteric glycopeptide Teicoplanin covalently bound to a 5µm spherical silica gel through multiple covalent linkages. Teicoplanin contains 23 chiral centers surrounding four pockets or cavities with hydrogen donor and acceptor sites readily available. This type of arrangement is known to be highly favorable for a number of enantiomeric separations. Isocratic elution of the enantiomers was accomplished by an unbuffered mixture of methanol and acetonitrile containing a small amount of formic acid providing an optimal coupling with the following mass spectral analysis. Figure 6.1 shows the total ion current (TIC) chromatogram obtained for the analysis of 100 pg/µl standard mixture solution showing a good peak shape for both analytes. Several injections of the standard mixture were carried out to ensure reproducibility of retention times.

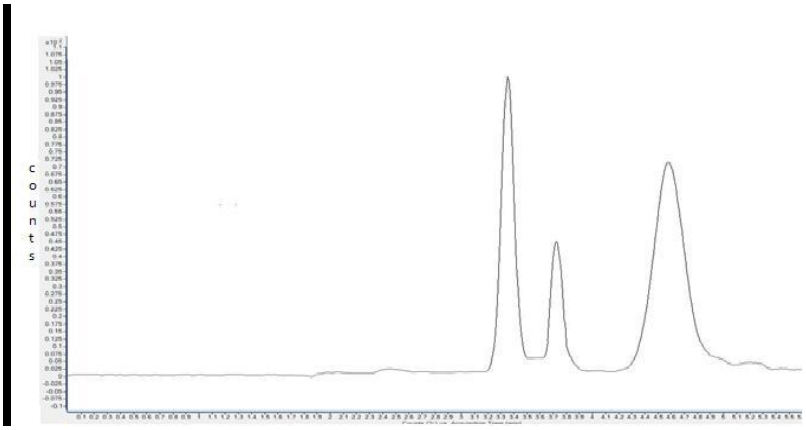


Figure 6.1- Total ion current (TIC) chromatogram for the analysis of 100 ng/ml standard mixture solution of D-Asp, L-Asp and NMDA

6.3.2 Mass spectral parameters selection

Next step consisted in the selection of the best mass spectral parameters for the SRM detection of the analytes. D-, L-Asp and NMDA were individually infused to establish the optimal instrument settings for each compound. Experimental tuning was used to decide ionization polarity, to select the best product ion (Q3 ion) and to optimize both the collision energy (CE) and the declustering potential (DP). All tuning data were manually examined to ensure proper selection of ionization polarity and product ion; the results are summarised in Table 6.1.

Compound Name	Precursor Ion (m/z)	Product Ion (m/z)	Dwell (ms)	Fragmentor (V)	Collision Energy (V)	Polarity
L Asp	134,00	116,00	200	81	1	Positive
	134,00	88,00	200	81	8	Positive
	134,00	74,00	200	81	9	Positive
D Asp	134,00	116,00	200	81	1	Positive
	134,00	88,00	200	81	8	Positive
	134,00	74,00	200	81	9	Positive
NMDA	148,00	88,00	200	81	9	Positive
	148,00	42,00	200	81	25	Positive

Table 6.1 Mass spectral parameters for the three analytes .

The SRM transitions (namely the best fragment ions) were properly selected for each metabolite by the MassHunter Optimizer software during direct infusion of analytes dissolved in the mobile phase. Figure 6.2 shows the SRM chromatograms obtained for D-Asp, L-Asp and NMDA showing a good selectivity for all analytes, with all transitions correctly occurring at the same retention time.

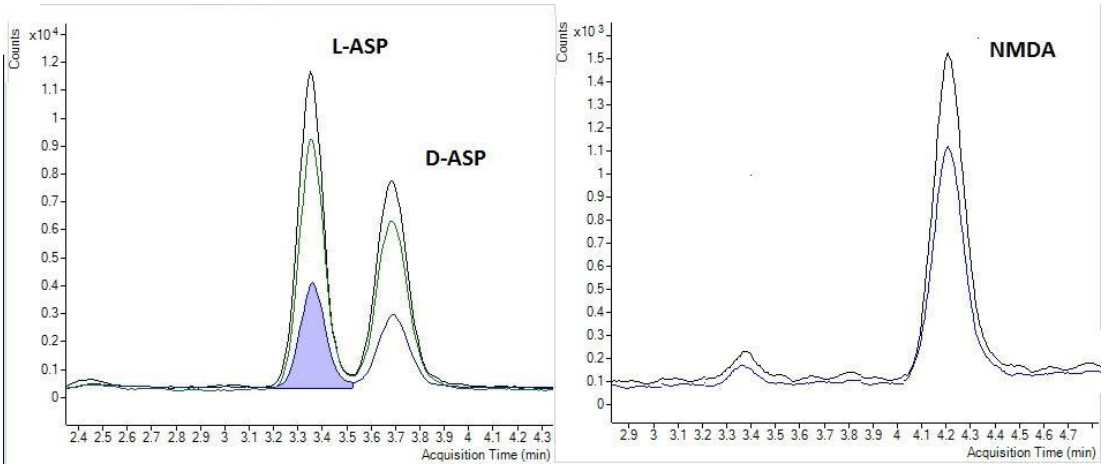


Figure 6.2. LCMSMS chromatogram showing the SRM transitions for D-Asp, L-Asp and NMDA.

6.3.3 Sample treatment

First, a reliable sample extraction procedure had to be optimised keeping sample manipulation to a minimum because of the low concentration of D-Asp occurring in most biological samples. Target analytes were extracted from freeze-dried specimens of mouse brain tissues following a two-steps procedure consisting in sonication of the sample and protein precipitation prior to LC-MS/MS analysis. Different buffers including Ammonium Bicarbonate 10mM pH7.5, Ammonium Bicarbonate 50mM pH 8.0, Tris-HCl 50mM pH 8.0, Hepes 50mM pH 8.0, and sonication parameters (from 10 to 120 min with 10 min increments) were evaluated. Each test sample was then divided in three aliquots and the analytes extracted in the supernatant by precipitation of the protein components using different solvent systems (acetonitrile, ethanol, methanol).

The different conditions explored were reported in Table 6.2. All test samples were then analysed by the SRM LC-MS/MS procedure to evaluate the best conditions for sample preparation and the recoveries. The best results were obtained by dissolving the sample in Tris-HCl 50mM pH 8.0 followed by sonication for 10 min. Finally, methanol was selected as the best solvent for protein precipitation. Extending the precipitation time beyond 30 min (i.e., up to 2 hours) did not increase the analyte recovery. Experiments were performed in duplicate and averaged

.

Buffer	**Solvent**	**Recovery (%)**
Ammonium Bicarbonate 10mM pH7.5	methanol	38
	Ethanol	14
	acetonitrile	11
Ammonium Bicarbonate 50mM pH 8.0	methanol	41
	Ethanol	25
	acetonitrile	13
Tris-HCl 50mM pH 8.0	methanol	93
	Ethanol	36
	acetonitrile	19
Hepes 50mM pH 8.0	methanol	41
	Ethanol	26
	acetonitrile	18

Table 6.2: Different extraction conditions and averaged percentage (%) of recovery for the three analytes. Conditions yielding the highest recovery for all three analytes were selected.

Possible mass spectral interferences from biological specimens were investigated. Thus a control matrix only containing L-Asp was prepared by using a sample of adult mouse brain treated with D-Asp oxidase in order to completely remove endogenous D-Asp and NMDA. The absence of any amount of free D-Asp in the adult mouse sample used as matrix control was verified by LC-MS/MS analysis using the SRM method.

The absence of any amount of free D-Asp in the adult mouse sample used as matrix control was verified by LC-MS/MS analysis following the procedure outlined above.

Neither interferences nor carryover peaks (i.e., signal below LOD) were observed in the blank samples by SRM analysis..

6.3.4 Validation method

Linearity and matrix effect were studied using standard solutions and matrix matched calibrations. Matrix matched calibration curves were built by spiking control tissue with L-Asp, D-Asp and NMDA. Both standard and matrix matched calibration curves were constructed by plotting peak areas against concentration (pg/µl), and linear functions were applied to the calibration curves. Data were integrated by Mass Hunter quantitative software demonstrating that the method was linear ($r \geq 0.999$) in the calibration range for all molecules. The signal corresponding to L-Asp was corrected for the presence of the endogenous metabolite in the control matrix. As an example, Figure 3 shows the standard calibration curves obtained for the three analytes. The matrix effect was calculated as the percentage of the matrix matched calibration slope (B) divided by the standard calibration slope (A). We obtained a matrix effect calculation (B/A x 100) of 11-15% for the three analytes.

