3. Work strategy

3.1 Metabolomics

Metabolome is the characteristic profile of low molecular weight biomolecules present in a cell. A living organism must perform the continuous conversion of material and energy, which is accomplished through thousands of metabolic reactions and transport processes. A metabolic pathway can be defined as a sequence of feasible and observable biochemical reaction steps, whereas metabolic flux is the rate at which material is processed through that pathway [56].

The fundamental metabolic question that need to be answered revolve around the identity and/or quantity of the metabolites that are present in a biological system, the identification of the pathways that link them and the factors that allow the resulting metabolic fluxes to be controlled [57].

Most of the observed bioactivities can be correlated to certain metabolite groups. Abiotic environmental factors such as UV radiation, temperature or moisture and biotic factors such as xenobiotics (e. g. toxins, pesticides, pharmaceuticals), nutritional sources (e. g. limited nitrogen or carbon sources, salt stress, starvation), progressing diseases, organism development or genetic modifications lead to very specific qualitative and quantitative shifts in metabolites patterns.

Detecting and identifying pattern changes in this metabolic profile is fundamental in understanding underlying biochemical changes in an organism's metabolic status as a consequence of disease, intoxication, environmental stress, genetic manipulation or, in general, any external agent.

In our case, to investigate the complex metabolic effects of the genetic manipulation performed, non-selective, but specific, 'information-rich' analytical approach is required. In an integrated biosystem, it is axiomatic that the initiation of functionally connected gene expression events, cell signaling, protein-synthesis changes and metabolic responses to a stressor must be essentially sequential.

Although some environmental perturbations or genetic manipulations may not cause the changes in transcriptome and proteome levels, they can and do have significant effects on the concentrations of numerous individual metabolites. The dynamic metabolite levels in organisms must reflect the exact metabolic phenotypes under different cultural and genetic conditions [58].

While determining the concentration of individual and targeted metabolites has a long tradition in biochemistry, the measurement and interpretation of such in vivo metabolite dynamics at a systems level is one of the greatest challenges in molecular biology and reconcile these data with the structural knowledge about metabolism needs as yet to be done [59].

Of central importance to this field is the notion of cellular metabolism as a network. One of the early paradigms of metabolic regulation, i.e. the central role of a few rate limiting reaction steps being catalyzed by allosteric enzymes, that provided a basis for attempts to manipulate cellular metabolism during several decades, has recently been challenged by the new view of "multisite modulation". It is now clear that the regulation of biochemical reaction rates (*metabolic fluxes*) is usually distributed throughout the enzymes of the network so that more than a single enzyme exerts significant control for a given pathway, and the balanced distribution of regulation over several enzymes may well shift with a change in metabolic state [32].

The action of a metabolic network thus appears as a non-linear dissipative process of holistic nature. Consequently, regulation can hardly be tackled appropriately by a purely reductionistic approach, i.e. through the study of a whole system by detailed examination of the properties of its constituent parts, because it led to an understanding of what determines the material flows in different pathways but not of how the production/utilization of metabolites are kept in balance. Moreover it is unable to give information about metabolic regulation and quantitative description of the metabolic network. A metabolic map given by reductionist approaches can be compared to a town map, that is not useful for revealing how much traffic can flow through the streets [32,60].

In other words, an enhanced perspective of metabolism and cellular function can be obtained by considering the participating reactions in their entirety, rather than on an individual basis [33].

This research field is, therefore, multidisciplinary, drawing on information and techniques from biochemistry, genetics, molecular biology, cell physiology, chemistry, chemical engineering, systems science, and computer science [61].

So, there is a need for methodologies that can describe altered gene expression and cellular protein profiles in terms of their early metabolic consequences [62]. There are many approaches attempting to give detailed qualitative and quantitative metabolite overviews in whole organisms, exudates or extracts. About them exists an artificial terminology, which depends solely on the quality of conclusions drawn from the identified and quantified analytes [63].

Metabonomics is a whole system approach reaching for the "*quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification*" as defined in 1999 [64].

Metabolomics, introduced shortly thereafter as an additional term, has been defined in 2002 as "*a comprehensive analysis in which all the metabolites of a biological system are identified and quantified*" [65]. Practically, it describes the metabolic profile of a living organism (cellules, tissues, biofluids) or, in general, any matrix, in terms of the most abundant low molecular weight compounds present [66].

Analyze the metabolite composition of a tissue extract, determine the structure of a novel metabolite, demonstrate the existence of a particular metabolic pathway in vivo and localize the distribution of a metabolite in a tissue are all possible by NMR [32].

A major advantage of such NMR-based studies is the general ease and simplicity of the methodology. Characteristic signals can be detected from many metabolites and, in some cases, NMR analysis can even led to the detection of new or unexpected compounds [57].

