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NUTRIGENOMIC AND METHODOLOGIC REFLECTIONS ON METABOLOMICS

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TABLE OF CONTENTS

1. INTRODUCTION

Scientifically observed today means that we are in the time of postgenomic era characterized by the arrival of the omics-sciences together with its technologies. The OMICS-term is composed of genomics, proteomics, transcriptomics and metabolomics [GHOSH, 2010].

Genomics is involved with a systematic approach of high-throughput structure determination to create a complete collection of protein folds and structures for every gene product or protein and to once unravel functions for all proteins [MITTL & GRÜTTER, 2001]. Transcriptomics is busy as a rugged high-throughput technology qualified of simultaneously quantifying a multitude of defined mRNA species [HEGDE et al., 2003] and as such it provides a holistic registration of molecular actions and reactions in response to dietary interventions and shortly can be summed up as gene or RNA expression analysis [RIST et al., 2006]. Proteomics spans a wide range of technologies with the common aim to figure out the identity and quantity of expressed proteins in cells, their three dimensional structure including their interaction associates [CHO, 2007]; shortly it can be defined as protein expression analysis and its challenge lies in the extended complexness of proteomes compared to for instance transcriptomes. It offers markers and intervention targets such as enzymes or transporters and is the adequate method for the detection of bioactive food proteins or peptides [RIST et al., 2006]. And metabolomics focuses on high-throughput characterization of all measurable metabolites of the entire metabolome [ORESIC, 2009]. That´s why metabolomics is ideally positioned to be of outstanding avail for a multitude of areas of nutrition science and research [WISHART, 2008] and as such this work is dedicated to the complex world of metabolomics.

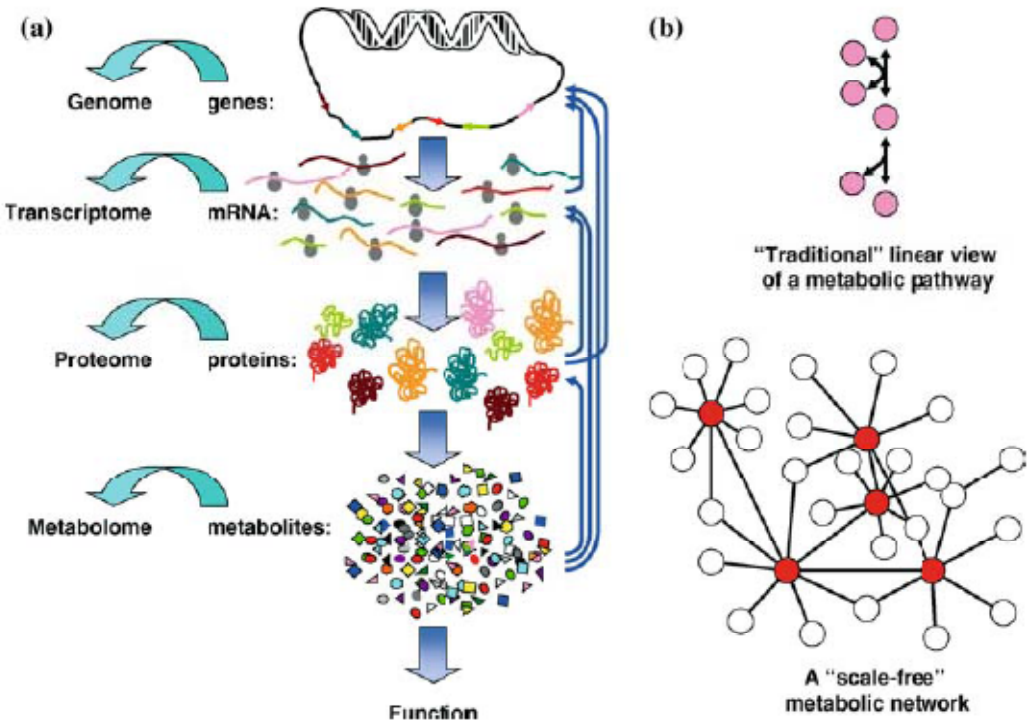

FIGURE 1: THE GENERAL SCHEMATIC OF THE OMIC ORGANIZATION

The principal information flow is from genes to transcripts to proteins to metabolites to phenotype; whilst blue vertical arrows indicate interplays navigating respective omic expression (a). Our traditional linear view of metabolomic pathway as well as the „scale-free" linkings within metabolite neighborhoods (b) [GOODACRE, 2005].

However, the application of these new methods today is integrated in laboratory practice and this circumstance plays a decisive role for enhanced examination of genetic effects in response to nutritional and environmental factors of influence. As they are minimizing classical detection limitations consequencing in the notable circumstance that all received multiple data can be taken together, they are capable to create new insights leading to encouraging scientific perspectives [GHOSH, 2010]. Hence with increased investigation options they are delivering promising material for updated nutrition research [RIST et al., 2006].

2. WHAT IS METABOLOMICS

The science of metabolomics is the discipline dedicated to the global study of metabolites.

Metabolites are the sequence of the interaction of the organism´s genome with its environment and are not solely the end product of gene expression but also participate in the regulatory system in an integrated way. They occur as small molecules intermediates and products of metabolism whose quantity depends on the biological system investigated. Their dynamics, composition, interactions and responses to interventions and to changes in the environment get studied in various areas of living systems such as cells, cell compartments, tissues and bioliquids. Metabolomics is concerned with the investigation but also qualitative and quantitative measurement of these metabolites [OLDIGES et al., 2007; GRIFFITHS et al., 2007; RIST et al., 2006] whereas again metabolomics research can be subdivided into two different areas of targeted and non-targeted methods. Fiehn in 2002 as a pioneer was one of the first who classified differences among metabolomics and who defined terms responding to the subareas [FIEHN, 2002]. These subvided metabolomic technologies covering also contrarian starting points are popular and it is well-known that they are enormously helpful to enhance investigation results and in parallel there already exist matured further strategies within the metabolomics field. Accordingly in the following the most established and applied ones will be discussed [HOLLYWOOD et al., 2006].

2.1. TARGETED METHODS

Contrary to the universal metabolomic approach dwelled on later, targeted metabolomics endeavors are focusing on the analysis of individual metabolite classes [GRIFFITHS et al., 2007]. Hence, the opulence of the entire metabolome information gets ignored because of the exclusive focus on specific compounds [OLDIGES et al., 2007].

To directly investigate the primary effect of a genetic alteration, studies often focus exclusively on the substrate and/or the specifically focused component. Since all other

metabolites are circumstantial during targeted approach, extensive sample clean-up should be utilized to burke interferences from other present metabolites [FIEHN, 2002]. Generally these targeted methods are akin to those applied in normal biochemical laboratories, but may differ as one has strived to evolve techniques capable to quantitatively cover metabolites which are not easy to cover in global approaches like eicosanoids [MURPHY et al., 2005], bile acids [HAGIO et al., 2009] or metabolites of central carbon metabolism [PILVI et al., 2008; ORESIC, 2009].

2.1.1. METABOLITE PROFILING

Metabolite profiling represents the roots of metabolomics science. At its origins, metabolite profiling studies appeared in the 1950s and developed during the decades whereas the first publications generally were about metabolites of specific compounds like particular pharmaceuticals [WILLIAMS et al., 1975; KRSTULOVIC et al., 1980] but soon were up to capture whole groups of compounds like catecholamines or oxylipins [MUSKIET et al., 1981; WEICHERT et al., 2002]. So generally metabolite profiling can be described to mainly focus on a specified group of metabolites such as for example lipids or those associated with a specific pathway; within clinical and pharmaceutical analysis, this is often termed metabolic profiling to track the destiny of a drug or metabolite [HOLLYWOOD et al., 2006].

2.1.2. METABOLIC PROFILING

Metabolic profiling rapidly classifies and elucidates the function of a whole pathway or intersecting pathways; it is often not a precondition to view the effect of a genetic alteration on all branches of the investigated metabolism. Instead, the analytical procedure can be restricted to the identification and quantification of a selected number of pre-defined metabolites in a biological sample [FIEHN, 2002]; for instance the quantification of preselected metabolic pathways or groups of metabolites with similar chemical properties. Knowledge about possible changes of the intra-cellular metabolite concentrations provides a direct entry for identification of enzymatic kinetics particularly to the in vivo kinetics of the underlying metabolic network

[OLDIGES et al., 2007]. Summed up, metabolic profiling deals with the quantitative analysis of a set of metabolites in a selected biochemical pathway of a specific class of components such as analytes as precursors or products of biological interactions [DETTMER et al., 2006] or a particular enzyme system that would be directly affected by abiotic or biotic perturbation [HOLLYWOOD et al., 2006].

2.1.3. METABOLIC FINGERPRINTING

Metabolic fingerprinting deals with the qualitative analysis of the endometabolome which means that all metabolites located inside the cell get studied [GLASSBROOK & RYALS, 2001]. It is a direct classification method of samples applying a technology that is rapid but which must not imperatively deliver specified metabolite information [GOODACRE, 2007]. In order to achieve unbiased, global screening numbers it is not always essential to determine the individual level of every metabolite. Instead it often suffices to rapidly classify samples according to the origin or their biological relevance in order to maintain a high throughput [FIEHN, 2002].

The ultimate ambition is to identify samples based on metabolite patterns of „fingerprints“ that change in response to disease, environmental or genetic perturbations [DETTMER et al., 2006]. Sometimes metabolic fingerprints have enough resolving power to distinguish between individuals.

2.1.4. METABOLIC FOOTPRINTING

A completely non-invasive approach is to measure the extracellular metabolites which have also gained popularity as the footprint or exometabolome [HOLLYWOOD et al., 2006]. In order to distinguish between intra- and extracellular metabolites the terms endo- and exometabolome have been coined [OLDIGES et al., 2007]. Hence, as already mentioned in contrast to metabolic fingerprinting investigating intracellular metabolites or the endometabolome, metabolic footprinting is concerned with the observation of metabolites consumed from and secreted into the growth medium by batch cultures of yeast utilizing direct injection MS, in which probes of culture media are injected directly into an electrospray ionization mass spectrometer. An excretion

maximization of the metabolites is achieved by the stimulation of the overflow metabolism because to the entirely defined medium diverse carbon components get supplemented that explore metabolically active network in equal mode as an engineer might prove an electrical circuit. Metabolic footprinting has showed to be valuable for discovering metabolites‘ patterns in single-gene-knockout strains in functional genomic analyses but also in mode-of-action investigations [KELL et al., 2005].

A notable advantage is that there is no need for exhausting quenching and extraction procedures when metabolites in the extra-cellular medium of bacterial cultures get studied even though the whole pattern of intra-cellular metabolome information such as phosphorylated components as well as further highly charged metabolites is improbable to reside outside which as a consequence is limiting this detection means compared to metabolic fingerprinting [OLDIGES et al., 2007].

2.2. NON-TARGETED METHODS

This branch is common under the topic „global metabolomics“ and is concerned with the dynamic, qualitative and quantitative investigation of all small molecules of the metabolome which can be found in cell-type, tissue, body fluid or organism [GRIFFITHS et al., 2007]. As it is not focused on a specific compound but interested in findings supporting a holistic overview, these non-targeted approaches aim to cover the metabolome as extensively as possible while sustaining the option to at least differentially quantify the metabolites. As a matter of fact such approaches not only offer characterization of changes in core metabolome generally included in targeted approaches but also support investigation of unsought or only sparsely explored metabolites [ORESIC, 2009].

2.3. THE METABOLOMIC CONJUNCTION WITH NUTRITION

One interesting factor is what happens to the organism when he or she is on diet in accordance to the already accepted association between nutrition and health. The field of nutrigenomics appears where an elucidation is required of how gene expression alters when a human is exposed to miscellaneous nutrient components.

Once this may allow the succesful employment of bioprotective nourishment. Metabolomics is supposed to be one decisive factor because nutrition by definition is targeting to maintain cellular and organism homeostasis. The homeostasis of human metabolism is influenced by a range of intrinsic and extrinsic factors. The intrinsic factors such as body composition, diurnal cycle and extrinsic factors including drugs or the microbiome are completely demonstrated in the figure below.

Metabolomics is expected to be capable to assist in the interpretation of disease mechanisms because a baseline healthy metabolome under varying nutritional conditions could be explored with the positive result of personalized metabolic assessment. Controlled aberrations of this baseline even would support increased understanding of how to keep human metabolism in homeostasis by tailoring alimentation [GOODACRE, 2007].

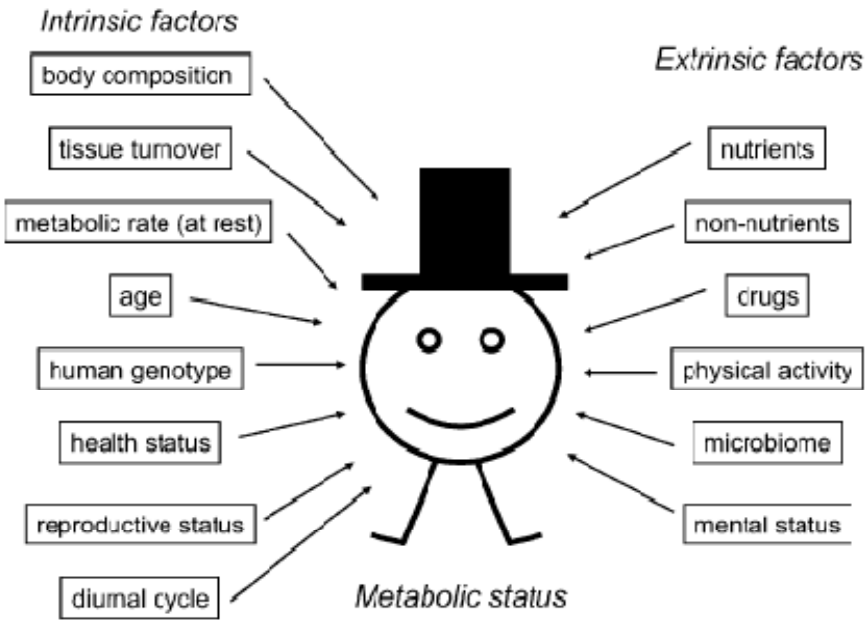

FIGURE 2: METABOLIC INFLUENCE FACTORS

Intrinsic as well as extrinsic factors own potential to bias human metabolic status. The status of these can be measured by utilizing metabolomics [GOODACRE, 2007].

3. NUTRIGENOMICS

3.1. FROM TRADITIONAL NUTRITION TO CURRENT AMBITIONS

Recently the holistic view on nutrition science has advanced initiated by the gained knowledge through illuminating studies that nutrients and other bioactive compounds in our nourishment are able to impact gene expression in various ways. Although a magnitude of mechanisms still are unexplored or not understood yet a range of human, animal and cell culture studies, which will be dwelled on by examples later, have approved clear-cut connections between food intake and biological mechanisms [GARCÍA-CAÑAS et al., 2010]. As a consequence there is an increasing interest in understanding the effect of diet on health by the investigation of mechanisms of nutrients and other biochemical food constituents at the molecular level [GARCÍA-CAÑAS et al., 2010]. Our daily food intake includes macronutrients and micronutrients both biasing effects at RNA, protein and metabolite level in cells when exhibited to food elements. Nutrients and non-nutrient constituents, diets and life habits have the potential to significantly influence each section in the genetic flow from gene expression to protein synthesis and protein degradation and as a result to modify metabolic activities [KUSSMANN et al., 2008]. So while traditional nutrition research mainly has focused to feed and sufficiently saturate populations, modern nutrition research is centering health maintenance and recovery as well as disease prophylaxis. Precondition to find and/or develop functional foods is to detect biologically active molecules together with their properties [KUSSMANN et al., 2006]. A priority for substantiated research achievements is not to deal with ‘single exposure-single time point’ type of studies but to embrace the interplay of complex accumulations of bioactive compounds. Considering the fact that this complexness of compounds means reality in nutritional toxicology, especially the low doses are required to get explored accurately [GHOSH, 2010].

The realization of the holistic ambition gets supported by the development of the OMICS-technologies whereas its sub-disciplines assist to molecularly allocate nutritional adjustments [KUSSMANN et al., 2008], but also the techniques have the

potential to discover specific markers, also popular as biomarkers, that respond effortlessly to a present nutrient, nonnutrient substance, diet or therapy in a satisfactory defined experimental setting [RIST et al., 2006]. Nutritional sciences can be understood as a cumulative item implying these three sub-disciplines whereas this work will be dedicated to metabolomic issues.

These new nutritional sciences are termed nutritional genomics or nutrigenomics and are based on the concept that food components may be capable to directly or indirectly change gene expression whereas the intake of certain ingredients could be either a hazard factor or a derogating factor according to the type of substance. The extent of dietary impact on health or disease balance may correlate with the distinct genetic make-up. Considering that illness and chronic afflictions may be eased, cured or obviated with dietary interventions accounting for individual´s specific nutrition status, demands but also genotype [KAPUT & RODRIGUEZ, 2004], it is worth to promote the identification of genes, proteins and metabolites that are modifiable by nutrients but also of genetic variations altering diverse nutrient-gene interplays [KORNMAN et al., 2004]. Hence the ultimate goal is personalized nutrition aiming to adapt nutritional components according to individual´s specific genetic makeup to achieve health benefits. Especially high-risk persons might once profit from targeted dose components to lower the likelihood of developing chronic diseases [KORNMAN et al., 2004].

3.2. NUTRITIONAL GENOMICS

The conceptual basis of the multidisciplinary science of nutritional genomics or nutrigenomics, can briefly be summed up by the following tenets established by the NCMHD Center of Excellence for Nutritional Genomics from California University;

1) Under certain circumstances and in some individuals, diet can be a serious risk factor for a number of diseases.
2) Common dietary chemicals can act on the human genome, either directly or indirectly, to alter gene expression or structure.
3) The degree to which diet influences the balance between healthy and disease states may depend on an individual’s genetic makeup.

4) Some diet-regulated genes (and their normal, common variants) are likely to play a role in the onset, incidence, progression, and/or severity of chronic diseases.
5) Dietary intervention based on knowledge of nutritional requirement, nutritional status, and genotype (i.e., "personalized nutrition") can be used to prevent, mitigate or cure chronic disease.

[CENTER OF EXCELLENCE FOR NUTRITIONAL GENOMICS, COPYRIGHT 2009]

Nutritional genomics again can be split into the two areas of nutrigenetics and nutrigenomics which are significantly differing from each other in essential points.

