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DEVELOPMENT OF METHODOLOGIES FOR FISH FRESHNESS ASSESSMENT USING METABONOMICS APPLICATIONS

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Esame finale anno 2013

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DIPARTIMENTO DI SCIENZE E TECNOLOGIE AGRO-ALIMENTARI

Assessment for admission to the final examination for the degree of PhD in Food and Biotechnology Science

Dr. Alessandra CIAMPA

The Ph.D. student focused his research work on the application of Nuclear Magnetic Resonance (NMR) spectroscopy to the analysis and to the quality control of seafood, based on a metabonomic approach. This study led to the following results:

1. Validation of Nuclear Magnetic Resonance (NMR) as an alternative methodology for the evaluation of fish freshness, replacing the traditional protocols (spectrophotometric and chromatographic methods), which often utilize hazardous and toxic reagents or require long run times and high manual dexterity (manuscript in preparation).

2. Characterization of specific metabolites that determine loss of freshness (trimethylamine (TMA-N), Inosine (HxR), hypoxanthine (Hx)) and that detect the nutritional value of fish (vitamins, amino acids, etc.). NMR approach can give, in one shot analysis, plenty of information on the freshness of fish by taking in consideration both storage time and temperature effects (Ciampa et al., 2012).

3. Principal Component Analysis (PCA) and Discriminant Analysis (PLS-DA) were used to develop the new indices of freshness through a fingerprint of the ^{1}H-NMR spectra of all metabolites present in the sample. Through the multivariate data analysis has been possible to classify groups of fish samples and identify the biochemical compounds that describe the evolution of their metabolic profile, during storage at 4°C and 0°C (manuscript in preparation).

In order to improve his knowledge on metabonomic and metabolomic approaches, Dr. Alessandra Ciampa has spent three months in Laboratory of Foodomics at the Institute of Food Science Research (CIAL-CSIC) of Madrid working on a project "Development of new Metabolomics methods in Foodomics. Metabolomics of the antitumoral activity of dietary polyphenols".
In addition, in order to improve his knowledge on the study of physiological aspects of fish freshness, with special regard to the metabolism of polar compounds in *Salmo salar* during chilled and iced storage, she has spent three months at the Norway University of Science and Tecnology (NTNU, Department of Biotechnology) of Trondheim.

The Board unanimously agrees that Dr. Alessandra Ciampa is qualified to sit the final exam for the doctorate degree in Food and Biotechnology Science.

Cesena, February 15th 2013

Coordinator of PhD Course
in Food and Biotechnology Science

Prof. Claudio CAVANI

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PREFACE

Assessment of seafood is based upon freshness and taste. Both are essential and vital to the seafood sector and to consumers, but freshness is the most important and often the most difficult to measure. The quality of seafood relies on a series of parameters regarding: safety, nutritional values, availability, freshness and edibility. There are a number of factors influencing these such as: handling, processing and storage from when the seafood is caught to the consumers' table.

Physical, chemical, biochemical and microbiological changes occur post-mortem in fish; and they result in a progressive loss of taste and quality which include changes in protein and lipid fractions, the formation of biogenic amines and hypoxanthine and microbiological spoilage.

Key factors essential to seafood quality are both the time and the temperature of storage. In fact, for the most part, fish spoilage depends upon the temperature that controls to a greater extent the bacterial and the autolytic breakdown. There are also other factors that can influence the rate of spoilage: such as the fish species, the sanitary conditions on board when caught, the amount of food in the fish guts and the origin of seafood (wild or farmed).

At present, no single instrumental method is reliable for the assessment of seafood freshness and spoilage. Thus, the European Community establishes the principles to control and to certify the quality warranty in seafood through sensory, chemical and microbiological analyses. (EU Regulation No. 2406/96).

The aim of this PhD thesis is to verify the possibility of using HR ^{1}H-NMR and chemometrics methods as an analytical instrumental method to detect all of the parameters in the assessment of fish freshness. Combining NMR with chemometrics indicates all of the metabolic consequences of the specific conditions on seafood (e.g., modifications due to storage temperatures, giving an overview of the evolution of freshness in time.)

The research presented in this thesis is the result of a three-year PhD study reporting the principle results of these two main activities:

A1) comparison between ^{1}H-NMR and traditional methodologies such as HPLC and colorimetric ones in the evaluation of the seafood freshness index such as TMA-N content and K-Index;

A2) application of multivariate chemometrics models in data analysis for the expression of a holistic fish freshness.

ABBREVIATIONS

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Abs: Absorbance

Ala: Alanine

ANCOVA: Covariance Analysis

AOAC: Association Official Analytical Chemists

APC: Aerobic Plate Count

ATP: Adenosine Triphosphate

ADP: Adenosine Diphosphate

AMP: Adenosine Monophosphate

CFU: Colony-Forming Units

COM: Common Organization Markets

DA: Discriminant Analysis

DHA: Docosahexaenoic acid

DMA: Dimethylamine

EC: European Commission

EU: European Union

FA: Fatty Acids

FAAs: Free Amino Acids

FAO: Food and Agriculture Organization

FID: Free Induction Decay

FT: Fourier Transformation

GABA: γ-Aminobutyrate

GC: Gas Chromatography

Gly: Glycine

HCA: Hierarchical Cluster Analysis

His: Histidine

HPLC: High-Performance Liquid Chromatography

HR-NMR: High Resolution – Nuclear Magnetic Resonance

Hx: Hypoxanthine

HxR: Inosine

Ile: Isoleucine

iECVA: Interval Extended Canonical Variable Analysis

IMP: Inosine Monophosphate

IRMS: Isotopic Ratios Mass Spectrometry

La: Lactate

Leu: Leucine

LOD: Limits of Detection

LOQ: Limits of Quantitation

LVs: Latent Variables

Met: Methionine

MvDA: Multivariate Data Analysis

NS: Number of Scans

PCA: Principal Component Analysis

Phe: Phenylalanine

PLS: Partial Leas t Squares

PP: Polypropylene

PUFA: Poly Unsaturated Fatty Acids

QIM: Quality Index Method

RG: Receiver Gain

RSD: Relative standard deviation

S/N: Signal/Noise

Ser: Serine

SNIF: Site-Specific Natural Isotope Fractionation

SSOs: Specific Spoilage Organisms

Tau: Taurin

TMA-N: Trimethylamine-Nitrogen

TMAO: Trimethylamine-Oxide

TCA: Trichloroacetic Acid

Trp: Tryptophan

TSP: 3-(Trimethylsilyl)Propionate-2,2,3,3-d4

TVB-N: Total Volatil Basic- Nitrogen

TVC: Total Viable Counts

UI: Under Ice

UV-Vis: Ultraviolet-Visible

Val: Valine

CHAPTER 1

Fish quality: an overview

1.1 FISH QUALTY: A GENERAL INTRODUCTION

Fish quality is a complex concept involving several factors such as safety, nutritional quality (due principally to high-quality fats, digestible proteins, essential minerals), availability, convenience, integrity, eating quality, physical attributes of the species, size and freshness (Botta, 1995; Cardello, 1995). Information about handling and storage techniques, including time/temperature histories, that can affect the freshness and quality of the products, is very important for all the parts of the food chain (Olafsdottir et al., 2004). Additionally, seasonal condition, the effects of fishing grounds and capture methods and the occurrence of various defects influence the overall quality.

One of the most unique characteristics of fish as food is that it is a highly perishable commodity. Consequently, the time passed after the catch and the temperature 'history' of fish are very often the key factors determining the final quality characteristics of a fish product (Olafsdóttir et al., 1997; Olafsdottir et al., 2004).

Availability is a traditional aspect of seafood quality because excellent freshness quality, excellent nutritional quality, and excellent seafood safety are irrelevant if the seafood product is not available (Pedraja, 1988).

According to seafood in general**, convenience and integrity** have become important aspects of seafood quality (McNutt, 1988; Pedraja, 1988) for three major reasons: first, the modern consumer is greatly affected by the widespread effects of time pressure. Second, many consumers do not frequently eat seafood and therefore lack confidence in preparing seafood at home. Third, frequently only one person in the family "likes" seafood, which means that there is a demand for seafood that can be prepared in small quantities. Thus, depending upon the consumer, convenience of seafood includes a variety of items such as (1) a simple packaging system that allows the consumer to rapidly and easily purchase, store, and unpack the product, (2) an appropriately sized package for the consumer's particular requirements, (3) simple quick recipes that allow the consumer to easily and rapidly prepare the product with confidence, and (4) achievement of all of this without sacrificing freshness quality (Botta, 1995).

Among all the previous factors that describe the quality of fish, **freshness**, together with safety, are the most fundamental. The state of freshness can be described by a variety of definite properties of the fish which can be assessed by various indicators (Bremner & Sakaguchi, 2000). These properties are dependent upon different biological and processing factors that influence the degree of various physical, chemical, biochemical and microbiological changes occurring *post mortem* in fish. Moreover, the examination of some of the various definitions of fresh fish and seafood (Table 1.1) indicates that freshness is defined also in terms of time (e.g. time since the seafood was caught, delivered to the store, etc.), how the seafood was processed (e.g. not canned, not cooked, not cured, and not frozen) and the intrinsic sensorial characteristics such as appearance, odor, flavor, and texture.

Table 1.1: definitions of fresh fish and seafood (Botta, 1995)

Reference	Definition of fresh fish and seafood
Rockland, 1992	"The term fresh indicates that the fish is not and has never been frozen, cooked, crude or otherwise preserved, and that it exhibits a clean, natural odor and physical characteristic representative of the species in good condition".
Martin, 1988	"Freshness reflects the degree to which microbiological spoilage or chemical deterioration has occurred".
Howgate, 1982	"Fresh seafood it not easy to define. Loss of freshness followed by spoilage". is a complex combination of microbiological, chemical and physical processes".
Waterman, 1982	"A seafood that has the characteristic of a newly harvested seafood, it is not the opposite of stale".
Dore, 1991	"A seafood that is the opposite of stale".
Dore, 1991	"A seafood that has not been frozen".
Gould, 1971	"A seafood is fresh from the point of death of the animal until the first detectable signs of spoilage".
Canada Dep.Fish. Oceans, 1992	"Fresh means natural raw fillets or minced fish which has not been changed to any other state by freezing, cooking, curing, etc".
Karmas, 1981	"Fresh designates the time the seafood is removed from the water and spoiled marks the state in which definite deterioration has taken place rendering the seafood inedible".
Regenstein, 1991	"Consumers consider the first point of freshness to be the time the seafood arrives at the store".
Wheaton, 1985	"Freshness is the degree of microbiological spoilage or chemical degradation to which the raw product has progressed".

1.2 FISH QUALITY: THE LEGISLATIONAL POINT OF VIEW

On January, 1 2002, the EU laws on fish labeling entered into force: in all European Union countries fresh or chilled, frozen, dried, salted or in brine products intended for the final consumer may be marketed only if they have marking or labeling containing the commercial designation of the species, the production method and the catch or breeding areas.

The labeling system introduced by EC Regulation No. 104/2000 in the reform of the common organization of the markets (COM) in fishery and aquaculture products and disciplined by Regulation No. 2065/2001, has been applied in Italy according to the procedures in the Decree of the Ministry of Agriculture and Forestry of March 27, 2002 and in the No. 21229 of December 21, 2001 and No. 21329 of May 27, 2002 Circulars (Table 1.2).

Table 1.2: list of fish products to which it applies the labeling referred to Regulation (EC) No. 104/2000 (Source: Regulation (EC) No. 104/2000 of the Council of December 17, 1999)

CN Code	Description of goods
0301	Live fish;
0302	Fish, fresh or chilled, excluding fish fillets and other fish meat of heading No. 0304;
0303	Fish, frozen excluding fish fillets and other fish meat of heading No. 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 fish, 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;

The label is "[...] the set references, indications, marks factory or trademarks, images or symbols relating to food and are listed directly on the package or on a stuck label or on the closing device or on boards or on rings or straps linked to product itself [...]" (Art.1 Decree No. 109/92).

Beyond the definition, the labels are affixed not only to provide the necessary information about the contents of the package placed on the market, but they also have a callback function which should attract the attention of the consumer by pictures.

The need to inform the consumer and, at the same time, to safeguard the fisheries' production are the main reasons that led the European Union to adopt the present normative system on the labeling. Both are finalized to increase the transparency of the market, the product recognition and, consequently, to increase the consumer confidence, and to protect the producers through the enhancement of their productions, coming from fisheries and aquaculture.

The legislation, on the obligatory information to be provided with labeling, distinguishes between the phase of retailing and the previous steps. Particularly, in retailing, the products listed in the Table 1.2 should include the following information:

a) the commercial designation of the species;

b) the production method (caught at sea or in inland waters or farmed);

c) the catch area.

The latter indicator implies for the catch at sea, the indication of one of the zones shown in Figure 1.1 (fishing areas defined by the FAO); for caught products in freshwater, the indication of Member State or Third Country of origin for the product and, finally, for farmed products, the indication of the Member State or Third Country of breeding, the final stage of the product's development from its young stage to the market.