Two related strategies have been developed for NMR metabolic data analysis. The most commonly used, termed "fingerprinting", is based on the analysis of an intact NMR spectrum that can be considered a "fingerprint" of unassigned signals arising from low molecular weight metabolites. The aim of such analysis is generally to classify groups of samples according to similarities and/or differences between their fingerprints, so the identity of the metabolites is secondary to this task. Metabolite fingerprinting by NMR is fast, convenient and useful for the discrimination between groups of related samples: multivariate analysis of unassigned NMR spectra can be used, for example, to compare the overall metabolic composition of wild-type and mutant yeasts strains and to assess the impact of stress conditions on the metabolome. If differences between spectra are found, then further methods will be used with the attempt of assigning those resonances within the most important regions of the NMR spectrum which are responsible for such discrimination [67].

The second strategy, termed "profiling", consists in the analysis of the cell metabolome allowing the characterization of metabolic phenotype under specific set of conditions [68]. It can be understood as a tool trying to discover pattern differences with the aid of statistical methods [69]. The NMR spectra of tissue extracts, in this case, are assigned, i. e. metabolites are identified and their concentrations calculated, providing an experimental profile to which multivariate methods can be directly applied. The advantage of this approach is that the results of these analysis are considerably more meaningful to the biochemists, as the variables that potentially discriminate two or more groups are reported as identified metabolites rather than unassigned resonances.

3.2 Applications

In the past few years, metabolomics has become a versatile approach, broadly used by industries and academia in medical, biological, environmental and food sciences. Especially in a systems biology context, metabolomics research has become so relevant that it has culminated in 2004 with the formation of a *Metabolomics Society* [70] as well as a dedicated journal called *Metabolomics* [35].

NMR-based metabolomic techniques have found a number of applications in both systems biology and biosafety and they are playing an integral and continually expanding role in the pharmaceutical industry, in particular for lead discovery, drug optimization and clinical validation.

Moreover, NMR spectroscopy is successfully being used to identify biomarkers from biofluids for early disease diagnosis of asthma, arthritis, cancer, cardiovascular, diabetes, neurodegenerative and pathogen infections [71]. The approach is straightforward in concept: compare biofluids from healthy and diseased individuals to identify metabolites uniquely correlated with the disease state itself. Furthermore, it has the advantage of being rapid and non-invasive, requiring the simple collection of urine, blood or saliva samples from patients.

NMR metabolomics is also becoming an essential tool for the identification and evaluation of drug toxicity. The approach is comparable to the identification of biomarkers: biofluids from animals or patients are analyzed before and after treatment with a drug candidate. Any differences in the metabolome that have been associated with serious toxic events, such as liver damage, would be used to identify a toxicity problem with the drug candidate. Similarly, NMR is currently making significant contributions to determine drug's *in vivo* efficacy and selectivity [71].

Most of NMR metabolomics studies have investigated metabolic fingerprint of biofluids, in particular plasma and urine [63,71,72]. However, in the last years metabolomics have received large acceptance also in food science [73-75].

Together with multivariate statistical analyses, NMR spectroscopy has been successfully applied to the characterization of various herbs and plant products for quality control, authentication, determination of geographical origin and for detecting adulteration of products. In the case of authentication and quality control of biological materials used as food, herbal medicinal products or nutraceuticals, changes of metabolite profiles can be associated with factors like adulteration or spoilage [63]. A similar approach was taken in a comparison of the geographical origin of olive oils. ^{1}H NMR was used to analyse 216 extra virgin olive oils collected over a three-year period from different Italian regions. The method was able to group the oils according to their location of production. In addition, a satisfactory differentiation between different years from the same location was achieved indicating the sensitivity of the approaches [76].

Metabolomics also represent a useful tool for functional genomic programs that contributes to our understanding of the complex molecular interactions in biological systems [58]. In order to elucidate an unknown gene function, genetic alterations are introduced in a system and by analyzing the phenotypic effect of such a mutation (i.e. by analyzing the metabolome), functions may be assigned to the respective gene.

Recently, genetic engineering has been employed as a new tool in applications including the improvement of environmental adaptability, disease resistance or tolerance to pesticides, by either modifying some endogenous genes or transferring some exogenous genes. Within the last years, NMR-based platforms have been successfully applied to investigate the metabolic consequences of genetic modification or strain differences in plants [77-79], animals [80,81] and yeasts [82].

Differential NMR metabolomics approach compares the metabolome of wild-type and mutant cell lines, for example under various environmental stress conditions. It can be used for functional genomic analysis of yeast mutants providing detailed quantitative information for the understanding of a biological network useful to identify the key genes for strain improvement. For example, metabolic analysis with low-resolution mass spectra and Fourier-Transform Infrared spectroscopy (FT-IR) was used to distinguish between different physiological states of wild-type yeast and between yeast single-gene deletion mutants [58].

Moreover, the differences in concentrations of some intracellular metabolites of *S. cerevisiae* were used to identify the phenotypes of several silent mutants.

3.3 From metabolomics to flux analysis

The incorporation of metabolomics into systems-biology opens the way to investigate the organization, regulation and control of metabolic networks experimentally and will profoundly contribute to the understanding of metabolism.

