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BIOMOLECULAR AND PROTEOMIC TECHNOLOGIES APPLIED TO SICILIAN AUTOCHTONOUS LIVESTOCK BREEDS BIODIVERSITY

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Purpose of the Doctoral Project

The aim of the doctoral project was the characterization of autochthonous Sicilian breeds, such as “Girgentana” goat breed, through the use of –OMICS technologies and, in particular, use of Proteomic methodologies applied to animal productions like milk and dairy products. The purpose of the PhD project was the development of advanced biotechnology methods and suitable proteomic protocols finalized to study Sicilian native livestock breeds, such as "Cinisara"(a bovine breed), "Comisana" (an ovine breed), “Girgentana" (a goat breed) breeds, through the authentication, exploitation and consequent preservation of typical dairy production obtained from native breeds raw materials.

The field of biotechnology applied to livestock production ranges from livestock animals genomic analysis to the use of molecular markers to improve the quality of breeds and livestock production, to kinship determination, individuals and breeds identification and animal products tracking and authentication, to animal biodiversity conservation.

Actually, in livestock production the actual growing problem is to increase, as part of the concept of sustainable agricultural and forest ecosystems management, the organoleptic properties and safety of animal origin products, improving transformation technologies and increasing quality production.

In this context a better knowledge of the importance of native livestock biodiversity (climate adaptation, morphological and functional differences between breeds, resistance to endemic diseases and ability to synchronize themselves to different dietary requirements related to environmental changes is required.

It is very important to protect native breeds because they might be a genetic resource for future selections, with objectives of adaptation to adverse condition, endemic diseases tolerance. It should be emphasized the close link between the need to protect native livestock biodiversity and the importance of produce unique local traditional products, due to their physic-chemical, microbiological and organoleptic properties.

The survival of Sicilian native breeds is strictly related to their characteristics, the best quality of the products obtained from them, the close link between local productions, territory, and tradition.

Actually, a real problem if we want to investigate animal breeds is represented by the poor data available in the databases concerning livestock breeds.

The extent of the PhD work was to develop proteomic protocols aimed to produce the first data available in the context of Sicilian livestock breeds as precursor studies able to enrich the actual knowledge related to animal biodiversity and local animal production.

The aim of a research work was to compare Protein profile of Girgentana breed whole bulk milk samples, taking into account different lactation periods and geographical areas by two dimensional difference in gel electrophoresis (2D-DIGE).

Milk proteomics has rapidly developed as an eligible approach and proteomic applications can vary from protein identification to complex characterization of protein post-translational modifications and protein pathways analysis.

This work was the object of an abstract presented during the Conference organized by the Scientific Association of Animal Production (ASPA). It allowed giving a general picture of Girgentana goat milk protein distributions over 3-10 pH range and it can be the initial study of following proteomic studies aimed to make the Girgentana goat milk reference map.

Another work related to milk from Sicilian breeds was finalized to analyze whole "Valle del Belice" sheep breed milk together with milk fractions.

The aim was to compare two different protocols to separate caseins fraction from whey proteins fraction, to better understand milk proteome.

In particular, one protocol based on Kjeldahl method (FIL-IDF) and a second one validated by the Scientific Association of Animal Production (ASPA) has been compared, in order to establish the most suitable protocol to follow.

The second part of the PhD research activities is related to the activities done during the mobility period, in Budapest. The aim of these research activities was the improvement of proteomic techniques "gel-based", sample preparation (protein spots "in-gel" digestion) and mass spectrometry applied to proteomics.

The acquisition of these skills can be perfectly translated in other fields of application, such as livestock production, since the proteomic techniques are very versatile and have a wide range of applications.

Animal foods such as Girgentana goat milk, represent complex food matrices containing different types of proteins and other components and the current available proteomic techniques, coupled to mass spectrometry exceed the current technical limitations that do not allow, for example, the study of membrane proteins, highly hydrophobic, and allow the study of multiple proteins, from several hundred up to several thousands.

HPLC and tandem mass spectrometry (MS/MS) are, in fact, systems able to separate and identify low abundance membrane proteins that would otherwise be impossible to study by traditional two-dimensional electrophoresis 2DE.

The depletion of abundant proteins from biological fluids can be of great help in decreasing proteome complexity, when the goal is the study of less abundant proteins or molecular markers discovery.

High resolution two-dimensional electrophoresis, combined with high performance liquid chromatography (HPLC) and mass spectrometry, represents a powerful tool for analyzing complex mixtures of several hundred proteins simultaneously.

Furthermore, liquid chromatography coupled to mass spectrometry allows the separation and identification of molecules of low molecular weight, goals that until a few years ago were considered very ambitious and impossible to reach.

The research activities done during the PhD mobility and performed in the laboratories of mass spectrometry applied to proteomics of the research center MTATTK of Budapest under the supervision of Professor Karoly Vekey as tutor were aimed to the validation of the repeatability of a method for depletion of high-abundance proteins using a commercially available kit based on affinity chromatography.

In this context, the main aim was to become familiar with methods finalized to solve proteome complexity and to evaluate in a critical way the repeatability of such a method.

As it is well known, a complication in the experiments of proteomics, which relates the experimental approach, is given by the presence of highly abundant proteins in biological samples, which makes difficult biomarkers discovery and identification, due to preponderant proteins that interfere with the analysis based on high performance liquid chromatography coupled to tandem-mass spectrometry (HPLC-MS/MS).

The removal of high abundance proteins such as human serum albumin (HSA), which constitutes more than 50% of plasma total protein content, may enable the identification of less abundant proteins. The principle is to make protein sample less complex, increasing the possibility of determining less abundant proteins.

As reported in literature, it is of great importance that a step of depletion is reproducible when included in a proteomic study.

This study was preparatory to further studies that will be pursued by Professor Karoly Vekey research group and are a part of a vaster study.

Within the mobility period of the PhD I was also involved in the research activities carried out at the laboratories of Proteomics Institute of Biology of the Faculty of Sciences of Budapest by Professor Gabor Juhasz research group.

Regardless of the subject of the research activity (the comparison between two different hypotheses about the connection between the circadian rhythm and neuronal plasticity of the brain in mammals), the aim was to improve techniques of Proteomics "gel-based" that I can also apply in livestock farming because they are very versatile. I also used tools for image analysis in proteomics (other than those I have previously used), methods of protein sample preparation (such as "in-gel" digestion of spot proteins), for the separation and identification of proteins through mass spectrometers and, finally, I used appropriate softwares useful in mass spectrometry applied to proteomics for the discrimination of the results.

1-PROTEOMICS

1.1: Biotechnologies Applied To The Study Of Sicilian Indigenous Livestock Breeds Biodiversity: State Of The Art:

The definition of biodiversity is to be traced back to the Forum in Washington in 1986. The term was used for the first time to indicate the complexity and universality of biological structures in a unique panoramic of biodiversity from the genetic and taxonomic points of view.

Biodiversity is a dynamic field and it can vary in space (we talk about geographical variation) and in time, with the increase of biodiversity due to mutations, allelic reassortment, intersections, or migration and with its reduction, due to phenomena of genetic isolation, genetic drift, self-fertilization, consanguinity, natural selection and anthropic selection. Biodiversity can be measured at the taxonomic, functional and genomic levels.

If we study biodiversity at the taxonomic level, we consider many indices, from the Linnaean classification to Simpson indices (number of species and relative abundance) and Shannon-Wiener indices (uniform distribution), to Weitzman approach.

Following the functional definition of biodiversity, a quantitative measure of variability is made, analyzing the causes of variability and considering heritability and repeatability.

At the genomic level, however, genomic markers polymorphism (microsatellite or STR, RFLP, SNP, mt DNA) and/or gene products (blood groups, serum proteins or erythrocytes, milk proteins, etc.) are considered. In addition, biodiversity at the genomic level can be measured in terms of intra-population variability and variability between populations (allele frequencies, taking into consideration Hardy-Weinberg expected and observed heterozygosity), for clustering and phylogenetic analysis and candidate genes or QTLs studies.

From a zootechnical point of view, biodiversity includes only thirty domesticated species, with approximately five thousands breeds due to geographical isolation or human action because of environmental, cultural, social, economic and religious needs.

The zootechnical meaning of biodiversity can be understood as adaptation ability to climate changes, functional diversity between species, resistance to endemic diseases, ability to synchronize with the changes of the food needs to environmental changes.

In addition, talk about zootechnical biodiversity means to speak about quality productions of our native breeds in terms of milk, dairy production and meat, to pay attention to the lipids of a product (low cholesterol levels, high proportions of polyunsaturated fatty acids) and to the proteins (essential amino acids branched, carnitine, glutathione etc).

Currently, in Europe about the fifty percent of the breeds risks the extinction and according to the FAO report in Europe have become extinct nearly one hundred domestic breeds.

Faced with the possible loss of livestock biodiversity as a result of trade, urbanization, "alien" animal breeds introduction (the "alien" breeds are more productive than the native ones, of high-quality and resistant to diseases), and use of pollutants, biodiversity conservation is an ethical problem.

The ONU association, in order to preserve species and natural habitats, signed international agreements for the protection of wetlands, the protection of the Mediterranean Sea, the protection of migratory species, the prohibition of endangered species, etc.

Many significant initiatives aimed to preserve zootechnical biodiversity are carried out by the FAO Organization (preservation and valorisation of animal genetic resources) and by the European Union (Natura 2000 and the Habitats Directive).

In Italy many actions are promoted to convince livestock producers of the benefits that could have the protection and recovery of indigenous breeds in future economic scenarios.

In order to preserve livestock biodiversity, livestock animals should be kept within their traditional production system, emphasizing the link between animal breed and environment, resistance to stress situations, resistance against endemic diseases and ability to provide high quality products (products with a high content of bioactive molecules.

Genetic variability preservation of native breeds is aimed to perform breeding programs for productive activities and uses the identification of possible genetic reservoirs (diversity or similarity indices), the evaluation of genetic purity, the identification and monitoring of the territory.

The field of biotechnology applied to livestock production is extensive, ranging from the genome analysis of livestock animals of particular economic interest to the use of molecular markers to improve the quality of breeds and livestock production, to kinship determination,

individuals and breeds identification and animal products tracking and authentication, to animal biodiversity conservation through the use of molecular markers.

In this context, the importance of identify and characterize molecular markers that are "unique" (in reference to an animal breed) and "typical" (in reference to a particular breed product).

The growing problem in livestock production is to increase, as part of the modern concept of sustainable agricultural and forest ecosystems management, the organoleptic and safety products of animal origin, improving transformation technologies, reducing the prices and increasing quality production. The context is that of a better knowledge of the importance of native livestock biodiversity, as climate adaptation, existence of morphological and functional differences between breeds, resistance to endemic diseases and ability to synchronize themselves to different dietary requirements related to environmental changes.

It is very important to protect traditional breeds because they might be a resource of genes for future selection works, with objectives of adaptation to adverse condition, endemic diseases tolerance, etc.

From a zootechnical point of view, it should be emphasized the close link between the need to protect biodiversity of native species and the importance of produce local products, due to their unique physic-chemical, microbiological and organoleptic properties.

The concerns are the same expressed in Malthus “Essay on the Principle of Population“, published in the late eighteenth century, highlighting the dramatic depletion of non-renewable natural resources (including biodiversity) because of industrialization spread.

The importance of the Conservation of breeds biodiversity is fundamental for livestock production and is now widely recognized and demonstrated by the Conservation programs promoted by many countries. Preserve biodiversity means not only to maintain biological diversities but also to defend immense unique cultural heritages in danger of disappearing due to the spread of the intensive animal farming management, with few highly productive breeds and without marginal agricultural areas. Actually, the survival of local breeds is strictly related to their characteristics and their economic value thanks to the best quality of the products obtained from them.

The trend in Italy and, in general, in Europe, is to produce great quality foods and this trend is a strong thrust to many Conservation efforts of local breeds through the development of typical food productions, characterized by a strong identity and strictly linked to the territory

and the tradition. Conservation of local livestock breeds is closely linked to the concept of sustainable development.

The current trend is to follow an "OMIC" strategy that uses biotechnologies to obtain typed local products by the identification and characterization of "molecular biomarkers" of "genetic uniqueness", in the case of the individual, and of "specificity" if we refer to food products. In particular, the analysis of DNA polymorphisms and proteomic profiles to estimate intra- and inter-population biological variability and to define a "molecular identity card" or "fingerprinting" as a valuable tool to support the current authentication systems and/or traceability system in food chain and to analyze the polymorphisms involved in determining both food qualitative and quantitative characteristics.

1.2: *The Post-Genomic Era*

In recent decades Genomics has answered to many important biological questions, representing the basis for scientific research and biotechnology in the years.

The Human Genome Project and other genome sequencing programs are turning out, in rapid succession, the complete genome sequences of specific species and thus, in principle, the aminoacid sequence of every protein potentially encoded by that species (Rowen & Mahairas, 1997; Fraser & Fleischmann, 1997). As expected, this revolutionary source of information, unprecedented in the history of biology, enhanced traditional research methods such as the biochemical approach and also catalyzed proceedings and developments of Omic sciences such as proteomics.

Thanks to the genome sequencing, for the first time in biology the complexity of a body from the information point of view has been realized (Williams & Hochstrasser, 1997).

But, as often it happens in Science, once obtained the first complete genomes it became evident the limit of the information they could provide (Nowak 1995). These limitations are not due to technological deficiencies but rather to the limit of information related to the molecular regulation and, as a consequence, to all the following processes that take place within the cell. The huge amount of the information contained in the genome provides a list of proteins that can be expressed from the genome in any cell that composes the organism studied, but it gives no information on proteins that will be expressed or activated at a given time or cellular compartment.

On the time scale of most biological processes, with the exception of evolution, the genomic DNA sequence can be viewed as static (Aebersold & Goodlett, 2001). Secondly, knowing the exact nucleotide sequence of a gene doesn't allow to predict the cellular type in which the encoded protein will be expressed, either it indicates in which development or differentiation stage or in response to which stimulus this expression will occur. In addition, the knowledge of the nucleotidic sequence of a gene does not allow either to predict post-translational modifications that occur after the synthesis of a given polypeptide. Besides, the only analysis of mRNAs doesn't allow obtaining indications on protein existence and its expression level, because there is no linear correlation between mRNA and protein amount (Gygi et al., 1999).

Intensive efforts are directed to assign "function" to individual sequences in sequence databases.

In this context, the importance of the analysis of linear sequence motifs or higher order structural motifs that indicate a statistically significant similarity of a sequence to a family of sequences with known function or the comparison of homologous protein functions across species (Hofmann et al., 1999; Henikoff et al., 1999; Skolnick et al., 2000; Marcotte et al., 1999; Enright et al., 1999).

1.3: *Proteome And Proteomics: The Dynamicity Of Life*

Currently proteomics is one of the most challenging post-genome research technologies, essential to highlight the role of the proteins expressed in a tissue in a defined time and in a determined physiological condition and the complex protein - protein interactions existing within an organism, for a better understanding of the cellular functions.

The proteome has been defined as the protein complement expressed by a genome (Wilkins et al., 1996; Wasinger et al., 1995; Hochstrasser, 1998; Loo et al., 1996).

This is a restrictive definition that implies a static nature of the proteome. In reality, the proteome is highly dynamic for its nature; the different types of expressed proteins, their abundance, state of modification, subcellular location, etc. being dependent on the physiological state of the cell or tissue in a well defined time. Therefore, the proteome reflects the cellular state or the external conditions encountered by a cell. As a consequence, proteome analysis can be viewed as a genome-wide assay to differentiate and study different cellular states and to determine the molecular mechanisms that control them (Haynes et al., 1998).

Proteomics is the study of the proteome, the protein complement of the genome. Proteomics can be defined as "the qualitative and quantitative comparison of proteomes under different condictions to understand cellular mechanism underlying biological processes" (Anderson & Anderson, 1996).

The terms "proteomics" and "proteome" were coined by Marc Wilkins and colleagues in the early 1990s. Until the mid-1990s, biochemists, molecular biologists, and cell biologists studied individual genes and proteins or small clusters of related components of specific biochemical pathways using the techniques available: Northern blots (for gene expression) and Western blots (for protein levels). Three developments made the foundation of the new biology.

The first was the growth of gene, expressed sequence tag (EST) and protein-sequence databases during the 1990s. These resources became ever more useful as partial catalogs of expressed genes in many organisms. The genome-sequencing projects of the late 1990s yielded complete genomic sequences of many model organisms and culminated in the complete sequence of the human genome. The second key development was the introduction of bioinformatics tools to extract information from these databases. The third key development was the oligonucleotide microarray, containing a series of gene-specific oligonucleotides or cDNA sequences on a slide or a chip.

By applying a mixture of fluorescently labelled DNAs from a sample of interest to the array, one can probe the expression of thousands of genes at once.

Gene microarrays offer a snapshot of the expression of many or all the genes in a cell. Unfortunately, the levels of mRNAs do not necessarily predict the levels of the corresponding proteins in a cell. Differing stability of mRNAs and different efficiencies in translation can affect the generation of new proteins. Once formed, proteins differ significantly in stability and turn-over rates. Many proteins involved in signal transduction, transcription-factor regulation, and cell-cycle control are rapidly turned over as a means of regulating their activities. Finally, mRNA levels tell us nothing about the regulatory status of the corresponding proteins, whose activities and functions are subjected to many endogenous posttranslational modifications and other modifications by environmental agents.

The extent and variety of modification varies with individual proteins, regulatory mechanisms within the cell, and environmental factors. Consequently, many proteins are present in multiple forms.

Current proteomic studies have mainly focused on two main areas: expression proteomics, which studies changes in protein levels (both qualitative and quantitative) under appropriate stimuli and/or physic-pathological conditions, and functional proteomics that attempts to identify components of cellular compartments, complex multiprotein and pathways of signal transduction.

Expression proteomics, in turn, is divided into two main lines: systematic proteomics, whose aim is the protein identification to construct the reference maps of the proteins expressed in the tissues; and differential proteomics, that provides the quantification of differentially expressed proteins in relation to changes in physiological and pathological conditions.

Both expression proteomics and functional proteomics are essentially based on two different analytical steps that, essentially, consist in the consecutive separation of the proteins that constitute the proteome and their subsequent individual identification through mass spectrometry procedures that are able to characterize their possible post-translational modifications and the network of interactions with other proteins.

1.4: *Proteomics Applications: A General Overview*

Recently, in the context of food science and nutrition, has been defined a new rapidly emerging discipline: foodomics, that studies food and nutrition fields through the application of advanced ”OMICS” technologies in order to improve people’s health.

In this context, foodomics has been defined as a new discipline that studies the food and the nutrition domains through the application of advanced omic technologies in order to improve consumer’s well-being, health and confidence (Cifuentes, 2009; Herrero et al., 2010).

As a consequence, foodomics is intended as a global discipline that includes all the emerging working area in which food (including nutrition), advanced analytical techniques (mainly omic tools) and bioinformatics are combined.

Foodomics involves multiple tools to deal with the different applications included in this field.

Through tools from the –omic sciences (genomics, epigenomics, transcriptomics, proteomics and metabolomics), foodomics offers new analytical methods to solve new challenges in food and nutrition science. MS-based techniques are fundamental for proteomics and metabolomics studies (Cifuentes, 2013).

The development of advanced omic technologies has given rise to extraordinary opportunities to increase our understanding about different issues: (I) to understand the mechanisms that underlie the beneficial and adverse effects of certain bioactive food components; (II) to compare different proteomic profiles among individuals in response to different diets; (III) to know the identity of genes involved in previous stages of diseases, detecting molecular biomarkers; (IV) to understand the stress adaptation responses of food-borne pathogens to ensure food processing and preservation; (V) to have the comprehensive assessment of food safety, quality and traceability; (VI) to understand the molecular basis of biological processes with agronomic and economic interest.

Proteomes differ among different individuals, cell types, and within the same cell according to cell activity and state.

Proteomic techniques are the most important means of identifying the protein complement of a cell. The cell space of an organism is packed with proteins, which are continuously synthesized and degraded according to the needs of the cell metabolism. Proteomic identification of proteins will therefore result in different proteomes depending on the time at which the protein complement of the cell is isolated. Proteomics remains the challenge to

characterize the structures, interactions, and functions of all the proteins in a cell at a defined state of development (Granvogl et al., 2007).

Initially defined to catalogue cells and tissues proteins, now proteomics has a wide range of application fields, including the systematic analysis of protein structure, expression, interaction, function, folding, purification and structural genomics (Abbott, 1999; Bennett et al., 2002; Graves & Haystead, 2002).

Proteomics encompasses four principal applications: 1) protein mining, 2) protein-expression profiling, 3) protein-network mapping, and 4) protein modifications mapping.

Protein Mining is simply the exercise of identifying all (or as many as possible) the proteins in a sample. The point of mining is to catalogue the proteome directly, rather than to infer the composition of the proteome from expression data for genes (e.g., by microarrays).

Protein-expression profiling is the identification of proteins in a particular sample as a function of a particular state of the organism or cell (e.g., differentiation, developmental state, or disease state) or as a function of exposure to a drug, to a chemical or physical stimulus.

Protein-network mapping is the proteomics approach to determining how proteins interact with each other in living systems. Most proteins carry out their functions in close association with other proteins. These interactions determine the functions of protein functional networks, such as signal-transduction cascades and complex biosynthetic or degradation pathways.

Proteomics approaches have been used to identify components of multiprotein complexes, which are involved in point-to-point signal-transduction pathways in cells.

Because proteomics consists not only in proteins identification and quantification but also in the study of their structure, localization, post-translational modification, networks, activities and functions, the data generated by a wide range of technologies are large-scale and multi-dimensional (Carbonaro, 2004).

Mapping of protein modifications is the task of identifying how and where proteins are modified. Many common posttranslational modifications govern the targeting, structure, function, and turnover of proteins. In addition, many environmental chemicals, drugs, and endogenous chemicals give rise to reactive electrophiles that modify proteins.

Proteomics shows an enormous potential in clarifying the mechanism of complex diseases at a molecular level (Dunn, 2000).

Identification of new diagnostic markers, therapeutic drugs and vaccine targets is taking more and more advantage of proteomics.

Besides, application of proteomic techniques to food quality research has revealed great

performances in underlying differences in food proteomes relevant for human nutrition.

Due to its high power in food products characterization, the employment of proteomics in food quality evaluation is expected to increase during the next years (Carbonaro, 2004).

In recent years, research on food safety and quality in the agri-food chain, in a context of increasingly rigorous quality control and monitoring procedures, has become a priority topic. We assisted to an increasing interest to ensure food safety in all aspects of food production, from farmers to consumers, dealing with the development of an environmentally friendly food production, clarifying the link between food and health, monitoring health risks food-related. An integrated production chain control system should be capable to support food quality assurance, to identify and document raw materials and the history of consumable products, actions in food processing, to assess and monitoring manage risks.

Quality is defined as "the totality of characteristics of an entity that bears its ability to satisfy stated and implied needs" (Raspor, 2005).

In the context of food quality, the importance of traceability, defined as the ability to document movements, processes, controls needed to define a product's life history.

Proteomic techniques have been used for raw materials and final products assessment and also for the development of new processes in food technology and biotechnology.

Proteomics offers a new powerful tool to characterize food protein component. Proteomics not only reveals proteins expressed in each tissue type but it also allows the investigation of the different protein composition, varying the tissues.

Whereas genomics provides information on the genome of an organism, proteomics reveals which proteins are expressed in each tissue type in a defined time (Clerens et al., 2012).

In addition, it's capable to characterize the proteome of tissues, detecting changes in the protein component during growth, maturation, post-mortem, before and after harvest/slaughter, and to evaluate the effect of downstream treatments such as cooking or curing (Clerens et al., 2012).

Besides, proteomics meets the requirement of having more and more precise and suitable methods required by consumer protection and law enforcement and, in particular, for the detection of allergens in food.

1.5: *Proteomics applied to animal productions*

The greatest challenges of OMIC-Sciences applied to animal production are: to better understand the aetiology and pathogenesis of disease, to enhance animal welfare, to improve production and to enhance quality and safety food (Roncada et al., 2012).

Proteomic techniques offer a new very promising approach to identify protein in food matrix and to study protein-protein interactions in foods, as well as interactions between proteins and other food components (Carbonaro, 2004).

Food of animal origin, namely meat, seafood, milk and dairy products, the main protein sources for human diet, is a very complex food mixture containing different kind of proteins and other components. The current available proteomic techniques enable the simultaneous study of several hundred up to several thousand proteins (Gaso Sokac et al., 2011).

High resolution two-dimensional electrophoresis (Patterson & Aebersold, 1995) coupled with mass spectrometry is a powerful tool to analyze complex mixtures of several hundreds of proteins simultaneously (Mann & Hendrickson, 2001).

High performance liquid chromatography is another technique of choice for proteomic studies, due to its ability to separate and identify lower molecular mass molecules.

Multidimensional HPLC and tandem MS coupled on-line are systems able to separate and to identify low-abundance membrane proteins that would otherwise be impossible through 2DE analysis (Smith, 2002).

Just as an example, in the last decade milk, a complex bioactive biological fluid of great interest has been studied from a proteomic point of view and great efforts have been done to enhance milk proteome knowledge. Because of the complexity and multiplicity of milk components, in the years different techniques have been combined to explore milk from different points of view: genetic aspects, molecular pathways, and cellular functions involved in milk production, quality, and safety and the main aim has been to gain tools to better understanding this complexity. Currently, through the rapid evolution of the so-called highthroughput technologies, is possible to generate large-scale data on the DNA, RNA, and protein levels in milk.

In addition, sophisticated computational tools help to integrate this data set to enhance information and, in particular, in comparative biology when the complete genome of some farm animals is not completely sequenced.

Milk is a complex body fluid useful for all newborn mammals because it contains many secreted proteins with different functions: nutrients, antimicrobials, cytokines and chemokines that contribute to post-partum environmental challenges such as infections (D'Alessandro et al., 2010; Lönnerdal, 2010).

Besides, milk is a high biological value resource that could be transformed into cheese and other dairy products of economic relevance for the dairy industry.

Mammalian proteomic milk is highly dynamic and it changes radically with time after birth from colostrum to mature lactation (Roncada et al., 2013).

Milk proteins have a wide range of functions: carriers of minerals, vitamins and other compounds, stabilisers of large aggregates or micelles of lipids and components of the immune defence system. Immune and innate defence proteins appear in milk during mammalian gland infections (Roncada et al., 2013).

Characterisation of bioactive milk components is a matter of enormous interest. The host defence proteins in milk and colostrum, for example, have the potential to add significant value to the dairy industry and many different techniques have been used to fractionate and analyse them using proteomic tools (Smolenski et al., 2007; Stelwagen et al., 2009).

Some of the most abundant proteins play a direct role in healthy growth and development of bones and other tissues, gastrointestinal tract, nervous system and blood functionality.

A number of milk proteins are involved in DNA repair activation, DNA replication and transcription (D'Alessandro et al., 2010).

Besides, milk proteins carry many post-translational modifications that promote the stability of secreted proteins (Roncada et al., 2013).

Recently, proteomic milk studies have been directed to the characterisation of the minor protein components, with a wide range of bioactive roles, such as control of inflammatory response and autoimmune disease (Kilara & Panyam, 2003; Madureira et al., 2010).

While the major protein components of both human and bovine milk have been biochemically characterized two decades ago (O'Donnell et al., 2004), proteomic analysis of the less abundant milk proteins have only just recently been reported for bovine (Bianchi et al., 2009; D'Amato et al., 2009; Reinhardt & Lippolis, 2006; Wu et al., 2010) and swine milk (Wu et al., 2010). Since 1982, when the investigation of milk through two-dimensional gel electrophoresis (Anderson et al., 1982) started, important progress has been made: PTMs characterization (for instance, glycosylation and phosphorylation); identification of protein profiles variations depending on the mammalian species or on the lactation period; detection

and identification of new proteins (such as the ones present in milk fat globule membrane). However, these last years a significant increase in the identification of the low-abundant milk proteins has been observed. These findings are useful for the characterization and understanding of lactation pathways and mechanisms and give also fundamental information on the biological activity and functionality of these important proteins.

An important task of proteomics is the investigation of major proteins, including caseins (CNs) (αs1-, α-, ß- and κ-casein) and whey proteins (ß-lactoglobulin, α-lactalbumin, bovine serum albumin). Caseins polymorphisms are key characteristics to be specifically considered in the cheese-manufacturing industry. Milk proteins are characterized by a great heterogeneity because of the presence of several isoforms in different ruminants.

Proteomics is, in particular, useful for finding different genetic variants, changes in the phosphorylation or glycosylation pattern and other PTMs. Moreover, milk contains a high number of interesting low abundance proteins, such as lactoferrin, immunoglobulins, glycoproteins, hormones and enzymes (Fox & Kelly, 2003). Milk and meat are major sources of protein incoming in the human diet

In fermented food of animal origin such as meat, milk and dairy products, proteomic changes of the substrate and in the starter microorganisms and their interaction play a fundamental role in the quality of the final products (Gagnaire et al., 2009; Johnson & Lucey, 2006; Dambrouck et al., 2003).

Proteomic studies have been aimed to detect and characterise protein markers suitable for assessing the development of meat tenderness during the *post-mortem* storage (Jia et al., 2009; Lametsch et al., 2003).

Proteomics techniques, in fact, can also be useful in studying changes in meat quality associated with post mortem aging and those induced by the interaction of muscle proteins with lipids, carbohydrates and other meat components (Carbonaro, 2004).

Bjarnadóttir et al. (2010) examined the insoluble protein fraction of meat (*longissimus thoracis*) from Norwegian Red cattle during the first foty-eight hour post-mortem period using two-dimensional gel electrophoresis and mass-spectrometry (MALDI-TOF MS/MS) and noted significant changes in thirty-five proteins related to three different predicted functions: metabolism, cellular defence/stress (proteins related mainly to the regulation and stabilisation of myofibrillar proteins) and structural (Clerens et al., 2012).

In particular, proteomic studies have shown that the reduced meat quality post-mortem seems to be related to post-mortem antioxidant and repair capacities, proteolysis and protein solubility (Laville et al., 2009).

Besides, proteomics is a powerful tool for identifying specific gene products involved in meat quality adulteration. For this reason, proteomic methods are considered key tools to identify meat origin. Due to the great economic importance of these products, most proteomic investigations have been done on beef (Han & Wang, 2008; Hollung et al., 2007; Cagnazzo et al., 2006) and pork meat (Hollung et al., 2007; Van der Wiel et al., 2007; Murgiano et al., 2010).

Murgiano et al. (2010) and Hollung et al. (2009) investigated the proteome in meat samples originating from pigs of different breeds.

In this study, protein extraction was performed and proteins were separated by two-dimensional gel electrophoresis and identified by mass spectrometry.

Two-dimensional gel electrophoresis, coupled with mass spectrometry, allows also the identification and characterization of marker proteins and enzymes expressed only in certain individual animals or produced under different conditions (Carbonaro M., 2004).

Besides, identification of either genes for allergic diseases or allergenic proteins is being successfully carried out (Beyer et al., 2002; Toda & Onos, 2002; Yu et al., 2003).

Today, food safety represents a challenge and robust analytical methods are needed to detect harmful compounds or organisms that might be present in meal at very low amount.

In this context, the importance of the use of mass spectrometry coupled to other analytical techniques, mainly separation methods that enable the simultaneous and sensitive quantification of different groups of compounds in different food matrices.

Based on the so-called bottom-up approach, two-dimensional gel electrophoresis, followed with image analysis and mass spectrometry or different variants of liquid chromatography coupled with mass spectrometry, is the most used analytical methodology used to study differentially expressed proteins in genetically modified organisms.

In addition, biomarkers-based proteomics is important for early diagnostics in veterinary medicine.

Biomarker studies on farm animals are needed to monitor animal health and welfare, state of disease, quality and safety of animal products (Bendixen et al., 2011).

Proteomics techniques have been applied to study bovine mastitis and to identify early biomarkers, investigating also proteome changes in mastitis milk (Danielsen et al., 2010; Hogarth, 2004; Smolenski et al., 2007) and plasma (Yang et al., 2009).

1.6: *Foodomics: Present and future challenges in food analysis.*

The development and application of analytical methods and techniques in food science has grown together with the consumers concerns about food content and food safety.

Ensuring safety, quality and traceability of food require the best modern analytical technologies able to offer better results in terms of increased sensitivity, precision, specificity, and/or time needed for the analysis.

The general trend in food science is to link food and health, in the light of a revised concept of food.

In fact, food is no more considered only a source of energy but also a way to prevent diseases (García-Cañas et al., 2012).

One analytical challenge that still remains in food safety is to present reliable results with respect to official guidelines as fast as possible and without impairing recovery, accuracy, sensitivity, selectivity and specificity (LeDoux, 2011).

More suitable analytical techniques are required by consumer protection and law enforcement and, in particular, for the detection of allergens in food. The importance of food allergy in food analysis is related to the critical consequence that can verify with low amounts of allergens in sensitized people.

Modern research in food science and nutrition is moving from classical methodologies to advanced analytical strategies in which MS-based techniques play a fundamental role (Herrero et al., 2012).

In recent years, the determination of allergenic proteins by liquid chromatography (LC) and mass spectrometry (MS) has greatly advanced and is routinely used for food allergens identification and quantization. (García-Cañas et al., 2012).

Food safety is today a challenging field in which modern analytical chemistry provide accurate, precise and robust methods to determine any harmful compounds of organism that might be present in food at very low concentrations.

The evolution of mass spectrometry and the application of Foodomics technologies improve the limits demanded by food safety legislation.

Proteomics techniques have been applied to assess food quality because protein profiling can give useful information on food composition, origin or adulteration (Carbonaro, 2004).

Proteomics profiling of wine reveals wine proteins considered very important to wine quality due to their capability to affect significant characteristics like taste, clarity and stability.

To give an example, an LC-MS/MS method was used (Kwon, 2004) to better know protein content of wine and the possible functions of the proteins present in wine.

This kind of applications might open new strategies and possibilities to detect any adulterations related to grape variety.

Protein profiling with mass spectrometry, in fact, can search for biomarkers able to characterize food samples according to their origin.

In organic wheat (Zorb et al., 2009), fish (Mazzeo et al., 2008) and shrimp (Ortea et al., 2009), precise biomarkers were found.

In another interesting research, Wang et al. 2009 developed a rapid method for the fingerprinting and barcoding of honey proteins with a MALDI-TOF mass spectrometer.

In this case, starting from the information collected by mass spectrometry, protein fingerprints were generated and translated in a database library of spectral barcodes.

Another example of proteomic application to food products with the aim to detect any adulteration is the development of an untargeted LC-QTOF-MS method for proteins analysis in skimmed milk powder (Cordewener et al., 2009), where can be possible to find a partial adulteration with soy or pea proteins.

1.7: *Proteomic Methodologies: Steps Of A Gel-Based Proteomic Analysis*

The phases of a standard proteomic study gel-based involve different steps and methodologies: samples preparation and solubilisation, protein separation (two-dimensional electrophoresis), proteins detection on two-dimensional maps by staining procedures, image acquisition, computer analysis of bidimensional maps images (gel matching and statistical analysis of the data), spot picking, proteins in-gel digestion, proteins identification from gel spots (N-terminal microsequencing, Mass Spectrometry-MALDI-TOF and Ms/Ms) and, finally, data processing (data searching and data mining).

Two-dimensional electrophoresis (2-DE) is the only technique that allows to obtain an expression profile of relative quantity of protein mixtures in parallel.

Trough 2-DE is possible to separate, in the same experiment, thousands of proteins.

This technique allows us to highlight any change in their level of abundance and identify specific protein isoforms and post-translational modifications that can't be predicted from the gene sequence.

However, a proteomic study is very complex due to the presence of proteins modifications (e.g., differential splicing and post-translational modifications), that increase terribly the number of proteins. As a consequence, is often necessary to reduce proteome complexity, simplifying the proteome at different levels of analysis, using high resolution separation techniques and through fractionation methods. As a result, many sub-proteomes are obtained and each sub-proteome is characterized by a defined pattern of proteins that share the same cellular localization.

1.7.1: Sample Preparation

Sample preparation is the most important and critical step in a proteomic analysis because its accuracy can affect the success of the entire experiment.

It doesn't exist a universal procedure to follow, due to the different nature of the samples examined. Each sample considered has its own nature and characteristics and, as a consequence, it's impossible to find a protocol valid for all the biological samples. To give an example, membrane proteins are hydrophobic and, then, are undetectable with the conventional two-dimensional electrophoresis because they are very basic and they tend to localize at the end of the strip of polymeric support after the first dimension, when they migrate subjected to an electric field and in a immobilized pH gradient and are separated according to their isoelectric point.

In addition, hydrophobic proteins are insoluble to sodium dodecyl sulfate (SDS), anionic detergent used in the second dimension (when proteins migrate according to their molecular weight), which masks the intrinsic charge of the proteins, thus favouring the formation of anionic complexes.

For this reason, it's important to use different protocols according to the different nature of protein samples. Bibliographic data can be useful, e.g., to make a theoretical map of the proteins potentially present in a given cellular compartment and, thus, to establish the optimal pH range to use in the first dimension.

An optimal sample preparation involves not only the extraction of biological samples but also the elimination of substances that may interfere with the analytical technique used and proteins solubilisation, to allow the break of non-covalent interactions between proteins and between these and the interfering substances.

The purpose of protein solubilisation is to reduce all the proteins to their primary structure, to inhibit proteins modifications, to inactivate proteases, and, especially, to prevent proteins reaggregation, breaking their disulfide and hydrogen bonds and exposing all of the groups to the lysis buffer. Solubilisation buffers contain different basic components: denaturing agents, detergents, reducing agents and carrier ampholites.

An optimal solubilisation buffer generally contains a high concentration of denaturing agents, a zwitteronic detergent and a reducing agent, to provide an effective denaturant environment.

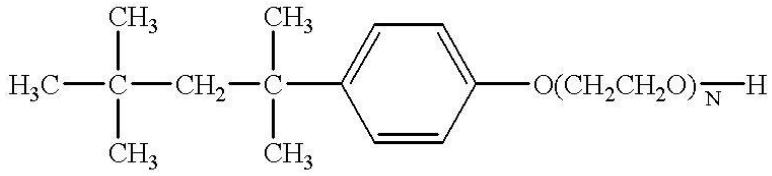
Urea is one of the most widely used denaturing agent, together with thiourea; it's a chaotropic agent and its function is to denature proteins, breaking *intra*- and *inter*- hydrogen bonds,

encouraging the unrolling in their primary structure and without interfering with the isoelectric focusing (urea is neutrally charged). The simplification of the proteins in their primary structure is a necessary requirement for making sure that each protein corresponds to a given isoelectric point.

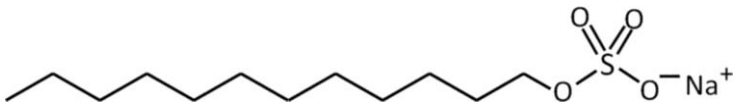
The detergents generally added to increase proteins solubility are the non-ionic detergents, such as Triton X-100 (Figure 1 (a)), or the zwitterionic surfactants such as CHAPS (Figure 1 (c)).

Figure 1: Chemical formula of the most common detergents used in the solubilisation solutions.