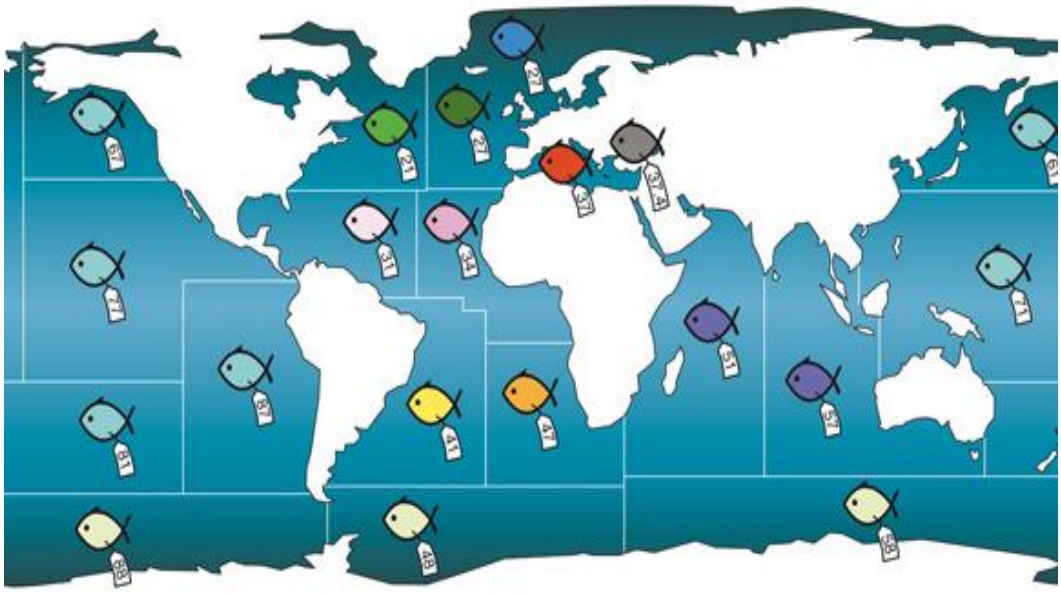

Figure 1.1: list of FAO catch areas: 21(North-West Atlantic), 27 (North- East Atlantic), 27 IIId (Baltic Sea), 31 (Central-Western Atlantic), 34 (Central-Eastern Atlantic), 41 (South-West Atlantic) 47 (South-East Atlantic), 37.1, 37.2, 37.3 (Mediterranean Sea), 37.4 (Black Sea), 51 and 57 (Indian Ocean), 61,67,71,77,81 and 87 (Pacific Ocean), 48, 58 and 88 (Antarctic). (Source: http://www.italiatavola.net

For product caught at sea it is permissible to omit the production method in retail, if is clear that it is a species caught at sea from the commercial designation and the catch area (for example Anchovies, Sardines, etc). Instead, for aquaculture products, there is the possibility to add the diction “Farmed ” to the term “Aquaculture Product”.

Although the Regulations 104/2000, 2065/2001 have been especially designed to increase the transparency of the fishing sector, there have been numerous infringements at consumer expense. Therefore in recent years a significant evolution of the regulatory in marketing and the control of fish products has taken place:

1 Council Regulation (EC) 104/2000 (OJ L17, p22, 21.01.2000) of December 17, 1999 on the common organization of the markets in fishery and aquaculture products.

2 Council Regulation (EC) 2065/2001 (OJ L278, p6, 23/10/2001) of 22nd October 2001 laying down detailed rules for the application of Council Regulation (EC) 104/2000 as regards informing consumers about fishery and aquaculture products.

3 Council Regulation (EC) No. 2318/2001 of November 29, 2001 laying down detailed rules for the application of Council Regulation (EC) No. 104/2000 as regards the recognition of producer organizations in the fishery and aquaculture sector.

4 Council Regulation (EC) No. 2369/2002 of December 20, 2002 amending Regulation (EC) No. 2792/1999 laying down the detailed rules and arrangements regarding Community structural assistance in the fisheries sector (OJ L 358 of 31.12.2002, p. 49).

5 Council Regulation (EC) No. 2371/2002 of December 20, 2002 on the conservation and sustainable exploitation of fisheries resources under the Common Fisheries Policy.

6 Council Regulation (EC) No. 2244/2003 of December 18, 2003 laying down detailed provisions regarding satellite-based Vessel Monitoring Systems.

7 Council Regulation (EC) No. 1198/2006 of July 27, 2006 on the European Fisheries Fund.

8 Council Regulation (EC) No. 1967/2006 of December 21, 2006 concerning management measures for the sustainable exploitation of fishery resources in the Mediterranean Sea, amending Regulation (EEC) No. 2847/93 and repealing Regulation (EC) No 1626/94.

9 Council Regulation (EC) No. 498/2007 of March 26, 2007 laying down detailed rules for the implementation of Council Regulation (EC) No. 1198/2006 on the European Fisheries Fund.

10 Council Regulation (EC) No 875/2007 of July 24, 2007 on the application of Articles 87 and 88 of the EC Treaty to de minimis aid in the fisheries sector and amending Regulation (EC) No. 1860/2004.

11 Council Regulation (EC) No. 710/2009 of August 5, 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.

12 Council Regulation (EC) No. 1224/2009 of November 20, 2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy.

13 Commission Implementing Regulation (EU) No. 404/2011 of April 8, 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.

In particular, the Regulation (EU) No. 1224/2009 in force since 2010, which will include the traceability from 2012, and labeling with the obligation of the capture date from January 1, 2015, has raised much controversy by the producers' and consumers' associations because it would penalize Italian operators more than international ones. In fact, according to Council Regulation (EC) No. 178/2002, food business operators have the responsibility and vigilance of the issuing, producing, processing and selling in the market. The operators are responsible, but the Member State adopts the food legislation and verifies its application (Figure 1.2). To this end, it organizes a system of official controls and determining measures and penalties applicable in case of violation (Liberati, 2012)

The legislative framework should ensure that the consumers and other relevant stakeholders have confidence in the decision-making processes within the food laws, on a scientific basis through the independence of the institutions protecting human health (Liberati, 2012).

Figure 1.2: controls system and "Pyramid of responsibilities" (Maria Severina Liberati, 2012)

To this end, in addition to labeling today it is becoming increasingly important to understand the history of a food product through its traceability, which according to Regulation EC No. 178/2002, has been in force since January 1, 2005. This makes tracing compulsory. For traceability is defined as "the ability to trace and follow a process of a food, feed, food-producing animal or substance that becomes part of a food or feed through all stages of production". For tracing is defined as "the ability to trace and follow a process of a food, feed, food-producing animal or substance that becomes part of a food or feed, through all stages of production, processing and distribution" (Reg. EC No. 178/2000). Essentially, the traceability system must satisfy the scheme represented in Figure 1.3:

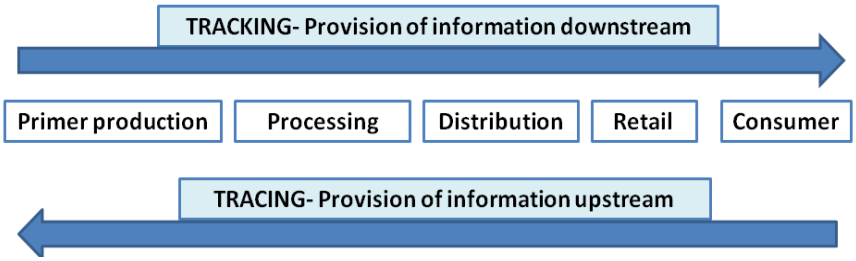

Figure 1.3: traceability scheme (Schwagele, 2005)

All food operators should implement a traceability system that enables the tracking of the product at any step in the chain in case of recall and the need for withdrawal. If needed, withdrawals should go to the consumer. This system shall also provide all the required and related information to identify the non-conformity and remove it.

Through a traceability system it is possible to unmask numerous commercial frauds, otherwise very difficult to identify. In Italy, there were 30,000 controls from naval units and more than 130,000 inspections at landing sites and business activities leading to the seizure of about 250,000 kg of fish products only in 2005. Many cases of Chinese fish sold as Italian were discovered on the Adriatic coast: "bianchetti" (*Crystallogobius linearis*) to mussels and clams with irregular labels.

In March-April 2006 at 162 retail points, located in 56 markets in 10 Italian regions chosen as samples, at least 10 sold species and their labels, for a total of 1620 labels containing information for the consumer were analyzed.

The survey results from the Citizen Defense Movement demonstrated that the fish benches were mostly part illegal as reported in Table 1.3.

In general, the most widely used information appears to be that on the commercial designation of the species (85.8%), following information about the catch or farming area (62.3%) and only 57.4% indicates the production method.

Consequently, in order to have a compliant food product labeling, (EC No. 1169/2011), it must contain the exact commodity denomination of products and their quality characteristics, so as to enable the consumers to effectively choose on an informed basis.

For example, information on the capture date, in accordance with the current EU regulations, which might include more calendar days, or a time period corresponding to more catch dates, should not be seen as a particularly insidious index to trade in fishing industry, but as precise indications useful to educate consumers about fresh fish.

For this reason, scientific information will help the consumer natural diffidence, thinking that fish is not fresh the day after its capture.

On the contrary, it is known to nutritionists and biochemists that the most important freshness requirements, expressed by analytical indicators, are

prolonged for more than few days, depending on the fish species and storage conditions.

The definition of the fresh fish shelf life has required a specific-species study, aimed at identifying the most simple and objective criteria and appropriate indicators to be adopted for freshness evaluation.

The study has been financed by the Italian Ministry of Agriculture within the Project “Fresh Fish”.

Table 1.3: data and elaboration: *Citizen Defense Movement* (Information given by seller of the benches)

Italian region	n° benches	Commercial designation of the species		Production method		Catch area	
		n°	%	n°	%	n°	%
Basilicata	15	15	100	14	93.3	15	100
Campania	14	9	64.3	3	21.4	5	35.7
Lazio	47	34	72.3	16	34	18	38.3
Liguria	6	6	100	6	100	6	100
Lombardia	17	17	100	10	58.8	12	70.6
Marche	27	26	96.3	14	51.8	17	62.3
Sicilia	9	5	55.6	5	55.6	4	44.4
Toscana	10	10	100	9	90	8	80
Umbria	5	5	100	5	100	5	100
Veneto	12	12	100	11	91.7	11	91.7
Total	162	139	85.8	93	57.4	101	62.3

1.3 FISH QUALITY: METHODS TO ASSESS THE FRESHNESS

As it has been written in par. 1.1, “quality” is closely associated with the state of “freshness” and the latter can be evaluated through different methodology such as sensory analysis, analytical determination of metabolites as histamine determination, TVB-N, TMA-N, physical determination like texture evaluation and microbiological analysis (Figure 1.4).

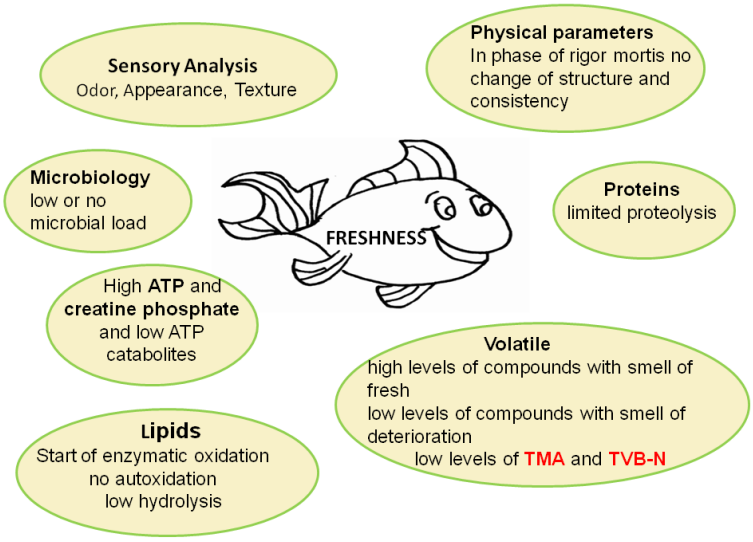

Figure 1.4: different parameters for the determination of fish freshness

1.3.1 Sensory analysis

Sensory methods rely on trained assessors. Thus objective sensory methods are required for use in quality control for evaluation of freshness and for determination of remaining shelf life of fish and seafood.

Currently in Europe, the official method for the sensory evaluation of seafood freshness (but applicable only for white fish, fish blue, some crustaceans, and only one cephalopod, the cuttlefish) is the scheme at four-levels regulated by EC Regulation 2406/96. In particular, levels or categories are the following: E (extra), A (good quality), B (satisfactory quality), C (not suitable quality) where E is the highest quality and below level B (called unfit or C) is the level where fish is discarded or rejected for human consumption (Table 1.4).

It has been indicated by scientists that the EU sensory schemes have disadvantages because information on remaining shelf life cannot be obtained directly from the freshness grades, and because the schemes are too complicated and may not be followed in practice (Huss, 1995; Lute & Martinsdòttir, 1997). Furthermore, it does not specify the different species under consideration.

Table 1.4: EU Scheme for white fish (Source: EU Regulation 2406/96)

Freshness category	Extra	A	B	Not admitted
Skin	Bright, iridescent pigment (save for redfish) or opalescent no discolouration	Pigmentation bright but not lustrous	Pigmentation in the process of becoming discoloured and dull	Dull pigmentation
Skin mucus	Aqueous, transparent	Slightly cloudy	Milky	Yellowish, grey, opaque mucus
Eyes	Convex (bulging), black, bright pupil, transparent cornea	Convex and slightly sunken; black, dull pupil, slightly opalescent cornea	Flat, opalescente cornea, opaque pupil	Concave in the centre, grey pupil, milky cornea
Gills	Bright colour, no mucus	Less coloured, transparent mucus	Brown / green becoming discoloured, thick, opaque mucus	Yellowish, milky mucus
Peritoneum on gutted fish	Smooth, bright, difficult to detach from flesh	Slightly dull, can be to detach from flesh detached	Speckled, comes away easily from flesh	Does not stick
Smell of gills and abdominal cavity	Seaweedy	No smell of seaweed, neutral smell	Fermented, slightly sour	Sour
Flesh	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

As an alternative to EU schemes, the quality index method (QIM) has been suggested. Originally, QIM was developed at CSIRO in Hobart, Australia (http://www.csiro.au), as a sensory method for a simple and rapid evaluation of whole or gutted fish of different species (Bremner, 1985). A relatively large number of fish quality attributes are evaluated in sequence by sight, smell and touch. Each attribute is scored from 0 to 3 (typically) by novice or experienced assessors with low scores indicating the best quality. The sum of all attribute scores is called demerit points, or QIM index points, and this value increases

linearly with storage time of a given fish. The direct relationship between QIM scores and storage time makes it easy to calculate remaining shelf life of fresh fish when stored at 0°C (Figure 1.5) (Hyldig & Green-Petersen, 2004).