The use of metabolite profiling for designing novel strategies to develop more efficient cell factories through metabolic engineering, form the basis for intelligent screening strategies to exploit the biotechnological potentials of yeast for the production of specific chemical structures or classes of chemicals [4]. However, the rational improvement of yeast strains for the production of primary and secondary metabolites requires a quantitative understanding of their metabolism [54].

Purpose of metabolic analysis is, often, explain the effect observed after a physiologic or genetic perturbation, to obtain an efficient tool for the prevision of the functioning of the metabolic network. The first fundamental step to explain the function of a biological system is identify its component and the interaction between them in their complexity; in other word understand the relationship between the structural complexity and the function of the metabolic network itself [83].

Compared with metabolomics in plant and biomedical science, microbial metabolomics had its own advantages in systems biology frameworks [58]. Microorganism such as *E. coli* and *S. cerevisiae* had about 600 metabolites compared with 200000 metabolites of plants. This will accelerate the processing and applications of metabolomic data in system biotechnology to improve microbial phenotypes.

However, how to get and how to use the metabolomic data in strain improvements are still big challenges so that the phenotype resulting from a rationally modified genotype will remain, at best, difficult to predict [32].

In fact, it can be argued that the metabolome is a fundamentally important biochemical manifestation of the genome; however, it has become clear that the complexity of the metabolic network is such that it is not yet possible to construct predictive models of the metabolic performance that allow rational metabolic engineering of the genomes for strain improvement.

The fundamental problem is a shortage of quantitative information on the components of the metabolic network and, mostly, on the interactions between them [67]. So deciphering the intricate web of metabolic networks of a complex system is a difficult challenge, which cannot be resolved fully by any metabolomic tool. Indeed, such analysis could give a fingerprint in which all the metabolites are revealed but not assigned, or a metabolic profile in which are identified and quantified only a subset of the entire metabolome [83].

Just note that even if the concentration of various metabolites in a given pathway is known, it is still difficult to deduce which are the perturbed reactions in the pathway without ambiguity. This is because a given metabolite (such as glutamate) can participate in many different reactions so that its total concentration could not discern changes in all - or even a few - reactions [84].

It is also important to keep in mind that metabolomic data represents pool sizes, i.e. intracellular metabolite concentrations. As such, they do not permit to make any assumptions as to the size or direction of fluxes. So while certain metabolite pool might be very small, there will still be a flux into and out of this pool which might be quite large [54].

Moreover, it is important to realize that there is not always a one-to-one relationship between a gene and a metabolite, so metabolites levels are usually a complex result of the expression of many genes and the function of many enzymes. It is therefore inherently difficult to interpret the patterns of the metabolites and particularly to infer something about gene functions only based on metabolite profiling [4]. To reveal cause and effect relationships it is important to gain deeper insight into the complex metabolic responses at the level of intracellular metabolite concentrations and fluxes [55].

Fluxes, indeed, most closely reflect the underlying metabolic phenotype, whereas other *omics* approaches only yield a sense of metabolic capacities (transcriptomics/proteomics) or thermodynamic driving forces (metabolomics) [85].

Whereas the fluxes represent a final, balanced manifestation of *all* components of the living system that influence metabolism, they are, in conjunction with the metabolite pool sizes, key observables that link the experimental knowledge about the network components and the theoretical description [32].

Concluding, metabolomic analysis of metabolite composition alone may be insufficient for a complete understanding of the metabolic phenotype, whereas flux measurements could provide a useful complementary parameter for the system-wide characterization of metabolic networks [83]. Indeed, although the metabolic pathways have been known for a number of decades, the quantification and regulation of metabolic networks is still not fully understood.

Thus, the understanding of the distribution of flux and its regulation in central carbon metabolism (i.e. a "quantification" of metabolism) is fundamental for the progress of metabolic engineering. With the knowledge of distribution of fluxes (e.g. of precursors, reduction equivalents, etc), an effective improvement in the yield of the biosynthetic production of primary and secondary metabolites could be achieved. [54].

3.4 Flux analysis

Metabolic flux can be defined as the flow of matter through the metabolic network. Fluxes measurement is essential for understanding the control and regulation of metabolic networks in microorganisms because intracellular metabolic fluxes are the manifestation of the metabolic pathway activity [83]. Indeed, each flux reflects the function of a specific pathway within the network and, as all biological activity is contingent on metabolic activity, the estimation of *in vivo* fluxes, i.e. the characterization of a cell metabolic state for a defined set of environmental parameters, delivers the phenotype of the organism [32]. Although measurement of individual flux is useful, it is important to underline that the metabolic phenotype of a cell is the *net* result of *multiple* fluxes through the metabolic network [83]. The entirety of all metabolic flux rates, the *fluxome*, is the physiologically most relevant description of metabolic activity.

Currently, there is particular interest in calculating such fluxes [57] and initial attempts to develop such methods for microbes focused on the technique of flux balancing, a method in which intracellular fluxes are inferred from measurements of metabolic inputs and outputs on the basis of an assumed network stoichiometry. However, the complexity of some metabolic network, particularly the existence of storage pools, duplication of steps in more than one sub-cellular compartment and opportunities for substrate cycling, compromises the application of flux balancing to such type of cells needing the use of more powerful techniques [83].