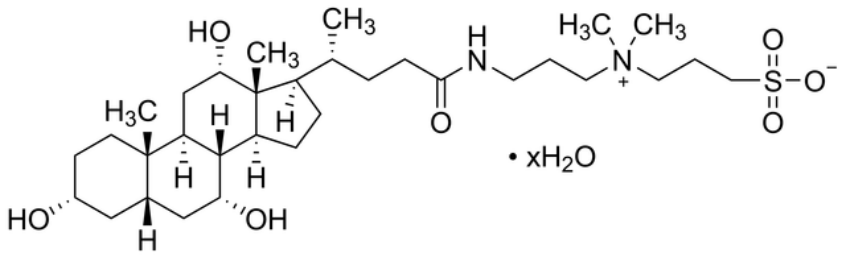
a) NON IONIC DETERGENTS: TRITON X-100



b) ANIONIC DETERGENTS: SDS



c) ZWITTERIONIC SURFACTANTS (CHAPS)



Another detergent commonly used in the solubilisation buffers is sodium dodecil sulphate (SDS) (Figure 1 (b)), an anionic detergent negatively charged able to bind to proteins.

Reducing agents, such as dithiothreitol (DTT) and dithioerythritol (DTE) and β-mercaptoethanol have the function to keep open the disulfide bridges, allowing the proteins to maintain their reduced state.

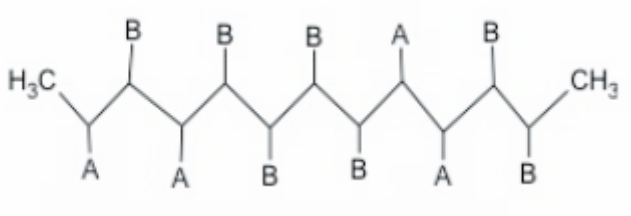
In addition to these compounds, proteases inhibitor can be added, to prevent polypeptides degradation by proteolytic enzymes.

Besides, carrier ampholites (Figure 2), complex mixtures of synthetic polyamino polycarboxylic acids highly soluble and amphoteric, are added to increase proteins solubility, to minimize proteins aggregation due to charge-charge interactions and to create a continuous pH range.

Many kinds of ampholites are commercially available and they differ in their pH range. At their isoelectric point the ampholites have their greatest buffering capability.

The choice of the ampholites depends on the pH range chosen for the isoelectric focusing (IEF), when proteins are separated according to their isoelectric point (pI) when an electric current is passed through the medium.

Figure 2: ampholites structure, where A is the acidic group and B is the basic group.



After protein solubilisation, samples should be quantified. This step is very important because many highly abundant proteins mask less abundant proteins and because a different amount of sample proteins is needed for sample loading according to the staining procedure to follow (each staining procedure has its sensitivity).

Accurate quantization of the samples to be analyzed is needed to ensure that an appropriate amount of protein is loaded and, in addition, it facilitates comparison between similar samples by allowing equal amounts of proteins to be loaded.

Accurate quantization of protein samples prepared for the following electrophoresis is, however, difficult because of the incompatibility of the reagents commonly used to prepare and solubilise samples (detergents, reductants, chaotropes and carrier ampholytes) with the common protein quantification assays.

Current spectrophotometric methods used for protein samples quantification rely either on Coomassie brilliant blue binding or protein-catalyzed reduction of cupric (Cu^{2+}) ion to cuprous (Cu^{+}). Dye-binding assays cannot be used in presence of reagents able to bind

Coomassie brilliant blue such as carrier ampholytes (Pharmalyte and IPG buffer) and detergents (CHAPS, SDS, and Triton X-100). Assays based on the reduction of cupric ions cannot be used together with DTT or other reductants or reagents such as thiourea or EDTA, able to form complexes with cupric ions.

Samples prepared for two-dimensional gel electrophoresis are usually difficult to quantify due to the possible presence of interfering carrier ampholytes, thiourea, detergents and reductants used in sample preparation.

For the accurate determination of protein concentration in samples prepared for two-dimensional gel electrophoresis 2-D Quant Kit is used to circumvent these limitations.

The procedure is mainly based on the combination of a precipitant and a co-precipitant to quantitatively precipitate sample proteins, leaving interfering substances (contaminants) in solution.

A pellet is formed by protein centrifugation and proteins are resuspended in an alkaline solution of cupric ions. The cupric ions present in the alkaline solution bind to the polypeptide backbonds of the proteins. A colorimetric reagent that reacts with the unbound cupric ions is, then, added. The resulting colour density that develops in the 2-D Quant Kit procedure is inversely related to protein concentration, with a linear response to protein in the range of 0-50 µg and a volume range of 1-50 µl. Protein concentration can be estimated by comparison to a standard curve obtained plotting the absorbance of the BSA standards against the quantity of protein (Figure 3).

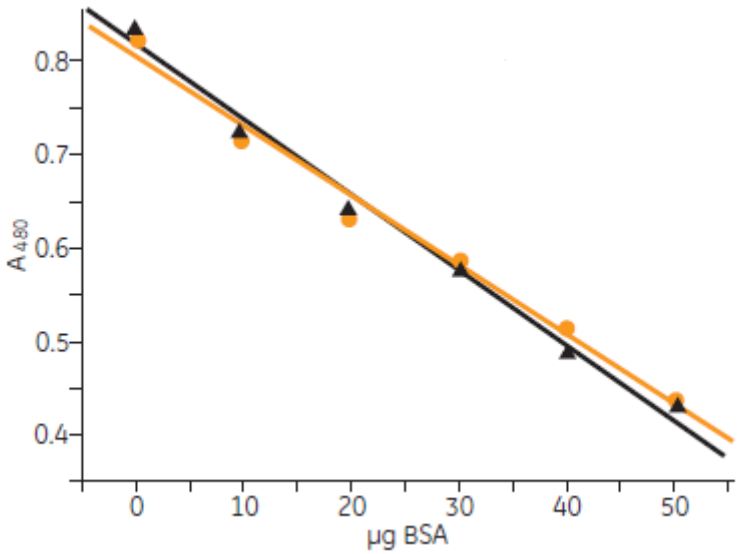


Figure 3: Standard curve with BSA dissolved in water (orange line) and in first-dimension sample solution (8 M urea, 4% CHAPS, 40 mM DTT, 2% Pharmalyte pH 3-10).

The optimal quantity of sample protein to load for electrophoresis depends on sample complexity, length and pH range of the Immobiline DryStrip gels and the different staining techniques of visualizing 2-D separation.

1.7.2: *Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) and Two-Dimensional Difference in Gel Electrophoresis (2D-DIGE)*

Two-dimensional polyacrylamide gel electrophoresis is the proteomic technique of choice because is the only technique able to separate thousands of proteins simultaneously (Görg et al., 2004).

The understanding of proteins network and quantitative variations depends on the ability to analyze many proteins at the same time and on the ability to quantify differences in the protein profile (Marcus et al., 2009).

In two-dimensional electrophoresis proteins are separated in two consecutive steps, corresponding to the I dimension, called isoelectric focusing (IEF), and the II dimension, called sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), taking advantage of two different properties of proteins: isoelectric point and molecular weight, respectively.

Each spot on the two-dimensional gel potentially corresponds to a single protein species in the sample. As a consequence, thousands of different proteins can be separated at the same time and information such as protein isoelectric point, apparent molecular weight and protein amount can be obtained.

At the beginning, the original technique used in the first dimension step carrier ampholytes containing polyacrylamide gels cast in narrow tubes to create a pH gradient (O'Farrel, 1975). In the first years, it was recognized the power of the two-dimensional gel electrophoresis as a biochemical separation technique but the technique wasn't successful, due to its low reproducibility, which made impossible to compare analysis of different laboratories, and because of its low resolution (an optimal resolution was obtained only for low amounts of proteins).

The technique was improved later with the introduction of Immobilines, that allowed to obtain an immobilized pH gradient inside the polymeric gel used in the first dimension or step of isoelectric focusing (IEF), also known as electrofocusing.

Later, the introduction of the Immobilized pH Gradient (IPG), polymeric supports in an immobilized pH range was used (Bjellqvist et al.,1982) and it was possible to obtain a stable pH gradient, polymerized together with the acrylamide matrix up a pH value of twelve (Görg et al., 2000). The introduction of immobilized pH gradients in the first dimension of

bidimensional electrophoresis (IEF) has made possible the achievement of superior resolution and reproducibility to the first dimension.

The high reproducibility of the results obtained in different laboratories made possible to compare gels, making electrophoretic reference maps.

Based on this concept, Angelika Görg and collegues developed the current used 2-D technique, where carrier ampholyte-generated pH gradients have been replaced with gel strips.

Two-dimensional difference in gel electrophoresis (2-D DIGE), first described in 1997 by Ünlü M. et al. 1997, is based on two-dimensional electrophoresis. Combining fluorescence, sample multiplexing and image analysis, it offers significant benefits over classical SDS PAGE because is an useful method to control system variations, allowing any biological variation and protein expression changes to be identified with statistical confidence. Multiplexing, up to three labeled protein samples can be run on the same 2-D gel in contemporary.

The multiplexing capability of 2-D DIGE, due to the use of CyDye DIGE Fluor dyes, enables the incorporation of the same internal standard (pooling equal amounts of all the samples in the experiment mixed together) on every gel, creating an intrinsic link across the gels and separating gel-to-gel variations (gel distortions, sample application variations and user-to-user variations) from biological variations. Besides, increased accuracy and reproducibility are reached. Due to the DIGE system, even small differences in protein expression levels can be determined, comparing the ratio from one fluorescent-labeled sample with another one. As a consequence, is possible to see less than the ten percent of the differences in protein expression between samples, with plus than the ninetyfive percent of statistical confidence. The automation of steps such as image analysis, spot picking, spot digestion and sample preparation for mass spectrometry measurements, have hallowed a significant increase in the throughput of protein analysis and identification.

Besides, due to the development of new mass spectrometry techniques, it’s possible to identify and characterize in a fast way very small amounts of peptides and proteins and rapid identification of genes encoding protein separated by two-dimensional gel electrophoresis is now possible thanks to the availability of genome sequencing data.

Two dimensional gel electrophoresis is also unique for its ability to detect post- and co-translational modifications, which cannot be predicted from genome sequences.

1.7.2.1: First Dimension Isoelectric Focusing (IEF)

Isoelectric focusing is an electrophoretic method used to separate proteins according to their isoelectric points (pI), the specific pH at which the net charge of the proteins (the sum of all the negative and the positive charges of amino acid chains and amino- and carboxyl- termini in the proteins) is zero. Proteins are positively charged at pH values below their isoelectric point and are negatively charged at pH values above their isoelectric point. If we plot protein net charge versus the pH of the surrounding environment, the obtained curve intersects the x-axis at the isoelectric point of the protein. A protein positively charged will migrate toward the cathode, becoming progressively less positive when it moves through the pH gradient until it reaches its isoelectric point. A protein negatively charged will migrate toward the anode, becoming progressively less negative until it also reaches a zero net charge. The presence of a pH gradient is critical to the isoelectric focusing technique (IEF).

Under the influence of an electric field and in presence of a pH gradient, proteins move through the pH gradient until they reach their isoelectric point.

The focusing effect of IEF concentrates proteins at their isoelectric point values and allows proteins separation on the basis of very small charge differences.

The original method for first-dimension IEF depended on carrier-ampholytes-generated pH gradients. Carrier ampholytes are small, soluble and amphoteric molecules with an high buffering capacity near their isoelectric point value.

In presence of an electric field, the carrier ampholytes that have the highest isoelectric point and the most negative charge move toward the anode, while the ones with the lowest isoelectric point and the most positive charge move toward the cathode. The other carrier ampholytes are distributed between the extremes, according to their isoelectric points. As a consequence, a continuous pH gradient is formed. To circumvent limitations associated with carrier ampholytes pH gradient, immobilized pH gradients (IPG) were developed. Görg et al. (2000) pioneered the development and use of IPG IEF for the first-dimension of 2-D electrophoresis.

An immobilized pH gradient is created adding to a polyacrylamide gel, at the same time it is cast, Immobilines, molecules with acidic or basic buffering groups linked to an acrylamide monomer and creating, thus, a pH gradient.

Immobilized pH gradients are created using two different solutions, containing both acrylamide monomers and catalysts but with differences. One of the two solutions contains a

relatively acidic mixture of acrylamido buffers and the other one contains a relatively basic buffers mixture.

During polymerization, the acrylamide portion of the buffers copolymerizes with acrylamide and bisacrylamide, forming a polyacrilamide gel.

The Immobiline DryStrip gels are cast onto a backing made of plastic (GelBond^{TM} PAGfilm). The gel is, thus, dried and cut into 3-mm-wide strips. The DryStrip gels obtained can be rehydrated through a rehydration solution.

First dimension Isoelectric focusing (IEF) is performed with Immobiline DryStrip gels, using a flatbed unit, such as IPGphor3, that allows a precise cooling, required during the isoelectric focusing. This is achieved using aluminium oxide ceramic Strip Holder or Manifold together with a Peltier temperature controlled system.

Using Immobiline DryStrip gels, it's possible to obtain bidimensional maps with superior reproducibility (the fixed pH gradient cannot drift) and resolution, in comparison with the previous tube gels using carrier ampholytes-generated pH gradient.

Through IPG technology the pH range on any Immobiline Dry Strip gel is increased and, thus, more very acidic and basic proteins can be separated.

Immobiline DryStrip gels allow an effective isoelectric focusing from very acidic proteins at pH 3 to very basic proteins at pH 11.

In addition, Immobiline DryStrip gels include a higher protein loading capacity.

Immobiline DryStrip gels are available with five different strip lengths (7, 11, 13, 18, 24 cm). The choise of the type depends on sample proteins to be loaded and on the sample quantity to be loaded.

Shorter strips (up to 13 cm) are used when the most abundant proteins are of highest interest, while the shortest IPG strips are used when sample load is limited and longer strips (i.e. 18 and 24 cm) are used for maximal resolution and loading capacity. Longer strips allow the detection of more spots but are very time-consuming in first- and second- dimension separations.

The different pH intervals allow to increase first dimension loading and to resolve crowded spots areas. The immobilized pH gradients ensure high reproducibility and a reliable comparison between the gels obtained. Immobiline DryStrip gels must be rehydrated prior to IEF.

Together with Immobiline DryStrip gels, it is possible to apply the sample directly. Alternately, protein sample can be applied by including it in the rehydration solution during

the rehydration loading. The rehydration loading allows to load and separate larger sample volumes (greater than 100 µl), larger sample amounts and diluted samples.

There are two general rehydration methods: a passive rehydration, without electric field and an active rehydration, under a voltage of 20-120 V, that facilitates the entry of high molecular weight proteins into the gel strips.

Immobiline DryStrip gels are rehydrated with a solution that generally contains urea, that solubilizes and denatures proteins; zwitterionic detergents, that solubilize hydrophobic proteins and minimize protein aggregation; DeStreak Reagent, that reduces the problem of streaking and extra spots due to proteins oxidation or DTT, a reducing agent; IPG Buffer, that improves protein separations; and a tracking dye (Bromophenol Blue), that allows to monitor IEF progress during the protocol; or with DeStreak Rehydration Solution, a ready-to-use solution containing urea, thiourea that, in addition to urea, further improve protein solubilization and CHAPS, a nondenaturing zwitterionic solubilizing detergent used for membrane proteins and the appropriate IPG Buffer.

DeStreak Reagent and DeStreak Rehydration Solution transfer thiol groups in proteins to stable disulfide groups preventing, thus, nonspecific oxidation during two-dimensional electrophoresis and reducing horizontal streaking and extra spots.

Isoelectric focusing is performed at high voltage (up to 10000 V, depending on immobiline DryStrips lengths) and very low currents (less than 50 µA per Immobiline DryStrip gel to avoid any damage of the immobiline DryStrip gels) due to the low ionic strength within DryStrip gels.

During protein migration to their equilibrium positions, the current decreases while the voltage increases gradually reaching the final desidered focusing voltage.

The low initial voltage minimizes sample aggregation and allows obtaining the parallel separation of samples with different salt concentration.

1.7.2.2: Immobiline DryStrip Equilibration

After IEF, the Immobiline DryStrip gels are equilibrated in equilibration solution (the SDS buffer required for the second-dimension separation) and applied on second dimension SDS-polyacrylamide gels for second dimension separation.

After IEF Immobiline DryStrip it is important to proceed immediately to DryStrip gels equilibration prior to second dimension run; alternatively, strip gels can be stored at -60°C or below for future proteomic analysis.

The equilibration solution contains Equilibration buffer that maintains DryStrip gels in an appropriate pH range; urea that together with glycerol reduces the effects of electroendosmosis due to fixed charges on the DryStrip gels in the electric field and can interfere with protein transfer from DryStrip gels to second-dimension gels; glycerol; DTT, reductant agent that preserves denatured unalkylated proteins in their reduced state; SDS, that denatures proteins, forming protein-SDS complexes negatively charged; iodoacetamide, that alkylates thiol groups on proteins preventing streaking; a tracking dye (bromophenol blue), that allows to monitor the electrophoresis.

Iodoacetamide alkylates residual DTT to prevent streaking and other artefacts. It is introduced in the second equilibration step to minimize unwanted reactions of cysteines.

1.7.2.3: Second Dimension or SDS-PAGE (PolyAcrylamide Gel Electrophoresis)

After IEF, the second-dimension SDS-polyacrilamide gel electrophoresis can be performed. SDS-PAGE is an electrophoretic method used to separate polypeptides according to their molecular weights. Polyacrilamide gels containing sodium dodecyl sulfate are used. SDS and proteins form complexes where SDS masks protein charge. A reducing agent such as DTT is also added to break the disulfide bonds present in the protein.

Denatured and negatively charged proteins (proteins complexed with SDS) are subjected to an electric field and will migrate towards the anode and according to their molecular masses through a "sieve effect" that the porosity of polyacrilamide gel creates. The recommended acrylamide percentage to be used in gel polymerization is related to proteins molecular weigth (Table 1).

RECOMMENDED ACRYLAMIDE PERCENTAGE	**PROTEIN MOLECULAR WEIGTH**
8 %	40-200 kDa
10 %	21-100 kDa
12 %	10-40 kDa

Table 1: recommended acrylamide percentage for gel polymerization and related protein molecular weigths

Increasing acrylamide percentage, smaller pores of SDS-PAGE gel will be obtained and, as a consequence, a better resolution of low molecular weight proteins will be reached. The migration speed depends on the electric field value, the total protein net charge. The degree of electrophoretic separation within the polyacrilamide gel depends largely on protein molecular weight and there's a linear relationship between the logarithm of the molecular weight and the relative distance of migration of SDS-protein complexes.

The most common buffer system used in second-dimension is the Tris-glycine system of Laemmli, that separates proteins at high pH values, with minimal protein aggregation and with a clear protein separation. A temperature control improves gel-to-gel reproducibility.

1.7.3.: Bioinformatics Applied To Proteomics

Proteomics has known an unparalleled growth, reflected by the amount of data generated from the experiments.

Proteomics analyses, indeed, generate an enormous number of data, expression information for thousands of proteins. As a consequence, bioinformatics is an essential part of data analysis, with special tools. The role of bioinformatics is to reduce the analysis time and to allow statistically significant results, in terms of high-throughput and statistical accuracy.Genomics and proteomics studies provide the platform for functional analyses (analyses of metabolism, protein-protein interactions etc).

Proteomics is considered the protein complement of the genome and involves several technologies to analyze all the proteins in a given sample (Aebersold & Mann, 2003; Pandey & Mann, 2000). Various questions concerning the proteins are addressed. What are the proteins contained in a biological sample? What is their concentration? Where are the proteins localized? How do protein expression levels alter in different compared samples? What are proteins posttranslational modifications (PTMs)? How proteins interact with other proteins or molecules (Gavin et al., 2006; Uetz et al., 2000)? The following questions concentrates on computational proteomics.

The rapid development of the proteomic techniques and the automation of the spot analysis by mass spectrometry requires faster bioinformatics tools to better analyze the data produced, for gel matching and spot identification.

A typical proteomic analysis involves the study of complex samples with many different proteins at varying concentrations (Rose et al., 2004). This complexity is one of the main problem for the currently available technology for identifying proteins from biological samples and, as a consequence, the majority of the low-abundance proteins are not observed. To circumvent this problem, a dominant proteomic technique is referred to as the ‘‘bottom-up’’ approach, in which proteins are digested into peptides by a proteolytic enzyme. The obtained peptides (and subsequently the proteins) are analyzed and identified by mass spectrometry (MS). The obtained peptides from proteins digestion are often a highly complex mixture, and a degree of separation can be achieved by peptide liquid chromatography (LC) prior to mass spectrometry.

Data elaboration for functional proteome

Functional proteome (May et al., 2011) is related to the identification of differentially expressed proteins comparing different samples and to the detection of proteins relative quantification.

Specific software are able to overlap gels scanned images and to detect the differentially expressed proteins.

In addition, recently high innovative shutgun technology based on high resolution liquid chromatography coupled to mass spectrometry have been developed and employed in the field of functional proteomics.

Peptide Mass Fingerprinting

Bidimensional electrophoresys (2-DE) produces numerous protein spots and it's possible to enzymatically digest proteins in situ and measures the obtained peptide masses by mass spectrometry (MS) technique. As a result, the mass spectrum obtained is signal-processed and a list of experimental peptide masses, referred to as the experimental spectrum, is generated. The data generated can be searched against an available protein database by comparing each protein sequence with the experimental peptide mass list. This comparison requires the computation of a theoretical mass spectrum by digesting the protein sequence "in silico" and calculating, thus, the theoretical peptide masses. A score is used to measure the correlation between experimental and theoretical data with highest-scoring sequence is assumed to be correct (Pappin et al., 1993; Magnin et al., 2004; Zhang et al., 2000). Peptide Mass Fingerprinting (PMF) concept clearly introduces the principle of mass spectrometry data identification by database searching. Nevertheless, when searching large databases, or when the number of available peptides is limited, the risk of false positive identification is high. In addition, the presence of modified (PTMs) or incompletely cleaved peptides contributes to reduces PMF data specificity.

Mass spectrometry provides additional information on each peptide and would be a marked improvement over peptide mass fingerprinting.

Peak Detection.

The software to extract a mass list from an experimental spectrum, usually provided by the mass spectrometer manufacturer, is essential in mass spectrometry data identification. Algorithm performance and quality of the data produced play an important role in both database searching and de novo sequencing.

Tandem Mass Spectrometry

From the data processing point of view, tandem mass spectrometry (MS/MS) can be considered as an additional level to mass fingerprinting. The set of fragment masses constitutes specific data.

By taking advantage of such peptide-specific mass sets, it is possible to identify the peptides. Given a peptide sequence, it is possible to compare theoretical and experimental MS/MS spectra during a database search. Protein identification can be obtained by mapping the observed peptides onto the protein sequences.

The ability to identify single peptides enables the analysis of complex peptide mixtures, as the peptides can be readily separated by liquid chromatography and the liquid phase containing the peptides is continuously introduced and ionized in the source of the mass spectrometer. The mass spectrometer, thus, ‘‘scans’’ the fluid for peptides by alternating between MS and MS/MS acquisitions. Peptide masses are acquired in MS mode, and the most intense peaks are selected for fragmentation in MS/MS mode. The instrument then returns to MS mode, and the alternating cycle continues.

In shotgun proteomics, protein separation is not performed and the entire sample is digested throught cleavage with proteases and analysed by multidimensional peptide liquid chromatography.

MS/MS Scoring Functions

The comparison of theoretical and experimental MS/MS spectra is performed by a scoring function, where the score is used to recognize the correct peptide from a database. The most intuitive notion of score is provided by the number of masses shared by experimental and theoretical spectra, named shared peak count (SPC).

In this case, all matched masses are weighted identically, although some are more informative than other masses.

Modified Peptides

It is possible that some aminoacids are modified (post translational modifications, chemical modifications), and the result is a mass shift. Such changes in mass are to be taken into account for a correct computation of theoretical MS/MS spectra. The simplest cases are fixed modifications, e.g., carboxyamidomethyl cysteine (þ57.02146 Da). All cysteine residues in a protein are reduced (i.e., protein disulfide bonds are broken) and the nominal amino acid mass is replaced by a shifted mass in all computations.

Post translational modification (PTM) detection and quantization is one of the most difficult challenge in proteomics. Dedicated bioinformatics tools have been performed to characterize phosforilation sites, considering peptides fragmentation spectra.

The MS/MS spectra of the theoretical modified peptides are first calculated and, thus, matched with the experimental ones. In this case, the similarity score is considered to identify peptides and the corresponding phosphorylation site.

Protein Identification

Protein identification have many troubles mainly caused by peptides shared by several proteins. When two or more sequences in the database have the same peptides, then it is impossible to identify with certainty which molecule(s) is(are) present in the sample. In such situation, to assign a score to a protein identification is a difficult question.

A standard procedure is to sum the highest score for each identified peptide.

A classical criterion used to accept a protein identification is to detect two distinct peptides above a reasonable peptide score (Cargile et al., 2004), with a very small number of generated false positive identifications. The choice of which protein database to use plays an important role in MS data identification.

NCBInr (http://www.ncbi.nlm.nih.gov) and Ensembl (Birney et al., 2004) are those most frequently used, whereas a commonly used curated database is UniProtKB/Swiss-Prot (Wu et al., 2006).

Peptide De Novo Sequencing

When the proteomic analysis is performed on a sample belonging to an organism whose genome is not completely sequenced (Shevchenko et al., 2001), it is necessary to predict peptide sequences directly (de novo peptide sequencing) and this is an obliged procedure when is no possible to search MS data against a protein sequence database because such a database is not available or is inappropriate.

New perspectives

Matching proteomic data with genome sequences is potentially possible to complement and correct genome annotations by mass spectrometry data.

The most challenging case is to search mass spectrometry data against a eukaryotic genome.

An alternative method is to use de novo sequencing predictions and to search the predicted sequences by homology. Besides, it is possible to combine gene structure predictions and MS data searches to reveal and validate splice sites (Colinge et al., 2005).

1.7.4.: Bioinformatic Tools For 2D-Gels Image Analysis

IMAGEMASTER 2D PLATINUM 7.0

Image Master offers flexible bioinformatic tools for visualization, exploration and analysis of 2-D gel data (Table 2). Gel images are displayed in sheets and panes.
It is possible to choose the actions to be performed during the gel image analysis.
From the menu it is possible to select spots, matches and annotations for detection and matching.

MENU	**DESCRIPTION**
FILE	Close, save, import, export, print and other basic operations.
EDIT	Undo/redo the last operations, show a history of operations, or edit (add, modify, delete) specific gels, spots, annotations or matches.
VIEW	Modify the settings for grid lines, profile or overview in the display, align images, show spot overlap, or change the way gels, spots, annotations or matches are visualized.
SELECT	Select specific spots. Spot sets, annotations or matches.
REPORTS	Display tabular or graphical information about selected gels, spots, annotations or matches, and compute differences and similarities between gel images. The data analysis is based on robust statistics, factor analysis and statistical tests.
TOOLS	Change display, quantification, and other options at the software level. Create and control a calibration tablet while working in the Image Pool.
HELP	Access documentation and obtain license, product, version or system information.

Table 2: Image Master tools and related description.

Sheets

Image Master is organized in sheets, were the sheets occupy the Display zone and each sheet has a tab with a name and an icon that represents the type: Image Pool, containing images for viewing and basic processing; Match Set, where is possible to carry out spot detection and matching; Class, for advanced expression analysis.

Panes

In each sheet there can be one or more panes, with named tabs. On the left side of each tab, one or more icons describe the match or class hierarchy.

Images

In the upper left corner of each image the gel name is displayed. The reference image for the matching has a name with a red corner. The current sheet reference has a darker green or darker gray image name than the other images. The images in the sheet are compared to the sheet reference.

Workspace

In Image Master software the workspace plays an important role. The workspace allows organizing the gels into projects, to specify the gels that are to be matched together and to define classes for statistical analysis. In the Workspace toolbar there are the commands to create new projects, add files to projects, remove, and backup and restore projects.

In the Workspace window there is the Workspace toolbar and the Navigator part, where are displayed files and folders to view (Image Pool) and to analyze (Projects). There's a hierarchical system of folders, subfolders and files. Images are opened through the Image Pool of the Navigator. To detect, match, carry out statistical analysis, the images to be analyzed must be transferred to a project. In the Navigator, each folder type has a specific icon (Image Pool, Project, Match, Class) and, in addition, images and match set folders have also icons that indicate the status. Through the Navigator icons is possible to distinguish DIGE gels from non-DIGE gels, know which gels have been detected and matched, and recognize gels used as references for matching.

Reports

Reports are practical for organizing and describing gel data and make much easier data processing (Table 3).

REPORT	DESCRIPTION
3D VIEW	A three-dimensional view of selected gel regions or areas around selected spots.
ANALYZE GEL	Information about each selected spot match such as its Match ID, value for each spot in the match, and chosen statistical measure calculated on all spots in the match. Scatter Plots also provide information that compare the spot values for two gels. The Match Statistics Table displays the number of matches and percentage of matches for each gel at the selected level of the match hierarchy. The DIGE Histogram displays the frequency distribution of the DIGE volume ratios.
ANALYZE CLASSES	Central tendency, dispersion, and overlapping measures for classes of gels computed for all selected matches. Differences between the spot values in several classes can also be quantified with Statistical Tests such as ANOVA, Mann-Whithney U test and Kolmogorov-Smirnov test.
GEL TABLE	Summarized information about the selected gels, such as Gel ID, file name and path, gel calibration data, gel resolution and size, number of detected spots etc.
SPOT TABLE	Specific information about selected spots such as Spot ID and coordinates, quantification values, attached labels etc.
ANNOTATION TABLE	Information about annotations, including the label content for each category, the annotation coordinates and Spot ID (if the annotation is linked to a spot).

Table 3: Image Master Reports and related description.

Data graphical representation as histograms, scatter plots and three-dimensional views are treated as reports. Each report is linked to its corresponding sheet.

All spots are represented in the reports but reports content can be limited to a subset of selected spots of interest.

The bidimensional gels must first be converted into an image file. During digitization, the gels are resolved into a two-dimensional matrix of squares, or pixels, where each pixel is characterized by its own coordinates and its signal intensity.

The scanning resolution of the gels plays a critical part because it influences the amount of details in the resulting images. The gel images are displayed in the Image Pool folder in the Workspace.

Gels remain in the Image Pool until they are added to a Project.

In the Image toolbar basic tools to process images are available and should be applied when it's necessary. Selected images can be rotated, flipped, cropped (new gels are created, with only the selected area).

It is possible to enter a gel description about the gel image, to be used to collect information such as sample type, gel running protocol, date of the experiment, name of the operator and other experiment details.

Gel descriptions can be displayed in a Gel Table. All gels, spots, matches, annotations, spots sets and other information are included in a project in the Workspace.

A project contains match hierarchies, each of which contains a match folder that describes how gels or gels populations (match set) should be matched together and a classes folder, where the biological question is stated.

Match hierarchies

Gels belonging to the same biological group are easier to match than images from different biological populations. As a consequence, is better to use hierarchical match structures to create more efficient match designs. The match hierarchy minimizes the number of difficult match combinations. Adopting hierarchical population matching instead of matching all images against a unique arbitrary reference image reduces time consuming on match editing. After defining one or two landmarks, the experiment is matched and matches are automatically propagated at each level of the match hierarchy.

Classes

A class for each set of gel is created. Comparing gel within the classes, we detect protein expression variations between different biological states.

1.7.5: Spot Detection Parameters

Image Master software offers the possibility to adjust spot detection parameters in real time.
The parameter **Smooth** fixes the number of times the software smooths the gel image before detecting spots, using a smooth-by-diffusion algorithm. The Smooth parameter is able to detect all real spots and split as many overlapping spots as possible while noise spots can be filtered out with the Saliency and Min Area parameters.
Saliency, is an efficient quantity for filtering spots based on the spot curvature and it indicates how far a spot stands out with respect to its environment. Real spots have high saliency values whereas artefacts and background noise have small saliency values. Although the Saliency is a measure for filtering spots, it is also highly dependent on images resolution and depth). For correct filtering it's possible to estimate the saliency range to use with gel images, looking at the saliency value given for a spot that we would like to suppress. The spot detection algorithm discards the spots with saliency values smaller than the specified threshold value.
After setting an appropriate Saliency value to filter out all noise spots, there may still be noise in the gels, due to dust particles. To cope with these artefacts it's possible to use the **Min Area** parameter that eliminates spots with a smaller area than the specified threshold (expressed in number of pixels).

SPOT QUANTIFICATION

Image Master computes in an automatic way the protein amount for each spot and, in such a way, the software is more robust and reproducible when calculating protein expression variation levels as relative quantification (Figure.4).

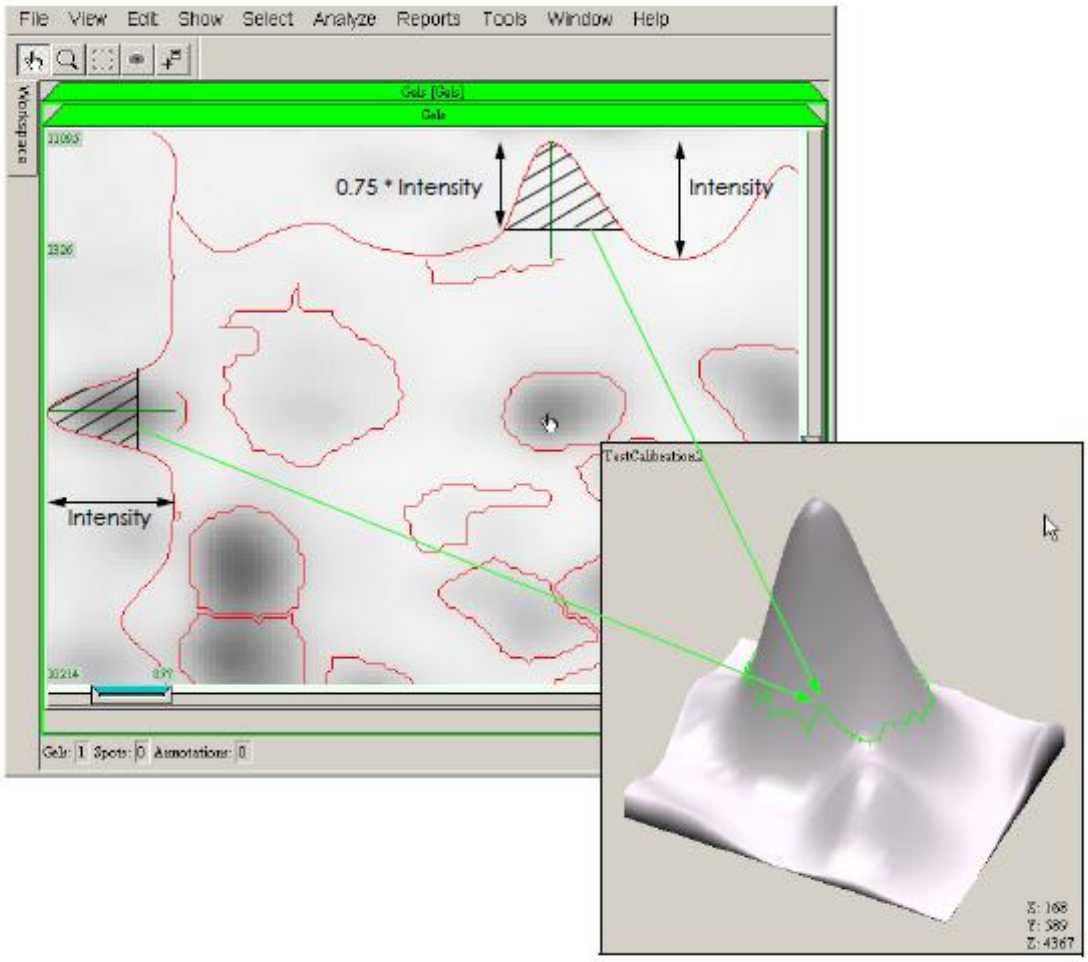


Figure 4: image taken from Image Master 2D platinum 7.0 – User manual.
The figure illustrates protein spot quantification performed by the software. The 3D View in the software reflects the spot shape and volume of what will effectively be quantified. The spot outline is the area at 75% of the spot height when measured from the spot peak.

Intensity

Spot intensity is calculated, based on the highest calibrated pixel intensities in the spot from which the background value (defined as the minimum pixel value in the area surrounding the spot) has been subtracted.

Area

To calculate spot area, the software computes the area at 75% of the spot intensity, as measured from the spot peak. The spot outlines in the display exactly surround the computed spot area expressed in mm^2.

Vol

Image Master calculates the spot volume as the volume above the spot outline situated at 75% of the spot height. The volume values depend on pixel intensity calibration.

%Vol

The relative spot volume is a normalized value that is relatively independent from protein loading and staining variations in the gels.

This value is calculated according to the following formula:

$$\%Vol = \frac{Vol}{\sum_{S=1}^{n} Vol_S} \times 100$$

where Vol_s is the volume of spot S in a gel containing n spots

SPOT CO-DETECTION ALGORITHM

The co-detection algorithm is designed to process one (single spot detection), two (double spot detection) or three images (triple spot detection) related to one gel at the same time.

Single spot detection is usually performed in the case of post-stained preparative gels for spot-picking, when a single image is associated with the gel; while, double and triple spot detection are used when 2D-DIGE analytical gels are made. In this second case, co-runned DIGE gels images are merged together to include all spots in a unique image. The following steps are spots detection and spot boundaries definition, with a resultant spot map that is, thus, overlaid back onto the original image file.

SPOT DETECTION PARAMETERS

During DIGE images detection, an estimation of the spot number is done. This value, as recommended, should be an overestimation in order to compensate the non detected protein spots.

SPOT QUANTIFICATION

In spot quantification, spots parameters such as volume, area, intensity, slope, and volume ratio for individual spots are calculated by the software.

Vol: spots volumes are calculated as sum of the pixels intensities within the spot boundaries, with background subtraction.

Vol ratio: Volume ratio values are calculated as ratio between the current image spot and the volume of DIGE reference image spot. The value obtained in this way is the change in spot volume comparing two different image gels.

SPOTS PROPAGATION

It is possible to propagate selected spots from one image to the other matched images to allow the quantification of identical spots areas in all gels.

For matched spots: the spot of the destination image is replaced with the spot of the source image.

For non-matched spots: the spot from the source image is copied to the equivalent location in the destination image and the position is calculated from the surrounding match vectors. Original and propagated spots are matched automatically.

GEL MATCHES

The software compares gel images to find matches between related spots that represent the same protein in the gels. Matches are propagated automatically at each level of the hierarchy.

LANDMARKS DEFINITION

When the gels are much distorted, gels matching is very difficult and the definition of few landmarks is needed. Landmarks can be defined as points that relate corresponding spots in the gels to be matched. Landmarks should only be defined on spots that clearly represent the same protein form.

As a general rule, it is good to validate one or two landmarks on all the images of hierarchy. As a consequence, the landmarks validated are useful to match all levels.

MATCHES EDITING

The software allows manually adding or deleting matches after the automatic matching procedure. All matches are created in a hierarchical manner. Spots may be properly matched in one match set level.

DATA ANALYSIS

It is possible to study protein expression variations among a series of gels that should be matched together, being part of the same match hierarchy. Data analysis can be performed at two different levels:

Analyze Gels: Study protein expression changes considering a set of gels. The analytical methods used include scatter plots, descriptive statistics, histograms, and factor analysis.

Analyze Classes: Find significant protein expression changes between classes of compared gels. This analytical methods used include descriptive statistics per class, histograms, overlapping measures, and statistical tests.