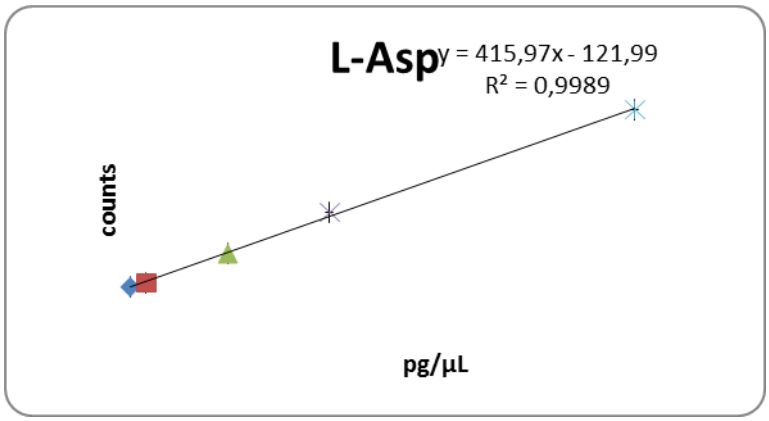
The limits of detection (LODs), were defined as the lower limit of concentration below which the sample could not be revealed and were determined by making 10 replies of blank samples spiked with low concentrations of each analyte. Calculations were made according to the following formula: LOD = 3*STD (STD=standard deviation).

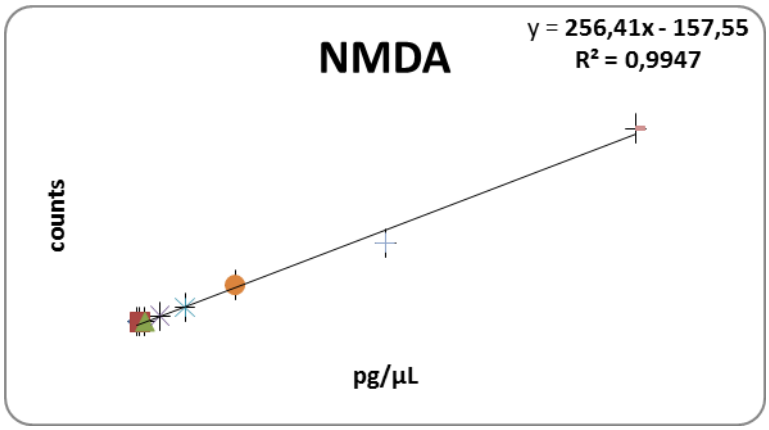
The LODs of D-Asp, L-Asp and NMDA resulted to be 0,52pg/µl for D-Asp, 0,46 pg/µl for L-Asp and 0,54 pg/µl for NMDA, respectively.

The precision (%RSD = 3.1–9.0) and accuracy (%RE =9.6–10.3) at different concentration levels, as reported before for each metabolite were calculated and resulted to be, reliable for the quantitative measurement of these metabolites in brain tissues. Data were collected over a period of 10 days and each measurement represents the average of 3 experiments. Table 3 reports the determined parameters for L-Asp, D-Asp and NMDA. The coefficients of determination (R2) were greater than 0.99 for all analytes.

In order to define the recovery in matrix and the LOQ, standard solutions were then prepared by spiking known amount of D-Asp, L-Asp and NMDA in the control mouse brain sample. Tissue samples were weighed and sonicated using the conditions previously defined; the metabolites were extracted and analysed by the LC-MS/MS procedure. The endogenous background of each analyte was corrected in the Mass Hunter software by calculating the non-spiked signal. LOQ was calculated as s/n=10, and resulted to be 1.57 pg/ul for D-Asp, 1.41 pg/ul for L-Asp and 1.64 pg/ul for NMDA, respectively. Recovery was calculated by comparing the extraction yield before and after the standard addition of the different analytes into the control tissue samples

Quantitation was achieved by using the calibration curves. Recovery for each analyte was better than 90% (see Figure 6.3)





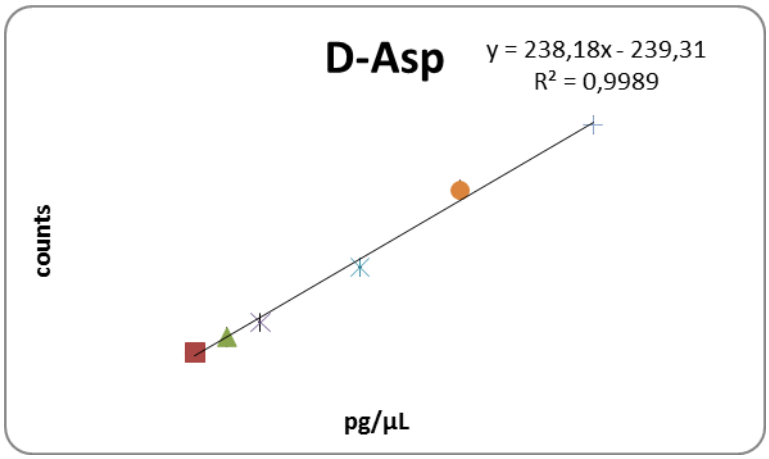


Figure 6.3: Standard calibration curves obtained for the three analytes.

Compound	**LOD (pg/ul)**	**LOQ (pg/ul)**	**Linear range (pg/ul)**	**50 pg/ul**	**100 pg/ul**	**200 pg/ul**
d-Asp	0,52	1,57	1-125	73.9±1.1	87.8±9.4	106.8±1.3
L-Asp	0,46	1,41	1-125	75.9±1.3	88.1±9.8	108.2±1.1
Nmda	0,54	1,64	0,25-50	74.9±1.5	91.4±9.1	104.1±1.6

Table 6.3. Validation parameters of the analytical method developed for L-Asp, D-Asp and NMDA determination

The reliability of the developed procedure and its applicability to routine practice were evaluated by determining the concentration of D-Asp, L-Asp and NMDA in several mouse brain tissues at different times of development as reported in Table 4. Freeze-dried specimens from mouse brain tissues were sonicated and precipitated according to the simple developed method, avoiding heavy sample handling, and directly analysed by tandem mass spectrometry in SRM scan mode. The analytes concentrations were calculated in pg/µl and then expressed in nmol/g of brain tissue in order to perform a comparison with data presented in the literature.

As an example, Figure 6.4 shows the TIC (Figure 6.4A) and the SRM transitions (Figure 6.4B) registred for the three molecules in a sample from prefrontal cortex of knockout mouse. Following SRM analysis, the three metabolites could be easily quantitated (see Table 6.4).

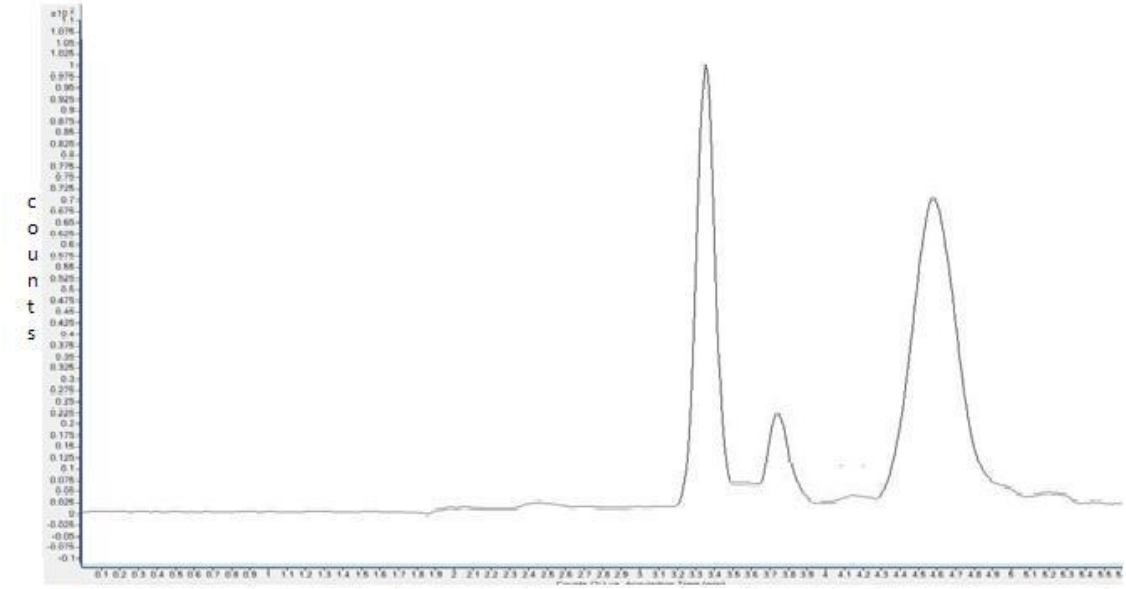


Figure 6.4A: TIC registred for the three molecules in a sample from prefrontal cortex of knockout mouse.