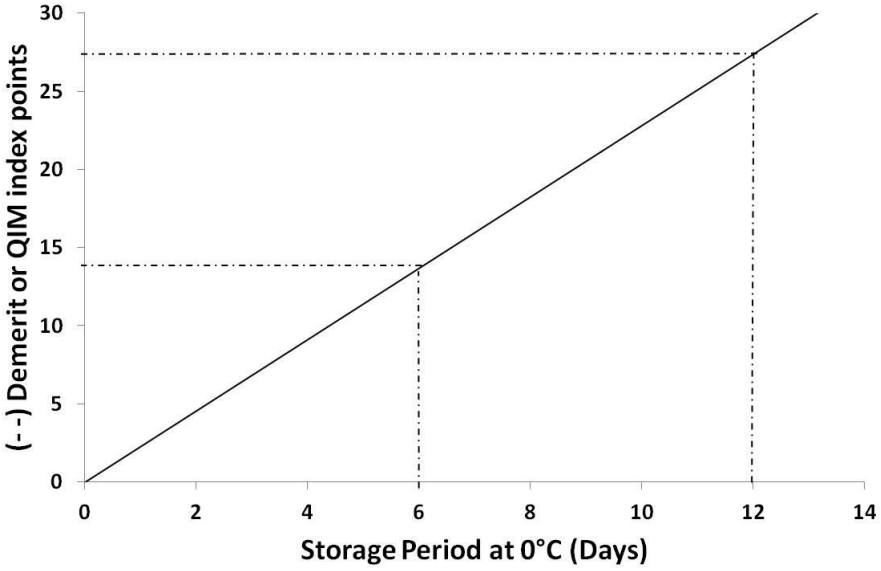

Figure 1.5: typical evolution of sensory scores as determined by a QIM scheme

To date, the system incorporates fresh herring (*Clupea harengus*) and cod (*Gadus morhua*), red fish (*Sebastes mentella/marinus*), Atlantic mackerel (*Scomber scombrus*), horse mackerel (*Trachurus trachurus*) and European sardine (*Sardina pilchardus*), brill (*Rhombus laevis*), dab (*Limanda limanda*), haddock (*Melanogrammus aeglefinus*), pollock (*Pollachius virens*), sole (*Solea vulgaris*), turbot (*Scophtalmus maximus*) and shrimp (*Pandalus borealis*), gilthead seabream (*Sparus aurata*) and farmed salmon (*Salmo salar*) (Sveinsdottir, Martinsdottir, Hyldig, Jørgensen & Kristbergsson, 2002).

1.3.2 Microbiological analysis

Spoilage of fish and fish products is a result of the production of off odors and flavors mainly caused by bacterial metabolites (Gram & Huss, 1996).

The numbers of specific spoilage organisms (SSOs) and the concentration of their metabolites can be used as objective quality indicators for determination of the shelf life of seafood.

Microbiological analyses of seafood involve testing for the presence or the absence of pathogens such as salmonellas and the determination of numbers of colony-forming units (CFU) named “total viable counts (TVC)” or “aerobic plate count (APC),” or numbers of CFU of indicator organisms such as Enterobacteriaceae, Coliforms, or Enterococci (Corry, Jarvis, Passmore & Hedges, 2007). Microbial growth models can be used to determine the effect of various time/temperature combinations on the shelf life of fish in the production and distribution chain. In particular mathematical models have been well established for the growth of spoilage bacteria such as *Photobacterium phosphoreum*, *Shewanella putrefaciens* (Dalgaard, 1995), *Brochothrix thermosphacta* (McClure et al., 1993), *Listeria monocytogenes* (Carrasco, Valero, Pérez-Rodríguez, García-Gimeno & Zurera, 2007), and *Clostridium perfringens* (Juneja, Huang & Thippareddi, 2006), which were shown to correlate with the remaining shelf life of the product and also correlated better than classical TVC measurements.

1.3.3 Chemical, biochemical analysis

Changes in seafoods during storage due to microbial activity, autolytic enzymes or chemical reactions can be useful indices of quality or spoilage.

K-Index: Adenosine triphosphate (ATP) is degraded into adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) during the processing and storage of fresh and lightly preserved seafood. IMP is formed by autolytic enzymes whereas spoilage bacteria contribute to HxR and Hx formation. Hx has a bitter taste which may be part of the off-flavour in stale fish. The K-Index (Eq. 1.1), called also K-value, was suggested by Japanese researchers in 1959 as an objective index of fish freshness (Saito, Arai & Matsuyoshi, 1959; Vázquez-Ortiz, Pacheco-Aguilar, Lugo-Sanchez & Villegas-Ozuna, 1997).

$$K - Index(\%) = \frac{\{[HxR]+[Hx]\}}{\{[ATP]+[ADP]+[AMP]+[IMP]+[HxR]+[Hx]\}} * 100 \qquad \text{(Eq. 1.1)}$$

In Japan, a K-Index of 20% is used as a critical limit for fish to be consumed raw. In most fish, K-Index increases linearly during the first days of chilled storage and

it is often an excellent index of freshness. However, the K-Index cannot be used in general as an index of spoilage because maximum values, for many fish species are reached long before sensory rejection. It may be worth mentioning that K-Index in lightly preserved seafood, e.g. cold-smoked salmon and cooked and brined shrimps, can be above 20% even for newly processed products of high sensory quality.

Ratios of catabolites like the K-Index have been indicated to be less prone to fish-to-fish or species-to-species variability than single compound quality indices. However, extensive analyses are required. The H-Index (Eq. 1.2), or H-value, which is quantitatively similar to the K-Index, reduces this problem (Karube, Matsuoka, Suzuki, Watanabe & Toyama, 1984).

$$H - Index(\%) = \frac{\{[HxR] + [Hx]\}}{\{[IMP] + [HxR] + [Hx]\}} * 100 \qquad \text{(Eq. 1.2)}$$

Concentrations of the adenine nucleotides required for calculation of the K- and H-Index can be determined in fish and seafood by chromatographic methods. Research into new biosensors for simple and rapid determinations of adenine nucleotides have been substantial in recent years and they may soon be available for practical use in process control and seafood inspection. K- and H-Index have not been included in regulations of the European Union.

TVB-N, TMA and other volatile amines. In seafood, total volatile basic nitrogen (TVB-N) primarily includes trimethylamine (TMA-N), ammonia, and dimethylamine (DMA). All these compounds, as well as total levels of TVB-N, are useful indices of spoilage in different fresh and lightly preserved seafood. The European Commission (Council Regulation No. 95/149/EEC of March, 1995) specified TVB-N to be used if sensory evaluation indicates doubt about freshness of different fish species. Critical limits of 25, 30 and 35 mg-TVB-N/100g were established for different groups of fish species. In processed, lightly or semi preserved seafood levels of TVB-N at sensory product rejection are more variable.

TMA-N is a microbial metabolite and it can only be used as an index of spoilage and not as an index of freshness (Martin, Franco, Molist & Gallardo, 1987).

Development of TMA-N in seafood depends primarily on the content of the substrate trimethylamine-oxide (TMAO) in the fish raw material, which is reduced into TMA-N (Fig. 1.6) by some species in the bacteriological flora of spoiling fish as *Shewanella putrefaciens*, *Photobacterium phosforeum*, and *Vibrionaceae* (Barrett & Kwan, 1985; Gram & Dalgaard, 2002; Gram et al., 1996).

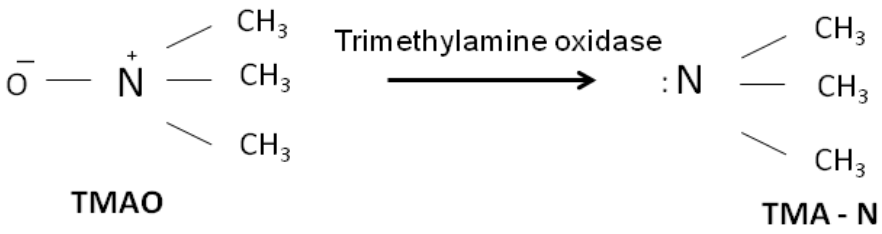

Figure 1.6: microbial reduction of TMAO into TMA-N by Trimethylamine oxidase

Biogenic amines. Many seafood spoilage bacteria produce one or more of the biogenic amines agmatine, cadaverine, histamine, putrescine, spermidine, spermine and tyramine. Since biogenic amines are heat stable, they are therefore appropriate also for the evaluation of freshness of raw material used in canned products.

Production of biogenic amines in seafood depends on the concentrations of the free amino acid substrates and is, therefore, strongly species dependent. For example, arginine is easily converted to agmatine as a result of bacterial activity, while lysine into cadaverine. Tyramine, tryptamine and 2-phenylethylamine formed from tyrosine, tryptophan and phenylalanine, respectively. Ornithine is the precursor of putrescine (Table 1.5).

Table 1.5: biogenic amines and their chemical precursor

Biogenic amine	Precursor
Istamine[1]	Histidine
Agmatine [2]	Arginine
Putrescine[2]	Ornithine
Cadaverine[2]	Lysine
Tyramine[3]	Tyrosine
Tryptamine[1]	Tryptophan
β-Phenylethyalamine[3]	Phenylalanine

[1] - heterocyclic amine, [2] - aliphatic amine, [3] - aromatic amine

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CHAPTER 2

A metabonomics approach to assess fish quality

2.1 METABONOMICS: A GENERAL INTRODUCTION

The term metabonomics is often confused with the term metabolomics and sometimes both are also used interchangeably. Even if they include approaches as metabolite profiling, metabolite target analysis and metabolic fingerprinting, they explain different concepts. To understand the term metabonomics and the other related terms (metabolomics, metabolic profiling, and "metabolic fingerprinting"), a short description of these terminologies is listed in Table 2.1 (Kashif, 2011).

Table 2.1: Descriptions of some useful metabolomics related terminologies (Kashif, 2011)

Terms Description	Description
Metabolome	The complete complement of small molecules present in an organism
Metabolomics	The technology geared towards providing an essentially unbiased, comprehensive qualitative and quantitative overview of all the metabolites present in an organism.
Metabonomics	A non plant term generally defining the technology used to measure quantitatively the metabolic composition of body fluids following a response to pathophysiological stimuli or genetic modification.
Metabolic profiling	Quantitative analysis of set of metabolites in a selected biochemical pathway or a specific class of compounds. This includes target metabolite analysis i.e. analysis of a very limited number of metabolites, e.g. single analytes as precursors or products of biochemical reactions.
Metabolic fingerprinting	High throughput qualitative screening of the metabolic composition in an organism or tissue with the primary aim of sample comparison and discrimination analysis. Generally no attempt is initially made to identify the metabolites present. All steps from sample preparation, separation and detection should be rapid and as simple as is feasible. Often used as a forerunner to metabolic profiling.
Metabolic footprinting	Fingerprinting analysis of metabolites that are excreted by cells (extra cellular) to the culture medium.

All of these approaches focus on the metabolic content of samples, but while metabolomics obtains a *"complete set of metabolites/low-molecular-weight intermediates, which are context dependent, varying according to the physiology, developmental or pathological state of the cell, tissue, organ or organism"* (Oliver, 2002), metabonomics is defined as *"the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification"* (Nicholson, Lindon & Holmes, 1999).

Even if the basic concept is different, metabolomics and metabonomics approaches can easily coexist in same study and one is strictly connected to the other in a double sense: the metabolomics exploration (metabolomics profile)

can help to understand the response of a biological system to extern stimuli (metabonomics profile) and *vice versa* (Fig. 2.1).

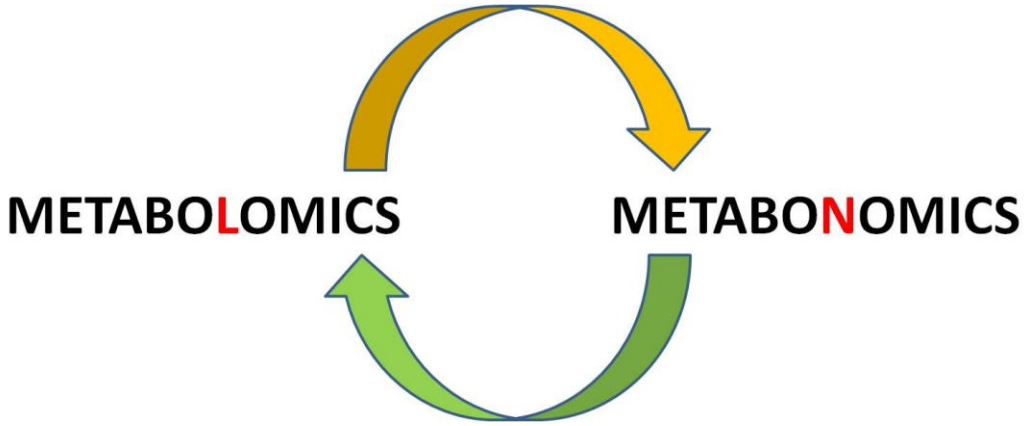

Figure 2.1: coexistence between metabolic and metabonomics approaches

Thus, considering the definition given in Table 2.1, **metabonomics** is the study of metabolites and their role in various physiological states. It is a holistic approach aimed to detect, to quantify and to catalogue the time related metabolic processes of an integrated biological system. Ultimately, it relates such processes to the trajectories of the physiological and pathophysiological events.

However, a metabonomic study can provide significant results only if the metabolic changes in a target group is significantly different from the biological variation of the relative control group (Abdel-Farid, Jahangir, van den Hondel, Kim, Choi & Verpoorte, 2009). Complementary to proteomics and genomics, first metabonomics has been widely applied to a wide range of problems in diverse biomedical research areas to understand the metabolites' behavior under certain exogenous conditions.

In recent times, due to the increasing interest in studying the effects of foods on the biochemistry of human and associated microflora, metabonomics also finds a role in nutritional science and in food chemistry, also leading to the birth, of other "omics" sciences such as genomics, transcriptomics, proteomics and a new science called **foodomics** (Capozzi & Bordoni, 2013).

Thus, metabonomics becomes a key tool in human nutrition opening the door to studying different aspects of ''molecular nutrition'' more strictly connected to **food quality** (Fig. 2.2), including:

1. food component analysis;
2. food quality/authenticity detection;
3. food consumption monitoring;
4. physiological monitoring in food intervention or diet challenge studies;

In fact, traditionally food component analysis involves identifying and classifying food constituents into very broad categories such as proteins, fats, carbohydrates, fiber, vitamins, trace elements, solids and/or ash. However, with the advent of metabolomics and metabonomics, foods and beverages are now being analyzed with considerably more chemical detail (Wishart, Querengesser, Lefebvre, Epstein, Greiner & Newton, 2001) with hundreds or even thousands of distinct chemical identities being detected and/or identified in certain foods.