GEL ANALYSIS

To analyze similarities between matched gels or experimental changes due to different stain intensities, protein sample loading or image acquisition problems, it's possible to produce Scatter Plots (Figure 5).

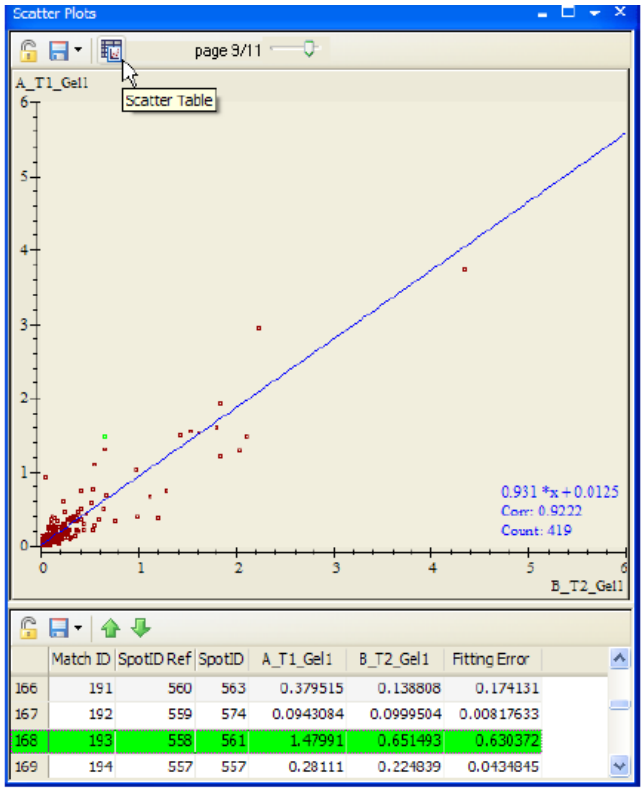


Figure 5: Example of Scatter plot, taken from Image Master 2D platinum 7.0 – User manual

Scatter plots are useful to have an idea of the relationship between the spot values from two gels by searching for the best-fit line through the data points, the linear dependence between the spot values of one gel (variable X) and the corresponding values in the sheet reference (variable Y).

The software offers the possibility to visualize a scatter plot for each gel in the sheet versus the sheet reference, together with the best-fit line, correlation coefficient and the number of matches displayed.

The software provides three statistical tests: ANOVA, Mann-Whitney U test, and the Kolmogorov-Smirnov test to analyze differences in protein expression between classes of gels.

The aim is to draw conclusions about the significance of the protein expression changes. It is possible to display the desired statistical values related to variations in protein expression between two samples populations for each match in the Class Analysis Table.

One-way ANOVA

Analysis of Variance (ANOVA) is one of the most important statistical tests available for biologists. When comparing two means, ANOVA test gives the same results as the t-test for independent samples.

One-way ANOVA tests the null hypothesis that all populations have the same means. It generates a P value. It is based on these assumptions:

• The samples are randomly selected from the larger populations.

• The two samples are independent, with no relationship.

• The data are sampled from populations that have an approximate Gaussian distribution

Mann-Whitney or Wilcoxon test

The Mann-Whitney U test is the non-parametric substitute for the two-sample t-test when the assumption of normality (Gaussian bell-shaped distribution) is not valid. It is equivalent to the Wilcoxon rank sum test. It should only be used for comparing two unpaired samples. The assumptions of the Mann-Whitney U test are:

• The variable of interest is continuous,
• The distributions of the two samples are identical,
• The two samples are independent.

Kolmogorov-Smirnov test

The Kolmogorov-Smirnov test tries to determine if two data sets differ in a significant way. It is used to test if two samples may come from the same distribution. The assumptions of the Kolmogorov-Smirnov test are:

• The probability distributions are continuous,
• The measurement scale is at least ordinal,
• The two samples are mutually independent.

DeCyder 2D 7.0

DeCyder™ 2D Software is automated image analysis software which allows detection, quantification, matching and analysis of two-dimensional DIGE gels, where complex protein mixtures are labelled with CyDye™ DIGE Fluor dyes. The multiplex labelling enables detection of small changes in protein levels as well as inclusion of an internal standard, a pool of all the samples (in equal amounts) within the experiment and therefore contains all proteins included in the experiment. By using an internal standard, gel-to-gel variation can be eliminated, quantification is accurate and it is possible to separate system variations (variations due to experimental conditions) from biological variations.
The software offers the possibility to perform three types of analysis, step by step:
Differential In-Gel Analysis (DIA); where protein spots are co-detected and quantified on a set of images from a 2D-DIGE experiment gel, Biological Variation Analysis (BVA); where multiple images from different gels are matched together to provide statistical data (univariate analysis) on differential protein expression levels between different experimental sample

groups, Extended Data Analysis (EDA); where multivariate analysis of protein expression data derived from Biological Variation Analysis is done.

From a statistical point of view, apart from univariate analysis, the software allows to perform PCA-analysis, pattern analysis, discriminant analysis and biological interpretations of the obtained results.

A Batch Processor allows the automation of the DIA and BVA module and it is recommended if many gels are to be analyzed. The processor detects spots, matches multiple gels, and performs statistics and filter proteins in an automated way.

Gel images must first be uploaded to the database through Image Loader tool before they can be analyzed. Image Loader automatically groups up to three images into one gel if the images are named with: a gel number, a description of the content in parentheses and the dye used.

The DIA (Differential In-Gel Analysis) is the initial step in a typical DeCyder analysis and performs spot co-detection and quantification (normalization and ratio calculation) and, in addition, it removes most of the system variations. A DIA workspace contains information from one gel.

It is possible to adjust in this step the contrast/brightness settings in case of image disturbances such as dirty glass plates or dark preparative gels, which might affect spot detection and gel matching.

The DIA module processes up to 3 gel images from a single gel, where each image is generated from samples labelled with different DIGE Cy-dyes fluors.

The DIA algorithm detects spots on a combined image derived from merging individual images from an in-gel set of images. This co-detection ensures that all spots are represented in all images. DIA quantitates spot protein abundance for each image and expresses these values as a ratio, thereby indicating changes in expression levels by direct comparison of corresponding spots.

During spot detection, it is recommended to select as estimated number of spots an overestimation of ten thousands spots to compensate for the detection of non-protein objects on the image, e.g. dust particles, that will be *excluded* from the analysis while it is recommended to select thirty thousand value for volume (recommended exclusion filter for normal quality gels), to filter spots.

The Biological Variation Analysis (BVA) processes multiple gel images, performs gels matching, multiple gels quantitative comparisons of protein expression. It is organized into four modes with different functions: **Spot map (S)**, with the function of setting up

experimental design; **Match (M)**, for gel-matching; **Protein (P),** to examine statistics of *all* proteins, **Appearance (A)**, that examines statistics of *one* protein. The spot maps in the workspace can be viewed and sorted in various ways.

When moving to Protein mode, the spot maps are by default sorted after experimental groups. Generally, landmarks are created, following few recommendations. It's better to select isolated, well-shaped (circular) spots and with a medium abundance.

The software allows using **Protein filter** to assign protein of interest. Recommended filter settings are useful to filter for differentially expressed proteins between two selected experimental groups. To give an example, the filter can be restricted to proteins which are present in at least 9 out of 12 spot maps (3 of 4 gels).

Extended Data Analysis (EDA) is used to perform multivariate analysis of protein expression levels data derived from the BVA module or the Batch Processor.

As an addition to univariate analysis, it is possible to perform PCA-analysis, pattern analysis, discriminant analysis and biological interpretations of the results. The first step after creating the EDA workspace is to create a base set, further analyzed with different calculation methods, resulting in new sets of data.

The software analyzes the base set to find differentially expressed proteins. A set with significantly differentially expressed proteins will be created. Principal Component Analysis and Hierarchical Clustering can be performed on the new set.

Bruker Protein Scape

Protein Scape is a bioinformatics platform to process and organize data for large proteomic projects. It integrates LC-data, gel data, mass spectrometry data, search results and quantification results. The platform comprises sophisticated tools for the analysis and evaluation of proteomic data, with data evaluation tools for mass-spectrometry based protein identification, characterization, and quantization.

Proteins Identification

ProteinScape identifies proteins through peptide mass spectra from protein digests (Figure 6). Peptide mass spectra are analyzed, assigned a sequence, and protein databases are used to search the peptide sequences of the candidate proteins. The higher the scores of the peptide sequences identified, the higher the confidence in the identification.

Identifying Glycans

ProteinScape is also able in identifying glycans in glycoproteins. It analyzes mass spectra for the presence of characteristic patterns and fragments that correspond to entries in glycan databases.

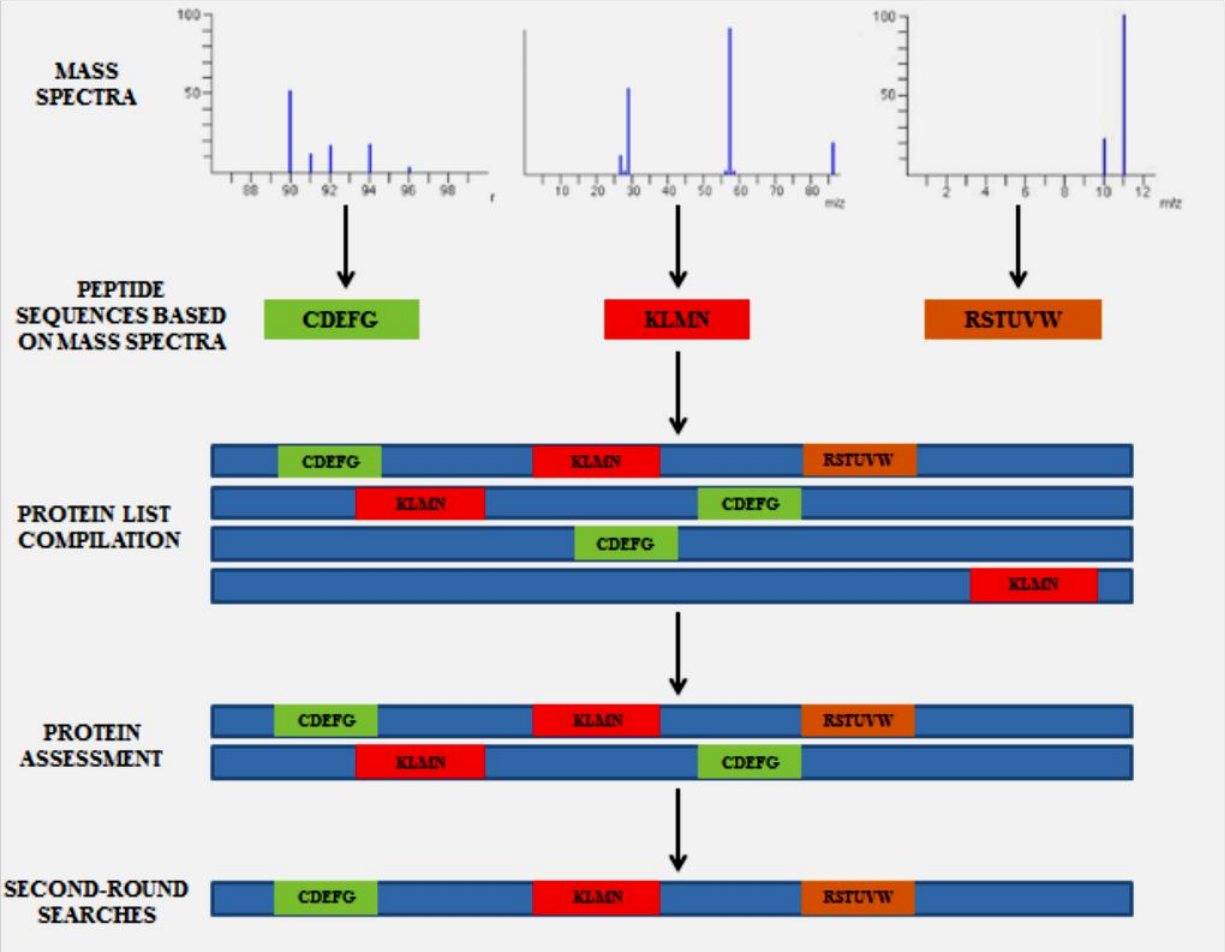


Figure 6: Identifying proteins using ProteinScape.

Proteins are identified through peptide mass spectra from protein digests.

Peptide mass spectra are analyzed, assigned a sequence, and protein list is generated. Proteins are assigned correctly. Searches are repeated using less stringent parameters.

Handling MS Data

It is possible to import into ProteinScape three different types of MS data set: single MS spectra (for example, MALDI TOF PMF), single MS/MS spectra (for example, MALDI TOF/TOF MS/MS), combined data (multiple MALDI TOF/TOF MS/MS spectra), MS/MS spectra without LC context (for example, a plain .mgf file), an LC run containing a number of MS/MS spectra with or without the associated MS spectra (for example, a Bruker DataAnalysis XML file).

Combined MS/MS data sets contain a sublevel with individual compounds: a precursor mass (*m/z*), a retention time, an MS spectrum, and one or more MS/MS spectra.

Identifying Proteins

ProteinScape performs the following steps to identify proteins: peptide identification, protein list compilation (MS/MS searches only), protein assessment, ID refinement.

Protein Searches Dialog

The **Protein Searches** dialog is a multipage dialog used to define parameters for peptide identification, protein list compilation (MS/MS data only) and protein and peptide assessment.

Protein Search Methods

A protein search method defines the parameters used to search for peptide and protein matches in ProteinScape. It defines the search engine used, modifications to take into account, the charge of peptides, mass tolerances, and enables filtering of results according to peptide or protein score.

ScoreBooster

A feature currently only available for MS searches is ScoreBooster, an algorithm based on a Calibrant list (Mass Control List, MCL) of known background and calibrant masses. It labels known peaks as background (contaminants) so they are not submitted to searches. It also performs an internal calibration using either the masses of the Mass Control List or the peptides of an identified protein as calibrants.

Protein-Specific Views

Five Views can be launched from the Protein Table menu: Sequence View, Alternative Proteins View, Quantization Statistics View, Protein GO Comparison View, and Protein Structure View.

Protein Info View

The Protein Info View displays a summary of protein database information such as accessions, gene names, sequence for each protein selected in the protein table.

Sequence View

The Sequence View displays the sequence coverage map of the protein selected in the Protein Table. The sequence map consists of gray bars that indicate identified peptides. Data such as the relative peak intensity, MS/MS score or Peak Quality Factor are indicated by the darkness of the shading; the darker the shading, the higher the respective value.

For peptides identified by MS/MS, the sequence boundaries of fragment ions that support the corresponding amino acid sequence are indicated by red vertical separators in the gray peptide bars.

Moving the mouse pointer over a peptide launches a ToolTip that displays the calculated *m/z*, Range, Sequence, Modifications and Peak Intensity values of the respective peptide in the Peptide Table (Figure 7).

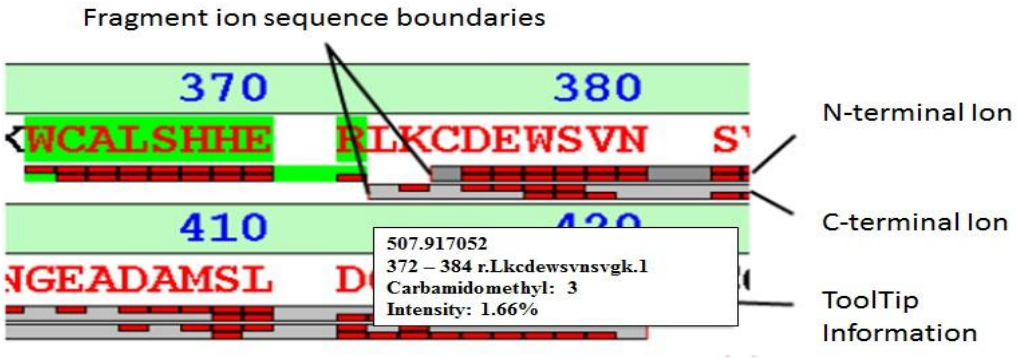


Figure 7: Sequence map information.

Alternative Proteins View

The Alternative Proteins View shows for a given protein all equivalent matches that cannot be discriminated on the basis of the underlying MS data and subset matches that contain a subset of the peptides of the best hit.

Protein Gene Ontology (GO) Comparison View

The Protein GO Comparison View enables ontological comparison of two or more proteins selected in the protein table.

It indicates the number of compared proteins associated with the respective ontological classification, divided into three groups: biological processes, cellular components and molecular functions.

Three-dimensional (3D) Structure View

The 3D Structure View displays the three-dimensional structure of a protein selected in the protein table. Identified peptides are displayed in blue and modified amino acids in red and this enables easy validation of the plausibility of a peptide match.

The correct representation of identified peptides and modified amino acids is based on residue numbers.

Protein Quantitation

ProteinScape supports different protein quantization workflows; including various isobaric, non-isobaric, chemical labelling (SILE), and metabolic labelling (SILAC) workflows and multiplex quantization experiments.

ProteinScape also supports label-free (LF) quantization workflows, in which data sets from a high-quality LC-MS system are quantified against each other, without internal quantization standards.

2 - MASS SPECTROMETRY

2.1: *Introduction*

Mass spectrometry is an important tool in biochemical research. The Human Genome Project and other genome sequencing programs have provided, in rapid succession, the complete genome sequences of specific species and thus, the amino acid sequence of every protein potentially encoded by that species (Rowen et al., 1997; Fraser & Fleischmann, 1997). As expected, this revolutionary source of information, unprecedented in the history of biology, enhanced traditional research methods such as the biochemical approach and also catalyzed proceedings and developments of Omic sciences such as proteomics.

Due to ionization sources (electrospray ionization and matrix-assisted laser desorption/ionization-MALDI), mass spectrometry has become an irreplaceable tool in biological sciences (Siuzdak, 2003).

In the previous years, scientists have developed and established Mass Spectrometry into a highly sensitive tool that is capable of analyzing small and large molecules (Siuzdak, 1996).Mass spectrometry may well be the technique of choice if you want to analyze gases, to reveal and identify drugs of abuse, pharmaceuticals, environmental pollutants, search for explosives at airports, sequence peptides from proteins, or if you are studying cancer and you want to better understand the role of complex carbohydrates in cancer (Greaves & Roboz, 2014). Mass spectrometry is also used to check in real time the breathing of patients by anaesthesiologists during surgery, to determine the composition of molecular species detected in space, to determine if the honey was adulterated with the use of sugary syrups, to locate oil deposits in the rocks by measuring precursors, to check continuous fermentation for the biotechnology industry, to determine the presence of dioxins in contaminated fish, to determine the genetic damage due to environmental causes, to establish the elemental composition of semiconductor materials, to identify the structure of biomolecules, such as carbohydrates, nucleic acids and steroids, to establish the sequence of biopolymers such as proteins and oligosaccharides, to determine "how" medicines are used by the body, to perform analysis in forensics (i.e., the confirmation and measurement of the quantity of drugs and their abuse), to establish age and origin of geochemical and archaeological samples, to identify and quantitatively determine the components of complex organic mixtures.

Mass spectrometry has been described as the smallest scale in the world because of the the size of the molecules that it weighs (Siuzdak, 2003).

In the past decade, remarkable technological improvements allowed for its application to protein, peptides, carbohydrates, DNA, drugs and many other relevant molecules (Siuzdak, 2003).

John B.Fenn, the 2002 Nobel Laureate in Chemistry, gave an exhaustive definition of what mass spectrometry is: *"Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields. Clearly, the* sine qua non *of such a method is the conversion of neutral analyte molecules into ions. For small and simple species the ionization is readily carried by gas-phase encounters between the neutral molecules and electrons, photons, or other ions. In recent years, the efforts of many investigators have lead to new techniques for producing ions of species too large and complex to be vaporized without substantial, even catastrophic, decomposition"* (Siuzdak, 2003).

2.2: *Mass Spectrometer: principles and instrumentation.*

Mass spectrometer is a versatile instrument that is capable to analyze, both qualitative and quantitative, a huge rage of compounds.

A mass spectrometer is an analytical instrument that determines the mass of chemical compounds by separating molecular ions according to their mass-to-charge ratio *(m/z)*, using electric or magnetic fields or a combination of both.

A mass spectrometer contains: a sample introduction system, an ion source where the analytes are vaporized and ions are generated, a mass analyzer, which separates the ions according to their mass-to-charge ratio, an ion detector, where the signal intensities of each *m/z* value are determined, a vacuum system to prevent ion losses through collisions with neutral gas molecules and with the wall of the mass analyzer, the detector and the ion source, the computer to control the instrument and to record and process the data generated (Figure 8).

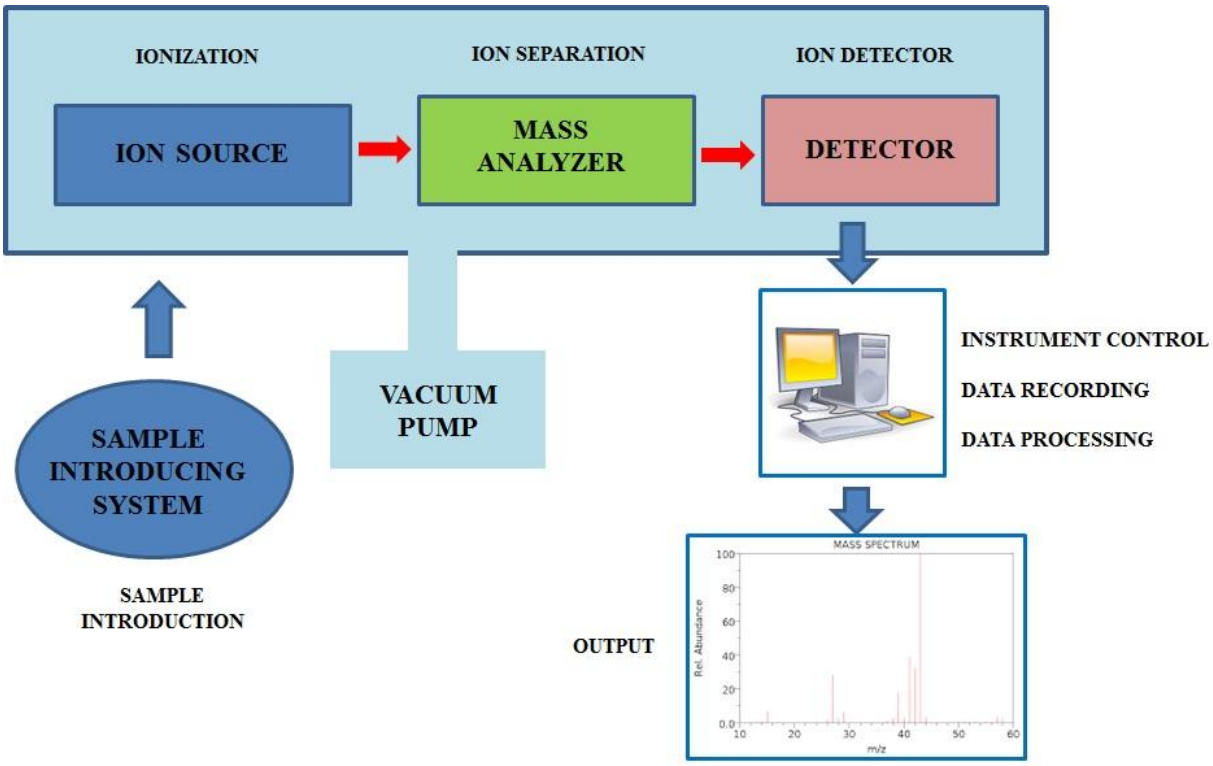


Figure 8: Block diagram of a mass spectrometer (basic sequence of events).

Each component has various forms and multiple types of each component can be mixed and matched, e.g., different ion sources with different analyzers (Greaves & Robot, 2014). In general, the mass analyzer, whose role is to separate ions according to their *m/z* values and to focus and to transfer them onto a detector, is the core component of mass spectrometers. For this reason, the choice of which analyzer to use is a critical choice because it affects various aspects of the data, including mass resolution, mass measurement accuracy and dynamic range (Greaves & Robot, 2014).

The mass analyser is, literally and figuratively, central to the technology. In the context of proteomics, its key parameters are sensitivity, resolution, mass accuracy and the ability to generate information-rich ion mass spectra from peptide fragments.

The ions are generated by inducing either the loss or the gain of a charge from a neutral species. Once the ions are formed, ions are electrostatically directed into a mass analyzer where they can be separated according to the ratio m/z and finally detected by an ion detector (such as an electron multiplier).

The result of molecular ionization, ion separation, and detection is a mass spectrum that can provide information like molecular mass or structural information (Siuzdak, 2003).

A mass spectrum is a two-dimensional representation of the distribution and intensity (abundance) of the ions introduced in the mass spectrometer and, in particular, in the mass analyzer, and separated and recorded according to the *m/z* ratio; it's the two-dimensional representation of signal intensity (ordinate) versus *m/z* (abscissa).

The intensity of a *peak*, as signals are usually called, directly reflects the abundance of ionic species of that respective *m/z* ratio which have been created from the analyte within the ion source (Gross, 2003).

Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two techniques most commonly used to volatize and ionize the proteins or peptides for mass spectrometric analysis (Fenn et al., 1989; Karas & Hillenkamp, 1988). ESI ionizes the analytes out of a solution and is therefore readily coupled to liquid-based (for example, chromatographic and electrophoretic) separation tools (Figure 9).

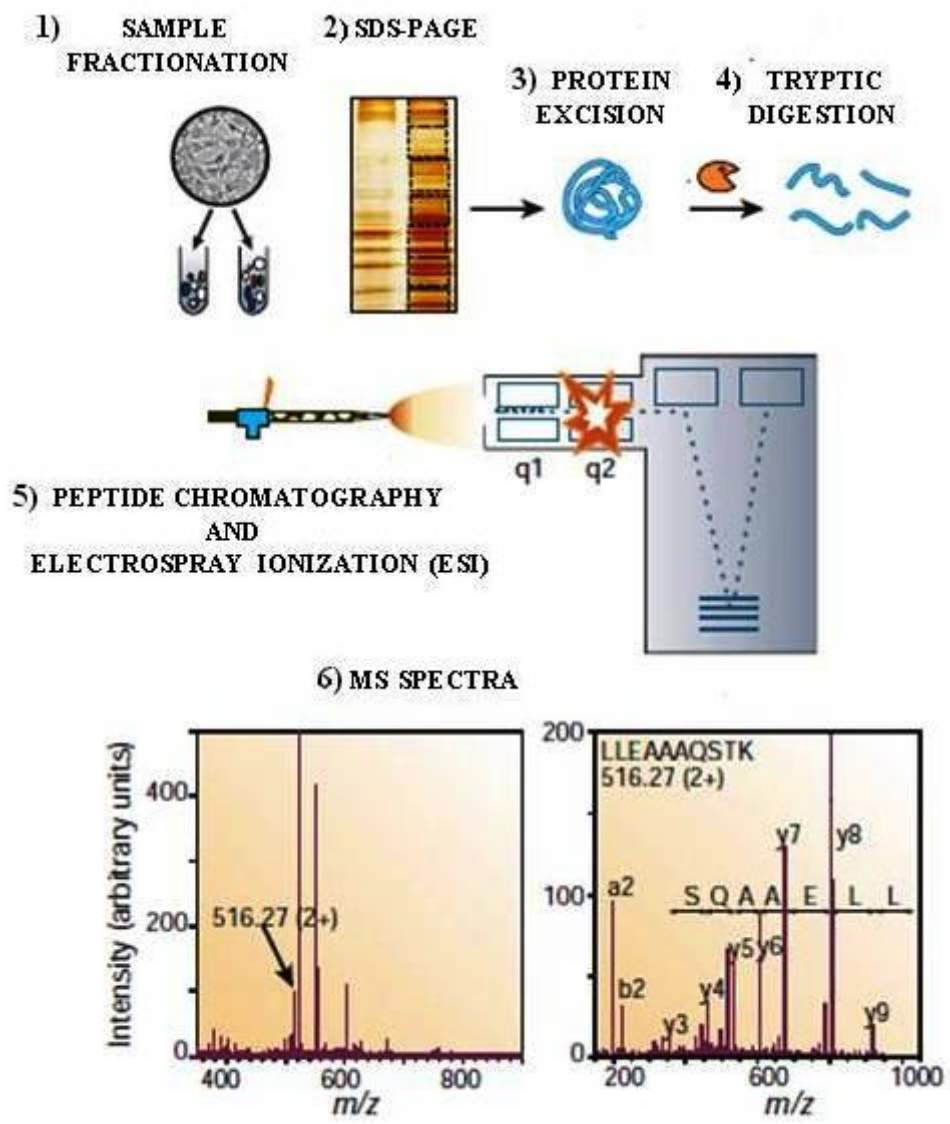


Figure 9: A panoramic of a generic mass spectrometry (MS)-based proteomics experiment, consisting of five stages. (1) In the first stage, the proteins to be analysed are isolated from cell lysate or tissues by biochemical fractionation or affinity selection. (2) The second stage is represented by an SDS-PAGE experiment that defines the 'sub-proteome' to be analysed. (3) In the third step, proteins are degraded enzymatically (usually by trypsin) to peptides because MS of whole proteins is less sensitive than peptide MS and the mass of the intact protein by itself is not sufficient for identification. As results, peptides with C-terminally protonated amino acids are obtained, providing an advantage in subsequent peptide sequencing. (4) In the fourth stage, the peptides obtained are separated by high-pressure liquid chromatography (HPLC) in very fine capillaries and eluted into an electrospray ion source where they are nebulised in small, highly charged droplets. After evaporation, multiply protonated peptides enter the mass spectrometer and, in stage (5), a mass spectrum of the peptides eluting at this time point is taken. The computer generates a prioritized list of these peptides for fragmentation and a series of tandem mass spectrometric or 'MS/MS' experiments ensues. These consist of isolation of a given peptide ion, fragmentation by energetic collision with gas, and recording of the tandem or MS/MS spectrum. The MS and MS/MS spectra are stored for matching against protein sequence databases.

MALDI sublimates and ionizes the samples out of a dry, crystalline matrix via laser pulses. MALDI-MS is normally used to analyse relatively simple peptide mixtures, whereas integrated liquid-chromatography ESI-MS systems (LC-MS) are preferred for the analysis of complex samples.

There are also software packages for handling the huge amount of data generated by the mass spectrometer during the measurements.

In addition, database matching of MS/MS data is possible because peptide molecular ions fragment preferentially at certain points along the backbone (Papayannopoulos et al., 1995; Paizs & Suhai, 2005).

The more widespread approach to database searching of MS/MS data is to skip the interpretation step and let the search engine try to match calculated mass values directly. This method was pioneered by John Yates and Jimmy Eng at the University of Washington, Seattle, who used a cross correlation algorithm to compare an experimental MS/MS spectrum against spectra predicted from candidate peptide sequences. Their ideas were implemented as the Sequest program (Eng et al., 1994).

2.3: Mass Spectrometry In Proteomics

Proteomics, in general, deals with the large-scale determination of gene and cellular function directly at the protein level.

Proteomics attempts to explain the information contained in genomic sequences in terms of the structure, function, and control of biological processes and pathways, by the systematic analysis of the proteins expressed in a cell or tissue in a defined time.

Mass spectrometry (MS) currently is the most important proteomic's tool (Aebersold & Goodlett, 2001).

Mass spectrometry (MS) has increasingly become the method of choice for analysis of complex protein samples. MS-based proteomics has established itself as an indispensable technology to interpret the information encoded in genomes.

MS-based proteomics is a discipline made possible by the availability of gene and genome sequence databases and technical and conceptual advances in many areas, most notably the discovery and development of protein ionization methods, as recognized by the 2002 Nobel Prize in chemistry (Aebersold & Mann, 2003).

During the decade of the 1990s, changes in MS instrumentation and techniques revolutionized protein chemistry and changed the analysis of proteins. These changes were catalyzed by the development of the two ionization methods: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization-MALDI (Fenn et al, 1989; Cole, 1997; Karas & Hillenkamp, 1988). These methods solved the difficult problem of generating ions from large, nonvolatile analytes such as proteins and peptides without significant analyte fragmentation.

Numerous reports document the success MS has enjoyed in studies into the protein primary structure or linear sequence of amino acids, the protein secondary structure or the folding of stretches of amino acids into defined structural motifs, the protein tertiary structure or the three-dimensional fold, and the quaternary structure or the spatial arrangement of folded polypeptides in multiprotein complexes (Aebersold & Goodlett, 2001).

Mass Spectrometry, in particular its application coupled with high-performance separation techniques such as HPLC, has had a dramatic effect on the sensitivity and the speed with which the primary structure of proteins and peptides can be determined (Aebersold & Goodlett, 2001).

Traditionally, proteins have been identified by de novo sequencing, most frequently by chemical degradation (Edman degradation) of proteins or isolated peptide fragments (Hewick et al., 1981; Aebersold et al., 1987).

These partial sequences are assembled to obtain the complete protein sequence from overlapping fragments but more frequently for the generation of probes for the isolation of the gene coding for the protein from a gene library.

With the growing size of sequence databases, it became clear that even relatively short and imperfect sequences (with gaps) were useful for the identification of proteins. This was possible correlating information obtained experimentally from the analysis of peptides with sequence databases. The concept of identifying proteins by correlating information extracted from a protein or peptide with sequence databases rather than by de novo sequencing was significantly enhanced when it was realized that mass spectrometers were ideally suited to generate the required data. Furthermore, the methods initially developed for the isolation of small amounts of proteins and peptides for Edman sequencing were compatible with peptide analysis by LC-MS and LC-MS/MS. This further accelerated the implementation of mass spectrometric methods for protein identification (Aebersold et al., 1987; Larive et al., 1999; Lamond & Mann, 1997; Patterson & Aebersold, 1995; Moritz et al., 1995; Aebersold & Leavitt, 1990; Aebersold et al., 1986). Correlation of mass spectrometric data with sequence databases also depended on the development of novel search algorithms. The availability of complete sequence databases, the development of mass spectrometric methods, and the sequence database search algorithms therefore converged into a mature, robust, sensitive, and rapid technology that has dramatically advanced the ability to identify proteins (Aebersold & Goodlett, 2001).

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. The ability of mass spectrometry to identify and, increasingly, to precisely quantify thousands of proteins from complex samples can be expected to impact broadly on biology and medicine (Aebersold & Mann, 2003).

2.4.: Principle of Peptide Mass Mapping

Peptide mass mapping is based on the insight that the accurate mass of a group of peptides derived from a protein by sequence-specific proteolysis (i.e., a mass map or fingerprint) is a highly effective means of protein identification.

MS sequence data provide the most powerful and unambiguous approach to protein identification (Liebler, 2002).

The principle behind protein identification by mass mapping was implemented by several research groups in an independent way and at approximately the same time (Patterson & Aebersold, 1995; Henzel et al, 1993; James et al., 1993; Yates et al., 1993; Pappin, 1997). Proteins of different amino acid sequence will, after proteolysis with a specific protease, produce groups of peptides the masses of which constitute mass fingerprints unique for a specific protein.

Therefore, if a sequence database containing the specific protein sequence is searched using the observed peptide mass fingerprint, then the protein is expected to be correctly identified within the database. This process has been automated through various methods, which have been developed and reviewed in the years (Patterson & Aebersold, 1995) but they share the same sequence of steps: 1) Peptides are generated by digestion of the protein sample with sequence-specific cleavage reagents that allow to obtain residues at the carboxyl- or aminoterminus to be considered fixed for the search. For example, the enzyme trypsin, popular for mass mapping, leaves arginine (R) or lysine (K) at the carboxyl-terminus and the N-termini of the tryptic peptides obtained (except for the N-terminal one) are expected to be the amino acid following a K or R residue in the protein sequence. 2) Peptide masses are measured as accurately as possible in a mass spectrometer, where an increase in mass accuracy causes an increase of the stringency of the search. 3) The proteins contained in the database are "digested" *in silico*, using the rules applied to the proteolytic method used in the experiment to generate a list of theoretical masses that are to be compared to the set of measured masses. 4) An algorithm is used to compare the set of measured peptide masses against those sets of masses predicted for each protein in the database and to assign a score to each match that ranks the quality of the matches. Both protein and DNA sequence databases are equally suited. If DNA sequence databases are being used, the DNA sequences are translated into protein sequences prior to digestion. The approach is best suited for genetically

well-characterized organisms where either the entire genome is known or extensive protein or cDNA sequence exists (Aebersold & Goodlett, 2001).

The mass mapping method is used routinely for protein identification from microbial species for which complete genome sequences have been determined and for use with protein purification by two-dimensional gel electrophoresis, where protein molecular weight and isoelectric point information can be used to aid identification.

It is often combined with tandem MS of peptides to resolve the identification of any ambiguous remaining masses (Aebersold & Goodlett, 2001).

The single most critical experimental parameter for protein identification by mass mapping is the accuracy of the peptide mass measurement (Fenyo et al., 1998; Jensen et al., 1996; Clauser et al., 1999; Takach et al., 1997).

Increased mass accuracy not only increases search speeds but also increases reliability in the score (Jensen et al., 1997; Jensen et al., 1996; Takach et al., 1997; Bruce et al., 1999). For peptide mass mapping data are commonly obtained via MALDI-TOF analysis from samples which are usually tryptic digests from proteins separated by two-dimensional gel electrophoresis (2DE). Today, 2DE and protein MS represent an integrated technology by which several thousand protein species can be separated, detected, and quantified in a single operation, and hundred of the detected proteins can be identified by sequential analysis of the peptide mixtures generated by digestion of individual gel spots. It is commonly assumed that 2DE-MS is a suitable technology for global proteome analysis based on its ability to display, quantify, and identify thousands of proteins in a single gel (Shevchenko et al., 1996).

In realty, an in-depth examination of the proteins routinely identified by proteomics techniques suggests that 2DE-MS does not represent a truly global technique because of the existence of specific classes of proteins (very acidic or basic proteins, excessively large or small proteins, and membrane proteins) known to be absent or underrepresented in 2D gel patterns (Aebersold & Goodlett, 2001).

In addition, by examining codon bias values of proteins identified from 2D gels, it has been shown that the 2DE-MS approach is incapable of detecting low abundance proteins without pre-gel enrichment (Gygi S. P. et al., 1999). Codon bias is a measure of the propensity of an organism to selectively utilize certain codons, which results in the incorporation of the same amino acid residue in a growing polypeptide chain. It is also thought to be a good measure of protein abundance because highly expressed proteins generally have large codon bias values (Kurland, 1991).

Furthermore, accurate quantization of proteins separated by 2DE is complex and of limited dynamic range, particularly in the case of high-sensitivity staining methods (Corthals et al., 2000). Clearly, the detection and quantification of low abundance proteins (transcription factors, protein kinases, and other proteins of regulatory function) is an important component of proteomics and incompatible with the standard combined 2DE-MS approach.

These limitations and the emerging ability to identify the components in protein mixtures using data-dependent, automated LC-MS/MS and sequence database searching have catalyzed the development of new, chromatography-based methods for the identification of the proteins contained in complex mixtures without separating the mixture into the individual protein components (Link et al., 1999; Ducret et al., 1998; Washburn & Yates, 2000; Tong et al., 1999; Jensen et al., 2000).

For proteomics, in general, the separation sciences continue to make great strides in analyzing complex mixtures and offer the potential for circumventing gel electrophoresis as a preparative tool for MS (Aebersold & Goodlett, 2001).