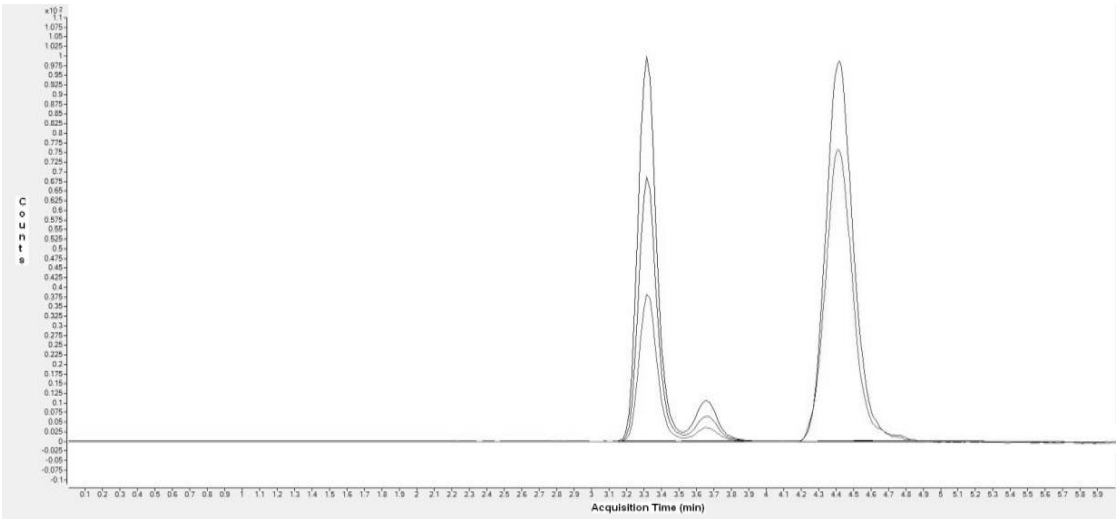


Figure 6.4B: SRM registred for the three molecules in a sample from prefrontal cortex of knockout mouse.

L-Asp	***nmol/g***		***errore***
Pfc1 *Ddo*$^{+/+}$	8183,1	±	581,21
Pfc2 *Ddo*$^{-/-}$	9827,78	±	923,81
Pfc3 *Ddo*$^{+/+}$	10592,36	±	995,68
Pfc4 *Ddo*$^{-/-}$	9985,22	±	873,84

D-Asp	***nmol/g***		***errore***
Pfc1 *Ddo*$^{+/+}$	112,82	±	0,21
Pfc2 *Ddo*$^{-/-}$	5144,59	±	192,3
Pfc3 *Ddo*$^{+/+}$	127,99	±	1,41
Pfc4 *Ddo*$^{-/-}$	5325,89	±	274,45

NMDA	***nmol/g***		***errore***
Pfc1 *Ddo*$^{+/+}$	1,17	±	0,36
Pfc2 *Ddo*$^{-/-}$	2,93	±	1,54
Pfc3 *Ddo*$^{+/+}$	1,8	±	0,26
Pfc4 *Ddo*$^{-/-}$	2,29	±	0,34

Table 6.4. Determination of L-Asp, D-Asp and NMDA in samples from mouse prefrontal cortex by SRM analysis. Ddo+/+ , wild type mouse; Ddo-/-, D-aspartate oxidase (DDO) knockout mouse.

Finally, the same analysis was performed on prefrontal cortex samples from both wild type and knockout mice for *Ddo* gene (*Ddo*$^{+/+}$ and *Ddo*$^{-/-}$,respectively). Figure 6.5 showed the results obtained by the LC-MS/MS procedure demonstrating that in *Ddo*$^{-/-}$ mice sample the D-Asp levels are much higher compared to *Ddo*$^{+/+}$ littermates.

Moreover, the D-Asp/L-Asp ratio determined by LC-MS/MS (0.012 for *Ddo*$^{-/-}$ mice and 0.441 for *Ddo*$^{+/+}$, see Fig 6.6) was approximately coincident with those previously obtained by HPLC measurements (0.019 for *Ddo*$^{-/-}$ mice and 0.624 for *Ddo*$^{+/+}$,manuscript in preparation).

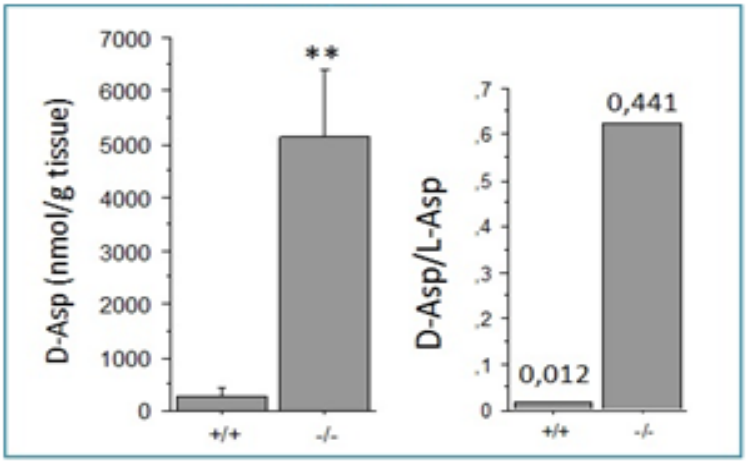


Figure 6.5 The results obtained by the LC-MS/MS procedure are reported. Ddo-/- mice sample showed a much higher of d-Asp levels when compared to Ddo+/+ littermates.

6.4 Discussion

Many *in vivo* and *in vitro* studies have suggested that D-Asp has a crucial role in NMDA receptor-mediated neurotransmission playing very important functions in physiological and pathophysiological processes. Evidence collected in mouse models with increased levels of D-Asp has shown that this D-amino acid enhances hippocampal NMDAR-dependent synaptic plasticity, dendritic morphology and spatial memory**.** In line with the hypothesis of a hypo function of NMDARs in schizophrenia, it has been shown that increased D-Asp levels can improve brain connectivity and protect against sensorimotor gating deficits and abnormal activation of brain circuits induced by psychostimulant drugs**.** In healthy humans, genetic variation predicting reduced *DDO*mRNA levels in post-mortem prefrontal cortex is associated with prefrontal phenotypes relevant to schizophrenia (F. N. Errico 2012,)**.**

These crucial biological functions trigger the need for a simple, reliable and effective methodology to quantitative the levels of d-Asp and its derivative NMDA in brain tissues, as the alteration of their amounts would be of invaluable clinical and diagnostic value.

We describe a simple and rapid method for the simultaneous direct measurement of free D-Asp, L-Asp and NMDA in brain tissue based on chiral fractionation of enantiamers and tandem mass spectrometry in multiple reaction monitoring mode avoiding any derivatization step. As D-Asp occurs at very low concentration in most biological samples, a reliable extraction procedure with minimum sample handling was first optimised (Fontanarosa, 2016).

Detection of D-Asp, L-Asp and NMDA by mass spectrometric methodology has a number of advantages. Purification of the samples to remove organic impurities in tissues prior to HPLC detection was unnecessary in the LC-MS/MS method, making the analytical procedure simpler and faster.

The SRM method allowed us to discriminate the target metabolites within the very complex mixture originated from tissue extracts. D- and L-Asp were distinguished by their retention time on chiral chromatography and all three compounds could be unambiguously identified by their specific SRM transitions thus avoiding any derivatization step. All the analytical parameters, LODs, LOQs were determined.

Finally, the developed procedure was applied to the quantitative determination of D-Asp and L-Asp from prefrontal cortex samples from both Ddo+/+ and Ddo-/-mouse, an animal model in which the Ddo gene has been genetically deleted**,** and the results were compared with the same measurements carried out by HPLC. Determination of D-Asp and L-Asp could be easily obtained with no interference or decrease in sensitivity. As expected, D-Asp levels were much higher in the knockout mouse than in wild type. Moreover, measurement of the D-Asp/L-Asp ratio determined by LC-MS/MS was in very good agreement with that obtained by HPLC (Karakawa S 2015).

In conclusion, the developed method provided to be a reliable and effective procedure for direct measurements of free D-Asp, L-Asp and NMDA in brain tissues and was successfully applied as a rapid screening procedure for detection of these metabolites levels in different mouse brain districts (manuscript in preparation).

Low reagent costs together with the relative simplicity of sample preparation make the LC-MS/MS SRM method well suited, not only for research work but also in those laboratories with a tandem mass spectrometer, for the measurement of routine clinical samples. The methodology we developed allows simultaneous D-Asp, L-Asp and NMDA profiling but can be easily extended to different class of metabolites, and easily expandable at clinical samples screening.