3.2.1.TERM OF NUTRIGENETICS

Nutrigenetics examines the impact of genetic differences on the response to a specific dietary model, functional food or supplementation on health effects [FENECH 2008].
It achieves to enlarge the gene-based differences in response to dietary constituents and to develop nutraceuticals matched to individual´s genetic initial position makeup [SUBBIAH 2006]. It is also known under the term personalized nutrition and especially aims to identify those genes, and nucleotide variants within these, that are associated with the various responses to nutrients [COSTA et al., 2008]. Nutritional components may have different impacts on individuals with differing gene variations [KORNMAN et al., 2004]. It studies how genetic variability impacts the body's response to a diet or a nutrient [RIMBACH et al., 2008].

3.2.2. TERM OF NUTRIGENOMICS

In contrast the area of nutrigenomics is concerned with the examination of diet or specific nutrient effects on gene expression. It investigates nutrient intake manipulating genome stability and gene expression which as a consequence has an impact on genome status [FENECH 2008].
It particularly concentrates on the effect of both micronutrients such as vitamins and minerals and macronutrients (carbohydrates, fats and proteins) on the genome,

proteome and metabolome [COSTA et al., 2010]. Hence nutrigenomics and nutrigenetics can be regarded as closely related disciplines which one should avoid to confuse because although they have the same aim to achieve increased health, they explore the interplay of diet and genes from the contrarious initial positions [RIMBACH et al., 2008].

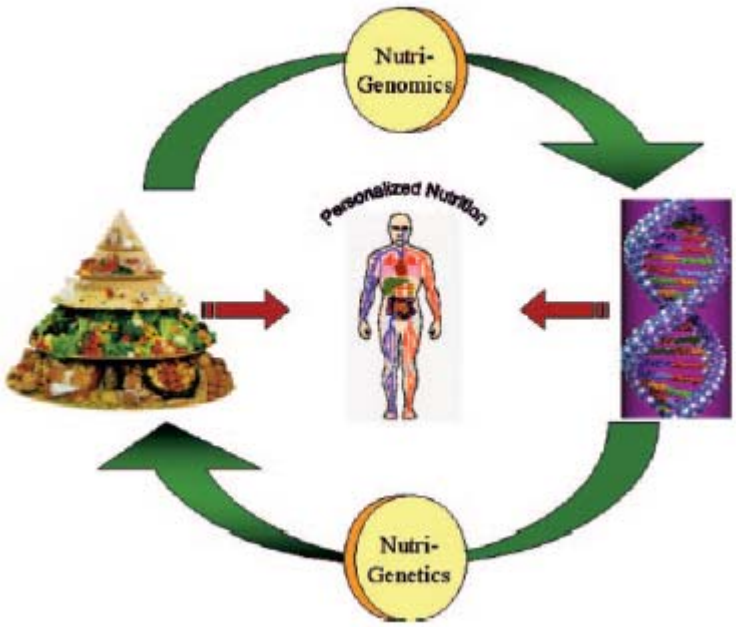

FIGURE 3: NUTRIGENOMICS AND NUTRIGENETICS: THE TWO SIDES OF A COIN
To fulfill requirements for successful personalized nutrition on the one hand the effects of diet on whole-body metabolism such as genes, proteins, metabolites and on the other hand the influence of genotype on nutritionally related disease must be considered [MUTCH et al., 2005].

3.3. GENETIC CHANGES ASSOCIATED WITH NUTRITION

In case of prevention of those diseases resulting from genome damage the two factors have to be considered that on the one hand inappropriate nutrition intake can alter gene expression needed for genome maintenance and lead to significant levels of genome mutation and on the other hand diverse genetic polymorphisms possess the

ability to change genes´ activity but also affinity for micronutrient cofactors in key enzymes which are part of DNA metabolism and repair [FENECH 2008].

3.3.1. POLYMORPHISMS

3.3.1.1. SINGLE NUCLEOTIDE POLYMORPHISMS

In recent years research was focused on identification of sequence variation in the human genome. Already comparatively well-known sorts of genetic variation are the single nucleotide polymorphisms (SNPs) [OSADA et al., 2003] occurring at a rate of 1 per 1300 bases and from which about 1.4 million SNPs have been tagged and localized [LANDER et al., 2001].

SNPs are specified as variations of lone base pairs in a DNA string determined by a change in a single DNA nucleotide whereas majority of variations occur by SNPs. In human they are estimated to appear in millions [DEBUSK et al., 2005]. SNPs can be compared with variations in a recipe. Each gene is a recipe for a specific protein or protein group that either coordinates biological functions or attends as structural building blocks for tissues such as collagen. Some SNPs modify the recipe for the gene so that either another quantity of the protein gets created or the structure of the protein molecule is changed [DEBUSK et al., 2005].

So single nucleotide polymorphisms in the human genome are substantial sites for phenotypic versatility with their ability to loan individuals their uniqueness on the basis of genome plasticity and of the genes´ interplay with environment [COSTA et al., 2010]. Hence according to the location of a SNP this probably leads to various effects at the phenotype level. SNPs in the coding gene areas changing the mission or texture of the encoded proteins are accountable due to a plentitude of known recessively or dominantly inherited monogenic dysfunctions. These SNPs are routinely analyzed for diagnostics. A different category of SNPs are those altering a protein´s primary structure involved in drug metabolism making those SNPs targets for pharmacogenetic analyses. Studies already have evidenced that small alterings in gene sequence are associated with various diseases known to be influenced by diet such as phenylketonuria, celiac disease, diabetes mellitus, lactose intolerance or

cardiovascular diseases [GUTTLER & GULDBERG, 2000; CASTELLANOS-RUBIO et al., 2010; DORIA et al., 2008; TROELSEN, 2005; CORELLA & ORDOVAS, 2005]. For the latter, in course of a study concerned with the systematic survey of SNPs in the coding regions of human genes, 106 genes were identified relevant to cardiovascular disease by screening an average of 114 independent alleles. There is a great demand for follow-ups of such studies in order to once reach the position status of directly testing the disease-common variant hypothesis [CARGILL et al., 1999].

The majority of SNPs however can be found in non-coding regions of the genome and without common influence on individual phenotype [SYVANEN 2003]. Although they do not result by themselves in a disease phenotype they supposedly determine genetic predisposition to once develop disease [COSTA et al., 2010]. Their scientific relevance lies in that they attend as useful aid for the identification of predisposing disease susceptibility genes. Additionally they assist in extending general comprehension of the molecular transactions of mutation [ZHAO et al., 2003].
However, as our knowledge about gene variation nevertheless still is rather marginal and as the understanding of influence of various genetic variants on susceptibility to certain diseases is in its infancy, an enormous need persists to cumulate a substantial catalogue of single nucleotide polymorphisms in potential genes including collected association studies for particular diseases. The basic principle would be to genotype a compilation of SNPs that appear at usual intervals covering the entire genome to retrieve genomic regions in which the occurrences of the SNP alleles vary between patients and controls. Today it is supposed that the SNP alleles are inherited in combination with the disease-predisposing alleles through the generations since very often they occur in physically close areas. The disease-predisposing genes could then be localized and isolated, and proteins encoded by them would serve as approach to develop adapted therapies [SYVANEN 2003].

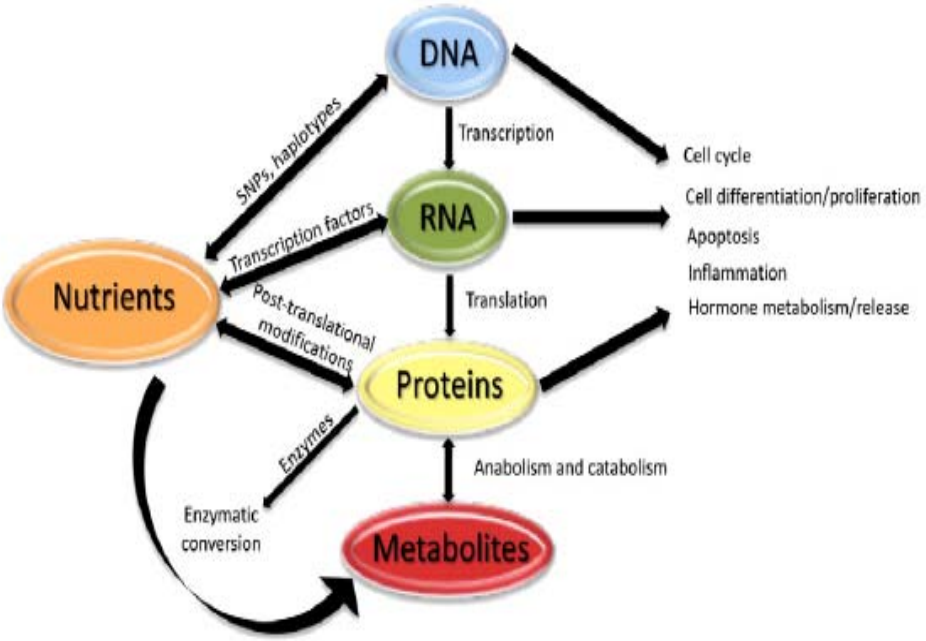

FIGURE 5: NUTRIENTS AND THEIR PARTICIPATION IN PHENOTYPE CHANGES.

Fig. 1. Nutrients and their participation to phenotype changes. Schematic representation of the molecular targets of dietary factors, and their assistance to generate such a phenotype. Nutrients may have direct and indirect interactions at various cellular levels. For example, in the presence of SNPs in key metabolic enzymes, dietary chemicals may be not completely metabolized or may significantly accumulate within the cells, or nutrients may directly affect gene transcription and protein translation. A changed catabolism of nutrients, creating a variety of metabolites, may also are involved in the onset of disease phenotypes working through global effects on diverse cellular processes such as cell differentiation/proliferation but also inflammation [COSTA et al., 2010].

So to briefly draw a balance due to already conducted studies evidencing that diet plays an essential role on developing risk of already mentioned disease types basing upon genetic predisposition, as a matter of fact it is obvious that one essential nutrigenomic challenge for the present naturally means the identification of the specific polymorphisms associated with modified risk of disease or interference to diet [MÜLLER & KERSTEN, 2003]. SNP determination can be useful if characteristics such as

frequency in the interested target of investigation and navigating proteins at the entrée of physiologic succession are provided. Additionally they need to have suitable biomarkers able to offer alternate measures of clinical effects. Although momentarily there are only sparse SNPs meeting these requirements, from those which are capable, research can gain plenty of insights. Revealing examples are methylenetetrahydrofolate reductase (MTHFR) and interleukin 1 (IL-1) demonstrating the relevance of coding and noncoding SNPs with respect to health management [GILLIES 2003].

3.3.1.1.1. INTERLEUKIN 1

In order to test this nutrigenomics hypothesis the field of inflammation serves as appropriate approach. On the one hand this is a key mechanism in the development of chronic diseases and on the other hand it is already common that certain nutrients manage to change expression of inflammation mediators [KORNMAN et al., 2004].

Inflammation means the central controlling process in its function as the body´s answer to harm induced by for example injury. Aiming to remove all disturbances it does not differentiate between those within the body (for example autoimmune disease) and those from outside (for example infections) so that one can say that acute and chronic diseases are modulated by inflammation. The complex and mulitlaying interplay between beneficial and noxal arms is termed by the inflammatory reply [VODOVOTZ et al., 2009] where the IL-1 family is closely connected to the inflammatory reply [DINARELLO, 2009]. It is particularly involved in various infectious, inflammatory and immunologic challenges [DINARELLO, 1985]. Thus inflammation plays a significant part in disorders as a sequence of aging processes but also chronic diseases whereas genetic differences in the inflammatory reply seem to navigate differing disease developments among individuals. Additionally these key cytokine genes have variations that appear to impact the differential expression of inflammation mediators including the course of the chronic diseases since they offer a descriptive instance of the role of inflammatory genetics [KORNMAN, 2006]. At the beginning of inflammation the genes for interleukin 1 (IL-1) and tumor necrose factor

(TNF) are the first genes activated in response to a challenge [KORNMAN, 2006]. These two cytokines are capable to mobilize each other wherefore they play a dominant role in inflammatory processes. The fundamental characteristic of IL-1 in biological working process is the capability to activate cyclooxygenase type 2 and inducible nitric oxide consequencing in important expression of prostaglandin E2 and nitric oxide by cells exposed to IL-1. Further essential attributes are the controlling of collagen, bone synthesis and breakdown but also initiation of adhesion molecules. Recombinant drugs blocking the activity of TNF or IL-1 have succeeded to decrease inflammation in plenty of treated patients suffering from rheumatoid arthritis which has coevally pointed out the critical part of the cytokines [KORNMAN et al., 2004]. Complex diseases occur by interplay between environmental influences and multiple genes and clinical outcomes presumably are the consequence of involvements of component causes and modifying factors. The genetic variations seem to provoke the progression of the mentioned disorders and that is why they represent an ideal starting point for nutritional interventions in order to positively influence variations and as a consequence chronic diseases [KORNMAN, 2006]. A recent study in overweight men has shown that an antiinflammatory dietary mix has the potential to modulate inflammation and oxidative and metabolic stress [BAKKER et al., 2010]. Miscellaneous regulatory processes capable to alter transcription factors and gene expressions and being involved in expression of the IL-1 genes or IL-1 activation of other inflammatory mediators have been demonstrated to be modifiable by various nutrients. For instance previous studies have approved that diet supplementation with omega-3 fatty acids or tocopherols have the potential of reducing IL-1 levels [MEYDANI, 1991; DEVARAJ & JIALAL, 2000]. The main target of research is to realize a reduction of the risk to once develop a chronic disease with gene-tailored nutrition, individually adapted for specific high-risk patients [KORNMAN et al., 2004].

3.3.1.1.2. METHYLENETETRAHYDROFOLATE REDUCTASE

Elevated levels of homocysteine stay in conjunction with cardiovascular diseases whereas the originators for the status of hyperhomocysteinemia are dietary deficiency

factors such as lack of vitamin B (B2, B6, B9, B12) but also genetic variations [DUCROS et al., 2002; KUMAR et al., 2009]. Methylenetetrahydrofolate reductase (MTHFR) is integrated in the regulation of folate and methionine metabolism [GILLIES, 2003] catalyzing the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which acts as a methyl donor within the process converting homocysteine to methionine [TRABETTI; 2008, ROTHENBACHER et al., 2002]. In 1988, Kang et al. discovered a variant of MTHFR associated with decreased enzyme activity and increased homocysteine concentrations [KANG et al., 1988].

Today after plenty of studies have been made, it is widely accepted that mutations which are caused by two SNPs on different positions of the gene (677C>T und 1298A>C) are associated with reduced MTHFR specific activity resulting in increased homocysteine levels [VAN DER PUT et al., 1998]. And an already ascertained causality between elevated homocysteine and cardiovascular disease exists [WALD et al., 2002]. Various studies demonstrated that polymorphisms in genes coordinating homocysteine metabolism in the homocysteine pathway (MTHFR 677 C>T, MTHFR 1298 AC and CBSins) seem to be capable of modifying the efficacy of the pharmaceutical pravastatin in lowering risk of cardiovascular troubles [DUCROS et al., 2002; MAITLAND-VAN DER ZEE et al., 2008].

To achieve successful treatment of homocysteinema it is advisable to aspire folate-rich and folic acid supplementation [ASHFIELD-WATT et al., 2002]. Additional dietary supplementation with adequate minerals and vitamins could in specific cases aid to surmount metabolic blocks in key DNA maintenance pathways. Increased administration of a cofactor should turn out as effective when a polymorphism in a gene hinders the binding affinity for its cofactor sequencing in a reduced reaction rate. Micronutrient impacts on DNA are observed for example in common mutations in the methylene-tetrahydrofolate-reductase (MTHFR) gene and other genes in the folate/methionine cycle because study results have evidenced that there are essential interactions between the MTHFR C677T polymorphism, its cofactor riboflavin and folic acid referring to chromosomal instability [FENECH 2008; KIMURA et al. 2004].

This is demonstrated by reduced nuclear bud frequency in T677T homozygotes relative to C677C homozygotes and by the knowledge that high riboflavin concentration heightens nuclear bud frequency when low folic acid conditions are given presumably by increasing MTHFR activity. This again diverts folate away from deoxythymidine triphosphate synthesis, enhancing prospects for uracil incorporation into DNA synthesis, the generation of BFB cycles and consecutively gene amplification but also nuclear bud formation [FENECH, 2003].

3.3.1.1.3. SNPs AND ADULT HYPOLACTASIA

A spectacular example of how SNPs change gene expression is a polymorphism that affects tolerance to dietary lactose in milk. Usually in adult mammals the lactase activity declines after the suckling period. Probably with the domestication of the cattle and the strong survival benefit, the genetic trait of lactase persistence has been suggested to originate from a mutation event 5000 – 10000 years ago predominantly in Northern Europeans [TROELSEN 2005]. Two particular SNPs are closely associated with adult-type hypolactasia. The DNA variant C_{-13910} upstream of the lactase gene is 100% established and a second variant, G_{-22018}, is more than 95% associated with adult-type hypolactasia as studies from Finnish families and individuals could reveal [ENNATAH et al., 2002]. 13910 as well as -22018 polymorphisms are located in introns of MCM6 which is situated in 3kb upstream of the activation zone of the human lactase gene [TROELSEN 2005]. They are supposed to change regulatory protein-DNA interactions that are responsible for gene expression. However it is verified today that the molecular difference between lactase persistence or nonpersistence is induced by a mutation at position -13910 and probably -22018 depending on cis-acting elements and the respective allele at position -13910 and/or -22018 [HOLLOX et al, 1999; TROELSEN et al., 2003].

3.3.1.1.5. FURTHER FAMIILIAR POLYMORPHISMS

Other familiar polymorphisms such as the manganese superoxide dismutase alanine to valine change in the -9 position which hinders transport of this enzyme to the

mitochondrion where it is usually located, is under suspicion to promote oxidative stress and breast cancer risk. Individuals with this mutation might benefit more than those without from a higher supply of food components such as specific vitamins or minerals in individually adapted dosages and amounts [AMBRASONE et al., 1999; FENECH, 2003].

In vitro studies on the interactive impacts of folic acid deficiency and inherited polymorphisms in the MTHFR, BRCA1 and BRCA2 genes showed that moderate deficiencies in folic acid influence genome instability more than the inherited mutations. Although those mutations, which are closely explored recently because of diverse spectacular study insights, should not be underestimated, it has to be taken into account that such research findings underline the enormous potential of diet to impact genome maintenance [KIMURA et al.,2004; FENECH et al.,1999].