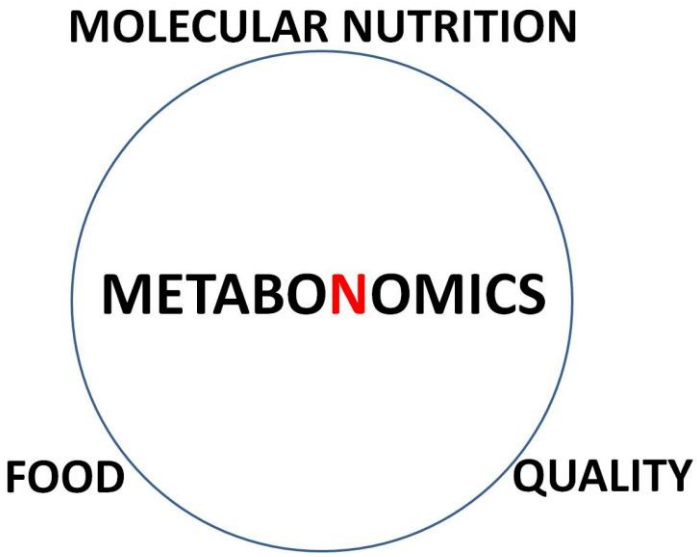

Figure 2.2: connection among molecular nutrition, food and quality

2.2 HR ^{1}H-NMR AND CHEMOMETRICS IN SEAFOOD SCIENCE

In food science and in general in metabonomics study, high resolution proton nuclear magnetic resonance (HR ^{1}H-NMR) spectroscopy is uniquely suited to detect a large range of endogenous low molecular weight metabolites in an organism. This technique is rapid and rich in structural and quantitative information and allows the metabolites to be analyzed simultaneously (Gartland, Beddell, Lindon & Nicholson, 1991; Wang et al., 2003).

In particular, the NMR technique has the advantage to supply detailed information on the molecular structure of the biological material observed,

reflecting at the end the metabolomic status of a biological living system, without losing important information on the system. This spectroscopic technique is generally used to detect hydrogen atoms in metabolites (^{1}H-NMR). Thus, in a typical sample (biological fluid in medical research or organic extract in food research) all hydrogen-containing molecules (almost all metabolites) will give an ^{1}H-NMR spectrum, as long as they are present in concentrations above the detection limit.

In this way, the NMR spectrum is the superposition, also commonly called **fingerprint** (Picone, 2009), of the spectra of all of the metabolites in the sample (Fig. 2.3). This fingerprint evaluates, in one shot, the entire metabolites' pattern variation due to external factors for example, such as, considering seafood stuff, storage time and temperature.

Figure 2.3: fish's pictures through the NMR spectrum

Nuclear magnetic resonance has also other unique advantages over other metabonomics techniques such as chromatography and MS methods. First, it is a more uniform detection system and can be used directly to identify and to quantify metabolites, even in vivo. The most promising features of NMR are its non-destructive nature, in a relatively shorter time or even direct measurement of the samples, e.g. urine. Another major advantage of NMR is that quantification is easy for all compounds as with a single internal standard all the

detected metabolites can be quantified without the need of calibration curves for each single compound. At the end, NMR sample does not require any physical or chemical treatment prior to the analysis, but only the solution conditions such as the temperature, pH and salt concentration have to be adjusted. In this last case, the preparation of a generic sample involves three fundamental steps:

1) the first step is to rapidly collect and freeze the sample to quench metabolism and preserve the metabolites. Samples are typically stored a -80°C to prevent any decay;
2) the second step involves the extraction methods. The choice depends on the polarity of the metabolites that are required. Solvents that extract only polar metabolites include perchloric acid, methanol or acetonitrile. If both polar and lipophilic metabolites are desired, then extraction using methanol and chloroform can be used to fractionate the metabolite classes;
3) the third step is to optimize the solution for high resolution NMR spectroscopy. This typically entails buffering the sample pH to minimize variation in the chemical shifts of the NMR resonance (e.g 100 mM phosphate buffer, pH = 7.0), adding D_2O to provide a frequency lock for the spectrometer and adding an internal chemical shift (and intensity) standard like sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP).

Since nearly no sample pretreatment is required in NMR spectroscopy, the inherent properties of the sample are well kept.

One the other hand, the preparation of the NMR sample does not require particular procedures; but on the other its intrinsic dynamic nature leads to the choice of an appropriate pulse sequence able to extract for the sample much more information as possible.

Generally, 1D ^{1}H-NMR with water presaturation, commonly called PRESAT or zgpr sequence, is a standard pulse sequence used in the NMR metabonomics approach (Figure 2.4). It is a simple two-pulse experiment that utilizes a relatively long, low power RF pulse to selectively saturate a specific frequency, typically water, and a non-selective 45-90° pulse to excite the desired resonances (H). This pulse sequence is particularly useful for aqueous samples or those with a single large solvent signal. With proper optimization, the resulting spectrum can be mostly free of the solvent signal and lead to improved Signal-to-Noise (S/N) for

solute resonances due to the reduction in dynamic range and subsequent increase in available gain.

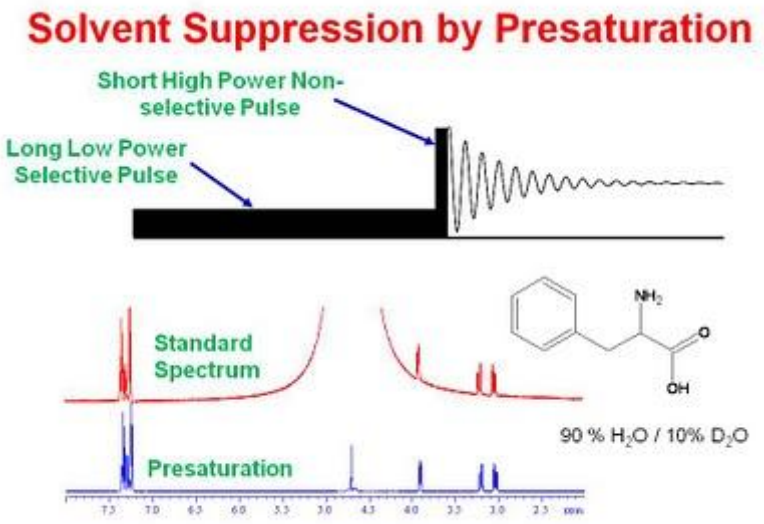

Figure 2.4: solvent (water) suppression by Presaturation

HR ^{1}H-NMR has been used to perform quantitative measurements of water-soluble metabolites (Ciampa, Picone, Laghi, Nikzad & Capozzi, 2012; Picone, Balling Engelsen, Savorani, Testi, Badiani & Capozzi, 2011; Savorani, Picone, Badiani, Fagioli, Capozzi & Engelsen, 2010), but also a total of n-3 Fatty Acids (FA) and of the DHA levels. This analysis can be carried out with a high degree of automation and gives a rapid fingerprint of the polar and lipid profile.

Other important information derives from the ^{13}C-NMR experiment which gives information about FA composition and the positional distribution of poly unsaturated fatty acids (PUFA) in triacylglycerols and phospholipids.

Different information, but yet interesting, comes from the ^{31}P-NMR experiments used to evaluate degree of freshness of loach muscle depending on metabolic changes of the high energy phosphate compounds.

2.2.1 Chemometrics

NMR spectrum, is capable to detect all metabolites in a sample. It is full of information (variables), part of which most of the time results to be redundant.

For this reason, it is important in a metabonomics research to compress these variables in order to have only those that contain the useful information.

This kind of approach is commonly called chemometrics (approach) and it can be defined as “How to get chemically relevant information out of measured chemical data, how to represent and display this information, and how to get such information into data” (Wold, 1995).

Chemometrics is the field of extracting information from multivariate chemical data using the tools of statistics and mathematics. It is typically used for one or more of three primary purposes:

1) to explore patterns of association in data;
2) to track properties of samples;
3) to prepare and use multivariate classification models.

Exploratory data analysis can reveal hidden patterns in complex data by reducing the information to a more comprehensible form. Such chemometrics analysis can expose possible outliers and indicate whether there are patterns or trends in the data. Exploratory algorithms such as principal component analysis (PCA) and hierarchical cluster analysis (HCA) are designed to reduce large complex data sets into a series of optimized and interpretable views. These views emphasize the natural groupings in the data and show which variables greatly influence those patterns.

Formally, PCA is a way of identifying patterns in data, expressing them in such a way as to highlight their similarities and/or differences (Rodŕiguez-Delgado, González-Hernández, Conde-González & Pérez-Trujillo, 2002; Silva, Andrade, Martins, Seabra & Ferreira, 2006). The advantage of this technique is the capability to reduce multidimensional data set (a data matrix) into a new set of uncorrelated (i.e., orthogonal) variables by performing a covariance analysis (ANCOVA) between factors.

The PCA works by decomposing the X-matrix (containing the original data set) as the product of two smaller matrices, which are called loading and score matrices (Figure 2.5).

The loading matrix (V) contains information about the variables: it is composed of a few vectors (Principal Components, PCs) which are (obtained as) linear combinations of the original X-variables.

The score matrix (U) contains information about the objects. Each object is described in terms of its projections onto the PCs, (instead of the original variables) (Eq. 2.1).

$$x = V^T * U \qquad \text{(Eq. 2.1)}$$

The information not contained in these matrices remains as "unexplained X-variance" in a residual matrix (E) which has exactly the same dimensionality as the original X-matrix.

The PCs, among many others, have two interesting properties:

1. they are extracted in decreasing order of importance. The first PC always contains more information than the second, the second more than the third and so on.
2. they are orthogonal to each other. There is absolutely no correlation between the information contained in different PCs.

In PCA, is possible to decide how many PCs should be extracted (the number of significant components, i.e. the dimensionality of the model).

Each new PC extracted further increases the amount of information (variance) explained by the model. However, usually the first four or five PCs explain more than 90% of the X-variance. Anyway, there is not a simple nor unique criterion which decides how many PC to extract so two kinds of considerations should be taken into account. From a theoretical point of view, it is possible to use cross - validation techniques to decide the number of PCs to include. Since data patterns can be hard to find in data of high dimension, like spectroscopic ones, where most of the time the information is redundant, PCA is a powerful tool for analyzing them.

In ^{1}H NMR, redundancy means that some of the variables are correlated with one another because they are measuring the same construct (different picks for the same molecule). Therefore, this redundancy can reduce the observed variables into a smaller number of artificial variables (principal components or latent factors) that are a linear combination of the original ones and will account for most of the variance in the observed variables without losing information.

In this way, by using a few components, each sample (spectrum) can be represented by relatively few numbers instead through values for thousands of variables (spectral data points). Then, samples can be plotted making it possible

to visually assess similarities and differences between samples and determine whether samples can be grouped (Ringnér, 2008).

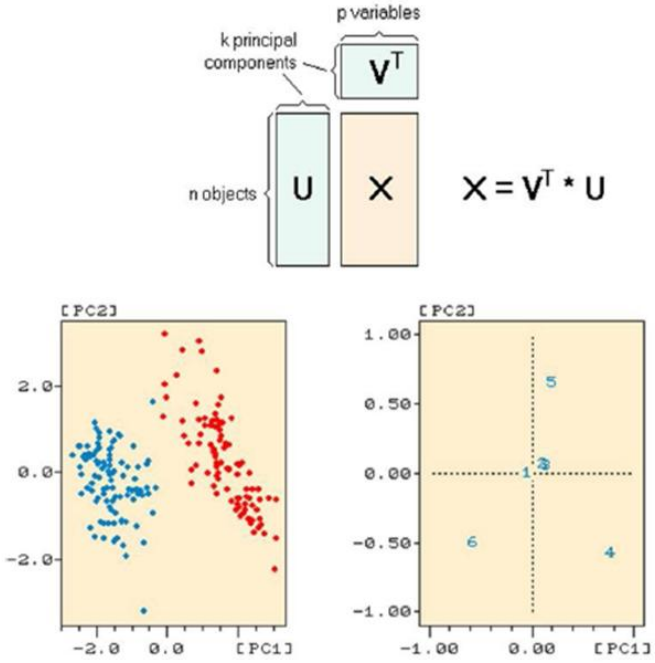

Figura 2.5: the two matrices V and U are orthogonal. The matrix V is usually called the loadings matrix and the matrix U is called the scores matrix. There are a few common plots which are always used in connection with PCA: 1) the scores/scores plot (left part of the Figure below) and 2) the corresponding loading/loading plot (right part of the figure below) (Figures is adapted from H., Lohninger (1999). Teach/Me Data Analysis. Springer-Verlag, Berlin-New York-Tokyo)

As a clustering technique, PCA is most commonly used to identify how one sample is different from another, which variables contribute most to this difference, and whether those variables contribute in the same way (i.e. are correlated) or independently (i.e. uncorrelated) from each other.

In contrast to PCA, **PLS** and **PLS-DA** (de Noord, 1994; Kleinbaum, 2007; Martens & Næs, 1992) are supervised classification techniques that can be used to enhance the separation between groups of observations by rotating PCA components so that a maximum separation among classes is obtained (Chevallier, Bertrand, Kohler & Courcoux, 2006).

The purpose of Discriminant Analysis is to classify objects (people, customers, foods, genes, things, etc.) into one of two or more groups based on a set of features that describe the objects (e.g. gender, age, income, weight, preference score, genotypes, metabolites' content etc.). In general, is assigned an object to

one of a number of predetermined groups based on observations made about the object. For example, if one wants to know whether a soap product is good or bad, this judgment is based on several measurements of the product such as weight, volume, people's preferential score, smell, color contrast etc. The object here is soap. The class category or the group (“good” and “bad”) is what is looked (it is also called dependent variable). Each measurement on the product is called features that describe the object (it is also called independent variable).
Thus, indiscriminant analysis, the dependent variable (Y) is the group and the independent variables (X) are the object features that might describe the group. The dependent variable is always category (nominal scale) variable while the independent variables can be any measurement scale (i.e. nominal, ordinal, interval or ratio).

