ALMA MATER STUDIORUM – UNIVERSITA' DI BOLOGNA

DIPARTIMENTO DI SCENZE E TECNOLOGIE AGRO-ALIMENTARI
CAMPUS DI CESENA

Corso di Laurea Magistrale in

SCIENZE E TECNOLOGIE ALIMENTARI

^{1}H NMR-based metabolomics investigation on the impacts of feeding in aquaculture of Gilthead sea bream (*Sparus aurata*)

Tesi in:

Chimica strutturale dei sistemi biologici

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Anno accademico 2020-2021
Sessione unica

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1. INTRODUCTION

The present thesis work was carried out at the laboratories of “Chimica dei Sistemi Biologici” and “Bio-NMR” at the Department of “Scienze e Tecnologie Agro-Alimentari” of “Università di Bologna”, placed in Cesena.

The research team of this laboratory uses the principles of Metabolomics to detect the modifications that occurs in biological matrices, such as animal or vegetal food products, caused by technological processes or different production methods, using the molecular profile obtained NMR spectroscopy. In this type of studies, the Nuclear Magnetic Resonance (NMR) spectroscopy is very advantageous compared to other techniques, as it allows the simultaneous characterization of a wide number of components present in the sample mixture as a whole, without the necessity of a separation step. Moreover, it is an untargeted method, so is not necessary to decide previously the molecules to focus on, as this technique enables to see everything present in the sample which is beyond the detection threshold and soluble in the solvent used for the extraction.

The aim of this thesis work is to define if there are differences in the metabolic profile of three groups of Gilthead sea bream (*Sparus aurata*) fed with different diets. To do that it has been used ^{1}H-NMR spectroscopy and a metabolomic approach.

This work fits well with the actual trend of development of the aquaculture, that is seen as an interesting source to provide food and to improve food security, and, at the same time, with the tendency of attention towards sustainability, quality and consumer compliance of the products, which are nowadays the most important global trends about foods. Sustainability is fundamental animal products, not coming from intensive farming, are more and more demanded as to be produced in an eco-friendlier way. Quality is crucial even in the side related to the health; this aspect is very important in fish products, which are perceived as healthy foods thanks to the good content of some beneficial compounds, as discussed below. Consumer compliance is significant as the foods must be economically accessible, available on the local market and safe.

In the recent years we witnessed a large development of aquaculture, and this leads to the need of searching strategies to improve the efficiency, productivity, but even sustainability of these systems. So, there has been even a grater increase of the research about all the aspects of aquaculture and the impact of different diets on the metabolism of fishes is one of that.

Obviously, the diet impacts the whole metabolism of fishes, and so it could affect efficiency of growth, survival rate, susceptibility to diseases, general state of health, resilience to a wide range of stressing conditions and so on; so is important to understand these aspects in order to improve the production for what concerns efficiency and productivity.

On the other hand, the diet also impacts the body composition of fishes and consequently the nutritional value, but even the taste, odour, texture, and sensory parameters in general; so, the understanding of these aspects can lead to obtain products of improved quality, understood as quality related to health, but even to sensory properties.

Nowadays an increasingly critical aspect is also sustainability, that regards all the aspect of the production and so even the production of feed for the aquaculture.

2. NMR SPECTROSCOPY

The 1H Nuclear Magnetic Resonance (NMR) spectroscopy allows the simultaneous characterization of a wide number of components in the sample as it generates a large quantity of spectral data that are informative of every molecule present in a mixture, if it contains hydrogen atoms: practically all organic substances! This technique enables to detect all the molecules in the sample which are both beyond the detection threshold (**LOD** "limit of detection" which accounts for 10^{-6} mol/dm^3) and soluble in the solution used for the extraction.

Moreover, this technique allows to obtain detailed information about the structure of the molecules, as it observes the behaviour of atomic nuclei surrounded by a given chemical environment, within a magnetic field.

By NMR spectroscopy, different states of the matter are suitable to investigation:

- Solid: NMR consents to obtain qualitative information.
- Solid dispersed in a liquid: low resolution spectra but with more quantitative information
- Solutions: NMR enables to obtain high resolution structural information; for this reason, these samples are preferred.

2.1. THEORETICAL PRINCIPLES

NMR spectroscopy is based on the magnetic properties of nuclei that have an odd mass number or an even mass number but odd atomic number; all nuclei with a nuclear spin quantum number (*I*) equal to 0 own a nuclear magnetic moment (μ) and so generate a tiny magnetic field, the magnitude of μ is proportional to the nuclear spin (*S*) of the nucleus and to the gyromagnetic ratio (γ), which is an intrinsic property of nuclei and determine its magnetic strength, indeed we can write: $\mu = \gamma * S$ (E. Hatzakis, 2018).

The NMR instrument generates a magnetic field (B_0) that interacts with the nuclear magnetic moment of the nuclei and thus they can be studied with NMR; nuclei with spin $I = {}^1/_2$ have convenient NMR properties and for this reason some of them are the most used in NMR studies, like ^{1}H, ^{13}C and ^{31}P (E. Hatzakis, 2018).

Nuclear spin is also characterized by the magnetic quantum number (m_I), which takes values from -*I* to *I* in steps of one and so generates 2*I* + 1 possible values, or orientations; each value corresponds to an energy state and normally the orientations have the same energy, but the application of an external magnetic field (B_0) separates the states and a

splitting between various nuclear spin levels occurs; the difference between the energy states depends on the magnetic field strength and gyromagnetic ratio (E. Hatzakis, 2018).

The hydrogen nucleus, for example, has a spin equal to $^1/_2$ and so it could have two orientations: up and down; when the nucleus is in a magnetic field the two orientations have a different energy because the state aligned with the magnetic field ($m_I = {}^1/_2$) has a slightly lower energy than the state opposite to the field ($m_I = -{}^1/_2$); the difference between the energies of the two states is: $\Delta E = h*v$, where h is the Planck's constant and v is called Larmor frequency and corresponds to: $v = B_0 * \frac{\gamma}{2\pi}$, so when the magnetic field increases the Larmor frequency increases, and so the difference between the energy of the two states increases (J. Keeler, 2013).

Transition among the energy states, called resonance phenomenon, can be achieved by the irradiation of the nucleus with a electromagnetic wave in the radiofrequency (RF) region, which can induce this transition because it has an energy equal to the difference between the energy of the two levels (E. Hatzakis, 2018).

When a sample is placed into the NMR instrument, nuclei spins have the tendency to align to the external magnetic field, because this alignment has the lowest energy, the nuclei that are able to align establish the equilibrium net magnetization, that can be represented as a vector along the z-axis. Then is applied a second field (B_1) as an RF pulse orthogonal to the main field, and that turn the magnetization toward the xy plane; afterward, the magnetization starts oscillating and induces a voltage to a coil oriented in the xy plane (E. Hatzakis, 2018).

Interrupting the transmission of the pulse the system relaxes to the equilibrium returning energy to the environment and the magnetization vector, will come back to the initial position; the process through which the energy, absorbed by nuclei, is transferred to neighbouring atoms is called relaxation. There are two ways by which the system can relax:

- Longitudinal relaxation (or spin-lattice): defined by the time T_1, this depends on the transfer of energy from excited protons to the surrounding ones.
- Transverse relaxation (or spin-spin): defined by the time T_2, this depends on the transfer of energy through the dipoles of the neighboring molecules.

Because of relaxation the magnetization on the xy plane decreases until it disappears, so even the voltage detected by the coil decreases. The detected voltage is called free induction decay (FID) and is the NMR raw signal; then, using the Fourier transformation *(FT), the FID is converted to a frequency domain signal, that is the NMR spectrum which*

represents the signal intensity as a function of the frequency at which the nucleus resonates and that is normally used in NMR studies (E. Hatzakis, 2018).

2.2. OBSERVABLES

The most common NMR observables that are used in food science studies are chemical shifts (δ), scalar coupling (J coupling), and signal area (E. Hatzakis, 2018).

2.2.1 *Chemical shift*

The frequency of the one-dimensional NMR plot (x-axis) is expressed with chemical shift, so that determines the location of a signal in the NMR spectrum. Chemical shift is expressed in parts per million (ppm) and conventionally is considered as 0 ppm the frequency in which there is the peak of an internationally recognized internal standard, such as trimethylsilylpropanoic acid (TSP) or tetramethylsilane (TMS) (E. Hatzakis, 2018).

The reason for using chemical shift to express the position of the peaks is that the frequencies of NMR peaks are directly proportional to the magnetic field strength, so using the chemical shift allows comparing spectrum obtained with NMR instruments that operate at different field strength (J. Keeler, 2013).

Mathematically the chemical shift is given by:

$$\sigma \text{ (ppm)} = 10^6 * \frac{v - v_{ref}}{v_{ref}}$$

where v is the frequency of the NMR peak; v_{ref} is the frequency of the peak of the reference compound, so is 0 ppm and the multiplication for 10^6 is used to express the results in ppm (J. Keeler, 2013).

The chemical shift gives information about the chemical environment around a nucleus because the differences in the electron density among various nuclei modify the resonance frequency absorbed by them. Usually, the chemical shift considered in a ^{1}H NMR spectra is from 0 to 10 – 12 ppm (E. Hatzakis, 2018).

In a ^{1}H NMR spectrum, the chemical shift allows to understand which chemical group hydrogen belongs to, as summarized in Figure 2.2.1

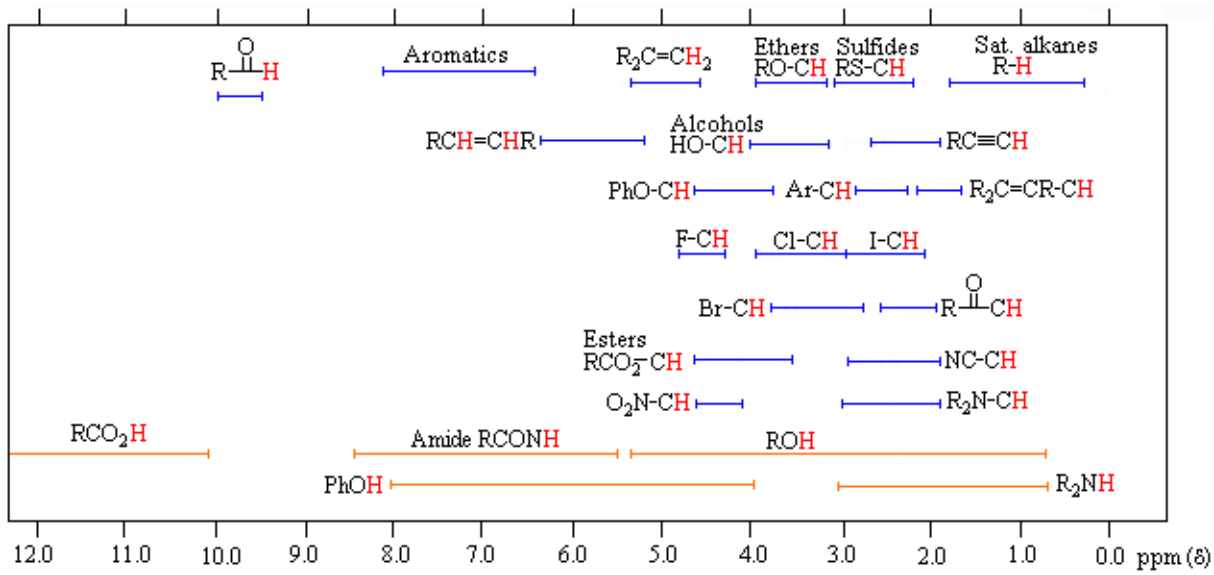

Figure 2.2.1: chemical shift of H belonging to different chemical groups (adapted by www2.chemistry.msu.edu)

When the hydrogen is linked with oxygen or other atoms, which allows it to make hydrogen bonds, the range is wider, properly because the hydrogen nuclei magnetization is affected even by the hydrogen bonds with the solvent.

2.2.2 *J coupling*

It is responsible for the signal splitting in the NMR spectra; in general, this is due to the fact that the nuclear spin of a nucleus polarizes the spin of the bonding electrons which then polarize the spin of the neighboring nuclei. The magnitude of J coupling depends on the distance and the dihedral angle between nuclei and so it is very important for structural characterization (E. Hatzakis, 2018).

The splitting pattern of a nucleus of interest is determined with the following equation: $2nI + 1$, in which n represents the number of neighboring protons and I is the nuclear spin quantum number, so for nuclei with spin (I) $^1/_2$ the equation can be simplified as follow: $n + 1$ (E. Hatzakis, 2018). Due to this effect, NMR signals are not single peaks but doublets, triplets, up to multiplets, depending on the number of hydrogen atoms interconnected by a few bonds.

2.2.3 *Signal area*

The area of an NMR signal is proportional to the number of nuclei that give the signal; moreover the signal area can be used even for quantitative NMR analysis because that is proportional even to the concentration of the nuclei and so it is possible, using an internal

standard at known concentration, to compare the signal areas to define the concentration of a compound (E. Hatzakis, 2018).

2.3. SPECTRA

The NMR spectra can be monodimensional (1D) or multidimensional, depending on how many frequency dimensions are considered, usually in food science applications are utilized 1D and 2D NMR spectra; in 1D NMR spectrum there is only one frequency dimension, instead of in 2D NMR spectrum there are cross-peaks, also called correlations peaks, that have two frequencies indicating a correlation between two different nuclei, This type of spectra have a better resolution than 1D NMR spectra, but they require also a longer experimental time, because they are formed by hundreds 1D NMR spectra (E. Hatzakis, 2018).

The position and the shape of the peaks give structural information because they are specific to each molecule.

2.4. NMR INSTRUMENTATION

Generally, the NMR system is composed by:

- Magnet: that generates the static magnetic field;
- NMR probe: that hosts the sample, and it is used to spread the RF pulse;
- Console: that contains the electronic components;
- Computer: that is used to control the acquisition parameters and to processing the obtained data.

2.4.1 *Magnet*

The magnet is formed by some concentric tanks, in the inner one there is a superconducting solenoid coil made of niobium-titanium, which generates the main magnetic field, this coil is kept in a liquid helium bath that holds the temperature at about 4 K, because at this temperature the coil resistance is close to zero and thus it is in the superconductive state, which is essential to generate a strong magnetic field, necessary to obtain high spectra resolution and sensitivity. The inner tank is surrounded by a tank in which is made vacuum while the external tank contains liquid nitrogen (E. Hatzakis, 2018).

Since the magnetic field is not perfectly constant, in the instrument there is a lock system that constantly irradiates the sample at the resonance frequency of the deuterium, or some other nuclei, and measures the frequency of it. If the magnetic field variates, the deuterium

does not absorb any more the radiofrequency; so, in this case, electric current is added or subtracted to bring it back in resonance. Moreover, is fundamental even the field homogeneity, that is obtained using other coils (shim coils) which surround the sample and produce magnetic fields, which can be regulated in order to compensate the inhomogeneity of the magnetic field (E. Hatzakis, 2018).

2.4.2 *NMR probe*

The NMR probe is positioned in in the centre of the magnet through a bore. Inside the probe there are the RF and the receiver coils generating and recording the signal, respectively. There are some different types of NMR probes and the most common way to categorize them is “inverse” or “observe”: in inverse probes the inner coil, the one closer to the sample, is used to excite proton frequencies, while the outer coil is used for heteronuclei; instead, observe probes have opposite structure. In 1D ^{1}H NMR experiments inverse probe is recommended (E. Hatzakis, 2018).

For the NMR probe are necessary some adjustment operations in order to obtain a good quality spectrum; the probe sensitivity changes with the signal frequency and a frequency for a maximum sensitivity of the sample does exist. The first adjustment operation is the tuning which sets the probe so that the frequency of maximum sensitivity is the principal frequency.

Another important adjustment operation is the matching, which ensures that the maximum amount of power directed to the base of the probe is transmitted to the coil located towards the top of the probe.

2.5. DATA PROCESSING

Before the acquisition of the 1D ^{1}H NMR raw spectrum, some optimization operations are applied aimed to improve the quality of the spectrum itself.

Frequently is necessary to adopt a procedure called solvent suppression, such as water, because the solvents are small symmetrical molecules which gives a strong NMR signal. For instance, water is present at 7-8 order of magnitude higher concentrations with respect to the molecule of interest. Thus, their suppression is crucial for foods’ NMR analysis, because, if solvent suppression is not done, the strong signal of water can cover the signals of less concentrated molecules making impossible to detect them (E. Hatzakis, 2018).

The most common water suppression techniques are presaturation, WATERGATE, and 1D version of the NOESY experiment (E. Hatzakis, 2018).

2.5.1 *Data transformation*

This first operation is done automatically by the control program and is the Fourier transformation from FID to spectrum; alternatively, this transformation can be manually done with the command "*efp*". Often, after the conversion, the spectra can be out of phase, so is used the command "*apk0.noe*" in order to make automatic phasing, calibration and line base correction.

2.5.2 *Line width evaluation*

It is possible to evaluate the goodness of the spectrum considering the line width, that is the width of the signal measured at the half of the height: the lower is its value, the higher is the resolution. This parameter is influenced by a wide range of factors, among which NMR tube quality, sample concentration, presence of paramagnetic impurities and a not adequate shimming. To measure the line width is considered the range that goes from -0.004 ppm to 0.004 ppm of the spectra, where there is the TPS's peak, which has been added as an internal standard, and is execute the command "*lb 0; efp; peakw; lb 1; efp*", which is a complex command that gives us back a numerical value which corresponds to the line width of the TPS's peak, a good line width is less than 1.0 Hz.

2.5.3 *Shift evaluation*

It is possible to evaluate the shift between the same peaks in different samples, that is due to external factors. The main cause of this shift is the variation of pH, indeed the control of them is fundamental in NMR studies, because even small variations of pH produce a shift in some peaks of the spectrum. This is due to the fact that the metabolites have acid-base properties and so the simultaneous presence of protonated and no protonated compounds makes the NMR analysis pH dependent.

In order to evaluate the shift, can be considered a peak in the region of the spectrum in which there are the peaks of the hydrogens that are part of aromatic groups, because these are the groups more strongly influenced by the variations of pH; holding zoomed on the chosen peak is used the command "*pps*", which allows to see the exact ppm that corresponds to the center of the peak, thus it is possible evaluate how much this peak shifts in the different spectra.

To solve the problem of the shift it can be added a buffer solution to the sample, in order to have the small variation of pH as possible.

2.6. QUALITATIVE AND QUANTITATIVE ANALYSIS

A fundamental step for metabolomics studies is the identification of the molecules present in the sample, by means of its molecular profile. As discussed above the peculiar characteristics of NMR signal are:

- Intensity: related to the concentration and to the number of protons;
- Chemical shift: related to the functional group and to the chemical environment;
- J coupling: that represents the multiplicity of a signal.

The peak's assignment to a specific molecule is made by taking into account these characteristics and it could be make through:

- Online databases: in which, indicating the chemical shift of a signal, is obtained a list of possible molecules that could give this peak.
- 2D NMR spectroscopy: as discussed above this type of spectra could give some useful information to the peak's identification.
- Software: allows to load the NMR spectrum and matching it with possible molecules' spectra.

2.7. CHEMOMETRICS TECHNIQUES

The spectra obtained by a consistent number of measurements contain a lot of information that is difficult to be understood with conventional univariate statistical methods, which consider only one variable at a time; for this reason, normally is used multivariate statistical analysis (MVSA or chemometrics) which enables to fully characterize a food product analysed (E. Hatzakis, 2018).

The application of MVSA allows to analyse a large data set with a lot of variables by reducing its dimensionality, so these techniques can represent a table with thousands of variables with a two- or three-dimensions graphs, in order to extract the maximum amount of significant information from the data set (E. Hatzakis, 2018).

Often, before the application of chemometrics techniques, is required a pre-processing procedure to make data from different samples comparable to each other and ensure reliable analysis; the most common steps of this pre-processing are spectral alignment, binning, sample scaling or normalization, data scaling, and spectral editing (E. Hatzakis, 2018).

Spectral alignment: the chemical shift can be affected by pH, presence of paramagnetic cations, and ionic strength and, even when are used buffers or chelators, some variations

could remain; in this case is necessary to use a signal alignment method, normally applied with software, as like ICOSHIFT (E. Hatzakis, 2018).

Binning: in this step the spectrum is segmented in areas of equal size, called bins, in order to reduce the data; this involves even a reduction in spectral resolution (E. Hatzakis, 2018).

Normalization: this step corrects the minor variations due to the little differences that could occur in the experimental conditions between the samples; usually the normalization is made multiplying the spectral intensities with a constant, derived from the internal standard or from the sum of the intensities in the spectrum itself (E. Hatzakis, 2018).

Mean-centering: this step consists of subtracting the mean values of the variables from the data and it is often applied before the scaling methods (E. Hatzakis, 2018).

Data scaling: this step is applied to reduce the impact of the most concentrated molecules in the sample, because often the compounds that show larger variability among samples are not the most concentrated. In food metabolomics are often used, as scaling methods, Auto-scaling or Pareto-scaling; in Auto-scaling the scaling factor is the standard deviation and this method is more effective for minimize the effects of high-concentrated metabolites, instead in Pareto-scaling each variable is divided by the square root of the standard deviation (E. Hatzakis, 2018).

After pre-processing the data are ready for statistical analysis and the most used methods are:

- Principal component analysis (PCA): it is an unsupervised method, in which the analysis is conducted without previous knowledge about the samples.
- Partial least squares (PLS-DA): it is a supervised method, in which is necessary a previous knowledge of the samples.

(E. Hatzakis, 2018).

2.8. METABOLOMICS

Metabolomics is the study of changes on the entire metabolites set of certain cells, tissues, organs or organisms, so it can be a helpful tool for studying living organisms and for this reason has been applied to various living systems to study different aspects; moreover, during the past few years, the application of metabolomics even in food systems has been growing a lot and this type of application is called "**food metabolomics**" (S. Kim, 2016).

Food metabolomics can be applied to all food system processes, including production of raw materials, food processing and food intake by humans (S. Kim, 2016). A food metabolomics analysis is composed by sample preparation, metabolite extraction,

derivatization, metabolite separation, detection, and data treatment, in any case not all these steps are always required; indeed, the necessary steps are selected based on type of study, kind of sample (solid or liquid), method used for the separation (gas chromatography or liquid chromatography), and detection method (mass spectrometry or NMR) (J.M. Cevallos-Cevallos et al., 2009).

- Sample can derive from foods or humans and the preparation method depends on what aspect is going to be studied (S. Kim, 2016).
- Metabolite separation and detection can be done using some different techniques both for separation, such as chromatography, gas or liquid, or capillary electrophoresis, and for detection, such as mass spectrometry, nuclear magnetic resonance or near infrared spectrometry (S. Kim, 2016 & J.M. Cevallos-Cevallos et al., 2009).
- Data treatment is usually composed of compounds' identification and multivariate statistical analysis (J.M. Cevallos-Cevallos et al., 2009).

In general, we can say that food metabolomics has a high potential to help to perform agriculture, food industries, and human health (S. Kim, 2016).
Foods are very complex matrices consisting of a multitude of compounds produced by the metabolism of animals, plants, and bacteria; additionally, the constituents of foods interact with each other and are modified by external factors such as raw material management, industrial processing, and distribution; finally, even the utilization of foods in human metabolism can create additional molecules (E. Hatzakis, 2018).
Some of the most important applications of food metabolomics in food system are food resource production, industrial food processing, and food intake by humans, as sown in Figure 2.8.1 (S. Kim, 2016).

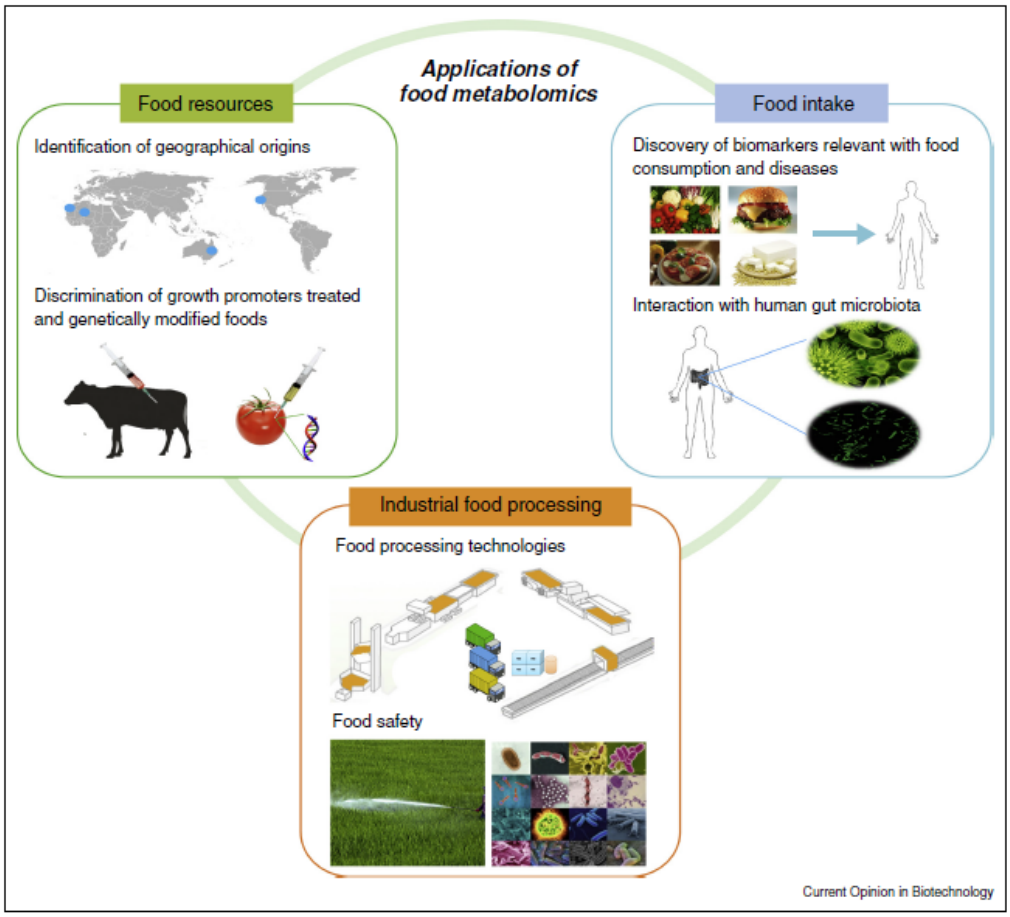

Figure 2.8.1: application of food metabolomics (Source: S. Kim, 2016)

Food resource production: Metabolomics has been used to investigate the foods' characteristics that depend on environmental and genetic factors.

For what concerns the environment, Metabolomics can help to determine the geographical origin of some products, allowing traceability and authenticity determination; moreover, Metabolomics can help to understand the influence of climatic and seasonal factors on the product's quality (S. Kim, 2016).

Instead, for what concerns genetic factors, food metabolomics could help to evaluate the characteristics of genetically modified foods and to compare them with their corresponding non-genetically modified counterparts (S. Kim, 2016).

Industrial food processing: Metabolomics can be used: 1) to understand the food changes caused by the industrial processing technologies; to detect chemical contaminants and foodborne pathogens in foods, which, however, can also be present in the raw materials; 2) to study food packaging materials in various aspects, such as monitoring possible contaminants and spoilage in packaging materials, evaluating packaging types and monitoring freshness of foods inside them; 3) to support the development of bio-based food

packaging materials providing some useful information for metabolic engineering of microorganisms and plants used for producing them (S. Kim, 2016).

Food intake by humans: Metabolomics is used to monitor specific food intake and dietary patterns in humans, showing that these significantly affect various metabolisms and to investigate food related diseases, revealing the alterations associated with them, identifying biomarkers of human diseases, and understanding metabolism of human diseases. Metabolomics can also be useful to understand the metabolism and functional status of the human gut microbiota, that is fundamental for the health of humans (S. Kim, 2016).

An example of this type of application is shown by the paper of Simonetti *et al*. in which an integrated genomic-metabolomic approach to the classification of acute myeloid leukemia defines a subgroup with *NPM1* and cohesin/DNA damage mutations (G. Simonetti et al., 2019).

Another way to classify the applications of food metabolomics is:

- Determination of food quality, for what concerns both raw materials and processed foods.
- Study of the food safety, to determinate the presence of pathogen or alterative microorganisms and to study some not understood effects of genetic modification.
- Compliance with food regulation, because metabolomics can be a useful tool to understand the origin and authenticity of the food, to detect the presence of genetically modified foods, which are not allow by much country regulations, and to check the labelled ingredients.
- Study of food microbiology, to identify bacterial contamination of foods, to understand microbial metabolism and its products in foods, and to test new antimicrobial compounds and the response of microorganisms to them.
- Study of food processing, to detect the changes in food components caused by them and to understand the suitability of certain varieties for processing.

(J.M. Cevallos-Cevallos et al., 2009).

As mentioned above, the NMR analysis can be used for metabolomics studies; the two main approaches in these types of studies are:

- Targeted analysis: biomarkers are predefined, and the aim is to quantify as many metabolites as possible (E. Hatzakis, 2018); this type of analysis is important for understand the behaviour of specific compounds in the sample under determined

conditions, this analysis typically requires a selective extraction of metabolites and higher level of purification (J.M. Cevallos-Cevallos et al., 2009).

- Untargeted analysis: the metabolites are not necessarily identified and quantified and to discriminate between samples and groups a comparison among the spectral patterns is used (E. Hatzakis, 2018); this type of analysis has been used for the identification of possible fingerprint of biological phenomena (J.M. Cevallos-Cevallos et al., 2009).

Based on the objective of the analysis and data manipulation metabolomics studies can also be classified as:

- Discriminative: aimed to find differences between samples even without creating statistical models or evaluating possible pathways; for this type of analysis are usually exploit multivariate data analysis techniques.
- Informative: focused on the identification and quantification of metabolites; these types of analysis are used in the development and update of metabolite databases (for example Human Metabolome Database).
- Predictive: based on the creation of statistical models to predict some variables that are difficult to quantify by other means; these models are usually created by partial least square (PLS) regression (J.M. Cevallos-Cevallos et al., 2009).

Usually, the steps involved in metabolomics analysis are at least:

- Pre-processing of spectroscopic data, such as baseline correction, calculation of intensity values on each point or peak or summed over segmented regions, obtained with binning.
- Production of a data table with rows, in which there are the samples, and columns, in which there are variables, frequencies or integrals.
- Normalization of the data, it is an operation that acts on the rows.
- Scaling of the data, it is an operation that acts on the columns.
- Multivariate statistical analysis of the data.

(A. Craig et al., 2006)

3. SEAFOOD PRODUCTS

Since the last years, several scientific studies on fish and seafood have highlighted the importance of these products in human diet and health, because of its very interesting composition for what concerns essential and beneficial compounds; even for this reason the consumption of these products has increased a lot in recent years.

3.1 CAPTURE AND AQUACULTURE PRODUCTION

The capture fishery production is relatively static since the 1980s: whilst the aquaculture is in continuous growth, the global fish production in 2016 was at about 171 million tons, of which 90.9 million tons from capture; from 1961 to 2016 the average annual increase in global fish consumption is 3.2 % against an average population growth of 1.6 %. This means that the average per capita consumption of fish is growing. Indeed, it has passed from 9.0 kg per capita in 1961 to 20.2 kg per capita in 2015 (FAO, 2018).

In 2015 fish was about the 17 % of animal proteins consumed by the global population and even this datum is growing, as the average annual increase in global consumption of meat from terrestrial animals (always from 1961 to 2016) is 2.8 %: so less that the fish one (FAO, 2018).

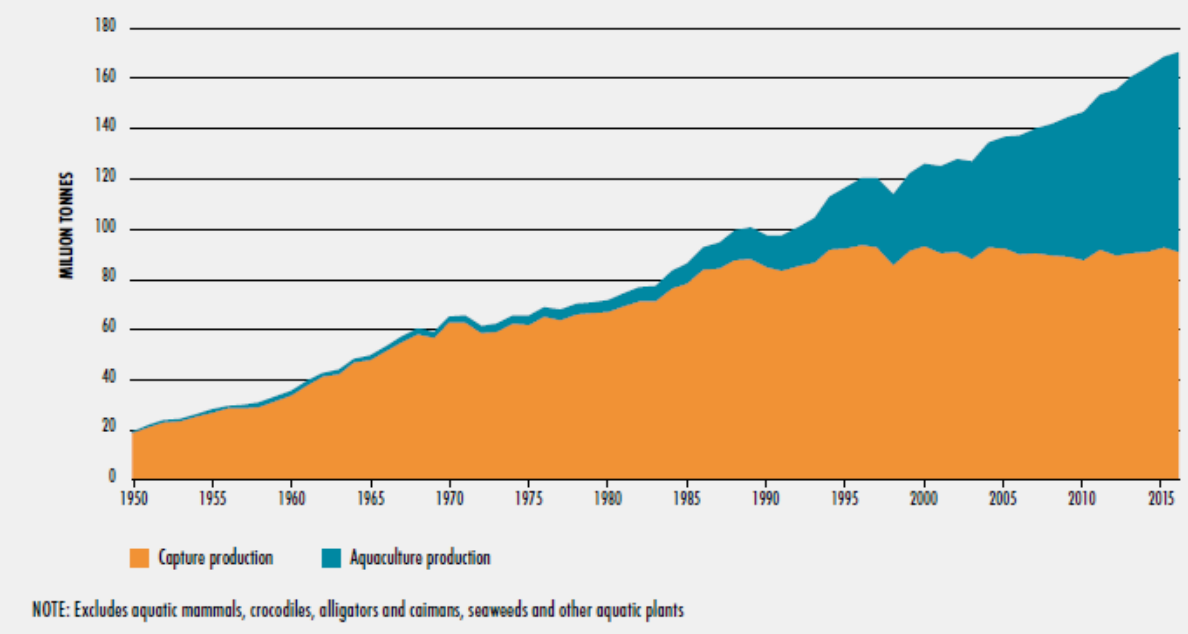

Figure 3.1.1: world capture fisheries and aquaculture production (FAO, 2018).

3.1.1 Capture fisheries production

The capture fisheries production can be divided into marine capture production and inland waters capture production.

Marine capture production: in 2016 it represents 87.2 % of the total global capture fishery production (FAO, 2018).

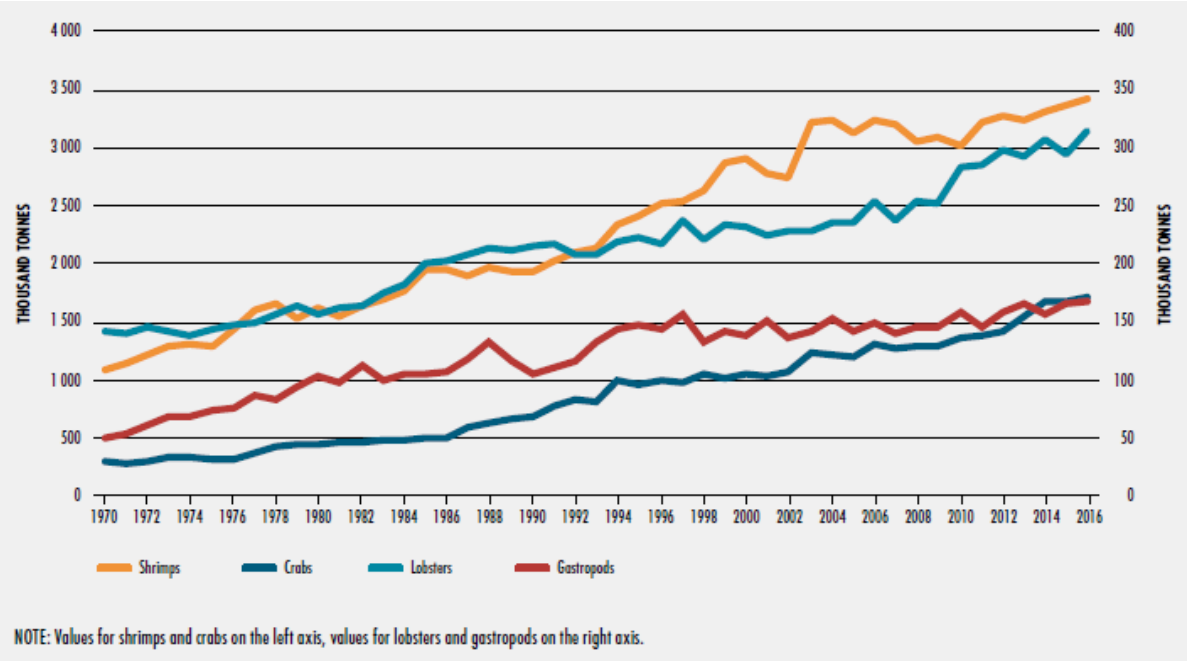

Figure 3.1.2: catch trends of valuable species groups (FAO, 2018).

For what the status of fishery resource concerns, the fish stocks can be divided into two categories:

- Fished at biologically unsustainable levels: are too much fished and so they do not have the time to restore themself, and in this case, we talk of overfished species.
- Fished at biologically sustainable levels: the individuals fished are equal or less than the ones born, and in this case, we talk respectively of maximally sustainably fished or underfished.

The state of marine fishery resources is declined in a constant way in the last years, with a progressive increase of the percentage of overfished species (from 10.0 % in 1974 to 33.1 % in 2015) and a specular reduction of stocks fished at biologically sustainable levels (from 90.0 % in 1974 to 66.9 % in 2015) (FAO, 2018).