3.3.2. EFFECTS OF NUTRITION COMPONENTS ON GENE EXPRESSION

Various ways exist for altering the activity of specific genes and their variants by bioactive dietary compounds. Principally they can impair gene expression directly or indirectly. At the cellular level nutrition compounds may:

1) serve as ligands for transcription factor (TFs) receptors
2) be metabolized by primary or secondary metabolic pathways, thereby changing substrate or intermediate concentrations, or (FATTY ACIDS)
3) positively or negatively influence signal pathways [KAPUT & RODRIGUEZ, 2004]

For enhanced exemplification of the complexness of „simple“ foods, the components of corn oil are shown in the following illustration. The plurality and concentrations of triglycerides, fatty acids, sterols and their esters, and tocopherols are able to effect mechanisms in different directions considering that dietary chemicals have several options to enter a cell [KAPUT & RODRIGUEZ, 2004].

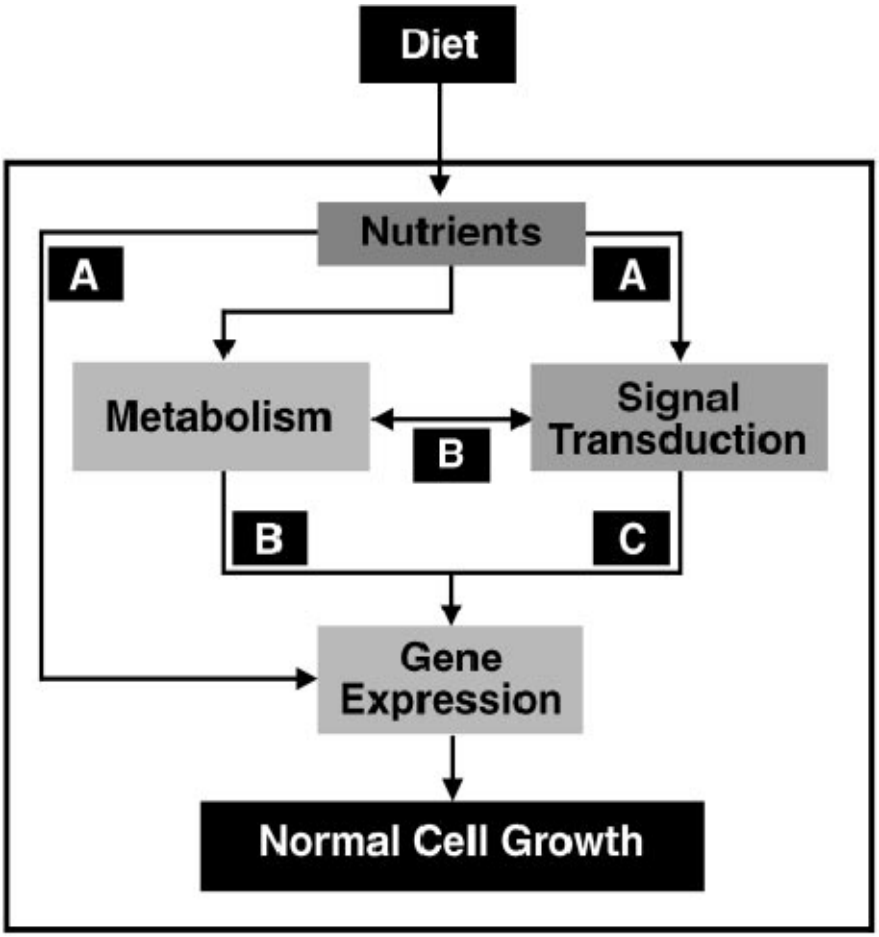

FIGURE 4: FATE AND ACTIVITIES OF NUTRIENTS WITHIN A CELL

Nutrients can attend directly as ligands for transcription factor receptors (pathway A), can be metabolized by primary or secondary metabolic pathways changing concentrations of substrates or intermediates (pathway B) and be involved in gene regulation or cell signaling, or also can change signal transduction pathways as well as signaling (pathway C) [KAPUT & RODRIGUEZ, 2004].

3.3.2.1. METABOLIZATION

For example fatty acids are metabolized via the beta-oxidation pathways in order to produce cellular energy (pathway B). An extended fatty acid-rich diet is accompanied by highest rates of beta-oxidation which can manipulate the intracellular energy balance and which as a consequence influences gene expression because of changes in the NAD+/NADH ratio [COSTA et al., 2010; LIN & GUARENTE, 2003].

3.3.2.2. SIGNAL TRANSDUCTION PATHWAY

Dietary chemicals can also develop the ability to directly bias signal transduction pathways (pathway C).

Signal transduction can be described as a process of information transmission initiated by an extracellular signal from the plasma membrane into the cell and along an intracellular chain of signaling molecules to excite a cellular reply [BODE & DONG, 2003].

3.3.2.2.1. 11-EPIGALLOCATECHIN-3-GALLATE

One example is a component of green tea which is a polyphenol called 11-epigallocatechin-3-gallate (EGCG). EGCG reduces signaling via the phosphatidylinositol 3-kinase, Akt kinase to NF-κB pathway because of inhibition of basal Her-2/neu receptor tyrosine phosphorylation. As NF-κB pathway plays a major role in forms of breast cancer the green tea polyphenol could offer potential benefit [PIANETTI et al., 2002].

The transcription factor NF- κB also is famous for its importance in inflammation processes [HOU et al., 2004]. Remarkable is also the observation from normal human epidermal keratinocytes, that lower concentrations of EGCG (smaller than 1 µM) stimulated cell proliferation resulting in increased thickness of the skin but hindered UV-induced apoptosis of epidermal keratinocytes - probably because of initiation of ERK and Akt pathways and altering the Bc1-2-to-Bax ratio. EGCG-induced ERK phosphorylation was identified to be critical for the phosphorylation of Ser112 in Bad protein, and the EGCG-induced activation of the Akt pathway was found to participate in the phosphorylation of Ser136. Additionally an enhancement in the Bc1-2-to-Bax ratio could be detected because EGCG increased Bc1-2 expression but lowered Bax expression [CHUNG et al., 2003].

A further study exploring effects of polyphenols pointed out that EGCG could be utilized for treatment of wounds or diverse skin conditions associated with changed cellular activities or metabolism. Scientists came to these conclusions from aged

keratinocytes with already decreased basal cellular activities which have been cultured in growth medium for approximately 25 days. Afterwards renewed DNA synthesis could be observed and activated succinate dehydrogenase up to 37-fold upon exposure to either EGCG or the polyphenols [HSU et al., 2003].

3.3.2.2.2. RESVERATROL

Resveratrol is a phytoalexin enriched in the skin of grapes but also found in berries or peanuts and is involved in p53-dependent processes. The p53 is one of the most important tumor-suppressor genes critical for apoptosis and lack of p53 expression is associated with an increased risk of tumor format-ion. Hence it seems to be very promising for cancer prevention as studies showed that the antitumor activity of resveratrol occurs through extracellular-signal-regulated protein kinases (ERKs) and p38 kinase-mediated p53 activation and induction of apoptosis. Furthermore studies have exhibited that resveratrol-induced activation of p53 and apoptosis depends on the activities of ERKs and p38 kinase and their phosphorylation of p53 at serine 15 which plays a critical role in the stabilization, up-regulation but also functional activation of p53 [HOU et al., 2004; HUANG et al., 1999; DONG, 2003].

3.3.2.3. NUTRIENT COMPONENTS LINKING TO TRANSCRIPTION FACTORS

Furthermore in some cases dietary chemicals themselves serve as exogenous ligands and thus possess the ability to directly modify gene expression for specific nuclear receptors of the transcription factor superfamily (pathway A) [COSTA et al., 2010]. In case of transcription factors (TFs) cells interact with their surrounding environment of verifying extracellular molecules with specific receptors located on their surface area [DEBUSK et al., 2005]. This docking process accesses the cascade process of signal transduction whereas the molecules entering the nucleus and connecting with specific DNA sequences are known as TFs. The particular DNA sequences which they are binding are accordingly called TF binding sites, also common as DNA elements. Sometimes the extracellular molecule itself even enters the nucleus to link to a receptor with the consequence that the arisen complex serves as the TF. But the TF

also has the ability to bind one or more further molecules to create the TF complex which afterwards binds to the DNA. The binding of the TF leads to a conformational change in the DNA and promotes or hinders transcription according to cellular and environmental requirements [DEBUSK et al., 2005]. This may also be the case for bioactive dietary components because they also are not the TFs themselves but bias the ability of TFs to bind to their DNA elements [DEBUSK et al., 2005]. In majority of cases nutrients bias gene expression by activating or suppressing specific transcription factors whereas the most essential group is the superfamily of nuclear receptors involved in affecting gene transcription. This group is composed of 48 members in the human genome and can be distinguished into six subfamilies of which the NR1 family is the most leading in nutrition metabolism. Nuclear receptors coordinate gene expression via various distinct processes that include both activation and repression of DNA transcription. After sitespecific DNA binding, their final transcriptional activity depends on coactivators and corepressors which are associated proteins [BUNGER et al., 2007].

3.3.2.3.1 NUTRIENT SENSORS

Transcription factors are mainly expressed in metabolically active organs such as adipose tissue, liver or intestine and they can be seen as the main agents by which nutrients bias gene expression. Because of their ability to alter the level of DNA transcription of specific genes in response to nutrient changes they are also called nutrient sensors. Nuclear receptors are obligatory in coordinating biological mechanisms like nutrient metabolism, cellular proliferation and differentiation. Nutrients are able to activate these receptors and consequentially they are also able to influence a wide range of cellular functions [AFMAN & MÜLLER, 2006].

In nutrition one can find a range of examples influencing gene expression and thus changing genetic outcomes such as vitamin A that modifies expression of diverse genes with the retinoic acid receptor TF, or PUFAs impacting the peroxisome proliferator activator receptor/retinoic acid receptor complexes to link to their DNA elements. Flavonoids for example manage to coordinate miscellaneous genes with the aid of

following genes: nuclear factor kappaB, estrogen receptor and activating protein-1 [DEBUSK et al., 2005].

3.3.2.3.2. PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS

This group of receptors is acting as nutrient sensors of intracellular lipid and fatty acid concentrations [KUSSMANN et al., 2006] which navigate impact of dietary fatty acids on gene expression. The Peroxisome Proliferator Activated Receptors (PPARs) grouped as NR1C can be divided into the three isotypes α (NR1C1), δ1 (also called β) (NR1C2), and γ (NR1C3) whereas they get distinguished according to their specific biological properties. Transcriptional regulation by PPARs implies heterodimerization with the retinoid x receptor RXR; RN2B. After activation by a ligand the PPAR/RXR heterodimer leads to transcription through binding to DNA response elements which are located in and around the promoter of target genes. Additional to upregulating gene expression, PPARs also have the ability to repress transcription, known as transrepression, by interacting with other transcription factors and impeding their signaling pathways [BUNGER et al., 2007].

3.3.2.3.2.1 PPARγ ISOTYPE

PPARγ isotype is mainly expressed in adipose tissue and can be seen as the prime regulator of adipocyte differentiation. Hence, as an essential regulator of adipogenesis, PPARγ is involved in the coordination of systemic glucose and lipid metabolism. Additionally it acts as the receptor for the thiazolidinedione TZD class of insulin sensitizers to assist in glycemic control in type 2 diabetic patients [BUNGER et al., 2007; CHRISTODOULIDES & VIDAL-PUIG, 2010]. The PPARγ gene might play an essential role in the onset of Type 2 Diabetes Mellitus as some studies found out that a missense mutation (proline → alanine) in PPARγ 2 stays in connection with improved insulin sensitivity and a lowered risk of developing Type 2 Diabetes Mellitus. It could be demonstrated that the Pro12Ala polymorphism in the PPARγ 2 gene beneficially influences insulin resistance and the risk of myocardial infarction in conjunction with T2DM [LI et al., 2003; DONEY et al., 2004]. PPARγ activation leads to adipocyte

differentiation but also ameliorated insulin signaling of mature adipocytes [KUSSMAN et al., 2006].

3.3.2.3.2.2. PPARα ISOTYPE

PPARα can mostly be found in the liver and plays a key role in adaptive thermogenesis and lipid/energy utilization (PPARS and PPAR adipocytes) whereas activation of PPARα results in proliferation and increased beta-oxidation of fatty acids [KUSSMAN et al., 2006].

PPARα is essential during status of food deprivation and abrosia. During absence of fasting, free fatty acids get released from the adipose tissue and as a result move to the liver where they get partially or entirely oxidated. These fatty acids also bind PPARα which then leads to increased expression of a range of genes because of binding to specific sequences in their promoter regions. Additionally they achieve indirectly increased expression by genes that are directly influenced by PPARα. The PPARα-target genes are implicated in miscellaneous metabolic mechanisms in the liver such as fatty-acid oxidation, apolipoprotein synthesis or amino-acid metabolism. This is a smart pathway where the originating signal leading to adaptive changes in liver metabolism during fasting period arises from the adipose tissue and manages with a receptor, the expression of which is upregulated by fatty acids during periods of abstinence [MÜLLER & KERSTEN, 2003].

4. METHODOLOGY OF METABOLOMICS

4.1. CONSIDERATIONS BEFORE APPLICATION START

One beneficial characteristic of OMICS-disciplines such as genomics or proteomics is that they are alleviating analytic investigations. At least by theory they are responsive to single analytical platforms adverting to the fact that expressed genes within an analyte can be defined by a single DNA array [SCHENA et al., 1995] and proteins by a single polyacrylamide gel [AEBERSOLD & MANN, 2003].

However, this is not so for metabolites. In contrast, metabolomics sticks out from any other compound study in scale and biologic plurality. And its metabolites appear everywhere [MOCO et al., 2007] like inside and outside of cells, bioliquids and tissues; frequently as mélanges differing in concentration and prevalence. Hence no single analytical technique comprises the power to measure all metabolites coevally. That is why technologies more and more get conjuncted to satisfy researchers´ ambitious claims. Hence, as the subsequent work will focus on methodological techniques, plenty of combination constructions of single methods will be recurrent theme [GERMAN et al., 2004]. In order to choose the right method or the combination of a couple or even multiple methods, a range of issues should get thought through previously such as:

Which key metabolites are intended to be measured and should they get captured quantitatively or qualitatively? What kind of nonessential nutrients should be involved in course of analysis? Which components will need kinetic measurements or flux? Which cells, tissues and/or liquids exactly should get sampled? [GERMAN et al., 2004]

Depending on the specific focus one can choose between targeted or untargeted methods as well as metabolic finger- or footprinting whereas each analysis method is characterized by a range of typically applied techniques. This is illustrated in Figure 6 below *[HOLLYWOOD ET AL., 2006].*

After clarification of these issues there is a palette of current methodological options available for metabolomic research dealing with points like rapid sample collection, instant quenching and extraction of intracellular metabolites but also quantification of these metabolites by applying modern technologies. The latter chiefly are

chromatographic techniques coupled to mass spectrometry and nuclear magnetic resonance spectroscopy [MASHEGO et al., 2007] which are going to be discussed in the following.

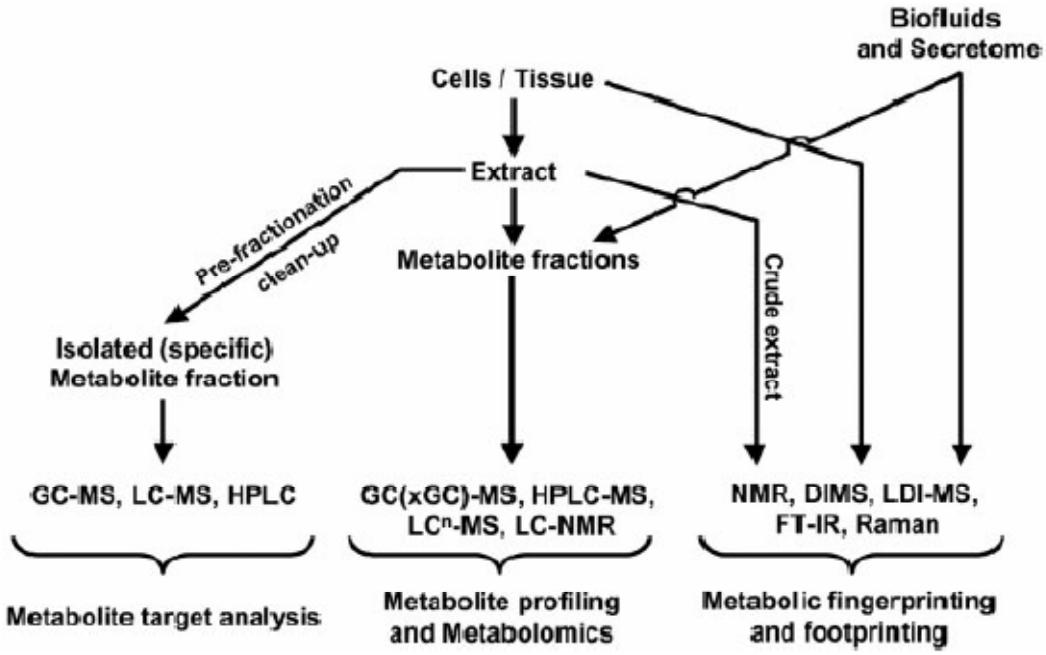

FIGURE 6: SELECTION OF TECHNOLOGIES APPLICABLE IN COURSE OF METABOLOMIC INVESTIGATIONS [HOLLYWOOD ET AL., 2006]

4.2. TECHNIQUES

4.2.1. MASS SPECTROMETRY

The investigation of the whole metabolome as the sum of all traceable components in the sample rather than analysis of each individual metabolite is performed by the metabolomics approaches. To monitor in parallel hundreds or even thousands of metabolites, high-throughput techniques are required [TOLSTIKOV et al., 2007].

Metabolomic studies are rather rare which are applying chromatographic separations with for example GC or HPLC on its own and which take retention time as the only major parameter component detection. Instead an enhancement of research possibilities has arisen by connecting two techniques [ROESSNER et al., 2000] which

was additionally supported by the fact that the outstanding complexity of differing components comprises upstream fractionation [METZGER et al., 2009]. Thus mass spectrometry got coupled to miscellaneous chromatographic techniques where ions are generated but also extracted into the analyzer field of the MS where their separation according to their mass-to charge (m/z) ratios takes place. Then these separated ions get discovered and through signal transmission the signal reaches the data system, the location where the m/z ratios are collected together with their relative abundance for final illustration as a mass spectrum [ALLWOOD et al., 2007].

4.2.1.1. GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Gas chromatography-mass spectrometry gets used for a majority of metabolomic analyses because it offers high sensitivity that reduces significantly the number of raw biological material which are obligatory for precise measurements, optimized component separation in the gas than in the liquid phase and for extended compound data collections and experimental protocol [KANANIA et al., 2008].