Partial Leas t Squares (PLS) is useful when a (very) large set of independent variables have to be predicted. It originates in the social sciences but becomes popular also in all branches basing on chemometrics methods, including food science (Poveda & Cabezas, 2006; Szydłowska-Czerniak, 2007). It is a multivariate regression method allowing establishing a relationship between one or more dependent variables (U) and a group of descriptors (T).
T- and U-variables are modeled simultaneously to find the latent variables (LVs) in T that will predict the latent variables in U and at the same time account for the largest possible information present in T; Figure 2.6 gives a schematic outline of the method.
The overall goal (shown in the lower box of Figure 2.6) is to use the factors to predict the responses in the population. This is achieved indirectly by extracting latent variables T and U from sampled factors and responses, respectively.
The extracted factors T (also referred to as X-scores) are used to predict the Y-scores U, and then the predicted Y-scores are used to construct predictions for the responses (Manetti et al., 2004).

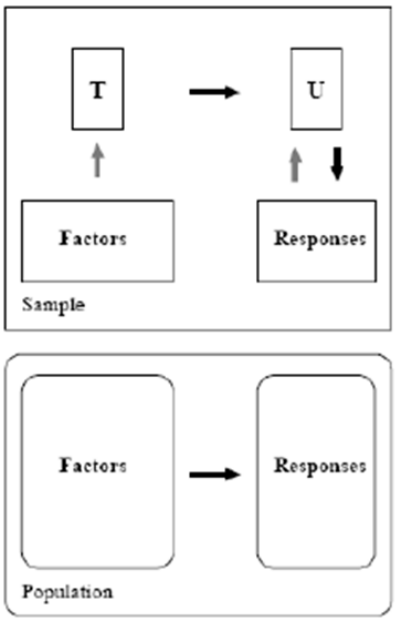

Figure 2.6: schematic outline of PLS method. Hence the PLS method is popular in industries that collect correlated data on many x-variables, known as predictors. For example, multivariate calibration in analytical chemistry; spectroscopy in chemometrics. The PLS method extracts orthogonal linear combinations of predictors, known as factors (**T** or **X-Scores**), from the predictor data that explain variance in both the predictor variables and the response (**U** or **Y-Scores**) variable(s) (Figure is adapted from D. T., Randall D. Tobias (2004). *An Introduction to Partial Least Squares Regression*. SAS Institute Inc., Cary, NC and S., Wold (1994). *PLS for Multivariate Linear Modeling QSAR*: *Chemometric Methods in Molecular Design. Methods and Principles in Medicinal Chemistry*. Van de Waterbeemd H (Editor) Verlag-Chemie)

So, in this case the latent variables are selected on the basis of explaining contemporarily both descriptors and predictors. These latent variables are similar to the principal components calculated from PCA. The first one accounts for the largest amount of information followed by the other components that account for the maximum residual variance. As for PCs, the last LVs are mostly responsible for random variations and experimental error. The optimal number of LVs, i.e. modelling information in X useful to predict the response Y but avoiding overfitting, is determined on the basis of the residual variance in prediction.

Cross - validation techniques are adopted for evaluating the predictive ability and for selecting the optimal number of latent variables.

PLS was contrived to model continuous responses but it can be applied even for classification purposes by establishing an appropriate Y related to each sample

belonging to a class. In this case it is called Partial Least Squares - Discriminant Analysis (PLS-DA).

In the case of proteomic data, one response variable for each group of samples is usually adopted. Each response variable is assigned a 1 value for the samples belonging to the corresponding class and a 0 value for the samples belonging to the other classes.

In general, a PLS analysis consists of the stages:

1. calculate a PLS model using a high number of factors (more than is likely to be required);
2. determine the number of factors to include in a fitted model by either:

- analysing information calculated during the process of extracting factors;
- calculating a prediction accuracy estimate based on, e.g. , cross - validation;

3. fit the model with the determined number of factors by calculating parameter estimates of the linear regression;
4. given a set of predictors and responses used to fit a PLS model, and a suitable number of factors used to calculate parameter estimates, estimate response values to new predictor data.

As noted previously, chemometrics approaches like PCA and PLS-DA, on their own, do not permit the direct identification or quantification of compounds. In the other approach to metabonomics (quantitative metabolomics or targeted profiling), the focus is on attempting to identify and/or to quantify as many compounds in the sample as possible.

This is usually done by comparing the spectroscopic data (obtained from the sample's NMR or MS ones) spectroscopic data reference library obtained from pure compounds (Spratlin, Serkova & Eckhardt, 2009; Weljie, Newton, Mercier, Carlson & Slupsky, 2006; Wishart, 2008). Once the constituent compounds are identified and quantified, the data are then statistically processed (using PCA or PLS-DA) to identify the most important biomarkers or informative metabolic pathways (Weljie et al., 2006).

Depending on the objectives and instrumental capacity, quantitative metabolomics may be either targeted (selective to certain classes of compounds) or comprehensive (covering all or almost all detectable metabolites).

2.2.2 NMR data preprocessing preprocessed for chemometrics data analysis

The discipline of chemometrics originates in chemistry; thus, typical applications of chemometrics methods are the development of quantitative structure activity relationships or the evaluation of analytical - chemical data. The data flood generated by modern analytical instrumentation (like spectroscopic technologies) is one reason that analytical chemists, in particular, develop applications of chemometrics methods. While most other types of spectroscopic data can be subjected to chemometrics analysis directly from the spectrometer, NMR data often need to be preprocessed in several ways in order to conform to the prerequisites for chemometrics data analysis:

1. **Fourier transformation** (Keeler, 2011) (Figure 2.7).

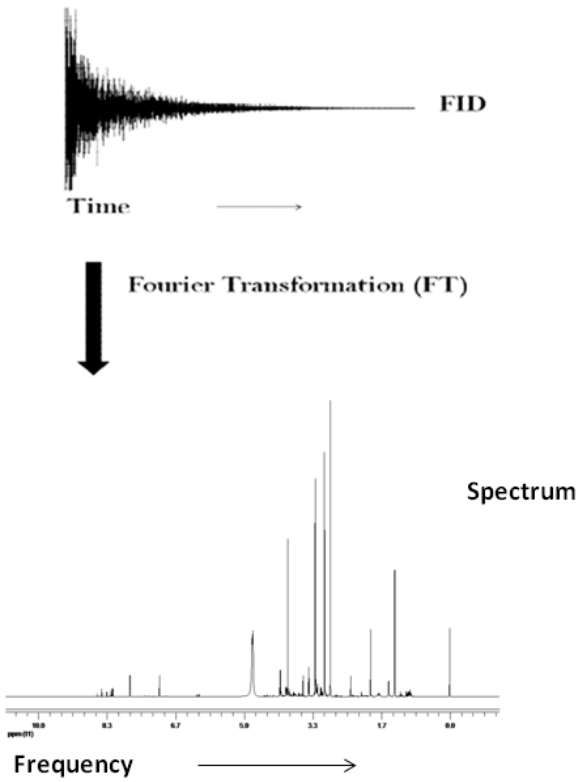

Figure 2.7: Fourier transformation of a FID obtained from a fish muscle (Bogue, *Boops boops*) sample's extract. The FT process takes the time domain function (the FID) and converts it into a frequency domain function (the spectrum). The 1H-NMR spectra was recorded at T= 300K on a Avance spectrometer, operating at 1H frequency of 600 MHz, Bruker; 512 scans were acquired, with data collected into 16K data points with a spectral width of 12 ppm, a pulse angle of 90°, a recycle delay of 4.0 s, and acquisition time of 2.347 s with a constant receiver gain. Free induction decay (FID) was Fourier transformed, with the MestReC Software (http://www.mestrec.com/), by performing an exponential multiplication with a 0.5 Hz line broadening

In NMR spectroscopy, a Fourier transformation (FT) is required to convert the time domain data (free induction decay or FID, an electrical signal oscillating at the NMR frequency), obtained from the spectrometer, to the frequency domain (NMR spectrum). Naturally, quantitative methods require that parameter settings for the Fourier transform (choice of zero-filling and apodization function) are equal for all samples to be evaluated, since they may influence the finer details in the spectra.

2. Phase errors (Keeler, 2011) (Figure 2.8).

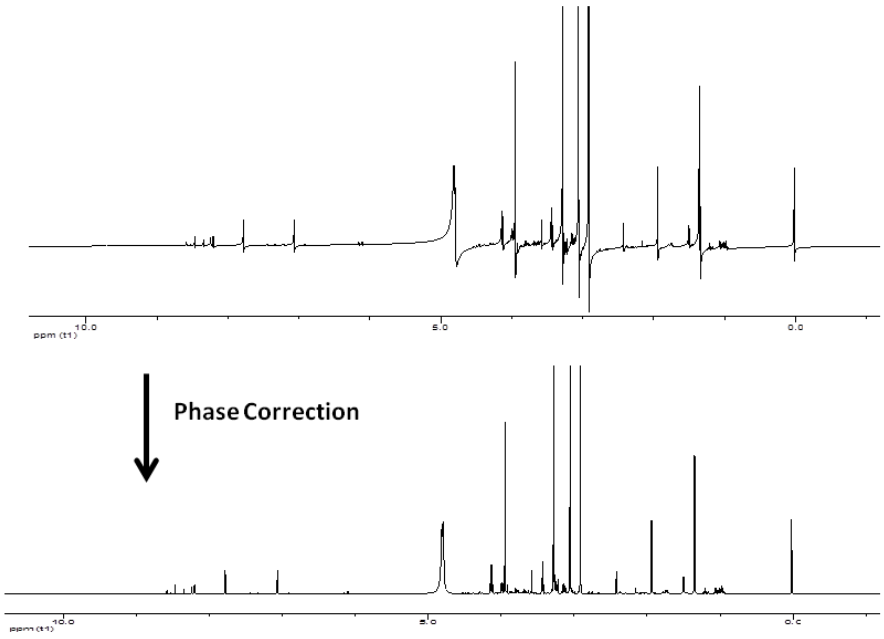

Figure 2.8: phase Correction of a spectrum obtained from a fish muscle (Bogue, *Bops boops*) sample's extract. The ^{1}H-NMR spectra was recorded at T= 300K on a Avance spectrometer, operating at 1H frequency of 600 MHz; 512 scans were acquired, with data collected into 16K data points with a spectral width of 12 ppm, a pulse angle of 90°, a recycle delay of 4.0 s, and acquisition time of 2.347 s with a constant receiver gain. The phase error correction was made with the MestReC Software by performing a manual correction on both zero and first errors orders (http://www.mestrec.com/)

A difficult problem encountered with NMR data is the existence of phase errors of two orders: one and zero. In the real experiment, after FT, the spectrum line shapes are a mixture of absorpitive and dispersive signals. They are related to the delayed FID acquisition that is commonly called first order phase. The delayed acquisition is a consequence of the minimum time required to change

the spectrometer from transmit to receive mode. During this delay, the magnetization vectors process according to their chemical shift frequencies.

The zero order phase error arises because of the phase differences between the magnetization vectors and the receiver. Manual phase correction is usually implemented in the instrument software, but this process is very time consuming, especially for the large data sets that are often analyzed using chemometrics. More importantly, manually phase-correcting a series of spectra will lead to suboptimal results due to the subjective evaluation of the correction necessary for individual spectra.

3. Data normalization (Figure 2.9).

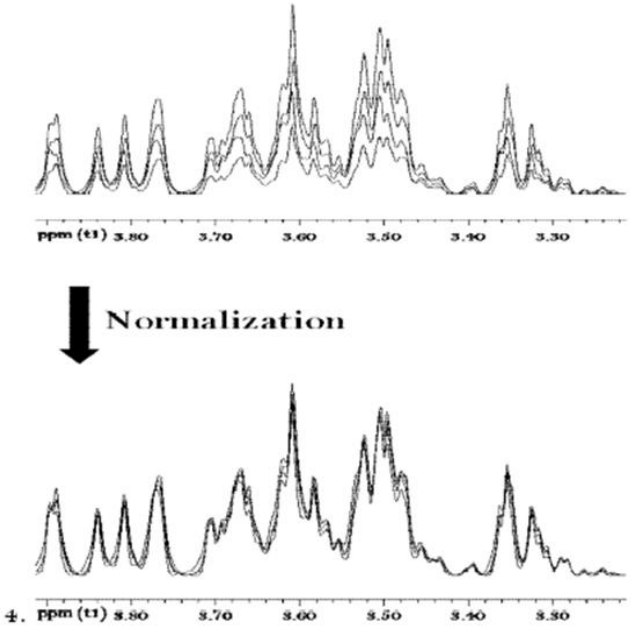

Figure 2.9: normalization of a set of spectra obtained from a fish muscle (Bogue, *Boops boops*) samples' extract. In this Figure is shown part of the midfield 1H NMR region (from 4.0 to 3.3 ppm). The ^{1}H-NMR spectra were recorded at T= 300K on a Avance spectrometer, operating at 1H frequency of 600 MHz; for each spectrum 512 scans were acquired, with data collected into 16K data points with a spectral width of 12 ppm, a pulse angle of 90°, a recycle delay of 4.0 s, and acquisition time of 2.347 s with a constant receiver gain. The normalization was made with the MestReC Software (http://www.mestrec.com/)

Data normalization is an important step for any statistical analysis.

The objective of data normalization is to allow meaningful comparisons of samples within the dataset. It is a row operation that is applied to the data from each sample and comprises methods to make the data from all samples directly

comparable with each other (Craig, Cloarec, Holmes, Nicholson & Lindon, 2006). In this way it is possible to minimize most of the differences introduced with the effect of variable dilution and spectral data acquisition and processing.

Normalization can be done using an internal “housekeeping” metabolite for example, an inner standard like TMS or, in this case, normalize each spectrum to (divide each variable by) the sum of the absolute value of all variables for the given sample. It returns a vector with an unit area (area = 1) "under the curve" (Wise & Gallagher, 1998).

One of its common applications is to remove or to minimize the effects of variable dilution of the samples (Capozzi, Ciampa, Picone, Placucci & Savorani, 2011)

4. **Chemical shift variations**: the last preprocessing problem to be mentioned here and which occurs only in HR-NMR spectroscopy is the chemical shift variations that may occur from sample to sample or even from peak to peak. The overall sample-to-sample variations are due to small variations in spectrometer frequency, while the peak to peak chemical shift variations are due to variations in, for example, pH. In this last case, a data reduction in the form called binning (Craig et al., 2006)is a pragmatic solution to the problem.