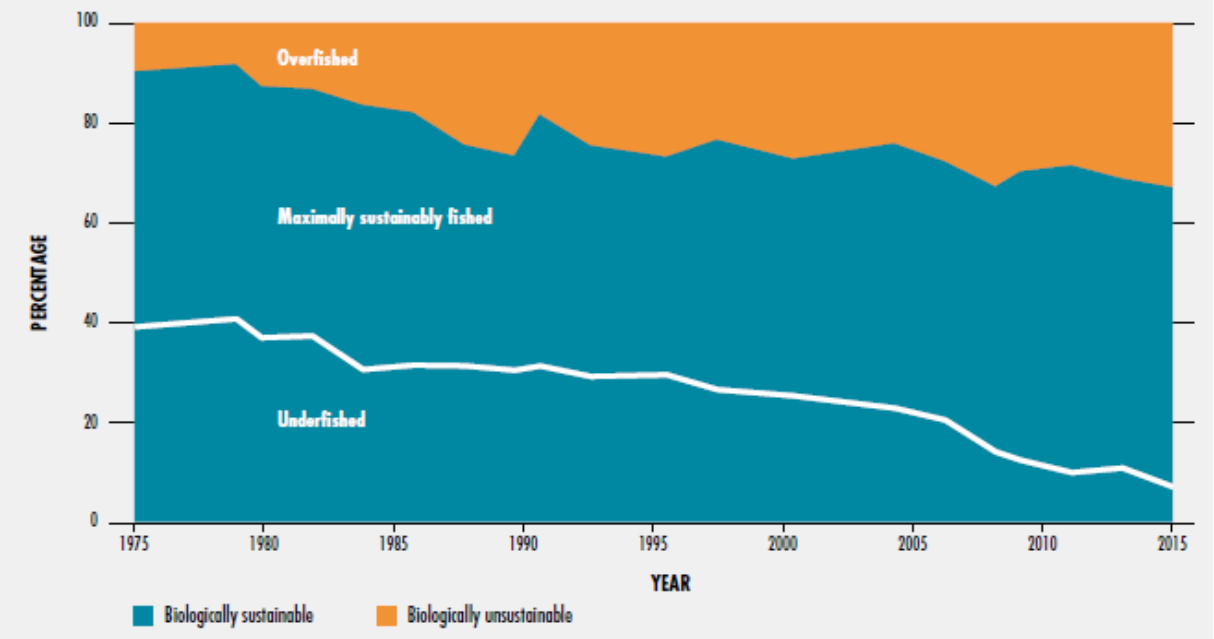

Figure 3.1.3: global trends in the stet of the world's marine fish stocks, 1974 – 2015 (FAO, 2018).

Considering the percentage of fish stocks overfished, in 2015, the higher values are in Mediterranean and Black Sea (62.2 %), followed by Southeast Pacific (61.5 %) and Southwest Atlantic (58.8 %), while the lowest values (13 – 17 %) are in most of the areas of the Pacific (FAO, 2018).

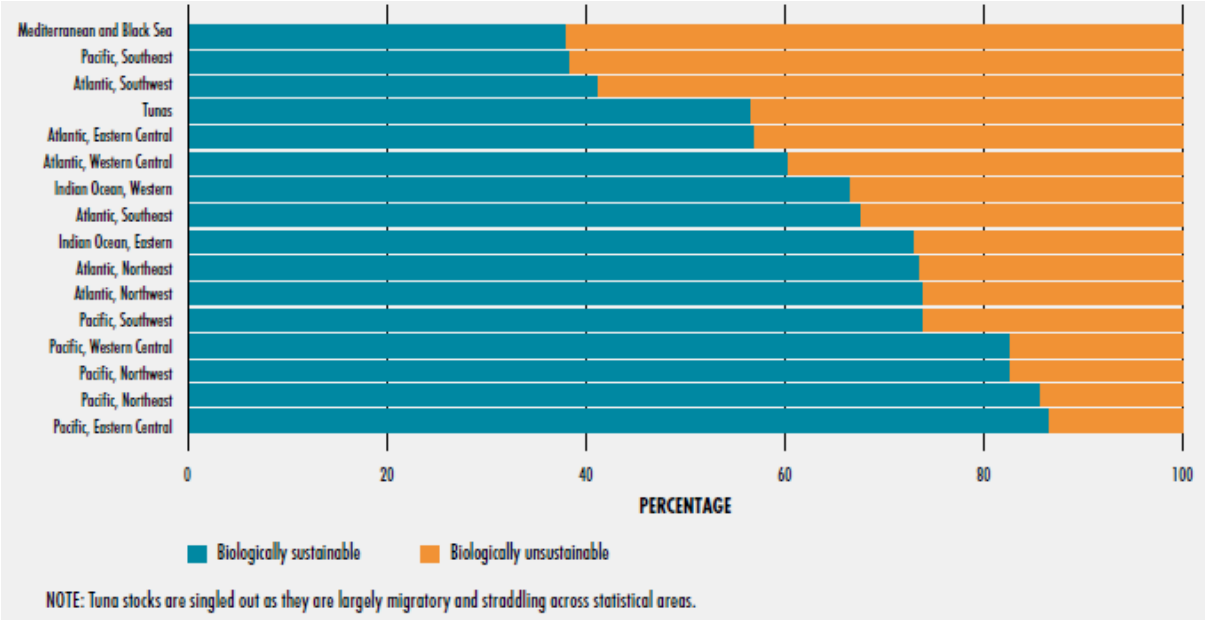

Figure 3.1.4: percentage of stocks fished at biologically sustainable and unsustainable levels by FAO statistical area, 2015 (FAO, 2018).

The situation of the overfished species is cause of concern, because it has first of all negative ecological consequences, but even negative social and economic consequences due to the reduction of fish production in the long term; for this reason it is trying to do

something, even at legal level, for example with stopping fishing during the reproduction period and limiting the quantity that can be fished, but it is not easy to control these aspects and the rebuilding of stocks that are currently overfished requires time (FAO,2018).

Inland waters capture production: in 2016 it represents the 12.8 % of the total global capture fishery production. It is necessary to consider that the available data concerning the inland waters production are much less accurate that the ones concerning the marine capture production, and this is because about the 95 % of the world's inland fishery is carried out in developing countries and 90 % of this production is consumed in the developing world (FAO,2018).

3.1.2 Aquaculture production

Aquaculture is growing faster than other major food production sectors (FAO, 2018).

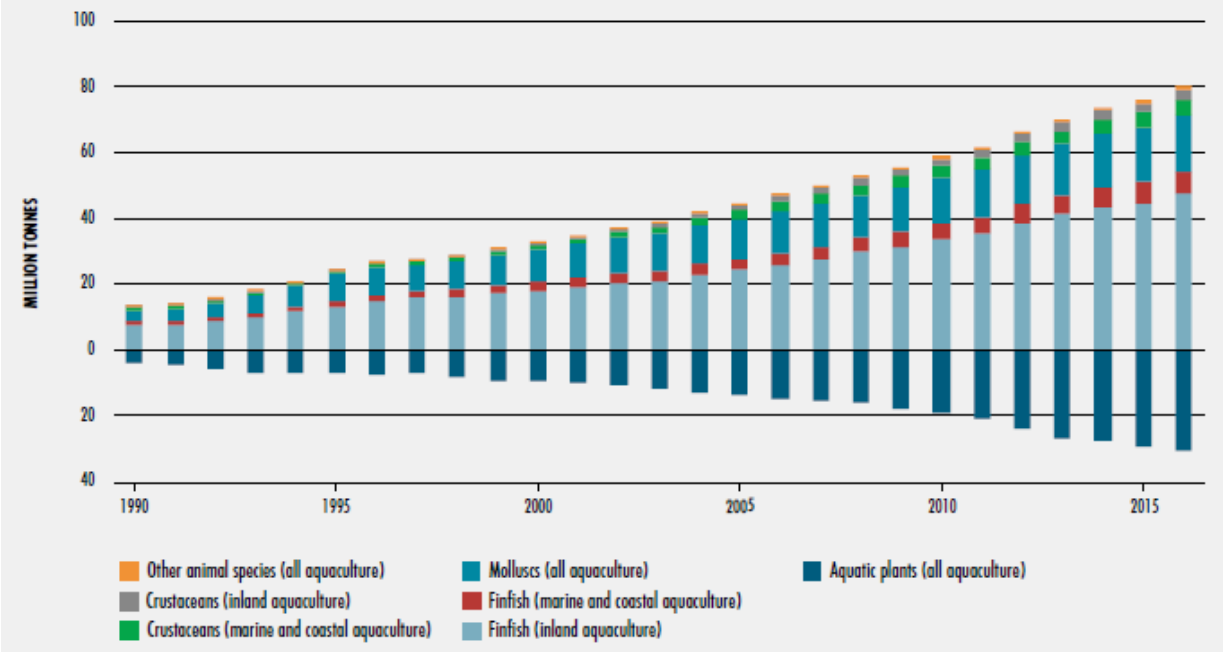

Figure 3.1.5: world aquaculture production of food fish and aquatic plants (FAO, 2018).

In 2016 aquaculture covered the 29.6 % of the total global fish production (excluding China), at regional level aquaculture share was 17 – 18 % of total fish production in Africa, the Americas and Europe; 12.8 % in Oceania; and 40.6 % in Asia (excluding China) (FAO, 2018).

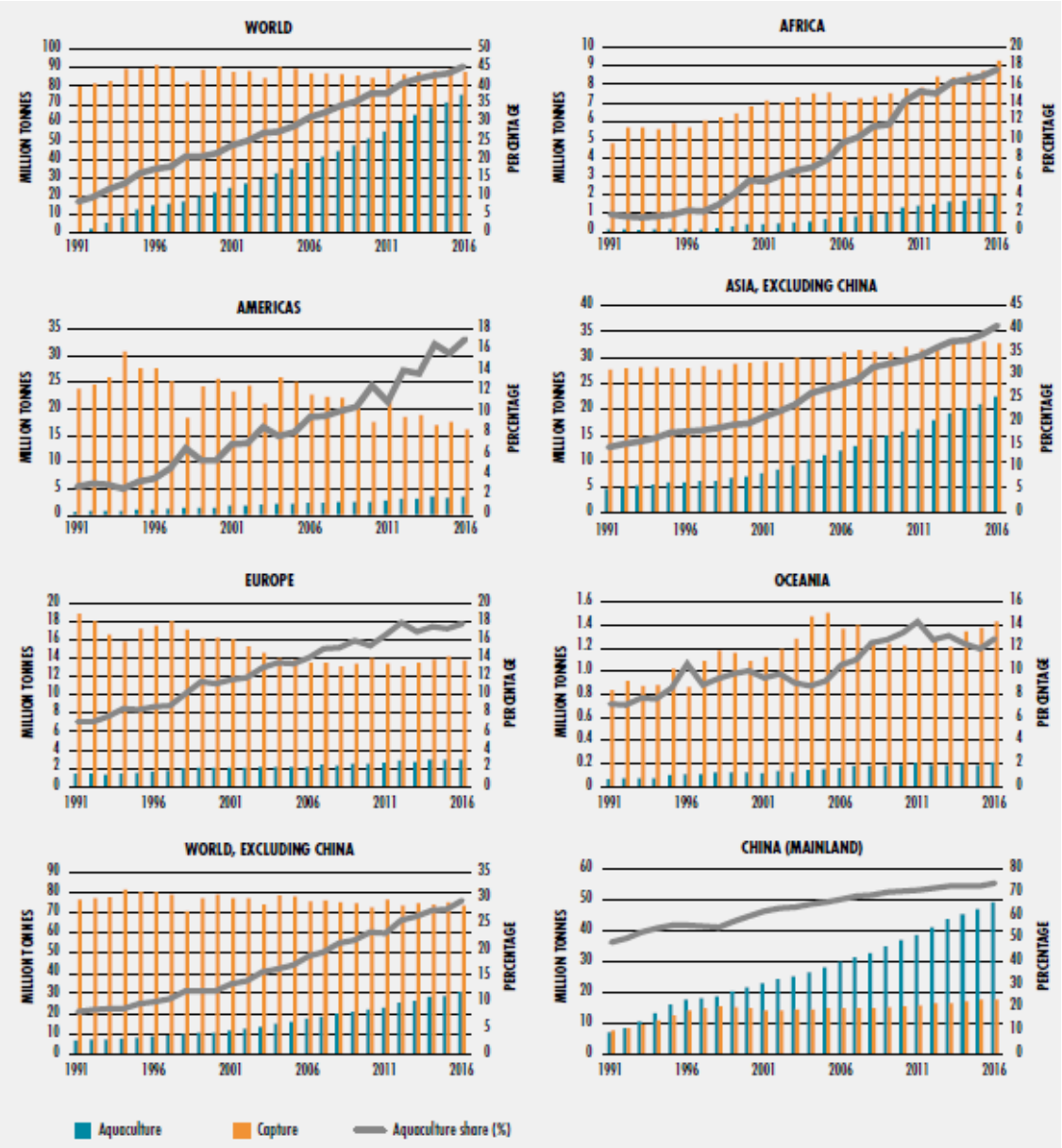

Figure 3.1.6: aquaculture contribution to total fish production (excluding aquatic plants) (FAO, 2018).

Aquaculture production can be divided into inland aquaculture and marine and costal aquaculture.

Inland aquaculture: world production of farmed food fish relies increasingly on inland aquaculture, that could be done in some different ways:

- Freshwater environments, usually earthen ponds: this is the most common typology.
- Saline – alkaline water: this is used in a small number of countries (e.g., China and Egypt) usually in areas where the soil conditions and the chemical properties of available water are not suitable for conventional crops or pasture.
- Tanks, above – ground tanks, pens and cages: where the local conditions allow the use of these methods.

In 2016 inland aquaculture was the 64.2 % of the total global production of farmed fishes; with inland aquaculture are farming mainly finfish (92.5 % in 2016), but even other species groups, as crustaceans in Asia, particularly shrimps, crayfish, and crabs; in inland aquaculture production can be farmed even some marine shrimps, that can growth in freshwater or in saline – alkaline water after acclimatization (FAO, 2018).

Marine and coastal aquaculture: marine aquaculture is also known as mariculture and is practiced in the sea, therefore precisely in marine water; instead, coastal aquaculture is practiced in structures completely or partially made by humans in areas near the sea, such as coastal ponds and gated lagoons, an important aspect is that in this last type of aquaculture the salinity is less stable than in mariculture, due to the rainfall or evaporation (FAO, 2018).
With marine and coastal aquaculture are produced mainly shelled molluscs (58.8 % in 2016), followed by finfish and crustaceans (together 39.9 % in 2016) (FAO, 2018).

Another important aspect that must be considering about aquaculture is the difference between production with and without feeding. Indeed, some farmed species need to be fed by the farmer and so we talk of fed species, instead some other species are able to get nourishment directly from the environment in which they are farmed, so we talk of unfed species.
The share of unfed aquaculture is decreasing because, even if it is increasing in absolute terms, the increase is slower than the one of the fed species (FAO, 2018).
In Asia, Central and Easter Europe and Latin America are also diffused multispecies polyculture farming systems, in which are used filter-feeding carps (filter-feeders are sub-group of suspension feeding animals that feed by straining suspended matter and foods particles from water, typically by passing the water over a specialized filtering structure), these systems use natural food and improve the water quality in the production system; recently was introduced, particularly in China, even Mississippi paddlefish (*Polyodon spathula*) as another filter-feeding species for this type of systems (FAO, 2018).
Another possibility is the use of extractive species, as marine bivalves, which extracts organic matter for growth, and seaweeds, which grow by photosynthesis by absorbing dissolved nutrients, these species, when farmed in the same area with fed species, are beneficial, because they remove waste materials and lows the nutrient load; for this reason,

the culture of extractive species with fed species in the same mariculture sites is encouraged (FAO, 2018).

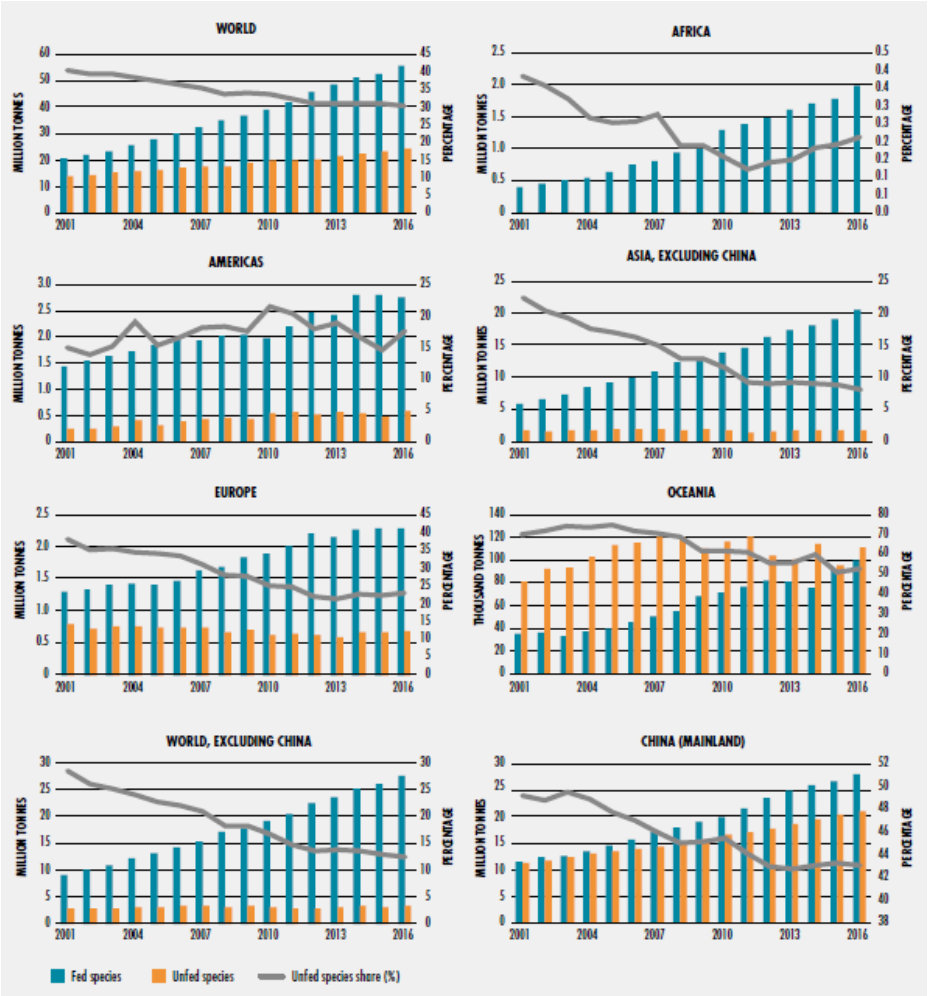

Figure 3.1.7: fed and non-fed food fish aquaculture production, 2001 – 2016 (FAO, 2018).

WORLD FISHERIES AND AQUACULTURE PRODUCTION AND UTILIZATION (MILLION TONNES)[a]

Category	2011	2012	2013	2014	2015	2016
Production						
Capture						
Inland	10.7	11.2	11.2	11.3	11.4	11.6
Marine	81.5	78.4	79.4	79.9	81.2	79.3
Total capture	**92.2**	**89.5**	**90.6**	**91.2**	**92.7**	**90.9**
Aquaculture						
Inland	38.6	42.0	44.8	46.9	48.6	51.4
Marine	23.2	24.4	25.4	26.8	27.5	28.7
Total aquaculture	**61.8**	**66.4**	**70.2**	**73.7**	**76.1**	**80.0**
Total world fisheries and aquaculture	**154.0**	**156.0**	**160.7**	**164.9**	**168.7**	**170.9**
Utilization[b]						
Human consumption	130.0	136.4	140.1	144.8	148.4	151.2
Non-food uses	24.0	19.6	20.6	20.0	20.3	19.7
Population (*billions*)[c]	7.0	7.1	7.2	7.3	7.3	7.4
Per capita apparent consumption (*kg*)	18.5	19.2	19.5	19.9	20.2	20.3

[a] Excludes aquatic mammals, crocodiles, alligators and caimans, seaweeds and other aquatic plants.
[b] Utilization data for 2014–2016 are provisional estimates.
[c] Source of population figures: UN, 2015a.

Figure 3.1.8: World fisheries and aquaculture production and utilization (FAO,2018).

Another important aspect that has to be taking into account is the fact that different types of aquaculture systems exist. Similarly to terrestrial animals, the breeding systems can be divided into intensive, extensive and semi-intensive (or semi-extensive); in the **intensive** aquaculture systems the fishes are breeding in cages in the sea or tanks inland, which are relatively small in size, the diet is mainly based on feed and there is a continuous management of biological cycles and environmental parameters.

While **extensive** aquaculture systems are made in natural or artificial large environment in which the human intervention is limited to capture, restocking, juveniles' management and possible control of the hydraulic regime of the waters, so the reproduction and alimentation are made in natural way, without the human intervention, usually this type of aquaculture is made in protected coastal areas, lagoon environment or in lakes and dams.

Lastly exists even a **semi-intensive** (or **semi-extensive**) aquaculture system, that is an intermediate form between the two seen before, in which the breeding areas are smaller compared to the ones of extensive aquaculture systems and the feeding is supplemented with feed in some periods of the year.

In the Mediterranean areas is also widespread the **valliculture**, which is an extensive or semi-intensive aquaculture system made in basins of lagoon areas delimited by embankments or fences, generally is made in environments where the rivers flow into the sea. This system is based on the natural migration of euryhaline fishes which, during the reproduction period, approach the coasts, after the hatching the larvae enter the lagoon in

search of food and shelter and are “trapped” thanks to the use of canal systems and locks, so they grow in the basin feeding on what they find in this environment. This system allows to have a varied production, but the time necessary to reach the commercial size is long, for this reason have been introduced semi-intensive variations of this system, in which are introduced in the environment fries and feed is given, resulting in the possibility to increase the fishes’ density.

Another type of aquaculture system is the breeding whit **organic method**, which is regulated by the Council Regulation (EC) 834/2007 on organic production and labelling of organic products, which regulates the organic production in general and by the Commission Regulation (EC) No 710/2009 laying down detailed rules on organic aquaculture animal and seaweed production, with which are introduced specific rules for the aquaculture production.

3.2 PHYSIOLOGY

According to the International Standard Statistical Classification of Aquatic Animals and Plants (ISSCAAP), developed by FAO the fishes are first divided into **cartilaginous** and **bony**, depending on the conformation of the skeleton; the bony fishes are further divided, depending on the environment in which they live, into **freshwater fishes**, which live in freshwater environments, **diadromous fishes**, which live a part of them life in freshwaters and the other part in marine waters and **marine fishes**, which live in marine environments. In the table below are summarized the species which are part of these categories.

Group	Species	ISSCAAP code	FAOSTAT code
FRESHWATER FISHES		1	1501
	Carps, barbels and other cyprinids	11	1401
	Tilapias and other cichlids	12	1402
	Miscellaneous freshwater fishes	13	1403
DIADROMOUS FISHES		2	1502
	Sturgeons, paddlefishes	21	1404
	River eels	22	1405
	Salmons, trouts, smelts	23	1406
	Shads	24	1407
	Miscellaneous diadromous fishes	25	1408
MARINE FISHES		3	1503
	Flounders, halibuts, soles	31	1409

	Cods, hakes, haddocks	32	1410
	Redfishes, basses, congers	33	1411
	Jacks, mullets, sauries	34	1412
	Herrings, sardines, anchovies	35	1413
	Tunas, bonitos, billfishes	36	1414
	Mackerels, snoeks, cutlassfishes	37	1415
	Sharks, rays, chimaeras	38	1416
	Miscellaneous marine fishes	39	1417

Table 3.2.1: species belonging to the different categories of fishes (Adapted from: http://www.fao.org/waicent/faostat/agricult/fishitems-e-e.html).

The marine fishes are further divided in **demersal**, which live near the seabed and move very little during their life and **pelagic**, which usually live away from the seabed, swims along all the water column and does not necessarily stay in the same areas, so they are fishes which usually move a lot.

The different environment in which these fishes live implicates even differences in their biologic characteristics, the principal difference is in the osmoregulation mechanisms that these fishes uses to maintain the equilibrium between the environment and them body fluids; marine fishes live in an environment in which the solutes are quit concentrated, so they tend to become dehydrated, for this reason they drink continuously and they produce low nitrogenous catabolites (urea and trimethylamine), which are kept in biofluids because in this way the solutes concentration in biofluids is near to the one in the external water and so they prevent the dehydration. On the other hand, freshwater fishes are subjected to the reverse mechanism, as they live in an environment in which the solutes are very low concentrated, so they tend to eliminate frequently the nitrogenous catabolites in order to maintain a low concentration of solutes even in their organism.

Another way to categorize the fishes is based on the capability to resist to changes in salinity; among the marine fishes there are some species called **euryhaline**, because they can live in a wide range of salinity; instead, the diadromous fishes can be divided into **anadromous**, which lives the juvenile period in the sea and when they must reproduce they go up the rivers to lay their eggs, and **catadromous**, which lives the first stages of their life in freshwaters and then they migrate to the sea, in which happens the reproduction.

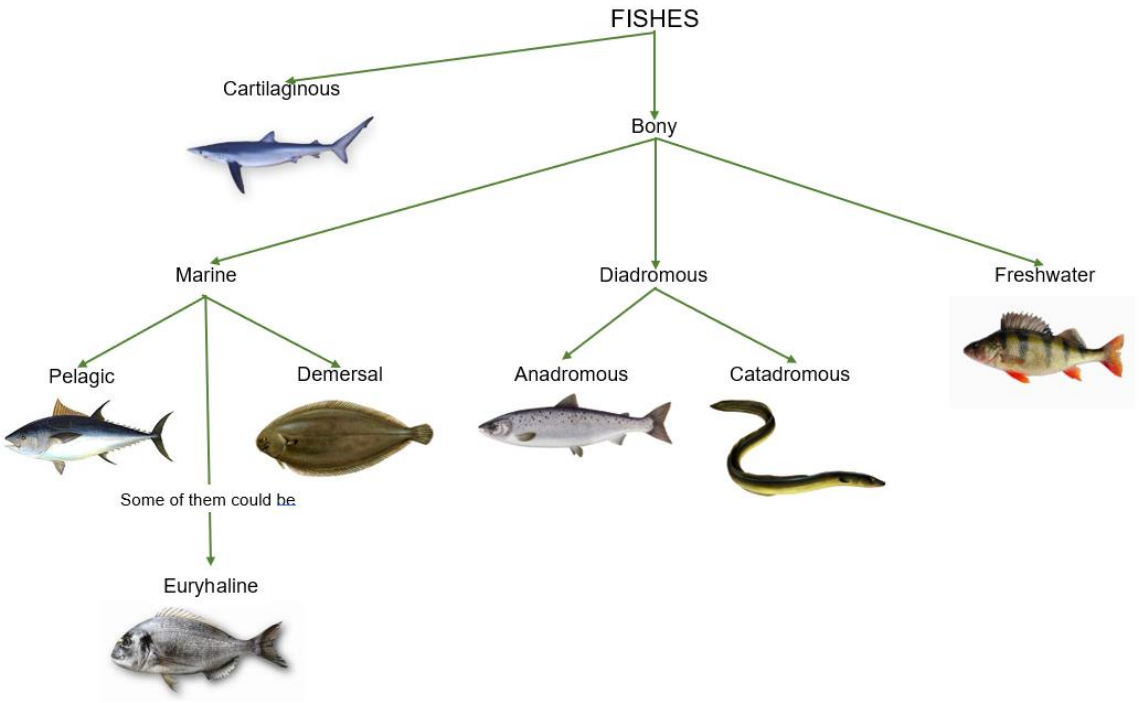

Figure 3.2.1: Classification of fishes.

3.3 COMPOSITION

The edible part of fishes is represented by skeletal muscle and changes in its composition may affect the quality of the product (K. Grigorakis, 2007), usually the body composition of the fishes is different in the various categories (discussed above in the paragraph 3.2).

	Pelagic fishes	Demersal fishes
Water (%)	65-75	75-81
Proteins (%)	16-19	18-21
Lipids (%)	5-15	< 2
Carbohydrates (%)	0.5	0.5
Minerals (%)	1-2	1-2

Table 3.3.1: average composition of pelagic and demersal fishes (Adapted from: V. Venugopal, 2005).

In general, the carbohydrates are always very low, and the pelagic fishes have more lipids because they swim a lot, so they need to have more energy reserves. Normally, when the lipids content increases the water and proteins content decreases.

3.3.1 Proteins

Usually in these products there are on average 17 – 22 % of proteins, that are high-quality, due to the high favourable essential amino acids pattern, as fish proteins are particularly rich in methionine and lysine; the digestibility of these proteins is more than 90 %, with a bioavailability approximately 5-15 % higher than proteins from plant sources (FAO, 2018 & Y.A.A. Bernardo et al., 2020 & V. Venugopal, 2005). The higher digestibility of fish proteins compared with the one of land animals is also because in fish muscle are less present strong collagenous fibres and tendons (V. Venugopal, 2005).

In the seafood products the quantity of non-protein nitrogen compounds (NPN) is higher than in the flesh of terrestrial animals and can variate from 10 to 40 %, these compounds include free amino acids, peptides, trimethylamine oxide, which is converted *post-mortem* in trimethylamine, creatine and creatinine, guanidine compounds and nucleotides; moreover, the marine fishes contains usually more of these compounds than the freshwater fishes as they are used in the cells in order to maintain the osmoregulation (V. Venugopal, 2005).

Several of these compounds contributes even to the taste and flavour of the fishes, for example, some amino acids have a specific taste (glycine has a sweet taste, while glutamic acid is a flavour enhancer), trimethylamine has a strong odour associated with the loss of freshness and urea, which is particularly concentrated in selachii fishes and is converted *post-mortem* in ammonia, has a bitter and slightly salt taste. Freshwater fishes have a more neutral flavour and are less perishable even for the less concentration of these compounds. Moreover, the ability of proteins to retain water is also important in determining the juiciness of the product and the quantity of muscle connective tissue protein, because its reduction, usually due to a long-term fasting before the death, can cause poor texture of the cooked flesh (K. Grigorakis, 2007).

3.3.2 Lipids

The lipids have some different biological functions, so it is possible to categorize them into:

- Storage lipids that are used as an energy reserve and are mainly triglycerides.
- Cellular lipids that have a structural function and are mainly phospholipids, glycolipids and cholesterol.
- Hormones and pigments that have specific biological functions and are mainly the unsaponifiable fraction of lipids.

According to the fat content in the fillet the fishes are divided into four categories:

- Very thin, less than 1 % of lipids
- Thin from 1 to 3 % of lipids
- Semi-fat from 3 to 10 % of lipids
- Fat (or oily) over 10 % of lipids

Very thin and thin fishes are usually demersal, in which the lipids are present almost exclusively in the liver, while in semi-fat and fat fishes there are adipocytes in the subcutaneous tissue, in the abdominal cavity and in the muscle deputies to the movement of tail and fins (V. Venugopal, 2005). Moreover, in a single fish the lipid content increases from tail to head, with higher deposition of fat in belly flap and dark muscle (V. Venugopal, 2005).

In general, lipids are the most important components in determining the product's quality, in very thin and thin fish species even a small increase in the fat may heavily affect the quality, while the same variation in semi-fat and fat species does not influence the quality and acceptability of the product, as these fishes store more fat in the muscle and so the variation is not so evident (K. Grigorakis, 2007).

Lipids in the flesh affects the sense of taste and the sensation in the mouth giving by the consumption of the product, moreover, they are important precursors of flavor compounds which characterize the fish. Talking about quality are very important the perivisceral and peritoneal fat, which are found respectively in and around the peritoneal cavity; perivisceral fat has mainly a visual importance and affects negatively the consumers' impression about the product, nevertheless this fat is usually removed with intestines when the fish is consumed fresh; instead, the peritoneal fat remains in the product as it is located behind the peritoneum, so it contributes to the taste of the product (K. Grigorakis, 2007).

The seafood products are an important source of ω-3 fatty acids, especially of eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), which are essential for the humans as the organism is not able to synthetize them, so they must be introduced with the diet; generally, the fishes are so rich in ω-3 fatty acids (V. Venugopal, 2005) because they are monogastric animals, so their body composition in fatty acids depends on what they eat and their diet is rich of ω-3 fatty acids which derive from the base of the food chain, in other terms from the phytoplankton which, unlike the terrestrial vegetables, is able to synthetize ω-3 fatty acids; successively, the phytoplankton is eat from zooplankton, that accumulates lipids and which is on turn eat from little fishes and so on until the fishes which are used for the human consumption.

There are a lot of evidence in literature that the composition of the muscle and the fat deposition is affected by feeding characteristics, fasting, photoperiod, weight and size, season, dietary fatty acids composition, gonadal maturation and spawning, salinity and water temperature and it is also necessary to consider that the composition variates even in the same fillet, as there are fat and musculature differences, which can lead to different organoleptic properties even in the same fillet (K. Grigorakis, 2007).

Even if all these factors are very important in the fishes from aquaculture, the fatty acids composition depends mainly on the diet, usually the cultured fishes are fatter than the wild ones and are richer in ω-6 fatty acids because they are feed with vegetal flours and oils which are in turn richer of these compounds.

Some other important factors which affect the fatty acid composition of fishes are:

- Season and life cycle: in the reproduction season they use most of the energetic reserves and proteins of the body to produce eggs or spermatozoa, so after this season the muscle contains less proteins and lipids, which are been replaced from water, so it is of less quality for the consumption.
- Water temperature: generally the species which live in colder waters are richer in unsaturated fatty acids, which are necessary to maintain the fluidity of tissues and cell membranes; even in the same species seasonal variations in the water temperature can lead to a modification in the fatty acid composition (V. Venugopal, 2005).

3.3.3 Other compounds

Carbohydrates: are usually less than the 1 % and mainly represented by glycogen.

Minerals: the total content of minerals in raw marine fish muscle are roughly in the range from 0.6 to 1.5 % wet weight; these minerals include sodium, potassium, calcium (which is particularly concentrated in the bone, but it is also present in the flesh), magnesium, phosphorus, iron (which is especially present in the fish which has dark-colored flesh), zinc, copper, selenium (which is important even because it protects against mercury and cadmium toxicity, that, unfortunately, could be present in the fish itself), fluorine, iodine (which is presents mainly in marine fishes), cobalt, manganese, and molybdenum (V. Venugopal, 2005).

Vitamins: both liposoluble, such as A, D, E and K, and water-soluble, such as B group and C (FAO, 2018 & Y.A.A. Bernardo et al., 2020). Vitamin A is especially concentrated in fish liver oils, while the concentration of vitamin E is related to the diet of the fishes as they are unable to synthetize that; in the fish are also present thiamin (vitamin B1), biotin (vitamin

B6), folic acid, niacin (vitamin B3), pantothenic acid (vitamin B5), riboflavin (vitamin B2) and vitamin B12 (V. Venugopal, 2005).

Bioactive compounds: a diverse array of them is present in fishes, to name one of them is of particular interest chitin, which is a nonsoluble polymer of N-acetyl glucosamine, that can be deacetylated into chitosan, which is a water-soluble cationic compound used in pharmaceutical, water purification, cosmetics, and packaging sectors, but it is even be studied to produce edible coatings which enhance the shelf-life of fresh fish products (V. Venugopal, 2005).

3.4 CONSUMPTION

3.4.1 Fish utilization and processing

Fish is a food commodity and there are a lot of species that can be prepared in a wide range of ways; is necessary to consider that fish can spoil more rapidly than many other foods so the process until the consumer requires care to maintain its quality and nutritional value and to avoid losses and wastes; anyway, the fish can be distributed and marketed worldwide in a wide range of product forms thanks to preservation and processing (FAO,2018).

In the recent years there has been an expansion in consumption and commercialization of fish products, so has been growing even the interest in food quality and safety, nutritional aspects, and waste reduction; to ensure the food safety and the protection of the consumers increasingly stringent hygiene measures have been adopted at national and international trade levels (FAO,2018).

In 2016 the 88 % of the total global fish production was used for direct human consumption, and this share has increased in the recent years (from about 67 % in the 1960s); for what the remaining 12%, used for non-food porpoises, concerns, the majority was utilized as fishmeal and fish oil (74 % in 2016), while the remaining part was used for direct feeding in aquaculture and raising of livestock and fur animals, in culture, in pharmaceutical uses and for ornamental. The most common, preferred and highly priced form of commercialization is live, fresh or chilled (45 % in 2016), followed by frozen (31 % in 2016), prepared and preserved (12 % in 2016) and cured, for example dried, salted, in brine, fermented smoked (12 % in 2016) products; anyway, there are significant differences in the utilization of fish and processing methods between regions and countries in the world; moreover is necessary to consider that actually the loss or wastage of fish is estimated at about 27 %, despite some improvements in fish processing and distribution, to reduce wastes and losses has been made in the past few years (FAO, 2018).

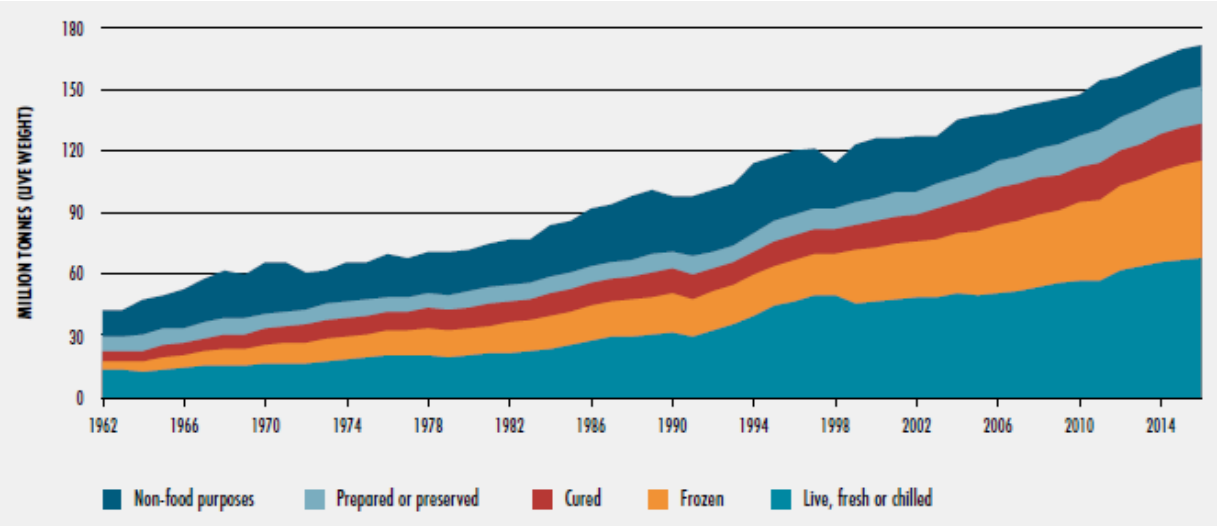

Figure 3.4.1: utilization of world fisheries production, 1962 – 2016 (FAO, 2018).