In the context of the progresses in the study of proteins, it has not to be neglected the contemporary advancement of bioinformatics which allows to manage and store the data generated at the genomic and proteomic level (Domon & Aebersold, 2006).

2.5: *Analysis of protein expression*

The genomics revolution has also catalyzed a new research method termed discovery science (Aebersold et al., 2000). Discovery science enumerates the elements of a biological system irrespective of any hypotheses of how the system functions. It complements the traditional hypothesis driven method to biological research, and proteomics is an essential component of discovery science. The initial efforts of proteomics have been focused on the identification of the proteins expressed by a cell or tissue a process that can be described as descriptive proteomics. More recently, the focus has shifted to the development of methods capable of measuring, on a proteome-wide scale, properties of proteins that reflect the function and dynamics of proteins. These include the quantity, the state of modification, the specific activity, and the association of a protein with other macromolecules.

Many of these methods depend on MS and are currently being rapidly further developed. The potential for method refinement, for developing methods to uncover new types of information, and for the power of the current methods to dissect biological systems at a

molecular level make MS and proteomics among the most exciting, dynamic, and important research themes at the present time.

A promising and exciting new use for MS in proteomics involves not just protein identification but also the determination of protein expression levels (relative quantity) between two different pools of proteins. Obtaining expression data for proteins is important because protein expression levels often are diagnostic for a given cellular state and are not directly related to levels of mRNA expression (Gygi et al., 1999).

2.6.: Post-translational modifications

MS is the method of choice for the detection and identification of post-translational modifications (PTMs). In principle, the methods used for protein identification are also applicable to the analysis of PTMs. For various reasons, PTM analysis is, however, significantly more complex than simple protein identification: (i) Proteins are frequently modified to a low stoichiometry only. Therefore, a high sensitivity of detection for the modified peptides is required. (ii) While proteins can be identified by the sequence or the CID spectrum of a single peptide, the identification of PTMs requires the isolation and analysis of the specific peptide that contains the modified residue(s). (iii) The bond between the PTM and the peptide is frequently labile. It may therefore be difficult to find conditions that maintain the peptide in its modified state during sample work up and ionization. (iv) More than 200 different types of protein modifications have been described (Krishna & Wold, 1998). The total sequence space containing all the potential modified protein sequences is therefore enormous.

Among the large number of PTMs described to date, only a few have been shown to be reversible and thus potentially of regulatory importance in biological processes. Of these, protein phosphorylation has received the most attention and is the best understood with respect to both the enzymes involved catalyzing the phosphorylation/dephosphorylation reactions and the functional consequences of protein phosphorylation. The most common type of protein phosphorylation studied involves the formation of phosphate esters bonds with the hydroxyl side chains of serine, threonine, and tyrosine (Aebersold & Goodlett, 2001).

Two counteracting enzyme systems, kinases and phosphatases, catalyze protein phosphorylation and dephosphorylation, respectively. The structures, specificities, and

regulation of the most common of these is well-studied and reviewed (Charbonneau & Tonks, 1992; Fischer & Krebs, 1989; Hunter, 1987).

There are assumed to be hundreds of protein kinases/phosphatases differing in their substrate specificities, kinetic properties, tissue distribution, and association with regulatory pathways. Protein phosphorylation generally exerts its regulatory function by altering the structure and thus the function of target proteins (Hunter, 1987; Duclos et al., 1991; Johnson L. N. et al., 1996).

A single phosphorylation event involving a serine residue close to the N-terminal induces a long-range allosteric change and thus a change in the catalytic activity (Joung et al., 1995).

The principal aims of essentially any protein phosphorylation study are: 1) to determine the amino acid residues that are phosphorylated in vivo in a protein present in a cell in a given biological state, 2) to identify the kinase/phosphatase(s) responsible for catalyzing the specific reaction, 3) to understand the functional significance of the observed phosphorylation events for the biology of the cell (Aebersold & Goodlett, 2001).

MS would most efficiently identify the precise residues phosphorylated in a proteome if phosphorylated peptides could be selectively isolated from a digest of the proteins contained in a sample, separated, and analyzed by tandem mass spectrometry. Although the progress reached by the technique, no proteome-wide phosphorylation studies have been reported to date. One of the reasons is that proteins are frequently phosphorylated to low stoichiometry (i.e., only a small fraction of a given protein may be phosphorylated and at multiple sites, generating differentially phosphorylated forms of the same protein), it is frequently difficult to isolate quantities of in vivo phosphorylated proteins that are sufficient for analysis by even the most sensitive MS methods (Aebersold & Goodlett, 2001).

The actual determination of the phosphorylated residue(s) generally consists of the following steps: (I) detection and purification of the phosphoprotein, (ii) enzymatic or chemical cleavage of the phosphoprotein into peptides, (iii) isolation of the phosphopeptides from nonphosphorylated peptides or at least phosphopeptide enrichment, and (iv) characterization of the phosphopeptides by MS (Aebersold & Goodlett, 2001).

Methods for determining which amino acid residue-(s) in a peptide are phosphorylated fall into two general themes. The first relies on the chemical lability of the phosphoester bonds in phosphoserine, -threonine, and -tyrosine which can easily be induced to fragment in a collision cell or the ion source of an ESI instrument or in a MALDI-MS, resulting in loss of

phosphate from the peptide. Phosphopeptides that lose phosphate can then be identified by any of several phosphate-specific diagnostic ion scans.

The second theme relies on the detection of the mass added to a peptide by the phosphate group. Typically, in protein phosphorylation studies, the amino acid sequence of the protein investigated is known.

Therefore, phosphopeptides derived from the protein can, in principle, be detected by a net mass differential of 80 u that occurs when phosphate is added to serine, threonine, or tyrosine. Thus, a peptide mass map of the proteolytically fragmented phosphoprotein can potentially identify the phosphorylated peptide by comparison to the known protein sequence (Aebersold & Goodlett, 2001).

The most comprehensive databases of protein modifications are Unimod (http://www.unimod.org), which focuses on modifications relevant to mass spectrometry, and RESID (http://www.ebi.ac.uk/RESID/), which concentrates on natural modifications, mostly post-translational. In database searching, modifications are handled in two ways. First, there are the quantitative modifications, usually called fixed or static. An example would be the efficient alkylation of cysteine. Since all cysteines are modified, this is effectively just a change in the mass of cysteine. It carries no penalty in terms of search speed or specificity. In contrast, most post-translational modifications do not apply to all instances of a residue. For example, phosphorylation might affect just one serine in a peptide containing many serines. Non-quantitative modifications, usually called variable or differential, increase the time taken for a search and reduce its specificity. This is because the software has to permute out all the possible arrangements of modified and unmodified residues that fit to the peptide molecular mass. As more and more modifications are considered, the number of combinations and permutations increases geometrically; a so-called combinatorial explosion (Cottrell, 2011).

2.7.: *Protein-protein interaction*

Most proteins exert their function by way of protein–protein interactions and enzymes are often held in tightly controlled regions of the cell by such interactions.

Bioinformatics methods, correlation of MS data with those obtained by other methods, or iterative MS measurements possibly in conjunction with chemical crosslinking (Rappsilber et al., 2000) can often help to further elucidate direct interactions and overall topology of multiprotein complexes.

Considerable efforts have been devoted to developing tagging systems optimized for analysis of protein complexes (Aebersold & Mann, 2003).

Sample preparation by in-gel digestion

Proteins and protein complexes characterization from cells and cell organelles represents the next challenge for investigation of the cell. The protein mixtures must be separated into single species, broken down into peptides, and, then, identified by mass spectrometry.

Most scientists engaged in proteomics separate proteins by electrophoresis. For characterization and identification of proteomes, mass spectrometry of peptides is the method of choice. To combine electrophoresis and mass spectrometry, sample preparation by "in-gel digestion" has been developed, because it ensures the proteins are specifically degraded into fragments that fit the effective molecular mass separation range of the mass analyzers (Granvogl et al., 2007). To date, many procedures are available for "in-gel digestion".

The method of in-gel digestion was established by Rosenfeld et al. (1992). The basic components of the method have been retained although several changes have contributed to increasing peptide yield and improving the quality of mass spectrometric data. Destaining, reduction, and alkylation of cysteines, enzymatic cleavage of proteins into peptides, and extraction of peptides from the gel are essential steps to obtain high-quality mass spectra (Figure 10) (Granvogl et al., 2007). Mass spectra quality is critically dependent on the amount of sample introduced into the mass spectrometer and on the sample purity. It has been demonstrated that there's a direct correlation between peptide concentration, signal intensity, and ease of spectra interpretation (Washburn. et al., 2001; Granvogl et al., 2006).

Although mass spectrometers sensitivity, the MS user cannot affect protein/peptide loss within the mass spectrometer. Losses must be prevented during protein preparation, separation, purification, and in-gel digestion. Protein or peptide loss usually may occur during destaining, by adsorption on surfaces of pipette tips and digestion tubes, during samples drying in a vacuum concentrator, as a result of peptides incomplete extraction from the gel after digestion, and during ionisation (Speicher et al., 2000; Stewart et al., 2001). The amounts of losses reported vary between 15 and 50% and it depends on peptide properties and concentration (Stewart II et al., 2001). Optimization and reduction of the processing steps is, then, useful to minimise peptide loss during the in-gel digestion process (Granvogl et al., 2007).

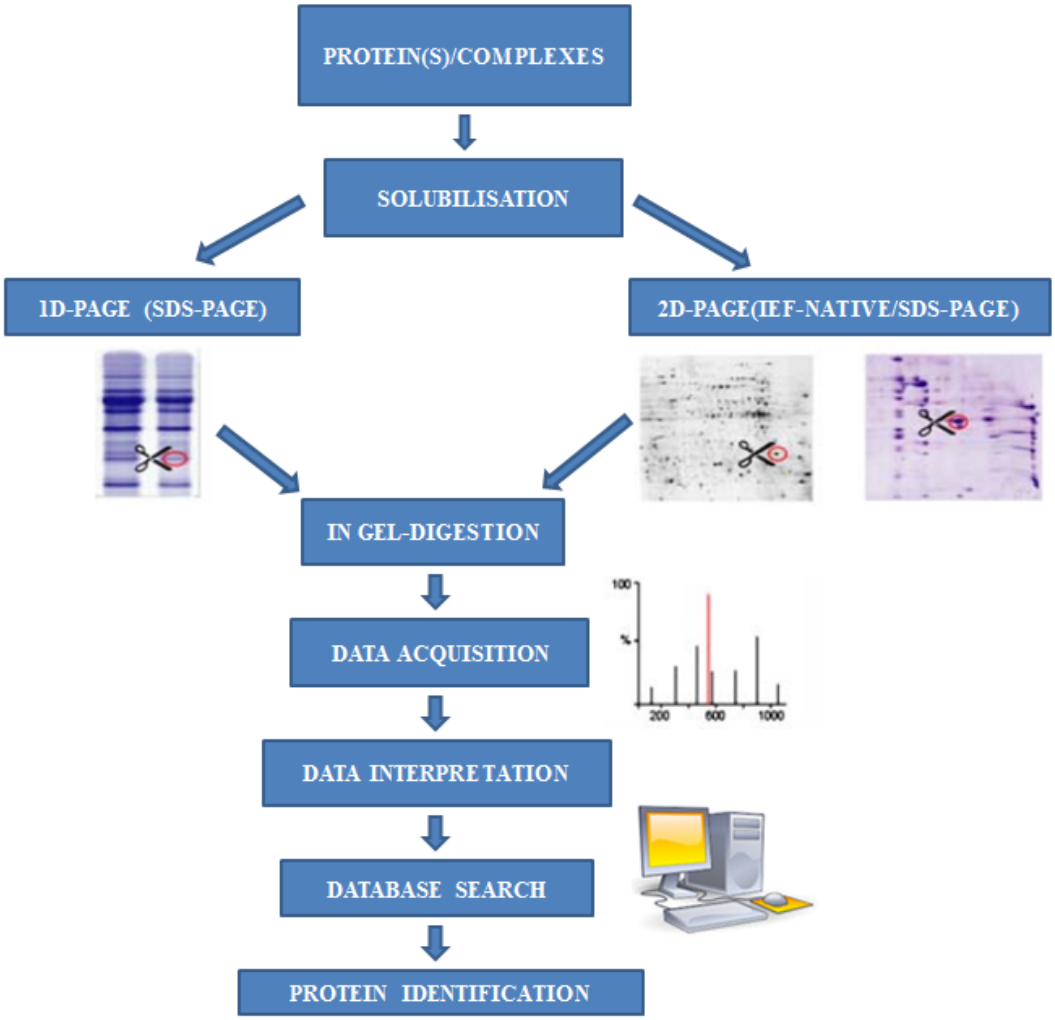


Figure 10: Typical workflow for sample processing, in-gel digestion, and mass spectrometry-based identification of a protein. The proteome of an organism or organelle is separated by 1D or 2D electrophoresis after solubilisation of the protein or protein complexes. After the gel is stained, proteins of interest can be excised from the gel and processed by in-gel digestion, which production of peptides that are processed for mass spectrometric analysis by MALDI-MS or ESI-MS. The protein is, then, identified by comparison of peptide mass fingerprint (PMF) or MS–MS spectra with proteome database entries, or de novo sequencing (the amino acid composition of the peptides is analyzed directly).

The in-gel digestion technique comprises different steps:

1) Staining of proteins for isolating samples from an electrophoresis gel:

Proteins separated by electrophoresis and intended for in-gel digestion can be visualized for isolation by use of several staining methods. Well known techniques for visualizing proteins are the Coomassie, silver, and fluorescence methods. The type of staining used is mainly dependent on the amount of protein in the sample (Table 4).

Although silver staining is undoubtedly more sensitive than Coomassie staining, the latter is still popular in research laboratories. Reasons are the numerous steps during silver staining, the precise timing required for reproducible staining intensity, and limited compatibility with mass spectrometry (Granvogl et al., 2007). In addition, with the invention of "Blue Silver" a Coomassie-based technique for high-sensitivity detection of proteins is now available. With "Blue Silver" approximately 1 ng protein can be detected (Candiano et al., 2004). Destaining of Coomassie Brilliant Blue (CBB) stained proteins is easily achieved by incubation of excised gel bands in a mixture of an organic solvent and ammonium bicarbonate. In recent years different fluorescent dyes have been introduced which combine the high sensitivity of silver staining and compatibility with mass spectrometry (Berggren et al., 2000; Mackintosh et al., 2003).

Dyes are based on complexes containing an organic compound and a heavy metal component (e.g. "Sypro Ruby") or are purely organic probes (e.g. "Deep Purple").

STAIN	TIME (H)	DETECTION LIMIT (ng/protein band)	LITERATURE
Coomassie colloidal (G-250)	15	1–16	Neuhoff et al. 1988, Berggren et al. 2000, Mackintosh et al. 2003, Candiano et al. 2004.
Coomassie R 250	12–48	30–100	Patton 2002, Mackintosh et al. 2003.
Silver	0.25 – 3	0.5	Heukeshoven and Dernick 1985, Merril et al. 1986.
Fluorescent dyes:			
SyproRuby	5.5–12	0.5–5	Berggren et al. 2000, Nishihara and Champion 2002, Lilley and Friedman 2004.
Deep Purple	3.5	0.1	Mackintosh et al. 2003.
DIGE	0.75	0.025	Marouga et al. 2005.

Table 4: Commonly used stains for visualization of gel-separated proteins.

Despite the advantages of fluorescence staining techniques, these methods require expensive hardware to detect the fluorescent signal and manual spot excision is complicated (fluorescence is not directly visible for the human eye). In addition, a fluorescence stain is not permanently light sensitive and, as a consequence, gels cannot be stored for future use if necessary.

To obtain highest sensitivity and dynamic range for protein detection, pre-electrophoretic covalent labelling of fluorescent dyes, called “Difference gel electrophoresis” (DIGE), has been established during the last ten years as an alternative to the post-electrophoretic reversible binding of dyes to protein (Unlu et al., 1997). The DIGE technique uses the selective chemical reactivity of the e-amino group of lysines to label approximately 2–5% of the lysines in the protein (minimal labelling) and all the sulfhydryl groups of the cysteines (saturation labelling).

2) Reduction and alkylation of proteins within electrophoresis gels:

Reduction and alkylation steps are included in most published in-gel digestion procedures. If not performed before electrophoresis of the proteins, reduction of disulfide bridges (e.g. with dithiothreitol) and successive alkylation of the SH groups is conducted after staining and destaining of the proteins (Figure 11). On alkylation with iodoacetamide, cysteine is transformed into carboxyamidomethylcysteine, with a proteolytic digestion (Speicher et al., 2000; Granvogl et al., 2007; Borchers et al., 2000; Shevchenko et al. 2001; Havlis et al. 2003). In addition, quantitative alkylation of cysteines is not achieved after PAGE, because

acrylamide monomers may also modify cysteines (PAM–Cys), and even cysteines, during electrophoretic separation (Hamdan et al. 2001; Mineki et al. 2002).

To generate mass spectra with consistent signals for cysteine-containing peptides, alkylation must be homogenous. This is best achieved when reduction and alkylation are performed before denaturing electrophoretic separation of the proteins (Sechi & Chait, 1998; Herbert et al., 2002).

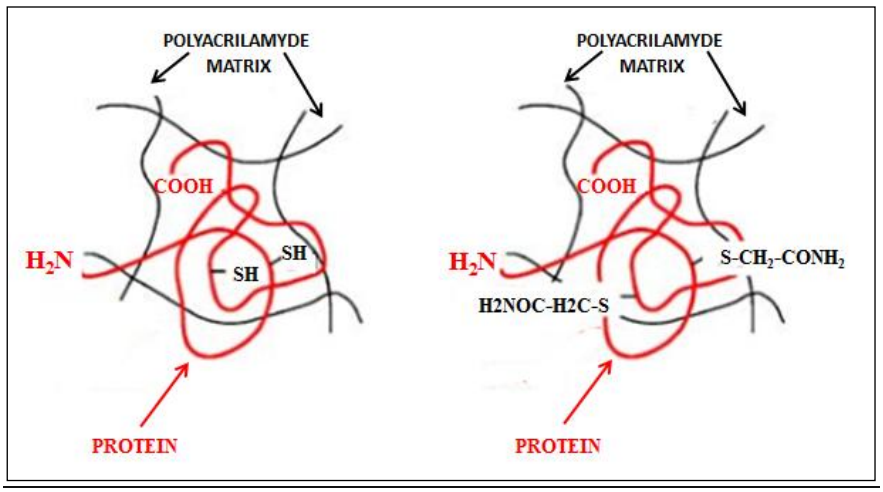


Figure 11: Reduction (on the left) and alkylation (on the right) to increase digestion efficiency and sequence coverage proteins.

3) In-gel digestion of proteins within the electrophoresis gel:

The in-gel digestion of proteins with specific proteases leads to a defined number of characteristic peptides with different molecular masses. The mass of these peptides is useful to identify the protein mass in spectrometry. The peptide yield after in-gel digestion is reported to be approximately 50–85% of the amount obtained after in-solution digestion (Hellman et al., 1995). At the level of uncleaved proteins the matrix of a polyacrylamide gel is an ideal sieve for accomplishing separation of proteins from low-molecular-weight components (Castellanos-Serra et al., 2005). Subsequent washing and dehydration are needed to remove reactants from the sample.

For generation of peptides, two general procedures are commonly used: chemical cleavage or enzymatic digestion with endoproteinases.

4) Extraction of peptides from polyacrylamide gels

In-gel digestion is the method of choice for preparing peptides for mass spectrometry.
Sometimes, however, it might be necessary to isolate the whole protein from the gel for further investigation, to determine the mass of the protein and to digest the isolated protein in solution (Granvogl et al., 2007).
Proteins can be extracted directly from the polyacrylamide gel by treatment with a solvent mixture comprising formic acid, acetonitrile, isopropanol, and water in an ultrasonic bath (Ehring et al., 1997). This method is reported to be efficient for extraction of small proteins up to 25 kDa at the low picomole level.

5) Concentration and desalting of peptides

Supernatants from extraction contain peptides of the digested proteins and salts from the buffers and from the electrophoresis gel. Salts are a major cause of low signal-to-noise ratio and sensitivity in electrospray ionization (ESI). Although MALDI-MS is usually regarded as tolerant of impurities in the samples, the quality of MALDI mass spectra can be significantly improved by use of desalted sample material (Bagshaw et al., 2000).
Purification and concentration of the peptide solution are therefore advantageous for all mass spectral applications.
Concentration of a peptide sample after digestion is achieved by vacuum centrifugation. If a volatile buffer salt (e.g. ammonium bicarbonate) is used, the salt can also be partially removed during the evaporation process. Salt removal is not complete, however, so consecutive cycles of water addition and evaporation are required to remove volatile buffer salts from the protein digest (Maynard et al., 2004). Reversed-phase (RP) microcolumns are commonly used for concentration and desalting of peptide mixtures before mass spectrometric analysis (Stewart et al., 2001; Terry et al., 2004; Wa, 2006; Lim et al., 2003).
Binding of peptides to the alkyl-chain (C4–C18) of the matrix is a result of hydrophobic interactions.

In MALDI-MS, desalting and concentration of peptide samples can be performed directly after spotting on the target. α-Cyano-4-hydroxycinnamic acid (CHCA) is commonly used as matrix.

6) Identification of proteins by mass spectrometry

For identification of proteins in a mass spectrometer after in-gel digestion, peptides must be charged and ionized before separation and detection by a mass analyser/detector system is possible. A rapid and very simple method to determine the identity of a protein by mass analysis is the recording of a peptide mass fingerprint (PMF) (James et al., 1993; Cottrell, 1994).

The molecular masses detected in the mass spectra after MALDI are compared with theoretical masses generated by an *in silico*-generated protein database and a likelihood number for identification of that protein is given.

Often, however, the information from PMF measurements is insufficient to identify the proteins (the number of assigned peptides is low, and no correlation can be achieved if the protein is not present in the database, if more than one protein is present in the excised gel spot, or if modification of the amino acids shifts the detected mass values relative to the in silico-generated database information). Another approach uses raw MS–MS data sets for direct protein identification in databases (e.g. Sequest) (Eng et al., 1994; MacCoss et al., 2002). Potential peptide sequences are identified in a protein database by matching the molecular mass of the acquired peptide with amino acid sequences from the database entries. Information of fragment ion signals is compared with the predicted fragment ions of the sequences derived from the database and best fits are scored and ranked in a list.

MS–MS data are used for identification of target proteins by de novo sequencing of peptides (Mann M. & Wilm, 1994; Zhang & McElvain., 2000). In principle, the amino acid sequence of a peptide is more constraining than its mass for protein identification (Zubarev et al., 1996). The technique of de novo sequencing is capable of assigning peptide sequences to fragments in mass spectra without the need for any information from databases.

Information obtained from de-novo-sequenced peptides can be used for the design of primers or for protein identification. For the latter, de novo sequencing is a reliable method for analysis of data resulting from organisms with incompletely sequenced genomes.

3 - MATERIALS AND METHODS

3.1.: *Evaluation of the repeatability of a method for depletion of high- abundance proteins using a commercially available kit based on affinity chromatography*

The aim of this study was the evaluation of the repeatability of a commercial kit based on a method of affinity chromatography used to deplete high abundance proteins.

As reported in literature, human blood plasma is one of the most studied biological fluids and is the main type of sample used for disease diagnosis because it can be correlated with specific physiological or pathological states.

The proteomic analysis of plasma sample is analytically difficult due to its highly abundant constituent protein concentration range (Luczak et al., 2014; Anderson & Anderson, 2002). The most common approach used to facilitate plasma proteomic analysis is to reduce plasma complexity by samples fractionation, enriching the sample in low-molecular-weight protein fractions, important biomarkers sources (Tirumalai et al., 2003). The masking effect of well-characterized high-abundant proteins in serum has been a main obstacle in the detection of low-abundant proteins that may be proteins of interest for new biomarker identification. Human blood samples belonging to healthy people were considered. Equal amounts of samples were collected and centrifuged at 250 g for ten minutes at room temperature to separate the plasma from the blood cells. The supernatants were frozen at -80°C until use.

Plasma samples were processed to decrease plasma complexity *via* depleting highly abundant proteins using the "Human Serum Albumin and Ig G Multiple Affiniy Removal System" (Agilent), designed to remove two interfering high-abundant proteins from human samples (such as plasma, serum, urine or cerebrospinal fluid). Removal of these highly abundant proteins improves LC/MS and electrophoretic analysis of the samples by increasing the dynamic range of the proteomic analysis.

The kit is based on a cartridge, an affinity ligand-modified resin that removes human albumin and Ig G from human biological samples. In addition, an optimized two buffers system of unknown composition allows for highly reproducible re-use of the spin-cartridge for over two hundred runs.

The Agilent Multiple Affinity Removal System combines the specificity of antibody-antigen recognition and the efficiency of standard liquid chromatography (LC) instrumentation for the depletion of highly abundant proteins and, in particular, for the depletion of human serum albumin and Ig G. The ready-to-use high abundant proteins removal system, consisting of an affinity column and two buffers system (Buffer A and Buffer B) is able to remove multiple proteins from human serum samples at the same time, in a single step. In addition, the two buffers system has been optimized to minimize the removal of proteins not targeted by the immobilized antibodies. In this way, as a result, it is possible to detect less-abundant proteins by loading up to ten times more low-abundant proteins mass onto gels for analysis than before depletion.

Human plasma samples (aliquots of 30 µl) were processed in three replicates, put into filter tubes and diluted to 200 µl with "Buffer A" (Agilent Technologies). Samples were, then, centrifuged at 11750 g for five minutes at 4°C. After the centrifugation, the cartridge was washed with Buffer A, using a siringe.

Samples have been passed through the cartridge and were spun at 250 g for ten seconds.

800 µl of Buffer A were, then, added to the samples.

The cartridge was washed with two ml of "Buffer B" (Agilent Technologies) and equilibrated with Buffer A.

The samples were, then, resuspended in an equal volume of Citric Acid and Potassium phosphate dibasic, vortexed and centrifuged more then three times at 12000 g for 15 minutes at 4°C, obtaining an average sample volume of 30 µl.

All the samples were put in HPLC tubes of 0.5 ml. A sample volume of 15 µl was considered for protein separation with high performance liquid chromatography.

During the separation, only three fractions were taken into account for each sample to be analyzed. The selected fractions were collected through an automated fractionator and lyophilized.

3.2: *Homeostatic changes in the synaptic proteome of rats induced by sleep*

For mammalian brain sampling, twelve male adult Wistar rats were sacrificed, kept in enriched environment. The investigation was carried out on the cerebral cortices of the twelve animals. Initially only two rats were sacrificed for two bidimensional gel preparations and from these subjects the cerebral cortex was taken. Subsequently, twelve rats were sacrificed and, respectively, six rats were sacrificed at 8 am, at the end of the awake period and other six rats were sacrificed at 6 pm, during the end of the sleeping period.

From the twelve rats, only the pre-frontal cortex was taken for analytical gels preparation. Samples were processed with a pH based differential extraction of synaptic membranes protocol.

Post-mortem brain tissues were dissected and homogenized in a solution containing 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.32 M sucrose, supplemented with protease and phosphatase inhibitor cocktails. Samples were, thus, centrifuged at 28000 rpm (100.000*g) for three hours in a SW 40 Ti rotor, using a Beckmann L7 Ultracentrifuge and Beckmann ultraclear tubes. The band at the interface (the synaptic membrane fractions) was collected with a needle, diluted with 0.1 mM $CaCl_2$ and centrifuged at 12000 rpm (15000*g) for twenty minutes. A pellet was obtained and solubilised in 20 mM Tris pH 7.4, supplemented with protease and phosphatase inhibitors, and sonicated.

Samples were added with a combination of three detergents, 0.5 % digitonin, 0.2 % sodium cholate and 0.5% NP-40 , which would be henceforth referred to as triple detergent, and the samples were left for end over end shaking for 60 min in 4C°.

Aliquots of synaptic membrane fractions were diluted with ice cold 0.1 mM $CaCl_2$, followed by the addition of 40 mM Tris-HCl pH 6 supplemented with 2% Triton-X and protease and phosphatase inhibitors, bringing the final volume with 20 mM Tris and 1% Triton-X 100.

The samples were left in cold room rocker for 30 minutes and centrifuged at 18000 rpm (35000*g) for twenty minutes.

The supernatant obtained, designated as the vesicular fraction (SV) was, then, diluted with chilled acetone and left in -20°C overnight.

Samples were resuspended in a lysis buffer, containing 7 M urea, 2M thiourea, 4% CHAPS, 20 mM Tris, 5 mM Mg $(Ac)_2$, 0.2 % Bromophenol Blue (BPB). The samples were, then, sonicated and quantified.

Samples were quantified using 2D-Quant Kit, used for the accurate determination of protein concentration in samples to be analyzed by high resolution electrophoresis techniques such as two-dimensional electrophoresis (2-D electrophoresis), Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) or Isoelectric focusing (IEF). In this step, an accurate quantification is needed because it facilitates comparison among similar protein samples by allowing identical amounts of protein to be loaded.

The procedure works by quantitatively precipitating proteins while leaving interfering substances in solution. The procedure uses a combination of a unique precipitant and co-precipitant to quantitatively precipitate sample proteins while leaving interfering contaminants in solution. The quantification assay is based on the specific binding of copper ions to proteins. The proteins are pelleted by centrifugation and resuspended in an alkaline solution of cupric ions. The cupric ions bind to the polypeptide backbones of any protein present in the sample. A colorimetric agent which reacts with unbound cupric ions is, then, added and unbound copper is measured with a colorimetric agent. The colour density is inversely related to protein concentration. Protein concentration can be accurately estimated by comparison to a standard curve.

The assay doesn't depend on protein amino acid composition, on reaction with protein side groups. As a consequence, there is only little protein to protein variation in the response of this assay. Protein samples can be quantified with the 2-D Quant Kit directly in electrophoresis sample solution, simplifying sample preparation and reflecting the protein concentration of the sample in an accurate way.

As first step, a standard curve was made, using the 2 mg/ml bovine serum albumin (BSA) standard solution provided with the kit. 500 µl precipitant were added both to standards and to protein samples, vortexing briefly. Samples were left 2–3 min at room temperature and 500 µl co-precipitant were added to each tube (to standards and samples), mixing briefly by vortexing or inversion. The tubes were centrifuged at a minimum of 10 000 × g for 5 min to sediment the protein. After the centrifugation a small pellet was visible.

The supernatants were decanted, avoiding resuspension or dispersion of the pellets. The tubes were repositioned in the microcentrifuge as before, with the cap-hinge and pellet facing outward. Another centrifugation was performed to bring any remaining liquid to the bottom of the tube and a micropipette was used to remove the remaining supernatant. The next step was the addition of 100 µl of copper solution and 400 µl of distilled or de-ionized water to each tube, vortex briefly to dissolve the precipitated proteins.

1 ml of working color reagent was added to each tube, ensure instantaneous mixing by introducing the reagent as rapidly as possible, mixing by inversion.

Samples tubes and standards were incubated at room temperature for 15–20 min. The absorbance of each sample and standard was read within 40 min of the addition of working colour reagent (as recommended) at 480 nm, using water as the reference.

The absorbance of the assay solution, unlike most protein assays, decreases with increasing protein concentration.

A standard curve was generated by plotting the absorbance of the standards against the quantity of proteins to determine the protein concentration of the samples.

After sample protein quantification, two cortex samples were considered for two-dimensional electrophoresis procedure, in order to obtain two preparative gels.

In the first dimension or step of isoelectric focusing (IEF), Immobiline™ DryStrip gels with pH interval of 3-10 NL were put in strip holders, subjected to the following voltage values:

30 V for 2 hours

500 V (gradient) for 4 hours

1000 V (gradient) for 6 hours

4000 V (gradient) for 2 hours

8000 V (gradient) for 3 hours

8000 V for 7 hours

500 V for 10 hours

Immobiline™ DryStrip gels have been rehydrated with a rehydration solution containing 8 M urea, 4% CHAPS, glycerine, Dithiothreitol (DTT), 0.5 % IPG Buffer and Bromophenol Blue (BPB).

The step of isoelectric focusing was performed using IPGphor 3 Isoelectric Focusing Unit and each sample was rehydrated individually in the IPGphor Strip Holder.

Two bidimensional gels were made, preparing a 12.5% solution containing acrylamide (29:1 Acrylamide/Bis-acrylamide), 1.5 M Tris-Cl pH 8.8, water, 10% SDS, 10% APS, 10% TEMED.

The gels were casted and covered with an N-butanol solution. Immobiline IPG Strip gels after Isoelectric focusing (IEF) were equilibrated in a SDS equilibration solution containing urea, glycerol, SDS, Tris, BPB.

The IPG Strips were equilibrated before in an aliquot of equilibration solution, adding β-mercaptoethanol (disulfide bonds reduction step) and after in an aliquot of equilibration solution, adding 2-iodoacetamide (alkylation step).

After the polimerization of the two preparative gels, second dimension or SDS-PAGE was performed. IPG strip gels were placed at the top of the bidimensional gels and casted in the Ettan Dalt six for the second dimension, using running buffers containing Tris, glycine, SDS.

After the second dimension or SDS-PAGE, the preparative gels were fixed with a solution containing H_3PO_4 and methanol and stained in a staining solution containing H_3PO_4, $(NH_4)_2SO_4$ and bleached to remove excess dye from the gels and stored for protein spots picking.

In the case of the analytical gels, twelve pre-frontal cortex samples were considered and six bidimensional gels were made.

Samples were processed with the same pH based differential extraction of synaptic membranes protocol used for the previous samples.

Samples were, then, solubilised in a lysis buffer containing 7 M urea, 2M thiourea, 4% CHAPS, 20 mM Tris, 5 mM Mg $(Ac)_2$, 0.2 % Bromophenol Blue (BPB), sonicated and the pH was measured, adjusting the pH to 8 in some cases.

Cortex samples were quantified using 2D-Quant Kit, as described before.

A two-dimensional electrophoresis 2D-DIGE was performed, using a mixture of equal amounts of the samples as standards and 24 cm IPG Strip gels of 3-10 NL pH range.

Six polyacrylamide gels were obtained and they represented two different conditions: cortex samples collected before sleep and cortex samples collected after sleep, in triplicate.

The six bidimensional gels obtained were scanned with Typhoon FLA 9500 laser scanner and the scanned gel images were analyzed through DeCyder 7.0 software.

The software allows modifying the images, adjusting contrast and brightness, to perform differential in gel analysis, selecting the estimated number of spots and parameters such as slope, area, volume and peak height of the protein spots.

In addition, the software is able to do the spot statistics, calculating average ratio and Student's t-test for two different sample groups compared. It is also possible to analyze the biological variations among the gels compared, to merge spots, to create a new one.

To allow the automatic gel matching and to overcome experimental troubles such as gel distortions, more than one hundred spots were matched manually.

Only the most statistically significant spots were considered and among these, only the ones easy for the spot picking, isolated were taken into account. In particular, 48 spots were selected for the picking from the preparative gels.

The preparative gels were left at 4°C for one day before the spot picking and after they were destained, as previous step before proteins spot picking.

A destaining solution containing pH 6.5 Tris was used to destain the two preparative gels, to eliminate the remaining Coomassie staining.

The destaining solution was, then, discarded and a second solution containing 25% methanol was added and after discarded. A Stabilization/ (NH_4) SO_4 solution was added and the preparative gels were left for two hours to avoid protein diffusion.

The next step was, therefore, the manual excision of protein spots selected from preparative gels. Protein spots were selected according to the statistical values obtained and, in particular, only the most significant spots (according to t-test value) and easier to pick up were selected (the isolated spots).

After the picking, protein spots were put in Eppendorf tubes containing acetic acid.

Sample "in-gel" digestion was performed. The first step was the destaining. Acetic acid was discarded and protein spot samples were destained first with a mix MilliQ-ACN (Acetonitrile) and after with acetonitrile and dried with a speed vacuum for about 45 min. to eliminate the solution from the samples.

Then, the enzymatic digestion was performed, using trypsin enzyme (Trypsin Gold-mass spectrometry grade). The enzyme was added to each sample and the samples were put at 4°C for 30 minutes to avoid trypsin autocatalysis. After half an hour, the remaining enzyme solution which was not absorbed by the gel plug was discarded and NH_4HCO_3 was added.

Three washing steps were followed: the first one with 1% formic acid, the second with a solution containing a mix of 25% acetonitrile and 0.1% formic acid and the last washing step with acetonitrile. Samples were, thus, dried through the speed vacuum and resuspended in 0.1% formic acid and transferred to HPLC vials for proteins separation by high performance liquid chromatography and identification by mass spectrometer Bruker Maxis ETD (Electron-Transfer Dissociation).

Data obtained from the mass spectrometer were analyzed using the software Protein Scape 3.1, a bioinformatics platform with sophisticated tools for the analysis and evaluation of proteomic data, suitable for mass-spectrometry based protein identification, characterization, and quantization.

Protein identification data were exported to an Excel file for a full list of proteins. Proteins with a peptide number less than two were eliminated from the protein list.

3.3: Two-Dimensional Difference In Gel Electrophoresis (2D-DIGE) for milk proteins characterization in the Sicilian native "Girgentana" goat and "Valle del Belice" ovine breeds

3.3.1: Proteomics Analysis Of Goat Milk Samples

Samples collection and processing

Samples of bulk milk belonging to the Sicilian native goat breed "Girgentana" were collected. For this purpose, two geographically distant farms have been considered and, in particular, a site in the province of Palermo and another one in the province of Agrigento.
During the sampling, also the month of sampling was taken into account, in order to assess any change in the protein profile due to the sampling area, and the month of sampling. After samples collection, the milk samples were transported at a controlled temperature in laboratory and subsequently frozen at -80 ° C. The milk samples were, then, thawed in melting ice by adding some enzymes important for the maintenance of samples quality: protease inhibitors (to avoid any degradation of proteins by enzymes or other endogenous bacterial enzymes released in solution), DNase and RNase (for nucleic acids removal).

Proteomic analysis

After determining protein concentration and quality of the goat milk samples by one-dimensional electrophoresis, a preparative two-dimensional electrophoresis 2D-PAGE was performed in order to refine the protocol to follow in the experimental procedure. The run was carried out using the following experimental conditions: a pH window of 3-10 for I dimension protein mixture separation and II dimension step or SDS-PAGE electrophoresis at 12% for proteins resolution.
After the run, the preparative gels were stained with Coomassie staining and acquired digitally, using Typhoon FLA 9500 laser scanner.

Image acquisition and analysis

The images obtained scanning the gels were then analyzed qualitatively using the image analysis software Image Master 2D Platinum v 7.0 through special algorithms. Gels images were overlayed and compared, detecting protein spots and determining their abundance, expressed as the ratio between the spots volumes in a gel image and the volumes of the corresponding spots of the internal standard.

During protein spots detection on the map, or "spot detection", was possible to adjust few parameters in a very easy way (smooth, saliency and minimum area) to distinguish the real protein spots from the "background noise". After protein spots detection, the image processing was performed.

In that phase, an important step was represented by the establishment of gel anchors or "landmarks", ie protein spots present in all gels that act as reference points and allow the overlapping and comparison of the gels images in one or more experiments.

Two-dimensional gel spots quantification is an automated process, during which the"background" is automatically corrected by subtracting the lowest value near each spot. Protein spots with a fold change value > 1.5 and with an ANOVA value statistically significant ($P \leq 0.05$) were considered differentially abundant.