2.3 CONTRIBUTION OF METABONOMICS IN SEAFOOD RESEARCH

In the last decade, several scientific studies on fish and seafood, including wild and farmed fish and shellfish, both of marine and freshwater origin, have shed light on the importance of these products in the human diet as healthy products (Cahu, Salen & de Lorgeril, 2004; Kadam & Prabhasankar, 2010; Lee, O'Keefe, Lavie & Harris, 2009; Mansfield, 2011; Oomen et al., 2000). The main quality attribute is related to the nutritional value especially due to the presence of essential amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, plenylalanine, threonine, tryptophan and valine), highly digestible protein (Friedman & Birch, 1997), vitamins (A, D and B complex), minerals and a high content of polyunsaturated fatty acids (PUFA). The latter represent the only source of ω-3 fatty acids, whose importance in prevention of human diseases has been largely discussed and abundantly presented in literature (Burr et al., 1989;

Farzaneh-Far, Lin, Epel, Harris, Blackburn & Whooley, 2010; Kris-Etherton, Harris, Appel & Committee, 2002; Simopoulos, 1991).

Moreover the studies of fish metabonomics, found in the literature, cover a wide range of species and different topics: fish physiology and development, pollutant effects on fish, fish condition and disease, and fish as foodstuffs.

For example in the fish physiology, NMR-based metabonomics has been used to investigate in juvenile steelhead trout (*Oncorhnchus mykiss*) metabolic effects of chronic temperature stress by comparing fish exposed to 15° (ambient) or 20°C (elevated temperature) for 10 weeks (Werner, Viant, Rosenblum, Gantner, Tjeerdema & Johnson, 2006). In particular, heat-shock protein induction, growth rate and metabolic status by measurement of traditional biomarkers (phosphocreatine, ATP, ADP, AMP and glycogen) are determined. Metabonomics provides a picture of the metabolic changes induced by temperatures stress, whereas no such trend is evident in the instant growth rate. In addition, the discovery-driven nature of NMR based metabonomics permitted the finding of an unusual, histidine-like metabolite in trout muscle which was decreased in the 20°C.

Different studies have shown the utility of ^{1}H-NMR also in the area of fish development. Podrabsky et *al*. have investigated the effects of hypoxia on killifish (*Austrofundulus limnaeus*) embryo development (Podrabsky, Lopez, Fan, Higashi & Somero, 2007).

These embryos have high anoxia tolerance enabling them to survive in ephemeral oxygen-deprived ponds. Together with GC-MS-base metabolomics, ^{1}H-NMR shows that embryo metabolism is dominated by glycolytic production of lactate, with minor contributions from succinate and alanine during hypoxia.

An additional observation is the large accumulation of the neurotransmitter γ-aminobutyrate (GABA) in hypoxic embryos. Taken together, this information has contributed to understanding the extreme anoxia tolerance ability of this species. Relating to this topic, Pouliquen et al. have studied the content of 15 metabolities from three batches of turbot (*Psetta maxima*) eggs in five developmental stages, from unfertilized ova to pre-hatching (Pouliquen, Omnes, Seguin & Gaignon, 1998). Free amino acids are present at all stages, with a sharp decline in concentration during the first 8 h of development. The levels of amino

acids continue to decrease reaching a minimum just prior to organogenesis, followed by a slight rise until pre-hatching. This pattern is in line with previous findings suggesting free amino acids as the main energy substrates for newly fertilized eggs. Relatively large variations between batches of eggs from different females were found in this study, as well as in the study by Turner et al (Turner, Viant, Teh & Johnson, 2007)

In another study by Viant (Viant, 2008) the concept of using a "**metabolic trajectory**" introduced by Homes et al. (Holmes et al., 1992) in the PCA scores plot to summarize changes in the metabolite pattern of developing medaka (*Oryzias latipes*) eggs is illustrated. Metabolites profiles of eggs sampled at the same developmental stage are similar, and therefore clustered together in the scores plot. The trajectory of development could then be followed in the plot through the various stages in a time-dependent fashion. In this article, the author suggestes that metabolic trajectories will become a powerful tool for studying effects of toxicants, or other stressors, during embryogenesis.

For example, the benefits of NMR-based metabonomics in risk assessment, related to fish and seafood, are also demonstrated in another paper by Viant et *al*. where the effects of three pesticides (dinoseb,diazinon and esfenvalerate) on eyed eggs and alevins of Chinook salmon (*Oncorhynchus tshawytscha*) are studied by NMR and HPLC-UV-based metabonomics (Viant et *al*., 2006). Again, NMR is more sensitive than traditional toxicology parameters (mortality, hatching success, myoskeletal abnormalities) for dinoseb and diazinon-exposed eggs. Dose-responses effects on both eggs and alevins are observed with each pesticide inducing specific metabolic responses.

In addition to its existing application in human toxicology, metabonomics is also used for the investigation, diagnosis and monitoring of various human diseases.

The first steps towards evaluating this approach in health monitoring of wild and farmed fish have been done recently.

In 2005, Stentiford et al. published a study on dab (*Limanda limanda*), which is a sentinel species within the UK National Marine Monitoring Programme to explore proteomics, metabolomics and metabonomics as a useful complement to histopathology in distinguishing liver tumour tissue from non-tumour tissue (Stentiford, Longshaw, Lyons, Jones, Green & Feist, 2003). In other study Solanky

et al. have applied NMR-based metabonomics 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 (Solanky, Burton, MacKinnon, Walter & Dacanay, 2005). The main differences between healthy and infected fish are in the levels of lipids and lipoproteins, whereas amino acid and carbohydrate levels are fairly unchanged.

Metabonomics proved to be considerably more accurate and less-time-consuming than the current methods (clinical microbiology and stress-test, respectively), and the authors of this study suggest that NMR-based metabonomics has the potential of becoming a useful approach in routine clinical diagnosis in aquaculture.

To summarise, in certain settings, metabonomics has shown to be more sensitive, more accurate or faster than methods traditionally used for monitoring the health of fish populations, both wild and farmed.

Other prospective application areas for metabolomics and metabonomics are food safety, food fraud, food authentication, food storage and processing, nutrition value, development of novel food and health care products.

For example, the groups of Sacchi and Falch have shown the utility of ^{13}C NMR-based metabonomics for the determination and quantification of lipid classes, free fatty acids, fatty acid composition of lipids in muscle from Albacore tuna (*Thunnus alalunga*) and roe and milt from cod (*Gadus morhua*) respectively (Falch, Størseth & Aursand, 2006; Sacchi, Medina, Aubourg, Giudicianni, Paolillo & Addeo, 1993). The unique feature of the ^{13}C NMR approach is the possibility of determing the positional distribution of fatty acids in lipids in one single measurement without pre-treatment of the lipid sample.

For example, Standal et al. (Standal, Axelson & Aursand, 2009) in the ^{13}C-NMR spectra of muscle lipids from Atlantic salmon (*Salmo salar L.*), mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) calculate the distribution of omega-3 polyunsaturated fatty acids between the sn-1,3 and sn-2 glycerol chains from the carbonyl region. Their results show that there are significant differences in the sn-2 position specificity of the fatty acids 22:6n-3, 20:5n-3 and 18:4n-3 among the species investigated. The most pronounced difference is that herring has a higher proportion of its 22:6n-3 in the sn-2 position in comparison to the two

other species. In this study, ^{13}C-NMR appears to be a superior technique compared to gas chromatography for classification purposes. The reason for this is most likely that ^{13}C gives a lipid profile, which, in addition to the acid-fatty composition (Aursand, Bleivik, Rainuzzo, Leif & Mohr, 1994; Aursand, Jørgensen & Grasdalen, 1995; Aursand, Rainuzzo & Grasdalen, 1993) contains further information about lipid classes (Gunstone, 1996; Gunstone, Chakra Wijesundera & Scrimgeour, 1978) and positional distribution of fatty acids in triacylglycerols, in phosphatidylcholine and phosphatidylethanolamine (Falch et al., 2006)

In a pilot study, Rezzi et al. have shown that ^{1}H-NMR lipid metabonomics and multivariate data analysis can be used to distinguish between wild and farmed Gilthead seabream (*Sparus aurata*) with a classification success of 100% (Rezzi et al., 2007). NMR-based metabonomics has proved to be a faster and simpler method for making wild/farmed discriminantions than previously used methods like site-specific natural isotope fractionation NMR (SNIF-NMR), isotopic ratios mass spectrometry (IRMS) and lipid extraction and fractionation followed by HPLC or GC analysis. The discriminatory capacity of the NMR approach appears somewhat less powerful when applied to the classification of farmed Gilthead sea bream according to its geographical origin (Italy, Greece, Croatia or Turkey).

Although clear trends can be seen in the data, more elaborate studies including larger number of samples from each farm and additional feeding trials assessing the effects of different feeds used at the farms are needed to confirm the suitability of metabonomics and metabolomics for this application.

The work of Savorani et *al*. (2010) (Savorani et al., 2010) is also very interesting; It describes a metabolic profiling study of Gilthead sea bream from three different aquaculture. In particular, a new multivariate classification tool, *i*ECVA, reveals several metabolites which are important biomarkers for characterizing the three different aquaculture systems: glycogen (stress indicator), histidine, alanine and especially glycine.

In summary, NMR-based metabolomics offers the direct study of both metabolic structure (Lipid and protein for example) and metabolic profiles in fish and is an alternative technique for classification of fish samples according to farming method and processing method. The use of metabonomics and metabolomics in the industries of food and fish farming is expected to expand in the near future,

especially in the area of food authentication and food quality. It could become a useful tool for regulatory authorities in terms of authenticating food labeling of fish products (e.g. production method, catchment area/fish farm of origin, nutritional value, storage and processing methods). The benefit to aquaculture industries could be to assess the health status of the fish or to investigate the effects of breeding conditions (for example feed, temperature, medication, pollutants) and nutritional quality (e.g. content of *n*-3 fatty acids) of the final product depending on storage and processing conditions. In the healthcare industry, metabonomics and metabolomics could assist in locating compounds in various fish species especially interesting to human health and this could potentially be used in the development of functional foods and health care products.

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CHAPTER 3

MATERIALS

3.1 EQUIPMENTS

3.1.1 Lab supplies

- Pipettes Standard – Gilson's Pipetman® P (P10, P20, P100, P200 and P1000. Range of volumes from 10 µl to 1000 µl) with suitable tips (Diamond® precision tip);
- Single use only sterile syringes in Polypropylene from (PP, Henke - Sass);
- Micro syringes in Polypropylene (PP) for HPLC from 100 µl;
- SARTSTEDT Polypropylene (PP) 50 and 15 ml Conical and Round Bottom Centrifuge Tubes, Falcon ™ Type;
- Eppendorf ® Safe-Lock® microcentrifuge tubes volume 0.5 and 1.5 ml;
- Bottle in polypropylene (PP) from 100 - 250 - 500 - 1000 ml;
- Laboratory ceramic mortar grinder with pestle;
- Laboratory glassware;
  - Beakers, low form, with spout (50, 250, 500 ml) by Simax
  - Beute from 50 ml (SCHOTT DURAN)
  - Vacuum flasks from 1000 ml
  - Cylinders from 50 – 1000 ml (PIREX)
  - Calibrated flasks from 25 -1000 ml (DURAN)
  - Funnels in glass from 35-70 mm (DURAN)
  - Separating funnel from 250 ml (BISTABIL)
  - Quartz cuvets from 3 ml (EXACTA)
  - Corning® Disposable Pasteur Pipettes, Bulk Pack, Non – sterile, (SIGMA ALDRICH®)
  - Graduated pipette from 5 to 20 ml (class A)
  - Screw capped (Teflon lined) culture tubes (20 x 150 mm)
- Inox steel Spatulas;
- Inox steel Scalpels ;
- Whatman N° 4 and N° 1 filter paper;
- Filter in cellulose with diameter 0.20 µm;

- Pipette controller;
- AMPOL NMR sample Tubes for use up to 700 MHz NMR (203 mm, round bottom);

3.1.2 Safety and protection supplies

- Safety Eyewear Glasses;
- Natural Latex Powdered and Powder - Free Exam Gloves;
- White lab coat;

3.2 REAGENTS

- Trichloroacetic acid (CCl_3COOH, 163.39 g/mol, TCA) 6.1 N, SIGMA - ALDRICH;
- Picric acid (C6H3N3O7, 229.1 g/mol) 1% in H_2O, SIGMA-ALDRICH;
- Trimethylamine ($(CH_3)_3$-N · HCl, 95.57 g/mol, TMA-N) 98%, ALDRICH;
- Adenosine 5'-triphosphate ($C_{10}H_{16}N_5O_{13}P_3$, 507.18 g/mol, ATP) 98%, ACROS;
- Adenosine 5'-diphosphate ($C_{10}H_{15}N_5O_{10}P_2$, 427.201 g/mol, ADP) 98%, ACROS;
- Adenosine 5'- monophosphate ($C_{10}H_{12}N_5O_6P$, 329.21 g/mol, AMP) 99%, ACROS;
- Inosine 5'- monophosphate ($C_{10}H_{13}N_4O_8P$, 392.17 g/mol, IMP) 97% , ACROS;
- Inosine ($C_{10}H_{12}N_4O_5$, 268.23, HxR) 99%, ACROS;
- Ipoxanthine ($C_5H_4N_4O$, 136.11 g/mol, Hx) 99.5%, ACROS;
- Formaldehyde (COH_2, 30.03 g/mol) 37.3%, SIGMA – ALDRICH;
- Potassium hydroxide (KOH, 56.11 g/mol), SIGMA – ALDRICH;
- Toluene (C_7H_8, 92.14 g/mol) 99.7%, SIGMA – ALDRICH;
- Sodium sulfate (Na_2SO_4, 142.04 g/mol) granular anhydrous, SIGMA – ALDRICH;
- Potasssium dihydrogen phospate (KH_2PO_4, 136.09 g/mol), PANREAC;

- Potasssium hydrogen phospate anhydrous (K_2HPO_4, 174.18 g/mol), PANREAC;
- Ethanol (C_6H_5OH, 46.07 g/mol) 99.5% purity, MERK;
- Hydrochloric Acid (HCl, 36.01 g/mol) 37% purity, SIGMA – ALDRICH;
- Milliq demineralized water, SIGMA-ALDRICH;
- Deuterium oxide (D_2O, 20.04 g/mol, 99.9% purity), BRUKER;
- 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt**;** ($(CH_3)_3SiCD_2CD_2CO_2Na$, 127.27 g/mol TSP), 98 atom % D, BRUKER.