Precondition for successful GC-MS analysis is analyte volatility and thermal consistency [HALKET et al., 2005] which may be the drawback of this technique because chemical derivatization prior to quantitative analysis is required. At long sight this exigency could be taken as the advantage of exhausting selective chemical enrichment and fractionation helpful to profile trace compounds within the metabolite masses [KOPKA, 2006].

Metabolites can be classified into two classes; on the one hand there exist volatile metabolites not requiring chemical derivatization to realize elution through gas chromatographs and on the other hand there are non-volatile metabolites needing chemical derivatization. The analysis of non-volatile metabolites has extensively been deployed for metabolic profiling where various classes of metabolites such as amines, lipids, amino and organic acids or others are elicited in the course of analyses. Sample preparation is more extensive and involves sample drying which can lead to a waste of volatile metabolites [DUNN et al., 2005].

Sample extraction in tissues generally is achieved by methanol, in contrast lipophilic components need to be treated with chloroform. For effective exploration of urine samples such as organic or amino acids and sugars, the use of urease accompanied by trimethylsilylation after drying assists to remove large amounts of urea which can conceal other components and reduce the chromatographic resolution.

Although there do exist some metabolites suiting to GC-MS without derivatization usually for the majority chemical derivatization is required to minimize the polarities of the functional groups while facilitating their separation by GC and beneficially biasing their mass spectral properties. Organic acids commonly get esterified by reaction with diazomethane, whereas silylation, predominantly trimethylsilylation or tertbutyldimethylsilylation, gets more and more favored. In order to achieve improvement of their GC features and to avoid enolization reactions which could introduce a lot of products, keto- as well as oxo- groups normally are oximated. As measured by silylation and oximation reactions, tert-Butyldimethylsilylation has profiled to be most suitable for metabolite profiling applications and furthermore exhibits the benefit that the derivatives behave less sensitive to the hydrolytic effects of moisture than the corresponding TMS derivatives [HALKET et al., 2005].

4.2.1.1. 1. COMPREHENSIVE TWO-DIMENSIONAL (2D) GAS CHROMATOGRAPHY

One possibility to enhance separation and identification of analytes in complex samples is the usage of comprehensive two-dimensional (2D) gas chromatography (GC × GC) [ADAHCHOUR et al., 2005]. This technique is characterized by two columns of varying polarity and shorter length than customary afforded rapid and high-resolution separations. Normally the first column comprises a non-polar stationary phase and the second is a short polar column. As the two different column selectivities are capable to impact retention processes, as a consequence those can lead to high peak capacities. A magnificent characteristic is that intact samples can be analyzed [PURSCH et al., 2002].

The two columns are conjoined by a modulator which is the most essential instrumental component in GC x GC as modulation is precondition for the sample´s transfer from the first column to the second. Hence the modulator works as a

continuous injector for the secondary column where primary thermal modulation gets achieved with aid of a resistively heated trap. Though there are several possibilities to achieve modulation one can differentiate three diverse modulation devices that mainly are in frequent use so far; slotted heater–thermal sweeper modulation, cryofocusing modulation and diaphragm-valve modulation. Slotted heater–thermal sweeper modulation works with a rotating heating device, also termed slotted heater, where from first column eluted samples get collected on a short segment of a thick-film capillary column and subsequently get detached by activation of a heat pulse. In cryogenic modulators each unique fraction gets cryo-trapped by means of CO_2 or N_2 cooled jets, focused and then after rapid heating transmitted into the second column as a narrow band. For Diaphragm-valve modulation a six-port diaphragm valve gets assembled as a modulator because rapid valve-switching produces pulses of sample that are injected into the second column. The sample eluting from the first column gets differentiated after the valve and prior the second column [PURSCH et al., 2002].

The very fast separation in the short and narrow two-dimension column entails in peak widths of mostly between 80–600 ms at the baseline. To achieve appropriate monitoring of these narrow peaks, quick detectors together with a small internal volume, a brief detector rise time and a high data acquisition rate are a must [ADAHCHOUR et al., 2006].

As always this method also owns its problems and two main drawbacks of the GC-MS technique for the investigation of polar compounds in chemical samples are the rather extended time for sample preparation and analysis as well as the restrictions induced by the requirement to deal with sample volatility. Many compounds can be derivatized so that they become more suitable for GC, but one has to be aware about the fact that not all compounds are amenable to this [RAMAUTAR et al., 2005].

GC-MS ion formation is typically provided by electron impact (EI). EI involves the independent elimination of the sample's solvent prior to the vaporized sample being swept into the ionization source where it is impacted by a steady flow of electrons with sufficient energy to ionize the vaporized molecule. Mass detection is realized by single quadrupole, TOF or ion-trap-based mass analyzers. A single quadrupole mass

analyzer requires chromatographic times of more than an hour to give satisfying separation of a complex sample mixture. By employing a high-speed TOF mass analyzer coupled with deconvolution software this can be minimized by approximately 75% [ALLWOOD et al., 2008]. TOF-MS with its capability of producing mass spectra of excellent quality within an enormously short time span of only few milliseconds combined with high sensitivity [SANTOS & GALCERAN, 2003] offers the rapid scan speed requested for coverage of the spectra of the narrow peaks nourished in GC×GC. With characteristically 200 ms width at peak base it is a proper means for identification of the sharp peaks in GC x GC [PURSCH et al., 2002] by sampling ions on bushes, which are expedited within a linear electric field into a field-free tube. Parallel to their velocity ions move a specific distance in the field attaining kinetic energy. Considering that speediness means to be inversely proportional to the square root of m/z, the ions get portioned so that lighter ions cross acceleration and drift zones earlier than the heavier ions. A planar detector is employed to transform the ion arrival situation into an electrical signal whose time with regard to the beginning event is measured [SANTOS & GALCERAN, 2003].

Nevertheless most of primary samples are so extraordinarily complex leading to differences in peak shapes, retention-time drift and variation as an answer to compound diversity that it is clear that deconvoluting metabolomics using conventional manual means would be too long-winded. As a result specialized, automated software tools have evolved and are already in the field.

4.2.1.1. 2. DATA INTERPRETATION

The chromatogram deconvolution can take place with a range of various software packages where three of them (ChromaTOF, AnalyzerPro and AMDIS) have been compared for their applicability for data analysis from metabolite mixtures analyzed with GC-TOF-MS. ChromaTOF and AMDIS produce artifactual components corresponding to noise and which create duplicate or multiple peak assignments that again definitely correspond to a single chromatographic peak and chemical entity. While reconstruction of „pure-component“ spectra from complex chromatograms can

be acted out without serious complicacies, in contrast repeatability of all software is regrettably rather unsatisfying. For an upgraded and advanced repeatability new algorithms need to be located and at least existing algorithms have to get modified with a simplified protocol for sample preparation. Besides, the results also depend on analyte concentrations.

According to the experimental objectives ChromaTOF and AnalyzerPro are recommended if obtaining an accurate number of the metabolites in samples from mass spectra is preferred. In case precise mass spectra are preferred, AMDIS and ChromaTOF are the more advisable choices [LU et al., 2008].

4.2.1.2. LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Liquid chromatography-mass spectrometry (LC-MS) is another combined technique which varies from gas chromatography-mass spectrometry because of lower analysis temperatures and the fact that analytes are not requested to be volatile. Additionally derivatization is not a precondition which extraordinarily simplifies sample preparation [DUNN & ELLIS, 2005]. It is a rather universal separation system that can be used for the targeted analysis of specific metabolite groups but also deployed in a wide-ranging non-targeted way. It as well provides the added benefit of analyte detection by means of fraction collection and/or concentration, which are more demanding when utilizing GC separations [BEDAIR & SUMNER, 2008].

LC-MS-based methods are the optimal choice for metabolomic analysis for analytes like urine which can be injected directly onto the column whereas not pre-treatment has to be carried out other than the facile displacement of particulates. Blood plasma can also be explored with minimal sample pre-treatment characteristically based on the removal of proteins by solvent precipitation, and tissue extracts are also approachable to LC-MS-based analysis [THEODORIDIS et al., 2008].

Principally liquid chromatography (LC) defined in its simplest modes can be described as the component separation of a composition based upon the rates at which they elute from a stationary phase usually over a mobile phase gradient. Unequal affinities of the mixtures components for the stationary and mobile phases results in their

separation considering that specific components will be more attracted to the mobile phase to also elute quickly whilst the remaining will be retained by the stationary phase for longer and that´s why elute more slowly with the sequence of a later retention time (RT) [ALLWOOD & GOODACRE, 2009].

4.2.1.2.1. ATMOSPHERIC-PRESSURE INTERFACE AND ELECTROSPRAY IONIZATION

A major tool to realize metabolite separation is the atmospheric-pressure interface API as it can be linked with liquid chromatography and as it exhibits a range of amenable characteristics such as satisfying sensitivity, high dynamic range and versatility but also provides soft ionization conditions allowing access to the molecular mass of intact molecules from complex mixtures [WERNER et al., 2008]. The leading characteristic is the nebulization of the column effluent from the LC into an atmospheric-pressure ion source area. The gas-phase ions with solvent vapor and nitrogen bath gas get sampled by means of an ion sampling device into a first pumping stage. The composition of gas, solvent vapor and ions is supersonically expanded into this low-pressure region. Afterwards the core of the expansion including the ions and further neutral material of higher molecular mass, gets sampled by a skimmer into a second pumping stage at a pressure of between 0.1–1 Pa, possessing an ion focusing and transport device in order to ideally transfer and focus the ions in an appropriate manner to the mass analyzer region. In the majority of systems the ion transfer device is composed of an RF-only quadrupole, hexapole or octapole whereas it is insubstantial whether a high flow-rate or a low flow-rate of liquid is nebulized. From the ionization point-of-view it is also irrelevant if the ions are generated by ESI or APCI although varying tuning of voltages in the ion optics might be requested by reason of some differences in the ion kinetic energies [NIESSEN, 1999].

Electrospray ionization (ESI) is a simple and extraordinarily gentle ionization technique where the sample solution needs to get nebulized into electrically charged droplets in order to sequently liberate the ions from the droplets to analyze them after they have been transported from the atmospheric pressure ionization source region into the vacuum and mass analyzer of the mass spectrometer. The necessary positive charge

on droplets is achieved by the removal of negative charge via electrochemical discharge of negative ions against the metal wall of the spray capillary [BRUINS, 1998]. ESI can be deployed in both positive and negative ionization polarities optional to the capillary voltage that is utilized. In ESI+ mode, common adducts include protonated [M + H]+, sodiated [M + Na]+, and potassiated [M + K]+ whereas in contrast in ESI− mode the plurality of ions are deprotonated [M − H]−, although when chlorine is present within the analyte metabolites, negatively charged chlorine adducts can also be found. Various complex and diverse adduct species can be viewed in both ionization modes. Naturally occurring 13C isotope-containing compounds and double charging of the adduct species additionally advance the data complexity, although the use of high-purity solvents and formic acid can support a reduction of the amount of species observed for each analyte. Compared to alternative ionization techniques ESI involves plenty of key advantages including ionization across a large mass range appropriate for metabolome analysis, good sensitivity, soft ionization because it does not 'over' fragment target analytes and expanded adaptability. It is especially the mass range able to cover by ESI that has made it to develop as one of the most wide-stretched ionization techniques [ALLWOOD & GOODACRE, 2010].

Nevertheless APCI and ESI also are methods with operation limitations. APCI and ESI sources are suspected to work inefficiently at ionizing nonpolar compounds and may cause ion suppression because of competition for charge from differing analytes or additives. This issue especially concerns and is problematic for ESI. APCI can be described as a more previsible and stable ion source than ESI for small molecules which are thermally stabile and volatile. Positive mode APCI creates almost solely [M + H]+ ions in opposition to ESI forming not only protonated ions but also cation-adducted ions like for example sodium and potassium. APCI is more accordable with high HPLC flow rates than ESI. APCI is favored for high flow rate and low-molecular-weight compound analysis. ESI sensitivity is most suitable at low flow rates but can suffer from unreliable detection efficiency for small drug components [HANOLD et al., 2004].

As a sequence atmospheric pressure photo ionization (APPI) has developed as the last arrival in the group of atmospheric pressure ionization (API) methods to connect mass spectrometry (MS) with liquid-phase separation techniques. The basic notion aims to further enlarge the fields of LC-MS-application to those molecules that are not or only poorly accessible to traditional already discussed ionization methods [RAFFAELLI & SABA, 2003]. For this aim photoionization (PI) has profiled as an alternative ionization source. If a molecule is feasible to photoionize only it´s up to interplay of the molecule with a photon of owning enough energy in opposition to APCI and ESI, which depend on ion-molecule chemistry to form the focused ions. APPI ionizes meaningfully more component classes which lightens detection of those constituents not strongly responding on molecular polarity. Besides it seems to be less ominous to matrix-generated ion suppression as well as buffer-created chemical noise [HANOLD et al., 2004].

However to gain the information diversity from an analyte to achieve complete structural elucidation, it is an imperative to employ MS spectroscopy detectors. An MS briefly explained functions to ascertain the mass-to-charge ratio (*m/z*) and the plethora of the differing analytes generated during ionization of a sample extract or chromatographic fraction. Ionization is a major step considering that ions get far more easily biased and thus manipulated than neutral molecules. The three basic components included in all varieties of MS are an ionization source, a mass analyzer and a detector and all three parts are kept under vacuum conditions to assure optimal transmission of ions to the analyzer and detector [ALLWOOD & GOODACRE, 2009]. Regardless of which kind of type gets chosen mass analyzers mean to be the central midpoint of the application which differ from design and performance, strength and weakness from each other. These analyzers can work alone by themselves or fractionally even linked together to complement one another by benefiting from contrarian virtues [AEBERSOLD & MANN, 2003].

The (triple) quadrupole mass filter still is the most commonly preferred mass analyzer where a triple-quadrupole instrument in selected-reaction monitoring (SRM) mode is the determining element in routine and high-throughput quantitative bioexplorations.

Improvements in quadrupole manufacturing process and RF power supply stabilities widened mass resolution without serious leakage in ion transmission. Mass spectra with 0.1 Da full width at half maximum in spite of the normal 0.6 Da can now be maintained and for mass-defensive elements this resolution advancement renders possible a more selective quantitative assignation without problematic losses in signal-to-noise ratio.

Three-dimensional quadrupole ion trap mass analyzers particularly in structure illuminating investigations have been conspicuous which is because of their feature to perform multiple stages of MS-MS and to integrate the data-addicted procedure in the instrument control software. Essential novel introductions referring to three-dimensional quadrupole ion traps are the outcome of a MALDI source for an ion-trap instrument and more an atmospheric-pressure MALDI source and the deployment of infrared multiphoton photodissociation to generate fragment ions over an extended mass range. Furthermore relevant is the principle study of the fragmentation of alkali adducts ions as well as further metal complexation products accomplished by ion-trap MS-MS. As a sequence of the MALDI development as an ionization technique outstanding breakthroughs in TOF technology can now be acted out as resolution up to 20 000 can now be realized as a result of mass accuracies of better than 15 ppm for routine reflectron instruments in conjunction with MALDI and better than 5 ppm for orthogonal-acceleration TOF-MS in combination with ESI [NIESSEN, 2003].

Regrettably API-MS shows poor reproducibility and high interinstrument variability in the generation of fragmentation patterns which have hindered the growth of widespread databases as implemented with electron ionization mass spectrometry or with NMR. When the explored metabolites are satisfactory documented in databases and commercially obtainable mass spectrometry experiments are sufficing, in this case identification gets accomplished by matching the retention time and collision-induced dissociation (CID) spectra of the focused compound to those of the respective reference molecule. CID is the most common ion activation technique where collisions between a fast precursor ion and a neutral target gas results in a rise of internal energy of the ion because of the partial conversion of its translational energy into internal

energy. In very sophisticated situations such as when for example the metabolite is not described in the databases, the need for further analytical techniques such as NMR will increase [WERNER et al., 2008].

4.2.1.2.2. HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

Amplified separation option also can be accomplished with hydrophilic interaction liquid chromatography (HILIC). It is capable to generate a liquid-liquid segmenting between the bulk eluent or rather the mobile, aqueous-organic based phase like for instance water-methanol as well as the water-rich layer that is in some extent made immotile on the surficial area of the stationary phase. This stationary phase is bare silica or silica derivatized with variable polar functional groups [HAO et. al, 2008].

A characteristic silica-based HILIC column consists of a stationary phase that is hydrophilic like for instance sulfoalkylbetaine zwitterionic functional groups with a net charge of zero and covalently attached to silica particles. Sulfoalkylbetaine zwitterions are well-established to be potent osmolytes with a strong capability to bind water as water is related with such zwitterions at interfaces. The outstanding benefits of water-fixing attributes of the zwitterionic stationary phase together with a low surface charge not boosting strong ion exchange interactions offer a unique environment competent of solvating polar and charged components. Hence these circumstances allow high-performance HILIC separations [CUBBON et al., 2009]. The mechanism of separation and retention is supposed to predicate on the analyte portioning into the hydrophilic surroundings and the fragile electrostatic interactions with either the positive or negative charge of the respective functional group [CUBBON et al., 2009].

4.2.1.2.3. LIQUID CHROMATOGRAPHY-NUCLEAR MAGNETIC RESONANCE-MASS SPECTOMETRY

An additional possibility to ameliorate challenging mixture analysis offers LC-NMR-MS which benefits from the coupling of the powerful chromatographic techniques.

The plurality of LC–NMR–MS applications disposes of stopped-flow mode. In this mode the sample is stationary as in classic NMR. When the analyte reaches the active NMR probe volume, the flow is held to afterwards diverte the eluant either to waste or to on-line storage loops. Loop storage mode is a valuable benefit whereas chromatographic peaks are trapped online in a 36-loop cassette subject to a UV or MS detection threshold. Automation navigates the peaks to the loops inspecting the transmission of loop contents to the NMR flow probe for stopped-flow analysis. Loop storage evades peak diffusion on-column which decreases NMR sensitivity when various analytes request for extended NMR acquisition. Minor sample amount is required because all the analytes in a single injection are stored and acquired later under automation. If the Bruker NMR-Mass Spectrometry Interface is (BNMI) included then MS experiments can be received on the loop contents and in parallel the NMR acquires data. As a result loop storage mode can be seen as a prolific operation way.
The valve-switching interface BNMI is a computer-driven splitter and a double dilutor for offering an adequate make-up flow for ideal ionization in the MS. It also allows proton–deuterium exchange to facilitate MS spectra otherwise received in LC–NMR–MS [CORCORAN & SPRAUL, 2003].