From the differentially abundant protein spots, ten spots were picked out from preparative gels.

Protein spots excision and proteins identification

The picked out spots were sent to the laboratory of Mass Spectrometry of Porto Conte Research Center (Sassari) for proteins separation by liquid chromatography and identification by mass spectrometry.

Protein identification data have been filtered by applying the identification criterion based on a number of unique peptides ≥ 2.

3.3.2: Proteomic Analysis Of Ovine Milk Samples

Proteomic analysis has been extended to samples of *Valle del Belice* sheep bulk milk, having as objective the development of protocols for milk fractions preparation to reduce the complexity of the proteome to be analyzed.
For this purpose, 5 different types of sheep milk samples were considered: whole milk, skimmed milk and three milk fractions: fat globules, caseins and whey proteins.

Samples collection and processing

Bulk milk samples from individuals belonging to the Sicilian native "Valle del Belice" sheep breed were collected. During sampling, a farm located in Contessa Entellina, in the province of Palermo, was considered.
After samples collection, a suitable volume of a mixture of protease inhibitors, in the proportion inhibitors - milk 1: 100 (10 µl / ml) was added to prevent samples protein degradation which could originate spot artefacts and the loss of high molecular weight proteins.
Milk samples were, then, transported at a controlled temperature in laboratory and subsequently frozen at -80 ° C.
Samples were thawed in melting ice, avoiding sudden temperature changes that could damage the samples, invalidating proteomic analysis results.

Protein fractions preparation

Caseins have been extracted from skim milk samples by following two different protocols, in order to compare extracted proteins amounts and to determine the most appropriate protocol to follow.
The protocol of caseins precipitation based on the Kjeldahl method (IDF - FIL) was performed.
The initial step of the protocol was the addition of distilled water heated to 41 ° C to aliquots of 10 ml skimmed milk, and subsequently the addition of equal volumes of acetic acid and sodium acetate to milk samples (1 ml of each added reagent).
Milk samples were, then, left at room temperature for about one hour and filtered with Whatman filters. In this way, it was possible to separate whey proteins from caseins.

The second protocol used is the protocol validated by the Scientific Association of Animal Production (ASPA) and is based on three distinct steps: the addition of diluited rennet (0,8 ml of rennet added to 100 ml of distilled water), incubation at 37 °C in a ventilated stove and following centrifugation at maximum speed.

Fat globules membrane proteins have been extracted using the protocol developed by Pisanu (Pisanu et al. 2011), with some modifications.

Whole milk samples were aliquoted into two tubes and centrifuged in order to obtain fat globules and skimmed milk. After centrifugation, the tubes were inverted and placed in ice. Fat was removed and a double washing in phosphate buffered saline (PBS, Phosphate Buffered Saline) was performed. Each washing step was followed by centrifugation and removal of the aqueous phase. The fat globules obtained were, thus, crystallized at 4 ° C "overnight ".

Fat samples were heated to 45 °C to obtain the overall dissolution, and then centrifuged to separate fats from proteins.

Proteins quantification:

After obtaining milk fractions, the commercial 2D Quant (GE Healthcare) kit was used to quantify proteins. This kit, based on the specific binding of copper ions to proteins, allows the accurate determination of protein concentration following a procedure that allows proteins precipitation and the release of interfering substances in solution.

To quantify protein samples, a standard of known concentration, represented by bovine serum albumin (BSA), was used for the calibration line. Then, two solutions provided by the 2D Quant kit: precipitant and co-precipitant, were added to both standards and samples.

After two centrifugation, a proteins precipitate was obtained and it was dissolved in a solution of copper ions and distilled water, followed by a passage to the vortex.

Subsequently, a colorimetric reagent was added both to samples and standards.

The colorimetric reagent binds to the copper ions and the density of coloration is inversely proportional to proteins concentration.

As reported in the manual of the 2D Quant kit, samples absorbance must be read with a spectrophotometer at a wavelength of 480 nm and using distilled water as a blank. In addition, samples absorbance should be read within 40 minutes after the colorimetric reagent addition to avoid to affect the readings negatively.

Protein concentration was estimated using the calibration line obtained by plotting the absorbance of the standards and protein samples concentration.

A test was performed using different buffers in which samples were resuspended. Specifically they were used: distilled water and a lysis buffer containing urea, denaturing agent that acts by disrupting non-covalent and ionic bonds between amino acids; CHAPS, a solubilising detergent that prevents proteins aggregation due to hydrophobic interactions; Tris, a zwitterionic buffer and DTE, a reducing agent that breaks proteins disulfide bonds and maintains proteins in their reduced state.

Another test was performed resuspending the samples in three different lysis buffers, of different composition, in order to assess variations in the quantification of the samples to vary the buffer used. In particular, a buffer containing urea, thiourea, CHAPS and Tris was used; a second buffer containing urea, CHAPS and DTT and a third buffer, containing urea, thiourea, CHAPS, DTT were also used.

Milk fractions samples were quantified with the spectrophotometer according to the coefficients of the calibration line obtained using the software R with a linear regression between the amount of the standard BSA and the absorbance values of the standards.

Casein solubilization:

Due to casein high concentration and to the complex samples texture, which also made it difficult to select sample volumes for quantification, it was required to solubilise caseins to obtain a correct quantification.

Increasing amounts of a lysis buffer containing urea were added to the casein fraction obtained by precipitation, until reaching the optimum volume at which it is obtained their complete solubilization.

4 - RESULTS AND DISCUSSION

4.1.: Evaluation of the repeatability of a method for depletion of high-abundance proteins using a commercially available kit based on affinity chromatography

STATE OF THE ART:

Actually, a complication of proteomics experiments, which relates the experimental approach, is the presence of highly abundant proteins in biological samples, which makes difficult biomarkers discovery and identification, due to preponderant proteins that interfere with the analysis based on liquid chromatography coupled to mass spectrometry (LC-MS).

The removal of high abundance proteins such as serum albumin (HSA), in plasma samples, and caseins, in milk samples, may enable less abundant proteins identification. As reported in literature, it is of great importance that a step of depletion is reproducible when included in a proteomic study.

The principle is to make protein sample less complex, increasing the possibility of determining less abundant proteins.

RESEARCH OBJECTIVES:

The objective was to evaluate the repeatability of the commercial kit for depletion of high-abundance proteins "Multiple Affinity Removal System" (MARS) of Agilent Technologies, which offers the possibility to process several samples simultaneously and relies on the use of a polypropylene column containing a filter, a resin and two buffers ready for use of unknown composition (not revealed by Agilent Technologies).

The aim was to evaluate the repeatability of the depletion kit above, to consider its suitability as first step of a vaste study related to glycosilated proteins, in order to eliminate problems related to high abundance proteins that mask less abundant proteins.

RESULTS:

During the measurements only three peaks were considered and for each protein fraction retention time value and absolute peak intensity values were collected.

From the absolute intensity values three parameters were derived: sum of the absolute intensity and first and third relative intensities, obtained dividing, respectively, first and third peak intensity value by the sum of intensities. Sum of intensities, first and third relative intensities were estimated as percentage value comparing differences/variability among the four sample groups.

Considering the repeatability in the same day and with only one operator, the following values were found:

Sum of intensities: 9%; first relative intensity: 4%; third relative intensity: 3%. Variability values between different days and between individuals/operators were found. Comparing depletion values collected in different days the following variability values were obtained: Sum of absolute intensities: 15%; first relative intensity: 5%; third relative intensity: 3%. Comparing depletion values from different operators the following variability values were obtained:

Sum of absolute intensities: 20%; first relative intensity: 6%; third relative intensity: 4%. During measurements, the reproducibility of the relative intensities values (5-7%) and the absolute intensity values (1-2%) was found.

DISCUSSIONS:

Depletion experiments were performed by three different operators. Four groups of six samples were depleted in triplicates; with some reproduction at different data.

Samples were, then, separated by reverse-phase high performance liquid chromatography (only one fraction was studied) and measured by UV.

Three peaks from the UV were selected and their intensities were measured for each sample. The values were used to characterize reproducibility and variability.

This study was a preparatory step to further studies that will be pursued in the glycobiology field by Professor Karoly Vekey research group.

During the measurements, it was demonstrated a better reproducibility of the relative intensities values, compared to the absolute intensity values, less reproducible of the first.

Moreover the decision taking into accounts the relative intensities values and not the absolute intensity values. From the values obtained during the measurements it can be stated that there is ca. the 10% difference in the quantity of protein depleted while only 1-3% difference in relative abundances.

Variability of the sum of the intensities is mainly due to random errors and variability between different operators. Variability of the first relative intensity is mainly due to inter-individual differences while variability value of the third relative intensity is modest.

4.2.: *Homeostatic changes in the synaptic proteome of rats induced by sleep*

STATE OF THE ART:

As reported by Biggio & Mostallino (2013), neuronal plasticity is represented by the extraordinary ability of neurons to change in the short and long term their function and their morphology as a result of environmental stimuli, endocrine, or pharmacological and pathological insults to ensure the maintenance of their functionality in such situations. It was demonstrated the loss of trophism and, therefore, of neuronal plasticity in neurons of brain areas (hippocampus, cingulate cortex, etc.) in depressed subjects not treated promptly with drug therapy or resistant to the therapy.

The importance of neuronal plasticity, considered a rapid and long-term adaptability developed by neurons in response to environmental stimuli, has allowed during the evolution important achievements such as the acquisition of more and more sophisticated and functional properties that relate to the control of emotions, affective and cognitive processes etc. Neuronal plasticity, as reported in literature, is related to the circadian rhythm. As reported by Albrecht (2012), the circadian system coordinates the physiology and behaviour of mammals with the environmental light-dark cycle.

It places the sleep during inactivity time using various mechanisms that involve neurotransmitters, nuclear receptors and protein kinases.

In mammals, the day can be divided roughly into a phase of activity, during which the physical activity is predominant, and a resting phase, during which the repair mechanisms become active and brain function is altered in a state of sleep. However, sleep is not a simple shutdown of brain function but rather it is a highly regulated process that involves two main mechanisms: (1) a homeostatic process that regulates the increase in the propensity to fall asleep during the wakefulness and the decrease of sleep intensity and (2) a process which borders circadian sleep and wakefulness at appropriate times during the day and is largely independent from the previous episodes of sleep/wakefulness (Borbely, 1998).

Deciphering the molecular basis of sleep is very difficult as it is very likely that the circadian and homeostatic processes are interlaced to a certain extent, which makes it difficult to separate sharply the two processes and the mechanisms involved.

Sleep definition is focused on the brain and its activity, since sleep loss manifests itself at the beginning in a loss of interaction with the environment, due to changes in brain activity. Some studies were conducted on gene expression modifications in rats brain after long term sleep deprivation (Cirelli et al., 2006) and the results have shown a strong correlation between sleep deprivation and the occurrence of physiological changes such as a dramatic increase in energy consumption, a decrease in body weight and the death of animals after 2-3 weeks.

In addition, the results obtained from the analysis of "sleep-related" transcripts, which code for glia proteins, proteins involved in protein synthesis, in the synthesis of cholesterol, in membrane trafficking, in the synaptic "down-regulation" and in memory consolidation confirm the importance of sleep on gene expression. In contrast, studies concerning spontaneous wakefulness or sleep deprivation for short time demonstrated that transcripts expressed at high levels during these situations encode different mitochondrial proteins, chaperones and heat shock proteins and proteins involved in synaptic potentiation and glutamatergic *transmission* (Cirelli et al., 2006).

These results suggest that the continued wakefulness not only increases energy consumption by the brain but it’s also a cellular stress for neurons and/or glial cells.

However, despite numerous studies on the subject, sleep deprivation syndrome remains largely unresolved and synaptic protein turnover has not been investigated during sleep with high-throughput techniques to date.

RESEARCH OBJECTIVES:

The aim of the research was to compare two different hypotheses related to brain plasticity and circadian rhythm in mammals. One of these hypotheses affirms the absence of any form of neuronal plasticity during sleep.

According to this hypothesis, there would be no creation of new synapses at this stage while it would have significant changes at the neuronal level in the wakefulness, as demonstrated by the ability of "problem-solving" in wakefulness and not during sleep.

This hypothesis is also known as G. Tononi and Cirelli C. synaptic homeostasis hypothesis (Tononi & Cirelli, 2014) and affirms that sleep is for the brain the price to pay for plasticity, since its characteristic is a reversible disconnection from the surrounding environment, usually accompanied by immobility.

During sleep, synaptic efficiency is renormalized and homeostasis is restored. Increased synaptic strength, according to the authors, would have different costs at the cellular level and system, including increased energy consumption and a higher demand for distribution of energetic cellular needs to synapses, resulting in cellular stress, and associated changes in support cells such as glia.

In addition, an increased synaptic "robustness" would also reduce the selectivity of neurons responses and would saturate the ability to learn.

Normalizing synaptic "robustness", sleep reduces the price of neuronal plasticity restoring, at the same time, neuronal selectivity and the ability to learn, leading to memories consolidation and integration.

Briefly, according to the synaptic homeostasis hypothesis, neurons would achieve some winning strategies that are strictly related to plasticity:

1) new learnings would occur primarily through synaptic potentiation.

2) synaptic strengthening would occur mainly during the wakefulness, when the organism interacts with the environment and not during sleep, when it is disconnected. In this way, what the organism is able to learn is controlled from reality and not by the imagination.

3) restoring of the normal situation of synaptic "robustness" would compare mainly during sleep, when the brain is spontaneously in an "offline" mode.

As illustrated by several computational models, the synaptic homeostasis hypothesis provides a parsimonious explanation of several positive consequences of sleep on memory-related processes, including acquisition (the restoration of the ability to acquire new memories), consolidation (memory improving, by supporting new memories), the ability to extract

information from the environment (the propensity of the brain to form memories that last longer and do not change, such as faces, maps and examples of a particular encounter with the environment), the integration of information (for the brain is easier to remember new information if these are placed in patterns already learned, recorded in an organized structure of old memories), and the ability to forget the sufferings (important mechanism to cope with the inevitable accumulation of minor details).

Conversely, however, the hypothesis of the creation of new synapses during sleep and wakefulness. This hypothesis is summarized by Albrecht (2012) in the review entitled "Circadian rhythms and sleep - the metabolic connection". It affirms the importance of sleep, considered more than just a wakefulness switching off but rather a highly regulated process that involves two main mechanisms:

1) a homeostatic process that regulates the increased propensity to fall asleep during the wakefulness and the increased sleep intensity during sleep,
2) a circadian process that separates sleep and wakefulness, confining them in appropriate times of the day and largely independent from the previous episodes of sleep/wakefulness (Borbely, 1998).

The aim of the present study was to characterize the altering proteins in the synaptic proteome of rats due to sleep, using synaptosome preparations and gel-based proteomics.

The synaptic homeostasis hypothesis suggests that sleep maintains synaptic excitability. Based on this homeostatic hypothesis, the reduction of the robustness of synaptic connections occurs during sleep because one of the main roles of sleep is to regain plasticity of the brain. This idea is mainly based on whole tissue transcriptomics data, but these experiments don’t consider that synaptic protein levels are specific, due to local protein synthesis and degradation.

On the other hand, the synaptic protein turnover during sleep is not well understood to date.

In the present study, we wanted to clarify the mechanisms of synaptic proteome modifications during sleep.

RESULTS:

Bidimensional maps with thousands spots were obtained. The gels obtained resulted well resolved and with only a very small stricking. In the following pages the scanned images from the six analytical 2D-DIGE gels (Figures 12-3-4-5-6-7).

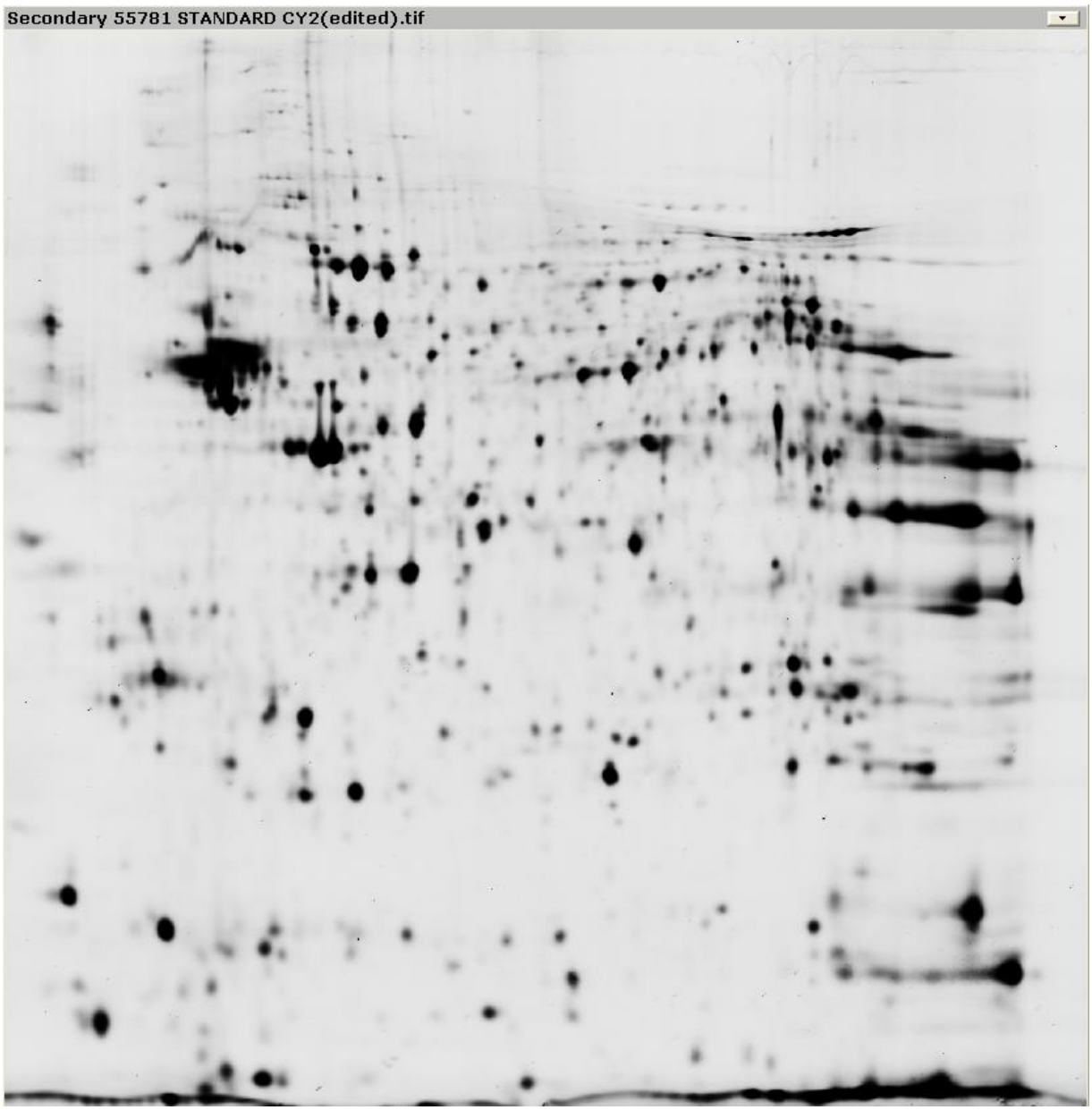


Figure 12: Gel 1. Proteomic map related to cortex samples taken from a first rat "sacrificed" before sleep and from a first rat "sacrificed" after sleep and internal standard (a mix in equal amounts of all the cortex samples present in the experiment).

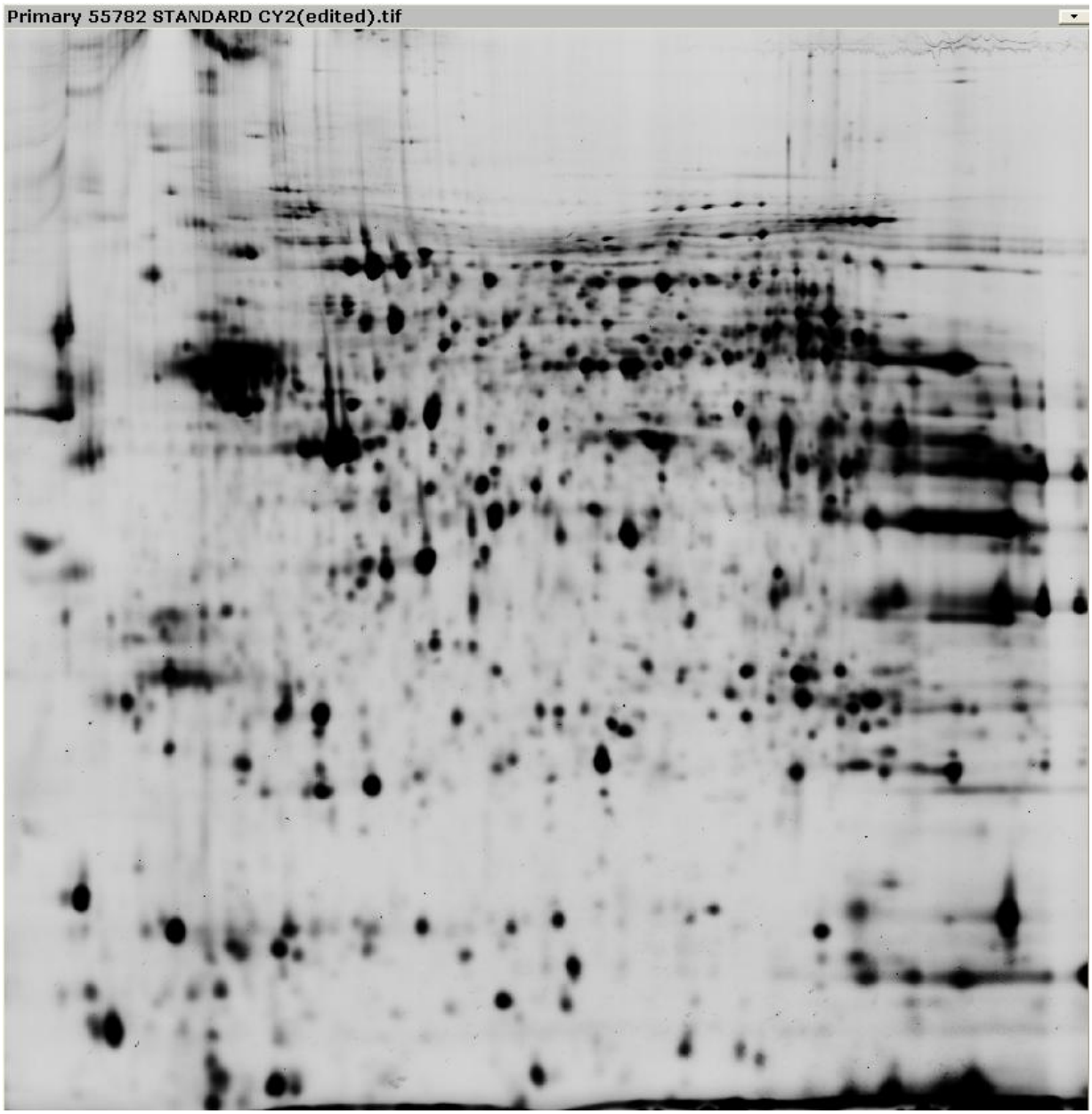


Figure 13: Gel 2. Proteomic map related to cortex samples taken from a second rat “sacrificed” before sleep and from a second rat “sacrificed” after sleep and internal standard (a mix in equal amounts of all the cortex samples present in the experiment).

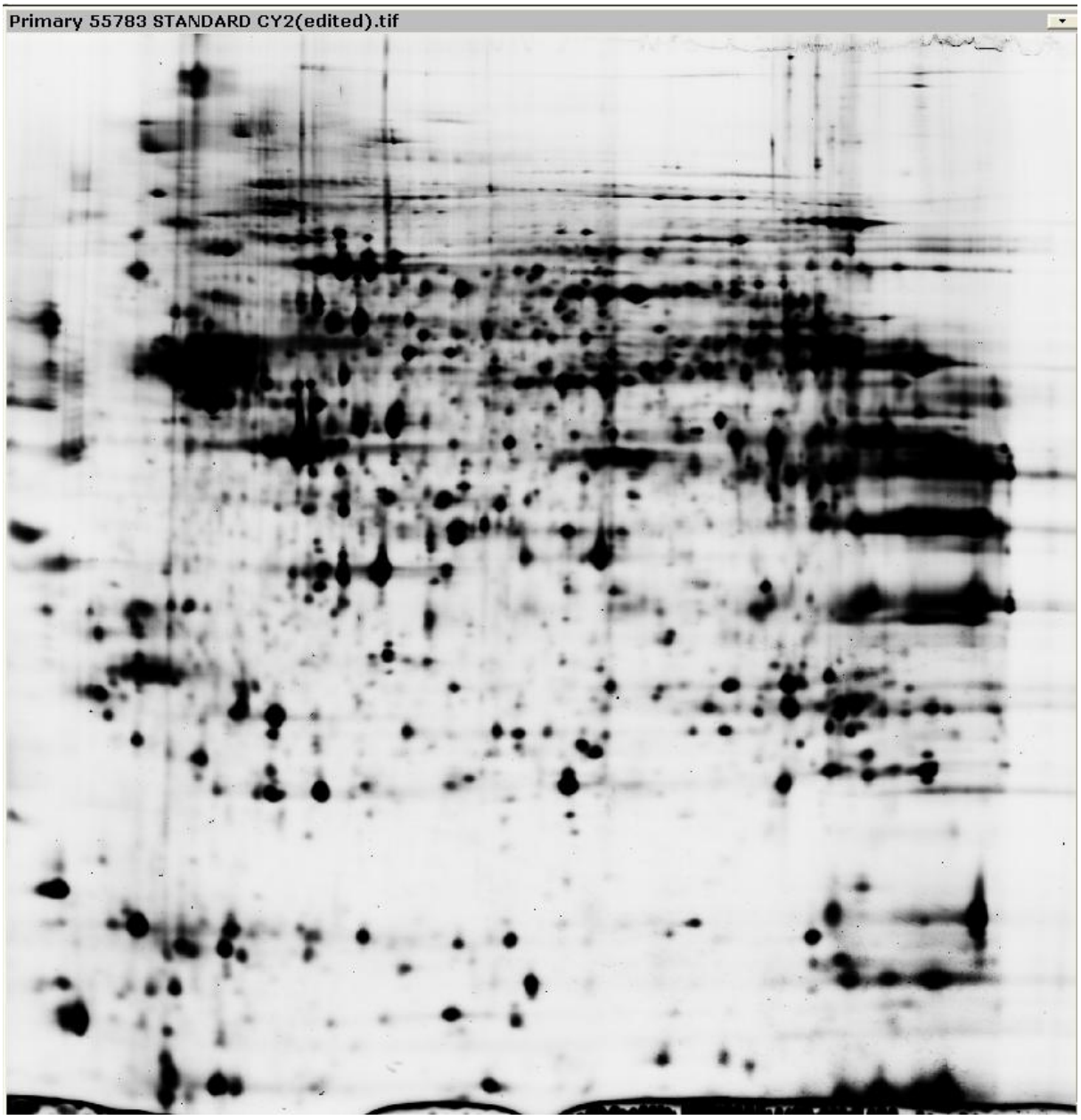


Figure 14: Gel 3. Proteomic map related to cortex samples taken from a third rat “sacrificed” before sleep and from a third rat “sacrificed” after sleep and internal standard (a mix in equal amounts of the entire cortex samples present in the experiment).

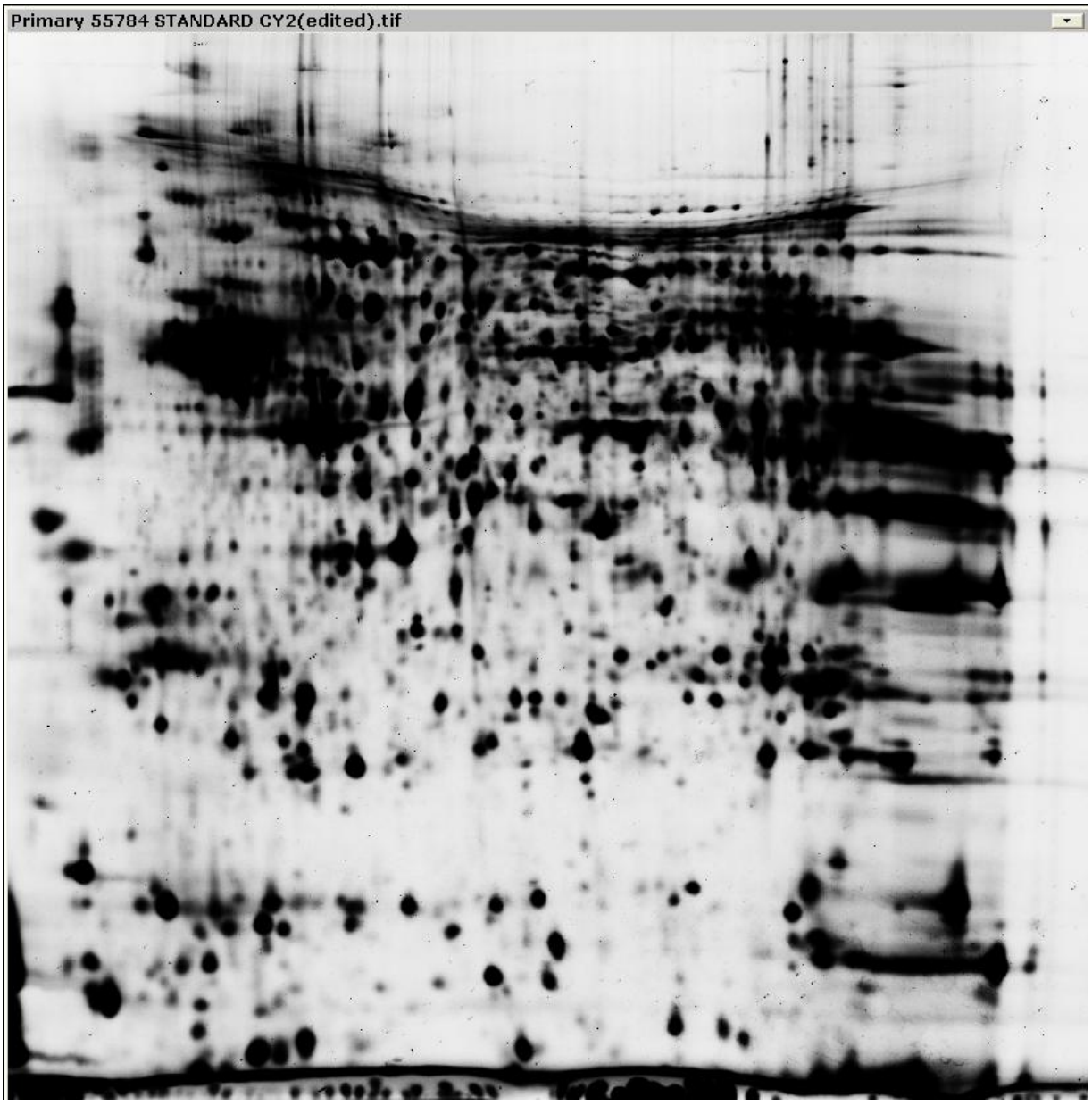


Figure 15: Gel 4. Proteomic map related to cortex samples taken from a fourth rat "sacrificed" before sleep and from a fourth rat "sacrificed" after sleep and internal standard (a mix in equal amounts of the entire cortex samples present in the experiment).

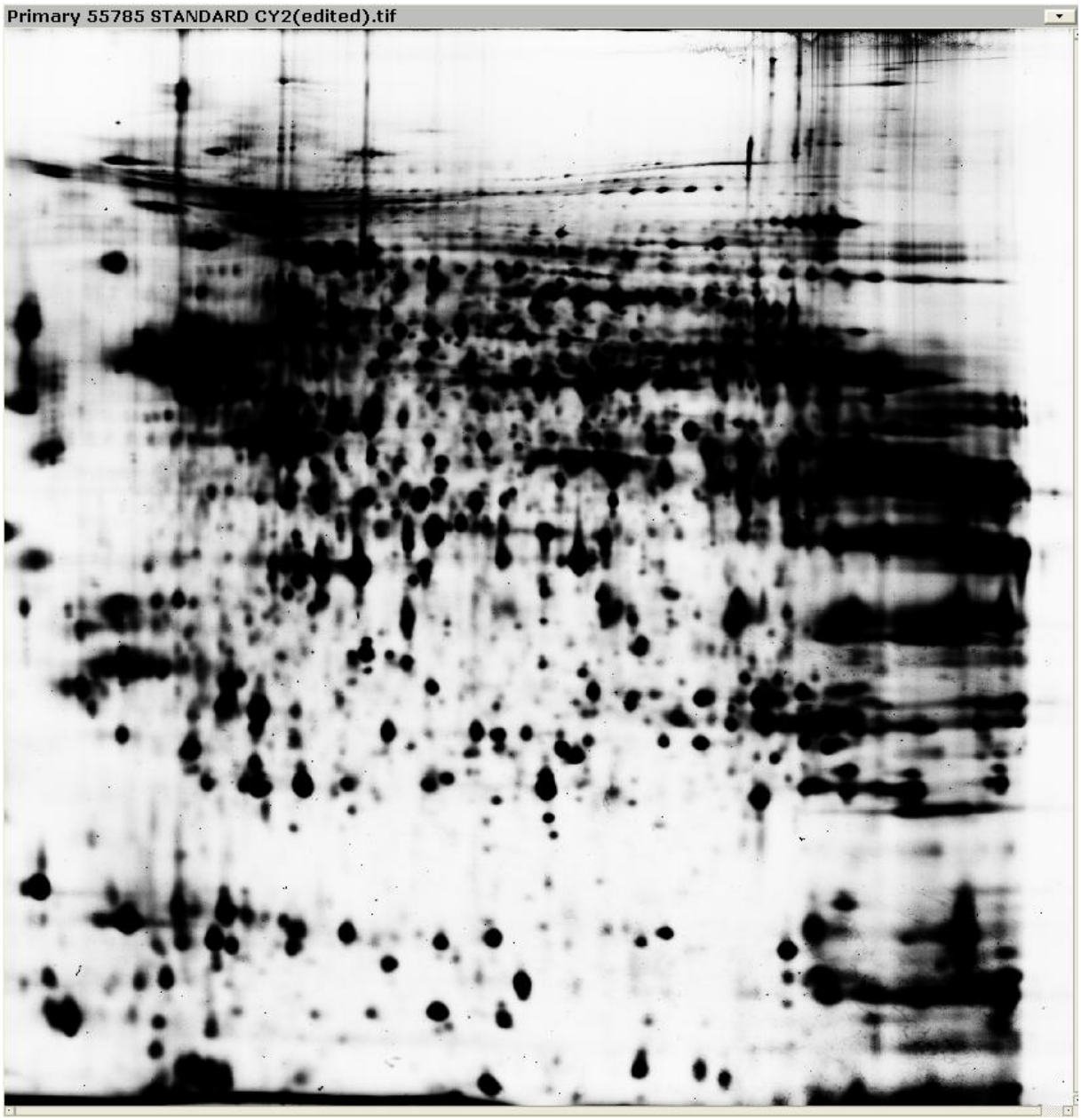


Figure 16: Gel 5. Proteomic map related to cortex samples taken from a fifth rat "sacrificed" before sleep and from a fifth rat "sacrificed" after sleep and internal standard (a mix in equal amounts of the entire cortex samples present in the experiment).

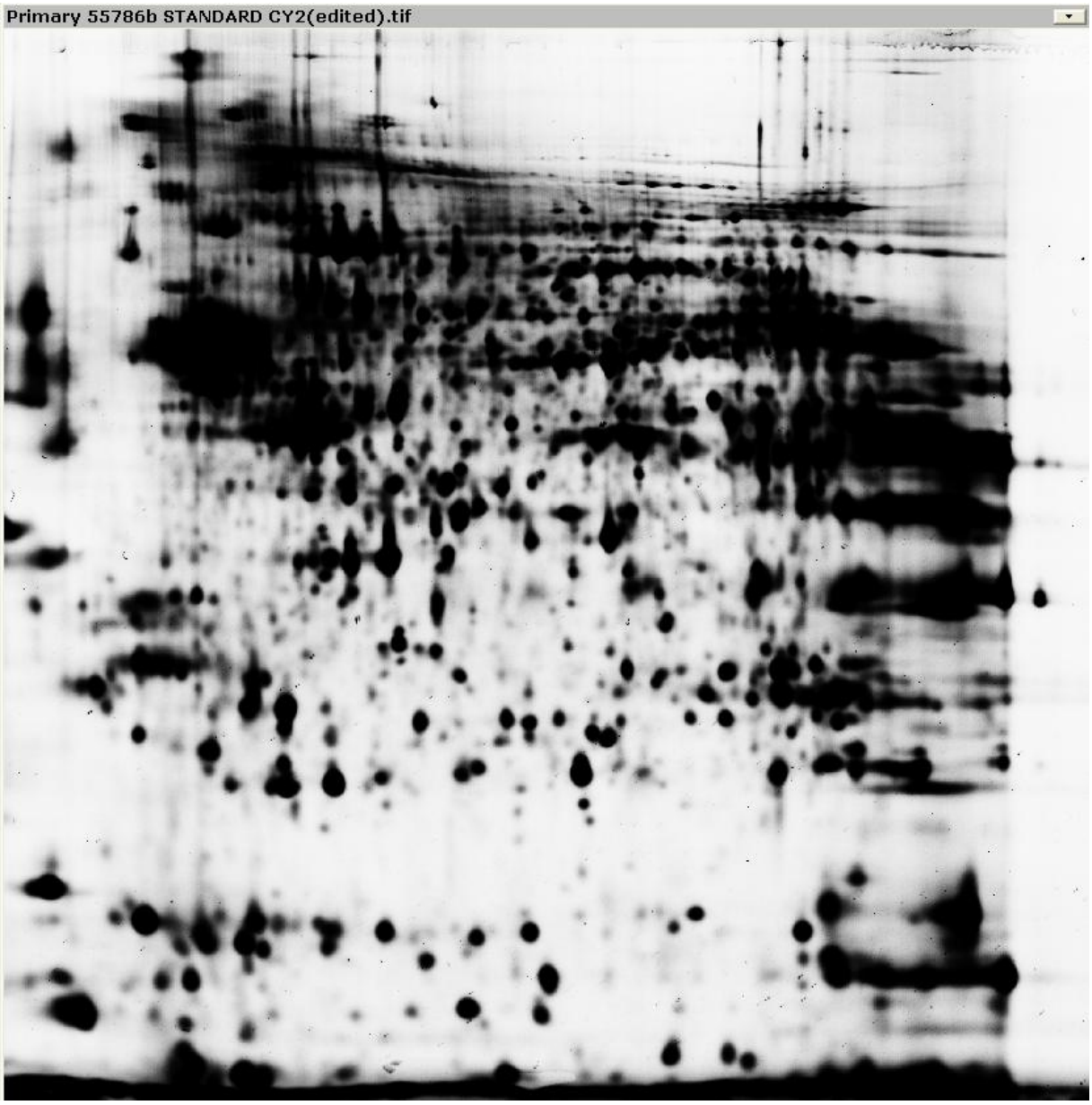


Figure 17: Gel 6. Proteomic map related to cortex samples taken from a sixth rat “sacrificed” before sleep and from a sixth rat “sacrificed” after sleep and internal standard (a mix in equal amounts of the entire cortex samples present in the experiment).