6.5 References

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7 Targeted proteomics: Insulin degrading enzyme, a case study

7.1 Introduction

Mass Spectrometry-based proteomics is actually considered a gold standard technique for protein analysis, ranging from global expression profiling to the identification of protein complexes and specific post-translational modifications. Recently, Selected Reaction Monitoring Mass Spectrometry (SRM-MS) has become increasingly popular in proteome research for the targeted quantification of proteins and post-translational modifications.

Targeted proteomics is a powerful tool for protein identification in complex biological samples, by limiting the number of target proteins to be monitored, focuses on optimization of chromatographic instrumental methods and acquisition parameters, to obtain higher sensitivity, selectivity and performance for protein analysis in complex mixtures (Liebler D. C. 2013). In particular, Mass Spectrometry Selected Reaction Monitoring (SRM) is emerging as a versatile platform for the systematic development of targeted protein arrays and alternative to the classical immune assay thus providing quantitative measures highly selective and specific for proteins useful as biomarkers, potential clinical tools useful for the diagnosis, prognosis and therapeutic monitoring (Picotti P. 2012). SRM assays enable the quantification of protein in a concentration range that goes from μg/mL to a few ng/mL without or reducing fractionation or enrichment strategies.

Insulin degradation enzyme (IDE) is a 110-kDa zinc metalloprotease found in the cytosol of all cells involved in the degradation of a number of small proteins (<6 kDa) including insulin and amyloid-β (Im 2007) . IDE has been intensively studied as a participant in the pathogenesis of diabetes and Alzheimer's syndrome (Duckworth 1998, Qiu 2006).

7.2 Material and methods

7.2.1 Chemicals and reagents

Recombinant human insulin degrading enzyme (IDE) were purchased from Sigma-Aldrich. All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis. All stock solutions were stored at −20 °C.

7.2.2 Preparation of standards solutions

A stock solution of IDE at 1 I0 μmol/mL) was prepared in 0.1% formic acid solvent and stored at −80 °C until use. Dilutions were subsequently performed to generate a calibration series at concentrations of 0.5 fmol/ μL, 1fmol/μL, 5 fmol/μL, 10 fmol/μL, 25 fmol/μL, 50 fmol/μL, 75 fmol/μL). All standards were kept at -20ºC before LC MS/MS analysis.

7.2.3 Reduction and carboamidomethylation

Dried protein and hepatocytes pellet were dissolved in denaturation buffer: Guanidine 6M, Tris HCl 0.3M, EDTA 10mM at pH 8.0.

Reduction was carried out by using a 10:1 DTT:cysteine molar ratio. After incubation at 37°C for 2 h, iodoacetamide was added to perform carboamidomethylation using an excess of alkylating agent of 5:1 respect to the moles of thiolic groups. The mixture was then incubated in the dark at room temperature for 30 minutes. The alkylation reaction was stopped by addiction of formic acid, in order to achieve an acidic pH.

The product was purified by precipitation, 100 μL volume of sample was treated by:

- 400 μL of CH3OH
- 100 μL of CHCl3
- 300 μL of water.

The resulting solution was centrifuged for 5 minutes at 12000 rpm.

The protein will be precipitated at the interface organic-inorganic phases. The supernatant was removed, and was added 300 μL of CH3OH. The resulting solution

was centrifuged for 5 minutes at 12000 rpm and it was clearly visible a protein pellet on the bottom of the Eppendorf. The supernatant was removed and sample was dried.

7.2.4 Tryptic digestion

Digestion of protein was carried out in AMBIC 10mM using trypsin at a 50:1 protein:enzyme mass ratio. The sample was incubated at 37°C for 16 h. Then sample was dried.

7.2.5 LC–MS/MS instrumentation and conditions

Peptide mixture was analyzed by LC-MS/MS analysis using a Xevo TQ-S (Waters) with an ionKey UPLC Microflow Source coupled to an UPLC Acquity System (Waters). The LC program is shown in pictures below:

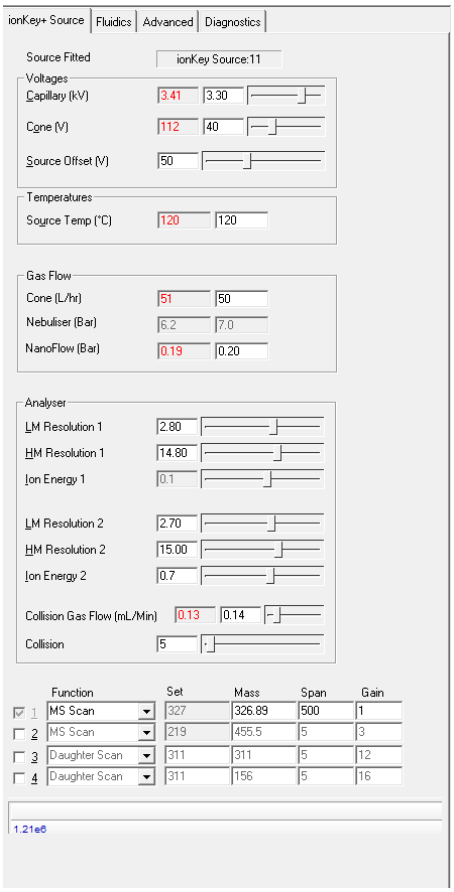

Figure 7.1 : Ionkey and Source Parameters.

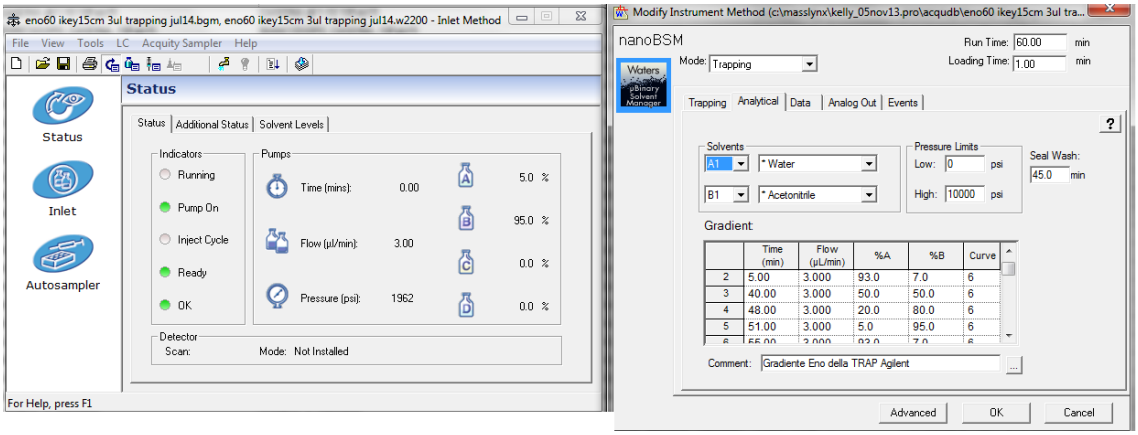

Figure7.2: LC method and Parameters.

7.2.6 Data processing

Proteotypic peptides, best transitions and collision energy were defined by Skyline software. Sample data acquisition, processing and quantitative analysis was carry out by The TargetLynx™ Application Manager Software.

7.3 Results

7.3.1 Mass spectrometer parameters optimization

The mass spectral parameters has been optimazed by using informatics tools as the software Skyline, which allows to maximize the sensitivity and specificity by identifying proteotypic peptide and for each peptide the three to five best transitions and provides a collision energy predictions for major instrument manufacturers of QQQ-MS. The results are shown in the table below:

PEPTIDE	PRECURSOR m/z	PRODUCT m/z	Dwell (ms)	Declustering Potential	COLLISION ENERGY
GWVNTLVGGQK [353-363]	**579,8**	915,5	50	80	20
		816,4	50	80	20
		702,4	50	80	20
		601,3	50	80	20
SLNNFR	**375,6**	663,3	50	80	13

[668-673]		550,2	50	80	13
		436,2	50	80	13
AFIPQLLSR [713-721]	**522,8**	973,5	50	80	18
		826,5	50	80	18
		713,4	50	80	18
		616,3	50	80	18
EQLGYIVFSGPR [826-837]	**683,3**	1108,6	50	80	24
		995,5	50	80	24
		938,5	50	80	24
		775,4	50	80	24

Table 7.1 Precursor ion, product ions and mass spectral parameters optimized for IDE.

7.3.2 Quantification and normalization

The transition and the experimental condition determinate in *silico* have been validated using a standard solution of the protein. For the realization of the calibration curve the *product ion* that having the greater signal intensity was chosen as *"quantifier"*. The other transitions ,*"qualifier"*, provide instead a confirmation of the correct identification of the molecule as it will have the same elution time.