4.2.1.3. HIGH PRESSURE-LIQUID CHROMATOGRAPHY

The technique of high pressure-liquid chromatography (HPLC) has been developed in order to enhance chromatographic performances referring to efficiency and speed. As a result liquid chromatography (LC) has recently emerged in the development of short columns packed with small particles of sub-2µm acting at high pressures of approximately 400 bar [NGUYEN et al., 2006]. Sub-2µm particles are frequently utilized to optimize the chromatographic resolution power along with short column lengths to achieve reduction of sample analysis times, backpressure and solvent consumption whereas the performance of HPLC separations is biased by the parameters of column length, small particle sizes but also pump pressure [GIKA et al., 2008; ALLWOOD & GOODACRE, 2009]. Usually HPLC separations are more suitable for the exploration of labile and high molecular weight compounds as well as for the study of nonvolatile

polar compounds in their natural occurrence [TOLSTIKOV et al., 2007]. The chromatographic separation performance of HPLC is up to the selectivity of the mobile phase and stationary phase system and the achievement potential of the HPLC column [FOUNTAIN et al., 2009]. The column can be viewed as the heart of each HPLC approach allowing component resolution based upon the selectivity and column efficiency. Usually HPLC columns consist of a tube made of stainless steel, glass or synthetic polymers filled with microparticulate porous silica [CABRERA et al, 1998]. The column efficiency depends on plenty of factors such as the diffusion coefficient of the sample in the mobile phase which again is down on the mobile phase viscosity and the molecular weight of the analyte but more essential on the column length and the packing particle size combined with the mobile phase speed [FOUNTAIN et al., 2009]. With monolithic columns satisfying ratios such as large-sized through-pores and as a consequence through-pore size / skeleton size ratios are available resulting in enlarged performance together with simple pressure-driven elution employing current instrumentation [TANAKA et al., 2002]. Worth mentioning is the frequently applied use of C18 monolithic silica capillary columns in HPLC coupled to ion trap mass spectrometry detection as it was explored on the model plant *Arabidopsis thaliana*. It could be shown that the application of a long capillary column is an easy and effective possibility to reduce ionization suppression by enhanced chromatographic resolution [TOLSTIKOV et al., 2003].

4.2.1.3.1. ADVANCED HIGH-PRESSURE-METHODS

Today HPLC-MS is the standard analytical tool in pharmaceutical qualitative and quantitative analysis of potential pharmaceuticals and their related metabolites for studies from initial discovery to large scale production. For the study area of metabolomics technological limitations of HPLC-MS constrained its use as metabolomics deals with the exploration of endogenous metabolites and not drug-related metabolites. As an alternative systems applying ultrahigh pressure LC systems have found their way as useful means in metabolomic investigations as the result of continuing developments of HPLC focusing on efficiency and velocity amelioration of

separations over traditional HPLC [DUNN et al., 2005]. In relation to conventional HPLC high-pressure methods such as ultra pressure liquid chromatography-mass spectrometry (UPLC-MS) utilizes particles of diameter less than 2µm in the column and optimal linear flow rate speeds of approximately 3,5-6mm per second at advanced pressures within a range of 6000 to 15000 bar. As a sequence separation ratio is enhanced together with band-broadening, better resolution and sensitivity by sharper peaks coupled with decreased analysis time [LI & LEDIGO-QUIGLEY, 2008]. A concrete example for its employment is the metabolomic nutrition study of human plasma where three protein precipitation methods and two anticoagulants were proved and compared. The method utilizing blood collection on heparin and methanol precipitation is analyzing by UPLC-MS and permits performing complete metabolic profiles while maintaining optimal discovery of selected metabolites [PEREIRA et al., 2009]. The recently coined and extended technique of ultra high pressure-mass spectrometry (UHPLC-MS) is connecting sub-2µm particle short length columns and HPLC instrumentation qualified of creating as well as withstanding constant pressures up to 15000 bar and hence producing highly stable mobile phase flow rates. Characteristics are the increased chromatographic separation options, resolution of chemical species not doable with HPLC and with elevated analysis times and minimized use and drop-off of solvents [ALLWOOD & GOODACRE, 2010].

In general these innovations allow to accomplish five to ten fold faster separations than classical liquid chromatography techniques while retaining or even increasing resolution. The enormous permeability and sizeable amount of theoretical plates per unit pressure drop associated with monoliths are features of their most essential and representative physical properties; the high porosity and short skeleton size of monolithic columns enable operation at high flow rates on relatively long columns employing classical LC systems for high-velocity and high-efficiency separations [SCHAPPLER et al., 2009]. The performance limits of conventional chromatographic packings have been upgraded with the progression of extraordinarily efficient and mechanically powerful 1.7µm bridged ethylsiloxane/silica hybrid particles. The utilization of smaller particle packing materials improve flow resistance allowing the

columns to work at larger backpressure synthesized accompanied by physical persistency without jeopardizing the mass loading capacity of the material referring to the bride surface zone, and stable to a wide array of chemical operating conditions whereas decreasing any secondary or mixed mode interplays with numerous analytes. Stability persistence over a broad pH acting range together with the diverse disposable bonded phases support flexibility for method improvement. Accordingly this young particle technology provides essential ameliorations in respect to sensitivity, resolution and sample throughput [GRUMBACH et al., 2005]. Additionally the combination of UHPLC with an MS detector appears to be an adequate method meeting key demands in terms of sensitivity, selectivity and peak-assignment certitude to realize quick analyte discoveries at low concentrations but challenging complex matrices. However because of the extremely narrow peaks - due to UHPLC - linking with MS devices might be a problem. For this possible occurrence specific quadrupole-based instruments owning more optimal acquisition rates have been developed for UHPLC hyphenation. With aid of this new analyzer generation full-scan acquisition could be enlarged up to 10000. Adjacent to quadrupole-based analyzers, TOF instruments are also capable to analyze and collect data over a wide mass range without affecting sensitivity [GUILLARME et al., 2010]. Triple-quadrupole instruments are especially well adapted for targeted analysis such as bioanalysis or multi-component screening and deliver marvelous sensitivity and selectivity. Modern MS2 instruments can achieve elevated signal-to-noise ratio even with only short SRM DT but a middle course should be arranged amongst sensitivity and acquisition rate taking into account the amount of MS2 transitions in a single SRM segment is restricted. TOF-MS analyzers offer an interesting alternative since they afford extended mass resolution including precision over a wide mass area. Furthermore TOF instruments are applicable for both targeted and non-targeted analysis. A substantial limitation of TOF-MS in contrast to MS means the slighter sensitivity but also the higher costs. Ultimately hybrid QqTOF-MS instruments involve the benefits of both techniques offering great possibilities for the confirmation and screening as well as the structural elucidation of unfamiliar components in complex matrices based on fragmentation information. As UHPLC

technology has significantly decreased run times but not decreased chromatographic resolution the major challenge now is sample preparation and data processing especially in complex applications like metabolomics. However at present the main limitation of UHPLC are equipment and consumables costs [GUILLARME et al., 2010].

4.2.1.3.2. MATRIX EFFECTS

The basic features such as high selectivity, sensitivity and throughput are main reasons why the development of atmospheric pressure ionization techniques have achieved frequent presence in laboratory procedures but with them also disturbances which gained popularity under the term matrix effects. Generally matrix effects can cause troubles in the course of the discovery of certain compounds which are not seen in the chromatogram but do provoke harmful influence on techniques´ precision and sensitivity. Matrix effects appear when molecules coeluting with the focused components manage to change the ionization performance of the electrospray interface [TAYLOR, 2005]. A comparison of the dimension of the matrix effect utilizing two differing interfaces, a heated nebulizer – HN and an ion spray – ISP showed, that the matrix effect definitely happened when ISP (ESI) interface was utilized but it was absent when the HN (APCI) interface was deployed. Although the roots of matrix effect are not identified in extenso, at present it is supposed that it may has its seeds in the challenge between an analyte and the coeluting, undiscovered matrix compounds responding to primary ions generated in the utilized interface. Optional to the ambiance in which the ionization and ion evaporation procedures are happening, this competition may effectively decrease (ion suppression) or increase (ion enhancement) the formation performance of the wanted analyte ions present at equal concentrations in the interface. A matrix effects minimization can be realized by altering and bettering sample extraction processes and by liquidating undiscovered matrix disturbances, acting out the assay under ameliorated chromatographic conditions to dissolve target analytes from undiscovered endogenous compounds that may bias the ionization efficiency of analytes, and evaluating and altering the HPLC-MS interface as well as the procedure of ionization of analytes. Any time when some change in the above

parameters is rendered the matrix effect should be reevaluated and its absence should be affirmed before the exploration of “real” analytes starts. However necessarily the precision and exactness of the technique should be assessed in any case applying biofluids from various sources and a relative matrix effect should be rated by analyzing biofluid extracts from different sources spiked with analytes after extraction [MATUSZEWSKI et al., 2003].

4.2.1.3.3. HIGH-PRESSURE COMBINED WITH ELEVATED TEMPERATURES

The area of high temperature-ultra performance liquid chromatography (HT-UPLC) is concerned with potential possibilities of using elevated temperatures and sub 2μm porous particle. In order to achieve sufficient effects one has to overcome a range of impediments. Absolute precondition is a thermally resistant stationary phase feasible to affront temperatures passing over 200°C. The same of course applies to those analytes which get exposed to high temperatures and hence need to keep their thermal stability referring on the time scale of the chromatographic run. Moreover the temperature discrepancy between the incoming eluent and the column must be reduced to mean smaller than 5°C because it would provoke peak broadening particularly in ultrafast separations. With the utilization of narrow-bore-columns the thermal difference defiance can be relevantly improved at elevated column linear speeds [THOMPSON & CARR, 2002]. As a consequence solvent temperatures higher than the boiling point of water up to 180°C as well as thermal gradients to minimize the use of organic solvent get employed whereas temperature acts as a variable; the water serves as a sole solvent in either isothermal or thermal gradient mode. Hot water including water above the boiling point up to its supercritical temperature as an eluent shows decreased viscosity and polarity but enhanced qualification to solubilizing non-polar components. These features lead to the liquid offering properties matchable to an organic solvent. As a sequence elevated temperatures can be used as a means to minimize the number of organic solvent requested or even to eliminate it completely. The capability of temperature to alter eluotropic strength implies that one can use thermal gradients to mimic the effects of solvent gradients.

Furthermore it is possible to drastically lower the analysis time without efficiency losing [GIKA et al., 2008; PLUMB et al., 2007; NGUYEN et al., 2007] and column performance at high flow rate can be meaningfully improved by elevating the column temperature. As the evaluation with high temperature for profiling components including compounds spread over a wide polarity range like those of the well-established model plant *Arabidopsis thaliana* has evidenced, the deployment of a generic linear gradient at high temperature is advantageous; the enormously enlarged peak capacity obtained at room temperature on long columns was kept at high temperature and in parallel the analysis time was decreased by a 2-fold factor [YAN et al., 2000]. The applicability and potential of using elevated temperatures was investigated in a further metabolomic study applying solvent temperatures higher than the boiling point of water up to 180°C from urine and blood plasma samples. They could be sustained from two strains of the Zucker rat, a normal strain as well as the obese strain. The biofluids were explored using high temperature chromatography and traditional reversed-phase LC with MS detection in full scan mode applying a hybrid triple quadrupole linear ion trap mass spectrometer. Ultimately it could be revealed that for urine although not for plasma chromatography at high temperatures supported better results than traditional reversed-phase LC with better peak asymmetry and higher peak effort [GIKA et al., 2008].

Despite all above described advantages HT-UPLC is not routinely used since it also includes several drawbacks. On the one hand the restricted availability of packing materials resistant at high temperatures means to be a serious challenge and on the other hand a feasible degradation of unstable compounds could happen [NGUYEN et al., 2006]. Moreover apparent improvements with respect to peak capacity have to be evaluated with regard to the fact that the application of a MS detector produces supplemental band broadening [GRATA et al., 2009]. Although the method has not taken root until now researchers believe that it may provide a useful alternative for metabolic profiling after realization of necessary developments [GIKA et al., 2008].

4.2.1.4. CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

Capillary electrophoresis-mass spectrometry (CE-MS) is an analytical separation technique feasible of high-resolution separation but also sensitive mass determination of a variety of charged metabolites over both positive and negative ionization modes [SATO et al., 2004]. It has especially proved to be suitable for the separation of polar and charged constituents. It is a more powerful technique than LC referring to separation efficiency and equipped with the benefit that the requested sample amount for analysis is rather little. A further attractive aspect is that costs for accessories are less than in LC due to very low organic solvent consumption or even none. Additional reasons are the slight number of reagents necessary and the application of previous capillaries instead of LC columns higher at price [RAMAUTAR et al, 2006]. Benefits over HPLC are velocity, resolution but also less request for solvent without or at least with only marginal organic solvent consumption and minimal solvent wastes. CE-MS gets deployed for both targeted as well as general metabolomic studies [ISSAQ et al., 2009]. The major disadvantage of CE is the rather small concentration sensitivity because of the limited number of analyte volume that can be introduced into the previous capillary and the low absorption path-length if UV detection gets utilized. Thus sample pre-treatment methods are frequently elementary to preconcentrate the sample. The hyphenation of extraction techniques eliminates the low sensitivity of CE. Coevally efficient clean-up of the sample can be maintained [RAMAUTAR et al., 2006]. Furthermore one has to consider the limited loading capacity. Whereas mL quantities can be loaded onto an LC column, a CE capillary can be filled with a maximum of approximately 1mL. But the limited loading efficiency has not developed too problematically yet due to the received information amount in the analyte which basically is extremely high [MISCHAK et al., 2009].

Currently CE-MS is only really adaptive to the exploration of polar compounds with the characteristic of extended water solubility. For instance the team of Sato [Sato et al. , 2004] unveiled significant complicacies during the study of non-water-soluble phospholipids and chlorophylls that coated the capillary walls leading to mass ion detection disturbances [ALLWOOD et al., 2008].

Anyhow in conjunction with electrospray ionization (ESI)-MS minimized discovery limitations are maintainable. Furthermore MS can offer identification of the metabolites. CE linked with ESI-MS therefore seems to own very promising characteristics as a complementary technique for metabolomic investigations. This coupling is more challenging than that of LC to MS as the very low flow rates in CE cause instability of the electrospray in case of use of a sheathless interface. This problem has been overcome with aid of a sheath-flow interface. Furthermore the CE system can be facilely grounded by sheath flow. Nevertheless such an interface has to deal with a serious drawback of negative impact on sensitivity [RAMAUTAR et al, 2006]. Considering the complexness of sample matrices an increased need for high resolution thus is constantly present and in this relation main classical strategies inducing peak efficiency, selectivity and resolution can be used. The main points biasing CE resolution are capillary dimension and nature, separation electrolyte composition like ionic strength, pH, salt nature and additives, deployed electric field as well capillary temperature. In order to fulfill them in the last years diverse strategies have grown to improve baseline resolution for electrokinetic separations whereas it is obligatory to respond to some non-conventional electrolytes such as non-aqueous electrolytes and quasi-isoelectric buffers.

Since the last 15 years organic solvents have been utilized for electrokinetic separations to ameliorate resolution either pure or in combination with other solvents. The array of usable solvents exhibiting various physico-chemical properties is very wide-spread and leads to a high variety and virtually all separation modes can apply organic solvents [VARENNE & DESCROIX, 2008]. The major separation modes applied in CE are capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC), capillary isotachophoresis, capillary gel electrophoresis and capillary isoelectric focusing whereas for metabolomics approaches CZE and MEKC are chosen most frequently. Accounting that the analysis of various types of endogenous metabolites in a biological system is the key task in metabolomics capillar electrophoresis-mass spectrometry should be applicable for the analysis of an extended range of chemical components with functional groups, widely varying

structures, physicochemical features and concentrations. Hence CE-MS is adequate for the exploration of a very wide variety of chemical compounds and molecules [KLAMPFL, 2004].

4.2.1.4.1. CAPILLARY ZONE ELECTROPHORESIS

Capillary zone electrophoresis (CZE) can be described as an essentially high voltage electrophoresis in free solution. The capillary is filled with the running buffer solution and the ionic analytes are dissolved with assistance of elevated electric fields of hundreds of volts per centimeter optional to their electrophoretic mobilities.

The brilliant Joule heat dissipation because of the favorable surface-to-volume ratio but also the intrinsic anticonvective features of the capillary allow the realization of wide efficiency separation even without the utilization of gels as anticonvective media [TAGLIARO et al., 1998]. Capillary zone eletrophoresis means to be a separation technique requiring low injection volumes which can be viewed situational both as benefit and a drawback. If for instance cell contents and tear fluid which are difficult to collect in huge volumes have to be sampled a small injection volume means to be an advantage. On the other side high concentration detection limits are maintained when small sample volumes are injected. An additional key-parameter for CZE separations is the large impact of the sample matrix like sample ions on the electrophoretic separation. High salt concentrations in the sample should be burked to minimize the possibility of high currents and heat generation. The latter can sequence in air bubbles and current breakdown. Also clogging of the capillary can bring problems. These disadvantages can be solved by the use of an adequate sample-preparation method before analysis begins [VISSER et al., 2005]. A CZE is associated with a stable constant flow without a gradient which results in changes in the optimal ionization parameters. Its low priced capillaries are quite robust and the technique is consistent with especially all buffers and analytes offering rapid separation and high resolution.

But one has to mark that the sample volume that can be loaded onto the CZE is restricted and only little amounts of analyte can be studied and hence emphasize the trouble of sensitive detection. CZE is particularly predestined due to the feasibility of

using volatile electrolytes. As a result the analyte separation within the electrical field of a capillary filled with electrolyte because of differences in the charge-to effective size-ratio, is especially suitable for the study of molecules with a broad range of size and hydrophobicity as found for peptides and proteins. In order to accomplish optimal CZE-separation in very short time high electric field strength is valuable. Furthermore peak broadening under high ionic strength environment is less obtained. By means of such ideal conditions grand currents are received [KOLCH et al., 2005].