During the gel matching, the image analysis software makes a gel reference called Master gel; a virtual gel image obtained adding all the spots from the gels of the gel matching.
As it's clearly visible (Figure 18) in the Master gel, there's a crowded region in the upper part of the gel, with hundreds spots. Only the isolated spots present in this region have been considered for the spot picking.

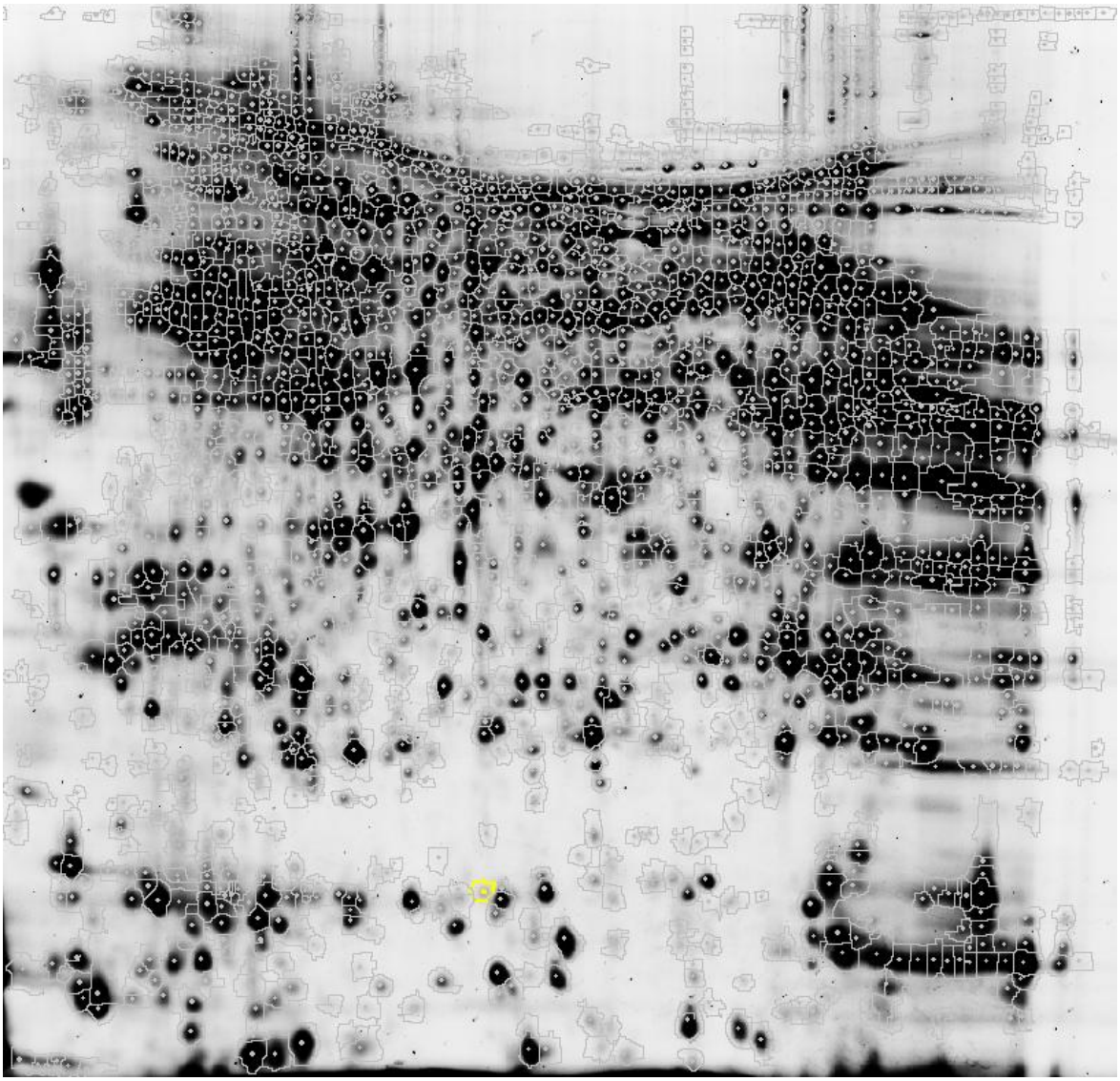


Figure 18: Proteomic map of the Master gel obtained after the gel matching.

RESULT AND DISCUSSION

In the following pages, the proteins list obtained after protein identification through mass spectrometer Q-TOF. On the left is reported the number of the spot assigned and on the right the list of proteins identified with the mass spectrometry for each spot sample analyzed.

Spot number	PROTEIN
1356	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial OS=Rattus norvegicus GN=Idh3a PE=1 SV=1
	Transaldolase OS=Rattus norvegicus GN=Taldo1 PE=1 SV=2
	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Rattus norvegicus GN=Ppp1ca PE=1 SV=1
	Tubulin beta-3 chain OS=Rattus norvegicus GN=Tubb3 PE=1 SV=1
	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1
	Serine/threonine-protein phosphatase PP1-gamma catalytic subunit OS=Rattus norvegicus GN=Ppp1cc PE=1 SV=1
1867	Calbindin OS=Rattus norvegicus GN=Calb1 PE=1 SV=2
	Ras-related protein Rab-3A OS=Rattus norvegicus GN=Rab3a PE=1 SV=1
	Ras-related protein Rab-14 OS=Rattus norvegicus GN=Rab14 PE=1 SV=3
	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1
	Tubulin beta-4B chain OS=Rattus norvegicus GN=Tubb4b PE=1 SV=1
	Tubulin beta-2A chain OS=Rattus norvegicus GN=Tubb2a PE=1 SV=1
	Ras-related protein Rab-5A OS=Rattus norvegicus GN=Rab5a PE=2 SV=1

	Ras-related protein Rab-8B OS=Rattus norvegicus GN=Rab8b PE=1 SV=1
	Rho-related GTP-binding protein RhoB OS=Rattus norvegicus GN=Rhob PE=1 SV=1
	Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform OS=Rattus norvegicus GN=Ppp2r1b PE=2 SV=1
	14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1
	Ras-related protein Ral-A OS=Rattus norvegicus GN=Rala PE=1 SV=1
1147	Selenocysteine insertion sequence-binding protein 2 OS=Rattus norvegicus GN=Secisbp2 PE=1 SV=1
2320	Syntaxin-binding protein 1 OS=Rattus norvegicus GN=Stxbp1 PE=1 SV=1
	WD repeat-containing protein 1 OS=Rattus norvegicus GN=Wdr1 PE=1 SV=3
	T-complex protein 1 subunit gamma OS=Rattus norvegicus GN=Cct3 PE=1 SV=1
	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial OS=Rattus norvegicus GN=Sdha PE=1 SV=1
	Dihydropyrimidinase-related protein 2 OS=Rattus norvegicus GN=Dpysl2 PE=1 SV=1
2341	Phosphoglycerate kinase 1 OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2
	Cytochrome b-c1 complex subunit 2, mitochondrial OS=Rattus norvegicus GN=Uqcrc2 PE=1 SV=2
	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2
	Creatine kinase U-type, mitochondrial OS=Rattus norvegicus GN=Ckmt1 PE=1 SV=1
	Citrate synthase, mitochondrial OS=Rattus norvegicus GN=Cs PE=1 SV=1
	Fructose-bisphosphate aldolase A OS=Rattus norvegicus GN=Aldoa PE=1 SV=2
	Dihydropyrimidinase-related protein 2 OS=Rattus norvegicus GN=Dpysl2 PE=1 SV=1
	ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1

Acetyl-CoA acetyltransferase, mitochondrial OS=Rattus norvegicus GN=Acat1 PE=1 SV=1

Glyceraldehyde-3-phosphate dehydrogenase OS=Rattus norvegicus GN=Gapdh PE=1 SV=3

1767 Phosphoglycerate mutase 1 OS=Rattus norvegicus GN=Pgam1 PE=1 SV=4

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 OS=Rattus norvegicus GN=Gnb2 PE=1 SV=4

Guanine nucleotide-binding protein subunit beta-4 OS=Rattus norvegicus GN=Gnb4 PE=2 SV=4

Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1

Adenylate kinase 4, mitochondrial OS=Rattus norvegicus GN=Ak4 PE=2 SV=1

Voltage-dependent anion-selective channel protein 1 OS=Rattus norvegicus GN=Vdac1 PE=1 SV=4

Vesicle-fusing ATPase OS=Rattus norvegicus GN=Nsf PE=1 SV=1

Creatine kinase U-type, mitochondrial OS=Rattus norvegicus GN=Ckmt1 PE=1 SV=1

2338 Hexokinase-1 OS=Rattus norvegicus GN=Hk1 PE=1 SV=4

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Rattus norvegicus GN=Gnb1 PE=1 SV=4

Haloacid dehalogenase-like hydrolase domain-containing protein 2 OS=Rattus norvegicus GN=Hdhd2 PE=2 SV=1

Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1

Tubulin beta-2A chain OS=Rattus norvegicus GN=Tubb2a PE=1 SV=1

Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1

1904 Ras-related protein Rab-14 OS=Rattus norvegicus GN=Rab14 PE=1 SV=3

Protein deglycase DJ-1 OS=Rattus norvegicus GN=Park7 PE=1 SV=1

Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1

2314	Dihydropyrimidinase-related protein 2 OS=Rattus norvegicus GN=Dpysl2 PE=1 SV=1
	Serum albumin OS=Rattus norvegicus GN=Alb PE=1 SV=2
	Syntaxin-binding protein 1 OS=Rattus norvegicus GN=Stxbp1 PE=1 SV=1
2323	Dihydropyrimidinase-related protein 5 OS=Rattus norvegicus GN=Dpysl5 PE=1 SV=1
	Pyruvate kinase PKM OS=Rattus norvegicus GN=Pkm PE=1 SV=3
	Dihydropyrimidinase-related protein 1 OS=Rattus norvegicus GN=Crmp1 PE=1 SV=1
	Dihydropyrimidinase-related protein 4 (Fragment) OS=Rattus norvegicus GN=Dpysl4 PE=1 SV=1
	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2
	Leucine-rich glioma-inactivated protein 1 OS=Rattus norvegicus GN=Lgi1 PE=1 SV=1
	Bifunctional purine biosynthesis protein PURH OS=Rattus norvegicus GN=Atic PE=1 SV=2
1764	14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1
	14-3-3 protein gamma OS=Rattus norvegicus GN=Ywhag PE=1 SV=2
	14-3-3 protein theta OS=Rattus norvegicus GN=Ywhaq PE=1 SV=1
	14-3-3 protein beta/alpha OS=Rattus norvegicus GN=Ywhab PE=1 SV=3
	14-3-3 protein eta OS=Rattus norvegicus GN=Ywhah PE=1 SV=2
	14-3-3 protein epsilon OS=Rattus norvegicus GN=Ywhae PE=1 SV=1
	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2
	Secretogranin-1 OS=Rattus norvegicus GN=Chgb PE=1 SV=2
	60 kDa heat shock protein, mitochondrial OS=Rattus norvegicus GN=Hspd1 PE=1 SV=1
1380	Guanine nucleotide-binding protein G(o) subunit alpha OS=Rattus norvegicus GN=Gnao1 PE=1 SV=2
	Protein SGT1 homolog OS=Rattus norvegicus GN=Sugt1 PE=2 SV=1

Spectrin alpha chain, non-erythrocytic 1 OS=Rattus norvegicus GN=Sptan1 PE=1 SV=2

ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2

Tubulin beta-2A chain OS=Rattus norvegicus GN=Tubb2a PE=1 SV=1

Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1

Adaptin ear-binding coat-associated protein 1 OS=Rattus norvegicus GN=Necap1 PE=1 SV=1

Dihydropyrimidinase-related protein 1 OS=Rattus norvegicus GN=Crmp1 PE=1 SV=1

1380 Dihydropyrimidinase-related protein 2 OS=Rattus norvegicus GN=Dpysl2 PE=1 SV=1

2365 Cofilin-1 OS=Rattus norvegicus GN=Cfl1 PE=1 SV=3

1828 Rho GDP-dissociation inhibitor 1 OS=Rattus norvegicus GN=Arhgdia PE=1 SV=1

Ubiquitin carboxyl-terminal hydrolase isozyme L1 OS=Rattus norvegicus GN=Uchl1 PE=1 SV=2

Tubulin alpha-1C chain OS=Rattus norvegicus GN=Tuba1c PE=1 SV=1

Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1

Tubulin beta-2B chain OS=Rattus norvegicus GN=Tubb2b PE=1 SV=1

Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1

Ras-related protein Rab-3A OS=Rattus norvegicus GN=Rab3a PE=1 SV=1

Rho-related GTP-binding protein RhoB OS=Rattus norvegicus GN=Rhob PE=1 SV=1

Glial fibrillary acidic protein OS=Rattus norvegicus GN=Gfap PE=1 SV=2

2328 Coronin-1A OS=Rattus norvegicus GN=Coro1a PE=1 SV=3

T-complex protein 1 subunit beta OS=Rattus norvegicus GN=Cct2 PE=1 SV=3

Dihydropyrimidinase-related protein 2 OS=Rattus norvegicus GN=Dpysl2 PE=1 SV=1

Rho GDP-dissociation inhibitor 1 OS=Rattus norvegicus GN=Arhgdia PE=1 SV=1

2179 Complexin-2 OS=Rattus norvegicus GN=Cplx2 PE=1 SV=1

	Visinin-like protein 1 OS=Rattus norvegicus GN=Vsnl1 PE=1 SV=2
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
	Hippocalcin-like protein 1 OS=Rattus norvegicus GN=Hpcal1 PE=1 SV=2
	Vesicle-associated membrane protein 3 OS=Rattus norvegicus GN=Vamp3 PE=1 SV=1
	Coactosin-like protein OS=Rattus norvegicus GN=Cotl1 PE=1 SV=1
	Selenocysteine insertion sequence-binding protein 2 OS=Rattus norvegicus GN=Secisbp2 PE=1 SV=1
2169	Nucleoside diphosphate kinase B OS=Rattus norvegicus GN=Nme2 PE=1 SV=1
	Rabphilin-3A OS=Rattus norvegicus GN=Rph3a PE=1 SV=1
1633	EF-hand domain-containing protein D2 OS=Rattus norvegicus GN=Efhd2 PE=1 SV=1
	Annexin A5 OS=Rattus norvegicus GN=Anxa5 PE=1 SV=3
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
670	Syntaxin-binding protein 1 OS=Rattus norvegicus GN=Stxbp1 PE=1 SV=1
	Aconitate hydratase, mitochondrial OS=Rattus norvegicus GN=Aco2 PE=1 SV=2
	Pyruvate kinase PKM OS=Rattus norvegicus GN=Pkm PE=1 SV=3
1524	Ribose-phosphate pyrophosphokinase 1 OS=Rattus norvegicus GN=Prps1 PE=1 SV=2
2140	Solute carrier family 12 member 5 OS=Rattus norvegicus GN=Slc12a5 PE=1 SV=2
2196	Peroxiredoxin-5, mitochondrial OS=Rattus norvegicus GN=Prdx5 PE=1 SV=1
	Ubiquitin-conjugating enzyme E2 variant 2 OS=Rattus norvegicus GN=Ube2v2 PE=1 SV=3
2326	Serine/threonine-protein phosphatase PP1-alpha catalytic subunit OS=Rattus norvegicus GN=Ppp1ca PE=1 SV=1
2326	Pyruvate kinase PKM OS=Rattus norvegicus GN=Pkm PE=1 SV=3
2220	Complexin-1 OS=Rattus norvegicus GN=Cplx1 PE=1 SV=1

	Vesicle-associated membrane protein 3 OS=Rattus norvegicus GN=Vamp3 PE=1 SV=1
1249	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial OS=Rattus norvegicus GN=Ndufa10 PE=1 SV=1
	Alpha-enolase OS=Rattus norvegicus GN=Eno1 PE=1 SV=4
	3-phosphoinositide-dependent protein kinase 1 OS=Rattus norvegicus GN=Pdpk1 PE=1 SV=2
1772	Phosphoglycerate mutase 1 OS=Rattus norvegicus GN=Pgam1 PE=1 SV=4
	Enoyl-CoA hydratase, mitochondrial OS=Rattus norvegicus GN=Echs1 PE=1 SV=1
	Triosephosphate isomerase OS=Rattus norvegicus GN=Tpi1 PE=1 SV=2
	Syntaxin-binding protein 1 OS=Rattus norvegicus GN=Stxbp1 PE=1 SV=1
2199	Superoxide dismutase [Cu-Zn] OS=Rattus norvegicus GN=Sod1 PE=1 SV=2
1889	Thioredoxin-dependent peroxide reductase, mitochondrial OS=Rattus norvegicus GN=Prdx3 PE=1 SV=2
	Proteasome subunit beta type-4 OS=Rattus norvegicus GN=Psmb4 PE=1 SV=2
	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial OS=Rattus norvegicus GN=Dlst PE=1 SV=2
1999	Phosphatidylethanolamine-binding protein 1 OS=Rattus norvegicus GN=Pebp1 PE=1 SV=3
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
2329	Malate dehydrogenase, cytoplasmic OS=Rattus norvegicus GN=Mdh1 PE=1 SV=3
	Dynamin-1 OS=Rattus norvegicus GN=Dnm1 PE=1 SV=2
	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic OS=Rattus norvegicus GN=Gpd1 PE=1 SV=4
1848 v	Triosephosphate isomerase OS=Rattus norvegicus GN=Tpi1 PE=1 SV=2
	Creatine kinase B-type OS=Rattus norvegicus GN=Ckb PE=1 SV=2

F-actin-capping protein subunit beta OS=Rattus norvegicus GN=Capzb PE=1 SV=1

2335 Eukaryotic translation initiation factor 5A-1 OS=Rattus norvegicus GN=Eif5a PE=1 SV=3

Actin, cytoplasmic 2 OS=Rattus norvegicus GN=Actg1 PE=1 SV=1

Vesicle-associated membrane protein 3 OS=Rattus norvegicus GN=Vamp3 PE=1 SV=1

776 Pyruvate kinase PKM OS=Rattus norvegicus GN=Pkm PE=1 SV=3

ATP synthase subunit alpha, mitochondrial OS=Rattus norvegicus GN=Atp5a1 PE=1 SV=2

Leucine-rich glioma-inactivated protein 1 OS=Rattus norvegicus GN=Lgi1 PE=1 SV=1

2334 Synaptotagmin-1 OS=Rattus norvegicus GN=Syt1 PE=1 SV=3

LIM and SH3 domain protein 1 OS=Rattus norvegicus GN=Lasp1 PE=1 SV=1

Serine/threonine-protein phosphatase PP1-gamma catalytic subunit OS=Rattus norvegicus GN=Ppp1cc PE=1 SV=1

NAD-dependent protein deacetylase sirtuin-2 OS=Rattus norvegicus GN=Sirt2 PE=1 SV=1

Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial OS=Rattus norvegicus GN=Idh3a PE=1 SV=1

1478 Malate dehydrogenase, cytoplasmic OS=Rattus norvegicus GN=Mdh1 PE=1 SV=3

Lys-63-specific deubiquitinase BRCC36 OS=Rattus norvegicus GN=Brcc3 PE=2 SV=1

Serine/threonine-protein phosphatase PP1-beta catalytic subunit OS=Rattus norvegicus GN=Ppp1cb PE=1 SV=3

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Rattus norvegicus GN=Gnb1 PE=1 SV=4

1044 C-terminal-binding protein 1 OS=Rattus norvegicus GN=Ctbp1 PE=1 SV=3

Septin-11 OS=Rattus norvegicus GN=Sept11 PE=1 SV=1

1160 Pyruvate dehydrogenase E1 component subunit alpha, somatic form, mitochondrial OS=Rattus norvegicus GN=Pdha1 PE=1 SV=2

	Phosphoglycerate kinase 1 OS=Rattus norvegicus GN=Pgk1 PE=1 SV=2
	Citrate synthase, mitochondrial OS=Rattus norvegicus GN=Cs PE=1 SV=1
	Creatine kinase U-type, mitochondrial OS=Rattus norvegicus GN=Ckmt1 PE=1 SV=1
	Synapsin-1 OS=Rattus norvegicus GN=Syn1 PE=1 SV=3
	Hyaluronan and proteoglycan link protein 1 OS=Rattus norvegicus GN=Hapln1 PE=1 SV=2
	Synapsin-2 OS=Rattus norvegicus GN=Syn2 PE=1 SV=1
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
	Argininosuccinate synthase OS=Rattus norvegicus GN=Ass1 PE=2 SV=1
	Glutamine synthetase OS=Rattus norvegicus GN=Glul PE=1 SV=3
	Isocitrate dehydrogenase [NADP] cytoplasmic OS=Rattus norvegicus GN=Idh1 PE=1 SV=1
	Cysteine desulfurase, mitochondrial OS=Rattus norvegicus GN=Nfs1 PE=2 SV=1
1172	Septin-5 OS=Rattus norvegicus GN=Sept5 PE=1 SV=2
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial OS=Rattus norvegicus GN=Ndufa10 PE=1 SV=1
	Neuronal-specific septin-3 OS=Rattus norvegicus GN=Sept3 PE=1 SV=2
	Tubulin alpha-1C chain OS=Rattus norvegicus GN=Tuba1c PE=1 SV=1
1191_2	Septin-5 OS=Rattus norvegicus GN=Sept5 PE=1 SV=2
1815	Ubiquitin carboxyl-terminal hydrolase isozyme L3 OS=Rattus norvegicus GN=Uchl3 PE=1 SV=1
	ATP synthase subunit beta, mitochondrial OS=Rattus norvegicus GN=Atp5b PE=1 SV=2
	Syntaxin-1A OS=Rattus norvegicus GN=Stx1a PE=1 SV=1
	14-3-3 protein zeta/delta OS=Rattus norvegicus GN=Ywhaz PE=1 SV=1

	Spectrin alpha chain, non-erythrocytic 1 OS=Rattus norvegicus GN=Sptan1 PE=1 SV=2
	14-3-3 protein gamma OS=Rattus norvegicus GN=Ywhag PE=1 SV=2
	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1
1856	Peroxiredoxin-6 OS=Rattus norvegicus GN=Prdx6 PE=1 SV=3
1872	Peroxiredoxin-6 OS=Rattus norvegicus GN=Prdx6 PE=1 SV=3
	Guanine nucleotide-binding protein G(o) subunit alpha OS=Rattus norvegicus GN=Gnao1 PE=1 SV=2
	Voltage-dependent anion-selective channel protein 1 OS=Rattus norvegicus GN=Vdac1 PE=1 SV=4
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
1217	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
	Endophilin-A1 OS=Rattus norvegicus GN=Sh3gl2 PE=1 SV=2
	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1
1504	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 OS=Rattus norvegicus GN=Gnb1 PE=1 SV=4
	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-2 OS=Rattus norvegicus GN=Gnb2 PE=1 SV=4
	Tubulin alpha-1C chain OS=Rattus norvegicus GN=Tuba1c PE=1 SV=1
	Selenocysteine insertion sequence-binding protein 2 OS=Rattus norvegicus GN=Secisbp2 PE=1 SV=1
2133	Nucleoside diphosphate kinase A OS=Rattus norvegicus GN=Nme1 PE=1 SV=1
2336	Annexin A3 OS=Rattus norvegicus GN=Anxa3 PE=1 SV=4
	Lambda-crystallin homolog OS=Rattus norvegicus GN=Cryl1 PE=2 SV=3
	Vesicle-fusing ATPase OS=Rattus norvegicus GN=Nsf PE=1 SV=1

	Voltage-dependent anion-selective channel protein 2 OS=Rattus norvegicus GN=Vdac2 PE=1 SV=2
2337	Peroxiredoxin-2 OS=Rattus norvegicus GN=Prdx2 PE=1 SV=3
	Dihydropyrimidinase-related protein 3 OS=Rattus norvegicus GN=Dpysl3 PE=1 SV=2
	Phosphatidylethanolamine-binding protein 1 OS=Rattus norvegicus GN=Pebp1 PE=1 SV=3
	Actin, cytoplasmic 1 OS=Rattus norvegicus GN=Actb PE=1 SV=1
	Creatine kinase B-type OS=Rattus norvegicus GN=Ckb PE=1 SV=2
	Tubulin beta-3 chain OS=Rattus norvegicus GN=Tubb3 PE=1 SV=1
	Tubulin beta-5 chain OS=Rattus norvegicus GN=Tubb5 PE=1 SV=1
	Tubulin beta-2A chain OS=Rattus norvegicus GN=Tubb2a PE=1 SV=1
	Ras-related protein Rap-2b OS=Rattus norvegicus GN=Rap2b PE=2 SV=1
2336v	CB1 cannabinoid receptor-interacting protein 1 OS=Rattus norvegicus GN=Cnrip1 PE=1 SV=1

I reported in the following pages the identified proteins with the relative functions.

KCIP-1 (14-3-3 protein beta/alpha)

KCIP-1, also known as prepronerve growth factor RNH-1 or protein kinase C inhibitor protein 1, is an adapter protein involved in the regulation of signalling pathways. It binds to a large number of partners, usually by recognition of a phosphoserine or phosphothreonine motif and, generally, modulating the activity of the binding partner. KCIP-1 is also a negative regulator of osteogenesis. It blocks the nuclear translocation of the phosphorylated form by AKT1 of SRPK2 and antagonizes its stimulatory effect on cyclin D1 expression, blocking the neuronal apoptosis elicited by SRPK2.

Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A beta isoform

PR65 subunit of protein phosphatase 2A, is a scaffolding molecule which coordinates the assembly of the catalytic subunit and a variable regulatory B subunit.

Aconitate-hydratase, mitochondrial

This protein is involved in the subpathway that synthesizes isocitrate from oxaloacetate.
This subpathway is part of the pathway tricarboxylic acid cycle, which is itself part of Carbohydrate metabolism.

Actin, cytoplasmic 1 and 2

Highly conserved proteins involved in various types of cell motility and ubiquitously expressed in all eukaryotic cells.

Serum albumin

Serum albumin, the main plasma protein, has a good binding capacity for water, Ca^{2+}, Na^{+}, K^{+}, fatty acids, hormones, bilirubin and drugs. It regulates, as main function, the colloidal osmotic pressure of blood. It is the major zinc transporter in plasma and typically binds about 8 ALDOA_RAT.

Fructose-bisphosphate aldolase A

Fructose-bisphosphate aldolase A plays a key role in glycolysis and gluconeogenesis.
In addition, may also function as scaffolding protein.

Annexin A3

Inhibitor of phospholipase A2, also possesses anti-coagulant properties.

Annexin A5

Annexin A5 is an anticoagulant protein that acts as an indirect inhibitor of the thromboplastin-specific complex, which is involved in the blood coagulation cascade.

Argininosuccinate synthase

Is indirectly involved in the control of blood pressure.

Synthase subunit alpha/beta, mitochondrial

Mitochondrial membrane ATP synthases (F_1F_0 ATP synthase or Complex V), produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain F-type.
ATPases consist of two structural domains, F_1 - containing the extramembraneous catalytic core, and F_0 - containing the membrane proton channel, linked together. During catalysis, ATP synthesis in the catalytic domain of F_1 is coupled via a rotary mechanism of the central stalk subunits to proton translocation.
These two subunits, alpha and beta, form the catalytic core in F_1.

Lys-63-specific deubiquitinase BRCC36

Metalloprotease that specifically cleaves 'Lys-63'- linked polyubiquitin chains. It is component of the BRCA1-A complex, a complex that specifically recognizes 'Lys-63'-linked ubiquitinated histones H2A and H2AX at DNA lesions sites, leading to target the BRCA1-BARD1 heterodimer to sites of DNA damage at double-strand breaks (DSBs).
In the BRCA1-A complex, it specifically removes 'Lys-63'-linked ubiquitin on histones H2A and H2AX, antagonizing the RNF8-dependent ubiquitination at double-strand breaks (DSBs).
BRCC36 is the catalytic subunit of the multiprotein complex BRISC that cleaves 'Lys-63'-linked ubiquitin in various substrates. It mediates the specific 'Lys-63'-specific deubiquitination associated with the COP9 signalosome complex (CSN), via the interaction of the BRISC complex with the CSN complex.

Calbindin

Calbindin buffers cytosolic calcium. It may stimulate a membrane Ca^{2+}-ATPase and a 3', 5'-cyclic nucleotide phosphodiesterase.

F-actin capping protein subunit beta

F-actin-capping proteins binds in a Ca^{2+}- independent manner to actin filaments, blocking the exchange of subunits at these ends. Unlike other capping proteins such as gelsolin and severin, these proteins do not sever actin filaments. It regulates cell morphology and cytoskeletal organization.

60 kDa heat shock protein, mitochondrial

60 kDa heat shock protein, mitochondrial is implicated in mitochondrial protein import and macromolecular assembly. May facilitate the correct folding of imported proteins. May also prevent misfolding and promote the refolding and proper assembly of unfolded polypeptides generated under stress conditions in the mitochondrial matrix.

Citrate synthase, mitochondrial

Involved in Citrate synthesis, it is also involved in the pathway of tricarboxylic acid cycle. This protein is involved in the subpathway that synthesizes isocitrate from oxaloacetate that is part of the pathway tricarboxylic acid cycle, which is it part of carbohydrate metabolism.

CB1 Cannabinoid receptor –interacting protein 1

It suppresses cannabinoid receptor CNR1-mediated tonic inhibition of voltage-gated calcium channels.

Cofilin-1

Cofilin-1 binds to F-actin and exhibits pH-sensitive F-actin depolymerizing activity. It regulates actin cytoskeleton dynamics and it plays a role in the regulation of cell morphology and cytoskeletal organization.

Required for the up-regulation of atypical chemokine receptor ACKR2 from endosomal compartment to cell membrane, it increases its efficiency in chemokine uptake and degradation.

Coronin-1A

May be a crucial component of the cytoskeleton of highly motile cells, involved both in the invagination of large pieces of plasma membrane, as well as in forming protrusions of the plasma membrane involved in cell locomotion.

Coactosin-like protein

Binds to F-actin in a calcium-independent manner. It acts as a chaperone for ALOX5 (5LO), influencing both its stability and activity in leukotrienes synthesis.

Complexin-1

Complexin-1 positively regulates a late step in synaptic vesicle exocytosis. It organizes the SNAREs into a cross-linked zigzag topology that, when interposed between the vesicle and plasma membranes, is incompatible with fusion, thereby preventing SNAREs from releasing neurotransmitters until an action potential arrives at the synapse. It is also involved in glucose-induced secretion of insulin by pancreatic beta-cells.

Complexin-2

Complexin-2 negatively regulates the formation of synaptic vesicle clustering at active zone to the presynaptic membrane in postmitotic neurons. It positively regulates a late step in synaptic vesicle exocytosis. It's also involved in mast cell exocytosis.

Lambda-crystallin homolog

It exhibits L-gulonate 3-dehydrogenase activity and NAD+ binding. Lambda-crystallin homolog is involved in fatty acid metabolic process, oxidation-reduction process and it participates in pentose and glucuronate interconversion pathway.

C-terminal-binding protein 1

It is a corepressor, targeting diverse transcription regulators such as GLIS2 or BCL6. It has dehydrogenase activity and it’s involved in controlling the equilibrium between tubular and stacked structures in the Golgi complex.
It functions also in brown adipose tissue differentiation.

Dihydropyrimidinase-related protein 1

Necessary for signalling by class 3 semaphorins and subsequent remodelling of the cytoskeleton. It plays a role in axon guidance, invasive growth and cell migration and it may participate in cytokinesis.

Dynamin-1

Dynamin-1 is a microtubule-associated force-producing protein involved in producing microtubule bundles and able to bind and hydrolyze GTP. It is most probably involved in vesicular trafficking processes. Involved in receptor-mediated endocytosis.

Enoyl-CoA hydratase, mitochondrial

This protein is involved in the pathway fatty acid beta-oxidation, which is part of lipid metabolism.

EF-hand domain-containing protein D2

It may regulate B-cell receptor (BCR)-induced immature and primary B-cell apoptosis. It plays a role as negative regulator of the canonical NF-kappa-B-activating branch. It controls spontaneous apoptosis through the regulation of BCL2L1 abundance.

Alpha-enolase

Multifunctional enzyme that, as well as its role in glycolysis, plays a part in growth control, hypoxia tolerance and allergic responses. It may also function in the intravascular and pericellular fibrinolytic system due to its ability to serve as a receptor and activator of plasminogen on the cell surface of several cell-types such as leukocytes and neurons.
It stimulates immunoglobulin production.

Glyceraldehyde-3-phosphate dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase has both glyceraldehyde-3-phosphate dehydrogenase and nitrosylase activities, thereby playing a role in glycolysis and nuclear functions, respectively. Glyceraldehyde-3-phosphate dehydrogenase is a key enzyme in glycolysis that catalyzes the first step of the pathway by converting D-glyceraldehyde 3-phosphate (G3P) into 3-phospho-D-glyceroyl phosphate. It modulates the organization and assembly of the cytoskeleton and it facilitates the CHP1-dependent microtubule and membrane associations through its ability to stimulate the binding of CHP1 to microtubules.

In addition, it also participates in nuclear events including transcription, RNA transport, DNA replication and apoptosis. Nuclear functions are probably due to the nitrosylase activity that mediates cysteine S-nitrosylation of nuclear target proteins such as SIRT1, HDAC2 and PRKDC. It is component of the GAIT (gamma interferon-activated inhibitor of translation) complex which mediates interferon-gamma-induced transcript-selective translation inhibition in inflammation processes. Upon interferon-gamma treatment assembles into the GAIT complex which binds to stem loop-containing GAIT elements in the 3'-UTR of diverse inflammatory mRNAs (such as ceruplasmin) and suppresses their translation.

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1

Guanine nucleotide-binding proteins (G proteins) are involved as a modulator or transducer in various transmembrane signalling systems. The beta and gamma chains are required for the GTPase activity, for replacement of GDP by GTP, and for G protein-effector interaction.

Rho GDP-dissociation inhibitor

It controls Rho proteins homeostasis and it regulates the GDP/GTP exchange reaction of the Rho proteins by inhibiting the dissociation of GDP from them, and the subsequent binding of GTP to them. It retains Rho proteins such as CDC42, RAC1 and RHOA in an inactive cytosolic pool, regulating their stability and protecting them from degradation. Actively involved in the recycling and distribution of activated Rho GTPases in the cell, it mediates extraction from membranes of both inactive and activated molecules due its exceptionally high affinity for prenylated forms. Through the modulation of Rho proteins, it may play a role in cell motility regulation. In glioma cells, inhibits cell migration and invasion by mediating the signals of SEMA5A and PLXNB3 that lead to inactivation of RAC1.

Glial fibrillary acidic protein

GFAP, a class-III intermediate filament, is a cell-specific marker that, during the development of the central nervous system, distinguishes astrocytes from other glial cells.

Glutamine synthetase

Glutamine synthetase is essential for fetal skin fibroblasts proliferation. This enzyme has 2 functions: it catalyzes the production of glutamine and 4-aminobutanoate (gamma-aminobutyric acid, GABA).

Guanine nucleotide-binding protein G (o) subunit alpha

Guanine nucleotide-binding proteins (G proteins) are involved as modulators or transducers in various transmembrane signalling systems. The G (o) protein function is not clear.

Glycerol-3-phosphate dehydrogenase [NAD (+)]; cytoplasmic

NAD-dependent glycerol-3-phosphate dehydrogenase (GPD) catalyzes the reversible reduction of dihydroxyacetone phosphate to glycerol-3-phosphate. It is a cytoplasmic protein, active as a homodimer, where each monomer contains an N-terminal NAD binding site.

Haloacid dehalogenase-like hydrolase domain-containing protein 2

The HAD-like hydrolase superfamily include phosphatases, phosphonatases, P-type ATPases, beta-phosphoglucomutases, phosphomannomutases, and dehalogenases, which are involved in a variety of cellular processes ranging from amino acid biosynthesis to detoxification.

Hippocalcin-like protein 1

It may be involved in the calcium-dependent regulation of rhodopsin phosphorylation.

Hyaluronan and proteoglycan link protein 1

It stabilizes the aggregates of proteoglycan monomers with hyaluronic acid in the extracellular cartilage matrix.

Hexokinase-1

It is an allosteric enzyme inhibited by its product Glc-6-P. This protein is involved in the pathway hexose metabolism, which is part of carbohydrate metabolism.

Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial

Isocitrate dehydrogenase (IDH) is an enzyme that catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate (α-ketoglutarate) and CO_2. This is a two-step process, which involves oxidation of isocitrate (a secondary alcohol) to oxalosuccinate (a ketone), followed by the decarboxylation of the carboxyl group beta to the ketone, forming alpha-ketoglutarate.

Eukaryotic translation initiation factor 5A-1

mRNA-binding protein involved in translation elongation. It has an important function at the level of mRNA turnover, probably acting downstream of decapping. Involved in actin dynamics and cell cycle progression, mRNA decay and probably in a pathway involved in stress response and maintenance of cell wall integrity. With syntenin SDCBP, functions as a regulator of p53/TP53 and p53/TP53-dependent apoptosis. It regulates also TNF-alpha-mediated apoptosis and it mediates effects of polyamines on neuronal process extension and survival. It may play an important role in brain development and function, and in skeletal muscle stem cell differentiation.

Adenylate kinase 4, mitochondrial

Involved in maintaining the homeostasis of cellular nucleotides by catalyzing the interconversion of nucleoside phosphates. It phosphorylates efficiently AMP and dAMP using ATP as phosphate donor, but it phosphorylates only AMP when using GTP as phosphatedonor. It also displays broad nucleoside diphosphate kinase activity.

Creatine kinase B-type and U-type, mitochondrial

It reversibly catalyzes the transfer of phosphate between ATP and various phosphogens (e.g. creatine phosphate). Creatine kinase isoenzymes play a central role in energy transduction in tissues with large, fluctuating energy demands, such as skeletal muscle, heart, brain and spermatozoa.

Pyruvate kinase PKM

Glycolytic enzyme that catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, generating ATP. Stimulates POU5F1-mediated transcriptional activation. It is involved in the subpathway that synthesizes pyruvate from D-glyceraldehyde3-phosphate.

LIM and SH3 domain protein 1

It plays an important role in the regulation of dynamic actin-based, cytoskeletal activities. Agonist-dependent changes in LASP1 phosphorylation; it may also regulates actin-associated ion transport activities, not only in the parietal cell but also in certain other F-actin-rich secretory epithelial cell types.

Leucine-rich glioma-inactivated protein 1

It plays a role in suppressing the production of MMP1/3 through the phosphatidylinositol 3-kinase/ERK pathway. It regulates voltage-gated potassium channels assembled from KCNA1, KCNA4 and KCNAB1. It slows down channel inactivation by precluding channel closure mediated by the KCNAB1 subunit. Ligand for ADAM22 that positively regulates synaptic transmission mediated by AMPA-type glutamate receptors.

Malate dehydrogenase, cytoplasmic

It reversibly catalyzes the oxidation of malate to oxalacetate using the reduction of NAD^+ to NADH. It's also involved in gluconeogenesis.

Nucleoside diphosphate kinase A

Major role in the synthesis of nucleoside triphosphates other than ATP. The ATP gamma phosphate is transferred to the NDP beta phosphate using a phosphorylated active-site intermediate. It possesses nucleoside-diphosphate kinase, serine/threonine-specific protein kinase, geranyl and farnesyl pyrophosphate kinase, histidine protein kinase and 3'-5' exonuclease activities. It's involved in cell proliferation, differentiation and development, signal transduction, G protein-coupled receptor endocytosis, and gene expression. Required for neural development including neural patterning and cell fate determination.