3.3 SOLUTIONS

- **Stock TMA Standard (1.00 mg TMA-N/ml):** Dissolve 0.6820 g TMA-HCl (dried overnight in dessicator) in distilled water. Dilute to 100 ml. Store refrigerated;
- **Working TMA Standard (10.0 µg TMA-N/ml)**: Pipet 1 ml TMA stock solution into a 100 ml volumetric flask and dilute to volume;
- **Formaldehyde (10%)**: Dilute 26.8 ml of 37.3% HCHO to 100 ml with distilled water;
- **KOH (25% w/w):** Carefully and with stirring dissolve 25g KOH in 75 ml distilled water;
- **KOH (1 M and 9 M**): Dissolve respectively 56.11 g and 504.99 g in 1000 ml distilled water;
- **Trichloroacetic acid (7.5%)**: Dissolve 7.5 g TCA in 92.5 ml distilled water;
- **Working picric acid Standard (0.02 % in toluene)**: Dilute 13.7 ml of picric acid solution in toluene (0,73%) in 486.3 ml toluene;
- **Mixture of ATP, ADP, AMP, IMP, HxR, and Hx at 0.166 mM each one:** 0.0229 g of ATP, 0.0195 g of ADP, 0.0144 g of AMP, 0.0163 g of IMP, 0.0111 g of HxR, 0.0056 g of Hx in 250 ml distilled water;
- **Solution HCl (1 M)**: Dissolve 82,85 ml of HCl (37% or 12.07 M) in 917.15 ml distilled water;

3.4 BUFFER SOLUTION

- Phosphate Buffer, 0.1M and pH 7.00;

3.4.1 Practical ways to make a buffer

Generally, three methods can be used to obtain a buffer solution:

1. the buffer pK_a method;
2. the two solution method;
3. acid and basic titration method.

For the $HPO_4^{2-}/H_2PO_4^-$ buffer the buffer pK_a method was used. In water the ion $H_2PO_4^-$ establishes an equilibrium with the conjugate base, HPO_4^{2-}. For $H_2PO_4^-$, the value of K_a equals 6.2×10^{-8} and pK_a equals 7.21. The magnitude s of the K_a and pK_a values of different weak acids give us a comparison of their relative strength. A weaker acid has less dissociation to the conjugate base and the equilibrium favors the undissociated weak acid form. This results in a smaller K_a value. A smaller K_a value corresponds to a larger pK_a. In other words, the weaker the acid, the larger the pK_a value. For experimental work in aqueous solutions, it is fundamentally important to be able to prepare a buffer solution at a desired pH.

The pK_a methods is based on the Henderson – Hasselbalch relationship (R.H.C. Strang, 1981) written in Equation 3.1.

$$K_a = \frac{[H^+]\ \ [A^-]}{[HA]} \qquad \text{(Eq. 3.1)}$$

The equation allows to calculate the correct ratio of basic form to acidic form which can be mixed to achieve the desired buffered pH. For the $HPO_4^{2-}/H_2PO_4^-$ buffer the equation 3.1, considering the definition of K_a and pH, is rearranged as shown in equation 3.2:

$$pH - pK_a = \log \frac{HPO_4^{2-}}{H_2PO_4^-} \qquad \text{(Eq. 3.2)}$$

Note that Henderson-Hasselbalch relationship indicates that the pH of a buffer solution does not depend on the total concentration of the buffering acid and conjugate base but only on the pK_a and the ratio of the concentration of these two species.

On the other hand, the buffering capacity of a solution quantifies the amount of H_3O^+ or OH^- the solution is capable of neutralizing before the acid or conjugate base form is saturated and the pH begins to fall or rise precipitously.

This will depend on the total concentration of the acid and conjugate base buffer ions. Also, the buffering capacity may be different towards addition of acid than towards base. This will be true unless the pH of the buffer solution is identical to the pK_a of the buffering acid – base equilibrium.

3.5 INSTRUMENTS

- Eletronic digital tecnica Balance (max 2200 g, d=0.01 g) SCALTEC (SBA 52) ;
- Eletronic digital analytical Balance (max 220g, d=0.0001g), SCALTEC (SBA 31) ;
- IKA® ULTRA-TURRAX® homogenizer T18, basic, AC input 115 V;
- JENWAY Model 3310 pH Meter with glass bodied combination electrode swing arm electrode holder & ATC;
- Heating magnetic stirrer mod. ARE, VELP Scientifica®;
- Beckman Coulter TM Microfuge® 18 Microcentrifuge (max 14000 rpm adjustable in 500 increments) ;
- FRAC-900, Amersham Pharamcia Biotech, high-pressure liquid chromatographic system, consisting of injection valve by a 20 µl capacity loop, and a UV-Vis absorbance detector;
- Spectrophotometer UV-Vis (UV-1601-Shimadzu) ;

- NMR Varian Mercury-plus AS400/54 (400MHz) spectrometer equipped with a 5mm PFG gradient 4 nuclei ($^{1}H/^{19}F/^{13}C/^{31}P$) probehead, with a 400 MHz (9.4 Tesla) superconducting magnetic system by Oxford Active Shielded and equipped with Sun BLADE 150 Host Workstation with Solaris 10 OS (80GB Hard Dish, 512 MB Ram, CD ROM SCSI Drive and VnmrJ 1.1D Software) ;
- FT-NMR Avance Bruker AvIII (600 MHz) spectrometer Ultra Shield Plus equipped with:
    - The electronic 3-channel RF consisting amplifiers from 100-Watt ^{1}H and broadband for X 300 Watt
    - Control unit gradients GCU
    - Control system of the temperature BVT3000
    - Probe 5 mm with Z grad ^{1}H-^{13}C-^{15}N
    - Autosampler with 60 holders.
    - Software Topspin 3.0

3.6 SOFTWARE

3.6.1 *NMR data processing*

Mestrec (www.mestrec.com), Magnetic Resonance Companion, "is a software package that offers state-of-the-art facilities for data processing, visualization, and analysis of high resolution nuclear magnetic resonance (NMR) data, combined with a robust, user-friendly graphical interface that fully exploits the power and flexibility of the Windows platform.

The program provides a variety of conversion facilities for most NMR spectrometer formats and includes all the conventional processing, displaying, and plotting capabilities of an NMR program, as well as more advanced processing techniques".

A pdf format tutorial designed to help to become familiar with Mestrec's features is available at http://nmr-aci.unihd.de/Anleitungen/mestrec/mestec.pdf

3.6.2 Chemometrics data processing

The multivariate statistical analysis were carried out using different statistical software due to thje different kind of analysis: R program, Matlab and Latentix 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 modeling, 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 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 non interactive language such as C or Fortran.

Latentix (http://www.latentix.com) is a new user –friendly stand-alone program for chemometric data analysis. It offers a raw data plot facility as well as comprehensive PCA and PLS modeling.

CHAPTER 4

HR ^{1}H-NMR spectroscopy for the determination of TMA-N and K-Index in different species of fish

4.1 INTRODUCTION

The AOAC Official Method (AOAC, 2000) recognizes as an official method for determining TMA-N the one based on Dyer's method (Dyer, 1945). This method encompasses a liquid–liquid extraction of TMA-N with toluene and its subsequent reaction with picric acid reagent to form a yellow complex, picrate, whose concentration can be calculated by measuring absorbance at 410 nm (UV). Due to the low analytical sensitivity achieved with this method, TMA-N determination in fish must be performed after a certain time of *post-mortem* storage; and as a result, it is applicable only to certain fish species (Pena-Pereira, Lavilla & Bendicho, 2010). Commonly, most of the methods described in the literature for the determination of TMA-N in fish samples includes: flow injection/gas diffusion systems with spectrophotometric (García-Garrido & Luque de Castro, 1997; Sadok, Uglow & Haswell, 1996), potenciometric (Adhoum, Monser, Sadok, El-Abed, Greenway & Uglow, 2003) or Fourier transform-infrared spectroscopy detection (Armenta, Coelho, Roda, Garrigues & de la Guardia, 2006); capillary electrophoresis with indirect UV-detection (Lista, Arce, Ros & Valcarcel, 2001; Timm & Jørgensen, 2002); gas chromatography with flame ionisation detector (FID) (Li, Zeng, Zhou, Gong, Wang & Chen, 2004; Veciana-Nogues, Albala-Hurtado, Izquierdo-Pulido & Vidal-Carou, 1996), nitrogen-phosphorus detector (NPD) (Béné, Hayman, Reynard, Luisier & Villettaz, 2001) or mass spectrometry (MS) detection (Chan, Yao, Wong, Wong, Mok & Sin, 2006). As far as the K-Index (par. 1.3.1), there are no official methods for their quantification. Several approaches have been proposed for the analysis of individual or a combination of nucleotide catabolites, but none are more reliable than the HPLC approach (Kaminishi, Nakaniwa, Kunimoto & Miki, 2000; Valle, Malle & Bouquelet, 1998). Therefore, the rapid methods for determination of this parameter can be the advantage in developing optimized processing of seafood.

In this thesis, HR ^{1}H-NMR is used in correlation to various chemical muscle proprieties (TMA-N and nucleotides concentration), as measured with standard determination methods with the aim of quantifying (QHNMR) the amount of TMA-N and the K-Index in order to build a calibration models able to predict the

variation of seafood quality parameters in different species of fish during their storage at 4°C. The potential of NMR spectroscopy for quantitative analysis of organic chemicals was first demonstrated two decades ago (Maniara, Rajamoorthi, Rajan & Stockton, 1998; Shoolery, 1977). Nowadays, many of the QHNMR measurements show relative standard deviations less than 5%, which is satisfactory for most analytical purposes (Schicho, Nazyrova, Shaykhutdinov, Duggan, Vogel & Storr, 2010).

4.2 MATERIAL AND METHODS

4.2.1 Biological material for the experimental work

Initially, in this first part of the research, Europen seabass (*Dicentrarchus labrax*) and Atlantic mackerel (*Scomber scombrus*) are used to set up the experimental work and to verify first the recovery of the all TMA-N during the extraction and to establish a good correlation between HR ^{1}H-NMR and standard fish quality determination methods. Once the experimental method was set up and the correlation observed, ^{1}H-NMR is used to evaluate the concentration of TMA-N and the K-Index value in wild fish samples, such as Bogue fish (*Boops boops*), and Red mullet (*Mullus barbatus*) (Fig. 4.1), stored at 4 °C during 11 days.

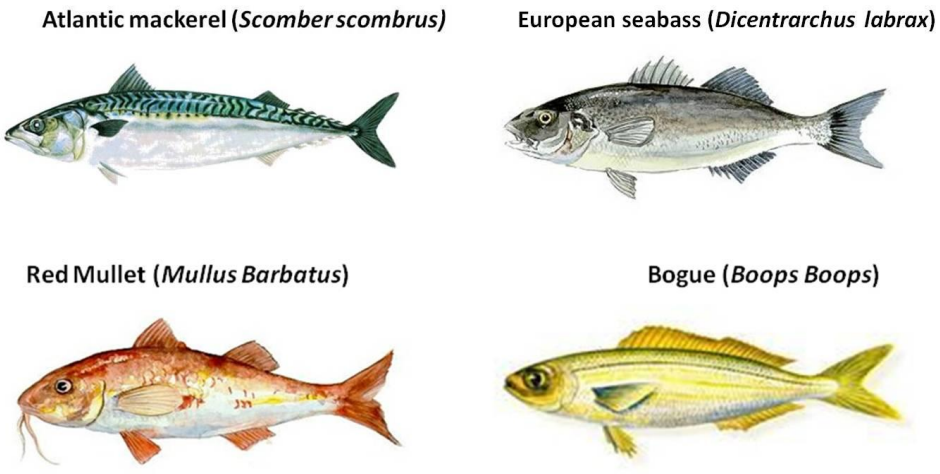

Figure 4.1: fish species used to set up the experimental work (Atlantic mackerel and European seabaa) and Red mullet and Bogue fish sampling performed in order to calculate TMA-N and K-Index during 11 days of storage at 4°C

4.2.2 Experimental design

Once the experimental method was set up and the correlation verified, Red mullet and Bogue fish are sampled for 11 days, as shown in fig. 4.2.

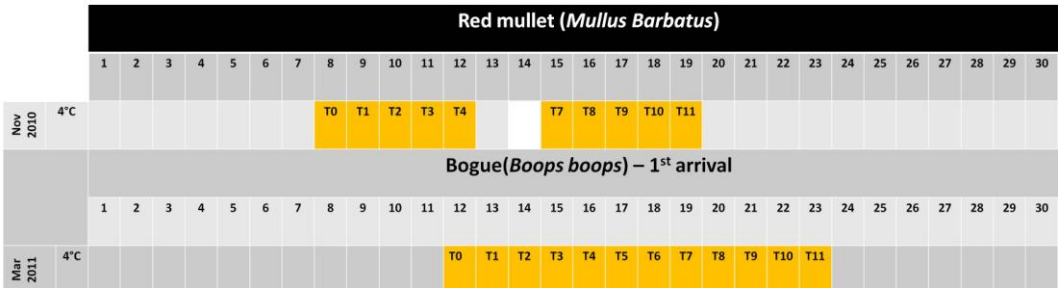

Figure 4.2: Red mullet and Bogue fish sampling performed in order to calculate TMA-N content and K-Index value by using HR ^{1}H-NMR and standard methods (UV-spectrofotometry and HPLC) during 11 days of storage at 4 °C

4.2.3 Sample preparation for determination of TMA-N by Ultraviolet (UV) *spectrophotometry*

The picric acid method of Dyer & Mounsey (1945) as modified by Tozawa et al. (Tozawa, Enokihara & Amano, 1971) is used, according to the AOAC official method (AOAC, 2000): 25 g of fish muscle are blended with 50 ml of a 7.5% trichloroacetic acid (TCA) solution and filtered (Whatman #4). The filtrate is stored at -80°C until the analyses are performed.