4.2.1.4.2. MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

Micellar electrokinetic capillary chromatography (MEKC) is based on a micellar ‘‘pseudostationary’’ phase connected to the buffer which interacts with the solutes depending on partitioning actions in a chromatography-like mode. In this system EOF functions as the chromatographic ‘‘mobile phase’’ which is ideal as it minimizes the band broadening appearing during the separation process [TAGLIARO et al., 1998]. MEKC can be seen as a hybrid of electrophoresis and chromatography and includes the feasibility of certain surfactants to spontaneously generate aggregates. Mentionable advantages of MEKC involve high performance, rapid analysis time and a potent flexibility in rapidly tuning or altering the running buffer composition and subsequently the selectivity of the separation (PAPPAS et al., 2005]. Neutral analytes not possible to be separated by CZE are readily separated by MEKC through differences in analyte affinities between the micellar pseudostationary and the environmental aqueous phases. At present MEKC not only enables the separation of neutral species but also supports added selectivity in the separation of Ionic substances that are sometimes exhausting to separate; however it suffers from low concentration sensitivity as a sequence of the restricted sample volume and short path length for absorbance-based discovery [MOLINA & SILVA, 2002].

4.2.1.4.3. CAPILLARY ELECTROCHROMATOGRAPHY

Capillary electrochromatography (CEC) is a miniaturized separation technique that conjoins selectivity of HPLC with the efficiency of capillary electrophoresis.

Constituents get separated optional to differences in partitioning ratio between the stationary and the mobile phase but also to differences in electrophoretic mobility. High separation efficiencies can be maintained in CEC in comparison to high-performance liquid chromatography which is to trace back to the fact of the special characteristics of the electroendoosmotic flow [EELTINK et al., 2003]. This electroendoosmotic flow is initiated at the interface between silica - of the packing particles and of the capillary walls - and the buffer which significantly ameliorates the kinetics of partitioning equilibria and as a sequence separation performance [TAGLIARO et al., 1998].

Compared with HPLC this method offers a range of advantages referring to the absence of backpressure in CEC smaller particles and which can be used as stationary phase than in the pressure dominated HPLC. Furthermore the EOF is more homogeneous and less biased by packing differences than a pressure driven flow. If a porous material is employed as stationary phase a flow through the pores of the particles may further achieve to better the CEC separation efficiencies [EELTINK et al., 2003]. Depending on the differences in column format one can distinguish three modes of CEC. First group are the packed columns which were principally applied in the first years and have been followed by monolithic columns and the open-tubular format as a second and very powerful trend direction in CEC research. The application of monolithic stationary phase enables the benefit that due to their continuous consistence retaining frits are no longer essential once the material is anchored to the capillary wall [EELTINK & KOK., 2006].

4.2.1.4.4. NON-AQUEOUS CAPILLARY ELECTROPHORESIS

An alternative to aqueous CZE termed non-aqueous capillary electrophoresis (NACE) holds great potential as further separation technique presenting several notable characteristics. Nonaqueous electrolytes generally create lower electric current than aqueous ones which as a sequence makes Joule effects rarely problematic. As a consequence high voltages can be used within short capillaries permitting rapid analyses as well as outstanding peak efficiency increase. Nonaqueous solvents are also

fully assortative and even in diverse cases more adequate than water with plurality of the detectors hyphenated with CE like UV, fluorescence and LIF, electrochemical detection and MS [GEISER & VEUTHEY, 2007]. Though NACE is considered a serious alternative to aqueous CZE at the moment nevertheless MEKC is the major choice to solve selectivity and solubility questions. NACE owns great potential whereas reasons are multiple involving the analyte´s dissociation degree, ion-pairing formation and differing ion conductivities [PORRAS & KENNDLER, 2005]. Additionally a favorable sample introduction to the mass spectrometer referring to essential aspects like volatility, surface tension, flow-rate, and ionization will probably assist to continue the expanding use of non-aqueous CE [RIEKKOLA et al., 2000].

One of the most attractive features of NACE is wide range of organic solvents available in pure or mixture forms. Different solvents can be applied if they fulfill preconditions such as physical and chemical stability, satisfying compatibility with capillaries and detectors and the feasibility to solubilize the analytes but also the electrolytes. In order to achieve rapid analysis effects the relative permittivity values of the solvents should be maximized whereas in parallel their viscosities should be minimized. Equally like in aqueous CE the attendance of electrolytes is required to generate sufficient and reproducible separations with NACE. An adequate buffering is necessary to control effective and apparent mobility of investigated components [GEISER & VEUTHEY, 2009]. One of the most notable characteristics of organic solvents is that their physical and chemical properties differ widely both from each other and from water. To that effect selectivity manipulation in non-aqueous CE can be achieved easily by altering the organic solvent or varying the proportions of two solvents [RIEKKOLA et al., 2000].

In contrast to water, organic solvents may develop powerful absorbance of ultraviolet light so that indirect ultraviolet (UV) or other discovery means are needed. This circumstance may lead to enhanced sensitivity than is achievable in water. The replacement of water with organic solvents in CE electrospray ionization mass spectrometry (ESI-MS) virtually fulfills all requested criteria to realize increased detection by stable spray and high sensitivity. The special spray formation is preferred by the lower surface tension and vaporization hotness of the organic solvents.

Additionally the risk of electrical breakthrough is minored by means of lower spray onset voltage [RIEKKOLA et al., 2000].

Generally NACE gets chosen notwithstanding its inherent solubility or selectivity complicacies. Anyhow NACE is infrequently applied now for other purposes but considering its quite existent and tempting benefits - referring to the generation of decreased electric current -, the option to achieve rapid analyses or the efficient linking with a plurality of diverse detectors it probably will not take too long until this technique has established in laboratory´s workaday life [GEISER & VEUTHEY, 2009].

So there are some cases where NACE is the most appropriate approach. For instance if solubility or selectivity troubles arise, NACE should of course be used as the proper method of choice. It should be favored to MEKC chiefly because of its simpleness and the decreased electric current created. Regarding that NACE will hardly always lead to improved solubility or selectivity in contrast to aqueous CE anyway the wide palette of solvents and solvent mixtures increases likelihood it will reach its intention. Moreover and as already mentioned NACE is well adapted to act out efficient and rapid analyses. And ultimately NACE involves some essential advantages for its hyphenation with ESI-MS considering that plenty of background electrolytes (BGEs) are volatile and create low electric currents. As a consequence enhanced interest may be observed for NACE probably with ACN and MeOH as selected solvents [GEISER & VEUTHEY, 2007].

4.2.2. NUCLEAR MAGNETIC RESONANCE

4.2.2.2. NMR IN GENERAL

The application of nuclear magnetic resonance already dates from the early 1970s when scientists began investigating the ethanol metabolism in rats by NMR-method whereas this technique has gained a significant position in contemporary knowledge of the metabolome and its processes [WILSON et al., 197]. By the mid-1980s the quality feature of NMR developed sensitive enough to detect metabolites in unmodified biological liquids [NICHOLSON & LINDON, 2008]. Hence it enforced its application to its

capability to deal with difficult metabolite complexes without demanding for chemical derivatization or remarkable separation [NICHOLSON & WILSON, 2003].

Because samples for example from different kinds of biofluids do not request for any kind of chemical or physical treatment before the analysis as a result there is no need to separate metabolites from the liquid. Noting the fact that in a single measurement NMR typically ejects a real opulence of information of the metabolites in the sample, it means an outstanding advantage to work without the need of preselection of analytes focused to detect. Additionally it enables identification of the metabolites´ structures but also to measure the molecules´ relative and absolute concentrations. And as it does not destroy analytes , it is particularly predestinated for investigating metabolite levels in intact tissues like biopsy samples which afterwards can be used in the course of following experiments. Furthermore it allows the dynamics and sequestration of metabolites in tissue samples to be found [NICHOLSON & LINDON, 2008]. Generally one can call it a non-biased and non-destructive method easily to quantify an abundance of spectral data results and which even permits the identification of novel compounds as a result of working procedures [NICHOLSON & WILSON, 2003]. This study of absorption of radiofrequency radiation by nuclei in a magnetic field is nuclear magnetic resonance dealing with the creation of strong magnetic fields and radio frequency pulses to the nuclei of the atoms. Elementary particles such as electrons or a nucleus are common to behave as if they rotate about an axis and thus have a property of spin. As each nucleus owns a charge, its spin gives rise to a magnetic field and the resulting nuclear magnetic moment oriented along the axis of spin possesses a characteristic value depending on the kind of particle. The spin can be figured as a small magnetic field causing the nucleus to generate an NMR signal whereas one has to mark that not all nulclei obtain the attribute of having a spin [KHANDPUR, 1989]. Furthermore not only some atomic nuclei hold a non-zero magnetic moment consequencing in the discrete energy states in a magnetic field after quantification. Nuclei like 1H, 13C, 15N, 19F and 31P can experience transitions between these states if radio-frequency pulses of right energy dimensions are deployed. The frequency of a transition is related to the type of nucleus and its electronic surrounding within a

molecule. So for instance 1H nuclei in a molecule give NMR peaks at chemical shifts according to their chemical environment. NMR especially is serving as a structural tool, and the interpretation of the chemical shifts illuminates information on the isomers and molecular conformations like splitting patterns appear as a sequence of nuclear interactions [NICHOLSON et al., 2002]. In case of data interpretation the opulent variety of outcoming data for sure poses a big challenge and as a consequence favorable methods are required to interpret them correctly and in a short time. The NMR spectrum of a sample can be categorized as an-dimensional object which delivers an n-dimensional metabolic fingerprint which characteristically changes corresponding to the metabolic process [NICHOLSON & WILSON, 1999]. Hence for a successful analysis and interpretation of the NMR data first the spectra get cumulated and processed. Then a primary data reduction is worked out that digitizes the one-dimensional spectrum into a series of typically 250 – 1000 integrated areas. After the removal of redundant signals and appropriate scaling, primary data analysis is applied to map the samples optional to their biochemical consistency. Therefore they occupy neighboring positions in the principal component space. One can describe it as an unsupervised mapping of data in a three-dimensional space. As the next step a supervised classification and calculation of confidence limits follows. Each class of samples is then modeled separately and class borders and confidence limits need to be calculated to gain a model for independent data prediction [NICHOLSON et. al, 2002].

4.2.2.2. ^{1}H-HMR

In a characteristical biological-liquid sample, all hydrogen-possessing molecules of the sample, which may be the case for virtually all metabolites, will exhibit an ^{1}H-NMR spectrum assumed that they appear in amounts above the detection limit [NICHOLSON & LINDON, 2008].

^{1}H-NMR spectroscopy of biofluids like plasma and urine already have gained a reputation in being extraordinarily successfully applicable to investigate various metabolic processes and diseases. Their metabolic formation changes according to circumstances of their variable role dependent on particular function but also the

whole organism´s common status. Hence each bioliquid is yielding its characteristic ^{1}H-NMR spectroscopic fingerprint. The relative concentrations of the solutes regulate the spectral intensity distribution and sometimes also intermolecular interactions play a pivotal role as they also can bias the distribution. For biochemical research, high-frequency ^{1}H-NMR spectroscopy sometimes is useful as it is a sensitive method considering the fact that an adequate instrumentation even allows low nanogram detection. Almost all metabolic intermediates possess unique ^{1}H-NMR signatures. An outstanding advantage is that one-dimensional spectra only request the buffering and adding of D_2O to generate a reference frequency within the short time span of few minutes. The annoying large interfering signal generated by water in all biofluids one easily can rid of by the use of adapted solvent-suppression. Metabolic analysis of bioliquids from urine, plasma and cerebospinal fluid illustrate the normal health status of an organism but also the sequence of toxicity and disease. Especially urine and plasma are suitable for clinical-trial monitoring and disease diagnosis referring to the circumstance that they can be obtained in a nearly non-invasive manner. Even a one-dimensional high-frequency ^{1}H-NMR spectra of 600 MHz or higher of urine includes an exorbitant number of sharp lines from a plentitude of metabolites. While in ^{1}H-NMR spectra of plasma low- and high-molecular-weight components are detectable, urine is dominated by rather low-molecular-weight-compounds. These provide a broad spectrum-band: protein and lipoprotein signals dominate simple one-dimensional ^{1}H-NMR spectra, with small-molecule fingerprints superimposed on them. Spin-echo experiments decrease the broad signal contributions from proteins, other macromolecules and micelles. One key factor for the successful deployment of NMR for sure is to find the most efficient way allowing to discover the metabolic information whereas detailed chemical analysis in order to obtain it is not requested. But indeed elaborated knowledge enabling to differentiate between „normal“ and „pathological“ states is required. Advanced knowledge and a statistical organization of facilitating biomarkers is for this concern auxiliary. NMR spectra of biofluids function in two dissimilar but kindred ways: as quantitative metabolic-fingerprinting tools and as an instrument to disturb metabolite biomarker structure. The application of biofluid

NMR spectrum means such a complex procedure that several NMR techniques may be needed implying 1H-1H and 1H-13C two-dimensional experiments [NICHOLSON et. al, 2002].

4.2.2.3. 1-D AND 2-D NMR

For investigations of the multicomponent metabolic compounding of biofluids, cells and tissues the use of 1-D and 2-D NMR-methods are already well established [NICHOLSON & WILSON, 1999]. Because of their simplicity, sensitivity and short acquisition times, 1D ^{1}H previous spectra are frequently collected for experimental studies. ^{1}H previous spectra of all biological samples are heavily dominated by the water resonance at 4.8ppm which can be suppressed by the use of simple presaturation methods [WISHART, 2008]. Besides ^{1}H for 1-D applications nuclei such as ^{13}C, ^{15}N and ^{31}P have also been utilized both in liquids and in solid state but considering the high prevalence in metabolites in ^{1}H and high natural abundance of 99.985%, as a matter of fact 1-D ^{1}H NMR spectra are dominating in majority of quantitative term studies. A major hindrance in a one-dimensional ^{1}H NMR spectrum is the restricted chemical shift range at even the highest magnetic fields and as the complexity of samples increases, the problem of resonance overlap easily degrades the accuracy of the analysis. Hence enhanced requirements existed to find methods that support satisfying resolution while retaining reasonable quantity realized with 2-D methods. These 2-D methodologies can be divided into two categories: proton –proton and proton–carbon correlation experiments. The most active area of development is for the ^{1}H -^{13}C heteronuclear single quantumcoherence (HSQC) experiment where carbon is applied to spread out and resolve peaks in the second dimension. The most decisive factor that can compromise quantitative results is resonance offset profile of ^{13}C channel radio frequency pulses. This dependency can be either compensated by deploying Q-CAHSQC together with coefficients to emend integration results, or when the ^{13}C chemical shift range is reasonable Q-CAHSQC modified with offset-tolerant composite pulses. Manipulating the NMR signals to create a two-dimensional spectrum would last longer than a simple one-dimensional experiment. Besides it

needs a more sophisticated detection scheme. However the enhanced information content of the spectrum can serve as an improved alternative for ameliorated metabolite detection [ISSAQ et al., 2009; KRISHNAN et al. 2005; KOSKELA et al., 2005].

4.2.2. 4. DATA INTERPRETATION

In order to gain revealing interpretation and successful classification of these maintaining complex data sets the deployment of pattern recognition (PR), expert systems and related bio-informatic tools are obligatory [NICHOLSON & WILSON, 1999].

4.2.2.4.1. PATTERN-RECOGNITION METHODS

Pattern-recognition methods demonstrate the difference between spectra of biofluids from individuals suffering from disease but also healthy subjects [NICHOLSON & LINDON, 2008]. Pattern-recognition as well as other similar multivariate statistical approaches often get applied when the detection of significant patterns in complex data sets is required and they are particularly in those special situations enormously suitable when in a data set are more variables than samples. First priority obtains the object classification such as for example ^{1}H-NMR spectra. Additionally one attempts by using this technique to forecast the origin of objects by tagging inherent patterns in a set of indirect measurements. Pattern-recognition methods reduce the measure of complexity with aid of two- or three-dimensional mapping procedures which simplify the illustration of inherent patterns [NICHOLSON et al., 2002]. NMR spectra appearing from a number of different biofluids have been used as input to PR examinations. For instance blood plasma offers a varying biochemical profile exhibiting resonances from both low molecular weight molecules and from lipoproteins even although these are damped in intensity due to their short T2 relaxation times [LINDON et al., 2001].

4.2.2.4.2. SUPERVISED AND UNSUPERVISED TECHNIQUES

In case of expert systems one has to distinguish between supervised and unsupervised techniques. Unsupervised techniques include principal components analysis (PCA) where principally no knowledge of the sample class is required and which base on the

calculation of the latent variables. Principal components (PCs) mean to be the linear combinations of the original descriptors [NICHOLSON et al., 2002]. They are created by linear combinations of the starting variables with matching weighting coefficients and can be termed as new variables. Their predicate is that each PC is orthogonal with all other PC whereas the first PC contains the most capacious part of information content by extreme data variance. The afterwards following PC in turn provides smaller amounts of variance analogously (that is PC1>PC2>PC3). Basically the plot of first two or three PCs offers the best representation referring to biochemical diversity within data conglomerations to be illustrated in two or three dimensions. The cardinal idea is to ameliorate the spotting potential of the original NMR-produced spectrum with aid of only a small collection of variables [NICHOLSON & WILSON, 1999; NICHOLSON et. al, 2002]. A further possibility is to model multiparametric data so that the class of an analyte from an autonomous data set can be forecasted on the basis of a range of mathematical models which again are derived from the primal data or training set. These methods are popular as supervised methods which use class information to achieve separation between classes. Supervised methods such as soft independent modeling of classification analogy (SIMCA), partial least squares (PLS) analysis as well as PLS discriminant analysis (PLS-DA) get deployed to forecast and discover unexplored components according to their NMR spectral properties [NICHOLSON et. al, 2002]. A rather well-established supervised method is the partial least squares (PLS) analysis. PLS is often in connection with discriminant analysis which means compassing and collecting linear feature combinations adequate to classify data into sets. The linking of PLS with discriminant analysis creates an area that can be set in a multidimensional plot to achieve separation of data into classes [NICHOLSON & LINDON, 2008].
Correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) applications offer spin–spin coupling connectivities and deliver information on which hydrogens in a molecule are narrow in chemical bond terms [BECKONERT et al., 2007].

One statistical approach worth to mention is statistical total correlation spectroscopy (STOCSY). It has widely been applied to plenty of problems in 1-dimensional NMR

metabolic profiling in order to support structural assignment. It is based on correlations found between the intensities of spectral peaks across a cohort of samples. This affords all given peaks from a metabolite to be identified and hence that the structure of that molecule can be ascertained. One determining advantage of STOCSY is that supplemental information can be assembled by appraising lower correlations between peaks or actually negative correlations, as these advert to connections between two or more molecules that are implicated in the same biochemical mechanism [NICHOLSON & LINDON, 2008]. Rather high numbers of spectra are precondition to reliably attest a structural relationship even though a few tens of spectra mostly are satisfactory for most purposes. Therefore when STOCSY gets deployed for structural classification, data sets offering high homogeneousity should get assembled together with a size larger than about 10 spectra in order to keep peak overlap and positional variation at a low level [EBBELS & CAVILL, 2009].