Nucleoside diphosphate kinase B

Major role in the synthesis of nucleoside triphosphates other than ATP. The ATP gamma phosphate is transferred to the NDP beta phosphate via a ping-pong mechanism, using a phosphorylated active-site intermediate. It negatively regulates Rho activity by interacting with AKAP13/LBC. It exhibits histidine protein kinase activity.

NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 10, mitochondrial

Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), that is believed not to be involved in catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone.

Adaptin ear-binding coat-associated protein 1

Involved in endocytosis.

Cysteine desulfurase, mitochondrial

It catalyzes the removal of elemental sulphur from cysteine to produce alanine. It supplies the inorganic sulphur for iron-sulphur (Fe-S) clusters. It may be involved in the biosynthesis of molybdenum cofactor.

Vescicle-fusing ATPase

Required for vesicle-mediated transport. Catalyzes the fusion of transport vesicles within the Golgi cisternae. Is also required for transport from the endoplasmic reticulum to the Golgi stack. Seems to function as a fusion protein required for the delivery of cargo proteins to all compartments of the Golgi stack independent of vesicle origin. Interaction with AMPAR subunit GRIA2 leads to influence GRIA2 membrane cycling.

Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial

The 2-oxoglutarate dehydrogenase complex catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and CO_2. It contains multiple copies of 3 enzymatic components: 2-oxoglutarate dehydrogenase (E1), dihydrolipoamide succinyltransferase (E2) and lipoamide dehydrogenase (E3).
This protein is involved in the subpathway that synthesizes glutaryl-CoA from L-lysine.

Pyruvate dehydrogenase E1 component subnit alpha, somatic form, mitochondrial

The pyruvate dehydrogenase complex catalyzes the conversion of pyruvate to acetyl-CoA and CO_2, and thereby links the glycolytic pathway to the tricarboxylic cycle.

Protein deglycase DJ-1

Protein deglycase DJ-1 repairs methylglyoxal- and glyoxal-glycated amino acids and proteins, and releases repaired proteins and lactate or glycolate, respectively. It deglycates cysteines, arginines and lysines residues in proteins, and thus reactivates these proteins by reversing glycation by glyoxals. It acts on early glycation intermediates (hemithioacetals and aminocarbinols), preventing the formation of advanced glycation endproducts (AGE). It plays an important role in cell protection against oxidative stress and cell death acting as oxidative stress sensor and redox-sensitive chaperone and protease; functions probably related to its primary function. It is involved in neuroprotective mechanisms like the stabilization of NFE2L2 and PINK1 proteins, male fertility as a positive regulator of androgen signaling pathway as well as cell growth and transformation through, for instance, the modulation of NF-kappa-B signaling pathway. Its involvement in protein repair could also explain other unrelated functions. Eliminates hydrogen peroxide and protects cells against hydrogen peroxide-induced cell death. Required for correct mitochondrial morphology and function as well as for autophagy of dysfunctional mitochondria. The protein plays a role in regulating expression or stability of the mitochondrial uncoupling proteins SLC25A14 and SLC25A27 in dopaminergic neurons of the substantia nigra pars compacta and attenuates the oxidative stress induced by calcium entry into the neurons via L-type channels during pacemaking. Regulates astrocyte inflammatory responses, may modulate lipid rafts-dependent endocytosis in astrocytes and neuronal cells. Binds to a number of mRNAs containing multiple copies of GG or CC motifs and partially inhibits their translation but dissociates following oxidative stress. Metal-binding protein able to bind copper as well as toxic mercury ions, enhances the cell protection mechanism against induced metal toxicity.

PkB kinase (3-phosphoinositide-dependent protein kinase 1)

Serine/threonine kinase which acts as a master kinase, phosphorylating and activating a subgroup of the AGC family of protein kinases. Its targets include: protein kinase B (PKB/AKT1, PKB/AKT2, PKB/AKT3), p70 ribosomal protein S6 kinase (RPS6KB1), p90 ribosomal protein S6 kinase (RPS6KA1, RPS6KA2 and RPS6KA3), cyclic AMP-dependent protein kinase (PRKACA), protein kinase C (PRKCD and PRKCZ), serum and glucocorticoid-inducible kinase (SGK1, SGK2 and SGK3), p21-activated kinase-1 (PAK1), protein kinase PKN (PKN1 and PKN2). It plays a central role in the transduction of signals from insulin by providing the activating phosphorylation to PKB/AKT1, thus propagating the

signal to downstream targets controlling cell proliferation and survival, as well as glucose and amino acid uptake and storage. The protein negatively regulates the TGF-beta-induced signaling by: modulating the association of SMAD3 and SMAD7 with TGF-beta receptor, phosphorylating SMAD2, SMAD3, SMAD4 and SMAD7, preventing the nuclear translocation of SMAD3 and SMAD4 and the translocation of SMAD7 from the nucleus to the cytoplasm in response to TGF-beta. Activates PPARG transcriptional activity and promotes adipocyte differentiation. It activates the NF-kappa-B pathway via phosphorylation of IKKB. The tyrosine phosphorylated form is crucial for the regulation of focal adhesions by angiotensin II. Controls proliferation, survival, and growth of developing pancreatic cells. Participates in the regulation of Ca^{2+} entry and Ca^{2+}-activated K^{+} channels of mast cells.

Essential for the motility of vascular endothelial cells (ECs) and is involved in the regulation of their chemotaxis. Plays a critical role in cardiac homeostasis by serving as a dual effector for cell survival and beta-adrenergic response. Plays an important role during thymocyte development by regulating the expression of key nutrient receptors on the surface of pre-T cells and mediating Notch-induced cell growth and proliferative responses. Provides negative feedback inhibition to toll-like receptor-mediated NF-kappa-B activation in macrophages.

Phosphatidylethanolamine-binding protein 1

It binds ATP, opioids and phosphatidylethanolamine.
Has lower affinity for phosphatidylinositol and phosphatidylcholine. Serine protease inhibitor which inhibits thrombin, neuropsin and chymotrypsin but not trypsin, tissue type plasminogen activator and elastase. It inhibits the kinase activity of RAF1 by inhibiting its activation and by dissociating the RAF1/MEK complex and acting as a competitive inhibitor of MEK phosphorylation.
HCNP may be involved in the function of the presynaptic cholinergic neurons of the central nervous system. HCNP increases the production of choline acetyltransferase but not acetylcholinesterase. Seems to be mediated by a specific receptor.

Phosphoglycerate mutase 1

Involved in the interconversion of 3- and 2-phosphoglycerate with 2,3-bisphosphoglycerate as the primer of the reaction. It can also catalyze the reaction of EC 5.4.2.4 (synthase) and EC 3.1.3.13 (phosphatase), but with a reduced activity.

Phosphoglycerate kinase 1

Kinase enzyme important for phosphoprotein glycolysis. This protein is involved in step 2 of the subpathway that synthesizes pyruvate from D-glyceraldehyde 3-phosphate.

Serine/threonine-protein phosphatase PP1-alpha catalytic subunit

Protein phosphatase that associates with over 200 regulatory proteins to form highly specific holoenzymes which dephosphorylate hundreds of biological targets. Protein phosphatase 1 (PP1) is essential for cell division, and participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. It's involved in regulation of ionic conductances and long-term synaptic plasticity. It may play an important role in dephosphorylating substrates such as the postsynaptic density-associated Ca^{2+}/calmodulin dependent protein kinase II. Component of the PTW/PP1 phosphatase complex, which plays a role in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase. It regulates NEK2 function in terms of kinase activity and centrosome number and splitting, both in the presence and absence of radiation-induced DNA damage. Regulator of neural tube and optic fissure closure, and enteric neural crest cell (ENCCs) migration during development. In balance with CSNK1D and CSNK1E, determines the circadian period length, through the regulation of the speed and rhythmicity of PER1 and PER2 phosphorylation. May dephosphorylate CSNK1D and CSNK1E.

Serine/threonine-protein phosphatase PP1-beta catalytic subunit

Protein phosphatase that associates with over 200 regulatory proteins to form highly specific holoenzymes which dephosphorylate hundred of biological targets. Protein phosphatase (PP1) is essential for cell division, it participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. Involved in regulation of ionic conductances and long-term synaptic plasticity. Component of the PTW/PP1 phosphatase complex, which plays a role in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase. In balance with CSNK1D and CSNK1E, it determines the circadian period length, through the regulation of the speed and rhythmicity of PER1 and PER2 phosphorylation. It may dephosphorylate CSNK1D and CSNK1E.

Serine/threonine-protein phosphatase PP1-gamma catalytic subunit

Protein phosphatase that associates with over 200 regulatory proteins to form highly specific holoenzymes which dephosphorylate hundred of biological targets. Protein phosphatase 1 (PP1) is essential for cell division, and participates in the regulation of glycogen metabolism, muscle contractility and protein synthesis. Dephosphorylates RPS6KB1.

Involved in regulation of ionic conductances and long-term synaptic plasticity. It may play an important role in dephosphorylating substrates such as the postsynaptic density-associated Ca^{2+}/calmodulin dependent protein kinase II. Component of the PTW/PP1 phosphatase complex, which plays a role in the control of chromatin structure and cell cycle progression during the transition from mitosis into interphase. In balance with CSNK1D and CSNK1E, determines the circadian period length, through the regulation of the speed and rhythmicity of PER1 and PER2 phosphorylation. May dephosphorylate CSNK1D and CSNK1E.

Peroxiredoxin-2

Involved in redox regulation of the cell. Reduces peroxides with reducing equivalents provided through the thioredoxin system. It may play an important role in eliminating peroxides generated during metabolism. Might participate in the signaling cascades of growth factors and tumor necrosis factor-alpha by regulating the intracellular concentrations of H_2O_2.

Thioredoxin-dependent peroxide reductase, mitochondrial

Involved in redox regulation of the cell. It protects radical-sensitive enzymes from oxidative damage by a radical-generating system. Acts synergistically with MAP3K13 to regulate the activation of NF-kappa-B in the cytosol.

Peroxiredoxin-5, mitochondrial

It reduces hydrogen peroxide and alkyl hydroperoxides with reducing equivalents provided through the thioredoxin system. Involved in intracellular redox signaling.

Ribose-phosphate pyrophosphokinase 1

It catalyzes the synthesis of phosphoribosylpyrophosphate (PRPP) that is essential for nucleotide synthesis.

Proteasome subunit beta type-4

The proteasome is a multicatalytic proteinase complex which is characterized by its ability to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. The proteasome has an ATP-dependent proteolytic activity. SMAD1/OAZ1/PSMB4 complex mediates the degradation of the CREBBP/EP300 repressor SNIP1.

Bifunctional purine biosynthesis protein PURH

Bifunctional enzyme involved in purine biosynthesis. Promotes insulin receptor/INSR autophosphorylation and is involved in INSR internalization.

Cytochrome b-c1 complex subunit 2, mitochondrial

This is a component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is part of the mitochondrial respiratory chain.

The core protein 2 is required for the assembly of the complex.

Ras-related protein Rab-14

Regulates, together with its guanine nucleotide exchange factor, DENND6A, the specific endocytic transport of ADAM10, N-cadherin/CDH2 shedding and cell-cell adhesion.

Involved in membrane trafficking between the Golgi complex and endosomes during early embryonic development. It regulates the Golgi to endosome transport of FGFR-containing vesicles during early development, a key process for developing basement membrane and epiblast and primitive endoderm lineages during early postimplantation development. May act by modulating the kinesin KIF16B-cargo association to endosomes.

Ras-related protein Rab-3A

Involved in exocytosis by regulating a late step in synaptic vesicle fusion. Could play a role in neurotransmitter release by regulating membrane flow in the nerve terminal.

Ras-related protein Rab-5A

The small GTPases Rab are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes. Rabs cycle between an inactive GDP-bound form and an active GTP-bound form that is able to recruit to membranes

different sets of downstream effectors directly responsible for vesicle formation, movement, tethering and fusion. RAB5A is required for the fusion of plasma membranes and early endosomes. Contributes to the regulation of filopodia extension.

Ras-related protein Rab-8B

The small GTPases Rab are key regulators of intracellular membrane trafficking, from the formation of transport vesicles to their fusion with membranes.
Rabs cycle between an inactive GDP-bound form and an active GTP-bound form that is able to recruit to membranes different sets of downstream effectors directly responsible for vesicle formation, movement, tethering and fusion. That Rab may be involved in polarized vesicular trafficking and neurotransmitter release. May participate in cell junction dynamics in Sertoli cells.

Ras-related protein Ral-A

Multifunctional GTPase involved in a variety of cellular processes including gene expression, cell migration, cell proliferation, oncogenic transformation and membrane trafficking. It accomplishes its multiple functions by interacting with distinct downstream effectors. Acts as a GTP sensor for GTP-dependent exocytosis of dense core vesicles. It plays a role in the early stages of cytokinesis and is required to tether the exocyst to the cytokinetic furrow. The RALA-exocyst complex regulates integrin-dependent membrane raft exocytosis and growth signaling. Key regulator of LPAR1 signaling and competes with ADRBK1 for binding to LPAR1 thus affecting the signaling properties of the receptor. Required for anchorage-independent proliferation of transformed cells.

Ras-related protein Rap-2b

Small GTP-binding protein which cycles between a GDP-bound inactive and a GTP-bound active form. Involved in EGFR and CHRM3 signaling pathways through stimulation of PLCE1. It may play a role in cytoskeletal rearrangements and regulate cell spreading through activation of the effector TNIK. May regulate membrane vesiculation in red blood cells.

Rho-related GTP-binding protein RhoB

It mediates apoptosis in neoplastically transformed cells after DNA damage.
It plays a negative role in tumorigenesis as deletion causes of tumor formation. It is involved in intracellular protein trafficking of a number of proteins. Targets PKN1 to endosomes and is involved in trafficking of the EGF receptor from late endosomes to lysosomes.
It is also required for stability and nuclear trafficking of AKT1/AKT which promotes endothelial cell survival during vascular development.
It serves as a microtubule-dependent signal that is required for the myosin contractile ring formation during cell cycle cytokinesis. Required for genotoxic stress-induced cell death in breast cancer cells.

Rabphilin-3A

Involved in protein transport. Probably involved with Ras-related protein Rab-3A in synaptic vesicle traffic and/or synaptic vesicle fusion. It could play a role in neurotransmitter release by regulating membrane flow in the nerve terminal.

Solute carrier family 12 member 5

It mediates electroneutral, potassium-chloride cotransport in mature neurons. Important for Cl-homeostasis in neurons. It is necessary for the ontogenic change in response to gamma-aminobutyric acid (GABA) from depolarization to hyperpolarization during neuronal development.

Secretogranin-1

Secretogranin-1 is a neuroendocrine secretory granule protein, which may be the precursor for other biologically active peptides.

Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial

Flavoprotein (FP) subunit of succinate dehydrogenase (SDH) that is involved in complex II of the mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (coenzyme Q). Can act as a tumor suppressor. This protein is involved in the synthesis of fumarate from succinate (eukaryal route).

Selenocysteine insertion sequence-binding protein 2

It binds to the SECIS element in the 3'-UTR of some mRNAs encoding selenoproteins. Binding is stimulated by SELB.

Septin 11

Filament-forming cytoskeletal GTPase. It may play a role in cytokinesis (potential) and a role in the cytoarchitecture of neurons, including dendritic arborization and dendritic spines, and in GABAergic synaptic connectivity.

Protein SGT1 homolog

It may play a role in ubiquitination and subsequent proteasomal degradation of target proteins.

Neuronal-specific septin-3

Filament-forming cytoskeletal GTPase. It may play a role in cytokinesis (Potential).

Septin-5

Filament-forming cytoskeletal GTPase. May play a role in cytokinesis (Potential). It may play a role in platelet secretion.

Endophilin-A1

Endophilin-A1 is implicated in synaptic vesicle endocytosis. It may recruit other proteins to membranes with high curvature.

NAD-dependent protein deacetylase sirtuin-2

NAD-dependent protein deacetylase, which deacetylates internal lysines on histone and alpha-tubulin and many other proteins such as key transcription factors. It participates in the modulation of multiple and diverse biological processes such as cell cycle control, genomic integrity, microtubule dynamics, cell differentiation, metabolic networks, and autophagy. It plays a major role in the control of cell cycle progression and genomic stability. In the antephase checkpoint it prevents precocious mitotic entry in response to microtubule stress agents, and hence allowing proper inheritance of chromosomes. It positively regulates the anaphase promoting complex/cyclosome (APC/C) ubiquitin ligase complex activity by deacetylating CDC20 and FZR1, then allowing progression through mitosis. It associates with both chromatin at transcriptional start sites (TSSs) and it is enhancers of active genes. NAD-

dependent protein deacetylase sirtuin-2 plays a role in cell cycle and chromatin compaction through epigenetic modulation of the regulation of histone H4 'Lys-20' methylation (H4K20me1) during early mitosis. It specifically deacetylates histone H4 at 'Lys-16' (H4K16ac) between the G2/M transition and metaphase enabling H4K20me1 deposition by SETD8, leading to ulterior levels of H4K20me2 and H4K20me3 deposition throughout cell cycle, and mitotic S-phase progression. Deacetylates SETD8 modulating SETD8 chromatin localization during the mitotic stress response. Deacetylates also histone H3 at 'Lys-57' (H3K56ac) during the mitotic G2/M transition. During oocyte meiosis progression, it may deacetylate histone H4 at 'Lys-16' (H4K16ac) and alpha-tubulin, regulating spindle assembly and chromosome alignment by influencing microtubule dynamics and kinetochore function. It deacetylates alpha-tubulin at ‘Lys-40’ and hence controls neuronal motility, oligodendroglial cell arbor projection processes and proliferation of non-neuronal cells. Phosphorylation at Ser-330 by a G1/S-specific cyclin E-CDK2 complex inactivates SIRT2-mediated alpha-tubulin deacetylation, negatively regulating cell adhesion, cell migration and neurite outgrowth during neuronal differentiation. It deacetylates PARD3 and participates in the regulation of Schwann cell peripheral myelination formation during early postnatal development and during postinjury remyelination. Involved in several cellular metabolic pathways. It plays a role in the regulation of blood glucose homeostasis by deacetylating and stabilizing phosphoenolpyruvate carboxykinase PCK1 activity in response to low nutrient availability. Acts as a key regulator in the pentose phosphate pathway (PPP) by deacetylating and activating the glucose-6-phosphate G6PD enzyme, and therefore, stimulates the production of cytosolic NADPH to counteract oxidative damage.

Maintains energy homeostasis in response to nutrient deprivation as well as energy expenditure by inhibiting adipogenesis and promoting lipolysis. Attenuates adipocyte differentiation by deacetylating and promoting FOXO1 interaction to PPARG and subsequent repression of PPARG-dependent transcriptional activity. It plays a role in the regulation of lysosome-mediated degradation of protein aggregates by autophagy in neuronal cells. Deacetylates FOXO1 in response to oxidative stress or serum deprivation, thereby negatively regulating FOXO1-mediated autophagy. Deacetylates a broad range of transcription factors and co-regulators regulating target gene expression. Deacetylates transcriptional factor FOXO3 stimulating the ubiquitin ligase SCF(SKP2)-mediated FOXO3 ubiquitination and degradation. Deacetylates HIF1A and therefore promotes HIF1A degradation and inhibition of HIF1A transcriptional activity in tumor cells in response to hypoxia. Deacetylates RELA in

the cytoplasm inhibiting NF-kappaB-dependent transcription activation upon TNF-alpha stimulation. Inhibits transcriptional activation by deacetylating p53/TP53 and EP300. Deacetylates also EIF5A. It functions as a negative regulator on oxidative stress-tolerance in response to anoxia-reoxygenation conditions. Plays a role as tumor suppressor.

Synaptosomal-associated protein 25

t-SNARE involved in the molecular regulation of neurotransmitter release. It modulates the gating characteristics of the delayed rectifier voltage-dependent potassium channel KCNB1 in pancreatic beta cells. It may play an important role in the synaptic function of specific function of specific neuronal systems. It associates with proteins involved in vesicle docking and membrane fusion. Regulates plasma membrane recycling through its interaction with CENPF.

Superoxide dismutase [Cu-Zn]

Destroys radicals which are normally produced within the cells and which are toxic to biological systems.

Spectrin alpha chain, non-erythrocytic 1

Fodrin, which seems to be involved in secretion, interacts with calmodulin in a calcium-dependent manner and is thus candidate for the calcium-dependent movement of the cytoskeleton at the membrane.

Syntaxin-1A

It plays a role in hormone and neurotransmitter exocytosis. It is potentially involved in docking of synaptic vesicles at presynaptic active zones. May mediate Ca^{2+}-regulation of exocytosis acrosomal reaction in sperm.

Syntaxin-binding protein 1

It may participate in the regulation of synaptic vesicle docking and fusion, possibly through interaction with GTP-binding proteins. Essential for neurotransmission and binds syntaxin, a component of the synaptic vesicle fusion machinery probably in a 1:1 ratio. Can interact with syntaxins 1, 2, and 3. May play a role in determining the specificity of intracellular fusion reactions.

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Synapsin-2

Neuronal phosphoprotein that coats synaptic vesicles, binds to the cytoskeleton, and is believed to function in the regulation of neurotransmitter release. It may play a role in noradrenaline secretion by sympathetic neurons.

Synaptotagmin-1

It may have a regulatory role in the membrane interactions during trafficking of synaptic vesicles at the active zone of the synapse. It binds acidic phospholipids with a specificity that requires the presence of both an acidic head group and a diacyl backbone. A Ca^{2+}- dependent interaction between synaptotagmin and putative receptors for activated protein kinase C has also been reported. It can bind to at least three additional proteins in a Ca^{2+}-independent manner; these are neurexins, syntaxin and AP2. It plays a role in dendrite formation by melanocytes.

Transaldolase

Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway.

Tubulin alpha-1C chain and Tubulin beta-2A chain

Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha chain.

Tubulin beta-5 chain

Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia. Known to play a role, in vitro, in the folding of actin and tubulin.

T-complex protein 1 subunit gamma

Molecular chaperone; assists the folding of proteins upon ATP hydrolysis. As part of the BBS/CCT complex may play a role in the assembly of BBSome, a complex involved in ciliogenesis regulating transports vesicles to the cilia. Known to play a role, in vitro, in the folding of actin and tubulin. It plays a role in the assembly of the von Hippel-Lindau ubiquitination complex. Interacts with DYX1C1.

Acetyl-CoA acetyltransferase, mitochondrial

Plays a major role in ketone body metabolism.

Triosephosphate isomerase

This protein is involved in the pathway gluconeogenesis, which is part of carbohydrate biosynthesis. It is involved in the subpathway that synthesizes D-glyceraldehyde 3-phosphate from glycerone phosphate.

Ubiquitin-conjugating enzyme E2 variant 2

The UBE2V2/UBE2N heterodimer catalyzes the synthesis of non-canonical poly-ubiquitin chains that are linked through 'Lys-63'. This type of poly-ubiquitination does not lead to protein degradation by the proteasome. It mediates transcriptional activation of target genes. Plays a role in the control of progress through the cell cycle and differentiation. Plays a role in the error-free DNA repair pathway and contributes to the survival of cells after DNA damage.

Ubiquitin carboxyl-terminal hydrolase isozyme L1

Ubiquitin-protein hydrolase involved both in the processing of ubiquitin precursors and of ubiquitinated proteins. This enzyme is a thiol protease that recognizes and hydrolyzes a peptide bond at the C-terminal glycine of ubiquitin. It also binds to free monoubiquitin and may prevent its degradation in lysosomes. The homodimer may have ATP-independent ubiquitin ligase activity.

Ubiquitin carboxyl-terminal hydrolase isozyme L3

Deubiquitinating enzyme (DUB) that controls levels of cellular ubiquitin through processing of ubiquitin precursors and ubiquitinated proteins. Thiol protease that recognizes and hydrolyzes a peptide bond at the C-terminal glycine of either ubiquitin or NEDD8. Has a 10-fold preference for Arg and Lys at position P3, and exhibits a preference towards 'Lys-48'-

linked Ubiquitin chains". Deubiquitinates ENAC in apical compartments, thereby regulating apical membrane recycling. Indirectly increases the phosphorylation of IGFIR, AKT and FOXO1 and promotes insulin-signaling and insulin-induced adipogenesis. Required for stress-response retinal, skeletal muscle and germ cell maintenance.

May be involved in working memory. Can hydrolyze UBB(+1), a mutated form of ubiquitin which is not effectively degraded by the proteasome.

Vesicle-associated membrane protein 3

SNARE involved in vesicular transport from the late endosomes to the trans-Golgi network.

Voltage-dependent anion-selective channel protein 1

It forms a channel through the mitochondrial outer membrane and also the plasma membrane. The channel at the outer mitochondrial membrane allows diffusion of small hydrophilic molecules; in the plasma membrane it is involved in cell volume regulation and apoptosis. It adopts an open conformation at low or zero membrane potential and a closed conformation at potentials above 30-40 mV. The open state has a weak anion selectivity whereas the closed state is cation-selective. It may participate in the formation of the permeability transition pore complex (PTPC) responsible for the release of mitochondrial products that triggers apoptosis.

Voltage-dependent anion selective channel protein 2

It forms a channel through the mitochondrial outer membrane that allows diffusion of small hydrophilic molecules. The channel adopts an open conformation at low or zero membrane potential and a closed conformation at potentials above 30-40 mV. The open state has a weak anion selectivity whereas the closed state is cation-selective.

Visinin-like protein 1

Regulates (in vitro) the inhibition of rhodopsin phosphorylation in a calcium-dependent manner.

WD repeat-containing protein 1

It induces disassembly of actin filaments in conjunction with ADF/cofilin family proteins.

DISCUSSIONS:

Changes in protein levels were quantified using two-dimensional differential gel electrophoresis (2D-DIGE), using fluorophors, and the altered proteins were identified by tandem mass spectrometry (MS/MS). We identified proteins that positively regulate a late step in synaptic vesicle exocytosis. For example, we found Complexin-1, that prevents SNAREs (Soluble NSF Attachment Protein Receptor) that mediate docking of synaptic vescicles with the presynaptic membrane in neurons from releasing neurotransmitters until an action potential arrives at the synapse.

Complexin-2 was also identified. It negatively regulates the formation of synaptic vesicle clustering at active zone to the presynaptic membrane in postmitotic neurons.

We also founds proteins like protein **deglycase DJ-1,** that is involved in neuroprotective mechanisms like the stabilization of **nuclear factor erythroid 2-related factor 2**, transcription activator that binds to antioxidant response (ARE) elements in the promoter regions of target genes and important for the coordinated up-regulation of genes in response to oxidative stress, and **serine/threonine-protein kinase PINK1, mitochondrial**, protein that protects against mitochondrial dysfunction during cellular stress by phosphorylating mitochondrial proteins and involved in the clearance of damaged mitochondria via selective autophagy (mitophagy) by mediating activation and translocation of **E3 ubiquitin-protein ligase parkin**, that participates in the removal and/or detoxification of abnormally folded or damaged protein by mediating 'Lys-63'-linked polyubiquitination of misfolded proteins such as PARK7 ('Lys-63'-linked polyubiquitinated misfolded proteins are recognized by HDAC6, leading to their recruitment to aggresomes, followed by degradation); mediates 'Lys-48'-linked polyubiquitination of ZNF746, followed by degradation of ZNF746 by the proteasome possibly playing a role in neuron death regulation; regulates cyclin-E during neuronal apoptosis; may protect neurons against alpha synuclein toxicity, proteasomal dysfunction, GPR37 accumulation, and kainate-induced excitotoxicity; may play a role in the controll of neurotransmitter trafficking at the presynaptic terminal and in calcium-dependent exocytosis.

The identified protein, **PARK7**, plays also a role in regulating expression or stability of the mitochondrial uncoupling **brain mitochondrial carrier protein 1**; that participates in the mitochondrial proton leak measured in brain mitochondria and **mitochondrial uncoupling protein 4;** that creates proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis and may play a role in thermoregulatory heat production and metabolism in brain; in dopaminergic neurons of the

substantial nigra pars compacta and attenuates the oxidative stress induced by calcium entry into the neurons via L-type channels during pacemaking. Regulates astrocyte inflammatory responses, may modulate lipid rafts-dependent endocytosis in astrocytes and neuronal cells.

Another identified protein, **serine/threonine-protein phosphatase PP1-gamma catalytic subunit** is involved in regulation of ionic conductances and long-term synaptic plasticity. It may play an important role in dephosphorylating substrates such as the postsynaptic density-associated Ca^{2+}/calmodulin dependent protein kinase II.

Solute carrier family 12 member 5, mediates electroneutral potassium-chloride cotransport in mature neurons and it's also important for Cl-homeostasis in neurons and necessary for the ontogenic change in response to gamma-aminobutyric acid (GABA) from depolarization to hyperpolarization during neuronal development.

Secretogranin-1 is a neuroendocrine secretory granule protein, which may be the precursor for other biologically active peptides.

Septin 11, filament-forming cytoskeletal GTPase, may play a role in the cytoarchitecture of neurons, including dendritic arborization and dendritic spines, and in GABAergic synaptic connectivity.

Endophilin-A1, is implicated in synaptic vesicle endocytosis.

NAD-dependent protein deacetylase sirtuin-2 deacetylates alpha-tubulin at 'Lys-40' and hence controls neuronal motility, oligodendroglial cell arbor projection processes and proliferation of non-neuronal cells. Phosphorylation at Ser-330 by a G1/S-specific cyclin E-CDK2 complex inactivates SIRT2-mediated alpha-tubulin deacetylation, negatively regulating cell adhesion, cell migration and neurite outgrowth during neuronal differentiation. Deacetylates PARD3 and participates in the regulation of Schwann cell peripheral myelination formation during early postnatal development and during postinjury remyelination.

Synaptosomal-associated protein 25 may play an important role in the synaptic function of specific neuronal systems. Associates with proteins involved in vesicle docking and membrane fusion.

Syntaxin-binding protein 1 may participate in the regulation of synaptic vesicle docking and fusion, possibly through interaction with GTP-binding proteins.

It is essential for neurotransmission and binds syntaxin, a component of the synaptic vesicle fusion machinery.

Synapsin-2 is a neuronal phosphoprotein that coats synaptic vesicles, binds to the cytoskeleton, and is believed to function in the regulation of neurotransmitter release. May play a role in noradrenaline secretion by sympathetic neurons.

Synaptotagmin-1 may have a regulatory role in the membrane interactions during trafficking of synaptic vesicles at the active zone of the synapse and plays a role in dendrite formation by melanocytes.

The analysis of the pathways revealed that there is a decrease on a large scale of many proteins involved in synaptic transport and metabolism of pyruvate while in very few proteins has been found an increase at the end of the sleeping period, compared to the awake state.

The results revealed that proteins involved in synaptic transmission and metabolism are widely regulated by sleep.

There is a direct evidence of a synaptic decreasing and a decreased "robustness" of synaptic activity in the samples examined.

The data obtained support the synaptic maintenance hypothesis of sleep, according to which sleep is a process of synaptic homeostasis.

The results, in fact, provide a further evidence of an homeostatic process that takes place in the synapses of the cortex during sleep and it’s involved both in synaptic plasticity and in metabolic processes.

4.3: Two-Dimensional Difference In Gel Electrophoresis (2D-DIGE) For Milk Proteins Characterization In The Sicilian Native "Girgentana" Goat And"Valle Del Belice Ovine Breeds.

State Of The Art:

The field of biotechnology applied to livestock production is extensive, ranging from the genome analysis of livestock animals of particular economic interest to the use of molecular markers to improve the quality of breeds and livestock production, to kinship determination, individuals and breeds identification and animal products tracking and authentication, to animal biodiversity conservation through the use of molecular markers.

In this context, the importance of identify and characterize molecular markers that are "unique" (in reference to an animal breed) and "typical" (in reference to a particular breed product).

The growing problem in livestock production is to increase, as part of the modern concept of sustainable agricultural and forest ecosystems management, the organoleptic and safety products of animal origin, improving transformation technologies, reducing the prices and increasing quality production.

It is very important to protect traditional breeds because they might be a resource of genes for future selection works, with objectives of adaptation to adverse condition, diseases tolerance, etc.

From a zootechnical point of view, it should be emphasized the close link between the need to protect biodiversity of native species and the importance of produce local products, due to their unique physic-chemical, microbiological and organoleptic properties.

Actually, only few data are available concerning livestock breeds and, as a consequence, the available databases are not so rich; this represents a problem if we want to investigate animal breeds. The data that I'm going to describe and "summarize" are referred to the extent to produce the first data available in the context of Sicilian livestock breeds as precursor studies able to enrich the actual knowledge related to animal biodiversity and local animal production.

The aim of a research work was to compare protein profile of Girgentana breed whole bulk milk samples between different lactation periods and geographical areas by two dimensional difference in gel electrophoresis (2D-DIGE).

The current trend is to follow an "omic" strategy that uses biotechnologies to obtain typed local products by the identification and characterization of "molecular biomarkers" of" genetic uniqueness ", in the case of the individual, and of " specificity " if we refer to food products.

Research Objectives:

The aim of the research activities done in the university of Palermo was the characterization of autochthonous Sicilian breeds through the use of biotechnologies and, in particular, use of Proteomics methodologies applied to animal productions.

The purpose of my research project was the development of advanced biotechnology methods and protocols finalized to authentication, exploitation and consequent preservation of typical dairy production obtained from the raw materials of Sicilian livestock breeds, such as "Cinisara"(a bovine breed), "Comisana" (an ovine breed), “Girgentana" (a goat breed), which are distinguished from cosmopolitan breeds for their added value, given by the close link between local production, territory, tradition and Sicilian native breeds, by proteomic approaches.

4.3.1: Milk Proteins Characterization In The Sicilian Native "Girgentana" Goat Breed

Results And Discussion:

After an experimental set-up, using 2D-DIGE performances was possible to compare the results obtained when two different farms geographically distant (one farm located in Agrigento province and another one located in Palermo province) were considered.
The proteomic analysis was performed both considering the same sampling time and different time periods. At the same time, during the protomic analysis were taken into account sampling done in a unique farm considering different time periods.
After two-dimensional electrophoresis, several spots on the gels appeared as strings, demonstrating that isoforms of differing charge, resulting from post-translational modifications, are present in milk.
Gel image analysis was performed and only the most statistically significant spots were considered and among these, only the ones easy for the spot picking, isolated, were taken into account.
Protein spots showing more than 1.5 fold change in spot volume (increased for up-regulation or decreased for down-regulation), with a statistically significant ANOVA value ($P \leq 0.05$), were considered differentially abundant. Among these, a set of spots were excised from preparative gels and identified by tandem mass spectrometry by "Porto Conte" Research Center. To assign protein identity, data were filtered following the application of identification criteria based on number of unique peptides ≥ 2.
From the differential analysis at the first sampling time, nine spots were considered for protein identification (Figure 19):

β-lactoglobulin fragment (spot n. 207), whey proteins belonging to lipocalins, a family of proteins which transport small hydrophobic molecules such as steroids, bilins, retinoids and lipids; lipocalins have been associated with many biological processes: immune response, pherormone transport, prostaglandin synthesis, retinoid binding and cancer cell interactions.

fragments of K-casein (spots n. 381, 395, 414), protein involved in a number of important biological processes, including casein micelle stability that prevents casein precipitation in

milk. K-casein is located on the surface of the micelle, functioning as an interface between the hydrophobic caseins of the micelle and the aqueous environment. It is also involved in thiol-catalyzed disulfide interchange reactions with whey proteins during heat treatments and (after rennet cleavage) in the facilitation of micelle coagulation;

***keratin-like* proteins** (spots n. 433, 507, 789), structural proteins that represent the main components of hairs, wool, horns. They also protect epithelial cells from damage or stress conditions;

αS1-casein (spots n. 939, 982), protein that plays an important role in the transport of calcium phosphate. αS1-casein is also required for the export of the other caseins from the endoplasmic reticulum and it plays a foundamental role in casein micelle biogenesis and casein transport in the secretory pathway.

From the differential analysis comparing different sampling periods, six different spots have been chosen for the identification (Figure 20).

- **"milk fat globule EGF factor 8"** protein (spots n. 17 e 736), indicated as MFG-E8 and also known as lactadherin, pleiotropic secreted glycoprotein of fat globules membrane involved in many biological processes: mammary gland morphogenesis, angiogenesis, tumor progression, inhibition of blood coagulation, tissue homeostasis and prevention of inflammation. It contributes to debris clearance and tissue repair in a variety of situations. In addition, it limits disease progression by promoting the engulfment of apoptotic bodies in atherosclerotic plaques and prion-infected brain by macrophages and microglia, respectively. MFG-E8 also reduces inflammation and disease progression in colitis by preventing Osteopontin from binding and activating Integrin alpha V beta 3.
- **-β-lactoglobulin** (spot n. 282), the major whey protein belonging to lipocalin family involved in the transport of small hydrophobic molecules. β-lactoglobulin, over-expressed in the lactating mammary gland of many species is mainly an important source of amino acids for the offspring of those animals;
- **-β-casein fragments** (spot n.183 e 737), produced by β-casein proteolysis, protein of the casein micelle that plays a foundamental role in milk enzymatic activities;
- **a *keratin-like* protein** (spot n.426), structural protein mainly component of hairs, wool, horns.

From the differential analysis comparing different sampling areas (different farms), one spot has been chosen for the identification (Figure 21).

- **serum albumin** (spot n.27), plasma carrier able to bind several hydrophobic steroid hormones and transport protein for hemin and fatty acids.

Milk fat globule EGF factor 8 protein variants, β-lactoglobulin, β-casein and serum albumin were successfully identified. 2D-DIGE allowed giving a general picture of milk protein distributions over 3-10 pH range. These preliminary results in addition to the identification of others interesting spots will be used to generate a reference proteomics map for the Girgentana goat breed.

Figure 19: Proteomic map of "Girgentana" whole milk samples and detected spots from the differential analysis at the first sampling month, comparing two different sampling area.