These more powerful techniques are based on introducing stable-isotope tracers into the network: the use of more than one labeled substrate can give increased flux resolution. This can be achieved using either the same substrate (usually glucose) with isotopic labeling in different positions, or different substrates [56].

Then, the redistribution of the label into various intermediates (metabolites) is analyzed, after the system has reached an isotopic steady state: this means that the labeling has reached steady state and that no complicating pathways are operating to alter the redistribution of the label. In this way, the role of several metabolic processes during the cell growth could be defined, allowing the exploration of metabolic pathways, leading to qualitative information on the links between labeled precursors and their products and quantitative information on metabolic fluxes [86].

Network flux analysis is the procedure for generating a map showing multiple fluxes in a metabolic network. The term is usually applied to a steady-state analysis of the redistribution of label from a ^{13}C-labeled substrate. Information on the pathways is gained both qualitatively, by interpreting the incorporation patterns of the ^{13}C into the different intracellular metabolites, and quantitatively, by establishing the flux distribution through alternative pathways. Each flux map provides a description of a fundamental cellular activity under the physiological conditions of the experiment and as such provides a definition of the metabolic phenotype of the biological system studied [83].

There have been relatively few applications of large scale flux analysis to eukaryotes [87]. The obvious complicating feature, respect to prokaryotes, is the sub-cellular compartmentalization of metabolism which leads to a dissection of central carbon metabolism in sub-networks localized in either the cytosol or in organelles, e.g. mitochondria or peroxisomes, so the analysis has to allow for the localization of the pathways and the transfer of specific metabolites between compartments.

In yeasts this problem is relatively straightforward, with little duplication of metabolism in the two principal compartments, the cytosol and mitochondrion, and only a small number of well defined transport fluxes across the inner mitochondrial membrane.

For this reason, yeasts are eukaryotic model organisms pivotal for many areas of biology, biomedicine and biotechnology and the main emphasis, nowadays, has been on extending the methods developed for bacterial systems to yeasts.

Quantifying fluxes is not an end in itself and it is *comparisons* between them that lead to metabolic insights. Ratios of metabolic fluxes determined by stable isotope-labeling experiments are of great interest for metabolic studies in biotechnological research because they directly reflect cells metabolic state: indeed, the resulting labeling pattern of the metabolites reflects the relative importance of the alternative pathways.

This makes these kinds of experiments an ideally suited technique to determine flux ratios [32]. Thus *flux ratios or differences* between fluxes within a metabolic network are likely to be useful and comparing the same fluxes in closely related systems can also be informative [57].

Comparisons can then be made between the fluxes obtained from different systems, or from the same system in different physiological states, and this can lead to insights into the integration and regulation of metabolism.

There was yet shown in literature [88] how flux measurements can be used to define metabolic phenotypes of heterotrophic cells and how a comparison of flux measurements on wild type and transgenic lines can lead to useful physiological conclusions about the operation of specific pathways.

3.5 Isotopomer analysis

Stable isotope analysis usually focuses on the identification of the isotopomer composition of the analyte. The term *isotopomer* is used to describe molecules with the same chemical structure that differ in isotopic composition. However the same term is used to indicate one of a set of molecules with the same chemical and isotopic composition, but differing in the positional arrangement of the isotopes: such molecules should be more correctly called *positional isotopomers* [83].

The most commonly used stable isotope for network flux analysis is ^{13}C because it provides the core information about the organic chemistry of life [32].

The technological demand for flux analysis is being met by recent advances in two available frameworks [89]:

1) **MS methods:** mass spectrometry (MS) detects mass differences between natural abundance isotopic distribution and isotopically enriched molecules being therefore able to detect any heavy isotope [90]. Typically the spectrum will contain lines corresponding to fragments of the detected molecule that may range from M (only ^{12}C atoms) to M+n (only ^{13}C atoms). For example, a three-carbon fragment will therefore give up to four lines, with masses m, m+1, m+2 and m+3, corresponding to the presence of zero, one, two or three atoms of ^{13}C, respectively. These lines correspond to the mass isotopomers of the fragment.

2) **NMR methods**: although restricted to nuclei which possess a nuclear magnetic moment (nuclear spin $I \neq 0$), this technique provide quantitative information about both the chemical identity of the labeled molecule and the distribution of the label within the molecule (positional isotopomers). It also offers the unique possibility to monitor the formation and breakage of covalent bonds within the bioreaction network via observation of ^{13}C-^{13}C spin–spin scalar or dipolar couplings in NMR spectra [32].

Using these two complementary analytical platforms, it is now practical to simultaneously identify and quantify a large number of metabolites [89]. An advantage of MS, used extensively in network flux analysis, is its relatively high sensitivity (requiring relatively small amount of samples, as compared to NMR), allowing to monitor that minor components typically undetected by NMR. It also gives rapid analysis but, conversely, typically requires an hybrid approach because of the low-molecular weight distribution of metabolites.