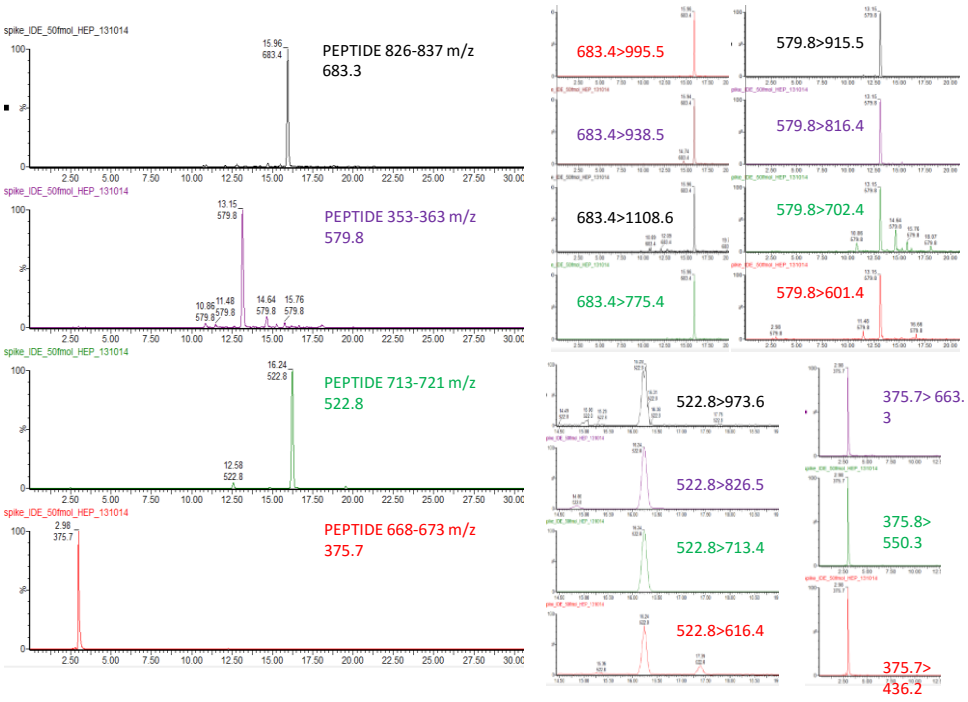

Figure 7.3 Proteotypic peptides and best transitions choosen for IDE protein.

Calibration curves were obtained by plotting chromatogram peak area against concentration (0.5 fmol/ µL, 1fmol/µL, 5 fmol/µL, 10 fmol/µL, 25 fmol/µL, 50 fmol/µL, 75 fmol/µL).

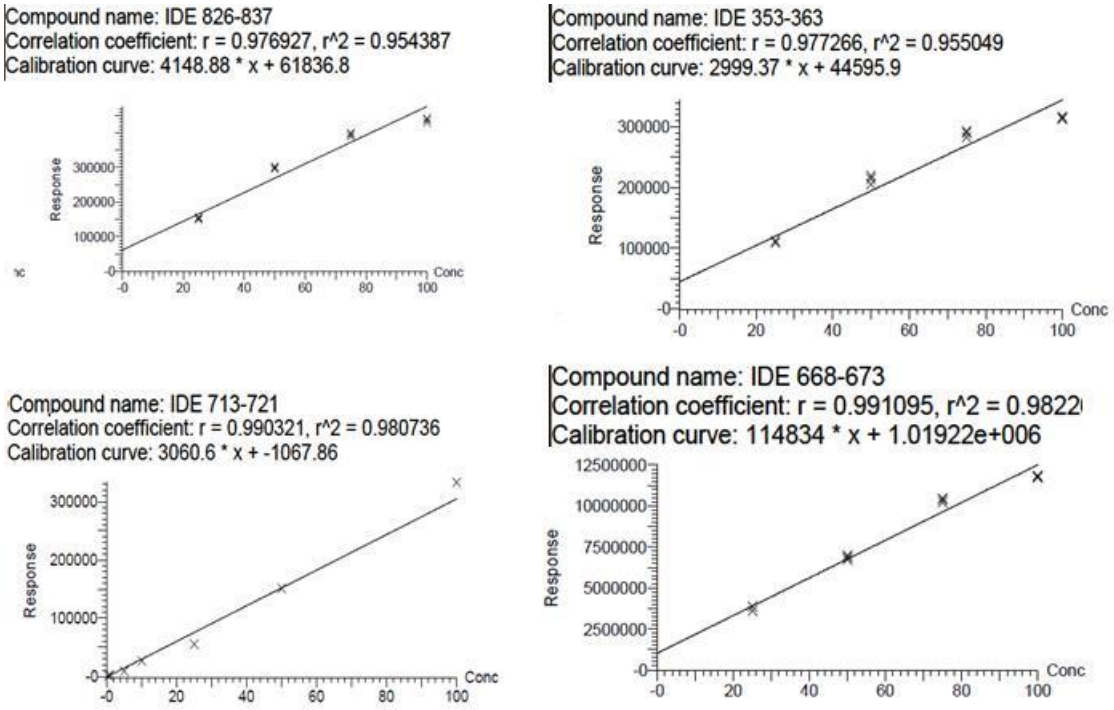

Figure 7.4 Calibration curves obtained for the four peptides.

The lower limit of detection (LOD,) and the LOQ are defined, respectively, as the concentration level at which the analyte can be reliably detected in the sample under consideration and as the level at which the analyte can be detected and measured with sufficient precision.

In order to account the matrix effect, serum samples were supplemented with known concentrations of standard protein (spike). In particular, Insulin Degrading Enzyme was spiked into human depleted serum in 10fmol concentration.

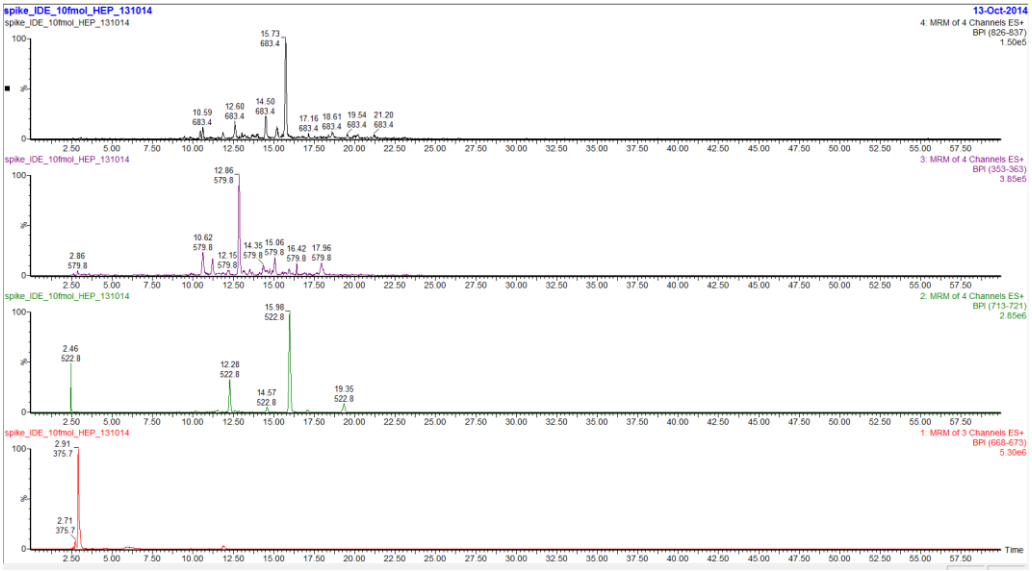

Figure 7.4 TIC chromatograms of proteotypic peptides in spiked matrix.

Recovery value (%) was also calculated (Tab7.2).

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PEPTIDE	Linear range (fmolL/ul)	Recovery %	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification
GWVNTLVGGQK [353-363]	**0,5-75**	75,2	0,8	2,5
SLNNFR [668-673]	**0,5-75**			
AFIPQLLSR [713-721]	**0,5-75**			
EQLGYIVFSGPR [826-837]	**0,5-75**			

Table 7.2 LOD, LOQ and recovery value for IDE protein.

7.3.3 Validation of the method

SRM targeted approach for IDE identification and quantification was applied to analyze three fraction of hepatocytes lysate. The epatocytes pellets were hydrolyzed as previously described, dissolved in 200 ul 0.1% Formic acid in water and analysed by LC–MS/MS. Quantification was achieved by the external standard method by interpolation on calibration curves, specific for each transition, using the TargetLynx™ Application Manager Software. The protein amount is calculated as the average value of the peptides and take into account the recovery values (Tab 7.3).

Concentration measured (ng/mg of Extract)
0,5-1,8

Table 7.3 Concentration range determined in hepatocytes extracts.