Thanks to technological developments, improved logistics and increased demand the commercialization of live fish has grown in the last years, even if it could be challenging because it is subject to health regulations, quality standards and animal welfare requirements; apart from this, other forms of commercialization have been growing, thanks to the improvements in processing, refrigeration, ice – making and transportation (FAO, 2018).

In the last years, the fish food sector has become more heterogenous and dynamic; frequently the fish processing is divided into high-value fresh and processed products and uniform-quality meals, usually ready or portion-controlled; despite the technical innovations in many countries, especially in less developed economies, yet there are problems of fish quality, due to the lack of adequate infrastructure and services for ensuring that (FAO, 2018).

As discussed above a part of fish is used to produce fishmeal and fish oil; fishmeal is a material that appear as a flour, it is rich in proteins and it is obtained from milling and drying fish or fish parts; fish oil, instead, is obtained pressing cooked fish and then spinning and separating the product and it is the richest available source of long-chain polyunsaturated fatty acids, that are fundamental in the human diet, nevertheless the majority of the fish oil produced in the world is used for aquaculture feeds; these products can be obtained from the whole fish, but even from fish trimmings or other fish by-products (FAO, 2018).

3.4.2 Consumption

As discussed above the consumption of fish has growth in the past years, due to the increased production, both with fisheries and aquaculture, that enhanced the possibility to consume diverse and nutritious foods, but the increase in consumption is also due to a

series of other factors, such as reduced wastage, better utilization, improved distribution, and growing demand (FAO, 2018).

Fishes and them products are a valuable source of nutrients and micronutrients that are fundamental for healthy diets, indeed even small quantities of fish can provide essential amino acids, fats, and micronutrients. Moreover, there are evidences that fish can be essential for pregnant women and very young children as it contributes to the neurodevelopment of unborn or young children; other researches instead shows the beneficial effects in mental health and prevention of cardiovascular diseases, stroke and molecular degeneration age-related; furthermore, experts agree that proper fish consumption has positive effect against the potential negative effects associated with contamination or other safety risks (FAO, 2018).

The dietary contribution of fish is significant mostly in terms of high-quality, easily digested animal proteins; in 2015 fish represented about 17 % of animal proteins and 7 % of all proteins consumed in the world, but it provides until 50 % of animal proteins intake in some countries; the average consumption of fish varies significantly across the world due to cultural, economic and geographic factors, this last aspect is very important because in the countries which have an outlet to the sea, even if they are poor countries, the fish consumption is more diffused only for these reason (FAO, 2018).

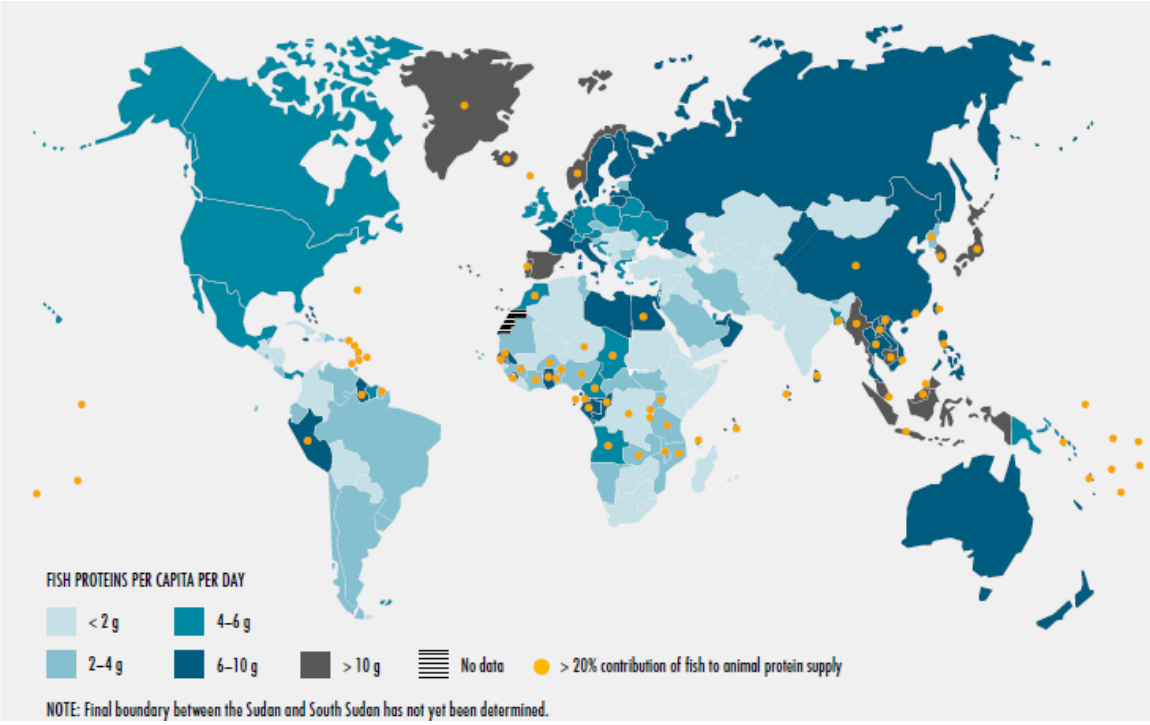

Figure 3.4.2: contribution of fish to animal protein supply, average 2013 – 2015 (FAO, 2018).

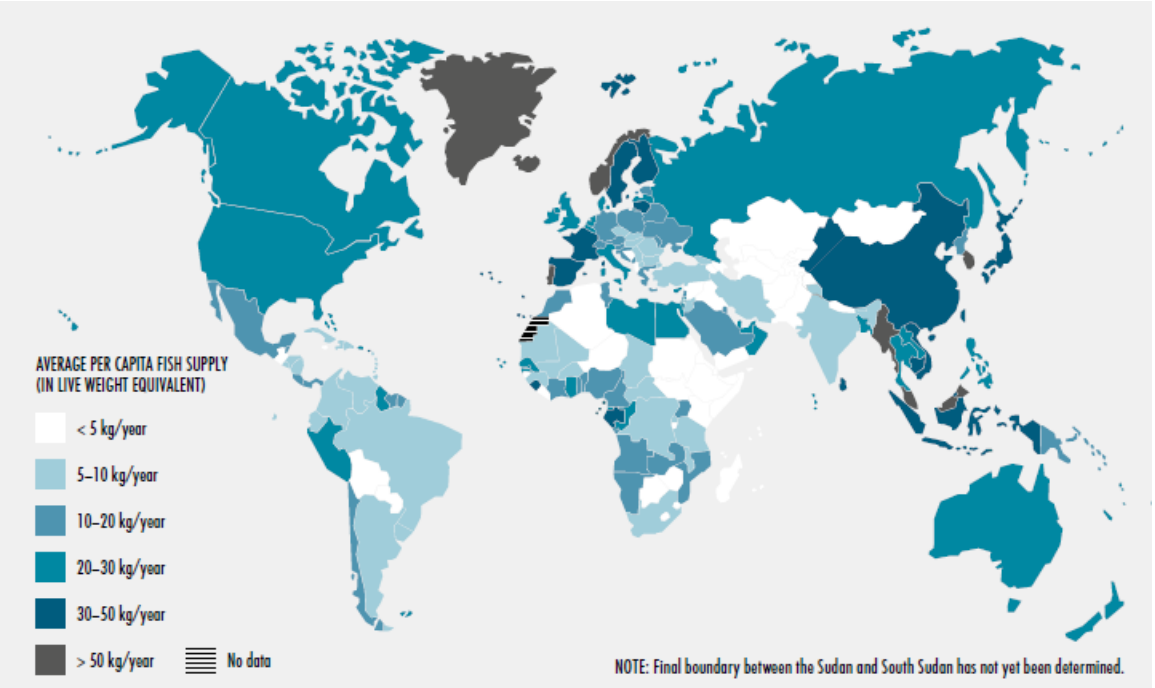

Figure 3.4.3: apparent fish consumption per capita, average 2013 – 2015 (FAO, 2018).

Which species products are made available to consumers is mostly due to natural resources and biological considerations, because fish is a highly perishable food, so long distances between where the fish is caught or farmed and where it is marketed involves significant logistic challenges and high costs; even for these reasons in the recent years there was a high development of the aquaculture, indeed aquaculture producers can better control the fish production process than capture fisheries, and so the share of farmed fish in human diets has increased quickly; moreover, aquaculture can be more efficient in take the fish from producer to consumer and it is more able than capture fisheries to guarantee the sustainability and product origin (FAO, 2018).

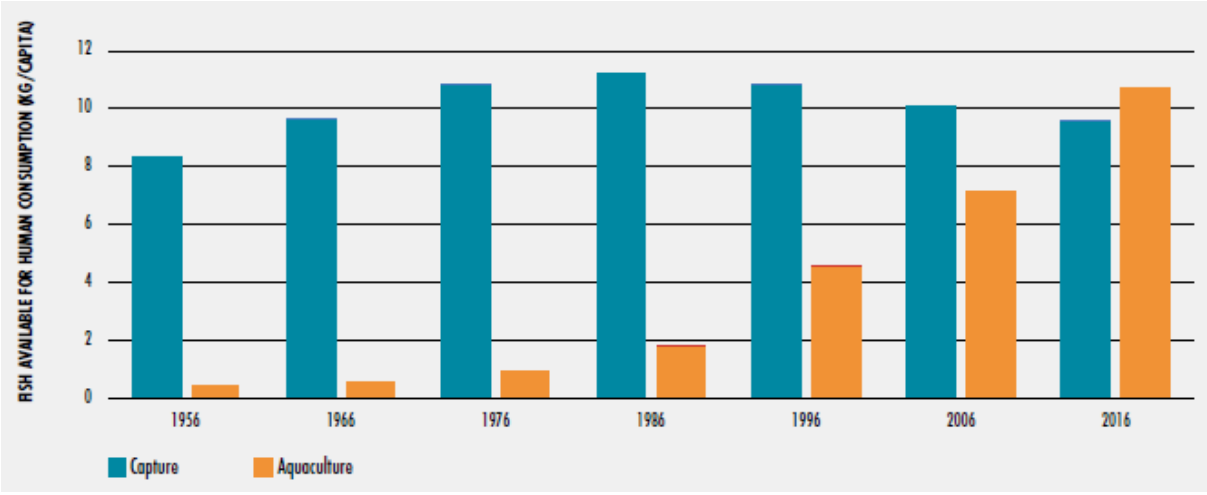

Figure 3.4.4: relative contribution of aquaculture and capture fisheries to fish human consumption (FAO, 2018).

The fish consumption is grown in the recent years even thanks to changes in the way consumers choose, purchase, prepare and consume fish products; the globalization has allowed consumer to access to a more wide range of fish species and has introduced new products and tastes; even seasonal variation is mitigated to international diversification and advances in preservation technologies; on the other hand the consumer is more careful to the origin of the products and so it is a driving factor for the development of traceability systems and certification schemes, which guarantee the sustainability and quality of the products (FAO, 2018).

Besides, even urbanization has modified the nature and extent of fish consumption and, indeed, the infrastructure of a city allows more efficient storage, distribution, and marketing of these products. In addition, urban inhabitants usually can spend more money in animal proteins, and they eat away from home more often, so the fish products prepared and marketed for convenience have been growing in popularity (FAO, 2018).

However, is important to mention that the overall levels of fish consumption also depend on market developments for other animal meats (FAO, 2018).

3.4.3 Food security and human nutrition

The fisheries and aquaculture sectors are crucial to improving food security and human nutrition and has an increasingly important role in the fight against hunger; moreover, the consumption and trade of fish products are rising (FAO, 2018). As discussed above fish and fish products has high nutritional value, due to its composition, even if usually, nutritional content is higher in small fishes and in fish parts that are not normally consumed, such as head, bones, and skin (FAO, 2018 & Y.A.A. Bernardo et al., 2020).

Fishes can be classified into three main categories: oily fish, white fish, and shellfish; especially oily fish contains a good quantity of vitamins and are rich in ω-3 fatty acids, which are important for brain and heart health, normal functionality of eyes, nerve development in babies and there are proofs that they reduce the risk of cancer and arthritis (P.K. Prabhakar et al., 2020).

In the recent years, there was a development of intensive aquaculture, in which are more and more widespread crop-based feedstuffs and lower fishmeal and fish oil are used, this influences the nutrient content of the products obtained, particularly fat content and fatty acid profile (FAO, 2018).

Fish processing co-products represent an underutilized source of nutrients and micronutrients, so in the recent years these co-products are increasingly used to produce

fishmeal and fish oil; in general improvement in feed formulations and feed manufacture can reduce the quantity of feed used per kilogram of farmed aquatic food produced, and so it can be improved the utilization of fishmeal and fish oil (FAO, 2018).

In this sector is necessary to implement the food safety control and inspection systems, because the consumption of fish can have a lot of beneficial effects on the human health, but it is also well known, for example, the health risks associated with specific chemical contaminants, such as mercury and dioxins, that may be present both in wild and farmed fishes (FAO, 2018).

3.5 QUALITY

3.5.1 General aspects

The total quality of the seafood products is defined as the set of features that can satisfy the organoleptic, healthy and convenience needs of the buyer/consumer, found with constancy in the product, obtained by proper management of the production chain, in the respect of animal welfare and environmental sustainability, disclosed in full transparency through traceability and labeling (S. Cautadella et al., 2011).

So, the quality of these products is influenced by a wide range of factors, including aspects that depends on the fishes, as like species, genetics, age, dimensions, gender and physiological state; aspects that depends to the breeding method, as like diet composition, level of rationing, meals frequency, finishing and fasting; and aspects that depends to the external environment and to the method of capture, in the case of caught fishes, as like chemical and microbiological contamination, temperature, salinity, caught method and post-caught proceedings. All these aspect influences the chemical characteristics and the body composition of the fishes, which in turn influences the nutritional and organoleptic characteristics of the final product.

Logically freshness and shelf-life are fundamental as they determine the product acceptability and so also its commercial value, but are even difficult to determine, since they are associated with food degradation and the reliability of the measures depends to the methods used; usually, freshness is associated with the best product conditions, so the ones it has immediately after the capture or harvest, the consumers evaluation of the quality of a food product depends even on personal expectations, that vary from different consumers and due to cultural or geographical influences. Moreover, it is necessary to consider that after buying the product, the consumers store it for a certain time before the consumption and is normally credited to the fact that at the consumption time they are more tolerant to

the defects of the product, in order to avoid wasting that; this behaviour can influence the estimated product shelf-life, which could be calculated basing on the alterations of sensory attributes that occurs in early stage of fish degradation, instead on the appearance of unacceptable taste characteristics, defining a smaller period than the maximum shelf-life estimated for a fresh fish, called rejection day (J. Freitas et al., 2021).
Normally the industries define the shelf-life as the moment in which the product is rejected by a certain percentage of consumers, clearly the freshness characterization is of increasing relevance to make better predictions of storage effects and distribution conditions on product shelf-life, so it is always more important to use accurate and reliable methods (J. Freitas et al., 2021).

Usually, seafood products are considered highly perishable products, in particular the ones which are sold fresh, this is due to the high content of free amino acids and other non-nitrogenous substances, which are used by microorganisms as a source of nutrients for them growth (Y.A.A. Bernardo et al., 2020). Generally, after the death of the fish some processes of modification of the structure and biochemistry of the product take place, such as oxidation and autolysis, which determine changes in the product, lead to loss of fish freshness and ease the spoilage of the fish, through the formation of compounds that contribute to that; one of the enzyme classes which starts to be active just after the dissolution of the *rigor mortis* are the endogenous proteases, which promotes the protein hydrolysis, that in turn leads to a loss of freshness (V.M. Ocano-Higuera et al., 2011 & Y.A.A. Bernardo et al., 2020 & J. Freitas et al., 2021).
Knowing the evolution of various descriptor and properties associated with the spoilage process can be useful to evaluate the quality and freshness of the fish products and estimate the capability of them to maintain these characteristics during the time can be useful to understand the remaining shelf-life time (J. Freitas et al., 2021). When the fish is sold as a whole product there is minimal industrial processing, composed by washing and cold storage, usually in ice, anyway the improvement of these operations can inhibit bacterial growth, enzymatic action, and oxidation, allowing a more longer freshness retention, a high safety, and a shelf-life extension (J. Freitas et al., 2021).
Usually, a fish product goes through four stages during the *post-mortem* period: *rigor mortis*, in which the muscle become stiff, dissolution of *rigor*, when muscle becomes again flexible, autolysis, that is associated with the loss of freshness, and bacterial spoilage. Normally, methods for estimating the freshness of fish are applied in the autolysis stage; the speed

with which the product passes through these stages depends on the species, the physiological conditions of the fish, the microbial contamination, and the temperature (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020).

The principal *post-mortem* changes that affect the quality of the product are decrease of the pH, ATP and protein degradation, lipid oxidation, which are usually due to the activity of endogenous enzymes and production of undesirable compound such as trimethylamine (TMA) and molecular low weight volatile bases (TVB), which are usually produced by the microorganisms. Moreover, in the muscle occur modifications of texture, water holding capacity (WHC) and color (V.M. Ocano-Higuera et al., 2011). Some other modification that could take place in the fish products are belly-bursting, due to the gasses produced by digestive enzymes; mucus production, which color passes from transparent to opaque when in the product are growing microorganisms; discoloration, due to the oxidation of the pigments and melanosis, that occurs only in the crustaceans.

The definition of fresh related to the fish indicates a patterner of typical characteristics, as shown in Figure 3.5.1, on the other hand, a deteriorated product has opposite typical characteristics, as shown in Figure 3.5.2.

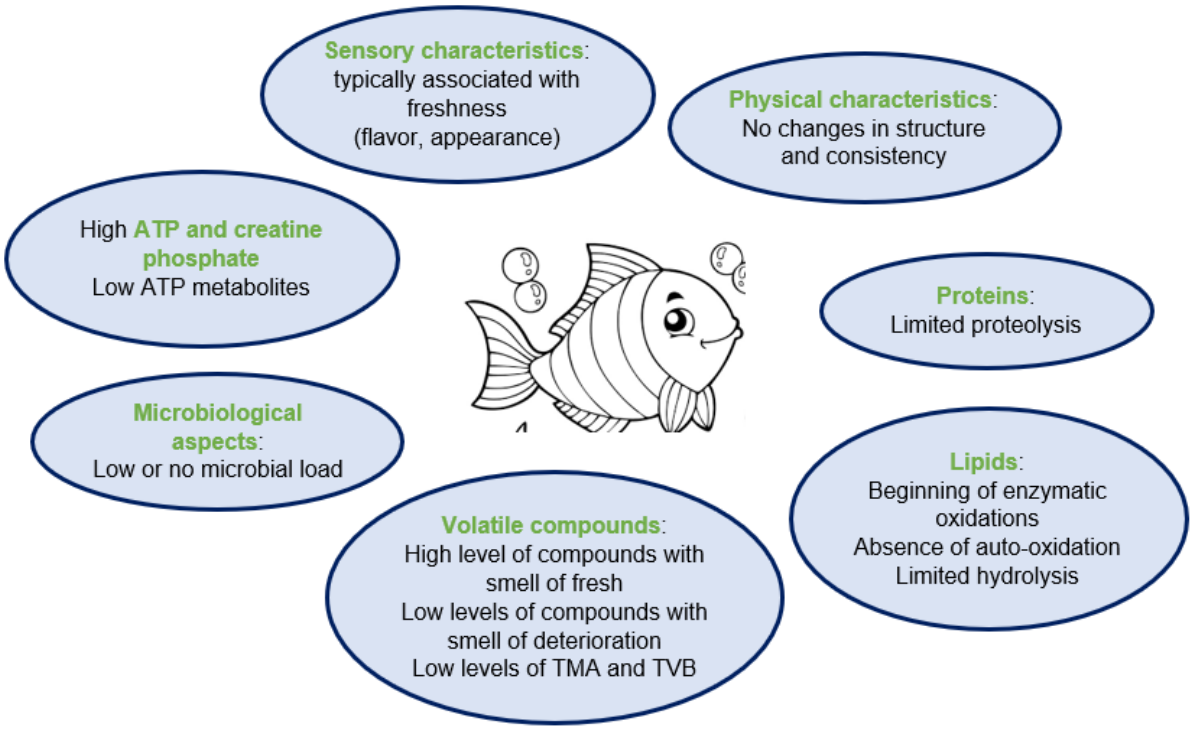

Figure 3.5.1: characteristics of fresh fish (Adapted from A. Ciampa, 2013).

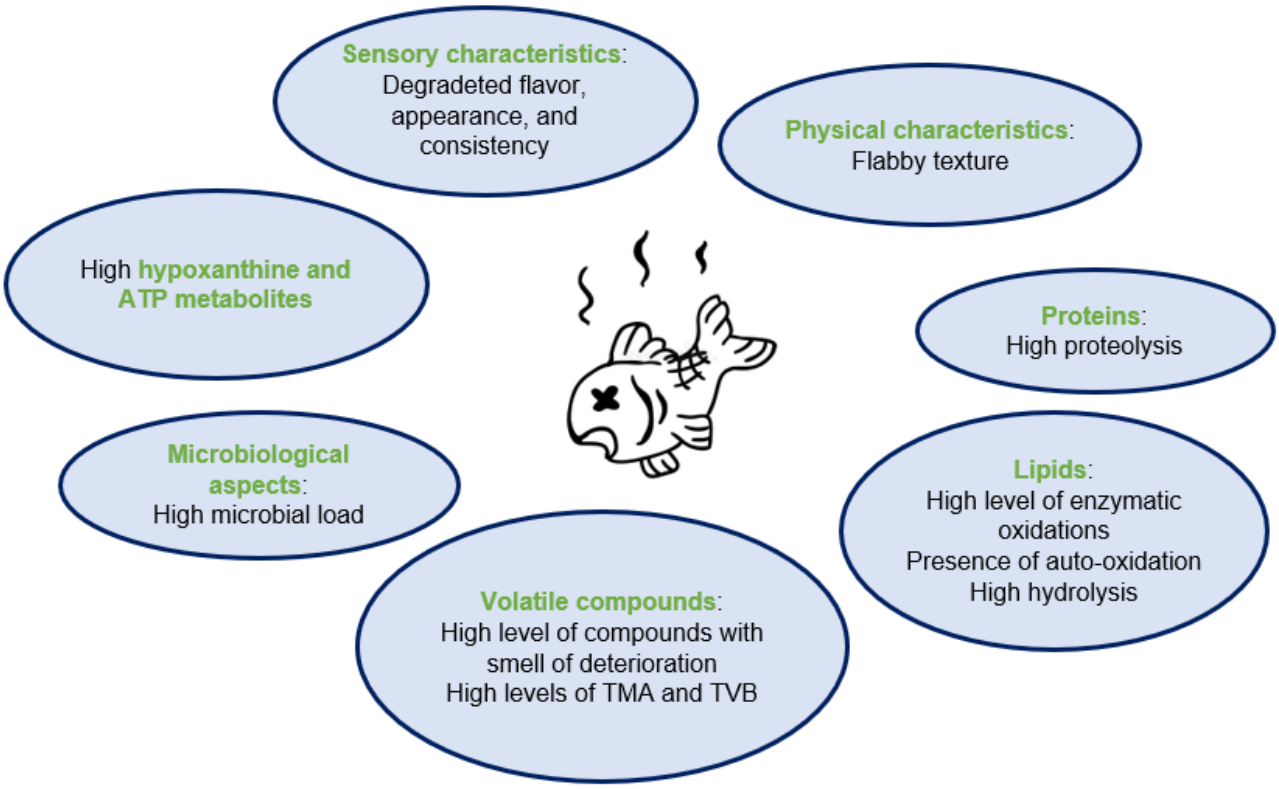

Figure 3.5.2: characteristics of degraded fish (Adapted from A. Ciampa, 2013).

3.5.2 The legislation

On December 13, 2014, the EU Regulation 1379/2013 entered in force which concerns the common organization of the markets in fishery and aquaculture (CMO); this Regulation is born from the necessity to inform the consumers and, at the same time, to safeguard the fisheries production and it is aimed at increasing the transparency of the market and the product recognition.

In the Regulation are clarified some definitions, as shown in Table 3.5.1.

	Definition
Fishery products	Aquatic organism resulting from any fishing activity or products derived therefrom
Aquaculture products	Aquatic organism at any stage of their life cycle resulting from any aquaculture activity or products derived therefrom
Aquaculture products	Any natural or legal person using means of production to obtain fishery or aquaculture products with a view to placing them on the market

Fishery and aquaculture sector	The sector of the economy which comprises all activities of production, processing and marketing of fishery or aquaculture products
Prepacked fishery and aquaculture products	Fishery and aquaculture products which are “prepacked food” as referred to point a) of Article 2(2) of Regulation (EC) No 1169/2011

Table 3.5.1: definitions from Reg. EU 1379/2013 (adapted from Reg. EU 1379/2013).

The fishery and aquaculture products covered by the Regulation are listened in the Table 3.5.2.

CN code	Description of the goods
0301	Live fish
0302	Fish, fresh or chilled, excluding fish fillets and other fish meat of heading 0304
0303	Fish, frozen, excluding fish fillets and other fish meat of heading 0304
0304	Fish fillets and other fish meat (whether or not minced), fresh, chilled or frozen
0305	Fish, dried, salted or in brine; smoked fish, whether or not cooked before or during the smoking process; flours, meals and pellets of fish, fit for human consumption
0306	Crustaceans, whether in shell or not, live, fresh, chilled, frozen, dried, salted or in brine; crustaceans, in shell, cooked by steaming or by boiling in water, whether or not chilled, frozen, dried, salted or in brine; flours, meals and pellets of crustaceans, fit for human consumption
0307	Molluscs, whether in shell or not, live, fresh, chilled, frozen, dried, salted or in brine; aquatic invertebrates other than crustaceans and molluscs, live, fresh, chilled, frozen, dried, salted or in brine; flours, meals and pellets of aquatic invertebrates other than crustaceans, fit for human consumption
1212 20 00	Seaweeds and other algae

Table 3.5.2: list of fish products to which it applies the labeling referred to Regulation (EC) n. 1379/2013 (Adapted from Reg. EU No 1379/2013).

According to the Reg. EU No 1379/2013 all these products can be sold to the final consumer or to a mass caterer only if appropriate making or labelling indicates:

a. the commercial designation of the species and its scientific name;
b. the production method, in particular by the following words “… caught …” or “… caught in freshwater …” or “… farmed …”;
c. the area where the product was caught or farmed, and the category of fishing gear used in capture of fisheries, as laid down in the first column of Annex III to this Regulation;
d. whether the product has been defrosted; this shall not apply to:

1. ingredients present in the final product;
2. foods for which freezing is a technologically necessary step in the production process;
3. fishery and aquaculture products previously frozen for health safety purposes, in accordance with Annex III, Section VIII, of Regulation (EC) No 853/2004;
4. fishery and aquaculture products which have been defrosted before the process of smoking, salting, cooking, pickling, drying or a combination of any of those processes.

e. the date of minimum durability, where appropriate.

Commercial designation: every Member State has a list of the commercial designations accepted in its territory, with indicate even the scientific names of the species, in Italy is actually in effect the Decree of the Ministry of Agriculture and Forestry of 13 January 2008.

Area where the product was caught or farmed: for products caught in the sea is necessary to indicate one of the zones as defined by FAO (see Figure 3.5.3); for products caught in freshwaters is necessary to indicate the Member State or third country of provenance of the product; for aquaculture products is necessary to indicate the Member State or third country in which the product has had the last stages of development, until reaching the market size (Reg. EU No 1379/2013).

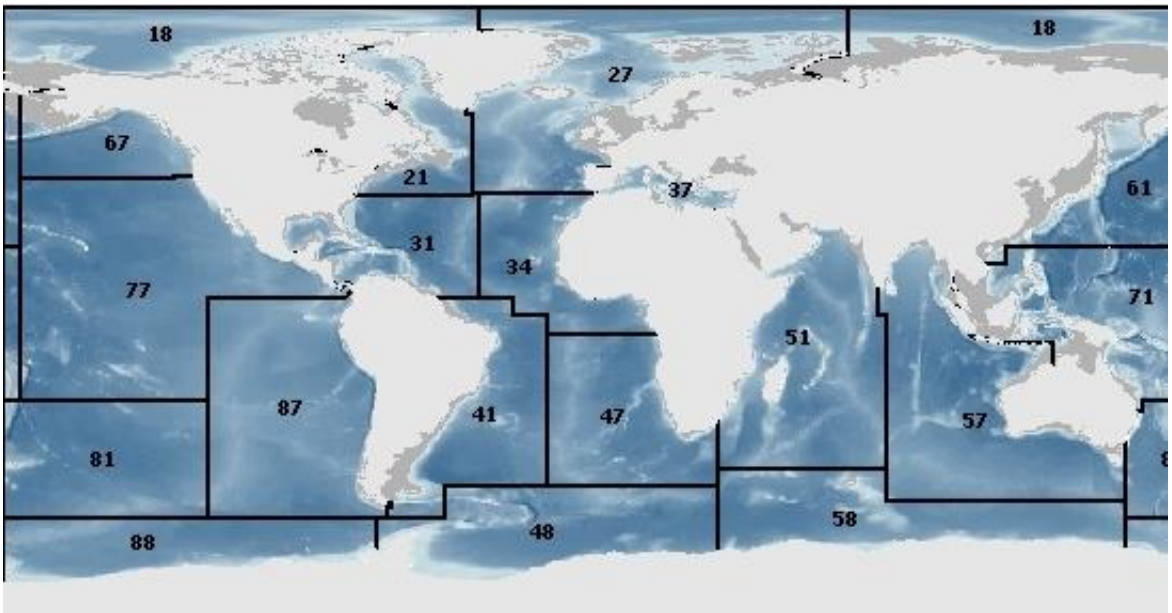

Figure 3.5.3: list of FAO catch areas Areas: 18 (Artic Sea), 21 (Atlantic, Northwest), 27 (Atlantic, Northeast), 31 (Atlantic, Western Central), 34 (Atlantic, Eastern Central), 37 (Mediterranean and Black Sea), 41 (Atlantic, Southwest), 47 (Atlantic, Southeast), 48 (Atlantic, Antarctic), 51 (Indian Ocean, Western), 57 (Indian Ocean, Eastern), 58 (Indian Ocean, Antarctic and Southern), 61 (Pacific, Northwest), 67 (Pacific, Northeast), 71 (Pacific, Wester Central), 77 (Pacific, Eastern Central), 81 (Pacific, Southwest), 87 (Pacific, Southeast), 88 (Pacific, Antarctic) (Source: http://www.fao.org/fishery/area/search/en).

Category of fishing gear used in capture of fisheries: as laid down in the first column of Annex III to this Regulation (see Figure 3.5.4)

Additional voluntary information: according to the Regulation No 1379/2013 can be included only if they can be verified and they are:

a. the date of catch products or the date of harvest of aquaculture products;
b. the date of landing of fishery products or information on the port at which the products were landed;
c. more detailed information on the type of fishing gear, as listened in the second column of Annex III (see Figure 3.5.4);
d. in the case of fishery products caught at sea, details of the flag State of the vessel that caught those products;
e. environmental information;
f. information of an ethical or social nature;
g. information on production techniques and practices;
h. information on the nutritional content of the product.

ANNEX III

INFORMATION ON FISHING GEAR

Mandatory information on the category of fishing gear	More detailed information on corresponding gears and codes, in accordance with Commission Regulation (EC) No 26/2004 (1) and Commission Implementing Regulation (EU) No 404/2011 (2)	
Seines	Beach seines	SB
	Danish seines	SDN
	Scottish seines	SSC
	Pair seines	SPR
Trawls	Beam trawls	TBB
	Bottom otter trawls	OTB
	Bottom pair trawls	PTB
	Midwater otter trawls	OTM
	Pelagic pair trawls	PTM
	Otter twin trawls	OTT
Gillnets and similar nets	Set (anchored) gillnets	GNS
	Driftnets	GND
	Encircling gillnets	GNC
	Trammel nets	GTR
	Combined trammel and gillnets	GTN
Surrounding nets and lift nets	Purse seines	PS
	Lampara nets	LA
	Boat operated lift nets	LNB
	Shore-operated stationary lift nets	LNS
Hooks and lines	Hand lines and pole lines (hand operated)	LHP
	Hand lines and pole lines (mechanised)	LHM
	Set longlines	LLS
	Longlines (drifting)	LLD
	Troll lines	LTL
Dredges	Boat dredges	DRB
	Hand dredges used on board a vessel	DRH
	Mechanised dredges including suction dredges	HMD
Pots and traps	Pots (traps)	FPO

(1) Commission Regulation (EC) No 26/2004 of 30 December 2003 on the Community fishing fleet register (OJ L 5, 9.1.2004, p. 25).
(2) Commission Implementing Regulation (EU) No 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy (OJ L 112, 30.4.2011, p. 1).

Figure 3.5.4: Annex III of Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 (Source: Regulation EU No 1379/2013).

In addition to labelling today is always more important to understand the history of the products, and this can be done through traceability, so the key global food chains, including seafood, are under increasing pressure to implement basic traceability systems in response to consumer concerns about food supply safety (J. Qian et al., 2020).

According to the Regulation (EC) No 178/2002, entered into force since 1st January 2005, traceability is defined as "*the ability to trace and follow a food, feed, food-producing animal or substance intended to be, or expected to be incorporate into a food or feed, through all stages of production, processing and distribution*" (Reg. EC No 178/2002). However, there are some different definitions of traceability, as the one gives by ISO-22005 or the one gives

by Codex Alimentarius, which is slightly different from the definition of the European Regulation, but the basic concept is the same; more in general traceability can be seen as a way to ensure food safety (J. Qian et al., 2020).

We can say that traceability systems have undergone an evolution during the years, due to the change of the requirements and the development of technologies, as schematized in Figure 3.5.5 (J. Qian et al., 2020).

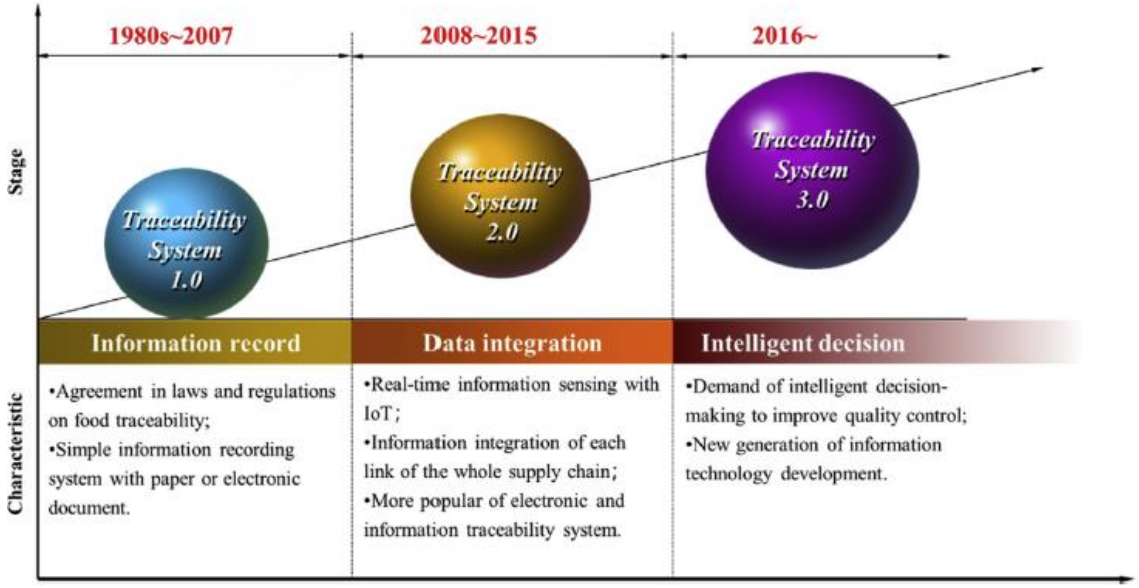

Figure 3.5.5: three development stages of traceability systems (J. Qian et al., 2020).

According to the Regulation (EC) No 178/2002 traceability is compulsory for all the food and feed operators in European Union and when all operators apply traceability the result is a fully traceable product.

According to a commonly accepted view traceability can be divided into tracking and tracing, where the first is the provision of information downstream, so during the movement of the product from the primary production to the consumer, instead the second is the provision of information upstream, so from consumer to primary production, in other words it is the possibility to identify the origin of a food product going backward to all the production stages (F. Schwagele, 2005).

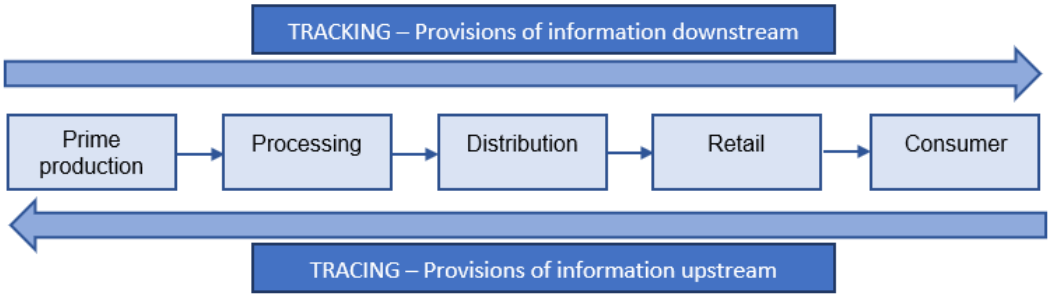

Figure 3.5.6: Traceability composed by tracking and tracing (adapted by F. Schwagele, 2005).