Including gas or liquid chromatography to separate compounds with similar molecular weight, the sample must be prepared by chemical derivation in order to obtain chargeable molecules (especially for GC-MS). Strong isotope effects have been observed in the retention time of differently labeled isotopomers in GC columns [91] and the natural abundance of other atoms than carbon leads to a disturbance of the mass isotopomer spectrum [71,92]. Moreover MS measurements lead to complex mass spectra, which are caused by partial fragmentation of the analyzed compound and isotope distribution [61]. Finally, MS techniques have the disadvantage of differentiating poorly between isomers with identical molecular masses [93]. In fact, MS measures the total mass of a given isotopomer fragment, but is not sensitive to the position of the label within its carbon skeleton: the relative intensities of the peaks at M+i (with i = 1...j…n) yield a mass isotopomer distribution and the intensity of a given peak at M+j reflects the abundance of all ^{13}C-isotopomers with j ^{13}C atoms, irrespective of their location in the carbon skeleton [32].

So, NMR has a particular advantage over MS, because it provides direct information on the positional distribution of the labeled atoms within a molecule. This arises because structurally unequal carbon atoms within the same metabolite are usually magnetically unequal as well and, therefore, give separate ^{13}C NMR signals [57].

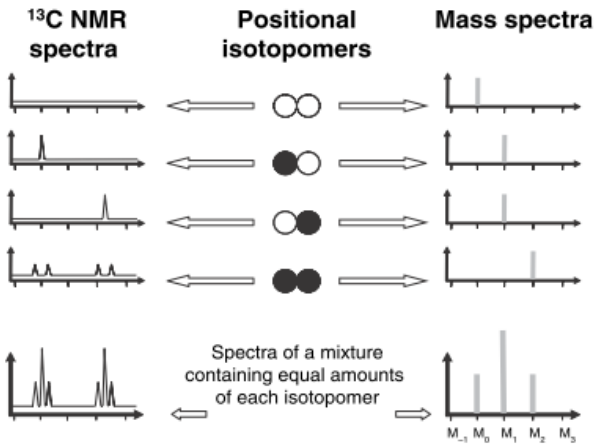

Figure 3.1: Comparison of ^{13}C NMR and MS spectra for the analysis of positional isotopomers [83].

Unfortunately, a completely unlabeled isotopomer produces no ^{13}C NMR signal (fig 3.1). This means that the observed NMR signals only reflect part of the isotopomer distribution and thus no percentage value can be assigned to each type of singlet, doublet, or double doublet peak. In MS spectra, instead, all isotopomers of a particular metabolite containing the same number of labeled carbon atoms are summarized under one peak signal. Because every isotopomer (especially the unlabeled isotopomer) contributes to exactly one peak in the MS spectrum, a percentage value can be calculated for each peak [92].

Anyway, NMR spectroscopy is one of the leading technologies used to measure metabolites levels and its use currently dominates metabolomics scientific literatures [94,95]. It is a versatile approach that can be applied to solid [96] or liquid samples, either *in viv*o or *in vitro*, often providing a very direct and convenient window on metabolism.

This is particularly true in the burgeoning field of stable isotope labeling [57]: a significant advance in this field occurred when spectral processing and multivariate methods were applied to NMR spectra, an approach pioneered by researchers at Imperial College [62,97,98].

The sensitivity of the experiment is extremely variable, since it is strongly dependent on the nature of the sample, the isotope that is detected, the magnetic field strength of the spectrometer and the way in which NMR signals are recorded [57].

For carbon, the relevant magnetic isotope is ^{13}C. This has a natural abundance of only 1.11%, contributing to a considerably lower sensitivity for ^{13}C NMR than ^{1}H NMR. Accordingly, the application of ^{13}C NMR in unlabeled systems is largely confined to the detection of the most abundant metabolites.

The first ^{13}C NMR spectroscopy study of a living organism was probably reported in 1972 [99]. Authors followed the metabolism of [1-$^{13}C_1$]-glucose by a eukaryotic cell system and concluded that the use of this pioneering technique "could have numerous applications for in vivo metabolic studies." Since then, ^{13}C NMR has developed steadily to a method routinely used in metabolic research with cells, perfused organs, animals and even humans [2].

This progress has been favored by the ability of ^{13}C NMR to perform repetitive, non-invasive measurements of metabolic processes as they proceed in their own intracellular environment and its capacity to measure unique physical properties not detectable by other methodologies, such as spin coupling patterns, isotopic shifts, or magnetic relaxation times (T_1 and T_2). These properties have been shown to provide valuable information on the operation *in situ* of specific metabolic pathways or the dynamics of some important biological assemblies. Moreover, ^{13}C resonances are distributed over a large chemical shift range and experience low relaxation rates, showing well-resolved narrow resonances distributed over large chemical shift range (~250 ppm), even in viscous media as those found in vivo [2].

The design of ^{13}C NMR experiments with selectively ^{13}C-enriched substrates is similar to the classical radiolabeling experiments using ^{14}C.