7.4 Discussionn

Targeted protein quantification by SRM ion mode has emerged rapidly over the past 10 years as one of the most challenging tasks in proteomic and biochemistry field research.

Proteomics quantification requires a more complex workflow respect to metabolomic, including an experimental design step to select the peptides suited for LC–MS measurements. The method is based on the identification of proteotypic peptides that uniquely and stoichiometrically represent the protein candidates of interest for quantitation. Several informatics tools such as the software Skyline, allows to maximize the sensitivity and specificity of the method by identifying proteotypic peptide and best transitions and provides a collision energy predictions.

The SRM assay for the analysis of IDE has demonstrated the high specificity and sensitivity and its applicability to quantify this protein in complex matrix.

7.5 References

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8 Targeted proteomics and metabolomics in Thyroide disease

8.1 Introduction

Biomarker research is one of the most interesting application in proteomics and metabolomics: different small molecule and protein/peptide present in clinical specimens such as tissue, proximal fluids and blood have been discovered as potential clinical tools for diagnosis, prognosis and therapeutic prediction of the disease of interest. For this reason, quantitative measurement of metabolites and proteins is one of the major goals of biochemistry (Liebler D. C. 2013).

Identification and validation of putative biomarkers in complex biological samples requires an instrumental method that is fast, highly selective, and sensitive. The gold standard techniques to analyze proteins and metabolites in different organisms, tissues or fluids, are immuneassay as Elisa and RIA. But antibodies also may not distinguish highly similar homologues and sequence variants arising from polymorphisms and mutations and they can't detect between different specific modification sites on proteins.

Selected reaction monitoring (SRM) has been established as an important methodology for biomarker quantification for its ability to target specific peptide sequences, including variants and modified forms, and the capacity for multiplexing that allows analysis of dozens to hundreds of peptides.

Congenital hypothyroidism with thyroid dysgenesis (TD) is a genetically heterogeneous disease. It's a frequent human condition characterized by elevated levels of TSH in response to reduced thyroid hormone levels. Congenital hypothyroidism is a genetically heterogeneous disease. In the majority of cases studied, no causative mutations have been identified and very often the disease does not show a Mendelian transmission. However, in approximately 5% of cases, it can be a consequence of mutations in genes encoding the TSH receptor or the transcription factors TITF1, FOXE1 or PAX8. We report here that in mouse models, the combination of partial deficiencies in the Titf1 and Pax8 genes results in an overt TD phenotype that is absent in either of the singly deficient, heterozygous mice. The disease is characterized by a small thyroid gland, elevated levels of stimulating

hormone thyroid (THS) levels, reduced secretion of thyroid hormones, triiodothyronine (T3), thyroxine (T4) and thyroglobulin biosynthesis, and high occurrence of hemiagenesis. The observed phenotype is strain specific, and the pattern of transmission indicates that at least two other genes, in additionto Titf1 and Pax8, arenecessary to generate the condition. These results show that TD can be of multigenic origin in mice and strongly suggest that a similar pathogenic mechanism may be observed in humans. (Amendola E. 2005).

Genomics is an entry point for looking at the other 'omics' sciences. The information in the genes of an organism, its genotype, is largely responsible for the final physical makeup of the organism, referred to as the phenotype. However, the environment also has some influence on the phenotype. Low molecular weight compounds are the closest link to phenotype.

For a holistic understanding of the biological behavior of a complex system, it is essential to follow, as unambiguously as possible, the response of an organism to a conditional perturbation at the transcriptome, proteome and metabolome levels (Oliver D. J. 2002) (Sweetlove L.J. 2003).

These three levels of expression profiling provide a complete picture of the RNAs, proteins and metabolites that enable one to: infer relevant associations between macromolecules; identify functional linkages between phenotypic expressions; and construct models that quantitatively describe the dynamics of the biological system. Broad phenotypic analyses are essential if we are to progress from prediction to experimental validation of gene function (Bino RJ1 2004).

In order to highlight the close link between genomics proteomics and metabolomics and emphasize the effects on phenotype, a method, useful to quantify murine serum concentrations of three biomarkers T3, T4 (Marotta P 2014) and THS involved in the regulation of endocrine activity of the thyroid, has been developed, in collaboration with the Department of Molecular Medicine and Medical Biotechnology , University of Naples Federico II and IRGS – Biogem.

8.2 Material and methods

8.2.1 Chemicals and reagents

Murine synthesized peptides were purchased from Primm (DFIYR, LFLPK). Triiodothyronine (T3) and thyroxine (T4) were purchased from Sigma-Aldrich All the solutions and solvents were of the highest available purity and were suitable for LC–MS analysis. All stock solutions were stored at −20 °C.

8.2.2 Preparation of standards solutions

Stock solution of two peptides were prepared at 10 mg/mL by adding 1 mL of 0.1% formic acid solvent to each peptides Dilutions were subsequently performed to generate a calibration series at concentrations of 0.5 fmol/ µL, 1fmol/µL, 5 fmol/µL, 10 fmol/µL, 25 fmol/µL, 50 fmol/µL, 75 fmol/µL, 100fmol/ul). Standard solutions of T3 and T4 were prepared at different concentrations (0.5, 1,0, 5, 10, 25, 50, 100 pg/µl) by serious dilution and were used for calibration curves.

8.2.3 Sample preparation

Blood samples were obtained from the tail vein, under light anesthesia, and collected in tubes without the presence of anticoagulant. After coat formation the samples were centrifuged and the recovered sera were kept frozen at −20 °C until assayed

8.2.3.1 Metabolites

100 µl of 145 samples of mouse serum were treated with 300 µl of cold ethanol. The mixtures were stored at -20°C for about 30 minutes followed by centrifugation at 10000 rpm for 10 min at room temperature. The clear supernatant, subsequently subjected to analysis, was transferred to a 1.5-mL tube and stored at -20°C. 1 ul of supernantant was used for analysis. In order to validate the performances of the method serum samples were spiked, prior to the extraction, with appropriate concentrations of the standard mixture of targeted analytes.

8.2.3.2 Protein

To eliminate major proteins interference in murine plasma, in particular Albumin (~45 mg/mL) and IgG (~10 mg/mL), representing approximately 65% and 15% of total plasma proteins, respectively, plasma samples were subjected to sample clean-up used immunoaffinity depletion.

8.2.3.3 Reduction and carboamidomethylation

Serum samples and standard peptides were dissolved in denaturation buffer: Guanidine 6M, Tris HCl 0.3M, EDTA 10mM at pH 8.0.

Reduction was carried out by using a 10:1 DTT:cysteine molar ratio. After incubation at 37°C for 2 h, iodoacetamide was added to perform carboamidomethylation using an excess of alkylating agent of 5:1 respect to the moles of thiolic groups. The mixture was then incubated in the dark at room temperature for 30 minutes. The alkylation reaction was stopped by addiction of formic acid, in order to achieve an acidic pH.

The product was purified by precipitation, 100 µL volume of sample was treated by:

400 µL of CH3OH
100 µL of CHCl3
300 µL of water.

The resulting solution was centrifuged for 5 minutes at 12000 rpm.

The protein will be precipitated at the interface organic-inorganic phases. The supernatant was removed, and was added 300 µL of CH3OH. The resulting solution was centrifuged for 5 minutes at 12000 rpm and it was clearly visible a protein pellet on the bottom of the Eppendorf. The supernatant was removed and sample was dried.

8.2.3.4 Tryptic digestion

Digestion of protein was carried out in AMBIC 10mM using trypsin at a 50:1 protein:enzyme mass ratio. The sample was incubated at 37°C for 16 h. Then sample was dried.

8.2.4 LC–MS/MS instrumentation and conditions

SRM protein analysis of standard solution and serum samples was carry out using a Xevo TQ-S (Waters) with an ionKey UPLC Microflow Source coupled to an UPLC

Acquity System (Waters). For metabolites analysis was performed using a 4000 QTrap ABSciex coupled to Eksigent expressHT-ultra system . For analysis the Eksigent 0.5 x100mm C18 HALO column was used at a flow rate of 40 μL/min with a 5% to 95% linear gradient in 6 min (A solvent 0.1% formic acid, 2% ACN in water; B solvent 0.1% formic acid, 5% water in ACN).

8.2.5 Data processing

The specific transitions and collision energy for the two peptides were defined by Skyline software. Sample data acquisition, processing and quantitative analysis was carry out by The TargetLynx™ Application Manager Software.

For metabolites, the acquisition methods were processed through software MassHunter Data Acquisition.

8.3 Results and discussion

8.3.1 Mass spectrometer parameters optimization

Best transitions and mass spectral parameters optimized are shown in the table 8.1A and B.