Through the traceability system is possible to locate commercial frauds, that are frequent and difficult to identify in the seafood sector, the most common types of them are the sailing of:

- a species different from the one declared on the label, usually of less value;
- thawed fish as fresh;
- fish that must be eaten uncooked non subjected to preventive freezing;
- framed fish as caught.

3.5.3 Method to assess the freshness and quality

For the seafood products the quality is strictly related to the freshness, that can be evaluated with a wide range of techniques, the most important categorizations of these techniques is the division into sensory and objective methods, where the second ones include chemical and biochemical, physical, microbiological (J. Freitas et al., 2021 & V.M. Ocano-Higuera et al., 2011), because the principal modifications that occurs in the product during the storage are of these types (P.K. Prabhakar et al., 2020), and novel, mainly spectroscopic, methods (J. Freitas et al., 2021 & V.M. Ocano-Higuera et al., 2011).

Generally, only a single index cannot describe the complexity of the changes that occurs during the spoilage, but they can complement to each other and lead to an acceptable estimation; it is important to keep in mind that every chemical or instrumental analysis must agree with sensory evaluation, because this is the type of evaluation that the consumer uses when he buys fresh fish (J. Freitas et al., 2021).

3.5.4 Sensorial methods

Usually, the seafoods' freshness is evaluated with empirical methods, which has been tried to standardize during the years, whit specific methods; sensory science is described as a "*quantitative discipline that uses the human senses for interpretation, measurement, and analysis of different environmental, physiological, processing, or conservation factors on food product characteristics*", so the procedures for sensory evaluation must be very well defined to make these assessments effective (J. Freitas et al., 2021).

The modifications that occur during the conservation has a high impact on the sensory attributes, which can be used to empirically evaluate the quality and/or freshness of the product; usually are evaluated: smell, eyes, gills, skin, and consistency.

Smell: is one of the most important parameters in the evaluation of the fish freshness (I. Fernandez-Segovia et al., 2008), it is due to a series of chemical compounds derived from

enzymatic and/or bacterial degradation of proteins, lipids, and other catabolites which leads to the formation of volatile fatty acids, ammonia, and so on; the intensity and peculiarity of these reactions can be related to a certain degree of freshness.

The olfactory examination is made usually on gills and abdominal cavity; the typical smell of a fresh product is of sea, instead the smell of degraded product is of acrid, rancid and ammoniacal.

Eyes: in a degraded fish the eye has lost convexity, brilliance of the pupil, and transparency of the cornea; in any case the alteration of the eye is due to a several factors and so the evaluation must be made for both the eyes for greater reliability.

Gills: in a fresh fish the color of the gills is bright red, but it tends to fade quickly during the storage. As for the eyes, the color of the gills can be due even to other factors and it can be artificially modified, so, for a greater reliability of the evaluation, are assessed even the presence of gill mucus and the smell.

Skin: usually is evaluated the integrity of the flakes, because in an altered fish the flakes tend to come off. Another important indicator is the appearance of the cutaneous mucus because it dries out over time and its appearance because it becomes from transparent to opaque when the microbial growth starts. Anyhow, as in eyes and gills, the presence of cutaneous mucus can be due even to other factors, such as bacterial pathologies or irritations due to some substances present in the water, so it is not an exhaustive index.

Consistency: is usually evaluated on the entire fish and not on the fillet; the consistency is strictly related to the evolution of the *rigor mortis* and the presence of the rigidity typically associated with them is considered as an index of a “ultra-fresh” fish.

The evaluations seen so far are completely empirical, so, in order to standardize the sensory evaluation, the European Commission has drawn up the Regulation 2406/96, in which are established some table for the assessment of the seafoods freshness. In this Regulation there are different tables for some categories of seafood products, in particular the species are divided in whitefish, bluefish, selachii, cephalopods, and crustaceans. The levels of freshness are “E” (extra), “A” (good quality), “B” (acceptable for consumption), and “C” (unfit for consumption) (J. Freitas et al., 2021), as an example in Table 3.5.3 and 3.5.4 are reported respectively the tables for the evaluation of white fishes and bluefishes.

	Criteria			
	Freshness category			Not admitted
	E	A	B	
Skin	Bright, iridescent (save for redfish) or opalescent; no discoloration	Pigmentation bright nut not lustrous	Pigmentation in the process of becoming discoloured and dull	Dull pigmentation
Skin mucus	Aqueous, transparent	Slightly cloudy	Milky	Yellowish grey, opaque mucus
Eye	Convex (bulging); black, bright pupil; transparent cornea	Convex and sightly sunken; black dull pupil; slightly opalescent cornea	Flat; opalescent cornea; opaque pupil	Concave in the center; grey pupil; milky cornea
Gills	Bright colour; no mucus	Less coloured; transparent mucus	Brown/grey becoming discoloured; thick, opaque mucus	Yellowish; milky mucus
Peritoneum (in gutted fish)	Smooth; bright; difficult to detach from flesh	Slightly dull; can be detached from flesh	Speckled; comes away easily from flesh	Does not stick
Smell of gills and abdominal cavity - **Whitefish other than plaice** - **Plaice**	 Seaweddy Fresh oily; peppery; earthy smell	 No smell of seaweed; neutral smell Oily; seaweddy or slightly sweetish	 Fermented; slightly sour Oily; fermented; stale; slightly rancid	 Sour Sour
Flesh extra criteria for headed anglerfish				
Blood vessels (ventral muscles)	Firm and elastic; smooth surface	Less elastic	Slightly soft (flaccid), less elastic; waxy (velvety) and dull surface	Soft (flaccid); scales easily detached from skin, surface rather wrinkled

Table 3.5.3: evaluation parameters for white fishes (Adapted from: European Commission Reg. 2406/96).

	Criteria			
	Freshness category			Not admitted
	Extra	A	B	
Skin	Bright pigmentation, bright, shining iridescent colours; clear distinction between dorsal and central surfaces	Loss of lustre and shine; duller colours; less difference between dorsal and ventral surfaces	Dull, lustreless, insipid colours; skin creased when fish curved	Very dull pigmentation; skin coming away from flesh
Skin mucus	Aqueous, transparent	Slightly cloudy	Milky	Yellowish grey, opaque mucus
Consistency of flesh	Very firm, rigid	Fairly rigid, firm	Slightly soft	Soft (flaccid)
Gills covers	Silvery	Silvery, slightly red or brown	Brownish and extensive seepage of blood from vessels	Yellowish
Eye	Convex, bulging; blue-black, bright pupil; transparent 'eyelid'	Convex and sightly sunken; dark pupil; slightly opalescent cornea	Flat; blurred pupil; blood seepage around the eye	Concave in the center; grey pupil; milky cornea
Gills	Uniformly dark red to purple. No mucus	Less bright colour, paler at edges. Transparent mucus	Becoming thick discoloured opaque mucus	Yellowish; milky mucus
Smell of gills	Fresh seaweed; pungent; iodine	No smell or seaweed. Neutral smell	Slightly sulphureous fatty smell, rancid bacon cuttings or rotten fruit	Rotten sour

Table 3.5.4: evaluation parameters for bluefishes (Adapted from: European Commission Reg. 2406/96).

However, the application of this evaluation presents some critical issues, in particular the procedures are not defined uniquely, so the evaluation is low reliable and reproducible, because it is difficult to applicate that in standardized way. Moreover, the parameters are not specific enough, their description is complex to understood and the method does not give information about the residual shelf-life of the product; for these reasons this scheme normally is not used in Quality Assurance control at industries level (J. Freitas et al., 2021).

From the necessity of standardize and simplify the sensory evaluation is born the Quality Index Method (QIM), which tries to objectify the evaluation of some characteristics and overcomes some limitations of the EU scheme. This method provides a table for each species and are now available 34 tables, in these ones are indicated a high number of attributes that must be evaluated (usually 10 – 15). This method is more flexible compared to the European scheme because the index of the quality, called Quality Index (QI) is calculated as a sum of the scores assigned to each single parameter, this scores are called

attributes demerit points and are usually from 0 to 3; the more the total score is near to zero, the fresher is the fish, indeed this value increases linearly with the storage time of the fish, this direct proportionality makes simple to calculate the remaining shelf-life of the product (J. Freitas et al., 2021 & Y.A.A. Bernardo et al., 2020), through the use of calibration curves.

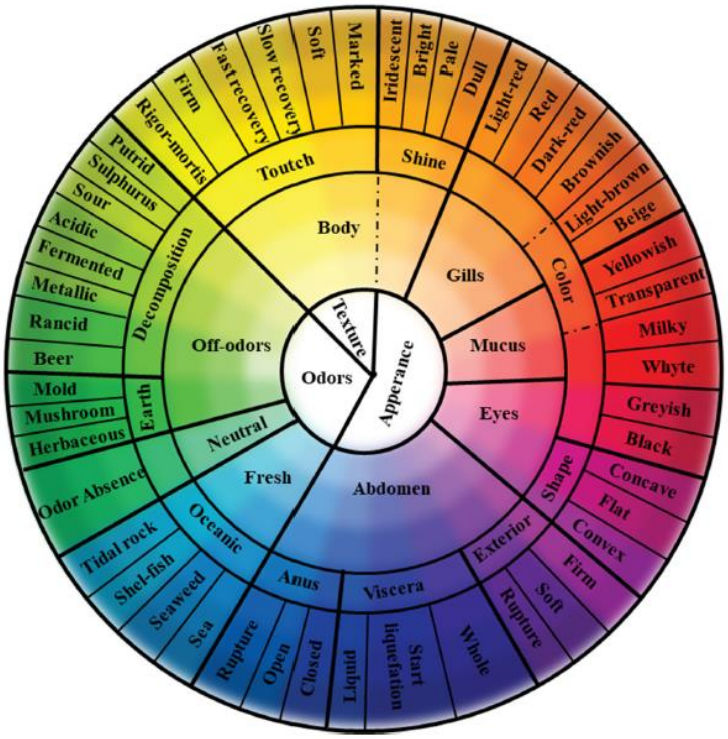

Figure 3.5.7: QIM sensory wheel, with the most common attributes evaluated and terminology used (J. Freitas et al., 2021).

The development of quality index occurs in three stages, two of training for assessors and the last one of validation, QIM provides a linear relationship between freshness and time of storage, so minimum differences attributed by assessors does not influence the result (Y.A.A. Bernardo et al., 2020). These characteristics make this method more adaptable than the European scheme, once it is developed and validated and its application is fast, non-destructive, species-specific and allows direct measurements of attributes and estimation of the shelf-life; anyway this method has also some weaknesses, such as the doubts about if is really necessary to evaluate a so large number of attributes, the impact of variability of the samples and the possible assessors' bias (J. Freitas et al., 2021), in addition to the fact that the score assigned by the assessor depends to how much they are trained and to the sensory method applied; some other factors which influence the final result are selection of parameters, sampling size, species specificity, storage conditions, experience of assessors, and bacteriological and chemical validation of the results (Y.A.A. Bernardo et al., 2020).

Selection of parameters which would compose the protocol is fundamental as if in the development of a QI protocol the parameters considered are different it will be unlike even the results obtained (Y.A.A. Bernardo et al., 2020).

Sampling size can influence the results obtained, because the procedure requires a large number of samples and a flexible and interactive experimental design (Y.A.A. Bernardo et al., 2020).

This method is also **species specific**, as the metabolic and microbial activities which cause the sensory changes occur in different way in different species of fishes, for this reason the procedure must be developed for every single species and it can be applied only for that one; this implicate that is necessary to develop a procedure for each single species, making the application of the method time consuming and costly (Y.A.A. Bernardo et al., 2020).

Storage conditions are fundamental as with the same period elapsed from the time of fishing the residual shelf-life of a product depends on the storage temperature and conditions, for example modified atmosphere packaging influences the sped of the reactions which leads to a loss of freshness in the product (Y.A.A. Bernardo et al., 2020).

Experience of assessors is relevant because one primary source of bias in this analysis is the incorrect construction of the panel group, indeed incorrect or insufficient training and low number of assessors may be responsible for the variation of the results obtained (Y.A.A. Bernardo et al., 2020).

Bacteriological and chemical validation of the results is necessary to ensure the reliability of the method; for chemical validation can be used K-value, TVB and TBA analysis as proposed by Y.A.A. Bernardo *et al*. (2020), which are three of the some common chemical analysis used for evaluate the fish freshness as discussed below; for bacteriological validation there is no agreement between authors about which bacteria should be monitored for fish quality assessment, because the specific spoilage microorganisms are different in different fishes, anyway even this aspect is discussed in a more detailed way below (Y.A.A. Bernardo et al., 2020).

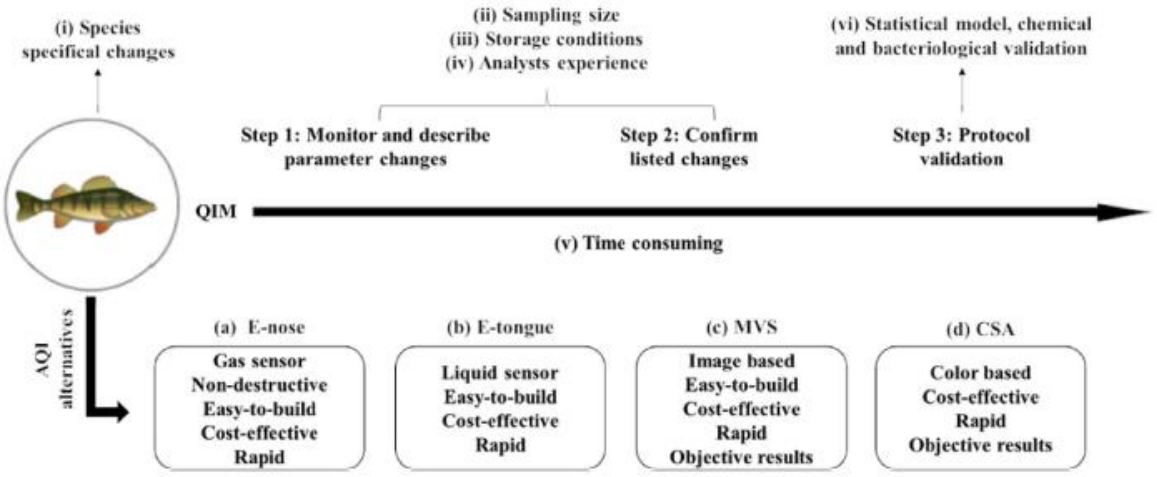

Figure 3.5.8: development of QIM in three stages and factors which influence the final result (Y.A.A. Bernardo et al., 2020).

The quality index can be evaluated even as “Artificial Quality Index” (AQI), in which the assessment of color and odor is made with artificial instruments, which are recently been classified as Sensory Bionic Technologies (SBT), discussed more in detail below (Y.A.A. Bernardo et al., 2020).

3.5.5 Analytical methods

In the several years there has been more and more interest in the development of reliable methods to assess the freshness of fish and to evaluate indexes of quality and spoilage (I. Fernandez-Segovia et al., 2008), even if usually, these methods require more time and are more expensive compared with the sensorial methods; as discussed previously these techniques can be divided into chemical and biochemical, physical, microbiological and novel methods.

3.5.5.1 Chemical and biochemical methods

These methods are based on the measurement of some parameters, such as: pH, ATP and its catabolites, TMA and TVB, presence of biogenic ammines, free fatty acids and thiobarbituric acid values and volatile compounds.

pH: it can be measured, but it is few indicative because the initial pH value depends to species, season, diet, level of activity, stress during catch and type of muscle (V.M. Ocano-Higuera et al., 2011); while the final pH depends more from what is happened before the death, than from the time elapsed since death; moreover, the range of variability is from 6.4

to 6.1 and it is difficult to make precise measures, especially when is considered a whole fish.

ATP and its catabolites: during processing and storage of seafood products adenosine triphosphate (ATP) is degraded in order into adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR), and hypoxanthine (Hx); until the formation of IMP the transformations occur by endogenous enzymes, while bacterial enzymes are responsible for the formation of HxR and Hx; the speed of the transformations depends on the species and type of muscle considered, due to the variability in the enzyme activity and in the initial nucleotide concentration (V.M. Ocano-Higuera et al., 2011 & I. Fernandez-Segovia et al., 2008).

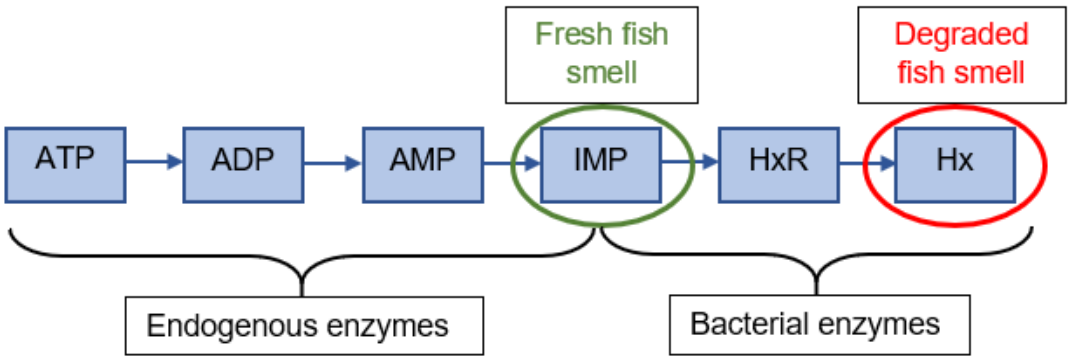

Figure 3.5.9: degradation of ATP

The relationship between these compounds is expressed by the K-value, also called K index, which is used to measure how fast these compounds degrade and which, in a wide range of fishes, correlates well with the loss of freshness and the storing time (V.M. Ocano-Higuera et al., 2011), this is normally used as an index of freshness before the bacterial spoilage begins (I. Fernandez-Segovia et al., 2008); the K-values is calculated as:

$$K\ (\%) = \frac{[HxR]+[Hx]}{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]} * 100$$

Considering all these compounds, instead of only one, has the advantage to reduce the differences among and within species, and so it increases the utility of this determination (V.M. Ocano-Higuera et al., 2011 & I. Fernandez-Segovia et al., 2008).

Usually is consider that a K-value less than 20 % indicates a fish of high quality, a K-value less than 50 % indicates a fish of acceptable quality, and a K-value over than 70 % indicates a fish unsuitable for the consumption (T. Saito et al., 1959); nevertheless, the maximum

values are often reached before the product is sensorially unacceptable, indeed some high quality processed seafoods can have a K-value even greater than 20 %. This parameter is considered one of the most significant in representing the freshness of fish but is necessary to consider that it is not so significant in some tropical fish species, for which are necessary some corrections, and it is dependent from temperature and storage duration (P.K. Prabhakar et al., 2020).

For simplifying the calculation of the index has been created even a simplified form:

$$\frac{[\mathrm{HxR}] + [\mathrm{Hx}]}{[\mathrm{IMP}] + [\mathrm{HxR}] + [\mathrm{Hx}]}$$

This can be used because ATP, ADP and AMP are rapidly degraded after the death by endogenous enzymes, so these compounds can only be found at relatively high level after killing and in fishes with minimal struggle before the death (I. Fernandez-Segovia et al., 2008).

Anyhow, both the forms of K-value are difficult to calculated and for this reason that index is normally used only for high value products or for research works.

TMA and TVB: traditionally are used as indicators of quality in fish products stored in ice (V.M. Ocano-Higuera et al., 2011), but it can be applied even for some other types of products.

The total volatile basic compounds (TVB-N or TVB) are volatile compounds that contain nitrogen, including trimethylamine (TMA), dimethylamine (DMA), ammonia (NH_3) and some other compounds, they develop during the storage and give smell of spoiled fish; usually the TVB value increase curvilinearly or linearly during the time, and in the majority of the fish products is considered that the maximum acceptable value is 30 – 35 mg muscle TVB-N/100 g, over that the fish is regarded spoiled and unfit for the human consumption. However, not always this cut-off value corresponds to a spoiled product, also because the TVB value depends on the species (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020); the European Commission has also fixed TVB limits for some species from 25 to 35 mg muscle TVB-N/100 g, with the Commission decision No 95/149. Usually, this is the preferred parameter for estimating the degree of decomposition of the fish muscle, but it indicates the later stages of spoilage and is considered less reliable for the measurement of the spoilage during the first steps, moreover, even the method used for the determination of these compounds can affect the results (P.K. Prabhakar et al., 2020).

Trimethylamine (TMA-N or TMA) is a biogenic amine, and it is formed from trimethylamine N-oxide (TMAO), which acts in the fish body as an osmoregulator. TMA if formed from TMAO through the microbial action or by an endogenous enzyme, but this last pathway of formation occurs only at temperatures lower than -18°C; usually the concentration of TMA in fish is used as an index of quality and as indicator of bacterial activity, but is necessary to say that this compound is product only by some species of microorganisms, present in a wide range of marine fishes but not in all, so in some species the correlation between the concentration of TMA and the bacterial contamination is not good because are not present these species of microorganisms (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020). This compound gives a pungent odor typical of spoiled fish and the rejection limit can variate from 5 – 10 mg to even 30 mg TMA-N/100 g muscle, depending on the storage conditions (aerobic or anaerobic); anyway, the presence of this compound in the fish depend even to species, season, location of catch, stage of spoilage, type of processing and storage and also from the method of analysis (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020).

Presence of other biogenic amines: these compounds are product mainly by the microorganisms that develop in the product during the storage, moreover, the seafood products contain a high level of free amino acids, so they are very susceptible to the development of biogenic amines. The principal biogenic amines that can be present in fishes are histamine, originated by histidine and mainly present in the fishes of the scombroid family; cadaverine, created by lysine and present in almost all fish species; putrescine, developed by ornithine and more rarely present in fishes; and tyramine, originated by tyrosine (P.K. Prabhakar et al., 2020). Based on the detection of these compounds can be calculated some indexes, such as chemical index, proposed by J.L. Mietz & E. Karmas (1977).

$$\text{Chemical index} = \frac{\text{histamine} + \text{putrescine} + \text{cadaverine}}{1 + \text{spermidine} + \text{spermine}}$$

Where spermidine and spermine naturally occurs in the product (P.K. Prabhakar et al., 2020).

Another index that can be used is the Amine Index (AI), proposed by G. Duflos, *et al*. (1999).

$$\text{AI} = \frac{\text{histamine} + \text{putrescine} + \text{cadaverine}}{\text{histamine} + \text{putrescine} + \text{cadaverine} + \text{tyramine} + \text{tryptamine} + \text{methylamine} + \text{spermidine} + \text{spermine}}$$

Or can be calculated even the Biogenic Amine Index (BAI), as:

$$\text{BAI} = \text{Histamine} + \text{putrescine} + \text{cadaverine} + \text{tyramine}$$

The determination of biogenic amines helps to understand the toxicity mechanism and the time of spoilage in a wide range of fish species, but them presence depends also to the species; another problem is that in the first stages of spoilage some of these compounds are still not present, so these indexes are more useful for the last stages of storage than for the first ones (P.K. Prabhakar et al., 2020).
The Commission Regulation (EC) No 2073/2005 of November 15 2005 defines limits of histamine presence in some fish products, in "*fishery products from fish species associated with a high amount of histidine*" it has been established that is necessary to test nine sample units, of which two could have a value of histamine between 100 and 200 mg/kg while the remaining sample units must have an histamine concentration under 100 mg/kg; in "*fishery products which have undergone enzyme maturation treatment in brine, manufactured from fish species associated with a high amount of histidine*" the concept is the same, but the limits are 200 and 400 mg/kg.

Free fatty acids (FFA) and thiobarbituric acid value (TBA): these compounds are product by the lipid oxidation, in which the lipids are broken in simpler compounds, such as these ones, so when they concentration increases the quality of the product decreases; the FFA value is measured in milliequivalents (mEq) peroxide per kilogram of lipid or as percentage of oleic acid equivalent; instead TBA is expressed in milligram of malonaldehyde per kilogram of lipid and it is measured as the absorbance at a fixed wavelength (P.K. Prabhakar et al., 2020).

Volatile compounds: it can be measured with techniques such as gas chromatography, eventually combined with mass spectrometry, or with electronic noses. The aroma is one of the most important indices of the fish freshness, as the modifications that determines a loss of quality usually produces unpleasant odor and aroma, so the measurement of volatile compounds can be used to evaluate the freshness and spoilage of the fish (I. Fernandez-Segovia et al., 2008).

3.5.5.2 Physical methods

These methods are based on the measure of some physical index, such as: stiffness index, contraction index, texture, dielectric properties and color.
Stiffness index: calculated with methods based on how much a fish bends when it is only partially leaning on a surface.

Contraction index: it depends to how much the muscle fibers contract during the *rigor mortis*.

Texture: analysed with some instruments such as a Texture Analyser; normally the fish product losses firmness during the storage time, due principally to the enzymatic degradation of muscle proteins, several researches have also associated the low muscle pH with tough texture and high drip loss (V.M. Ocano-Higuera et al., 2011), the modification of the texture has a direct impact on the fish freshness (P.K. Prabhakar et al., 2020).

Dielectric proprieties: are affected by injury, death, and decay, so they can be useful to understand some modification that occur in the product and to measure the quality of the product, especially when it is applied whit multivariate statistical analysis; generally, we can say that this method is versatile in the prediction of different variables associated with the loss of quality in fish (M. Kent et al., 2004).

Color: it can be evaluated even with a colorimeter, in order to standardize the empirical evaluation of this aspect. Usually, the color changes during the storage, affect the quality and the acceptance by the consumers (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020).

3.5.5.3 Microbiological methods

Usually, are carried out the total viable count (TVC) (P.K. Prabhakar et al., 2020) and the count of the specific spoilage organisms, called SSOs.

The fish microflora is composed by *Pseudomonas*, *Shewanella*, *Alcaligenes*, *Vibrionaceae*, *Serratia*, *Micrococcus* (P.K. Prabhakar et al., 2020), but even lactic acid bacteria (LAB), hydrogen sulfide producing bacteria (HSPB), *Brochothrix termosphacta*, *Photobacterium* spp. (Y.A.A. Bernardo et al., 2020), and *Listeria monocytogenes* (E. Carrasco et al., 2007); the majority of these microorganisms can produce the compounds associated with the fish spoilage, such as alcohols, biogenic amines, organic acids, amines, sulphides, aldehydes, and ketones (P.K. Prabhakar et al., 2020).

Frequently the microbial presence in the product is indirectly determined with the measure of TMA as discussed above, because a lot of microorganisms which are normally present in the fish can convert TMAO in TMA to obtain energy (for example *Aeromonas* ssp., *P. phosphoreum*, *Enterobacteriaceae* and *Vibrio* ssp.) (P.K. Prabhakar et al., 2020).

3.5.5.4 Novel methods

Are more innovative techniques that are very interesting for some aspects, the principal techniques of this category are biosensor techniques, sensory bionics technology and spectroscopic techniques (P.K. Prabhakar et al., 2020).

Biosensor techniques: are based on the detection of biological elements thanks to a biological recognizer, the information is then traduced by a transducer into a signal, that can be of three main types: electrical, thermal, or optical; the principal types of biosensors are electrochemical and enzyme (P.K. Prabhakar et al., 2020).

In electrochemical biosensors there are electrodes which are able to detect the presence of some chemical compounds that are formed during the fish deterioration, such as gasses like NH_3, TMA and DMA, in this case the detection is due to the ability of the electrodes to have redox reactions with the compounds detected; while the enzyme biosensors are able to determine the fish freshness thanks to the detection of the metabolites generated from the degradation of ATP, in literature can be found some examples of the application of these type of biosensors for the determination of the fish freshness (P.K. Prabhakar et al., 2020).

Sensory Bionics Technology (SBT): are composed of a sensor array which includes sensors able to detect different substances, some examples of this techniques are electronic nose or tongue, colorimetric sensor array and computer vision, which all can represent how the product is perceived by sensory organs of the humans (P.K. Prabhakar et al., 2020 & Y.A.A. Bernardo et al., 2020).

Electronic nose: our sense of smell gets tired easily and the use of human olfaction for the evaluation can generate errors, for these reasons are being developed electronic noses which allow a detection rapid, non-destructive, sensitive, cost-effective and easy-to-build; these instruments are composed by an array of heterogenous electrochemical gas sensors which can detect different gases and have pattern recognition systems (Y.A.A. Bernardo et al., 2020).

Electronic tongue: the application principle is the same as electronic noses, but in these is used liquid sensor array instead of a gas one (Y.A.A. Bernardo et al., 2020).

Colorimetric sensor array (CSA): also called optoelectric sensor, is an olfaction-visualization technology based on color changes in the sensors due to chemical bonds between chromogenic agents and gases (Y.A.A. Bernardo et al., 2020).

Computer vision: is mainly used to analyse the color of the samples, which is one of the most important food quality parameters because it affects the willingness to purchase of the consumer; it is also called Machine Vision System (MVS) or Computer Vision System (CVS), it is able to analyse digital images in order to obtain some information and it can be used to simulate the human eye perception (Y.A.A. Bernardo et al., 2020). This technique is interesting in fishes as they are susceptible to color changes related with the loss of freshness and caused mainly by the formation of metmyoglobin.
The principal problem of all the SBT is the fact that they detect chemical and microbiological changes that occur in the late stages of storage, so they say more about the state of spoilage than the state of freshness; moreover, they are novel technologies, so they are still affected by structural and methodological problems, which require more research to be overcome (Y.A.A. Bernardo et al., 2020).

Spectroscopic techniques: the interaction of electromagnetic radiations and food components is used to analyse food quality, this can be done thanks to the individuation of models that correlate the spectral fingerprint of the product, obtained by spectroscopy, with some parameters of freshness used in traditional techniques (P.K. Prabhakar et al., 2020).
Some techniques of this type are Vis/NIR spectroscopy, HIS (Hyperspectral imaging) techniques, fluorescence spectroscopy, Ramam spectroscopy and Nuclear Magnetic Resonance (NMR).
Vis/NIR spectroscopy: the production of some metabolites changes the Vis/NIR absorption spectrum and even the absorption intensity; comparing with the traditional techniques this one is more rapid and simple, and it is not destructive (P.K. Prabhakar et al., 2020).
HSI (Hyperspectral imaging) techniques: it provides a tridimensional dataset with spectral and spatial information, and it represents the characteristics of the samples along with their distribution; this type of analysis is rapid, non-destructive, and reliable for the evaluation of fish freshness (P.K. Prabhakar et al., 2020).
Fluorescence spectroscopy: it measures the fluorescence intensity as a function of emission wavelengths, actually there are several types of this spectroscopy, but the most common in the food science are Front Face Fluorescence Spectroscopy (FFFS), which is especially suitable for complex food matrices because it uses particular incidence angle that minimizes scattering, reflection and depolarization, and Fluorescence excitation-emission (EEM) or fluorescence landscapes; these analysis are rapid, non-destructive and relatively low cost compared to some other techniques that can be used (P.K. Prabhakar et al., 2020).

For example, FFFS coupled with chemometric tools has been used to monitor the structural changes in fish proteins and their physicochemical environment during storage and to differentiate between fresh and frozen-thawed fishes (R. Karoui et al., 2017).

Ramam spectroscopy: it has been used as a technique for the determination of food adulterations and authenticity and it measures fundamental vibrations in functional groups in the molecule providing detailed structural information in a non-destructive way. Moreover, if coupled with chemometric analysis, it can be even more useful (H.M. Velioglu et al., 2015), an example of application of Ramam spectroscopy for analysing fish is provided by H.M. Velioglu *et al*. (2015), which combines this technique with principal component analysis (PCA) in order to discriminate between fresh and frozen-thawed samples and between different species, thanks to the detection of differences in the fatty acids composition.

Nuclear Magnetic Resonance (NMR): it is very useful in the food quality evaluation because it allows the detection of a wide range of molecules and so it can be use, for example, to quantify ATP and its catabolites in order to calculate the K-value or to detect a wide range of other compounds which influence taste, consistency, quality and so on (E. Shumilina et al., 2015).

3.6 GILTHEAD SEA BREAM (*Sparus aurata*)

3.6.1 Morphology

Gilthead sea bream (*Sparus aurata*) is a fish belonging to the family *Sparidae* and to the genus *Sparus*. It has body with an oval shape, very high and laterally compressed and the head profile is convex with small eyes; the mouth has the mandible shorter than the maxilla. This fish color is silver-grey with a big dark spot at the beginning of the lateral line that also covers the upper part of the opercular bone, this fish can be easily recognized thanks to the characteristic golden frontal band between eyes. Furthermore, the dorsal fin is blue-grey with a median black line and the caudal fin is grey-greenish white with black tips (http://www.fao.org/).

3.6.2 Habitat and ecology

It inhabits seagrass beds and sandy bottoms as well as the surf zone, commonly to depths of about 30 m, but adults may occur at 150 m depth. It stands either solitary or in small aggregations and it is demersal fish, so is a sedentary fish. It is an euryhaline species and this means that it could live in a wide range of saline concentrations. Moreover, it is

hermaphrodite, so it is a functional male in the first two years and at over thirty centimeters in length becomes female (L. Sola et al., 2007).

Gilthead sea bream is mainly carnivorous, feeding on mollusks, particularly mussels, crustaceans, and fish, but it is accessorily herbivorous if it is necessary to survive (B. Russell et al., 2014).

It occurs naturally in the Mediterranean and rarely in the Black sea, and in the Eastern Atlantic, from the British Isles, Strait of Gibraltar to Capo Verde and around Canary Islands (L. Sola et al., 2007).

Figure 3.6.1: Distribution of Gilthead sea bream (L. Sola et al., 2007).

3.6.3 Breeding and fishing

Gilthead sea bream represents one of the most important fishes produced by aquaculture, with a global production around 158,839 tons; instead, the capture production is around 6,073 tons (http://www.fao.org/); it is a very important commercial species, and it is also one of the most important fishes used for aquaculture in the Mediterranean Sea, indeed most aquaculture production occurs in the Mediterranean, but there is also a lower production of this species in the Red Sea, the Persian Gulf and the Arabian Sea; instead, for what concerns the capture, it is fished most intensively from February to October and is caught with on line gear, trammel nets, bottom trawls, beach seines and traps. It is also an important sport fish, caught mostly by spearfishing (B. Russell et al., 2014).

The Gilthead sea bream has traditionally been cultured in Mediterranean costal lagoons and brackish/salt water ponds, especially in the northern Adriatic *valli* in Italy and the Egyptian *hosha*. These fishes are farmed extensively in lagoons or intensively in tanks or cages; most

production is from intensive farming, while extensive farming still remains a traditional activity in some regions, but with a very low impact on the market (L. Sola et al., 2007).
The breeding of gilthead sea bream is composed of various stages:

1. Reproduction: consists in the breeding of broodstock and it allows to obtain fertilized eggs.
2. Hatchery: consists in the breeding of larvae in tanks on the ground until obtaining fries.
3. Breeding: composed by a pre-fattening step in cages on the ground or in basins and a fattening step in floating cages.

Reproduction: on farms the majority of broodstock are kept for spawning for several years, but new males must be added every year, as they turn into females at about two years old, this implies that from 5 to 20 % per year renewal occurs, when possible, for that purpose are preferred wild fishes (L. Sola et al., 2007). One female can produce up to one million eggs and the normal fertilization ratio is 90 – 95 %. Fertility and egg quality are strictly related to a calm environment and a balanced diet. Spawning generally occurs from October to December and so once hormones were used to induce spawning, but now that are been replaced by modulation of environmental conditions, in particular temperature and photoperiod, that enabled to extent the spawning season to all year round (L. Sola et al., 2007). Incubation of the eggs lasts about two days at 16 – 17 °C (B. Russell et al., 2014).
Hatchery: larvae are breeding in cylindroconical tanks 3 to 6 m in diameter, at a stocking density of 100 to 250 individuals per liter. Larvae are fed with live food for the first period and then are weaned with increasing concentrations of artificial feed, until the complete replacement of live food. During fry production is essential to control environmental parameters such as temperature, salinity, dissolved oxygen, light intensity and photoperiod (L. Sola et al., 2007). The larval stage lasts about 50 days at 17.5 °C or about 43 days at 20°C (B. Russell et al., 2014).
Breeding: the fries are then placed in pre-fattening cages, in which they remain for 2 – 4 months, afterwards there is a fattening step which lasts for 10 – 12 months, until the fishes reach a weight of about 400 g.

The most important food needs of these fishes concern:

- Carbohydrates: the maximum amount of starch that can be present in feed is 15 %, better if that is extruded

- ❖ Lipids: minimum between 12 and 24 %; for what concerns the polyunsaturated fatty acids that are particularly important in the firsts phases of life of this fishes and others crucial aspects are also:
  - EPA/DHA ratio: that has to be 1:2 in the larval stage and between 1:1 and 2:1 after reaching the live weight of 1 g
  - Total n-3 fatty acids, DHA and EPA: that has to be 0.9 – 1 % in the juvenile stages and 1.5 – 2.7 % in the fattening stage
- ❖ Vitamins: deficiencies, especially of vitamin C, are often associated with malformations; some very important needs are:
  - Nicotinic acid: 63 – 83 mg/kg of feed
  - Thiamine (vitamin B1): 10 mg/kg of feed
  - Pyridoxine (vitamin B6): 1,97 mg/kg of feed
  - Biotin: 0,21-0,37 mg/kg of dry substance in the feed

3.6.4 Quality

This species is marketed fresh or frozen, but is also used for fishmeal and fish oil production and it is regularly present in the markets throughout the Mediterranean region (B. Russell et al., 2014); the flesh of this fish is highly esteemed, and it is considered the best tasting fish of the *Sparidae* family, which also includes other fishes, like common dentex (*Dentex dentex*), white bream (*Diplodus saragus*), bogue (*Boops boops*) and red pandora (*Pagellus bellotti and erythrinus*) that are mainly produced with fishing and whose meats are sometimes used to replace gilthead sea bream fraudulently.