A relevant difference is that ^{13}C precursors are administered in substrate amounts, whereas ^{14}C substrates are used in tracer amounts. ^{14}C method has the advantage of high sensitivity, allowing the labeling experiment to be a true tracer experiment, but it suffers from the major disadvantage that it usually requires extensive fractionation of the tissue extract to generate information on the metabolic fate of the label, and even then there is no easy way of identifying the carbon atoms that have been labeled [100].

The extension of ^{13}C-NMR approaches for metabolic studies in humans will be of high interest, in particular because stable isotope tracers offer a degree of medical safety that cannot be achieved with radioactive tracers and an ever-increasing amount of data from structural biology will give insight into the functioning of molecular processes at atomic resolution [32].

^{13}C NMR method, indeed, presents important advantages over the use of ^{14}C: the metabolism of the ^{13}C-labeled substrate can be followed in real time, in situ and non-invasively because of their safety in biological systems [93,101]. Moreover, the analysis by ^{13}C NMR of homonuclear spin-coupling patterns and isotope effects allows investigation if two or more ^{13}C atoms occupy contiguous positions in the same metabolite molecule [2], providing a wealth of information about the positional labeling of the carbon skeleton of a molecule [100].

In fact, the introduction of ^{13}C-labeled molecules into a complex bioreaction network yields non-randomly ^{13}C-labeled metabolites so that a number of *isotopomers* for each metabolic intermediate are generated [102]. The term isotopomer is a combination of the terms isotope and isomer and it means one of the different labeling states in which a particular metabolite can be encountered. Because a metabolite with n carbon atoms can be labeled or unlabeled at each carbon atom position, there can be 2^n different labeling states [92]. ^{13}C isotope abundance at a given atom position may in principle vary between 1,1% (natural abundance) and 100% [32]. Isotopomers are specified using a notation in which 0 and 1 indicate ^{12}C and ^{13}C, respectively [83].

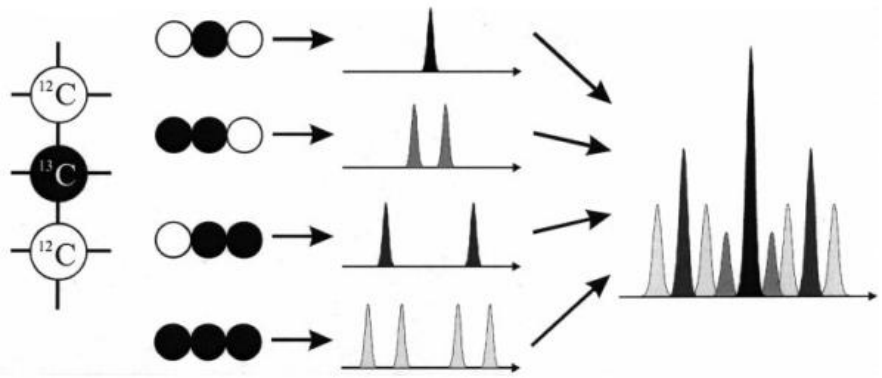

Figure 3.2: Typical ^{13}C NMR spectra of a metabolite with three carbon atoms. Observing the middle carbon atom we can have a singlet, two kind of doublets or a double doublet peak, depending on the labeling state of its neighbors. The isotopomer distribution of this pool produces several superimposed signals with different intensities [92].

Direct observation of the ^{13}C NMR spectrum allows identification of labeled metabolites and the position of labeling. In a ^{13}C NMR spectrum, in fact, the isotopomer distribution is resolved in detail because a labeled carbon atom produces different hyperfine splitting signals depending on the labeling state of its direct neighbors (fig. 3.2). In addition, by analyzing the ^{13}C homonuclear spin coupling patterns, a property unique to contiguously labeled compounds, the metabolic fate of substrates containing ^{13}C-^{13}C units can be followed [103]. Indeed, the value of ^{13}C-^{13}C coupling constants $^{1}J_{C\text{-}C}$ is indicative of the chemical environment of the carbon atoms involved, so, in biosynthetic studies, carbon-carbon couplings between ^{13}C atoms in geminal and vicinal positions, as well as those between carbon atoms directly attached to each other, provide useful information about the network [90].

For example, four different multiplets can be detected for carbon that is centrally embedded in a C_3-fragment, provided that the two scalar couplings with the central carbon are not equal (fig. 3.3): a singlet (s), a doublet split by a smaller coupling (d), a doublet split by a larger coupling (d*), and a doublet of doublets (dd). This situation is encountered, for examples, for the α-carbons of the proteinogenic amino acids, since $^{1}J_{C\alpha C=O}$ coupling constants (≈ 50-60 Hz) are significantly larger than $^{1}J_{CC}$ coupling constants between aliphatic carbons (≈ 35 Hz) [32].