The STOCSY practice has been widened to also comprise heterogeneous data groupings. In this mode the approach is common as statistical heterospectroscopy (SHY) which already successfully has been utilized for the coanalysis of both NMR and mass spectra. Consequently the advantageous outcome can be enjoyed due to the expanded assignment of biomarkers of the toxin effect by utilizing the correlated but complementary information which can be obtained from the NMR and mass spectra taken on a entire sample cohort [BECKONERT et al., 2007].

So generally observed NMR stands out for its congenial characteristic of rapid and precise measurements of analytes in biofluids without demanding for initial processing of separation but in contrast it is also known for its major weakness of meager dynamic range resulting in that only the most opulent constituents possible of being watched [WANT et al., 2005].

4.2.2.5. MAGIC ANGLE SPINNING

Complicacies such as sample heterogenity and short relaxation times resulting in resonance broadening and restricted spectral information can be avoided by an approach called magic angle spinning. It functions by the rapid spinning of the sample

with 5 kHz at the magic angle of 54.7° by what the information leakage induced by most of the line broadening effects viewed in non-liquid samples such as tissues gets meaningfully decreased . This broadening is generated by sample heterogeneity and residual anisotropic NMR parameters that are originally averaged out in free solution where molecules can turn quickly and isotropically [LINDON et al., 2009]. Principally magic angle spinning gets adopted for the exploration of intact cells or tissues. Additionally as deployment of HR MAS enormously heightened spectral resolution in the complex entire cell sample it has been utilized for the study of whole cells of the microalga *Thalassiosira pseudonana*. Especially in high-resolution NMR of solid and semisolid materials magic angle spinning is an extraordinarily favored method which firstly has been applied by Lowe [LOWE, 1959] and Andrew [ANDREW et al., 1958] where they exemplified independently from each other that rapid spinning of a solid sample at the magic angle relative to the applied magnetic field successfully decreases the NMR peak widths [CHAUTON et al., 2003]. However for samples where the constituent substances maintain a lot of molecular motion such as in tissue biopsies, the anisotropic interplays are fractionally-averaged and that´s why they get minimized. Considering that chemical shift anisotropies for protons are usually rather minor, the major reasons for line broadening in the ^{1}H NMR spectra of such samples are substantially averaged $^{1}H–^{1}H$ dipolar couplings and results because of variance in magnetic susceptibility across a sample. Thus spinning at the magic angle at moderate velocity in connection with the provision of a ^{2}H lock signal provides extraordinarily high-resolution spectra. To get rid of the line broadening effects in tissues utilizing MAS only demands for a spinning rate of a few hundred Hz, but this would generate spinning side bands with sizeable intensity with the potential to shroud other metabolite centre band signals. That´s why the pragmatic effort has been to spin the sample at the specific rate which puts the side bands outside the spectral window like for instance a rotation at 4000 Hz for 400 MHz observation [LINDON et al., 2009]. The use of HR-MAS ^{1}H NMR spectroscopy to the investigation of undifferentiated and differentiated pre-adipocyte as well as adipocyte cells presented improved spectral resolution and sensitivity versus static explores. MAS of cells enabled the detection

and also quantitation of a range of cellular metabolites that are not definitely resolved in non-spinning approaches and supported enhanced visibility of phospholipids. Diffusion-weighted MAS NMR spectra rendered possible compartment assignment as well as mobility determination for majority of metabolites. The undifferentiated and slighter pre-adipocytes kept their stability and exhibited no microscopic proof of cell lysis after 2 h of MAS at 3.5 kHz and more than 80% of remaining cells stayed viable by trypan blue exclusion. In opposition to the bigger, lipid-loaded differentiated adipocytes were encountered at approximately 17% showing degrees of cell lysis under MAS conditions [WEYBRIGHT et. al, 1998]. But it has also has proved as an effective means to explore the cell wall structures of intact bacteria. For example $^{1}H-^{13}C$ heteronuclear single quantum coherence HR-MAS NMR offers rapid analysis of the cell wall structure in living bacterial cells and in parallel permitting observation of for example species differentiation or environmental impacts [LI et al., 2005].

4.2.3. VIBRATIONAL SPECTROSCOPY

4.2.3.1. VIBRATIONAL SPECTROSCOPY IN GENERAL

Vibrational spectroscopy including techniques such as Infrared (IR) and Raman spectroscopy are applied in an expanding area of biological disciplines and thus the importance of IR and Raman spectroscopy as metabolic fingerprinting tool develops continuously [DUNN et al., 2005].

When light is incident on matter like for instance on a biological cell or tissue it is capable to interact with the atoms or molecules in various ways. Photons can be absorbed directly but also can be scattered. Light absorption is most probable if the wavelength of the radiation stands in the infrared or in the ultraviolet (UV) zone. The IR absorption entails in the excitation of vibrational modes of the molecules whereas the UV absorption is associated with the excitation of an electronic transition which is often accompanied afterwards by a radiative emission, fluorescence [PETRY et al., 2003]. After light gets scattered from a molecule, majority of photons are elastically scattered. The scattered photons possess the same energy and thus wavelength as the

incident photons. A small fraction of light of approximately 1 out of 108 photons is scattered at optical frequencies differing from the frequency of the incident photons. The process leading to inelastic scattering is popular as the Raman effect and provides the typical Raman spectra [PETRY et al., 2003].

IR and Raman spectra are contrary to each other and deliver vibration images of an atom´s compounds and this feature is the reason why both techniques are also referred to as vibrational spectroscopy.

4.2.3.2. INFRARED SPECTROSCOPY

Infrared spectroscopy investigations can be described as multidisciplinary studies [DUMAS & MILLER, 2003] which are based upon the collection, recording and measurement of molecular vibrations [QUINTEIRO RODRIGUEZ, 2000]. Infrared spectroscopy probes these molecular vibrations. Functional groups can be related to characteristic infrared absorption bands and which correspond to the elemental vibrations of the functional groups. Especially when a molecule exhibits a symmetry center, all symmetrical vibrations referring to the center behave infrared inactive. In opposition to the asymmetric vibrations of all molecules are discovered. This deficit of selectivity permits us to prove the properties of virtually all chemical groups within a sample, but also remarkably of amino acids and water molecules which can only marginally be viewed by different spectroscopic methods [BERTHOMIEU & HIENERWADE, 2009].

Vibrational transitions of molecules are elicited by the absorption of infrared radiation. In the mid- and far-infrared spectral regions this is on principle the case when the frequencies of light and vibration are the same and when the molecular dipole moment alters during the vibration. Considering that vibrational frequency and likeliness of absorption are up to the strength and polarity of the vibrating bonds, they are biased by both intra- and intermolecular factors. The rough position of an infrared absorption band gets defined by the vibrating masses and the type of bond, the precise position by electron withdrawing or donating effects of the intra- and intermolecular surroundings and by connecting with further vibrations. The strength of

absorption rises with advancing polarity of the vibrating bonds. Generally all polar bonds add to the infrared absorption which at the same time means to be both the advantage but also disadvantage of infrared technique. A drawback because of the spectrum of larger molecules is composed of many overlapping bands with the sequence that valuable information can be concealed behind board and featureless absorption bands. On the other hand it holds a potency due to almost all biomolecules are feasible to absorb infrared radiation whereas in parallel there is no need of marking biomolecules to turn them detectable [BARTH, 2007]. The IR spectra of most materials exhibit a huge amount of absorption bands having its seeds from the interaction between discrete light quanta and mechanical motions of the molecules affected by the absorption of IR radiation [BEEKES et al., 2007].

The IR region ranging from 1 to 100 µm is subdivided in three zones, the far-infrared region from 100 to 25 µm , the mid-infrared region from 25 to 2.5 µm, and near-infrared region from 2.5 to 1 µm. The mid-infrared region illustrates primary molecular vibrations and is the most popular and widely deployed zone for the differing molecules´ analysis. All compounds demonstrate specific absorbance peaks in a section region ranging between 1350 and 1000 cm^{-1}. Thus this physical property is viewed as a molecular fingerprint. In contrast the far- and near-IR do not get frequently employed as only skeletal and secondary vibrations appear in these regions producing spectra that are extremely complicated to interpret [QUINTEIRO RODRIGUEZ, 2000].

Further benefits of infrared spectroscopy are the extended application range from minor molecules to for example large membrane proteins as well as a high time resolution down to 1 µs and the small requested amount of sample [BARTH, 2007]. Additionally no reagents are needed. Many of the standard clinical chemistry tests insist on the supplementation of reagents to the sample to generate a multi-colored product and the product concentration is defined calorimetrically. This method is essential as virtually little occurring biological species in their native form own strong and specific UV or visible spectra. Otherwise all of the organic and a lot of the inorganic materials existing in biological fluids lead to infrared spectra without precondition of chemical modification. As a sequence the infrared spectrum of a

biological liquid is the addition of the absorptions developing from all of the IR active species in the fluid, weighted optional to the concentration of the particular species. This results in the next major advantage: as a fluid´s IR spectrum means is the sum of all IR active components present, the concentration of each IR active compound is encoded in each spectrum. That´s why it is generally possible to find out the concentration of miscellaneous analytes from a sole spectrum which again grants time as well as labor reduction [JACKSON et al., 1997].

4.2.3.2.1. FOURIER TRANSFORM INFRARED SPECTROSCOPY

Infrared spectroscopy got upgraded by the involvement of two novel components: by means of the interferometer and by application of a mathematical transformation, the Fourier Transform algorithm which permits the coeval discovery of the entirely transmitted energy [QUINTEIRO RODRIGUEZ, 2000; DUYGU et al., 2009].

Fourier transform infrared (FTIR) spectroscopy is a potent, nonperturbing technology adopted for enhanced detection and characterization of various components [LEWIS & McELHANEY, 2007] whereas it is a form of vibrational spectroscopy. In the past decades Fourier transform infrared spectrometers have almost comprehensively substituted dispersive instruments due to their better performance in almost all relations. The use of the FT technique has ameliorated the acquisition of IR spectra extremely [WARTEWIG & NEUBERT, 2005]. It is based upon the measurement of molecular vibrations. Molecules possess a set of resonance vibrations produced by thermal energy [QUINTEIRO RODRIGUEZ, 2000]. The Fourier transform infrared spectrum reflects both molecular structures but also molecular environment. In course of this technique the analyte gets irradiated with infrared radiation from an infrared source whereas the absorption of this radiation initiates vibrational motions by depositing energy quanta into vibrational modes [SACKSTEDER & BARRY, 2001]. That´s why a molecule after exposition to radiation created by the thermal emission of a hot source absorbs only at frequencies depending on its molecular vibration modes within the electromagnetic spectrum amongst visible and short waves [QUINTEIRO RODRIGUEZ, 2000].These changes in vibrational motion are leading to bands within

the vibrational spectrum and each spectral band is associated with its specific amplitude and frequency [SACKSTEDER & BARRY, 2001].

In the Fourier transform implementation of the infrared technique, an interferometer made up of a beam splitter, a mobile mirror and a fixed mirror, is applied to achieve modulation of the infrared radiation. An optical element divides the infrared radiation into two components whereas respectively one component is reflected by a fixed mirror and the other one by a moving mirror. The two components get reunited at the beam splitter with constructive or destructive interference. The interference dimension biases on the optical path discrepancy amongst the two mirrors. Intensity variation in the infrared radiation seeping through the analyte is measured as a function of optical path length. At continual moving mirror rate, this is homologous to a measurement in the time regime. This FT-IR method gives an amendment in signal-to noise due to the complete spectrum is discovered contemporaneously. The detected signal termed the interferogram, is Fourier-transformed to endow the signal as a function of infrared frequency which is announced in units of wave number. These data are then splitted by a background and rigged mathematically to lend the infrared absorption spectrum [SACKSTEDER & BARRY, 2001]. The Fast Fourier transform algorithm is here the means able to amplify, digitize, collect and ultimately transform the interference patterns of the interferogram signals into a spectrum. That´s why the Fourier transformation can be plainly viewed as a mathematical method of withdrawing the specific frequencies from the interferogram to illustrate them again in an IR spectrum [BEEKES et al., 2007]. After data manipulations the amplitude of each vibrational band in the FT-IR spectrum is given in units of absorbance [SACKSTEDER & BARRY, 2001].

With the arrival of Fourier transform-infrared spectroscopy the immanent problems of traditional dispersive IR spectroscopy could be obviated. To be contrary to classic dispersive IR spectroscopy FT-IR spectroscopy is not any longer forced to measure one wavelength after the other due to its option to deploy an interferometric modulation of radiation.

A further plus is that this method can be adapted to samples possessing consistencies like being powdered, dehydrated or aqueous. Furthermore the FT-IR spectrometer can be modified in order to render possible investigations of very small-sized samples. Worth mentioning also is its extreme sensitivity [SACKSTEDER & BARRY, 2001].

Outstanding advantages of FT-IR are enormous speed together with high specificity in opposite to traditional technologies and which as a sequence make them especially adaptive to various microorganisms permitting differentiations even below subspecies level. The particular power of the FT-IR technique is its characteristic to perform epidemiological case studies as well as extended screening investigations within a remarkably short time span [DUYGU et al., 2009].

4.2.3.2.2. DATA INTERPRETATION

After successful spectra receipt a number of data processing approaches stand by to gain qualitative but also quantitative information. This qualitative information can frequently be converted non-subjectively into diagnostic information by means of multivariate pattern recognition applications. Quantitative information can be nourished from IR spectra of biological liquids rather easy and accompanied by accuracy close to that of standard clinical chemistry techniques [JACKSON et al., 1997]. Multivariate statistical analysis (MSA) can utilize miscellaneous options for the pre-attendance, evaluation and illustration of difficult and high figures of spectral data. Considering the methods often adopted for pattern recognition in FT-IR spectroscopy one can mention factor analysis, hierarchical clustering and artificial neural networks [BEEKES et al., 2007]. Besides also multivariate regression is a frequently applied method to analyze one or multiple compounds in a complex sample where considerable signal overlapping takes place. A calibration model gets accomplished by means of an exercising data set with common concentrations and this enables realistic predictions of unacquainted concentration levels invoking on the generated model. Basically effectual preciseness and stability of classification but also predictive regression means have to be accurately examined with the aid of an adequate amount

of validation samples before the analysis of the nameless gets carried out [WANG & MIZAIKOFF, 2008].

4.2.3.3. RAMAN SPECTROSCOPY

Raman spectroscopy is an appropriate and famous means for exploring the relationship between structure, function and dynamics of bioactive molecules. The application of Raman spectroscopy in the life sciences had started in the late 1960s and early 1970s when reputable laser sources became disposable for sample excitation [SCHWEITZER-STENNER, 2005]. It alleviates increased illumination from structure to function because the Raman effect involves a close interplay between atomic locations, electron distribution and intermolecular strength, it is situated at the bridgehead between structure and function. Raman spectroscopy investigates the scattered photons from a laser beam focused in the sample solution [CAREY, 1999].

In the Raman effect, incident light gets inelastically scattered from an assay and shifted in frequency by the energy of its typical molecular vibrations [KNEIPP et al., 1999]. An energy transfer appears as a sequence of the coupling between the incident radiation and the quantized states of the scattering system. This exchange is distinguishing and gets measured with electromagnetic radiation of a specific wavelength, routinely a laser in the visible to mid-Infrared part of the electromagnetic radiation. The energy exchange results in a measurable Raman shift in the wavelength of the incident laser light and is also famous as the inelastic light scattering effect. Optional on the coupling, the incident photons either gain (anti-Stokes) or lose (Stokes) energy on the vibrational-rotational or electronic level. The scattered light with lower energy as compared to the incident laser light is termed Stokes-Raman scattering, and in contrast the radiation with higher energy is characteristic for the anti-Stokes-Raman scattering [PETRY et al., 2003; ELLIS & GOODACRE, 2006] whereas the anti-Stokes photons have more energy than those of the exciting radiation [VANKEIRSBILCK et al., 2002]. Generally the inelastic scattered photons are common as the Raman spectrum and they deliver information on molecular vibrations that again provide data about molecules´ conformation and surroundings [CAREY, 1999]. Based on the energy level

point of view the process of Raman scattering can be understood as the transition of a molecule from its ground state to an excited vibrational state, attended by the simultaneous absorption of an incident photon and emission of a Raman scattered photon. The Raman scattered light can be cumulated by a spectrometer and displayed as a spectrum, in which its intensity is displayed as a function of its frequency change. Considering that each molecular species possesses its own inimitable set of molecular vibrations, the Raman spectrum of a particular species will be composed of a series of peaks, each shifted by one of the typical vibrational frequencies of that molecule [HANLON et al., 2002]. Accordingly Raman spectra deal with measuring associated molecular vibration and rotational energy changes. Anyway the necessity for vibrational activity in Raman spectra is not a change in dipole moment, as it would be in IR spectra, but a change in the polarizability of the molecule. That´s why it is feasible to gain spectral information from a homo-nuclear molecule by Raman spectroscopy [VANKEIRSBILCK et al., 2002].

One of the advantages of this technique lies in its high sensitivity to subtle molecular and biochemical changes. Furthermore the Raman technique provides principally narrow bandwidths and is easily interfaced to fibre-optics for remote analysis in order to facilitate sampling; interfacing to separation techniques or remote sensing is usually straightforward [BAENA & LENDL, 2004]. Since it has developed a direct and non-invasive technique requesting just minor sample portions, it holds conveniences over conventional approaches [HERRERO, 2005]. So it can be deployed under ambient conditions in virtually every environs. Measuring a Raman spectrum does not urgently imply extra sample preparation techniques as opposed to infrared absorption spectroscopy [KNEIPP et al., 1999] which results in significant cost savings. Since water is a fragile Raman scatterer, Raman shows minimal sensitivity towards water-induced interference which enables to analyze aqueous solutions [VANKEIRSBILCK et al., 2002]. An enormous benefit offers Raman spectroscopy because it delivers detail from an essential site in a much bigger macromolecular complex [CAREY, 1999]. Optical fiber probes for routing excitation laser light to the sample and transporting scattered light to the spectrograph render possible remote discovery of Raman signals. Additionally

the spatial and temporal resolution of Raman scattering are defined by the spot size and pulse length according to the excitation laser [KNEIPP et al., 1999]. Nowadays in Raman scattering studies laser photons get used which hold an extended range of frequencies from the near-ultraviolet to the near-infrared region and which permit selection of optimum excitation status for every sample. With the choice of wavelengths exciting proper electronic transitions, resonance Raman investigations of selected elements of a sample or constituents of a molecule can be carried out [KNEIPP et al., 1999]. Worth mentioning is a fundamental rise of selectivity since measurements can be performed which are characteristic for a chromophore with a specific electronic absorption spectrum. So it is feasible to selectively ascertain the vibrational spectra of a chromophore with just minimized background annoyances [SCHLODDER et al., 2009]. Relating to instrumentation, most essential for Raman biospectroscopy is the adequate choice of the laser´s excitation wavelength. A single-wavelength-specific image already can offer interesting information about a sample. Considering that the contrast in vibrational images is solely deduced from spectral differences between biochemical compounds, as a sequence single-wavelength images demonstrate spatial distribution of the species which absorb at that wavelength [SHAW et al., 2000]. For analytical linear Raman spectroscopy mainly consecutive wave lasers with a fixed wavelength are in use. During the last two decades especially argon and krypton ion lasers were applied in plenty of Raman laboratories whereas two different Raman spectrometers, either a dispersive one or a Fourier transform spectrometer, are usually utilized for Raman biospectroscopy. Today the standard configuration for a dispersive spectrometer consists of either a triple monochromator or a single monochromator combined with sharp-cut filters, like for instance a multilayer dielectric interference filter or a diffraction filter. In a triple monochromator the first two stages (substrative mode) are utilized for the elimination of the Rayleigh scattered light, whereas the third monochromator, which also is known as spectrograph, is busy to disperse the collected Raman radiation onto a multichannel detector. As a sequence the Raman spectra can be recorded very narrow within only little wavenumbers to the Rayleigh line which results in a poor light transmittance.