Generally, the skeletal muscle in gilthead sea bream of commercial size depicts the 34.3 – 48 % of the total body weight and we can say that the fishes which come from aquaculture and the ones which are caught wild show some difference in body composition and in the appearance and organoleptic characteristics (K. Grigorakis, 2007).

For what the **body composition** concerns, the average compositions of wild and captured gilthead sea bream are different: in general gilthead sea bream from aquaculture have higher muscle fat and lower muscle moisture compared with wild ones; in particular, the fishes from aquaculture have higher quantity of deposit fat, both perivisceral and peritoneal; instead for what concerns the fatty acid composition there is a high variability, but are not found significant differences in wild and cultured fishes (K. Grigorakis, 2007), even because it is necessary to remember that the fatty acid composition depends from what the fishes eat, so this aspect, in aquaculture, is determined primarily by the type of feed used and if

the diet composition is similar to the ones of wild fishes this is the reason for which there are no significant differences.

Moreover there are no significant differences in the amino acids composition of wild and cultured gilthead sea bream; the same goes even for the total content of trace elements, even if are found some individual differences, indeed wild fishes show higher levels of iron (Fe) and aluminium (Al) and lower levels of titanium (Ti) and vanadium (V), but it is necessary to remember that trace minerals content depends from diet, environment and season, but even from sampling procedures and analysing techniques used (K. Grigorakis, 2007).

In gilthead sea bream there are also significant differences in the myosin subunits proportions between the red muscle of the two counterparts of fishes, these differences indicate different patterns in muscle growth (K. Grigorakis, 2007).

In general, in literature there are evidence that in gilthead sea bream the quantity of dietary lipids influences the fat deposition, in particular it seems that the muscle lipid increases with dietary lipids, instead the visceral lipid remains the same; moreover, even the protein intake influences the lipid deposition, probably because a part of proteins is used as energy source even for the lipid deposition (K. Grigorakis, 2007).

Furthermore, even the form of feed (extruded, pelleted) seems to influence the lipid composition in gilthead sea bream, indeed extruded feeds usually contain less lipids, but lead to an increased fat content; anyway, the muscle fat is correlated with the feeding intensity and it is important to say that the increase of ration size in fishes feed ad libitum overwhelms the compositional effects (K. Grigorakis, 2007).

Moreover, even fasting causes alterations in muscle composition and fat deposition, as fasting in general causes a mobilization of fat, in gilthead sea bream the mobilization involves first the liver fat and then the muscle fat (K. Grigorakis, 2007).

These aspects are influenced also by other factors, such as the photoperiod, indeed continuous light causes a reduced fat content, probably because long days lead to a grater activity of metabolism; fish size, as the muscle, perivisceral and peritoneal fat increase with fish size or weight; season, as fat depots are higher at the end of summer and beginning of autumn and lower after the winter, this trend is due to the feeding intensity and the gonadal maturation and spawning in wild fishes (K. Grigorakis, 2007).

Certainly the fatty acid composition of the diet strongly influences the fatty acid composition of gilthead sea bream muscle, in particular is interesting to notice how the substitution of fish oil with plant origin fats influences the fat composition of the muscle; obviously the

changes in the fat composition of the muscle depends to fatty acid composition of the plant origin fats used in the diet, usually the use of these fats in the diet of fishes leads to a EPA/DHA ratio reduction, EPA and DHA total quantity reduction and n-3/n-6 ratio reduction, while the monounsaturated fatty acids of plant origin increases and saturated fatty acids decreases; moreover, some evidences in literature seems to indicate a preferential deposition of certain fatty acids above some others in the body tissues (K. Grigorakis, 2007). Gonadal maturation and spawning have high impact on the muscle fatty acids composition, as the fishes use the body reserves for the reproduction, even age and size can influence the fatty acid pattern, feed utilization, hormone production and metabolism (K. Grigorakis, 2007).

Other factors that influence the fatty acid composition of gilthead sea bream muscle are salinity and water temperature, generally the unsaturation of fatty acids increases when the fishes live in cold waters (K. Grigorakis, 2007).

For what concerns the amino acids composition of the muscle there are very little information but seems that the muscle free amino acid pool increases in the juvenile fish by more dietary plant protein supply (K. Grigorakis, 2007).

For what the **external appearance and organoleptic characteristics** concern, wild gilthead sea bream has, on average, lower body height, sharper snout, smaller belly and sharper dorsal fins, it also shows paler color, thinner skin and bigger sharper teeth, moreover, it has softer smell; the fishes which come from semi-intensive aquaculture appears more similar to the wild ones (K. Grigorakis, 2007).

The body shape and the presence of scales are influenced by culturing conditions and mainly by stocking density, as it determines the ability of the fish to move but is very important even the genetic heritage, which obviously influences these aspects; regarding the skin color it is related to the intake and source of carotenoid, this could explain why the fishes come from semi-intensive aquaculture systems are similar to the wild ones, as they eat even natural food and not only feeds, moreover, fasting causes bleaching of skin; while muscle coloration is not affected by the carotenoids intake, as they are not incorporated in the muscle but it is related to the fat content of the muscle and when it increases the muscle became whiter (K. Grigorakis, 2007).

For what the organoleptic properties concern, some studies have found the wild gilthead sea bream having more pleasant taste and firmer texture than the cultured ones; moreover, the fishes which came from intensive aquaculture are found to be more fat, juicy and with higher fresh fish flavour, in general the most fatty fishes give a smooth and succulent mouth

sensation, so we can say that the juiciness of the product depends more from the fat content than from the moisture content of the muscle (K. Grigorakis, 2007).
In wild gilthead sea bream are present more volatile compounds, which contributes to the aroma and it could give a better and more delicate aroma; moreover, even the fat in the muscle contributes to the aroma as lipids have a slight taste and, especially unsaturated fatty acids, are precursors of volatile compounds, so the dietary history influences the fatty acid composition of the muscle and consequently influences even the taste of the final product (K. Grigorakis, 2007).

3.6.5 The FutureEUAqua project

The fishes analysed for this study come from the project FutureEUAqua, which aims is to identify the improvements, along the whole value chain, necessary to have aquaculture products of high quality, safe for a growing population and without harming the environment and compromising animal health and welfare. So, the overall objective of the project is to bring these aspects together and, in particular, is "*to effectively promote the sustainable growth of climate-change resilient, environmentally friendly organic and conventional aquaculture of main fish species and low trophic level organisms in Europe, to meet future challenges with respect to the growing consumer demand for high quality, nutritious and responsibly produced food. To this end, FutureEUAqua will promote innovations in the whole value chain, including genetic selection, ingredients and feeds, non-invasive monitoring technologies, innovative fish products and packaging methods, optimal production systems such as IMTA and RAS, considering socioeconomic aspects by the participation of a wide spectrum of stakeholders, training and dissemination activities*" (https://futureeuaqua.eu/index.php/media/).
FutureEUAqua project is divided into nine work packages (WP): sustainable breeding (WP1), sustainable and resilient feed and feeding strategies (WP2), consumer and regulatory activities (WP3), sustainable and resilient production system (WP4), internet of Things for healthy fish and environment (WP5), quality and safety of aquaculture products (WP6), training, capacity building and support to policy (WP7), dissemination and communication (WP8), project management (WP9).

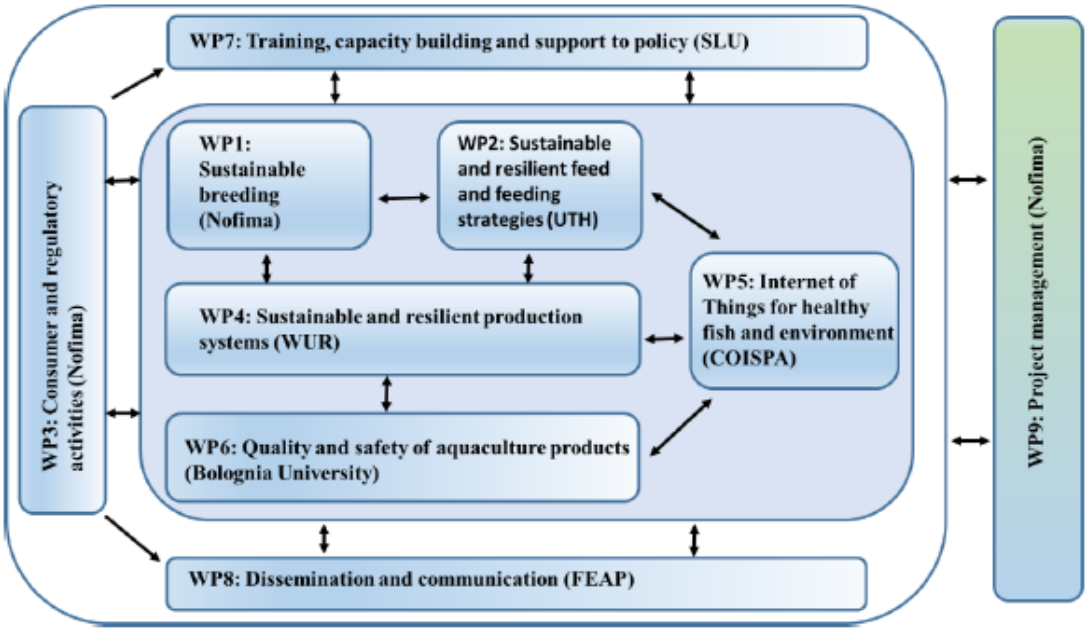

Figure 3.6.2: structure of FutureEUAqua project and relations between the work packages (WP) (adapted from https://futureeuaqua.eu/index.php/media/).

The main objective of the project is to support production and consumption of aquaculture products, so the expected impacts are to improve resilience and sustainability of aquaculture farming system and practices; genetic diversity of farmed algae and fish species; tailor made feeding formulas; integration of smart tools to monitor the farming environment and the animal health and welfare; nutritional quality, including digestibility, and safety of aquaculture products; mild and non-thermal technologies for processing food; novel efficient intelligent packaging; consumers' awareness, perceptions and acceptability of the European aquaculture products and methods; professional skills and competences of those working and being trained to work within the blue economy; and support the implementation of the EU Common Fisheries Policy (CFP) and contribute to policymaking in research, innovation and technology.

Inside this wide project the present study is focus on the aspects related to "*ingredients and feeds*", so WP2, indeed, analyses samples from fishes fed with three different diets, as discussed subsequently, in order to study if different feeds affect the composition and the quality of the product.

For what this aspect concerns, the aim of the project is to develop innovative feeds, both conventional and organic, which improve sustainability, resilience of the fishes, health, and product quality. The final purpose is to find tailor made aquafeeds that allows to improve the performances of fishes and maintain or enhancing nutritional value and environmental respect of the products.

3.7 METABOLOMICS IN SEAFOOD SCIENCE

^{1}H NMR-based metabolomics studies applied to fish are related to different species, tissues and application areas and have contributed new knowledge in a wide range of aspects such as physiology and development, effects of pollution, fish condition and disease and fish as foodstuffs (L. M. Samuelsson et al., 2008).

For what the **physiology of fish** concerns, NMR-based metabolomics has been used, for example, to study chronic temperature stress in juvenile steelhead trout (*Oncorhynchus mykiss*) (M. R. Viant et al., 2004). Here metabolomics provided a picture of the metabolic changes induced by temperature stress.

In other studies, has been evaluated the effect of the exposure to different sewage effluents (L. M. Samuelsson et al., 2011) or the effect of hypoxia in a moderately hypoxia-tolerant fish, the common carp (*Cyprinus carpio*) (I. Lardon et al., 2013).

Some studies have also shown the utility of ^{1}H NMR-based metabolomics in the analysis of fish **development**, as it helps to understand the metabolic events in the developmental process. For example, NMR was used to study the effect of elevated water temperature on the development of steelhead trout (*Oncorhynchus mykiss*) eggs (M. A. Turner et al., 2007), demonstrating that there is an evident effect on the metabolite profiles of eggs in the later developmental stages.

In another study was examinate the content of 15 metabolites from three batches of turbot (*Psetta maxima*) eggs in five developmental stages (D. Pouliquen et al., 1998), the results suggest that free amino acids are the main energy substrates for newly fertilized eggs.

Other paper by Viant (M. R. Viant, 2003) illustrates the concept of using a "*metabolic trajectory*" in PCA scores plot to summarize the changes in the metabolite pattern of developing medaka (*Oryzias latipes*) eggs. Metabolites profiles of eggs sampled at the same development stage were similar, so clustered together in scores plot. The trajectory of development could then be followed in the plot through the various stages in a time-dependent fashion. So, the author suggested that metabolic trajectories will become a powerful tool for studying effects of toxicants, or other stressors, during embryogenesis.

Metabolomics can also be useful in **environmental risk assessment**, to evaluate how physical, chemical, and biological stressors impact metabolism (S. Roques et al., 2020). As demonstrated, for example, in a paper on trichloroethylene-exposed developing medaka (*Oryzias latipes*) embryos (M. R. Viant et al., 2005), where ^{1}H NMR shows to be much more sensitive than traditional toxicology parameters (morphological abnormalities, hatching

success and mortality) to assess the impact of pollutants; information about affected metabolic pathways was obtained and visualized by using the metabolic trajectory concept. In another paper by Viant *et al*. the effects of three pesticides (dinoseb, diazinon and esfenvalerate) on eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) were studied by NMR- and HPLC-UV-based metabolomics (M. R. Viant et al., 2006). Anew NMR demonstrated to be more sensitive than toxicology parameters for dinoseb and diazinon-exposed eggs and it was also observed that each pesticide induces a specific metabolic response.

Furthermore, have been published even studies in which NMR-based metabolomics is used to assess the effect of ECDs (endocrine-disrupting chemicals) (L. M. Samuelsson et al., 2006), heavy metals (E. M. Santos et al., 2010), heavy oils (E. Kokushi et al., 2012) and cyanobacterial blooms (B. Sotton et al., 2017).

Metabolomics is also used for the study of human diseases and recently this approach was applied even for the investigation of fish **condition and disease** (L. M. Samuelsson et al., 2008). In general, metabolomics can be applied in fish health and welfare monitoring, and this could be very important to prevent losses related with farmed fish diseases due to pathogen infection (S. Roques et al., 2020).

For example, NMR-based metabolomics was applied for investigating differences in the plasma metabolite profile of Atlantic salmon (*Salmo salar*) infected with the Gram-negative bacteria *Aeromonas salmonicida* as an alternative to currently used methods (K. S. Solanky et al., 2005). From this study emerged that metabolomics is more accurate and less time-consuming than the current methods (clinical microbiology and stress-test, respectively).

In general metabolomics could be applied to study host–pathogen interactions (as in the study quoted immediately above); disease characterization, as in the article by Southam *et al*. in which are studied metabolic changes in flatfish hepatic tumors (A. D. Southam et al., 2008); treatment efficiency and stress response due to farming practices such as fish handling, as studied, for example, in a paper which concerns the characterization, whit ^{1}H NMR and mass spectrometry, of metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress (T. K. Karakach et al., 2009) and netting (M. Y. Mushtaq et al., 2014).

Some prospective application areas for metabolomics are food safety, food fraud, food authentication, food storage and processing, nutritional value, development of novel food and health care products (L. M. Samuelsson et al., 2008). For example, in a paper by Rezzi *et al*. has been demonstrated that ^{1}H NMR lipid metabolomics and multivariate data analysis

can be used to distinguish between wild and farmed Gilthead sea bream (*Sparus aurata*) (S. Rezzi et al., 2007). Another interesting study describes a metabolic profiling study of Gilthead sea bream (*Sparus aurata*) from three different aquaculture methods (F. Savorani et al., 2010), in that research a new multivariate classification tool, iECVA, revealed several metabolites which are important biomarkers for characterizing the three different aquaculture systems: glycogen (stress indicator), histidine, alanine and especially glycine.

Metabolomics can be useful even to study changes occurring during the storage of the product as demonstrated, for example, in a paper about the changes in the amino acid composition of bogue (*Boops boops*) during storage at different temperatures (A. Ciampa et al., 2012) or in another work about the monitoring of *post-mortem* changes in Atlantic salmon fillets stored at 0 and 4 °C (E. Shumilina et al., 2015).

Another important aspect that can be studied with metabolomics is the **effect of different diets** on the fish metabolome. For what the feed for fish concerns, the global trend is to replace the ingredients of animal origin, such as fish meal and fish oil, with plant-based ones. This leads to the need to analyse the new feed formulations to understand if they could be suitable to replace the conventional feeds. To do this, usually the analytical composition is characterized and the digestibility of new feeds is assessed to evaluate their effects on fish growth performances, feed consumption and other zootechnical parameters. Unfortunately, these conventional methods might be insufficient to understand the consequences of feeds on fish metabolism and do not provide information on the profile of small molecules and other nutritional and anti-nutritional factors. So, in order to obtain this information, is necessary to use numerous and costly analytical approaches (S. Roques et al., 2020).

Conversely, omics technologies enable a holistic vision, in particular metabolomics is focus on the global set of metabolites in a biological system and could provide information about metabolic activities. So, combining a feeding trial with metabolomic analysis of tissue and biofluids it is possible to have new insights into feed and nutrient effects (S. Roques et al., 2020).

As discussed above metabolomics analysis is composed by sample preparation, metabolite extraction, derivatization, metabolite separation, detection, and data treatment (J.M. Cevallos-Cevallos et al., 2009).

Sampling and analytical sample preparation require particular attention in fish nutritional studies, these types of studies are mainly focus on the analysis of muscle and, usually, is sampled the dorsal muscle without skin. Skeletal muscle is a complex tissue, which includes white and red muscle, that have different characteristics and so different metabolite profiles.

Depending on the aim of the study different parts are sampled. Rarely could be analysed even liver or biological fluids, such as urine, or more frequently, blood or even faeces or mucus (S. Roques et al., 2020).

Metabolomics can be useful to discriminate fish on their feeding habits, as that obviously impact the fish metabolism. This can be avail even to distinguish framed from wild fishes (S. Roques et al., 2020).

This technique can also be used to characterize the lipid profiles, identify or quantify valuable fatty acids, such as PUFA and to study the impact of feeds on the lipid profile of fish muscle (S. Roques et al., 2020). On the other hand, the polar fraction can provide information on changes in some characteristic of fish fillet quality, as some polar compounds (for example trimethylamine, hypoxanthine and inosine-monophosphate) can be related to freshness or other metabolic aspects (S. Roques et al., 2020).

Metabolomics can also be used to study how the diet can modulate the fish metabolism (S. Roques et al., 2020).

Metabolomics can contribute to fish nutrition studies through the evaluation of direct or indirect impact of diet on metabolism. It can be useful to classify fish according to diets, identify biomarkers of disturbed state, or understand the metabolic pathway affected; the variations in the metabolism induced by the diets can also be used to create classification models, for example metabolomics can contribute to assess fish origin based on muscle lipid profiling or on the identification of biomarker of metabolic modifications or nutritional deficiency (S. Roques et al., 2020).

Lastly, metabolomics may contribute to understand the mechanism involved in the utilization of novel feedstuffs and which precursors supply intermediary metabolism (S. Roques et al., 2020).

To summarize, metabolomics has proved to be useful for screening metabolites in different species and tissues, to study developmental changes in the metabolome, to understand the fish physiology and for studying time-related effects such as embryogenesis (L. M. Samuelsson et al., 2008).

It has also demonstrated to be more sensitive or at least as sensitive as more traditional ecotoxicological parameters and so it is promising for future applications in environmental risk assessment. It could be useful to determining no-effect concentrations, modes-of-action, dose-response relationship, to develop biomarkers of exposure and effect and to observing which developmental stages are the most sensitive to toxicants and/or determine

the duration of metabolic responses to temporary chemical exposures (L. M. Samuelsson et al., 2008).

In some applications metabolomics has proven itself more sensitive, more accurate and faster than traditional methods for monitoring the health of fish populations, despite this, differences between treatments or diseases are sometimes small, so complementary data may be necessary for robust and accurate classification. Application areas of metabolomics include diagnosis of disease, monitoring of disease progress and/or response to veterinary treatment, detection of early onset of disease, understanding host-pathogen relationship, classification of tumor types, and finding “health biomarkers” to aid as a diagnostic and prognostic tool for individual and ecosystem health (L. M. Samuelsson et al., 2008).

Metabolomics could become a useful tool for regulatory authorities in terms of authenticating food labelling of fish products. In aquaculture industries it could be used to assess the health status of the fish, investigate the effects of breeding conditions and nutritional quality of the final product depending on storage and processing conditions. While in healthcare industries metabolomics could assist in locating compounds in various fish species especially interesting from the human health perspective and which could potentially be used in the development of functional foods and health care products (L. M. Samuelsson et al., 2008).

Recently metabolomics has also been used to study the effect of different diets on the fish metabolome, to discriminate fish on their feeding habits, identify biomarkers of disturbed state, or understand the metabolic pathway affected (S. Roques et al., 2020).

4 OBJECTIVES

The aim of this thesis work is to assess whether there are substantial differences in the metabolic profile of three groups of Gilthead sea bream (*Sparus aurata*) fed with different diets. To accomplish this task, ^{1}H NMR spectroscopy and a metabolomic approach have been used.

In the experimental work, the liquid trichloroacetic extracts of samples of the muscle of fishes were analysed by NMR. The obtained spectra were processed, and then analysed with Principal Component Analysis (PCA), in order to appreciate a possible grouping of the fish population consequent to discriminant patterns of crucial metabolites that are individuated by an unsupervised approach. From a methodological point of view, nuclear magnetic resonance provides spectra that could be considered as a digital analogue of the molecular profile of aqueous fish extracts, to be analysed in the form of digital signatures, whatever the chemical nature of the substances they represent. This avoids creating interpretative biases related to the preliminary choice of a set of molecules expected to be relevant because of preconceived ideas. From the metabolomic evaluation we intend to extract descriptive parameters of the molecular quality of fish to be related with other evaluations of sensory, structural, technological and nutritional properties that are integral part of the FutureEUAqua project and that are carried out by other research groups. From the interpretation of the spectral information, it is expected not only to highlight any metabolic differences between populations of fish fed in different ways, but biomarkers able to establish the state of health or stress of the animals will be identified, and a strategy such as proper nutrition could be finely tuned to make fish more resistant to climate change and disease.

5 MATERIALS AND METHODS

5.1 EQUIPEMENTS

Lab supplies

- Pipetting Standard - Gilson's Pipetman® P (P100 and P1000. Range of volumes from 10 µL to 1000 µL) with suitable tips (Diamond® precision tip)
- BECTON DICKINSON Polypropylene 50 and 15 ml Conical Tubes, Falcon™ Type
- Spatula and spoons stainless steel
- Square, polystyrene and anti -static weighing Dishes (volume 7, 100 and 250 mL)
- Eppendorf® Safe-Lock® microcentrifuge tubes volume 1.5 ml
- Bottle in polypropylene (PP) (Volume 100, 250 and 500 ml)
- Laboratory glassware:
  - Beakers, low form, with spout (volume 50, 250 and 500 mL)
  - Disposable Glass Pasteur Pipettes, VWR international
  - Graduated pipette (10 ml, class A)
  - Glass rod
- Pasteur pipette rubber bulbs
- Pipette filler
- Magnetic stir bars
- NMR sample Tubes (standard 5 mm, round bottom), Hilgenberg
- Plastic caps for NMR sample Tubes

Safety and protection supplies

- Safety Eyewear Glasses
- Nitrile Powder - Free Gloves, BERICAH
- White lab coat

5.2 REAGENTS

- Trichloroacetic acid (CCl_3COOH, 163.39 g/mol, TCA), CHEM-LAB
- Deuterium oxide (D_2O, 20.04 g/mol, 99.9 % purity), EURISOTOP®
- Milliq demineralized water
- Sodium Hydroxide (NaOH, 39.997 g/mol)
- Hydrochloric Acid (HCl, 36.46 g/mol)
- 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt Cambridge Isotope Laboratories, Inc. ($(CH_3)_3SiCD_2CD_2CO2Na$, 172.27 g/mol TSP, 98 % purity)

Buffer solutions

- Trichloroacetic acid solution 7.0 % (w/v): dissolve 7.00 g of TCA in 100 ml of distilled water
- Standard solution, TSP 10 mM in deuterium oxide: dissolve 0.034 g of TSP in 20 ml of deuterium oxide

5.3 INSTRUMENTS

- Electronic digital technical Balance (max 2200 g, d= 0.01 g), SCALTEC (SBA 52)
- CyberScan 510 pH Meter with glass bodied combination electrode, swing arm electrode holder & ATC probe
- Heating magnetic stirrer mod. ARE, VELP Scientifica®
- PV-1 Vortex Mixer, Grant-bio
- D3024R Microcentrifuge (max 15000 rpm), Scilogex
- Z 366 K Centrifuge (max 16000 rpm), Hermle Labortechnik GmbH
- FT-NMR Avance Bruker AvIII (600 MHz) spectrometer Ultra Shield Plus equipped with:
  - The electronic 3-channel RF consisting of amplifiers from 100-Watt ^{1}H and broadband for X 300 Watt
  - Control unit gradients GCU
  - Control system of the temperature SmartCooler BCU I
  - Probe 5 mm with Z gard ^{1}H - ^{13}C - ^{15}N
  - Autosampler with 24 holders
  - Software TopSpin version 3.5 pl 6 (Bruker BioSpin, Karlsruhe, Germany)

5.4 SOFTWARE AND DATABASE

5.4.1 NMR data processing

TopSpin is the control and preliminary elaboration program used. It allows to make the operations necessary to stabilize the magnetic field, setting all the parameters of the acquisition and control the acquisition procedure. Moreover, after the acquisition, it allows to make some preliminary elaboration steps like timing, calibration and correction of the base line, but also evaluation of the line width and chemical shifts of the peaks.

ChenomX (https://www.chenomx.com/) is a software that works with comprehensive Spectral Reference Libraries and allows to recognize analytes comparing the signal obtained with

the expected spectrum of different analytes present in the database of the software itself, giving even the estimated concentration based on the known concentration of the internal standard. The software allows to search what compounds give a peak around an exact chemical shift, then identification of the compound is made by considering spin coupling and chemical shifts, as well as variations due to pH or ion presence. Once the compound that gives the peak has been identified the expected and real peaks can be fitted automatically or manually.

Human Metabolome Database (https://hmdb.ca/) is an online database containing detailed information about molecules and metabolites found in the human body, including spectra. So, it was used as a supporting tool in the identification of the analytes.

5.4.2 Chemometrics data processing

The multivariate statistical analysis were carried out using different statistical software due to the different kind of analysis: R program and Matlab were the software used for these porpoises.

R (http://www.r-project.org) is a language and environment for statistical computing and graphics. The language provides a wide variety of statistical (linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering, etc) and graphical techniques, and is highly extensible. One of its strengths is the ease with which well-designed publication-quality plots can be produced, including mathematical symbols and formulae where needed. R is available as Free Software under the terms of the Free Software Foundation's GNU General Public License in source code form.

Matlab (http://www.mathworks.com) is a high-performance language for technical computing. The name stands for matrix laboratory and was originally written to provide easy access to matrix software. It integrates computation, visualization, and programming in an easy-to-use environment where problems and solutions are expressed in familiar mathematical notation; this allows to solve many technical computing problems, especially those with matrix and vector formulations, in a fraction of the time it would take to write a program in a scalar noninteractive language such as C or Fortran. It is used for some preliminary alignment operations on the peaks of the spectra.

5.5 EXPERIMENTAL DESIGN

In the experimental work the muscle of farmed Gilthead sea bream (*Sparus aurata*) was analysed. The fishes have been farmed in Greece. Three groups of farmed Gilthead sea bream (*Sparus aurata*) were studied, each one was fed with a different composition feed:

- Commercial conventional: is a feed that represents the ones normally used in the farming of this species of fish;
- FutureEUAqua conventional: is an innovative feed;
- FutureEUAqua organic: is an innovative and organic feed.

The last two diets are also called "*futuristic*", as they are developed in the context of the project FutureEUAqua, so this name recalls that of the project.

Two hundred and sixty-one NMR samples were prepared from extracts of the muscle's tissue of eighty-seven fishes, twenty-nine for each diet already described.

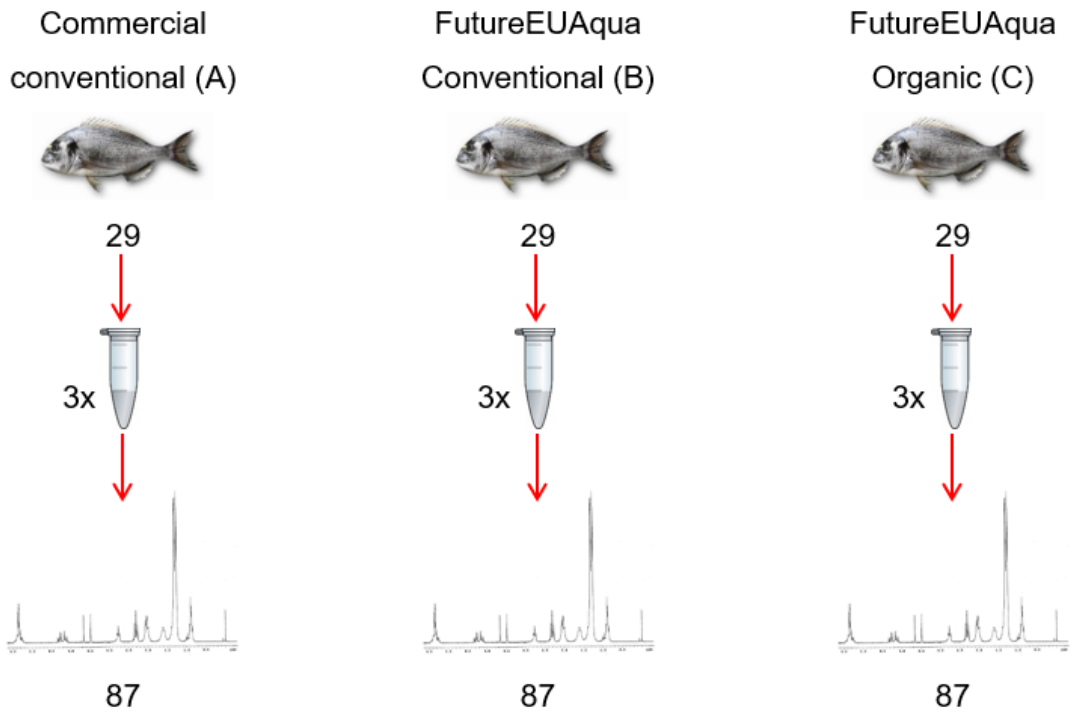

Figure 5.5.1: Gilthead sea bream sampling

5.6 SAMPLE PREPARATION

Four grams of muscle from different body parts of each fish were weighted with a technical balance inside 50 ml Falcon™ Type tube, then eight milliliters of 7 % trichloroacetic acid (i.e., 2 ml per gram of sample) were added to the muscle and mixed with Vortex and glass rod until complete homogenization is reached, this step is necessary to denature enzymes and to extract soluble compounds. Samples were centrifuged at 10000 rpm, for 10 minutes at 4°C, to separate the liquid extract from the solid part. The resulting supernatant, transferred into a beaker, was neutralized with NaOH at different concentrations to a final pH of 7.8 ± 0.05. The solution was dispensed in 1 ml aliquot in Eppendorf tubes, from each sample were obtained three replicas. Samples were centrifuged at 14000 rpm, for 10 minutes at 4°C, to remove the precipitate. The resulting supernatant was dispensed in 720 µl aliquot in Eppendorf tubes in which there have been added previously 80 µl of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP). Samples were centrifuged at 14000 rpm, for 5 minutes at 4°C. The resulting supernatant was dispensed in 650 µl aliquot in NMR sample tube.

As discussed above the principal components of fish muscle are protein and lipids, besides some other molecules less present such as carbohydrates, minerals, vitamins, and bioactive compounds.

To summarize, in this context, it is possible to identify three ways to analyse the samples: proteomic, which is the study of the proteins present in the samples or metabolomics, which is, instead, focuses on the analysis of metabolites. In turn metabolomics can be further divided into metabolomics applied to hydrophilic compounds and metabolomics applied to hydrophobic compounds.

The applied procedure allows to detect hydrophilic compounds, so mainly the ones of protein nature present in the samples. Moreover, it is necessary to notice that for the analysis of lipids is more widespread GC-MS, or some other similar techniques, even because NMR spectroscopy is more suitable for the analysis of aqueous solutions, while for hydrophobic compounds it is better to use different techniques.

This procedure is necessary because usually NMR spectroscopy requires liquid samples as it is impossible to analyse the whole fish in this type of instrument. Furthermore, taking the four grams for the analysis from different body parts of the fish muscle it is possible to obtain a representative sample as the composition of the muscle can change not only between different fishes but even in different parts of the same fish.

5.7 NMR DATA ACQUISITION

After the preparation, the sample is put in the carousel of the instrument and then the NMR spectra are acquired. The spectra are recorded in an NMR spectrometer that work at the protonic frequency of 600 MHz, equipped with an inverse probe and a refrigerated autosampler, which keeps the samples at 4°C. The samples spectra are acquired with 64 scans of 32K points, with a spectral width of 12 ppm, a time of acquisition of 2.27 s and a recycling time of 8.0 s. The control and preliminary elaboration program used is TOPSPIN, which applied an automatic process lasting for 22 minutes for each sample and consisting of: sample loading, temperature stabilization, tuning, matching, shimming, pulse calibration at 90° and acquisition. The pulse used is the sequence *noesygppr1d.comp*, which allow the pre-saturation of water. After the acquisition there is the processing, in which the digitalized signal is subjected to a mathematical manipulation treatment to improve the signal to noise ratio.

As previously discussed, there are a wide range of possible analysis which can be carried out on fish samples, but the majority of these are specific analysis, therefore of target type, in which is search a specific compound or at least few metabolites. While NMR spectroscopy is an untargeted analysis, so it enables to see everything present in the sample, which is both beyond the detection threshold and soluble in the solution used for the extraction.

Considering that the purpose of the present study was to detect differences between fishes feed with different diets was decided to use a method with the widest spectrum, that enables to have an idea of everything present in the sample, while this is not possible if it is use an analysis which search only for a limited number of compounds.

It is necessary to underline that the purpose of the present study was not to define if fishes feed with a specific diet are of better or worst quality compared to the other, but only to establish if there are any type of differences between them.

5.8 CHEMOMETRIC TECHNIQUES

The predominant chemometric technique used is **Principal Component Analysis** (**PCA**), that is the unsupervised analysis more used for analysing NMR spectra. In this method the analysis is conducted without previous knowledge about the samples, and it allows to understand if there are whatever kind of difference between the samples analysed. The outcome of this technique is a scatter plot, which displays the samples and provides an overview of the data, indicating any possible clustering; so, if no clustering appears in the PCA this is an indication of absence or presence of very small differences between the

samples. PCA reduces the dimensionality of the data, so it makes possible to go from a very high number of variables to a scatter plot that contains two principal components (PC axis) and data points that represents the samples; PCs are combinations of the original variables and explain a certain amount of variance. Furthermore, the technique gives a loadings plot, which shows the most important variables; so, it can be used to trace compounds that gives the greatest differences between samples (E. Hatzakis, 2018).

The PCA regards the total variance of the samples so if it is necessary to consider the variance due to a specific factor is necessary to use a supervised technique, the most used type of these techniques is the **Partial Least Squares Discriminant Analysis** (PLS-DA), since it is a supervised technique it always leads to a separation of the classes, even if there are no significant differences between them, so, for this reason, it must be used carefully (E. Hatzakis, 2018).

6 RESULTS AND DISCUSSION

6.1 NMR SPECTRA AND IDENTIFICATION OF COMPOUNDS

As previously discussed, the ^{1}H NMR spectroscopy provides spectra composed of signals belonging to hydrogen nucleus which are part of molecules. The position of signals in the spectra gives information about the chemical nature of the molecules in the sample. Generally, the spectrum of biological samples can be dived into three sub-regions, named upfield, midfield and downfield region.

The upfield region goes from 0.5 to 3 ppm and includes the signals belonging to aliphatic chains, so specifically to organic acids, sidechains of amino acids, lipids, which anyway are very little present in aqueous extracts, or, in general, hydrocarbon moieties of hydrophilic molecules which are not affected by nearby electron-attractor groups. The midfield region extends from 3 to 5.5 ppm and comprehends signals belonging to alcohols, sugars, alpha amino acids, or unsaturated compounds. The downfield region goes from 5.5 to 9 ppm and contains signals of aromatic compounds (G. Picone et al., 2011).

A typical ^{1}H NMR spectrum obtained during the present study is shown in Figure 6.1.1.