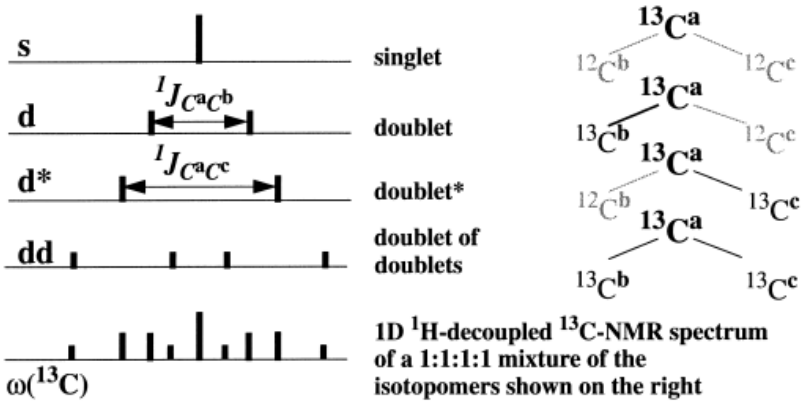

Figure 3.3: ^{13}C-NMR spectral pattern observable for the central carbon of a C_3-fragment, with different scalar coupling constant respect to the adjacent carbon atoms [32].

The isotopomer abundances generated during the labeling experiment are solely dependent on the natural ^{13}C isotope abundance, the fraction of ^{13}C-labeled precursor supplied and its degree of ^{13}C-labeling, the structure of the metabolic network and the corresponding fluxes.

So, the differential ^{13}C-labeled isotopomer profiles and abundance can serve as a fingerprint of the metabolic networks activity and could reflect both qualitative and quantitative differences in the metabolic pathways that lead to the synthesis of each metabolite [104].

In fact, the increased intensity of the signals observed in the ^{13}C-NMR spectra obtained after infusion of ^{13}C-labeled substrate, indicates that the label has been incorporated in the metabolites [2].

Measurements of ^{13}C label distribution in carbon skeleton of metabolic intermediates provided useful information about different biochemical pathways [103] because processing of the labeled carbon source through two (or several) alternative pathways to the same metabolite often involves different sequences of breaking and making carbon-carbon bonds; so the labeling pattern of that metabolite is often different depending on which pathway was followed [105].

For example, if pathway A incorporates one ^{13}C atom from the labeled substrate in position i of metabolite M, a single resonance will appear at frequency ω_i (fig. 3.4 A).

If by any chance the ^{13}C atom from the substrate is incorporated *through a different mechanism* (pathway B) in position i+1 of M, a *new* single resonance will appear, now located at a different frequency (ω_{i+1}) in the ^{13}C NMR spectrum (fig. 3.4 B). If pathways A and B are both active, ^{13}C atoms may be incorporated simultaneously at positions i and i+1 of the same metabolite molecule. In this case, a new interaction appears between the adjacent magnetic moments of contiguous ^{13}C nuclei. This interaction splits the original singlet resonances into doublets (fig. 3.4 C) [2]. So, based on the data obtained through the NMR analysis of the different isotopomers, it is possible to gain an insight into the topology of the metabolic network, i.e. identify which metabolic routes are more active at different environmental/genetic conditions.

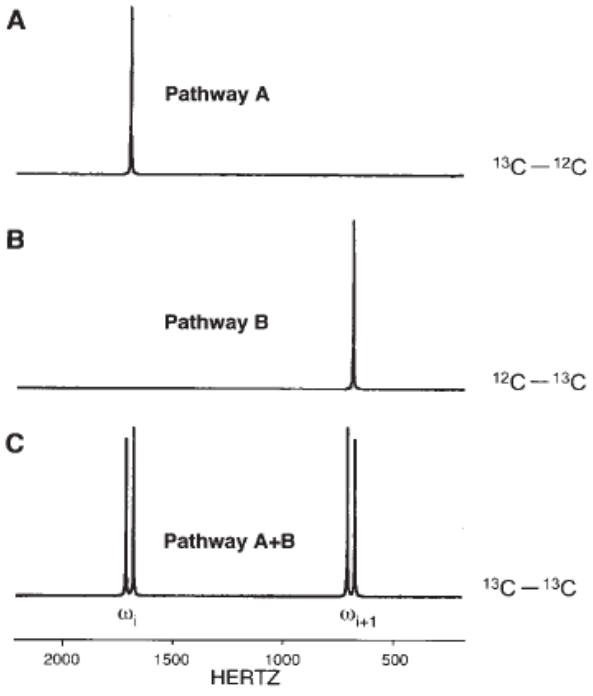

Figure 3.4: ^{13}C NMR signals for a specific carbon atom within a metabolite [2].
A – Only the pathway A is active.
B – Only the pathway B is active.
C – Both the pathway A and B are active.

Concluding, ^{13}C NMR spectroscopy associated with the use of ^{13}C-enriched substrates is a powerful tool to investigate intracellular metabolism because of the wealth of information contained in the distribution of isotopes in key metabolites [102]. Through the combination of the incorporation patterns analysis and metabolic models it is possible to quantify the fluxes through the different branches of the metabolic network.

3.6 General applications

Useful information can be obtained with this stable-isotope NMR-active labeling approach in a broader applications field (functional analysis of gene dysregulations in lung cancer [89], for example) and not only by the use of the ^{13}C isotope.