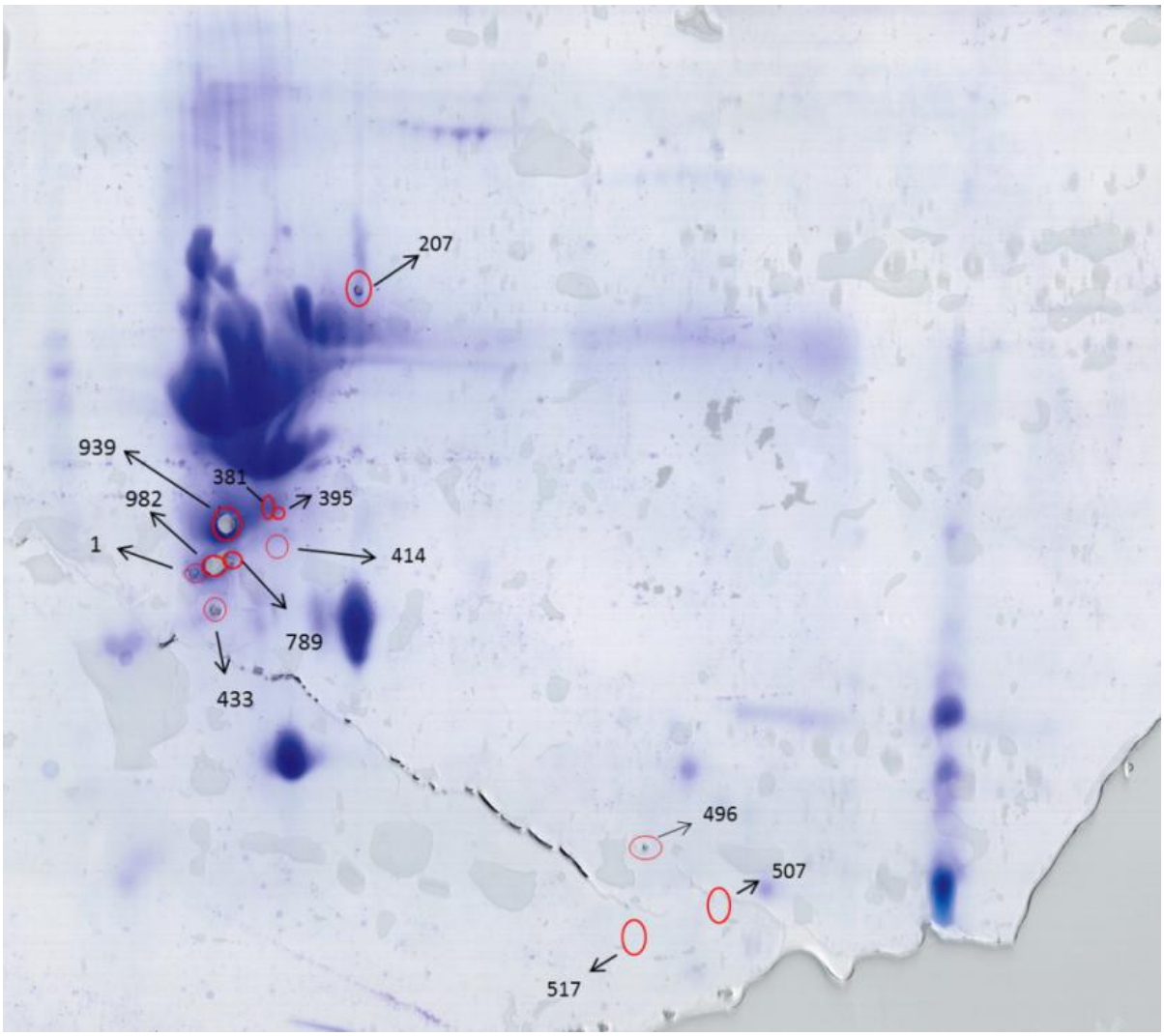


Figure 20: Proteomic map of “Girgentana” whole milk samples and detected spots from the differential analysis comparing different sampling time points.

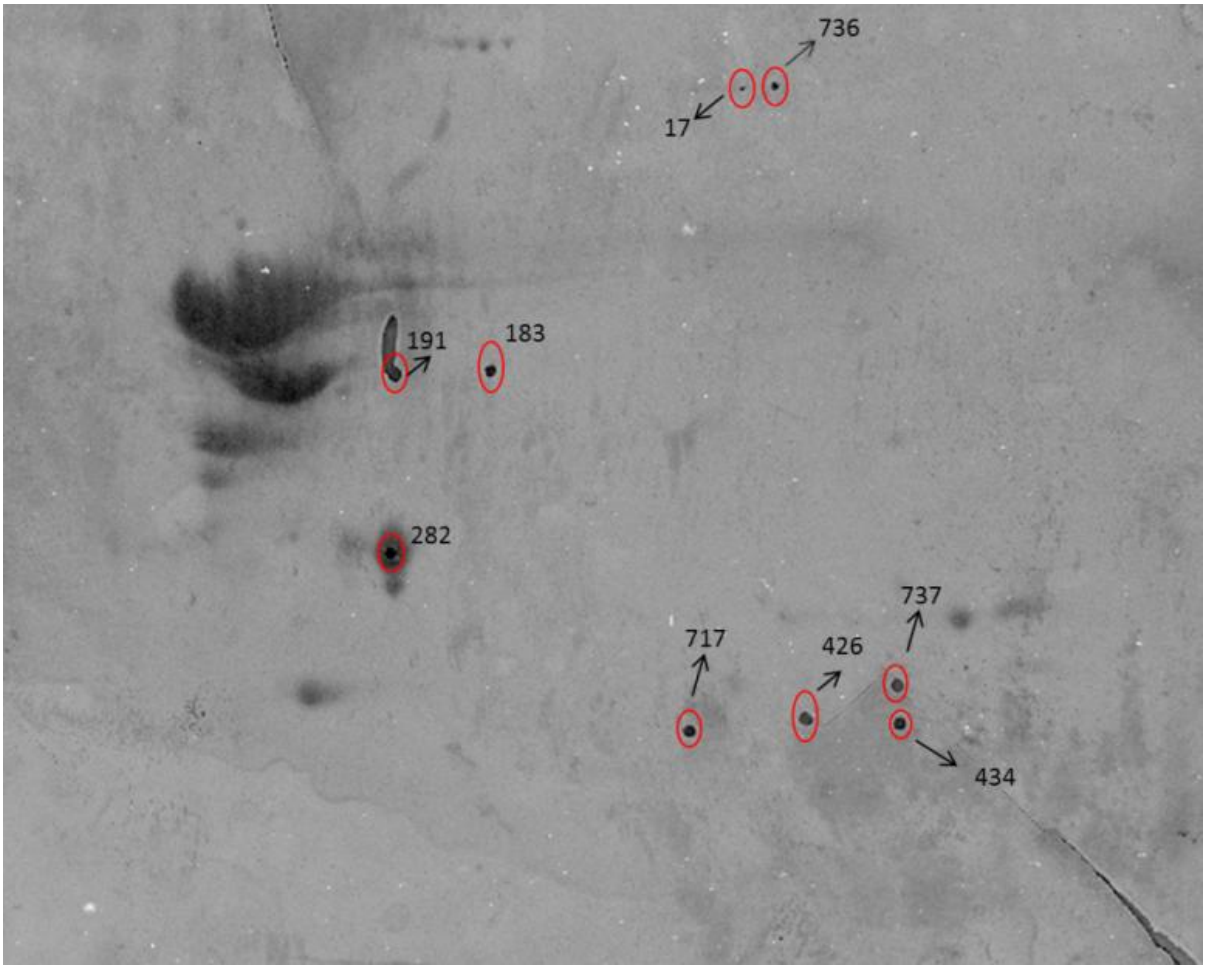
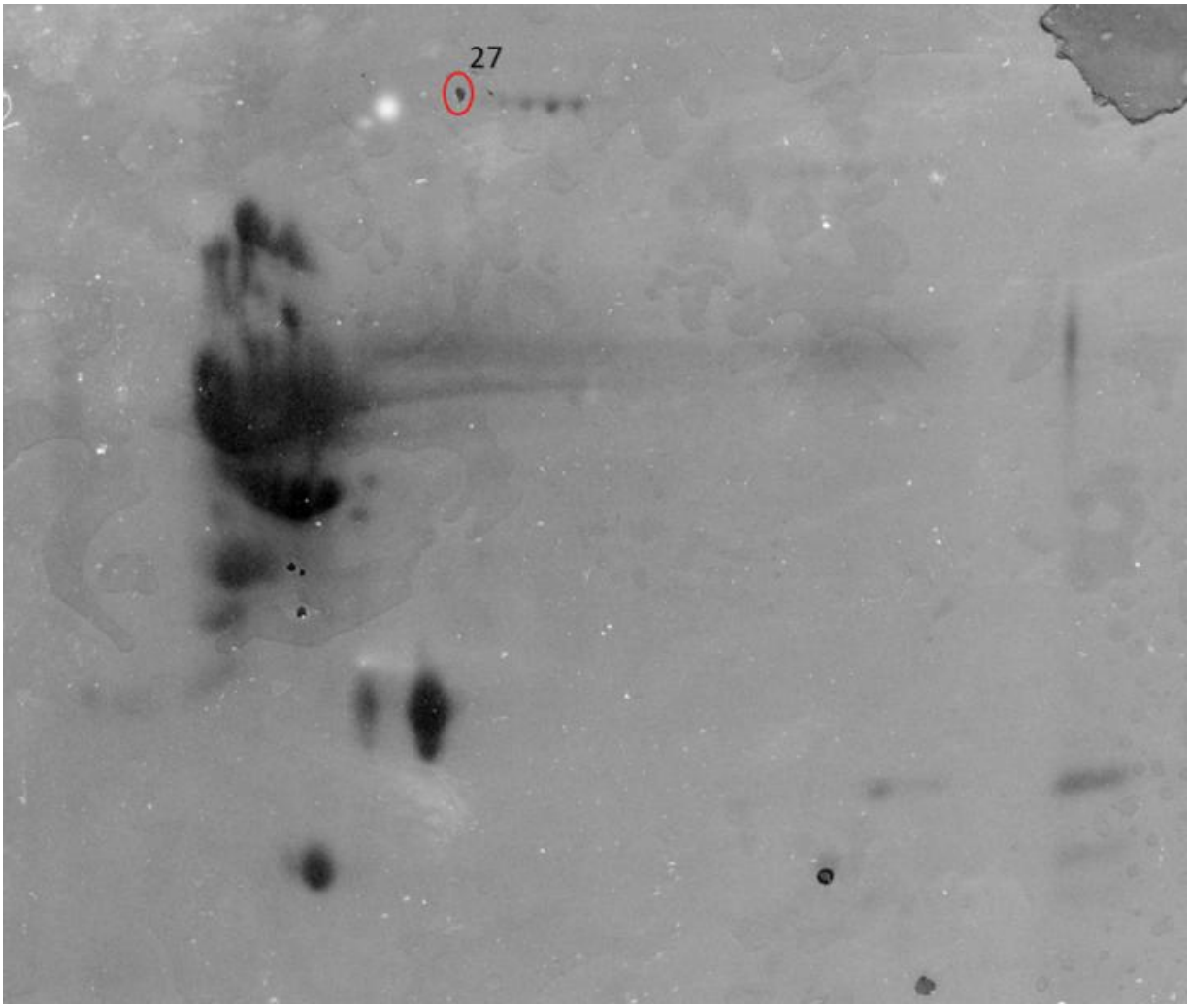


Figure 21: Proteomic map of “Girgentana” whole milk samples and detected spots from the differential analysis , comparing two different sampling area.



4.3.2: *Milk Proteins Characterization In The Sicilian Native "Valle Del Belice" Ovine Breed*

RESULTS:

Valle del Belice sheep bulk milk samples have been processed, having as objective the development of protocols for milk fractions preparation.

The aim was to reduce the complexity of sheep milk proteome to be analyzed. For this purpose, 5 different types of sheep milk samples were considered: whole milk, skimmed milk and three milk fractions: fat globules, caseins and whey proteins. Protein concentration was estimated using the calibration line obtained by plotting the absorbance of the standards and protein samples concentration.

To obtain milk fractions, two different protocols were considered. Caseins have been extracted from skim milk samples by following two different protocols: caseins precipitation protocol based on the Kjeldahl method (IDF-FIL) and the protocol validated by the Scientific Association of Animal Production (ASPA), in order to compare extracted proteins amounts and to determine the most appropriate protocol to follow in proteomic analysis.

Using the protocol validated by the Scientific Association of Animal Production (ASPA) a greater value of whey proteins quantification and a similar value of casein quantification (a little much more with Kjeldahl) were obtained. Due to practical reason, it was decided to follow the protocol validated by the Scientific Association of Animal Production (ASPA), faster than Kjeldahl protocol.

From the protein quantification the following average values have been obtained:

Whole milk: 35,5 µg/µl
Skimmed milk: 32,5 µg/µl
Fat globules: 25,05 µg/µl
Whey proteins: 11,33 µg/µl
Caseins: 22 µg/µl

A test was performed resuspending the samples in distilled water and in a lysis buffer containing urea, CHAPS, Tris and DTE and comparing the quantification results. The results obtained demonstrated no difference between the protein concentrations.
Another test was performed resuspending the samples in three different lysis buffers, of different composition, in order to assess any variation in protein samples quantification.
In particular, a buffer containing urea, thiourea, CHAPS and Tris was used; a second buffer containing urea, CHAPS and DTT and a third buffer, containing urea, thiourea, CHAPS, DTT were also used.
Milk fractions samples were quantified with the spectrophotometer according to the coefficients of the calibration line obtained using the software R with a linear regression between the amount of the standard BSA and the absorbance values of the standards.
Due to casein high concentration and to the complex samples texture, it was required to solubilise caseins to obtain a correct quantification.
Increasing amounts of a lysis buffer containing urea were added to the casein fraction obtained by precipitation, until reaching the optimum volume at which it is obtained their complete solubilisation.

5 - CONCLUSIONS

The doctoral project was aimed to characterize Sicilian native livestock breeds, such as “Girgentana” goat breed and “Valle del Belice” ovine breed, through the development of advanced biotechnology methods and proteomic protocols applied to milk and dairy products. The purpose of the PhD was to characterize Sicilian autochthonous breeds through the authentication, exploitation and preservation of native breeds raw materials such as milk. The application field of the –OMIC Sciences applied to livestock production ranges from the genomic analysis of livestock animals to the use of molecular markers to improve the quality of breeds and livestock production, to kinship determination, individuals and breeds identification and animal products tracking and authentication, to animal biodiversity conservation. In this context, the importance of identify and characterize molecular markers that are "unique", in reference to an animal breed and " typical ", in reference to a particular breed product. The actual growing problem in livestock production is to increase the organoleptic properties and safety of products of animal origin, improving transformation technologies and increasing quality production.

In this context, a better knowledge of the importance of the zootechnical meaning of native livestock biodiversity as climate adaptation, resistance to endemic diseases and ability to synchronize themselves to environmental changes is required.

Talk about zootechnical biodiversity means to speak about quality productions of our native breeds in terms of milk, dairy production and meat, to pay attention to lipids profile of a product (low cholesterol levels, high proportions of polyunsaturated fatty acids) and to the proteins (essential amino acids branched, carnitine, glutathione etc).

In addition, it should be emphasized the link between local animal breeds and territory, resistance to stress situations, resistance against endemic diseases and ability to provide high quality products with a high content of bioactive molecules.

Native breeds might be a genetic resource for future selections, with objectives of adaptation to adverse condition, endemic diseases tolerance. It should be emphasized the close link between the need to protect native livestock biodiversity and the importance of produce local traditional products with unique physic-chemical, microbiological and organoleptic properties.

Actually, the survival of Sicilian native breeds is strictly related to the quality of the products obtained from them, characterized by a strong identity and strictly linked to the territory and the tradition. Conservation of local livestock breeds is closely linked to the concept of sustainable development.

Actually, a real problem if we want to investigate animal breeds is represented by the poor data available in the databases concerning livestock breeds. The extent of the PhD work was to develop proteomic protocols aimed to enrich the actual knowledge related to Sicilian livestock breeds biodiversity and local animal production, producing the first data available as precursor studies. The aim of a research work was to compare the proteomic profile of Girgentana breed whole bulk milk, taking into account different lactation periods and geographical areas by two dimensional difference in gel electrophoresis (2D-DIGE).

Milk proteomics has rapidly developed as an eligible approach and proteomic applications can vary from protein identification to complex characterization of protein post-translational modifications and protein pathways analysis.

This work was the object of an abstract presented during the Conference organized by the Scientific Association of Animal Production (ASPA). It allowed giving a general picture of Girgentana goat milk protein distributions over 3-10 pH range and it can be the initial study of following proteomic studies aimed to make the Girgentana goat milk reference map. Samples of bulk milk belonging to the Sicilian native goat breed "Girgentana" were collected and processed for the proteomic analysis.

For this purpose, two geographically distant farms have been considered (one farm localized in the province of Palermo and another one localized in the province of Agrigento).

During the sampling, also the month of sampling was taken into account, in order to assess any changes in the protein profile due to the sampling area, and the month of sampling.

After determining protein concentration and quality of the goat milk samples by one-dimensional electrophoresis, a preparative two-dimensional electrophoresis 2D-PAGE was performed in order to refine the protocol to follow in the experimental procedure.

After the run, the preparative gels were stained with Coomassie staining and acquired digitally, using Typhoon FLA 9500 laser scanner.

The images obtained scanning the gels were analyzed qualitatively using the image analysis software Image Master 2D Platinum v 7.0 through special algorithms.

Gels images were overlayed and compared, detecting protein spots and determining their abundance.

After protein spots detection, the image processing was performed, with the establishment of gel anchors or "landmarks".

Protein spots with a fold change value > 1.5 and with an ANOVA value statistically significant ($P \leq 0.05$) were considered differentially abundant.

The picked out spots were sent to the laboratory of Mass Spectrometry of Porto Conte Research (Sassari) Center for proteins separation by liquid chromatography and identification by mass spectrometry.

Protein identification data have been filtered by applying the identification criterion based on a number of unique peptides ≥ 2.

The proteomic analysis was performed both considering the same sampling time and different time periods. At the same time, during the proteomic analysis were taken into account sampling done in a unique farm considering different time periods.

After two-dimensional electrophoresis, several spots on the gels appeared as strings, demonstrating that isoforms of differing charge, resulting from post-translational modifications, are present in milk.

Gel image analysis was performed and only the most statistically significant spots were considered and among these, only the ones easy for the spot picking, isolated, were taken into account.

Protein spots showing more than 1.5 fold change in spot volume (increased for up-regulation or decreased for down-regulation), with a statistically significant ANOVA value ($P \leq 0.05$), were considered differentially abundant. Among these, a set of spots were excised from preparative gels and identified by tandem mass spectrometry by “Porto Conte” Research Center. To assign protein identity, data were filtered following the application of identification criteria based on number of unique peptides ≥ 2.

From the differential analysis have been identified **β-lactoglobulin** , whey proteins belonging to the family of lipocalins, which transport small hydrophobic molecules (steroids, bilins, retinoids and lipids) and associated with immune response, pherormone transport, prostaglandin synthesis, retinoid binding and cancer cell interactions; **fragments of K-casein**, protein involved in casein micelle stability that prevents casein precipitation in milk and also involved in thiol-catalyzed disulfide interchange reactions with whey proteins during heat treatments and (after rennet cleavage) in the facilitation of micelle coagulation; ***keratin-like* proteins** structural components of hairs, wool, horns, that also protect epithelial cells from damage or stress conditions; **αS1-casein**, protein that plays an important role in the transport

of calcium phosphate and also required for the export of the other caseins from the endoplasmic reticulum and for casein micelle biogenesis and casein transport in the secretory pathway; **"milk fat globule EGF factor 8"** protein, indicated as MFG-E8 and also known as lactadherin, pleiotropic secreted glycoprotein of fat globules membrane involved in mammary gland morphogenesis, angiogenesis, tumour progression, inhibition of blood coagulation, tissue homeostasis and prevention of inflammation; **β-casein fragments**, produced by proteolysis of β-casein, protein of the casein micelle that plays a foundamental role in milk enzymatic activities; **serum albumin**, plasma carrier able to bind several hydrophobic steroid hormones and transport protein for hemin and fatty acids.

2D-DIGE allowed giving a general picture of milk protein distributions over 3-10 pH range. These preliminary results in addition to the identification of others interesting spots will be used to generate a reference proteomics map for the Girgentana goat breed.

Proteomic analysis has been extended to *Valle del Belice* sheep bulk milk samples, having as objective the development of protocols for milk fractions preparation.

The purpose was to reduce the complexity of ovine milk proteome to be analyzed.

For this purpose, 5 different types of sheep milk samples were considered: whole milk, skimmed milk and three milk fractions: fat globules, caseins and whey proteins

Bulk milk samples from individuals belonging to the Sicilian native "Valle del Belice" sheep breed were collected. During sampling, a farm located in Contessa Entellina, in the province of Palermo, was considered.

Caseins have been extracted from skim milk samples by following two different protocols, in order to compare proteins amounts and to determine the most appropriate protocol to follow.

The protocol of caseins precipitation based on the Kjeldahl method (IDF - FIL) was performed.

Milk samples were, then, filtered with Whatman filters and in this way, it was possible to separate whey proteins from caseins.

The second protocol used was the protocol validated by the Scientific Association of Animal Production (ASPA). Using the protocol validated by the Scientific Association of Animal Production (ASPA) a greater value of whey proteins quantification and a similar value of casein quantification (a little much more with Kjeldahl) were obtained.

Due to practical reason, it was decided to follow the protocol validated by the Scientific Association of Animal Production (ASPA), faster than Kjeldahl protocol.

Increasing amounts of a lysis buffer containing urea were added to the casein fraction obtained by precipitation, until reaching the optimum volume at which it is obtained their complete solubilization.

Fat globules membrane proteins have been extracted using the protocol developed by Pisanu et al. (2011), with some modifications.

After obtaining milk fractions, a test was performed using different buffers in which samples were resuspended: distilled water and a lysis buffer containing urea, denaturing agent that acts by disrupting non-covalent and ionic bonds between amino acids; CHAPS, a solubilising detergent that prevents proteins aggregation due to hydrophobic interactions; Tris, a zwitterionic buffer and DTE, a reducing agent that breaks proteins disulfide bonds and maintains proteins in their reduced state.

The quantification results have been compared and demonstrated no difference between the protein concentrations.

Another test was performed resuspending the samples in three different lysis buffers, of different composition, in order to assess variations in the quantification of the samples to vary the buffer used. In particular, a buffer containing urea, thiourea, CHAPS and Tris was used; a second buffer containing urea, CHAPS and DTT and a third buffer, containing urea, thiourea, CHAPS, DTT were also used.

The second part of the PhD research activities is related to the activities done during the PhD mobility period, in Budapest. The aim of these research activities was the improvement of proteomic techniques “gel-based”, sample preparation (protein spots “in-gel” digestion) methods and mass spectrometry applied to proteomics.

The acquisition of these skills can be perfectly translated in other fields of application, such as livestock production, since the proteomic techniques are very versatile and have a wide range of applications.

Animal foods such as Girgentana goat milk, represent complex food matrices with different types of proteins and other components and the current available proteomic techniques, coupled to mass spectrometry exceed the current technical limitations that do not allow, for example, the study of membrane proteins, highly hydrophobic, and allow the study of multiple proteins, from several hundred up to several thousand.

HPLC and tandem mass spectrometry (MS/MS) are, in fact, systems able to separate and identify low abundance membrane proteins that would otherwise be impossible to study by traditional two-dimensional electrophoresis 2DE.

The depletion of abundant proteins from biological fluids can be of great help in decreasing proteome complexity, when the goal is the study of less abundant proteins or molecular markers discovery.

High resolution two-dimensional electrophoresis, combined with high performance liquid chromatography (HPLC) and mass spectrometry, represents a powerful tool for analyzing complex mixtures of several hundred proteins simultaneously.

Furthermore, liquid chromatography coupled to mass spectrometry allows for the separation and identification of molecules of low molecular weight, goals that until a few years ago were considered very ambitious and impossible to reach.

The research activities done during the PhD mobility and performed in the laboratories of mass spectrometry applied to proteomics of the research center MTATTK of Budapest under the supervision of Professor Karoly Vekey as tutor were aimed to the evaluation of the repeatability of a method for depletion of high-abundance proteins using a commercially available kit based on affinity chromatography.

The main aim was to become familiar with methods finalized to solve proteome complexity and to evaluate in a critical way the repeatability of such a method.

It is of great importance that a step of depletion is reproducible when included in a proteomic study.

The objective was to evaluate the repeatability of the commercial kit for depletion of high-abundance proteins "Multiple Affinity Removal System" (MARS) of Agilent Technologies, which offers the possibility to process several samples simultaneously.

The aim was to evaluate the repeatability of the depletion kit above, to consider its suitability as first step of a vaste study related to glycosilated proteins, in order to eliminate problems related to high abundance proteins that mask less abundant proteins.

The aim of this study was the evaluation of the repeatability of the commercial kit based on a method of affinity chromatography used to deplete high abundance proteins.

As reported in literature, human blood plasma is one of the most studied biological fluids and is the main type of sample used for disease diagnosis because it can be correlated with specific physiological or pathological states.

Actually, a complication of proteomics experiments, which relates the experimental approach, is the presence of highly abundant proteins in biological samples, which makes difficult biomarkers discovery and identification, due to preponderant proteins that interfere with the analysis based on liquid chromatography coupled to mass spectrometry (LC-MS).

The removal of high abundance proteins such as serum albumin (HSA), in plasma samples, and caseins, in milk samples, may enable less abundant proteins identification. As reported in literature, it is of great importance that a step of depletion is reproducible when included in a proteomic study.

The principle is to make protein sample less complex, increasing the possibility of determining less abundant proteins.

The proteomic analysis of plasma sample is analytically difficult due to its highly abundant constituent protein concentration range (Luczak et al., 2014; Anderson & Anderson, 2002). The most common approach used to facilitate plasma proteomic analysis is to reduce plasma complexity by samples fractionation, enriching the sample in low-molecular-weight protein fractions, important biomarkers sources (Tirumalai et al., 2003). The masking effect of well-characterized high-abundant proteins in serum has been a main obstacle in the detection of low-abundant proteins that may be proteins of interest for new biomarker identification. Human blood samples belonging to healthy people were considered.

Plasma samples were processed to decrease plasma complexity *via* depleting highly abundant proteins using the "Human Serum Albumin and Ig G Multiple Affiniy Removal System" (Agilent), designed to remove two interfering high-abundant proteins from human samples (such as plasma, serum, urine or cerebrospinal fluid). Removal of these highly abundant proteins improves LC/MS and electrophoretic analysis of the samples by increasing the dynamic range of the proteomic analysis.

Thanks to the ready-to-use high abundant proteins removal system, it is possible to detect less-abundant proteins by loading up to ten times more low-abundant proteins mass onto gels for analysis than before depletion.

Depletion experiments were performed by three different operators. Four groups of six samples were depleted in triplicates; with some reproduction at different data.

Depleted samples were separated by reverse-phase high performance liquid chromatography (only one fraction was studied) and measured by UV.

Three peaks from the UV were selected and their intensities were measured for each sample. The values were used to characterize reproducibility and variability. This study was a preparatory step to further studies that will be pursued in the glycobiology field by Professor Karoly Vekey research group.

During the separation, only three fractions were taken into account for each sample to be analyzed.

The selected fractions were collected through an automated fractionator and lyophilized.

During the measurements only three peaks were considered and for each protein fraction retention time value and absolute peak intensity values were collected.

From the absolute intensity values three parameters were derived: sum of the absolute intensity and first and third relative intensities, obtained dividing, respectively, first and third peak intensity value by the sum of intensities. Sum of intensities, first and third relative intensities were estimated as percentage value comparing differences/variability among the four sample groups.

During measurements, the reproducibility of the relative intensities values (5-7%) and the absolute intensity values (1-2%) were found.

During the measurements, it was demonstrated a better reproducibility of the relative intensities values, compared to the absolute intensity values, less reproducible of the first. From the values obtained during the measurements it can be stated that there is ca. the 10% difference in the quantity of protein depleted while only 1-3% difference in relative abundances.

Variability of the sum of the intensities is mainly due to random errors and variability between different operators. Variability of the first relative intensity is mainly due to inter-individual differences while variability value of the third relative intensity is modest.

This study was preparatory to further studies that will be pursued by Professor Karoly Vekey research group and are a part of a vaste study.

Within the mobility period of the PhD I was also involved in the research activities carried out at the laboratories of Proteomics Institute of Biology of the Faculty of Sciences of Budapest by Professor Gabor Juhasz research group.

Regardless of the subject of the research activity, the comparison between two different hypothesis about the connection between the circadian rhythm and neuronal plasticity of the brain in mammals, the aim was to improve techniques of Proteomics "gel-based" that I can also apply in livestock farming because they are very versatile. I also used tools for image analysis in proteomics (other than those I have previously used), methods of protein sample preparation (such as "in-gel" digestion of spot proteins), for the separation and identification of proteins through mass spectrometers and, finally, I used appropriate softwares useful in mass spectrometry applied to proteomics for the discrimination of the results.

As reported by Biggio & Mostallino (2013), neuronal plasticity is represented by the extraordinary ability of neurons to change in the short and long term their function and their

morphology as a result of environmental stimuli, endocrine, or pharmacological and pathological insults to ensure the maintenance of their functionality in such situations.

The importance of neuronal plasticity, considered a rapid and long-term adaptability developed by neurons in response to environmental stimuli, has allowed during the evolution important achievements such as the acquisition of more and more sophisticated and functional properties that relate to the control of emotions, affective and cognitive processes etc.

Neuronal plasticity, as reported in literature, is related to the circadian rhythm.

As reported by Albrecht (2012), the circadian system coordinates the physiology and behaviour of mammals with the environmental light-dark cycle.

Sleep is not a simple shutdown of brain function but rather it is a highly regulated process that involves two main mechanisms: (1) a homeostatic process that regulates the increase in the propensity to fall asleep during the wakefulness and the decrease of sleep intensity and (2) a process which borders circadian sleep and wakefulness at appropriate times during the day and is largely independent from the previous episodes of sleep / wakefulness (Borbely, 1998).

Deciphering the molecular basis of sleep is very difficult as it is very likely that the circadian and homeostatic processes are interlaced to a certain extent, which makes it difficult to separate sharply the two processes and the mechanisms involved.

Some studies were conducted on gene expression modifications in rats brain after long term sleep deprivation (Cirelli et al., 2006) and the results have shown a strong correlation between sleep deprivation and the occurrence of physiological changes such as a dramatic increase in energy consumption, a decrease in body weight and the death of animals after 2-3 weeks.

In addition, the results obtained from the analysis of "sleep-related" transcripts, which code for glia proteins, proteins involved in protein synthesis, in the synthesis of cholesterol, in membrane trafficking, in the synaptic "down-regulation" and in memory consolidation confirm the importance of sleep on gene expression. In contrast, studies concerning spontaneous wakefulness or sleep deprivation for short time demonstrated that transcripts expressed at high levels during these situations encode different mitochondrial proteins, chaperones and heat shock proteins and proteins involved in synaptic potentiating and glutamatergic transmission (Cirelli et al., 2006).

These results suggest that the continued wakefulness not only increases energy consumption by the brain but it’s also a cellular stress for neurons and/or glial cells.

However, despite numerous studies on the subject, sleep deprivation syndrome remains largely unresolved and synaptic protein turnover has not been investigated during sleep with high-throughput techniques to date.

The aim of the research was to compare two different hypotheses related to brain plasticity and circadian rhythm in mammals. The hypothesis known as G. Tononi and Cirelli C. synaptic homeostasis hypothesis (Tononi & Cirelli, 2014) affirms that sleep is for the brain the price to pay for plasticity, a reversible disconnection from the surrounding environment, usually accompanied by immobility.

During sleep, synaptic efficiency is renormalized and homeostasis is restored.

Increased synaptic strength, according to the authors, would have different costs at the cellular level and system, including increased energy consumption and a higher demand for distribution of energetic cellular needs to synapses, resulting in cellular stress, and associated changes in support cells such as glia.

In addition, an increased synaptic "robustness" would also reduce the selectivity of neurons responses and would saturate the ability to learn.

According to the synaptic homeostasis hypothesis, neurons would achieve some winning strategies that are strictly related to plasticity:

1) new learnings would occur primarily through synaptic potentiation.
2) synaptic strengthening would occur mainly during the wakefulness, when the organism interacts with the environment and not during sleep, when it is disconnected.
3) restoring of the normal situation of synaptic "robustness" would compare mainly during sleep, when the brain is spontaneously in an "offline" mode.

The synaptic homeostasis hypothesis provides a parsimonious explanation of several positive consequences of sleep on memory-related processes, including acquisition (the restoration of the ability to acquire new memories), consolidation (memory improving, by supporting new memories), the ability to extract information from the environment (the propensity of the brain to form memories that last longer and do not change, such as faces, maps and examples of a particular encounter with the environment), the integration of information (for the brain is easier to remember new information if these are placed in patterns already learned, recorded in an organized structure of old memories), and the ability to forget the sufferings (important mechanism to cope with the inevitable accumulation of minor details).

Conversely, however, the hypothesis of the creation of new synapses during sleep and wakefulness.

The aim of the study was to characterize the altering proteins in the synaptic proteome of rats due to sleep, using synaptosome preparations and gel-based proteomics.

The synaptic homeostasis hypothesis suggests that sleep maintains synaptic excitability.

Based on this homeostatic hypothesis, the reduction of the robustness of synaptic connections occurs during sleep because one of the main roles of sleep is to regain plasticity of the brain.

This idea is mainly based on whole tissue transcriptomics data, but these experiments don't consider that synaptic protein levels are specific, due to local protein synthesis and degradation.

On the other hand, the synaptic protein turnover during sleep is not well understood to date.

In the present study, the purpose was to clarify the mechanisms of synaptic proteome modifications during sleep.

The investigation was carried out on the cerebral cortices of twelve adult Wistar rats.

Respectively, six rats were sacrificed at 8 am, at the end of the awake period and other six rats were sacrificed at 6 pm, during the end of the sleeping period.

From the twelve rats, only the pre-frontal cortex was taken for analytical gels preparation.

Samples were processed with a pH based differential extraction of synaptic membranes protocol.

Only the most statistically significant spots were considered and among these, only the ones easy for the spot picking, isolated were taken into account. In particular, 48 spots were selected for the picking from the preparative gels.

Protein spots were selected according to the statistical values obtained and, in particular, only the most significant spots (according to t-test value) and easier to pick up from the preparative gels were selected (the isolated spots).

Data obtained from the mass spectrometer were analyzed using the software Protein Scape 3.1, a bioinformatics platform with sophisticated tools for the analysis and evaluation of proteomic data, suitable for mass-spectrometry based protein identification, characterization, and quantization.

Protein identification data were exported to an Excel file for a full list of proteins. Proteins with a peptide number less than two were eliminated from the protein list.

Bidimensional maps with thousands spots were obtained. The gels obtained resulted well resolved and with only a very small stricking.

Changes in protein levels were quantified using two-dimensional differential gel electrophoresis (2D-DIGE), using fluorophors, and the altered proteins were identified by

tandem mass spectrometry (MS/MS). We identified proteins that positively regulate a late step in synaptic vesicle exocytosis. For example, we found **Complexin-1**, that prevents SNAREs (Soluble NSF Attachment Protein Receptor) that mediate docking of synaptic vescicles with the presynaptic membrane in neurons from releasing neurotransmitters until an action potential arrives at the synapse.

Complexin-2 was also identified. It negatively regulates the formation of synaptic vesicle clustering at active zone to the presynaptic membrane in postmitotic neurons.

We also found proteins like protein **deglycase DJ-1,** that is involved in neuroprotective mechanisms like the stabilization of **nuclear factor erythroid 2-related factor 2**, transcription activator that binds to antioxidant response (ARE) elements in the promoter regions of target genes and important for the coordinated up-regulation of genes in response to oxidative stress, and **serine/threonine-protein kinase PINK1, mitochondrial**, protein that protects against mitochondrial dysfunction during cellular stress by phosphorylating mitochondrial proteins and involved in the clearance of damaged mitochondria via selective autophagy (mitophagy) by mediating activation and translocation of **E3 ubiquitin-protein ligase parkin**, that participates in the removal and/or detoxification of abnormally folded or damaged protein by mediating 'Lys-63'-linked polyubiquitination of misfolded proteins such as PARK7 ('Lys-63'-linked polyubiquitinated misfolded proteins are recognized by HDAC6, leading to their recruitment to aggresomes, followed by degradation); mediates 'Lys-48'-linked polyubiquitination of ZNF746, followed by degradation of ZNF746 by the proteasome possibly playing a role in neuron death regulation; regulates cyclin-E during neuronal apoptosis; may protect neurons against alpha synuclein toxicity, proteasomal dysfunction, GPR37 accumulation, and kainate-induced excitotoxicity; may play a role in the control of neurotransmitter trafficking at the presynaptic terminal and in calcium-dependent exocytosis. The identified protein, **PARK7**, plays also a role in regulating expression or stability of the mitochondrial uncoupling **brain mitochondrial carrier protein 1**; that participates in the mitochondrial proton leak measured in brain mitochondria and **mitochondrial uncoupling protein 4;** that creates proton leaks across the inner mitochondrial membrane, thus uncoupling oxidative phosphorylation from ATP synthesis and may play a role in thermoregulatory heat production and metabolism in brain; in dopaminergic neurons of the substantial nigra pars compacta and attenuates the oxidative stress induced by calcium entry into the neurons via L-type channels during pacemaking. Regulates astrocyte inflammatory responses, may modulate lipid rafts-dependent endocytosis in astrocytes and neuronal cells.

Another identified protein, **serine/threonine-protein phosphatase PP1-gamma catalytic subunit** is involved in regulation of ionic conductances and long-term synaptic plasticity. It may play an important role in dephosphorylating substrates such as the postsynaptic density-associated Ca^{2+}/calmodulin dependent protein kinase II.

Solute carrier family 12 member 5, mediates electroneutral potassium-chloride cotransport in mature neurons and it's also important for Cl- homeostasis in neurons and necessary for the ontogenic change in response to gamma-aminobutyric acid (GABA) from depolarization to hyperpolarization during neuronal development.

Secretogranin-1 is a neuroendocrine secretory granule protein, which may be the precursor for other biologically active peptides.

Septin 11, filament-forming cytoskeletal GTPase, may play a role in the cytoarchitecture of neurons, including dendritic arborization and dendritic spines, and in GABAergic synaptic connectivity.

Endophilin-A1, is implicated in synaptic vesicle endocytosis.

NAD-dependent protein deacetylase sirtuin-2 deacetylates alpha-tubulin at 'Lys-40' and hence controls neuronal motility, oligodendroglial cell arbor projection processes and proliferation of non-neuronal cells. Phosphorylation at Ser-330 by a G1/S-specific cyclin E-CDK2 complex inactivates SIRT2-mediated alpha-tubulin deacetylation, negatively regulating cell adhesion, cell migration and neurite outgrowth during neuronal differentiation. Deacetylates PARD3 and participates in the regulation of Schwann cell peripheral myelination formation during early postnatal development and during postinjury remyelination.

Synaptosomal-associated protein 25 may play an important role in the synaptic function of specific neuronal systems. Associates with proteins involved in vesicle docking and membrane fusion.

Syntaxin-binding protein 1 may participate in the regulation of synaptic vesicle docking and fusion, possibly through interaction with GTP-binding proteins.

It is essential for neurotransmission and binds syntaxin, a component of the synaptic vesicle fusion machinery.

Synapsin-2 is a neuronal phosphoprotein that coats synaptic vesicles, binds to the cytoskeleton, and is believed to function in the regulation of neurotransmitter release. May play a role in noradrenaline secretion by sympathetic neurons.

Synaptotagmin-1 may have a regulatory role in the membrane interactions during trafficking of synaptic vesicles at the active zone of the synapse and plays a role in dendrite formation by melanocytes.

The analysis of the pathways revealed that there is a decrease on a large scale of many proteins involved in synaptic transport and metabolism of pyruvate while in very few proteins has been found an increase at the end of the sleeping period, compared to the awake state. The results revealed that proteins involved in synaptic transmission and metabolism are widely regulated by sleep.

There is a direct evidence of a synaptic decreasing and a decreased "robustness" of synaptic activity in the samples examined.

The data obtained support the synaptic maintenance hypothesis of sleep, according to which sleep is a process of synaptic homeostasis.

The results, in fact, provide a further evidence of an homeostatic process that takes place in the synapses of the cortex during sleep and it’s involved both in synaptic plasticity and in metabolic processes.

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