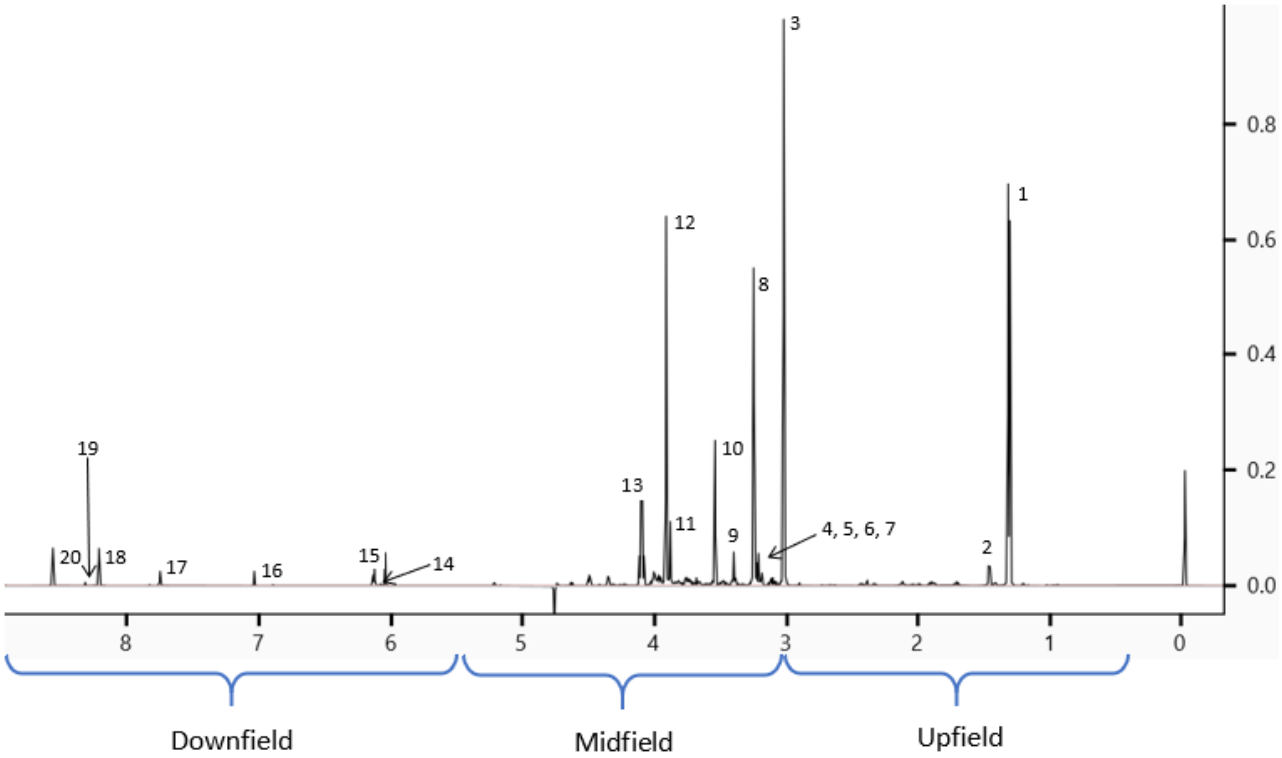

Figure 6.1.1: ^{1}H NMR spectrum of extract of Gilthead sea bream fillet, with the indication of the upfield, midfield and downfield regions.

As it is possible to notice, the peaks with the highest intensity are in the midfield region, except for one tall peak in the upfield one, while in the downfield region there are some clearly distinguishable peaks, but with an intensity lower than the ones in the midfield region. Every molecule present in the samples gives a specific pattern of signals which allows to identify the molecule thanks to deconvolution programs, in particular for this study has been used ChenomX software. The assignment of principal molecules in the sample is illustrated in the table below, are considered only the signals which are clearly distinguishable from the other ones.

	Metabolite	Signal (ppm)	Multiplicity
1	Lactate	1.3	d
2	Alanine	1.5	d
3	Creatine/Creatine phosphate	3.0	s
4	Choline	3.2	s
5	sn-Glycero-3-phosphocholine	3.2	s
6	Taurine	3.2	t
7	Trimethylamine N-oxide (TMAO)	3.3	s
8	Betaine	3.3	s
9	Taurine	3.4	t
10	Glycine	3.5	s
11	Betaine	3.9	s
12	Creatine/Creatine phosphate	3.9	s
13	Lactate	4.1	m
14	Inosine	6.1	d
15	Inosine 5-monophosphate (IMP)	6.1	d
16	Histidine	7.0	s
17	Histidine	7.7	s
18	Inosine 5-monophosphate (IMP)	8.2	s
19	Inosine	8.3	s
20	Inosine 5-monophosphate (IMP)	8.6	s

Table 6.1.1: Main metabolites in Gilthead sea bream extract.

Based on this type of table it could have been done a quantification of the compounds to detect differences between the samples. However, to obtain the maximum amount of information, an exploratory investigation has been used, with multivariate data analysis of the matrix of NMR spectral data, as it allows to analyse everything present in the sample, instead of only the identified compounds. For this purpose, Principal Component Analysis (PCA) has been used.

6.2 DATA PRE-TREATMENT

As quickly mentioned above (in paragraph 2.8) the application of multivariate statistical analysis (MVSA) usually requires a pre-processing step to make the data from different samples comparable to each other and ensure a reliable analysis (E. Hatzakis, 2018), these steps are critical and the most important objective of them is to minimize undesirable noise in order to make the following analysis more robust, accurate and precise (T. Vu et al., 2018), indeed this pre-treatment also allows to reduce as low as possible the artefacts due to sample preparation and instrumental errors.

For the samples considered are applied some steps of pre-processing, such as alignment, removal of some peaks, regional scaling, icoshift and binning.

The **alignment** of the spectra is done on the internal standard (TSP) peak, after that is necessary to **remove some parts of spectra and peaks**, so are removed i) the parts from 20 to 9 ppm and from 0.5 to -20 ppm in which there are no peaks; ii) the part from 4.96 to 5.00 ppm in which there is the peak of the water, this part is removed because the water can produce a high interference; iii) the part from 6.05 to 6.07 ppm in which there is a peak of a compound present in the TCA used and so it is removed because making the analytical blank is possible to see that this compound is present in the TCA and so it represents a contaminant, which is not present in the samples themselves.

After these first steps is applied a **regional scaling**, which is a type of normalization of the data, the more used normalization technique for the NMR spectra is the Probabilistic Quotient Normalization (PQN), in which is calculated a median spectrum of all the samples spectra and then this spectrum becomes the reference which is used to normalize the spectra, through a division of each one for a normalization factor (da T. Vu et al., 2018), this step is necessary to reduce the impact of some errors such as dilution errors, which can produce sample preparation artefacts or some types of instrumental errors, for example small differences in the tuning conditions of the spectrometer and imperfect baseline and phase corrections (A. Craig et al., 2006 & T. Vu et al., 2018). In the regional scaling applied in this study every spectrum is divided into the three zones previously discussed (upfield, midfield and downfield region), and the PQN is applied in every zone separately, after that the three zones are again reunited in a single spectrum; the regional scaling is used to reduce the impact of the most concentrated molecules, as often the compounds that shows the larger variability among samples are not the most concentrated (as already mentioned above). Anyway, it is necessary to notice that the treatment applied is not a proper regional

scaling, but only a procedure that could however reduce the impact of the most concentrated molecules, making possible to notice the differences in the less concentrated compounds.
After that, **icoshift**, a MatLab tool which allows to align manually the peaks which are shifted, has been used in order to represent a more refined alignment, with respect to the one on internal standard. This alignment could be particularly useful for the peaks in the aromatic area (at about 7 – 8 ppm) which are the ones that are more affected from the shift due to even small variations in the pH of the sample. This step is optional and sometimes is not used as it represents a forcing of the data set, but in this case has been decided to employ that after an evaluation of the results obtained from PCA carried out with one dataset without icoshift and one with that; the results are very similar and so it has been decided to use the dataset with icoshift because this one allows to simplify the work in the following steps.
The last step is the **binning**, which reduces the number of points considered, as we started from spectra of 65536 points each, the number of points is first reduced from the removal of some parts of the spectra and peaks after which remains over 44700 points. In the present study, with binning, every spectrum is segmented in areas of 100 points each, just called bins, so, finally, are obtained 447 bins, each including a range of 0.0183 ppm; this step allows to reduce the data and so to simplify the next multivariate data analysis, but it also reduces the spectra resolution.
Moreover, the binning step also provides a reduction of the variation of chemical shift among different spectra, generated by small differences in the pH of the samples (A. Craig et al., 2006).

6.3 MULTIVARIATE DATA ANALYSIS

As mentioned above the Principal Component Analysis (PCA) is the unsupervised technique more used for analysing NMR spectra (E. Hatzakis, 2018). This technique is born from the need to recognize similarities and differences inside a dataset, it is mathematic treatment of the data matrix, whose purpose is to represent a large number of variables using a much smaller number of factors, called principal components (PC). In fact, PCA minimizes the number of variables with a small loss of information, this is possible thanks to the creation of a new space in which representing the samples, created redefining the axes of the system using the principal components instead of the starting variables.
From the mathematical point of view PCA tries to understand what distinguishes every sample from all the others, so it tries to maximize the distance between this sample and all the other ones. Then it aggregates the variance associated with the same phenomenon and

uses that one to create the principal component, so each of this encloses the variance of every signal which is associated with the same phenomenon that variates. As a result, is obtained the principal component space, in which the intergroup variance, so the variance between different groups, is maximized and the intragroup variance, so the variance inside every group, is minimized. This is due to the fact that in trying to maximize the distance between different samples is obtained an approach of the more similar ones.

Each principal component is formed by a series of starting variables and explains a percentage of the total variance of the data, expressed with the eigenvalue. The first principal component (PC1) explains the maximum percentage of the variance, so it is the direction along which there is the greatest dispersion of data, the following principal components explain a progressive smaller percentage of the total variance, so the last principal components describe mainly the noise of the data, then they are not took into account. In the present study for the analysis of the samples have been considered the first two principal components (PC1 and PC2).

Then the analysis plans to label every sample depending on the membership group in order to see if there are sets of homogeneous samples. If every group has an own dispersion, which is displayed with the representation of ellipses, it indicates that there are significant differences between the groups of samples. Graphically the dispersions are different when the ellipses are not or only partially superimposed. Lastly, it is possible to identify the phenomena which are responsible to the differences between groups.

The position of the samples in the principal component space is described from coordinates called **scores**. Another important parameter of the PCA are the **loadings**, which are the cosines of the angels between the variable and the principal component, so the analysis of the loadings allows to understand which is the contribution of each variable to the principal component.

Moreover, the **scores plot** obtained from the analysis is particularly useful in order to identify the abnormal data, called outliers, as it could already be seen by eyes, or it could be identified with mathematical methods.

The influence of the loadings on the principal component are represented graphically with a **BarPlot** and are identified the loadings that influences the principal component the most, then from these loadings is possible to go back to the corresponding bin, which represents the starting variable as every bin derives from the points of the spectrum.

After that is applied an **ANOVA** (Analysis of Variance) **on the bins**, the ANOVA is a method used for multiple comparisons between means, whose purpose is to bring out differences between groups which are stronger than differences between various data of a single group. Thanks to the application of ANOVA on the bins it is possible to identify the significant ones ($p < 0.05$), after that it is also feasible to make another PCA only considering the significant bins, then, after the creation of BarPlots, are selected only the most **important loadings**, and it could be done by defining a threshold. When the value of the loading is higher than this threshold the loading is considered important, in this study the threshold used is defined as:

$$\text{Threshold} = |\text{mean} - \text{standard deviation}|$$

Afterwards from the important loadings are obtained the corresponding bins and the **range** of ppm associated, this is necessary to identify the compounds which determine the differences between the samples, indeed after this step the compounds have been identified with ChenomX.

The last step is the creation of **BoxPlots** using the Tukey test, it is a statistical test used when ANOVA indicates that there are significant differences. To understand between which groups there are these differences, it is necessary to see the distribution of the data and it is useful to understand how much the compound is significant in determining differences between the groups of samples.

6.4 METABOLOMES OF FISH FED BY DIFFERENT FEED

For the present study, the spectra have been divided in bins of 100 points each, corresponding approximately to 0.0183 ppm, as previously discussed. Two hundred sixty spectra have been considered and not two hundred sixty-one as previously mentioned as a spectrum obtained from a fish feed with the FutureEUAqua organic diet has been excluded from the dataset. This was done because during the acquisition of the spectra are being performed some PCAs on a partial dataset, just for locate outliers; the sample mentioned clearly turned out to be outlier and so it was immediately ruled out.

The PCA performed on significant bins, obtained with ANOVA, is shown in Figure 6.4.1.

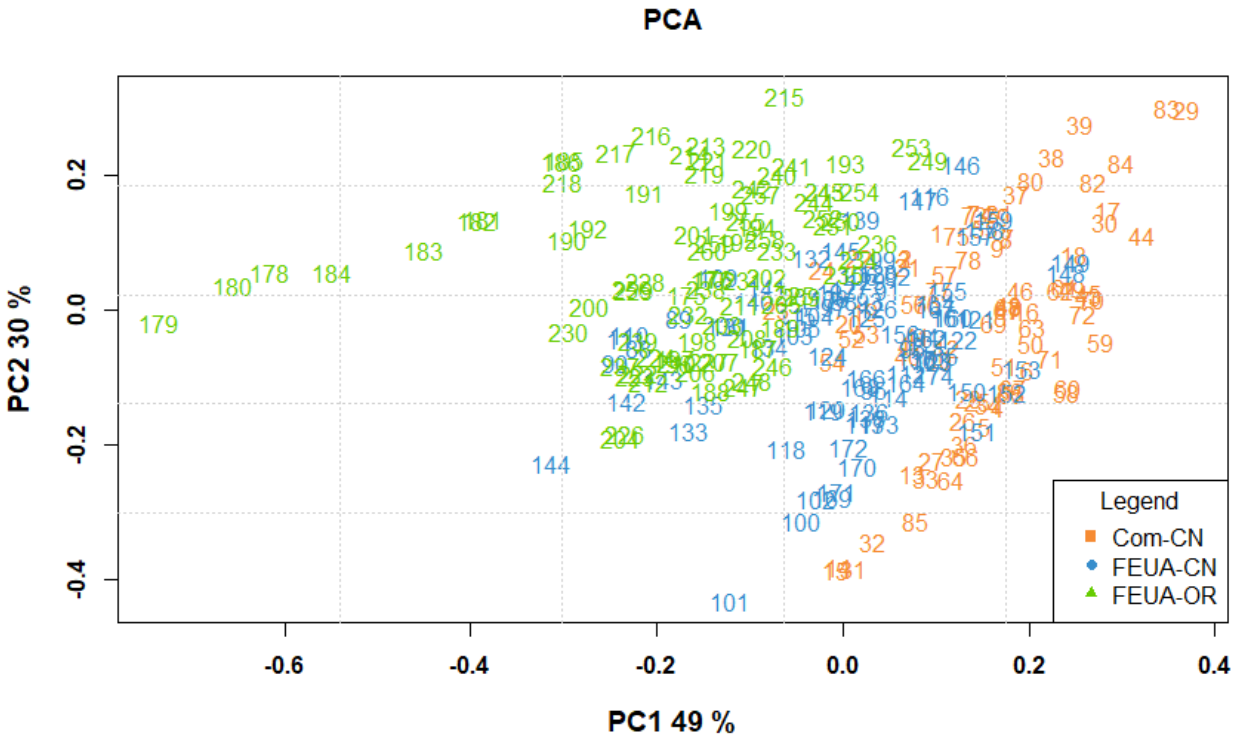

Figure 6.4.1: PC scores plot resulting from PCA on significant bin selected with ANOVA ($p<0.05$).

Considering this figure, it is possible to see that there are samples which are excessively distant from the others, so they are being deleted them from the dataset as they are outlier. Specifically, are removed the samples indicated in the figure as 101, 144, 178, 179, 180, 181, 182, 183, 184.

After that, another PCA is performed, always on significant bins selected with ANOVA, but considering the dataset without outliers, which is shown in Figure 6.4.2.

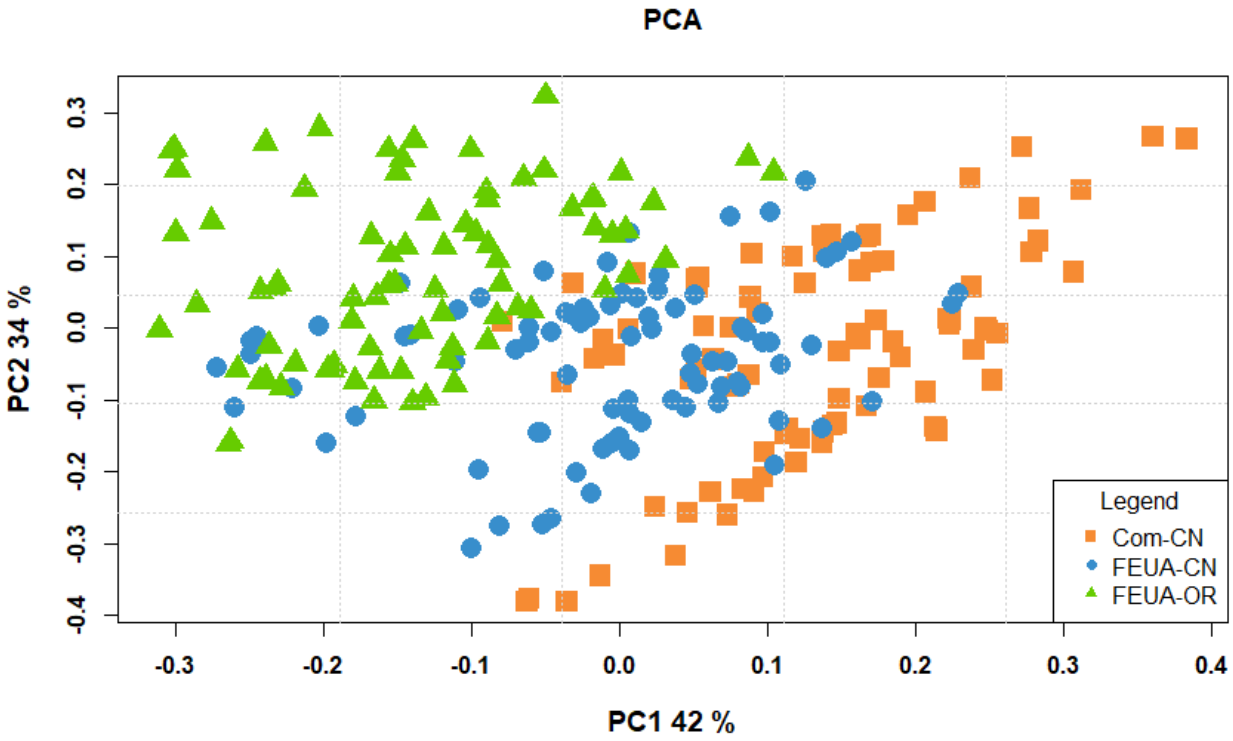

Figure 6.4.2: PC scores plot resulting from PCA on the dataset without outlier and on significant bin selected with ANOVA ($p<0.05$).

As it could be seen in Figure 6.1.3 PC1 and PC2 explain, respectively, 42 % and 34 % of the total variance. The combination of the first two PCs can cluster the samples according to the diet, as a clear separation could be observed among the three groups, highlighted in the figure with different colours. For a better illustration of the separation in Figure 6.4.3 are represented the ellipses of the three groups.

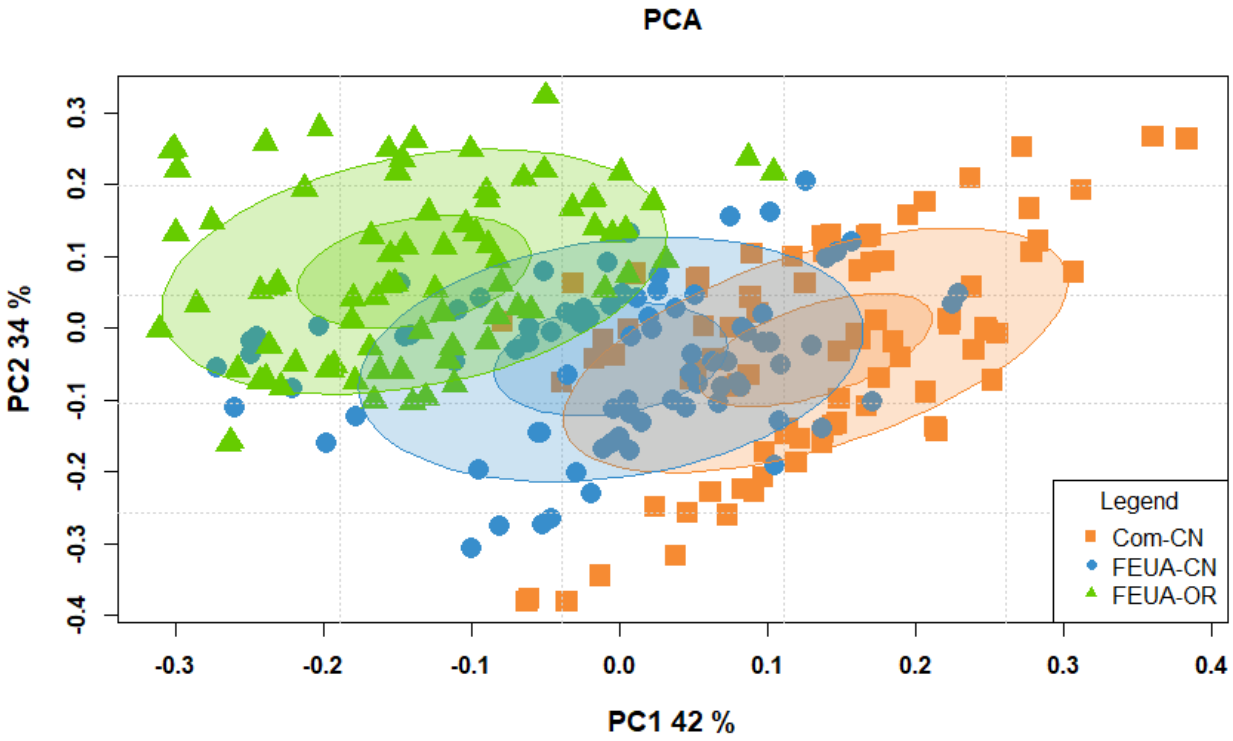

Figure 6.4.3: PC scores plot resulting from PCA on the dataset without outlier and on significant bin selected with ANOVA ($p<0.05$) with ellipses of the three groups.

As we can see, the ellipses corresponding to the three groups are only partially superimposed so we can conclude that there are clear differences between them.

Moreover, it is possible to see that, especially for what concerns PC2, there is a clear separation between the organic diet and the other two, while the conventional diets result less separated.

Some other information can be obtained from the analysis of PC loadings plot, as PC loadings indicate how much each NMR bin contributes to the description of every PC. PC1 and PC2 loadings are shown as bar plot in Figure 6.4.4. The height of each bar indicates how much, and in which direction, the spectral variable contributes to the PC, so that enables to deduce which are the bins that determine differences between the samples.

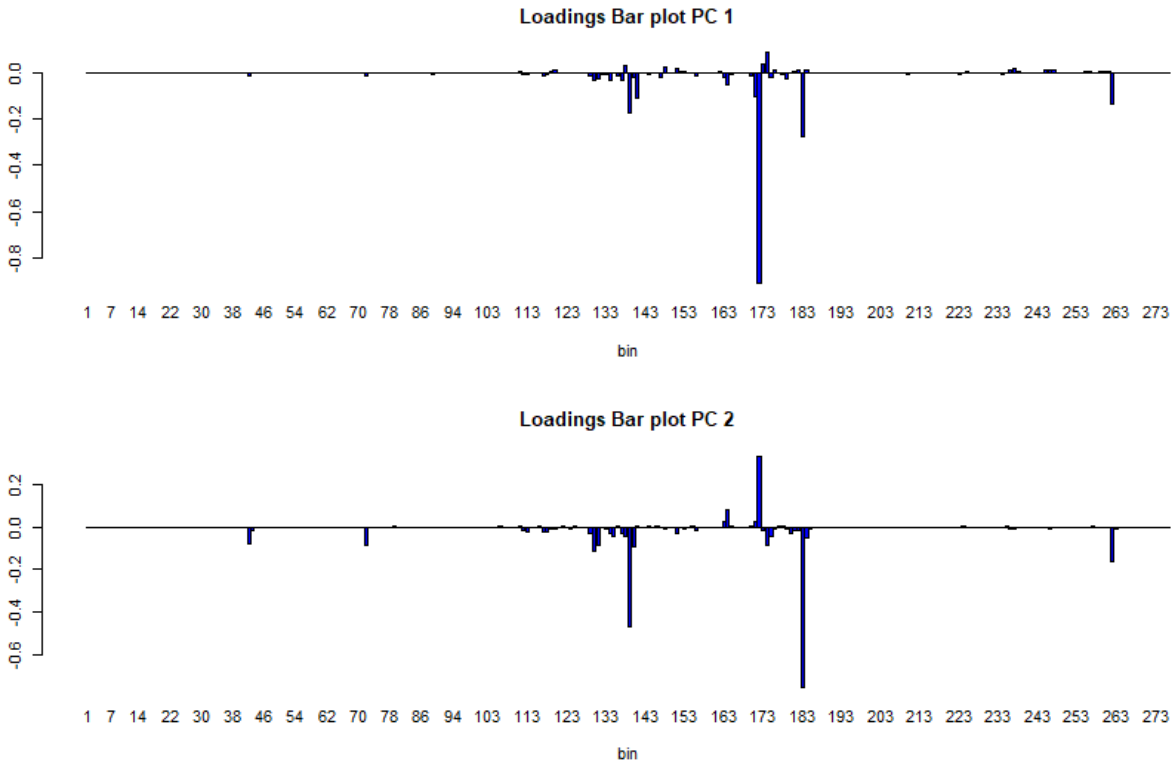

Figure 6.4.4: PC1 and PC2 bar plots of the importance of every loading.

Besides a visual observation of these graphs, is possible to select the loadings which have a value over a threshold in order to understand what are the most important, as discussed above. After the selection of the most important ones, it is possible to obtain the corresponding peaks since they have been assigned with ChenomX.

6.5 DIFFERENT CLASSES OF COMPOUNDS

In the table below, the bins corresponding to the most important loadings have been reported, with the centre, their range of chemical shift, and the assignment of the metabolite which gives that peak and the significance in PC1 and/or PC2.

Bin	Center	Range	Metabolites	Significant in:	
				PC1	PC2
42	7,765237	7,746937-7,783537	Histidine		✓
72	7,051851	7,033551-7,070151	Histidine		✓
130	4,124226	4,105926-4,142526	Lactate		✓
131	4,105933	4,087633-4,124233	Lactate		✓
139	3,941306	3,923006-3,959606	Creatine *	✓	✓
140	3,923014	3,904714-3,941314	Betaine		✓
141	3,90472	3,88642-3,92302	Betaine	✓	
164	3,410838	3,392538-3,429138	Taurine		✓
171	3,282795	3,264495-3,301095	Betaine	✓	
172	3,264503	3,246203-3,282803	Trimethylamine N-oxide	✓	✓
174	3,227918	3,209618-3,246218	Choline	✓	✓
183	3,044999	3,026699-3,063299	Creatine	✓	✓
184	3,026709	3,008409-3,045009	Creatine		✓
262	1,325557	1,307257-1,343857	Lactate	✓	✓

Table 6.5.1: bins corresponding to the most important loadings, with the center, the range of ppm of them, the metabolite which gives that peak and the significance in PC1 and/or PC2.

The assignment of the compounds is performed using the software ChenomX and in the Figures below (from Figure 6.5.1 to Figure 6.5.10) are reported the identification of most the significant ones as an example.

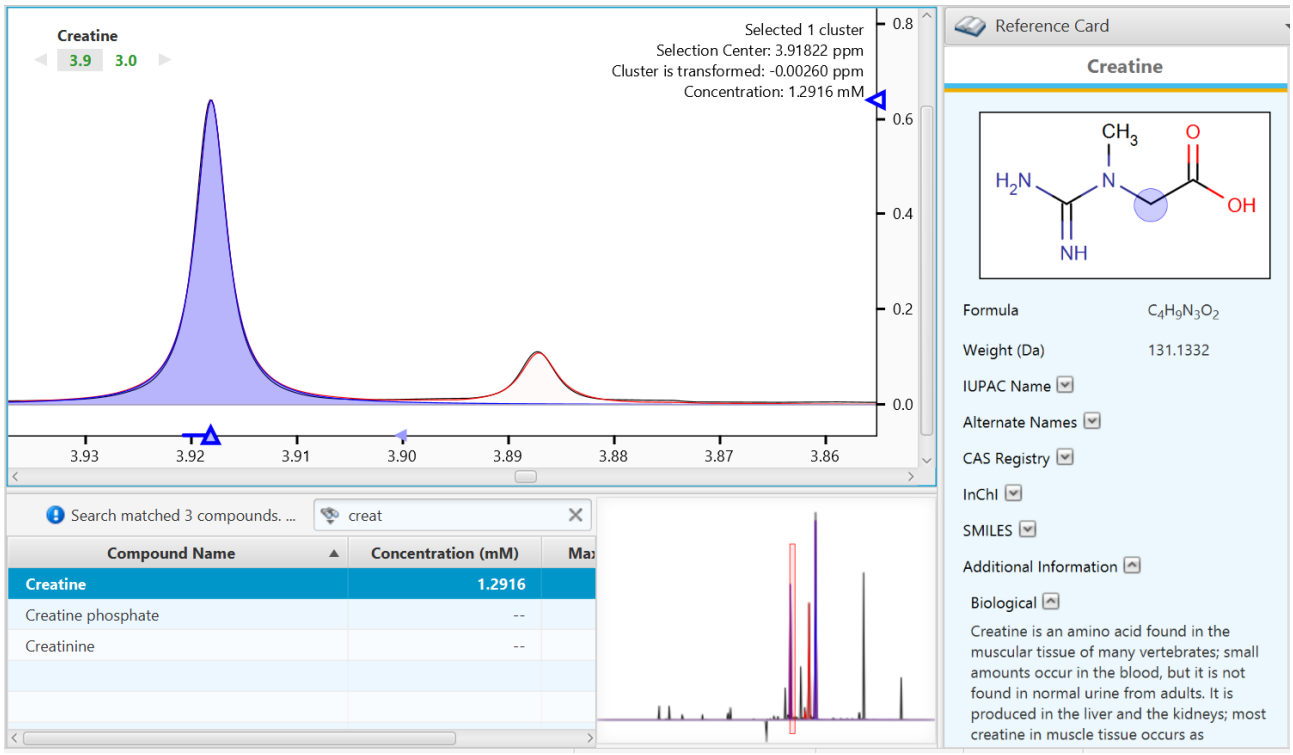

Figure 6.5.1: identification of creatine corresponding to bin 139.

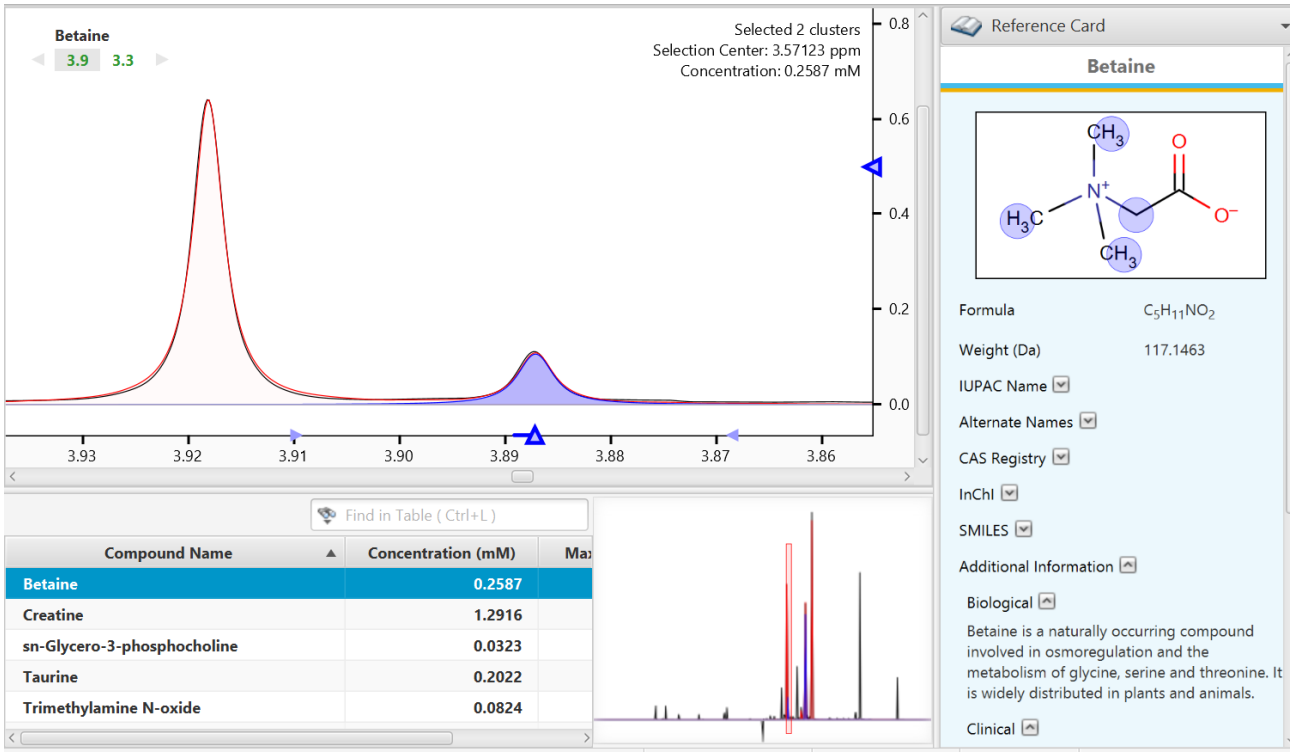

Figure 6.5.2: identification of betaine corresponding to bin 141.

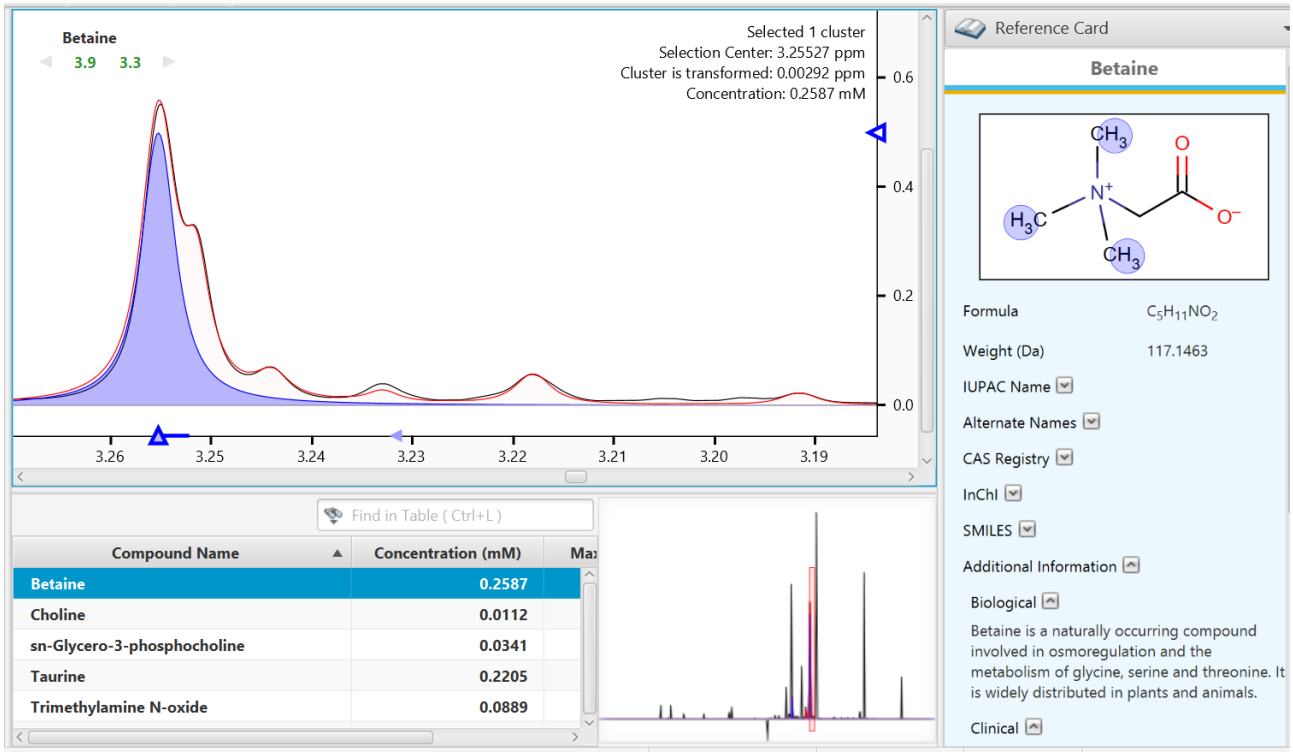

Figure 6.5.3: identification of betaine corresponding to bin 171.