A

Compaund	**Precursor Ion (m/z)**	**Product Ion (m/z)**	**Dwell (ms)**	**Declastering Potential (V)**	**Collision Energy (V)**	**Polarity**
DFIYR	357,1	598,3	250	90	12	Positive
		451,2	250	90	12	Positive
LFLPK	309,2	504,3	250	90	10	Positive
		357,2	250	90	10	Positive

. B

Compound Name	Precursor Ion m/z	Product Ion m/z	Dwell (ms)	Declustering Potential (V)	Collision Energy (V)	Polarity
T3	651,9	605,7	250	80	20	Positive

	651,9	197,1	250	80	80	Positive
T4	777,9	731,6	250	80	30	Positive
	651,9	322,8	250	80	90	Positive

Table 8.1 A and B. Specific transitions and instrumental parameters optimized

8.3.2 Quantification and normalization

The transition and the experimental condition determinate in *silico* have been validated using standard solutions of T3, T4 and TSH in LC MS MS.

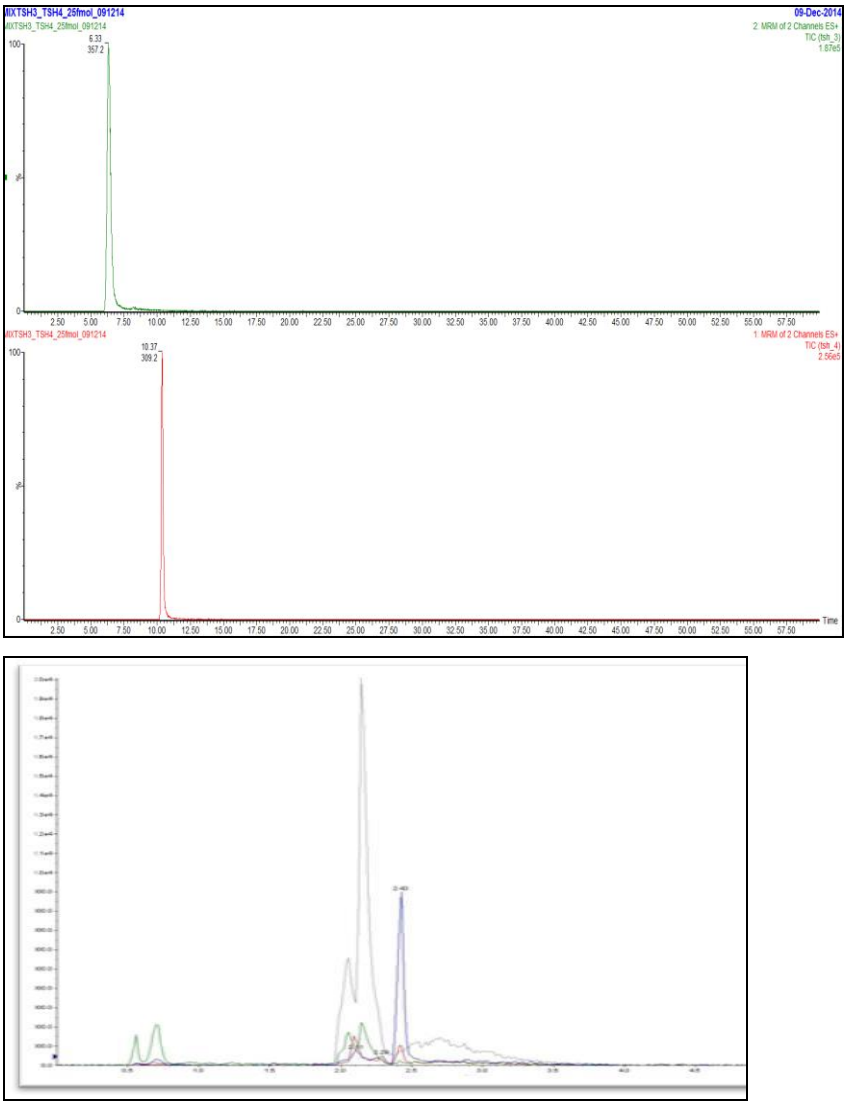
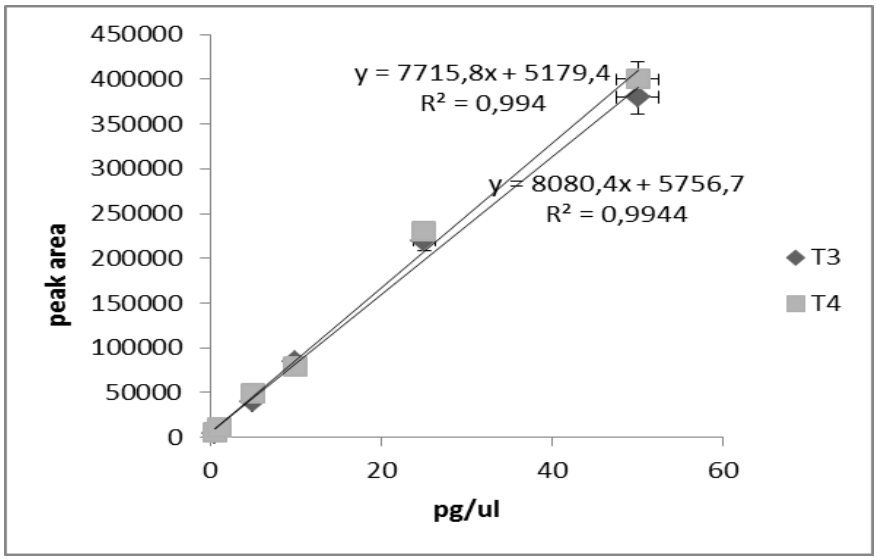


Figure 8.1.a) Total ion chromatogram for TSH peptides B) XIC cromathogram for T3 and T4.

The calibration curves were obtained by analysing all standard solutions at different concentrations. Good linearity (R2 > 0.9) is obtained for all compounds as shown in figure, both for metabolites and proteins.



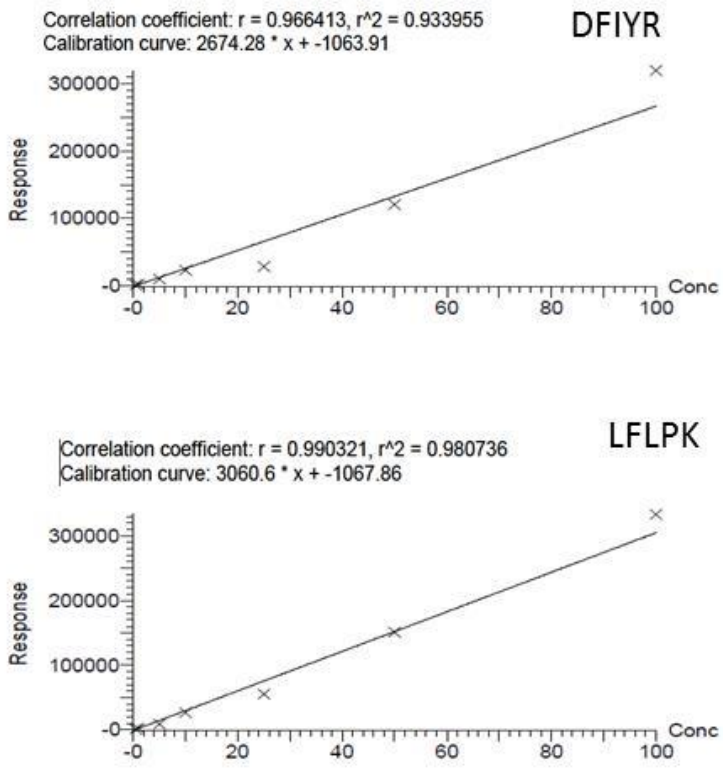


Figure 8.2 Calibration curves for standars mixture.

Limit of detection (LOD) , limit of quantification (LLOQ) and recovery were determined by addition of defined concentration of standard solution to the serum (Tab.8.2 and 8.3).

PEPTIDE	Linear range (fmolL/ul)	Recovery %	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification
DFIYR	0,5-100	86,80	2,50	7,50
LFLPK	0,5-100			

Table 8.2 LOD, LOQ and recovery value for protein peptides.

Compound Name	Polarity	Linear range (pg/ul)	Recovery %	LOD (pg/ul) Limit of detection	LOQ (pg/ul) Limit of quantification
T3	Positive	0,5-100	90	0,25	0,71
	Positive				
T4	Positive	0,5-100	95	0, 18	0,62
	Positive				

Table 8.3 LOD, LOQ and recovery value for metabolites.

8.3.3 Validation of the method

The SRM method so set up both for TSH and T3 and T4 has been applied to quantification of this specific compounds in 145 murine serum sample.