Although sensitivity is low and signals are broad, due to the nuclear quadrupole (I = 1), ^{2}H NMR is widely used to investigate the fate of deuterated precursors and to study mechanistic aspects of biosynthesis. Due to its low natural abundance (0.016%), ^{2}H is suitable for selective labeling and it is of special importance to unravel the stereospecificity of metabolic processes [90].

About to the ^{13}C nucleus, in particular, polypropylene (PP) samples, in which the three different carbon atoms along the chain were selectively labeled, were subjected to thermal aging and γ-irradiation by Mowery et al. [106,107]. By using solid-state ^{13}C NMR measurements at room temperature, they have been able to unambiguously identify the origin of the various oxidative products and their relative concentrations with consideration to the mechanisms of their formation [106]. Dramatic differences, indeed, were found in the type and distribution of oxidation products originating from the three carbon atom sites within the PP macromolecule (tertiary carbon, secondary carbon and methyl side group) showing how the isotopic labeling provides insight into chemical reaction mechanisms, since oxidation products can be traced back to their positions of origin on the macromolecule. This paper indicates the utility of isotopic labeling to provide more direct insight into the complex chemical reaction pathways by which polymers undergo oxidation, through the use of selective isotopic labeling of macromolecules. In general, this approach would be a promising new method for obtaining a more detailed molecular-level understanding of the complex mixture of oxidation products and chemical reaction pathways that underlie radiation-oxidation chemistry in polymeric materials [107].

The combination of ^{13}C NMR detection and substrates selectively enriched in ^{13}C at specific positions have made it possible to follow *in vivo* and *in vitro* the activity of a large variety of metabolic pathways in cells, animals and humans.

A large number of studies using *in vivo* ^{13}C NMR has been performed in the intact rodent or human brain, opening the field of *in vivo* ^{13}C NMR as one of the most powerful techniques in non-invasive studies of neurological processes. Several brain disorders have been investigated using ^{13}C NMR, which has already provided new and important clues to clinical diagnosis (mitochondrial brain disorders, the glutamate-glutamine cycle and glutamate neurotransmission in certain diseases and study of hepatic encephalopathy and impaired consciousness) [108,109]. A significant number of studies of cerebral metabolic compartmentation have used high-resolution ^{13}C NMR analysis of brain extracts supplying glucose or acetate contiguously labeled with ^{13}C as cerebral substrates [110-116].

In their study, Aureli et al. [117] reported some new aspects of acetyl-carnitine metabolism in an *in-vivo* animal model. In particular, the acetyl moiety of acetyl-carnitine appears to feed two distinct acetyl-CoA pools (intra- and extra-mitochondrial) involved in cholesterol synthesis. The peculiar ^{13}C labeling pattern observed results from the specific pathways involved in its synthesis. The analysis of the ^{13}C-labelling pattern in 3-hydroxybutyrate and cholesterol carbon skeleton provided evidence that the acetyl-carnitine-derived acetyl-CoA pool used for ketone bodies synthesis in mitochondria was homogeneous, whereas cholesterol was synthesized from two different acetyl-CoA pools located in the extra- and intra-mitochondrial compartment, respectively.

Isotopomer analysis of lactate, alanine, glutamate, proline, serine, glycine, malate and ribose-5-phosphate moiety of nucleotides has allowed original integrated information regarding the pentose phosphate pathway, TCA cycle and amino acid metabolism in proliferating human leukemia T cells to be obtained [86].

In particular, the contribution from the anaplerotic pathway versus the oxidative pathway, i.e the pyruvate carboxylase (PC) activity versus the pyruvate dehydrogenase (PDH) activity, in the synthesis of glutamate was calculated.

Dieuaide-Noubhani et al. [118] showed that glycolysis, the oxidative pentose phosphate cycle, sucrose turnover, polysaccharide synthesis and entry into the TCA cycle could all be detected in maize root tips by using suitable isotopomers of [^{13}C]-glucose and analyzing the specific enrichment of the carbons in a range of carbohydrates and amino acids.

^{13}C-labeled isotopomer profiles was also used to obtain important functional classification of 23 microbial strains isolated from a temperate and a tropical forest soil [104]. The microbial isolates were cultured with uniformly ^{13}C-labeled glucose as the C source such that all biochemical components synthesized from glucose were ^{13}C labeled. Differences in the chemical composition of microbial biomass, and thereby microbial community composition, can be related to the quantity and quality of the soil organic matter produced from their biomass.

We can conclude that NMR spectroscopy has a continuing role to play in the development of a quantitative understanding of metabolism and in the characterization of metabolic phenotypes [57].

Summarizing, the inherent flexibility of NMR as an analytical tool leads to a wide range of applications, including [100]:

- ✓ identify and quantify metabolites;
- ✓ define the intracellular environment in which metabolism occurs;
- ✓ characterize the pathways of primary and secondary metabolism;
- ✓ analyze the metabolic response to environmental stress and physiological perturbation;
- ✓ measure metabolic fluxes;