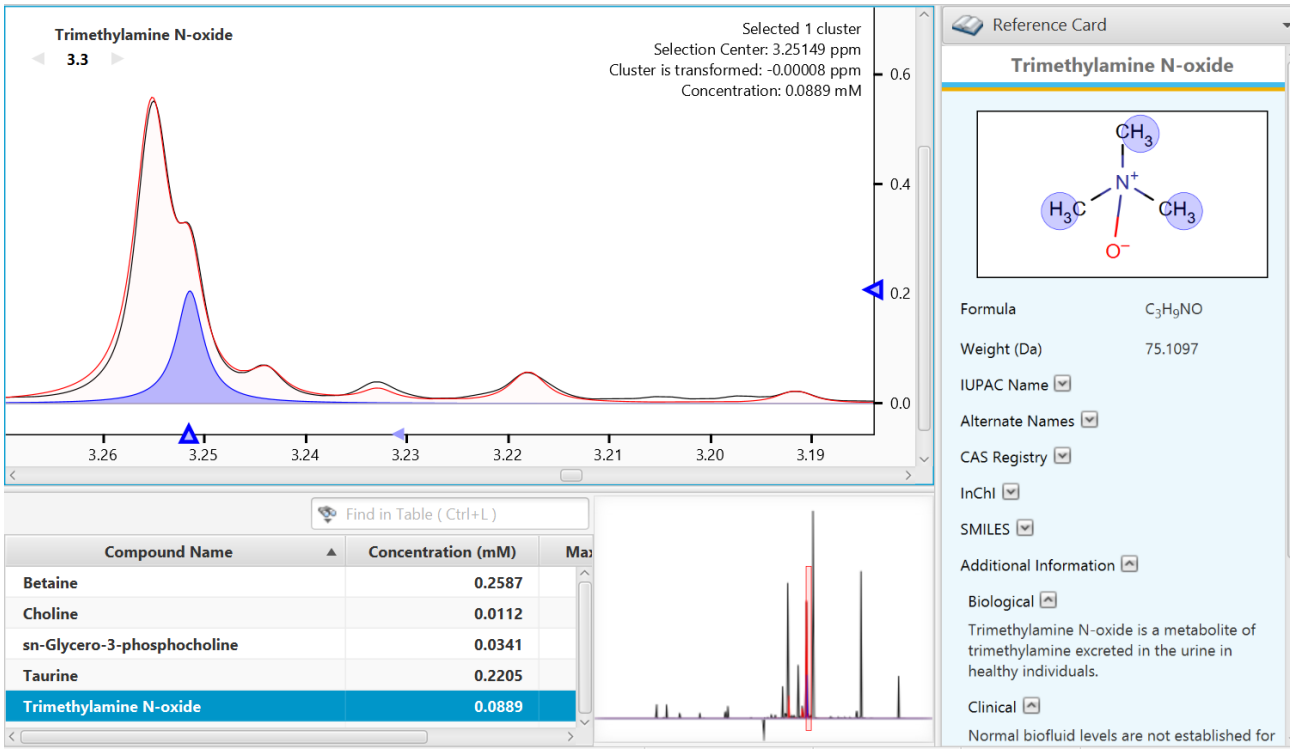

Figure 6.5.4: identification of trimethylamine N-oxide corresponding to bin 172.

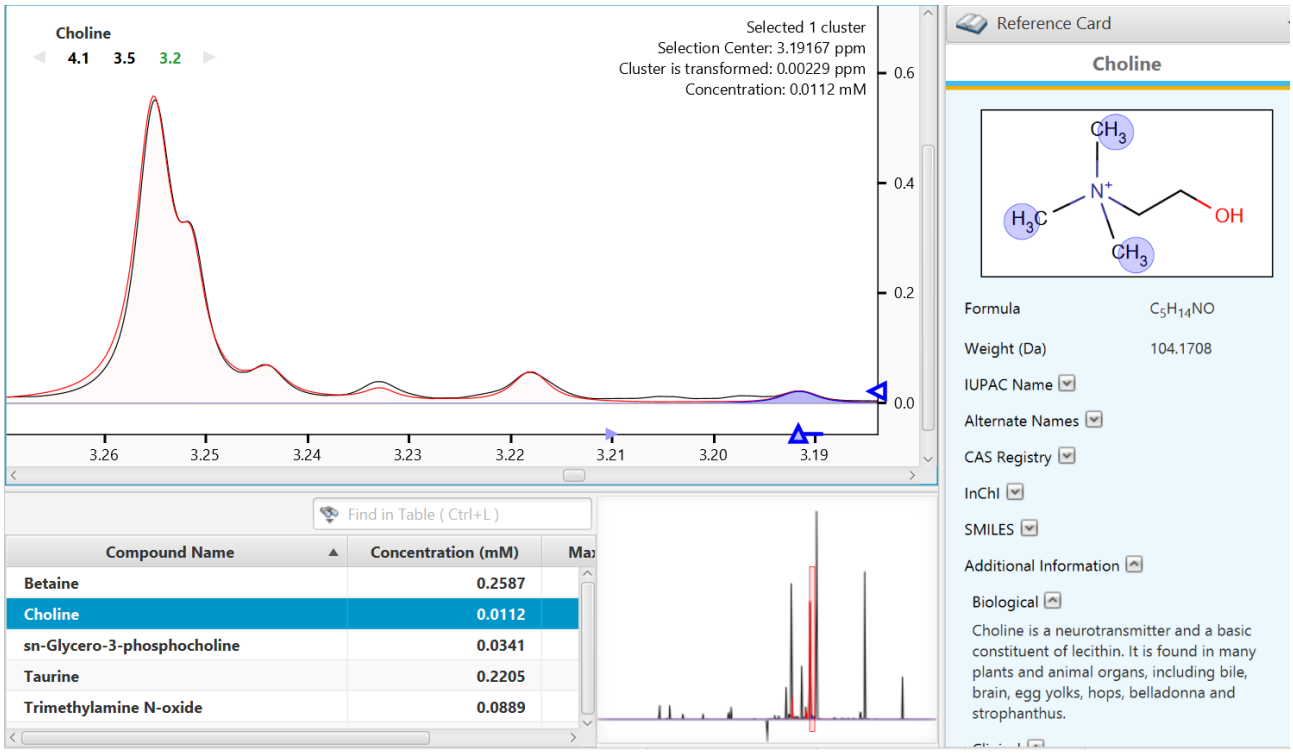

Figure 6.5.5: identification of choline corresponding to bin 174.

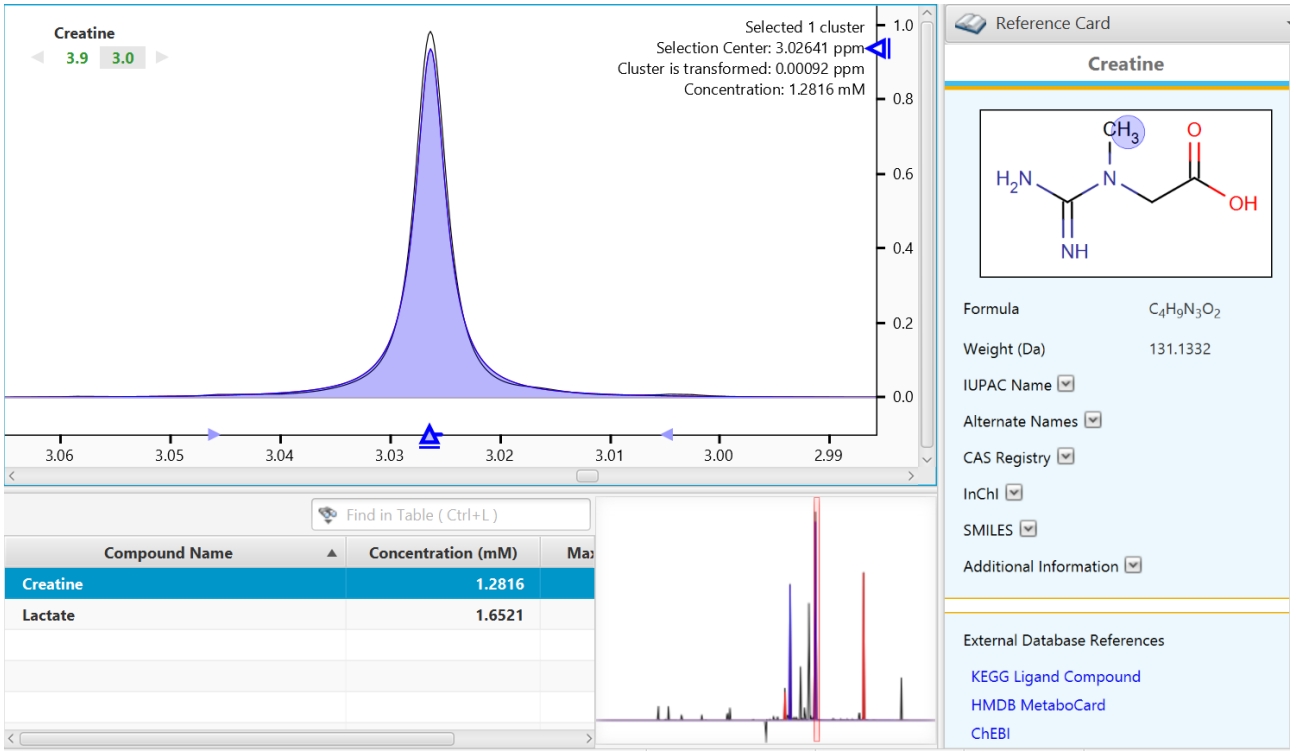

Figure 6.5.6: identification of creatine corresponding to bin 183.

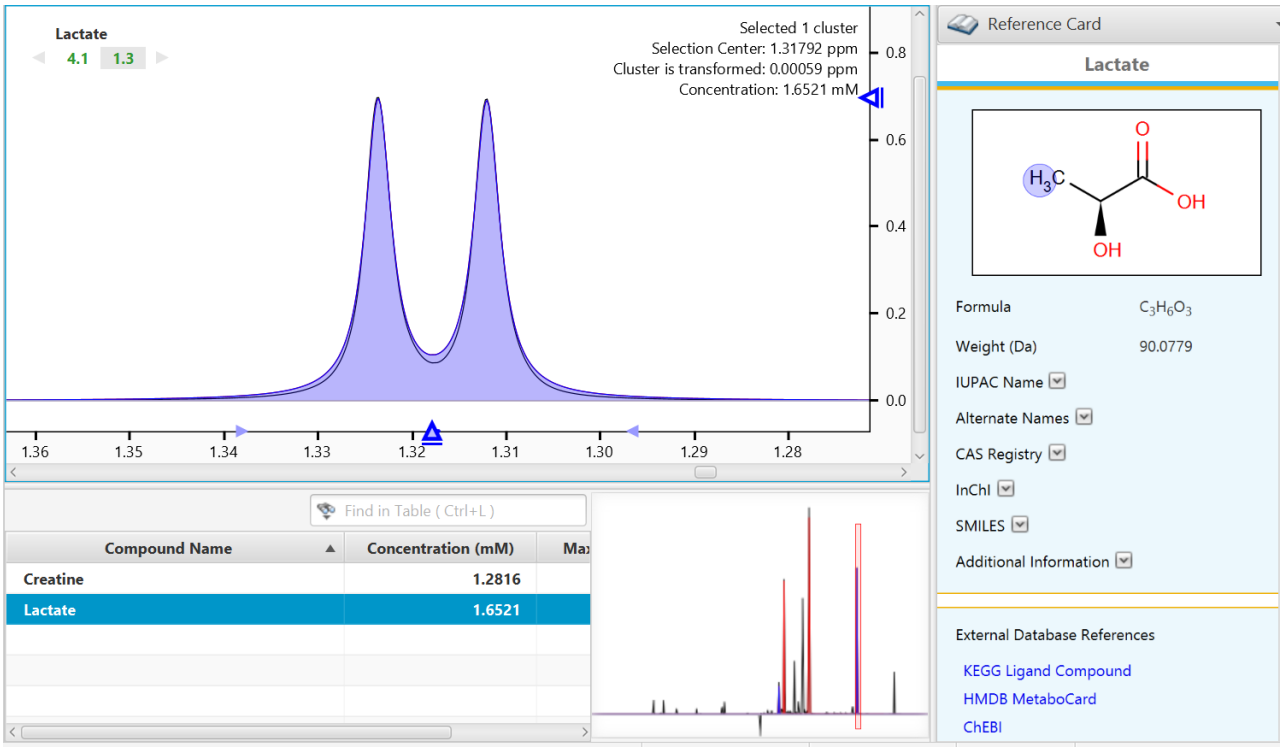

Figure 6.5.7: identification of lactate corresponding to bin 262.

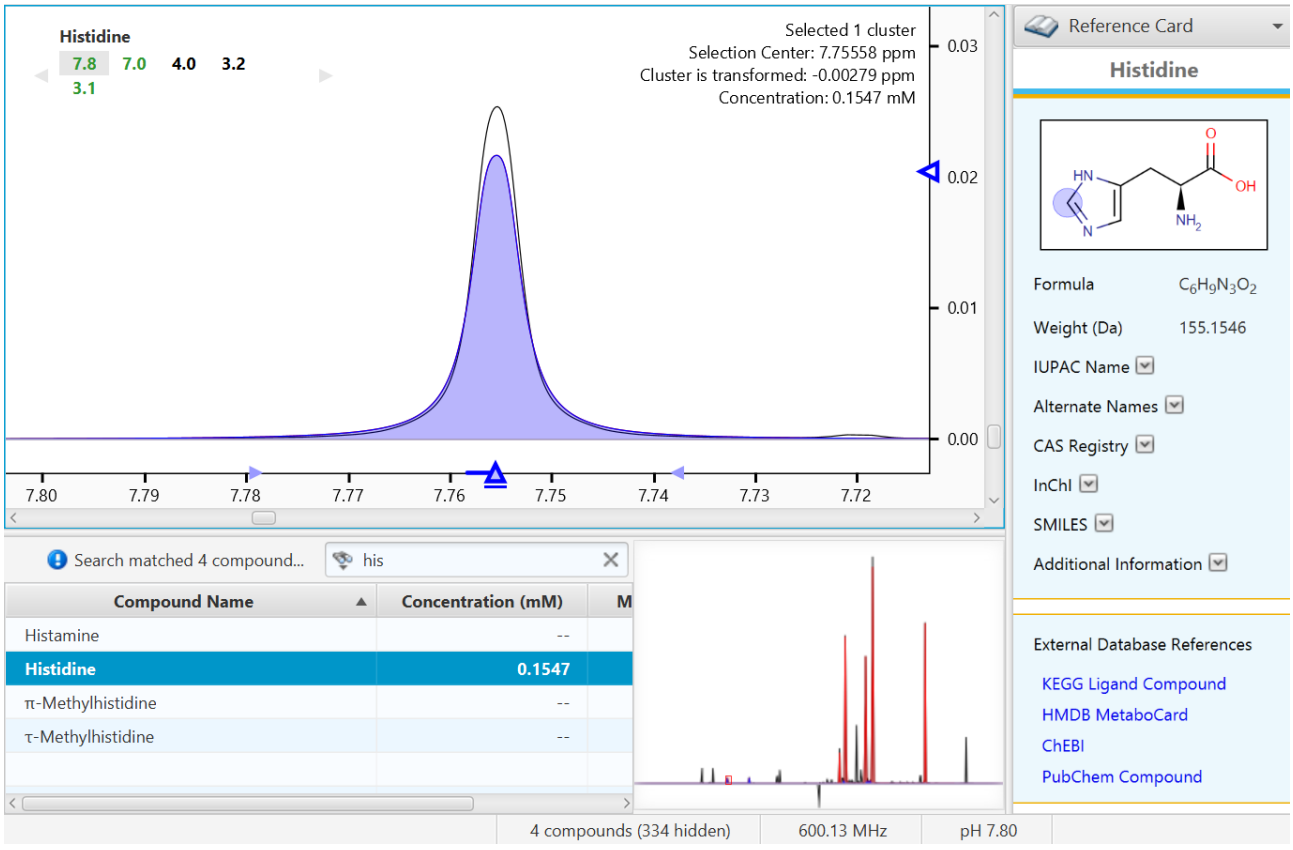

Figure 6.5.8: identification of histidine corresponding to bin 42.

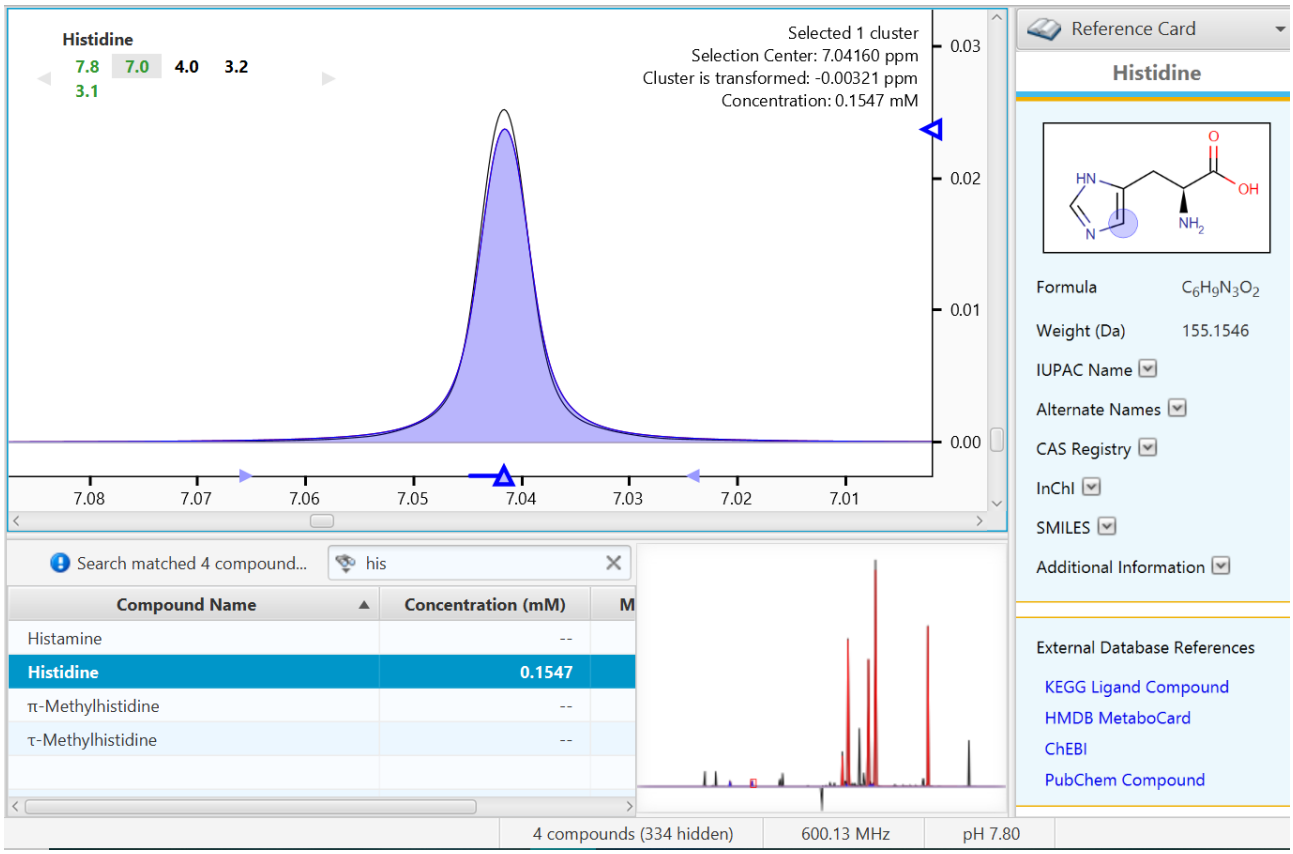

Figure 6.5.9: identification of histidine corresponding to bin 72.

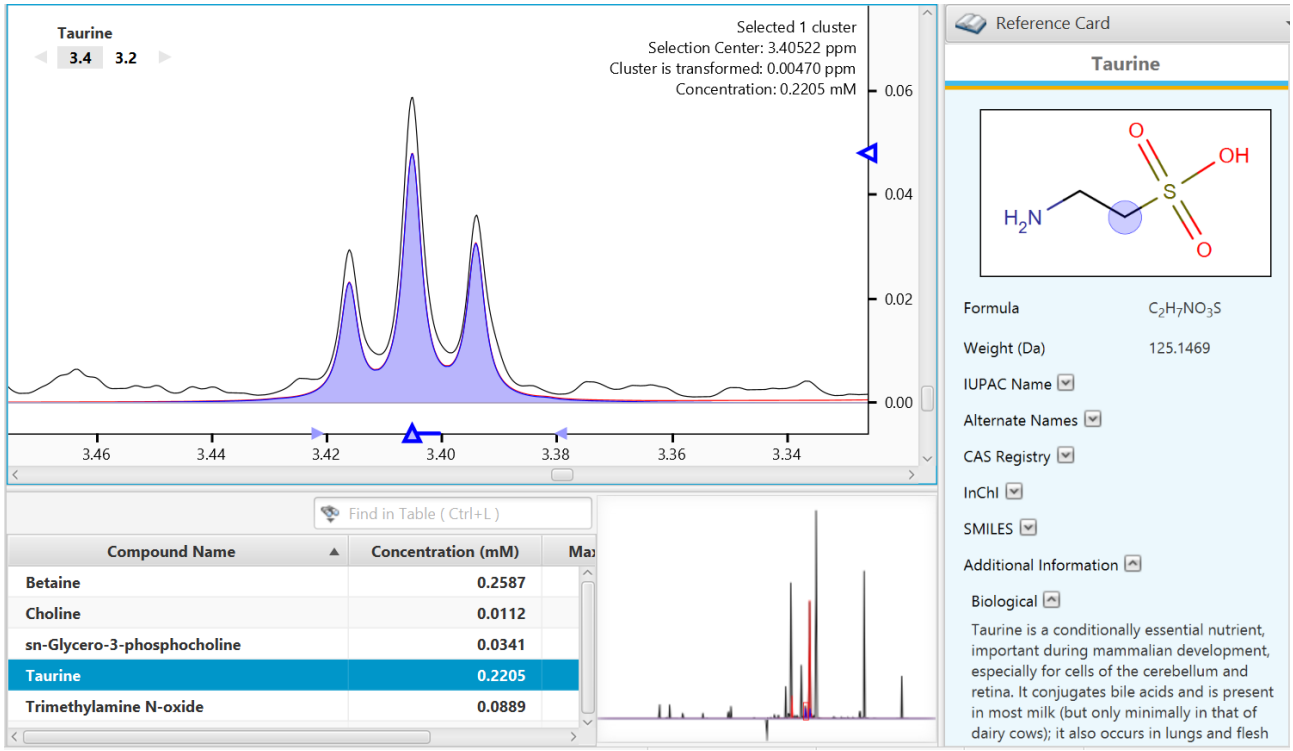

Figure 6.5.10: identification of taurine corresponding to bin 164.

To better understand the differences between the three diets considered, the concentration of the compounds which determine a separation along PC1 and PC2 are represented with BoxPlots, as reported in the Figures below (from Figure 6.5.11 to Figure 6.5.20).

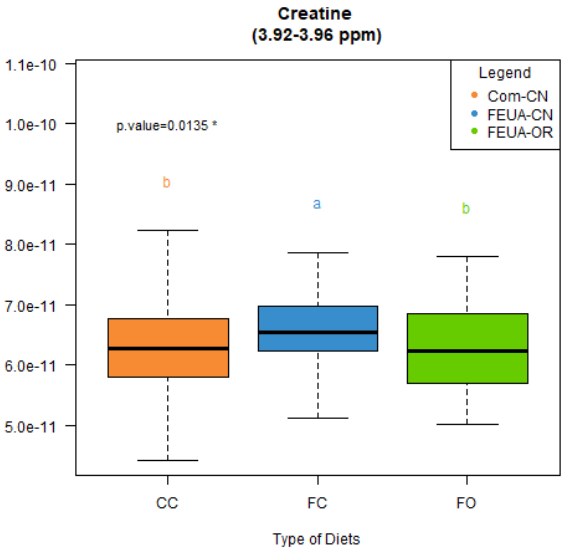

Figure 6.5.11: BoxPlot of creatine's peak corresponding to bin 139.

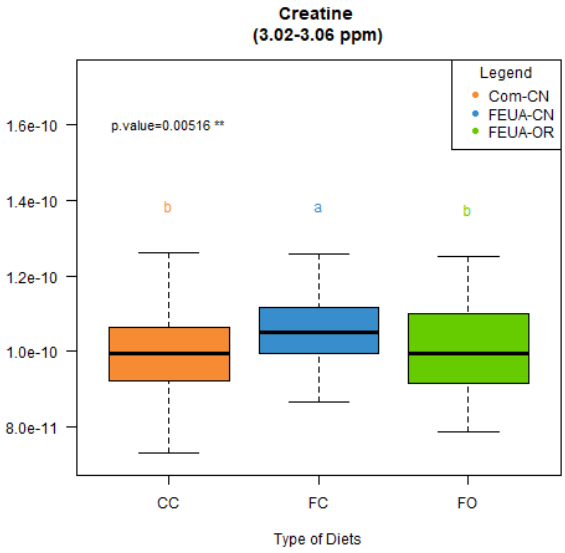

Figure 6.5.12: BoxPlot of creatine's peak corresponding to bin 183.

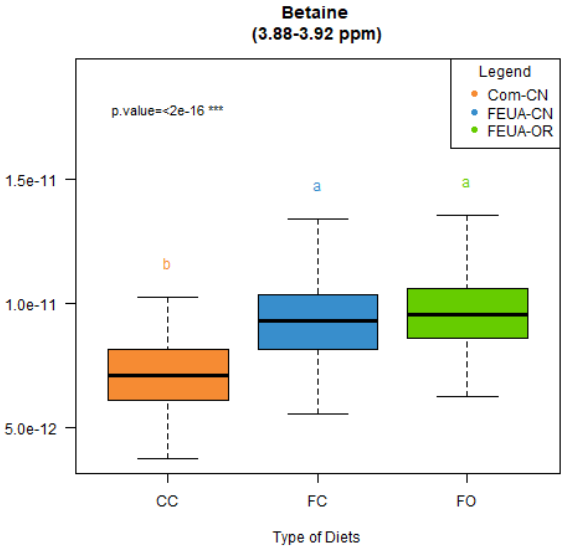

Figure 6.5.13: BoxPlot of betaine's peak corresponding to bin 141.

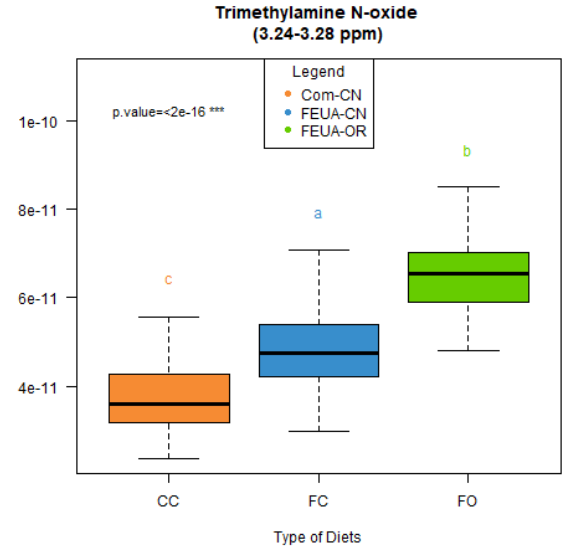

Figure 6.5.14: BoxPlot of trimethylamine N-oxide's peak corresponding to bin 172.

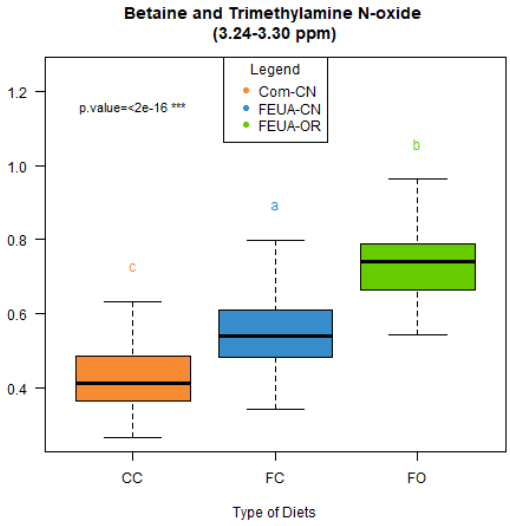

Figure 6.5.15: BoxPlot of betaine and trimethylamine N-oxide's peaks corresponding to bins 171 and 172. These two peaks are considered together as they are in a row.

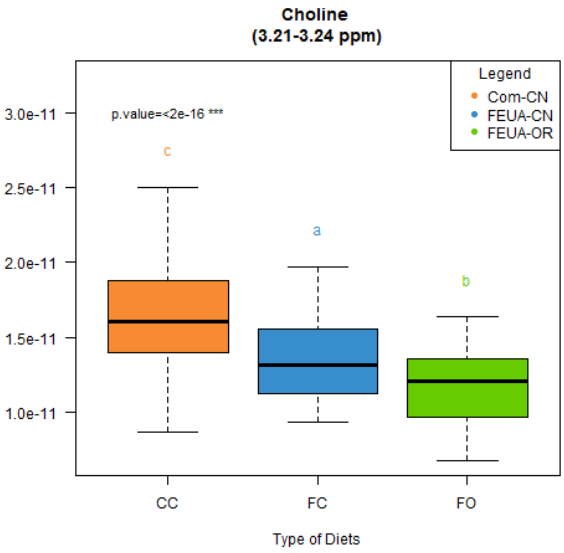

Figure 6.5.16: BoxPlot of choline's peak corresponding to bin 174.

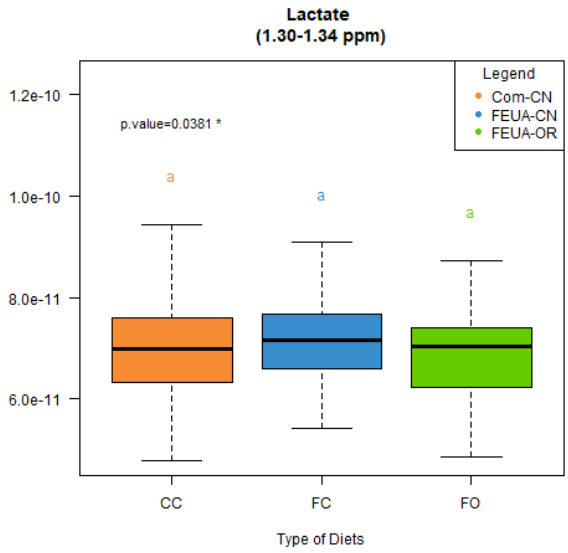

Figure 6.5.17: BoxPlot of lactate's peak corresponding to bin 262.

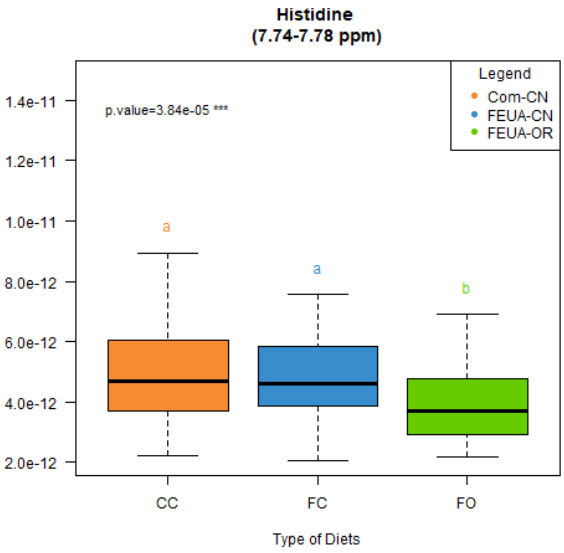

Figure 6.5.18: BoxPlot of histidine's peak corresponding to bin 42.

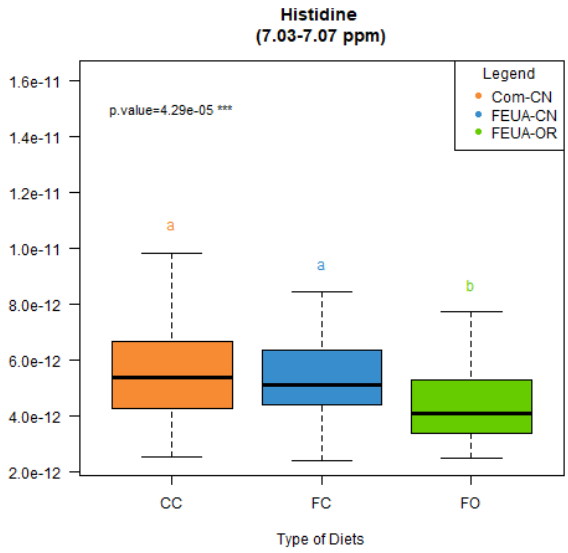

Figure 6.5.19: BoxPlot of histidine's peak corresponding to bin 72.

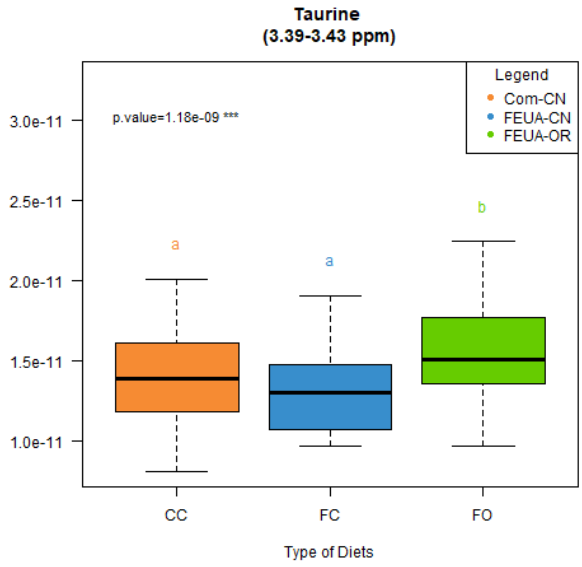

Figure 6.5.20: BoxPlot of taurine's peak corresponding to bin 164.

For what **Trimethylamine N-oxide** (TMAO) concerns, it is usually converted after the death in trimethylamine (TMA), as discussed above in paragraph 3.5.3.2.1, so ordinarily TMAO is used as freshness indicator as when the fish is deteriorate TMAO decreases and TMA increases. However, in this case there are no significant differences in the content of TMA, so the odds in the content of TMAO would not be related to the different state of freshness, but to a unlike endogen production in fishes feed with different diets. In the body of fishes TMAO acts as osmoregulator and differences in its content could be due to species, season and location of catch (V.M. Ocano-Higuera et al., 2011 & P.K. Prabhakar et al., 2020).

Another compound which has an osmoregulatory role in the body of fishes is **betaine** which acts as methyl donor to perform this function; increases of temperature and salinity stimulate the production of this compound in the mitochondria, as it is accumulated and helps the cells to bear changes in the osmotic pressure avoiding excessive water losses from them (A. Polat et al., 1999). This compound is fundamental in the diet of carnivorous fishes, so normally it is added in the feeds for breed fishes to cover the needs (A. Polat et al., 1999). Moreover, betaine is used in the feeds as an "attractant" as it makes the taste and smell more similar to what the fishes would eat in nature and so more appetizing for them (A. Polat et al., 1999).

Betaine could be converted in TMAO or donate methyl for the synthesis of creatine (A. Polat et al., 1999).

Moreover, in mitochondria betaine could be synthetized from **choline** (A. Polat et al., 1999), this compound has some important roles in the body such as maintenance of cell membranes structural integrity, cholinergic transmission, trans-membrane signal, prevention of hepatic carcinogenesis, lipids and cholesterol transport and metabolism; these functions are absolved even by the compounds formed from this molecule and so even by betaine (S. Das et al., 2021).

The assumption of this compound with the diet is fundamental for the fishes as they are even able to synthetize it, but usually the endogenous synthesis is not sufficient to cover the needs; given its many functions in the organism the deficiency of this compound may cause various ailments (S. Das et al., 2021).

Another significant compound found is **creatine.** In the muscle of fishes are present creatine and phosphocreatine as reserve of energy, in anaerobic conditions phosphocreatine is converted in creatine in order to produce ATP from ADP, so after the death of the fish phosphocreatine is quickly depleted (F. Savorani et al., 2010). This is the reason why we assume that the peaks of spectra are due only to creatine.

A greater presence of creatine in the muscle can indicate an increase in the production of ATP (R. Abro et al., 2014). The differences in the presence of this compound could be due to odds in the stress suffered before the catch, as the stress stimulates a grater production of ATP and so higher conversion of phosphocreatine in creatine (T. Morkore et al., 2008).

An acute stress before the catch also stimulates the *post-mortem* production of **lactate** through glycolysis (T. Morkore et al., 2008), indeed when the fishes struggling and try to escape before the death, they make rapid and intense movements, which bring to accumulation of lactate in the muscle. Anyway, whatever is the stress before the death after

it the glycogen present in the muscle is hydrolyzed in glucose, which enters in the glycolysis and allows the production of ATP and lactate (T. Morkore et al., 2008).
An interesting aspect related to lactate is the fact that its formation is connected to the reduction of pH after the death and this decrease in turns influences the release of lytic enzymes from lysosomes, so a greater production of lactate can cause a higher autolysis, and so degradation, of the product (A. Ciampa et al., 2012).

For what concerns PC2 it is possible to see that there are two additional compounds: taurine and histidine, which determine differences between the three groups.
Taurine is a sulphonic acid present in high concentration in the animal tissues and it has been demonstrated that is essential in the feeding of the most important fish species, as it could be synthetized from cysteine in the fish body, but not in sufficient quantities. The removal from feed of some ingredient rich in taurine, such as fishmeal, can lead to deficiency, which can reduce the growth and survival, increase the susceptibility to diseases and compromise the larvae development. Moreover, taurine influences the osmoregulation and the membranes stability, so its deficiency can lead to a series of physiological problems and tissues' changes (G.P. Salze et al., 2015).
In humans this compound is important from the nutritional point of view and fish is the main source of the free form of this amino acid, which can have some important roles even in the human metabolism, for example it has been demonstrated that it is related with the cholesterol metabolism (F. Savorani et al., 2010).
Histidine, as some other free amino acids, acts as osmoregulator in the fish body (A. Ciampa et al., 2012). It is an essential amino acid for fishes and its deficiency can reduce the growing performances and the feed conversion ratio (Y.-J. Gao et al., 2016).

7 CONCLUSIONS

The differences in the concentration of the metabolites detected cannot be attributed to the environmental conditions as it has been verified that the fishes were farmed during the same period in three cages where the environmental conditions were the same. So, it is possible to deduce that the differences are due to unlike metabolisms in the fishes feed with different diets.

Considering PC1 it is possible to notice that there is a clear separation between all the three diets. Regarding the metabolites illustrated previously **TMAO**, **betaine** and **choline** are the compounds which variate the most between fishes feed with different diets. These compounds are linked at the metabolic level. In the group where choline is less concentrated, corresponding to the fishes feed with the FutureEUAqua organic diet, TMAO and betaine are more concentrated and the other way around in fishes feed with the commercial conventional diet. Considering that choline is the precursor of both TMAO and betaine we can think that the fishes feed with the FutureEUAqua organic diet convert more choline in TMAO and betaine. It could indicate that these fishes are more able to synthetize osmoregulator compounds and so they are more adaptable to the environment as they can maintain the homeostasis more easily.