Alternatively in case intentions are directing in an elevated light throughput and high collection performance in place of recording of low-frequency modes close to the Rayleigh line, a single grating monochromator in combination with a notch filter can act as useful means. The detection of the Raman scattered light is usually done by the adoption of multichannel detector devices. The most preferred multichannel detectors are composed of either fortified diode arrays or charge coupled devices (CCD). Both detectors offer the multiplex benefit enabling simultaneous detection of a wide spectral range [PETRY et al., 2003]. Seasonably dispersive Raman spectrometers are frequently provided with silicon-based CCD multichannel detector systems, and laser sources with acting wavelength in the ultraviolet, visible or near-infrared area get applied [WARTEWIG & NEUBERT, 2005].

A confinement of Raman spectroscopy is that casually samples with high background signals appear whose removal turned out to be rather complicated. As a result this means an active investigation field since the source of the background is not explored satisfactorily. The most adequate target molecules for Raman difference spectroscopy are those possessing rather intense normal or non-resonance Raman signals, which principally means that they own widened phi-electron systems as they are polarizable and entail strong Raman scattering [CAREY, 1999]. Another major difficulty for Raman measurements lies in the high levels of fluorescence, intrinsic or generated by impurities, and which lead to an overlaying of the Raman bands. Although at times this can be avoided by shifting the laser wavelength to the NIR spectral region [VANKEIRSBILCK et al., 2002], unfortunately this is not obtainable in all cases. Even weak fluorescence signals can be intense enough to disguise the Raman signals. Moreover the conversion capability of the Raman effect is rather marginal. Only a minor amount of the laser photons is converted into Raman photons and thus restraining the detection of molecules for instance in cells or tissue with little concentration [PETRY et al., 2003].

4.2.3.3.1. RESONANCE SPECTROSCOPY

Most of described facts often hinder the recording of Raman spectra from biological samples. To circumvent the above-mentioned problems, specialized Raman techniques can be applied. Two approaches popular as the resonance Raman effect and surface-enhanced Raman scattering [PETRY et al., 2003], are able to enhance the intensity of Raman signals by multiple orders of power and to quench the fluorescence [BARANSKA & SCHULZ, 2008; PETRY et al., 2003] if the frequency of the excitation light is selected to act resonant with an electronic transition of the molecule (Resonance Raman) [SCHLODDER, 2009].

Resonance Raman spectroscopy is released when the excitation laser frequency is coincident or at least almost coincident with an electronic transition of the analyte. This can sequence in a 10^2 – 10^6 enhancement in the Raman signal matched to dispersive Raman spectroscopy [BAENA & LENDL, 2004]. In traditional Raman spectroscopy, the signal obliques only on the frequency v_0 of the light disposed for initiating the Raman effect. As a scattered signal the intenseness of the Raman signal will diversify optional to the fourth power of this frequency v_0^4 [ROBERT, 2009]. When the wavelength of the incident light is coincident or resonant with an electronic transition of the molecule, then the Raman scattering watched may be increased by various magnitude orders. This is the resonance effect. One substantial key to figure out resonance Raman enhancement is to keep in mind that the electronic transitions in a molecule are conducted by vibrational energy changes [BROWNE & McGARVEY, 2007]. It is thus realizable to selectively monitor a molecule in a complex medium given that it features an absorption transition, the energy of which adapts the energy of the incoming photons. This turns it possible to study molecular interactions whereas study of the resonance Raman-active modes watched upon excitation with a given electronic transition will provide information about the nature of this transition, about the participating nuclei and about the coupling between this transition and the varying vibrational modes of this molecule. Summed up, the nature but also position of the resonance Raman bands will give insights about the vibrational structure of the low-energy electronic states playing a part in the transition used for inducing the

resonance, whilst the intensity of these bands will deliver information about the coupling of these modes with the electronic transition. Naturally the information content of resonance Raman spectra will tightly be determined by the way how precisely the vibrational modes of the explored molecules can be calculated [ROBERT, 2009]. The Raman effect must be seen as a small probability process; a major drawback of Raman spectroscopy is that the signal measured usually is very minor which as a sequence is jeopardized to get contaminated with spurious fluorescence. In resonance conditions, the large yield in the Raman signal permits easier measurements on biological samples, although it is most often virtually impossible to excite photosynthetic aggregates in their red transitions, as their intrinsic fluorescence standard compromises the observation of the Raman signal [ROBERT, 2009]. But in order to realize resonance, visible or ultraviolet excitation must be utilized and disturbance from fluorescence is very frequently an occurring complication with Resonance Raman [BAENA & LENDL, 2004]. It often suffers from fluorescence background which has the potential to entirely conceal the Raman signals. Furthermore in real sample analysis background fluorescence from other constituents or from the matrix can also be a serious problem. Additionally many laser systems still are associated with lack of tunability. The freedom of excitation wavelength choice is necessary for optimum sensitivity and selectivity. Though fluorescence interference can be circumvented by use of very short wavelengths there anyhow remain a range of challenges to deal with [EFREMOV et al., 2008].

4.2.3.3. 2. SURFACE-ENHANCED RAMAN SPECTROSCOPY

The option to determine the size, shape and material of a surface has remade the area of surface-enhanced Raman spectroscopy, in short termed SERS. And as the excitation of the localized surface plasmon resonance of a nanostructured surface or nanoparticle can be found at the central point of this specific type of Raman spectroscopy, this technique has developed as potent means for the discovery of miscellaneous inorganic and biochemically relevant analytes. The extension mechanisms are so much sophisticated that they allow verification of even single

molecules. The main benefits of SERS is a $10^3 – 10^7$ enhancement of the Raman signal of an analyte when it is adsorbed to or near to the surface of certain noble metal structures with nanoscale attributes [STILES et al., 2008; BAENA & LENDL, 2004].

Principally the enhancement factor EFSERS = 10^6 can be understood as the SERS effect accompanied by two mechanisms: on the one side an electromagnetic extension mechanism associated with wide local fields as a result of surface plasmon resonance and on the other side a chemical enhancement mechanism which includes a resonance Raman-like process characterized by chemical interactions between the molecule and the metal surface. SERS mostly comes off as an intermixture of the both effects. These two mechanisms occur because the intensity of Raman scattering is parallel to the square of the induced dipole moment *μind* which again is the result of the Raman polarizability *α* and the magnitude the product of the incident electromagnetic field E. As a consequence of exciting the localized surface plasmon resonance of a nanostructured or nanoparticle metal surface, the local electromagnetic field is increased by a factor of 10 for instance [STILES et al., 2008; TIAN et al., 2002].

The electromagnetic field enhancement created from various metal nanostructures plays for the majority of noble-metal systems a substantial role. So collectively viewed, SERS involves complex couplings and interplays among molecules, nanoparticles as well as photons [TIAN et al., 2002]. Enduring irradiation of the laser beam over the SERS substrate can pander to decompose the sample analytes and are capable to significantly broaden or impair depending on intensity of monitored spectral bands. Furthermore the achievement of chemical or morphological changes in the SERS substrate is also possible. These effects can be reduced with the aid of a sample translation technique; by rapid spinning of the sample, the residence time of both analyte and substrate within the irradiated zone is extraordinarily lowered [BAENA & LENDL, 2004].

4.2.3.3. 3 DATA INTERPRETATION

For insightful interpretation of the gained vibrational spectra one has to differentiate that the output of FT-IR and Raman spectroscopy either is an infrared absorbance

spectrum or a Raman profile. These comprise many overlapping bands and that´s why data interpretation cannot be accomplished by ordinary visual inspection but adequate alternatives are required. Thus it is necessary that these vibrational spectroscopic measurements get linked with any of the rapidly growing plurality of diverse analysis strategies, in what seems to be the exponential phase of computational bioanalysis [ELLIS & GOODACRE, 2006]. Multivariate data analysis avails oneself mathematical, statistical and computer sciences to effectively receive valuable information from data generated by means of chemical measurements [WANG & MIZAIKOFF, 2008] whereas the multivariate data such as those originated from an infrared or Raman experiment are monitoring results of plenty different variables, wavenumbers or wavenumber shifts, for a number of individuals or objects like for example diseased or healthy ones. Every variable can be regarded as constituting another dimension in a way that if there are n variables of IR or Raman bands each object may be advised to stand at a position in an abstract also termed as n-dimensional hyperspace. This hyperspace is importantly demanding to visualize, and the elementary theme of multivariate analysis is therefore simplification or dimensionality decline. This dimensionality reduction happens by either applying unsupervised or supervised learning algorithms. Principally unsupervised approaches such as principal component analysis (PCA) or hierarchical cluster analysis (HCA) are in use to find out differences but also resemblances between these spectra [ELLIS & GOODACRE, 2006]. PCA is a feature reduction method and basis for multivariate data treatment which gets applied for data visualization. Its ability to reduce the number of variables and to demonstrate a multivariate data table in a low dimensional space is extremely fundamental. Hence the novel variables mean to be linear combinations of the original ones which can be construed like spectra [ROGGO et al., 2007]. Considering the majority of possible Infrared and Raman spectra and that the amount of samples in the analysis is very small compared to the number of variables, the deployment of multivariate analysis by PCA and HCA has proved as most suitable. These methods are adopted to structure the gained data by exhibiting equalities and diversities between the metabolic states. Thus they can be used to

accumulate samples into groups by fabricating scatter plots and tree-figured dendrograms [WERTH et al., 2010; ELLIS & GOODACRE, 2006].

Supervised pattern-recognition techniques such as linear discriminant analysis (LDA), partial least-squares discriminant analysis (PLS-DA) and soft independent modeling of class analogy (SIMCA) are diverse referring to their feature to allocate classes during and therefore permitting a more exact classification within the class pallet. As each means includes advantages but also disadvantages, it is hardly possible to render recommendation and thus the choice should depend on the specific starting point conditions. Notable is that meliorated classification performance could be realized by combining differing pattern-recognition methods. Multivariate regression gets often used to explore one or more components in a complex sample that are exposed to decisively overlapping analytical signals. A training data set with known concentrations of concern will be used to create a calibration model in order to find out concentration levels of unknown samples based on the accepted model.

However it is necessary to assure if sufficient exactitude and robustness of classification and predictive regression models are given by means of an adequate set of validation samples prior to the analysis of the unknowns [WANG & MIZAIKOFF, 2008]. But one should also assure about disposability of spectral libraries of vibrational spectra as it is of enormous significance for satisfying interpretation of spectra of unknown components. This is essentially a necessity in industrial laboratories where the payback time for the investment will be rather short. Also quantum chemical ab-initio calculations have become a serious tool in interpretation, but only recently its accuracy becoming more or less acceptable. Marginal differences in small molecules between calculated and experimental frequencies do not mean to be a hindrance for interpretation. But already in slightly bigger molecules the amount of vibrational bands might occur in an enormous extent and dense spectral regions that it is advisable to assign even the proper order of vibrational bands based on the calculations [MEIER, 2005].

CHAPTER 5 –NUTRIGENOMIC FUTURE OUTLOOKS

The increasing basic sensitivity towards nutrition needs and at the same time deepening science finding referring to genome interactions has probably expedited the arrival of the OMICS-disciplines. It has become apparent that nutrient chemicals hold key functions in altering gene transcritption, protein levels and functions as well as the metabolome on the basis of a given genome. Those recently gained „omical“ insights again might have opened the door for a new way of disease treatment – to prevent them already in advance. This theory gets supported by the conclusions of scientists such as branch-known Dr. Michael Fenech from CSIRO Health Sciences and Nutrition in Australia, Adelaide. In the following a verbatim citation of his argumentation:

„The technological advances have opened up a new opportunity in disease prevention based on the concepts that: (a) excessive genome instability, a fundamental cause of disease, is often an indication of micronutrient deficiency and is therefore preventable; (b) accurate diagnosis of genome instability using DNA damage biomarkers that are sensitive to micronutrient deficiency is technically feasible; (c) it should be possible to optimise nutritional status and verify efficacy by diagnosis of a reduction in genome damage rate after intervention. Given the emerging evidence that the dietary requirement of an individual may depend on their inherited genes, we can anticipate: (a) important scientific developments in the understanding of the relationships between dietary requirement and genetic background to optimise genome stability; (b) that the accumulated knowledge on dietary requirements for specific genotypes will be used to guide decisions by the practitioners of this novel preventive medicine in what might be called ‘Genome Health Clinics’. In other words, one can envisage that instead of diagnosing and treating diseases caused by genome damage, health and medical practitioners will be trained to diagnose and nutritionally prevent the initiating cause, i.e. genome instability itself.“ [FENECH, 2003]

And Peter J. Gillies, Ph.D. FAHA from the University of Toronto, USA, Department of Nutritional Sciences, declares (originally cited):

„One of the expectations of nutrigenomics and nutrigenetics is that a wide range of nutrient modifiable genes and related SNPs will be identified, validated, and incorporated into dietary strategies for the optimization of health and the prevention of disease.

Dietitians may have a special opportunity to redefine their role in the health care community. In this endeavor, dietitians could take on the role of "nutrigenetic counselors" with its attendant new training and responsibilities." [GILLIES, 2003]

CHAPTER 6 – CONCLUSIONS

Over the intervening years human attitude on nutrition for sure has moved from satisfaction of hunger to an increased sensitivity towards nourishment including elevated contingents of „healthy compounds“. This trend away from quantity with direction to more quality combined with new research findings concerning genetic interactions have pathed the way for the arrival of the omics-disciplines and out of it the discipline of nutrigenomics. This very young research area offers promising hope that once prevention of disease will successfully be realized by nutritional intervention optional to indiviual´s specific genome formation. This would be a medical sensation considering that various kinds of diseases would not need to occur to treat and eliminate them but they could be circumvented in advance. As a consequence people would not be forced to suffer from diverse illnesses – an idea which for sure animates to work hard. But to work ambitiously to approach the target should not get mixed up with premature and thus unrealistic dreams, enforced by financial interests of economic system. It is a fact that this indeed auspicious research field is in its infancy and that´s why majority of further studies have to get acted out to once seriously provide personalized nutrition treatment. Although research has made interesting findings promoting the final goal one can´t ignore also existing studies with contrary research issues. Marking that contradictions mean a „scientific accompaniment“ of nutrigenomic research workaday life, it should not more but also not less attend importance than it is: an indicator that we are dealing with an enormously complex field of genomic interactions which on its own already means to be a challenging universe, but not enough, we are investigating what kinds of all possible impacts of all possible nutrient components on all possible genes may could happen. A suspenseful field full of complexness demanding to rethink antiquated mindsets due to research findings and in parallel to fortify coherent conclusions due to research findings. As majority of scientists are keeping this aspects in attention not to over- but also not to underestimate achievements we can look forward for further nutrigenomic breakthroughs. The today available technical equipment for sure is multifaceted and

maturely developed as has been exemplified in case of metabolomics. And from the metabolomic point of view, already realized as well as ongoing endeavors do assist to accomplish the ultimate „omical“ goal to collect all gained research data from all omics-disciplines to once establish an accumulated fund of universal knowledge in the form of the OMICS-DATABASE.

CHAPTER 7 – LITERATURE

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CHAPTER 8 – LIST OF FIGURES

CHAPTER 9 - ABSTRACT

Scientifically observed we reside in the middle of the young OMICS-sciences and nutritionally observed this circumstance means a great chance to enhance our previous knowledge to probably once optimize nutrition attuned to the individual. This work is concerned with the spectacular area of nutrigenomics which desires to offer a molecular genetic understanding of how common dietary components influence health by changing the expression of structure of an individual´s genetic makeup. As a part of the entire OMICS-cascade the focus lies on metabolomics as a discipline dedicated to the global study of the whole metabolome. The metabolome again consists of metabolites which are small molecule intermediates or metabolism products with the characteristic potential to optionally appear everywhere within the body. In order to analyze them they get quantitatively but also qualitatively collected and measured by means of a range of techniques. The entire methodological pallet of metabolomic technologies assisting to illuminate nutrigenomic facts will be presented.

Wissenschaftlich betrachtet befinden wir uns gerade inmitten der Erforschung der jungen OMICS-Wissenschaften, was aus ernährungstechnischer Sicht die Chance birgt, möglicherweise in Zukunft optimierte Ernährungsempfehlungen - abgestimmt auf das jeweilige Individuum- anbieten zu können. Diese Arbeit beschäftigt sich mit der aufsehenerregenden Thematik der Nutrigenomik, welche die Wechselwirkungen zwischen Nahrungsbestandteilen und deren Einflußnahme auf die Genexpression untersucht. Der Schwerpunkt ist auf die Disziplin der Metabolomik gesetzt, die als Bestandteil der OMICS-Kaskade mit der Erforschung des gesamten Metaboloms betraut ist. Das Metabolom setzt sich aus Metaboliten zusammen, Metaboliten wiederum besitzen als Stoffwechselzwischenprodukte und/oder -endprodukte die Eigenschaft, im gesamten Körper vorzukommen. Um sie zu erfassen, werden sie mittels spezieller Technologien sowohl qualitativ als auch quantitativ gesammelt und ausgewertet. Die gesamte dafür zur Verfügung stehende methodische Palette wird im Rahmen dieser Arbeit vorgestellt.

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