Quantitation was achieved by using the calibration curves. Recovery was calculated by comparing the extraction yield before and after the standard addition of the different analytes into the control samples.

Analyte	Concentration range	
	Phenotype normal	Hypothyroidism
T3 (pg/µL)	0,9 - 10	<0,1
T4 (pg/µL)	9 -65	<0,5
TSH (fmol/ul)	1,5- 2,6	6,5 - 16

Table 8.4 Concentration mean value determined in murine serum.

Subsequently, mice are divided in three experimental groups (OFF untreated controls, 1wkON treated for a week and the serum was collected immediately, 1wkOFF were treated for a week and the serum was collected one week after the end of treatment), treated with a substance that induces hypothyroidism characterized by low T4 and high TSH.

A recovery of thyroid function resulted by T4 increase and TSH decrease was observed (Fig8.3).

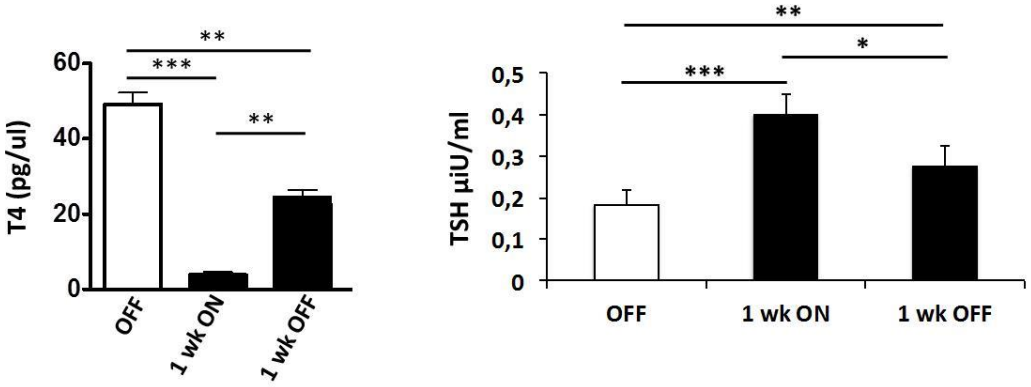


Figure 8.3 T4 and TSH trand in experimental groups under the influence of a thyroid inibhitor

8.4 Discussion

The identification of biomarkers for diagnosis, prognosis, therapy monitoring and toxicity is a major goal of biomedicine in the 21st century. These molecules are metabolites and proteins whose concentrations vary under the influence of endogenous and exogenous factors. For this reason the main objective of the analysis of diagnostic compounds is their detection and quantification in biological samples like human plasma or urine. Clinical application almost invariably uses an antibody-based diagnostic approach such as ELISA or dipstick.

Whilst this process has the potential to deliver clinically important markers, particularly within the cancer field, it may not represent the most efficient process since it requires the independent and sequential development of at least two analytical methodologies with the disadvantage of high costs.

Currently there is a growing interest in simultaneous analysis of multiple biomarkers characterizing a given condition. This means that multiwell screen will be needed with all the problems associated with the production of antibodies and cost.

Furthermore, patient heterogeneity, variation during sample acquisition and storage, sample stability, dynamic range, modification status of analytes, macromolecular interactions and other factors all deeply influence the observed qualitative and quantitative characteristics of a potential biomarker.

Confirmation and validation of putative biomarkers in complex biological samples requires an instrumental method that is fast, highly selective, and sensitive. Analytes quantification using selected reaction monitoring (SRM) has been established as an important methodology for biomarker quantification.

The application of LC/MS/MS in SRM ione mode for protein and metabolite analysis compared to the current antibody-based automated immunoassays presented some advantage. First, it provide excellent selectivity and specificity regardless of the complexity of the serum. Second, it has a LOQs lower of the usual concentration range of immunoassays. Third, the assay is simple and highly reproducible as well as being robust and able to accommodate high sample throughput.

The examples provided above clearly indicate the power of SRM methodology for sensitive quantification of proteins and metabolites biomarkers within complex mixtures in routine use.

The results of quantitative determination of T3, T4 and TSH have made possible the genotype- phenotype correlation confirming the hypothesis of a multigenic origin of the alteration of the thyroid.

The SRM assay for the analysis of TSH has demonstrated the high specificity and sensitivity of the method resulting in a viable alternative to the official method ELISA.

It has resulted particularly useful in research field thanks to its versatility for application in different organisms.

8.5 References

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9 Conclusions

9.1 SRM compared to other quantification techniques

Targeted metabolomics and proteomics by SRM have shown the potential of becoming the technique of choice for the identification and quantification of specific compounds in biological matrix.

A number of methods can produce metabolic signatures of biomaterials, including nuclear magnetic resonance (NMR), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), capillary electrophoresis-mass spectrometry (CE-MS), and so on.

GC coupled to MS has been extensively used in metabolome analysis because of its high separation efficiency and the easy interfacing of GC with MS. It can be used to analyze a wide range of volatile compounds and semi-volatile compounds through chemical derivatization. The volatile compounds include ketones, aldehydes, alcohols, esters, sulfides, some lipids, and so on. The semi-volatile metabolites include sugars, sugar-phosphates, sugar-alcohols, organic acids, amino acids, lipids, peptides, alkaloids, amines, amides, and so on. In addition to GC-MS, LC coupled to MS is useful tool for metabolomics studies. They can resolve and quantify multiple components in crude biological extracts in the nanomole to picomole and even femtomole range from as little as submicroliter volumes. LC-UV-MS is routinely used for metabolites quantification based on the peak area associated with UV signals. Currently LC-MS/MS is considered the workhorse of metabolites detection because of the fact that the derivatization of polar/on-volatile metabolites for GC-MS analysis is both time consuming and require fewer material compared to LC.

Analytical methods based on affinity reagents, such as antibodies, have been routinely used in biological and biomedical research to measure the presence and/or quantity of target proteins by exploiting the antibody specificity and the direct or indirect detectability of a tag such as radioisotopes, enzymes and fluorophores. The methods with the lowest limit of detection have the potential to detect, in principle, low-abundance compounds, down to zeptomole detection limits. However, the development of reagents of suitable specificity and affinity to support the

unambiguous measurement of the target protein in a complex background remains challenging, expensive and arduous. A step toward increased multiplexing of affinity reagent–based assays was the development of protein arrays in which microarrays, chips or beads allow for the parallel, multiplexed screening of large numbers of proteins, in an automated manner, and with minimal consumption of sample and reagents.

Another often used technique is the direct tagging of the target protein with a detectable and quantifiable label such as a fluorescent protein and its quantification by flow cytometry or microscopy techniques. The multiplexing capabilities are normally limited, and the introduced tag can potentially affect the localization, function, stability and expression of the target protein. These fusion proteins offer the possibility to quantify the amount of the target protein in single cells, thus revealing the cell-to-cell variability in expression and to provide information about the subcellular localization of proteins. Other techniques to quantify specific proteins in single cells are immunofluorescence, which can be applied to cells or tissue sections, and mass cytometry, which expands the multiplexing capabilities of single-cell probes by the use of multiatom elemental tags. However, both techniques rely on antibodies and thus have limitations associated with their use.

9.2 Advantage of SRM

In recent years there has been a rapid increase of targeted proteomics and metabolomics using SRM methods. As demonstrated in this thesis project, SRM offers several advantages, such as specificity, sensitivity, linearity and it ideally suits for multiple quantitative analysis.

The selective nature of SRM reduces the likelihood of interference from matrix and other target compounds, allowing for the rapid analysis of more compounds in a single run. In addition, the capacity of monitoring more transitions simultaneously allows for greater security in the identification of specific target compound, reducing false positive.

LC-SRM measurements showed that the range of analytes concentration accessible by direct LC-SRM is 4–5 orders of magnitude and that limits of detection at the low

attomole level can be routinely reached, even in complex samples at high reproducibility and throughput.

Another important advantage is that the SRM analysis does not require a laborious step of sample pre-treatment or a detailed chromatographic separation offering the possibility of reducing the time analysis, including the extraction and data processing.

SRM analysis are not limited to a single application area but it is a versatile analytical approach. Laboratories working in food safety, environmental monitoring and bioanalysis can all benefit from the advantages of a SRM analysis. Therefore, costly sample preparation procedures can be optimized to deliver an efficient lab oratory workflow.

Moreover, one can measure 50–100 analytes in parallel in the same LC-SRM run if a reasonable duty cycle for accurate quantification and a sufficient dwell time to maintain a low limit of detection are applied. Upon identity confirmation, the primary transitions are used to quantify the analyte. This approach has the potential to dramatically increase the multiplexing capabilities of SRM.

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IX ItPa Annual Congress 24-27/06/2014 Napoli