As already mentioned, the environmental conditions were the same for all the three groups feed with different diets, but it does not necessarily mean that there were not environmental stresses. So, it is possible to think that fishes feed with different diets react in unlike ways to the environmental stresses, so the metabolic responses triggered by these stresses are in turn different. This could explain why the concentrations of these three metabolites, which help to respond to stresses, are different in fishes feed with different diets.

For what concerns **creatine**, as we can see from the BoxPlots reported before, it is slightly more concentrated in fishes feed with the FutureEUAqua conventional diet, while its presence is approximately the same in the fishes feed with the other two diets, even if the overall differences are very limited. Always from the observation of BoxPlots it is possible to see that there are no significant differences in the presence of **lactate** between the fishes feed with different diets. Considering that this compound is related to the stress suffered before the death it is possible to deduce that the differences are due to this aspect and not to the diet, as it is possible that fishes from the same cage (feed with the same diet) have different levels of stress when caught. The same conclusion can be effective even for the creatine, since this is related to the stress suffered by the fish before the death too.

As discussed above, concerning PC2, it is possible to see that there are two additional significant compounds: **taurine** and **histidine** and that there is a clear distinction between the group feed with FutureEUAqua organic diet from the other two. From the observation of the BoxPlots it is possible to notice that the change in the metabolite concentration is coherent with that distinction, as both the compounds show to have the same concentration in the fishes feed with the two conventional diets, but a different amount in the ones nourish with the FutureEUAqua organic diet.

Generally, it is possible to notice that the fishes feed with commercial conventional and FutureEUAqua organic diets are extremely different, while the ones feed with FutureEUAqua conventional diet are somewhere in between the other.

8 REFERENCES

1. A. Ciampa (2013). *Development of methodologies for fish freshness assessment using metabonomic applications*. Doctorate thesis in “Scienze e Biotecnologie degli alimenti”, Alma Mater Studiorum – Università di Bologna
2. A. Ciampa, G. Picone, L. Laghi, H. Nikzad, F. Capozzi (2012). *Changes in the Amino Acid Composition of Bogue (Boops boops) Fish during Storage at Different Temperatures by ^{1}H-NMR Spectroscopy*. Nutrients, 4, pp. 542 – 553, DOI: 10.3390/nu4060542
3. A. Craig, O. Cloarec, E. Holmes, J.K. Nicholso, J.C. Lindon (2006). *Scaling and Normalization Effects in NMR Spectroscopic Metabonomic Data Sets*. Analytical Chemistry, 70, pp. 2262 – 2267, DOI: 10.1021/ac0519312
4. A. D. Southam, J. M. Easton, G. D. Stentiford, C. Ludwig, T. N. Arvanitis, M. R. Viant (2008). Metabolic Changes in Flatfish Hepatic Tumours Revealed by NMR-Based Metabolomics and Metabolic Correlation Networks. Journal of Proteome Research, 7(12), pp. 5277 – 5285, DOI: 10.1021/pr800353t
5. A. Polant, G. Beklevik (1999). *The importance of betaine and some attractive substances as fish feed additives*. In: J. Brufau (ed.), A. Tacon (ed.). *Feed manufacturing in the Mediterranean region: Recent advances in research and technology*. CIHEAM, cahiers Options Méditerranéennes, 37, pp. 217 – 220
6. B.L. Peachey, E.M. Scott, D.M. Gatlin III (2018). *Dietary histidine requirement and physiological effects of dietary histidine deficiency in juvenile red drum Scianeop ocellatus*. Acquaculture, 483, pp. 244 – 251, DOI: 10.1016/j.aquaculture.2017.10.032
7. B. Russell, K.E. Carpenter, D. Pollard (2014). *Sparus aurata*. *The IUCN Red List of Threatened Species* 2014: e.T170253A1302459. https://dx.doi.org/10.2305/IUCN.UK.2014-3.RLTS.T170253A1302459.en. [Downloaded on 16 March 2021]
8. B. Sotton, A. Paris, S. Le Manach, A. Blond, G. Lacroix, A. Millot, C. Duval, Q. Qiao, A. Catherine, B. Marie (2017). Global metabolome changes induced by cyanobacterial blooms in three representative fish species. Science of the Total Environment, 590–591, pp. 333 – 342, DOI: 10.1016/j.scitotenv.2017.03.016
9. Commission decision No 95/149 of the European Commission of 8 March 1995 on fixing the total volatile basic nitrogen (TVB-N) limit values for certain categories of fishery products and specifying the analysis methods to be used

10. Commission Regulation (EC) No 710/2009 of 5 August 2009 amending Regulation (EC) No 889/2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007, as regards laying down detailed rules on organic aquaculture animal and seaweed production
11. Council Regulation (EC) No 834/2007 of 28 June 2007 on organic production an labeling of organic products and repealing Regulation (EEC) No 2092/91
12. Council Regulation (EC) No 2406/96 of 26 November 1996 on laying down common marketing standards for certain fishery products
13. D. Pouliquen, M.-H. Omnes, F. Seguin, J.-L. Gaignon (1998). *Changes in the dynamics of structured water and metabolite contents in early developing stages of eggs of turbot (Psetta maxima)*. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 120(4), pp. 715 – 726
14. E. Carrasco, A. Valero, F. Perez-Rodriguez, R.M. Garcia-Gimeno, G. Zurera (2007). *Management of microbiological dafety of ready-to-eat meat products by mathematical modelling: Listeria monocytogenes as an example*. International Journal of Food Microbiology, 114, pp. 221 – 226, DOI: 10.1016/j.ijfoodmicro.2006.09.013
15. E. Hatzakis, (2018). *Nuclear Magnetic Resonance (NMR) Spectroscopy in Food Science: A Comprehensive Review*. Comprehensive Reviews in Food Science and Food Safety, 18(1), pp.189-220, DOI: 10.1111/1541-4337.12408
16. E. Kokushi, S. Uno, T. Harada, J. Koyama (2012). *^{1}H NMR-Based Metabolomics Approach to Assess Toxicity of Bunker a Heavy Oil to Freshwater Carp, Cyprinus carpio*. Environmental toxicology, 27(7), pp. 404 – 414, DOI: 10.1002/tox.20653
17. E. M. Santos, J. S. Ball, T. D. Williams, H. Wu, F. Ortega, R. van Aerle, I. Katsiadaki, F. Falciani, M. R. Viant, J. K. Chipman, C. R. Tyler (2010). *Identifying Health Impacts of Exposure to Copper Using Transcriptomics and Metabolomics in a Fish Model*. Environmental Science & Technology, 44(2), pp. 820 – 826, DOI: 10.1021/es902558k
18. E. Shumilina, A. Ciampa, F. Capozzi, T. Rustad, A. Dikiy (2015). *NMR approach for monitoring post-mortem changes in Atlantic salmon fillets stored at 0 and 4 °C*. food Chemistry, 184, pp. 12 – 22, DOI: 10.1016/j.foodchem.2015.03.037
19. FAO, 2018. *The State of World Fisheries and Aquacolture*. ISBN: 978-5-130562-1, ISSN: 1020-5489
20. F. Savorani, G. Picone, A. Badiani, P. Fagioli, F. Capozzi, S.B. Engelsen (2010). *Metabolic profiling and aquaculture differentiation of gilthead sea bream by ^{1}H NMR*

metabonomics. Food Chemistry, 120, pp. 907 – 914, DOI: 10.1016/j.foodchem.2009.10.071

21. F. Schwagele (2005). *Traceability from European prespective*. Meat Science, 71, pp. 164 – 173, DOI: 10.1016/j.meatsci.2005.03.002
22. G. Duflos, C. Dervin, P. Malle, S. Bouquelet (1999). *Use of Biogenic Amines to Evaluate Spoilage in Plaice (Pleuronectes platessa) and Whiting (Merlangus merlangus)*. Journal of AOAC International, 82(6), pp. 1357 – 1363
23. G.P. Salze, D.A. Davis (2015). *Taurine: a critical nutrient for future fish feeds*. Aquaculture, 437, pp. 215 – 229, DOI: 10.1016/j.aquaculture.2014.12.006
24. G. Picone, S.B. Engelsen, F. Savorani, S. Testi, A. Badiani, F. Capozzi (2011). *Metabolomics as a Powerful Tool for Molecular Quality Assessment of the Fish Sparus aurata*. Nutrients, 3, pp. 212 – 227, DOI: 10.3390/nu3020212
25. G. Simonetti, A. Padella, C. Mengucci, E. Fonzi, G. Picone, M. Pazzaglia, M. Perricone, M. Fontana, S. Bruno, M. T. Bochicchio, E. Franchini, J. Nanni, G. Marconi, I. F. do Valle, R. De Tommaso, A. Ferrari, M. Ghetti, R. Napolitano, C. Papayannidis, A. Ghelli Luseran di Rora, C. Cerchione, E. Ottaviani, G. Castellani, D. Remondini, F. Capozzi, G. Martinelli (2019). PF197 a new classification of acute myeloid leukemia based on integrated genomics and metabolomics. HemaSphere, 3(S1), p. 51
26. H.M. Velioglu, H.T. Temiz, I.H. Boyaci (2015). *Differentiation of fresh and frozen-thawed fish samples using Ramam spectroscopy coupled with chemometric analysis*. Food Chemistry, 172, pp. 283 – 290, DOI: 10.1016/j.food.chem.2014.09.073
27. I. Fernandez-Segovia, I. Escriche, A. Serra (2008). Evolution of Volatile Fraction and ATP Related Compounds During Storage of Desalted Cod (Gadus morhua). Food Science and Technology international, 14(5_suppl), pp.37-47
28. I. Lardon, M. Eyckmans, T. N. Vu, K. Laukens, G. De Boeck, R. Dommisse (2013). *^{1}H-NMR study of the metabolome of a moderately hypoxia-tolerant fish, the common carp (Cyprinus carpio)*. Metabolomics, 9(6), pp. 1216 – 1227, DOI: 10.1007/s11306-013-0540-y
29. J. Freitas, P. Vaz-Pires, J.S. Camara (2021). *Quality Index Method for fish quality control: Understanding the applications, the appointed limits and the upcoming trends*. Trends in Food Science & Technology, 111, pp. 333 – 345
30. J. Keeler, (2013). *Understanding NMR Spectroscopy*. Hoboken: Wiley.
31. J.L. Mietz, E. Karmas (1977). *Chemical quality index of canned tuna as determined by high-pressure liquid chromatography*. Journal of Food Science, 42(1), pp. 155 – 158

32. J.M. Cevallos-Cevallos, J.I. Reyes-De-Corcuera, E. Etxeberria, M.D. Danyluk, G.E. Rodrick (2009). Metabolomic analysis in food science: a review. Trends in Food Science & Technology, 20, pp. 557-566, DOI: 10.1016/j.tifs.2009.07.002
33. J. Qian, L. Ruiz-Garcia, B. Fan, J.I. Robla Villalaba, U. McCarthy, B. Zhang, Q. Yu, W. Wu (2020). *Food traceability system from governmental, corporate, and consumer perspectives in the European Union and China: A comparative review*. Trends in Food Science & Technology, 99, pp. 402 – 412, DOI: 10.1016/j.tifs.2020.03.025
34. K. Grigorakis (2007*). Compositional and organoleptic quality of farmed and wild gilthead sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) and factors affecting it: A review*. Aquaculture, 272, pp. 55 – 75, DOI: 10.1016/j.aquaculture.2007.04.062
35. K. S. Solanky, I. W. Burton, S. L. MacKinnon, J. A. Walter, A. Dacanay (2005). *Metabolic changes in Atlantic salmon exposed to Aeromonas salmonicida detected by ^{1}H-nuclear magnetic resonance spectroscopy of plasma*. DISEASES OF AQUATIC ORGANISM, 65, pp. 107 – 114
36. L. M. Samuelsson, B. Bjorlenius, L. Forlin, D. G. J. Larsson (2011). *Reproducible ^{1}H NMR-Based Metabolomic Responses in Fish Exposed to Different Sewage Effluents in Two Separate Studies*. Environmental Science and Technology, 45(4), pp. 1703 – 1710, DOI: 10.1012/es104111x
37. L. M. Samuelsson, D. G. Joakin Larsson (2008). *Contributions from metabolomics to fish research*. Molecular BioSystems, 4, pp. 974 – 979, DOI: 10.1039/b804196b
38. L. M. Samuelsson, L. Forlin, G. Karlsson, M. Adolfsson-Erici, D.G. J. Larson (2006). *Using NMR metabolomics to identify responses of an environmental estrogen in blood plasma*. Aquatic Toxicology, 78(4), pp. 341 – 349, DOI: 10.1016/j.aquatox.2006.04.008
39. L. Sola, A. Moretti, D. Corsetti, N. Karaiskou, A. Magoulas, A.R. Rossi, M. Rye, A. Triantafyllidis, C.S. Tsigenopoulos (2007), Gilthead seabream – Sparus aurata. Compendium of Genetic Impact of Aquaculture Activities on Native, 47-54
40. M. A. Turner, M. R. Viant, S. J. The, M. L. Johnson (2007). *Developmental rates, structural assimetry, and metabolic fingerprints of steelhead trout (Oncorhynchus mykiss) eggs incubated at two temperatures.* Fish Physiology and Biochemistry, 33, pp. 59 – 72, DOI: 10.1007/s10695-006-9117-2
41. M. Kent, J. Oehlenschlager, S. Mierke-Klemeyer, R. Knochel, F. Daschner, O. Schimmer (2004). *Estimation of the quality of frozen cod using a new instrumental method*. European Food Research and Technology, 219, pp. 540 – 544, DOI: 10.1007/s00217-004-0997-z

42. M. R. Viant (2003). *Improved methods for the acquisition and interpretation of NMR metabolomic data*. Biochemical and Biophysical Research Communications, 310, pp. 943 – 948, DOI: 10.1016/j.bbrc.2003.09.092
43. M. R. Viant, C. A. Pincetich, R. S. Tjeerdema (2006). *Metabolic effects of dinoseb, diazinon and esfenvalerate in eyed eggs and alevins of Chinook salmon (Oncorhynchus tshawytscha) determined by ^{1}H NMR metabolomics*. Aquatic Toxicology, 77, pp. 359 – 371, DOI: 10.1016/j.aquatox.2006.01.009
44. M. R. Viant, I. Werner, E. S. Rosenblum, A. S. Gantner, R. S. Tjeerdema, M. L. Johnson (2003). *Correlation between heat-shock protein induction and reduced metabolic condition in juvenile steelhead trout (Oncorhynchus mykiss) chronically exposed to elevated temperature*. Fish Physiology and Biochemistry, 29, pp. 159 - 171
45. M. R. Viant, J. G. Bundy, C. A. Pincetich, J. S. de Ropp, R. S. Tjeerdema (2005). *NMR-derived developmental metabolic trajectories: an approach for visualizing the toxic actions of trichloroethylene during embryogenesis*. Metabolomics, 1(2), pp. 149 – 158, DOI: 10.1007/s11306-005-4429-2
46. M. Y. Mushtaq, R. M. Marcal, D. L. Champagne, F. van der Kooy, R. Verpoorte, Y. H. Choi (2014). Effect of Acute Atresses on Zebra Fish (Danio rerio) Metabolome Measured by NMR-Based Metabolomics. Planta Medica, 80(14), pp. 1227 – 1233, DOI: 10.1055/s-0034-1382878
47. P.K. Prabhakar, S. Vatsa, P.P. Srivastav, S.S. Pathak (2020). *A comprehensive review on freshness of fish and assessment: Analytical methods and recent innovations*. Food Research International, 133.
48. R. Abro, A.A. Moazzami, J.E. Lindberg, T. Lundh (2014). Metabolic insights in Arctic charr (Salvelinus alpinus) fed with zygomycetes and fish meal diets as assessed in liver using nuclear magnetic resonance (NMR) spectroscopy. International Aquatic Research, 6 (63), DOI: 10.107/s40071-014-0063-9
49. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 on laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down in matters of food safety.
50. Regulation (EC) No 2073/2005 of the Commission of 15 November 2005 on microbiological criteria for foodstuffs.
51. Regulation (EU) No 1379/2013 of the European Parliament and of the Council of 11 December 2013 on the common organization of the markets in fishery and aquaculture

products, amending the Council Regulations (EC) No 1184/2006 and (EC) No 1224/2009 and repealing Council Regulation (EC) No 104/2000.

52. R. Karoui, A. Hassoun, P. Ethuin (2017). *Front face fluorescence spectroscopy enables rapid differentiation of fresh and frozen-thawed sea bass (Dicentrarchus labrax) fillets*. Journal of Food Engineering, 202, pp. 89 – 98, DOI: 10.1016/j.jifoodeng.2017.01.018
53. S. Cautadella, M. Spagnolo (2011). *Lo stato delle pesca e dell'acquacolture nei mari italiani*. MIPAAF
54. S. Das, A. Patra, A. Mandal, N.S. Mondal, S. Dey, D. Kole, A.K. Mondal, A.R. Ghosh (2021*). Study on impacts of direct supplementation of choline into semi-intensive fish culture system based on haematopoietic alterations*. Environmental and Sustainability Indicators, 9, 100089, DOI: 10.1016/j.indic.2020.100089
55. S. Kim, J. Kim, E.J. Yun, K.H. Kim (2016). Food metabolomics: from farm to human. Current opinion in biotechnology, 37, pp.16-23, DOI: 10.1016/j.copbio.2015.09.004
56. S. Rezzi, I. Giani, K. Heberger, D. E. Axelson, V. M. Moretti, F. Raniero, C. Guillou (2007). *Classification of Gilthead Sea Bream (Sparus aurata) from ^{1}H NMR Lipid Profiling Combinet with Principal Component and Linear Discriminant Analysis*. Journal of Agricultural and Food Chemistry, 55, pp. 9963 – 9968, DOI: 10.1021/jf070736g
57. S. Roques, C. Deborde, N. Richard, S. Skiba-Cassy, A. Moing, B. Fauconneau (2020). Metabolomics and fish nutrition: a review in the context of sustainable feed development. Reviews in Aquaculture, 12, pp. 261 – 282, DOI: 10.1111/raq.12316
58. T. K. Karakach, E. C. Huenupi, E. C. Soo, J. A. Walter, L. O. B. Afonso (2009). *^{1}H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (Salmo salar) to long-term handling stress*. Metabolomics, 5(1), pp. 123 – 137, DOI: 10.1007/s11306-008-0144-0
59. T. Morkore, P.I. Mazo T., V. Tahirovic, O. Einen (2008). *Impact of starvation and handling stress on rigor development and quality of Atlantic salmon (Salmon salar L)*. Aquaculture, 277, pp. 231 – 238, DOI: 10.1016/j.aquaculture.2008.02.036
60. T. Saito, K. Arai, M. Matsuyoshi (1959). A Nex Method for Estimating the Freshness of Fish. Bullettin of the Japanese Society of Scientific Fisheries, 24(9), pp. 749 – 750
61. T. Vu, E. Riekeberg, Y. Qiu, R. Powers (2018). *Comparing normalization methods and the impact of noise*. Metabolomics, 14(108), DOI: 10.1007/s11306-018-1400-6
62. V.M. Ocano-Higuera, A.N. Maeda-Martinez, E. Marquez-Rios, D.F. Canizales-Rodriguez, F.J. Castillo-Yanez, E. Ruiz-Bustos, A.Z. Graciano-Verdugo, M. Plascencia-

Jatomea (2011). *Freshness assessment of ray fish stored in ice by biochemical, chemical and physical methods*. Food Chemistry, 125, pp. 49 – 54

63. V. Venugopal (2005). *Seafood processing Adding Value Through Quick Freezing, Retortable Packaging and Cook-Chilling*. Chapter 15: Nutritional Value and Processing Effects. Taylor & Francis Group, CRC Press, DOI: 10.1201/9781420027396, eBook ISBN: 9780429121180
64. Y.A.A. Bernardo, D.K.A. Rosario, I.F. Delgado, C.A. Conte-Junior (2020). *Fish Quality Index Method: Principles, weaknesses, validation, and alternatives - a review*. Comprehensive reviews in food science and food safety, 19, pp. 2657 – 2676, DOI: 10.1111/1541-4337.12600
65. Y.-J. Gao, Y.-J. Liu, X.-Q. Chen, H.-J. Yang, X.-F. Li, L.-X. Tian (2016). *Effects of graded levels of histidine on growth performance, digested enzymes activities, erythrocyte osmotic fragility and hypoxia-tolerance of juvenile grass carp Ctenopharyngodon Idella*. Aquaculture, 452. Pp. 388 – 394, DOI: 10.1016/j.aquaculture.2015.11.019
66. http://www.fao.org/
67. https://futureeuaqua.eu/index.php/media/
68. https://www2.chemistry.msu.edu/faculty/reusch/orgpage/nmr.htm

9 APPENDIX

```
################################

rm(list=ls(all=TRUE))

################################
require(baseline)
require(ggplot2)
require(Rcpp)
require(Hmisc)
require(mixOmics)
require(R.matlab)
#require(xlsx)
#require(pca3d)
#require(agricolae)
require(multcompView)

setwd("C:\\Users\\giuli\\Documents\\NMR\\Dataset Orate - filetto 14 e campione C1M-2")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\Metabolomica_allinea.matrice_15.10.2012.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\Metabolomica_da.files.a.matrice_15.10.2012.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\MetabolomicA_area.picco_22.09.2014_2_ConZeri.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\MetabolomicA_scala.matrice_15.10.2012_ConPQN.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\Metabolomica_PCAplot_20.11.2014.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\MetabolomicA_rimuovi.picchi_15.10.2012.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\MetabolomicA_bindata_15.10.2012.R")
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\MetabolomicA_normalizza.matrice_29.08.2013 (1).R")

###############
# Load samples #
###############
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\Metabolomica_da.files.a.matrice_15.10.2012.R")
orata<-da.files.a.matrice()
plot(orata$x, orata$y[1,], xlim=c(-0.01,0.01), type="l")
orata$y<-orata$y/1E10

###################
# Tabel with names #
###################
row.names(orata$y)
substr(rownames(orata$y),1,3)
```

```
ciccio<-substr(rownames(orata$y),1,3)
substr(rownames(orata$y),4,5)
mimmo<-substr(rownames(orata$y),4,5)
tabella<-rbind(ciccio,mimmo)
sm<-edit(tabella)
orata[["tabella"]]<-tabella
row.names(orata$tabella)<-c("Tipo","Ripet")

##################
# Alignment on TSP #
##################
source("C:\\Users\\giuli\\Documents\\NMR\\Codici
R\\Metabolomica_allinea.matrice_15.10.2012.R")
Ua<-allinea.matrice("orata", riferimento=c(-0.5,0.5))
Ua[["tabella"]]<-orata$tabella

seq<-c("A","B","C")
area.picco("Ua", ppm=c(-20,20), n=c(1:260), yzoom=0.5, ss=c(0.003,0.02),col.sp=2,
gruppi=seq, cerca.in.tabella=1)

#####################################
# Delete some parts of spectra and peaks #
#####################################
Ur<-rimuovi.picchi("Ua", ppm=c(0.5,4.69,5,6.05,6.07,9), azione="prendi")
Ur[["tabella"]]<-orata$tabella
area.picco("Ur", ppm=c(-20,20), n=c(1:260), yzoom=0.5, ss=c(0.003,0.02),col.sp=2,
gruppi=seq, cerca.in.tabella=1)

#################
# Regional Scaling #
#################

Ur1<-area.picco("Ur",ppm=c(-20,3),ris=2)
Ur2<-area.picco("Ur",ppm=c(3,5.5),ris=2)
Ur3<-area.picco("Ur",ppm=c(5.5,20),ris=2)

QMed1<-apply(Ur1$y[,],2,mean)
QMed2<-apply(Ur2$y[,],2,mean)
QMed3<-apply(Ur3$y[,],2,mean)

QMedian1<-apply(Ur1$y[,],2,median)
QMedian2<-apply(Ur2$y[,],2,median)
QMedian3<-apply(Ur3$y[,],2,median)

QDev1<-apply(Ur1$y[,],2,sd)
QDev2<-apply(Ur2$y[,],2,sd)
QDev3<-apply(Ur3$y[,],2,sd)

Ur1bis<-list(x=Ur1$x,y=rbind(QMedian1,Ur1$y[,]))#Metto lo spettro mediano all'inizio della
matrice
```

```
Ur2bis<-list(x=Ur2$x,y=rbind(QMedian2,Ur2$y[,]))#Metto lo spettro mediano all'inizio della matrice
Ur3bis<-list(x=Ur3$x,y=rbind(QMedian3,Ur3$y[,]))#Metto lo spettro mediano all'inizio della matrice

UPQN1<-normalizza.matrice("Ur1bis",metodo="PQN")# PQN effettuata sullo spettro mediano
UPQN2<-normalizza.matrice("Ur2bis",metodo="PQN")# PQN effettuata sullo spettro mediano
UPQN3<-normalizza.matrice("Ur3bis",metodo="PQN")# PQN effettuata sullo spettro mediano

Unorm1<-area.picco("UPQN1", n=c(2:261), ppm=c(-20,20), ris=2)#Ho tolto lo spettro Mediano dalla mia matrice
Unorm2<-area.picco("UPQN2", n=c(2:261), ppm=c(-20,20), ris=2)#Ho tolto lo spettro Mediano dalla mia matrice
Unorm3<-area.picco("UPQN3", n=c(2:261), ppm=c(-20,20), ris=2)#Ho tolto lo spettro Mediano dalla mia matrice

Unorm<-list(x=Ur$x,y=cbind(Unorm3$y,Unorm2$y,Unorm1$y))
Unorm[["tabella"]]<-Ua$tabella

area.picco("Unorm", ppm=c(-20,20), n=c(1:260), yzoom=0.5, ss=c(0.003,0.02),col.sp=2, gruppi=seq, cerca.in.tabella=1) #ci premette di visualizzare solo una zona sello spettro

#########
# Icoshift #
#########

allin<-readMat("C:\\Users\\giuli\\Documents\\MatLab\\Spettri icoshiftati\\OrateAllineate.mat")
Urall<-list(x=Unorm$x,y=allin[[1]])
Urall[["tabella"]]<-orata$tabella
area.picco("Urall", ppm=c(-20,20), n=1:260, yzoom=0.5, ss=c(0.00,0.01),col.sp=2, gruppi=seq, cerca.in.tabella=1)

#########
# Binning #
#########
Ubin<-bindata("Urall", bin=100)#Optimize size bin is 0.01 ppm - #447 bins di 100 punti, cioè di 0.0183 ppm
Ubin[["tabella"]]<-orata$tabella
area.picco("Ubin", ppm=c(3,4.6), n=1:260, ss=c(0.003,0.02),col.sp=2, gruppi=seq,cerca.in.tabella=1)

################################
# Number of spectra in each group #
################################
campioniA<-grep("A",row.names(Ua$y))
campioniB<-grep("B",row.names(Ua$y))
campioniC<-grep("C",row.names(Ua$y))
```

```
Agroup<-c(length(campioniA))
Bgroup<-c(length(campioniB))
Cgroup<-c(length(campioniC))

length(campioniA)
length(campioniB)
length(campioniC)

#########
# ANOVA #
#########
class<-c(rep("A",Agroup),rep("B",Bgroup),rep("C",Cgroup))#
obs<-length(Ubin$x)
factors <- rep(NaN, obs)
for (i in 1:obs) {
prova<-anova(lm(Ubin$y[,i]~class))
factors[i] <-prova$Pr[1]
}
pr<-0.05
significativi<-c(1:length(factors))[factors<pr]
Ubins<-list(x=Ubin$x[significativi],y=Ubin$y[,significativi])
Ubins[["tabella"]]<-orata$tabella

##############################
#  PCA on significant bins #
##############################
pca<-prcomp(Ubins$y, scale=F, center=T)
imp<-round(summary(pca)$importance[2,1:3],2)
imp<-imp*100
imp
x<-c(pca$x[,1])
y<-c(pca$x[,2])

colori=c(rep("#F68B33",Agroup), rep("#388ECC",Bgroup), rep("#66CC00",Cgroup))
colori1=c(rep("F68B33",1), rep("388ECC",1), rep("66CC00",1))
simboli<-c(rep(15,Agroup),rep(16,Bgroup),rep(17,Cgroup))

plot(x,y, main="PCA", pch=simboli,xlab=paste("PC1 ", imp[1], " %",
sep=""),ylab=paste("PC2 ", imp[2], " %", sep=""),
cex=2,cex.lab=1.2, font.lab=2,font.axis=2,
xlim=c(min(pca$x[,1]),max(pca$x[,1])),ylim=c(min(pca$x[,2]),max(pca$x[,2])),
col=colori, lwd=3)
grid(nx=5, ny=5, col="lightgray", lty="dotted", lwd = par("lwd"))
legend("bottomright", legend=c("Com-CN","FEUA-CN","FEUA-OR"), pch=c(15,16,17),
col=c("#F68B33","#388ECC","#66CC00"), fill=NULL, border="white", title="Legend")

plot(x,y, main="PCA", pch="", xlab=paste("PC1 ", imp[1], " %", sep=""),ylab=paste("PC2 ",
imp[2], " %", sep=""),
```

```
cex=1,cex.lab=1.2, font.lab=2,font.axis=2,
xlim=c(min(pca$x[,1]),max(pca$x[,1])),ylim=c(min(pca$x[,2]),max(pca$x[,2])),lwd=3, col=
colori)
text(x,y,labels=c(1:260),col=colori)
grid(nx=5, ny=5, col="lightgray", lty="dotted", lwd = par("lwd"))
legend("bottomright", legend=c("Com-CN","FEUA-CN","FEUA-OR"), pch=c(15,16,17),
col=c("#F68B33","#388ECC","#66CC00"), fill=NULL, border="white", title="Legend")

############################
# Delete outliers # After ANOVA #
############################
row.names(Ubins$y)<-row.names(Unorm$y)
Ubinsout<-list(x=Ubins$x,y=Ubins$y[-c(101,144,178,179,180,181,182,183,184),])
tabellaout<-orata$tabella[,-c(101,144,178,179,180,181,182,183,184)]
Ubinsout[["tabella"]]<-tabellaout

####################################
# Number of spectra in each group #
####################################
campioniA<-grep("A",row.names(Ubinsout$y))
campioniB<-grep("B",row.names(Ubinsout$y))
campioniC<-grep("C",row.names(Ubinsout$y))

Agroup<-c(length(campioniA))
Bgroup<-c(length(campioniB))
Cgroup<-c(length(campioniC))

length(campioniA)
length(campioniB)
length(campioniC)

######################
#  PCA on significant bins #
######################

pca<-prcomp(Ubinsout$y, scale=F, center=T)
imp<-round(summary(pca)$importance[2,1:3],2)
imp<-imp*100
imp
x<-c(pca$x[,1])
y<-c(pca$x[,2])

colori=c(rep("#F68B33",Agroup), rep("#388ECC",Bgroup), rep("#66CC00",Cgroup))
colori1=c(rep("F68B33",1), rep("388ECC",1), rep("66CC00",1))
simboli<-c(rep(15,Agroup),rep(16,Bgroup),rep(17,Cgroup))

plot(x,y, main="PCA", pch=simboli,xlab=paste("PC1 ", imp[1], " %",
sep=""),ylab=paste("PC2 ", imp[2], " %", sep=""),
cex=2,cex.lab=1.2, font.lab=2,font.axis=2,
xlim=c(min(pca$x[,1]),max(pca$x[,1])),ylim=c(min(pca$x[,2]),max(pca$x[,2])),
col=colori, lwd=3)
```

```
grid(nx=5, ny=5, col="lightgray", lty="dotted", lwd = par("lwd"))
legend("bottomright", legend=c("Com-CN","FEUA-CN","FEUA-OR"), pch=c(15,16,17),
col=c("#F68B33","#388ECC","#66CC00"), fill=NULL, border="white", title="Legend")

plot(x,y, main="PCA", pch="", xlab=paste("PC1 ", imp[1], " %", sep=""),ylab=paste("PC2 ",
imp[2], " %", sep=""),
cex=1,cex.lab=1.2, font.lab=2,font.axis=2,
xlim=c(min(pca$x[,1]),max(pca$x[,1])),ylim=c(min(pca$x[,2]),max(pca$x[,2])),lwd=3, col=
colori)
text(x,y,labels=c(1:260),col=colori)
grid(nx=5, ny=5, col="lightgray", lty="dotted", lwd = par("lwd"))
legend("bottomright", legend=c("Com-CN","FEUA-CN","FEUA-OR"), pch=c(15,16,17),
col=c("#F68B33","#388ECC","#66CC00"), fill=NULL, border="white", title="Legend")

##########
# Ellipses #
##########
tab<-matrix(c(pca$x[,1], pca$x[,2]), ncol=2) # tab<-matrix(c(x, y), ncol=2)

I<-c(1:87)
II<-c(88:172)
III<-c(173:251)

A<-cor(tab[I,])
B<-cor(tab[II,])
C<-cor(tab[III,])

library(ellipse)

polygon(ellipse(A/(max(abs(pca$rotation))*1000), centre=colMeans(tab[I,]), level=0.95),
col=adjustcolor("#F68B33", alpha.f=0.25), border="#F68B33")
polygon(ellipse(B/(max(abs(pca$rotation))*1000), centre=colMeans(tab[II,]), level=0.95),
col=adjustcolor("#388ECC", alpha.f=0.25), border="#388ECC")
polygon(ellipse(C/(max(abs(pca$rotation))*1000), centre=colMeans(tab[III,]), level=0.95),
col=adjustcolor("#66CC00", alpha.f=0.25), border="#66CC00")

polygon(ellipse(A*(max(abs(pca$rotation))*0.005), centre=colMeans(tab[I,]), level=0.95),
col=adjustcolor("#F68B33", alpha.f=0.25), border="#F68B33")
polygon(ellipse(B*(max(abs(pca$rotation))*0.005), centre=colMeans(tab[II,]), level=0.95),
col=adjustcolor("#388ECC", alpha.f=0.25), border="#388ECC")
polygon(ellipse(C*(max(abs(pca$rotation))*0.005), centre=colMeans(tab[III,]), level=0.95),
col=adjustcolor("#66CC00", alpha.f=0.25), border="#66CC00")

##########
# BarPlots #
##########
nf<-layout(matrix(c(1,1,1,1,2,2,2,2),4,2, byrow=TRUE))
layout.show(nf)
ciccio<-c(1:276)
barplot(pca$rotation[ciccio,1], col="blue", main="Loadings Bar plot PC 1", xlab="bin",
ylab=" ",names.arg=ciccio)
```

```
barplot(pca$rotation[ciccio,2], col="blue", main="Loadings Bar plot PC 2", xlab="bin",
ylab=" ",names.arg=ciccio)

which.max(pca$rotation[,1])
which.min(pca$rotation[,1])
which.max(pca$rotation[,2])
which.min(pca$rotation[,2])

############################
# Loadings over the threshold  #
############################
pippo1<-abs(pca$rotation[,1])#prendo i valori dei loadings 1
pippo2<-abs(pca$rotation[,2])#prendo i valori dei loadings 2
soglia1<-abs(mean(pippo1)-sd(pippo1))
soglia2<-abs(mean(pippo2)-sd(pippo2))
loads1<-c(1:length(pippo1))[pippo1>=soglia1]
loads2<-c(1:length(pippo2))[pippo2>=soglia2]
loadstot<-sort(unique(c(loads1,loads2)))

print(loadstot)
print(loads1)
print(loads2)

##################################
# BoxPlot with Tuckey Test # example #
##################################
setwd("C:\\Users\\giuli\\Documents\\NMR\\BoxPlot\\Senza outlier")
int<-c(139)
Ubins$x[int]
Ubins$x[int]-0.0183
Ubins$x[int]+0.0183
I<-c(1:87)
II<-c(88:172)
III<-c(173:251)

gruppo<-c(rep("CC",length(I)),rep("FC",length(II)),rep("FO",length(III)))
integrale<-c(Ubinsout$y[,int]/1E10)#con un solo bin
dati=data.frame(gruppo,integrale)
model=lm(dati$integrale ~ dati$gruppo)
ANOVA=aov(model)
summary(ANOVA)

TUKEY<-TukeyHSD(x=ANOVA, 'dati$gruppo', conf.level=0.95)
dev.new(width=8, height=8)
plot(TUKEY , las=1 , col="brown", cex.axis=0.5)
generate_label_df<-function(TUKEY, variable){
    Tukey.levels<-TUKEY[[variable]][,4]
    Tukey.labels <- data.frame(multcompLetters(Tukey.levels)['Letters'])
    Tukey.labels$gruppo=rownames(Tukey.labels)
    Tukey.labels=Tukey.labels[order(Tukey.labels$gruppo) , ]
    return(Tukey.labels)
```

```
    }

LABELS=generate_label_df(TUKEY,"dati$gruppo")
coloriamo<-c("#F68B33","#388ECC","#66CC00")
dev.new(width=15, height=8)
tiff(paste("Creatine",".tiff"))
par(mar=c(6, 4.1, 4.1, 2.1))
par=(las=2)
a=boxplot(dati$integrale ~ dati$gruppo ,
ylim=c(min(dati$integrale),1.3*max(dati$integrale)) , col=coloriamo, ylab="",
main="Creatine \n(3.92-3.96 ppm)",las=1,outline=F,
xlab="Type of Diets")
over=0.1*max(a$stats[nrow(a$stats),])
text(c(1:3) , a$stats[nrow(a$stats),]+over, LABELS[,1], col=coloriamo)
text(1,(1.2*max(dati$integrale)),"p.value=0.0135 *", cex=.9, lwd=2)
legend("topright", legend=c("Com-CN","FEUA-CN","FEUA-OR"), pch=16,
col=c("#F68B33","#388ECC","#66CC00"), fill=NULL, border="white", title="Legend")
dev.off()
graphics.off()
```