Before determining the unknown quantity of TMA-N, a calibration curve is performed using 1.0, 1.5, 2.5 and 3.0 ml of standard solution of TMA-N (10 µg/ml). Through the calibration curve it has been possible to evaluate unknown TMA-N concentration in the following equation:

$$TMA-N(mg/100g) = \frac{(Abs-a)/b * V_1 * 0.1}{V_2 * W}$$

Where: N-TMA (mg/100g): trimethylamine expressed as mg N-TMA per 100g fish, Abs: absorbance at 410 nm, a and b: intercept and slope of calibration curve, V_1: volume (ml) of the extractant phase, V_2: volume (ml) of extract added to test tube and W= weight(g) of fish used in 1:2 extraction.

Different aliquots of extract are pipetted and they are between 0.1 - 4.0 ml so that the concentration of TMA-N in the extract is determined by appropriate aliquot size.

Water is added to each aliquot to bring total volume to 4.0 ml for both extracts and standards. For the blank 4.0 ml distilled water are used.

Just before analysis, 1 ml of 10% formaldehyde, 10 ml of toluene and 3.0 ml of 25% KOH were added, in the order, to the filtrate.

The reaction mixture is shaken vigorously for 30 min at 30 °C and after the separation of the phases, 7 ml of the upper toluene solution is moved to a new large dryer tube containing approximately 0.3 to 0.4 g anhydrous Na_2SO_4 and gently shaken until the solution is clear. Five milliliters of this solution are added to 5 ml of picric acid (0.02% in toluene) and mixed by swirling gently. Absorbance is measured at 410 nm in a UV/Vis spectrophotometer (Schimazu).

4.2.4 Sample preparation for determination of K-Index by High Performance Liquid Chromatography (HPLC)

An aliquot of the TCA extract (10 ml) is used also for the K-Index calculation. The extract are neutralized to pH 7.0 with 1M KOH and then filtered (Whatman #1) to remove insoluble potassium trichloroacetate (Márquez-Ríos, Morán-Palacio, Lugo-Sánchez, Ocano-Higuera & Pacheco-Aguilar, 2007). Twenty µl of the neutralized solution are injected. A FRAC-900, Amersham high-pressure liquid chromatographic system is used, consisting of injection valve with a 20 µl capacity loop and a UV-VIS absorbance detector. A Beckman C18 stainless steel column (25 cm x 4.6 mm i.d.) is used for nucleotides separation. The mobile phase is 0.1 M phosphate buffer pH 7.0 (0.04 M KH_2PO_4 and 0.06 M K_2HPO_4) with a flow rate of 1 ml/min. The eluant is monitored at 254 nm. Nucleotide standards include a mixture of ATP, ADP, AMP, IMP, HxR, and Hx at 0.166 mM each one.

4.2.5 Sample preparation for HR ^{1}H-NMR data acquisition

The pH of a 1 ml aliquot of aqueous extract in TCA is adjusted to 7.8 using 9 M KOH in microfuge tubes and centrifuged at 14K rpm for 5 minutes in order to remove potassium trichloroacetate precipitate. The supernatant is stored at -80 °C until ^{1}H-NMR measure.

The samples are prepared for NMR analysis by adding to the thawed samples 160 µl of a D_2O solution of TSP 6.25 mM (Figure 4.3).

^{1}H-NMR spectra are recorded at 298 K with a Varian Mercury spectrometer operating at a frequency of 400.13 MHz. Each spectrum is acquired using 32K data points over a 7211.54 Hz spectral width and adding 256 transients. A recycle delay of 4s and a 90° pulse of 6.30 µs are set up. Saturation of residual water signal is achieved by irradiating its frequency during the recycle delay at δ equal to 4.703 ppm. Each spectrum is processed with MestRe-C 4.9.8.0 (Mestreab Research SL, Spain) by manually adjusting the phase and the base-line and applying a line broadening factor of 0.5 Hz.

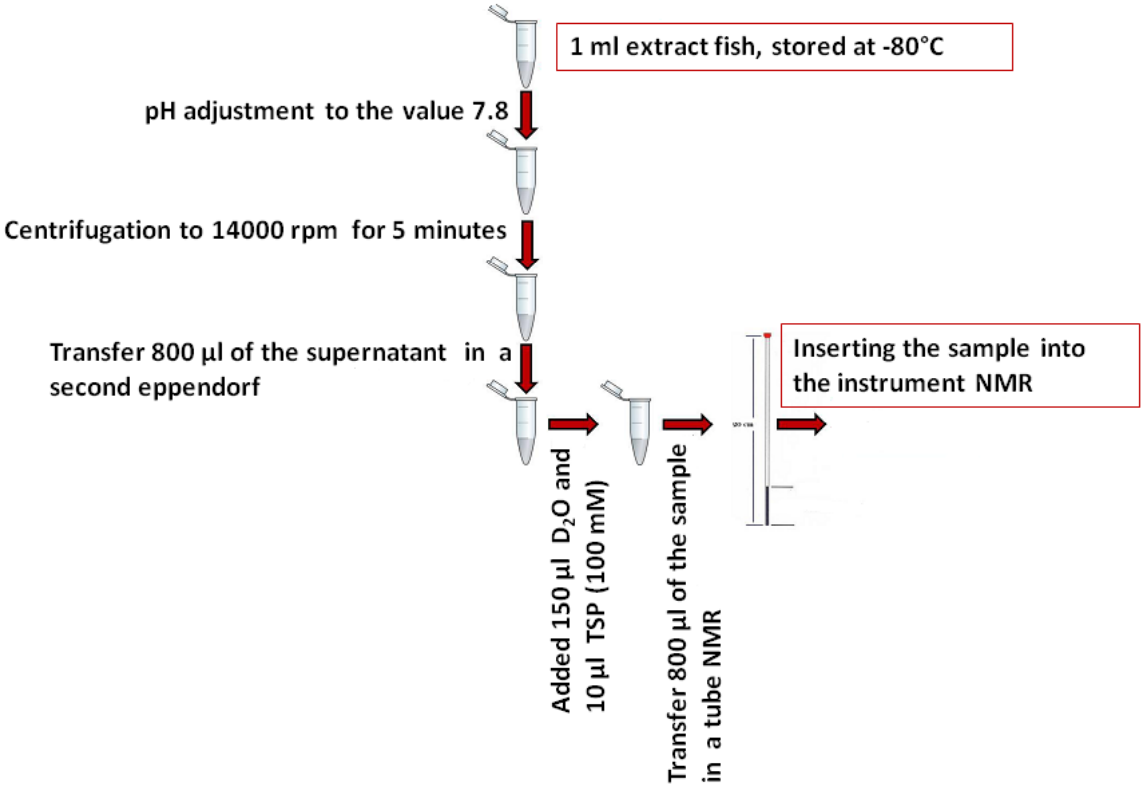

Figure 4.3: layout of sample preparation for the NMR analysis

Peaks related to TMA-N and nucleotides are assigned by comparing with the literature (Picone, 2009; Picone, Balling Engelsen, Savorani, Testi, Badiani & Capozzi, 2011; Savorani, Picone, Badiani, Fagioli, Capozzi & Engelsen, 2010) their chemical shift and multiplicity. When more peaks due to different protons of the same molecule are identified, both are employed for the quantification.

4.2.6 Statistical Analysis

ANOVA is performed in order to test the significance of some “effects” during the instrumental analysis. In particular, software SPSS, version 17.0 is used to determine the significant variance component due to analyst, acquisition-instrumental, sample preparation and the processing in the spectroscopic method, during data elaboration.

4.3 RESULT AND DISCUSSION

In conformity with the Eurachem guidelines (EURACHEM Guide, 1998), the proposed method is validated in terms of instrumental linearity, precision, analytical recovery, as well as limits of detection and quantitation. All of the parameters described below are determined considering three different preparations per fish sample, one spectral acquisition per preparation, and six repetitions of the data processing per spectrum, for a total of 18 measurements per point.

4.3.1 Instrumental Linearity and Accuracy

The instrumental linearity of the calibration curve for the picrate method is checked reporting the absorbance’s values versus the concentrations of TMA-N standard solutions (Fig. 4.4).

The regression shows good linearity. Least-squared analysis produces a correlation coefficient of 0.9946 and a regression equation of y= 0.0152x+0.0050. These results confirm a linear relationship between the TMA-N concentration and instrumental response in the range of 0−30 μg/ml.

The instrumental accuracy is tested performing three replicates for sample. Mean (x), relative standard deviation (RSD) and bias, obtained at four different concentration levels, are reported in Table 4.1.

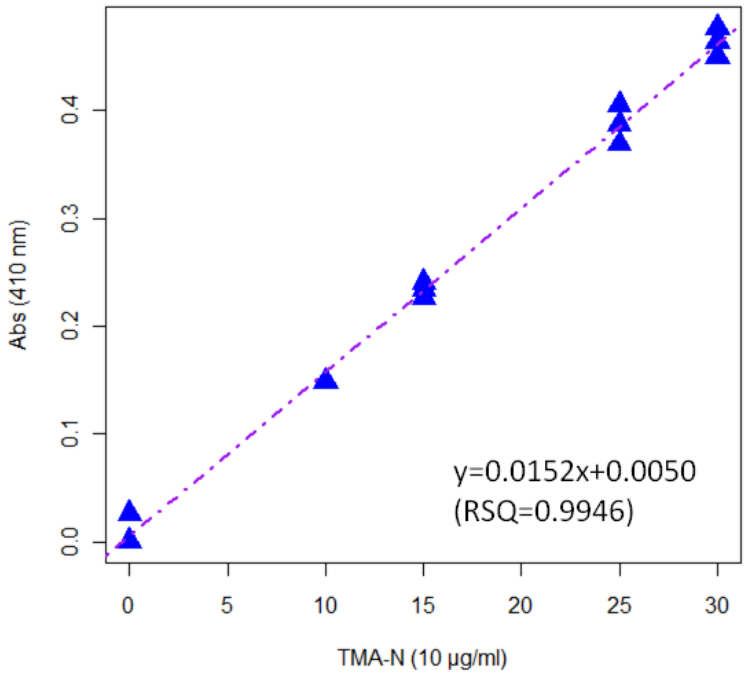

Figure 4.4: calibration curve for picrate method. The regression shows good linearity. Least-squared analysis produces a correlation coefficient of 0.9946 and a regression equation of y = 0.0152x+0.0050

The RSDs obtained are always satisfactory, and they are acceptable according to Horwitz's formula (RSDH) (Horwitz, 1982) and HorRat (Horwitz Ratio)[1] value (Linsinger & Josephs, 2006).

The calculation of TMA-N by ^{1}H-NMR spectroscopy is performed using an absolute method with reference to an internal standard compound without a calibration model.

[1] The Horwitz ratio (HorRat) is a normalized performance parameter indicating the acceptability of methods of analysis with respect to among-laboratory precision (reproducibility). It is the ratio of the observed relative standard deviation among laboratories calculated from the actual performance data to the corresponding predicted relative standard deviation calculated from the Horwitz equation. It is more or less independent of analyte, matrix, method, and time of publication (as a surrogate for the state of the art of analytical chemistry). It is now one of the acceptability criteria for many of the recently adopted chemical methods of analysis of AOAC INTERNATIONAL, the European Union, and other European organizations dealing with food analysis (e.g., European Committee for Standardization and Nordic Analytical Committee). The origin and applications of the formula are described. Consistent deviations from the ratio on the low side (values <0.5) may indicate unreported averaging or excellent training and experience; consistent deviations on the high side (values >2) may indicate inhomogeneity of the test samples, need for further method optimization or training, operating below the limit of determination, or an unsatisfactory.

Table 4.1: instrumental Linearity for colorimetric method (UV)

N-TMA (µg)[a]	Bias[b] (%)	RSD[c] (%)	RSDH[d] (%)	Horrat[e]	Accuracy[f]
10	-6.6	0.41	7.7	0.05	6.6
15	-0.2	3.55	7.1	0.50	3.6
25	0,1	5.46	6.6	0.83	5.5
30	0.2	3.41	6.4	0.53	3.4

[a]Mean of 3 measurements per point, obtained for 4 different aliquots trimethylamine standard solution.
[b](mc-nc) x 100/nc, where mc is the measured amount and nc is the nominal amount.
[c]Relative standard deviation. [d]Acceptable relative standard deviation according to Horwitz's formula for intralaboratory studies.
[e]Horrat=RSD measured/RSD Horwitz predicted.
[f]Accuracy =(p2 + b2)^{½} where p is precision (expressed as relative standard deviation, RSD) and b is the bias.

NMR is by definition (Malz & Jancke, 2005) a quantitative spectroscopic tool because the intensity of a resonance line is directly proportional to the number of resonant nuclei (spins). This fact enables, in principle, a precise determination of the amount of molecular structures and, hence, of substances in solids as well as liquids.

In this research work TSP was used as an internal standard by which it was possible to calculate TMA-N concentration on fish samples by using the following equation

$$TMA-N(g/100g) = \frac{I_{TMA-N} * E_{TMA-N} * [TSP] * 0.96 * V_1 * FW_{N(TMA)} * 100}{I_{TSP} * E_{TSP} * 0.80 * m_{fish}}$$

Where:

TMA-N (g/100g) = trimethylamine expressed as g TMA-N per 100 g of fish muscle, [TSP] = molar concentration (M) of TSP, I_{TMA-N} and I_{TSP} = peak areas of TMA-N and TSP, respectively, E_{TMA-N} and E_{TSP} = TMA-N and TSP equivalent proton. 0.96/0.80 = dilution factor, V_1 = volume (l) of acid trichloroacetic (TCA (7.5 %)) added for 1:2 extraction, $FW_{N(TMA)}$ = Nitrogen molecular weight in TMA-N and m_{fish} = weight (g) of fish used in 1:2 extraction.

4.3.2 Analytical recovery and repeatability

To evaluate the accuracy and the extraction efficiency of the proposed method, recovery experiments are performed on samples, European seabass and Atlantic mackerel, in which 0.1 mg/100 g TMA-N is detected, spiked at three

concentrations of standard TMA-N for a total of 4 samples for each fish species (Fig. 4.5). The results presented in Table 4.2 and 4.3 are satisfactory. Recovery close to 100% is observed at all spiking levels.

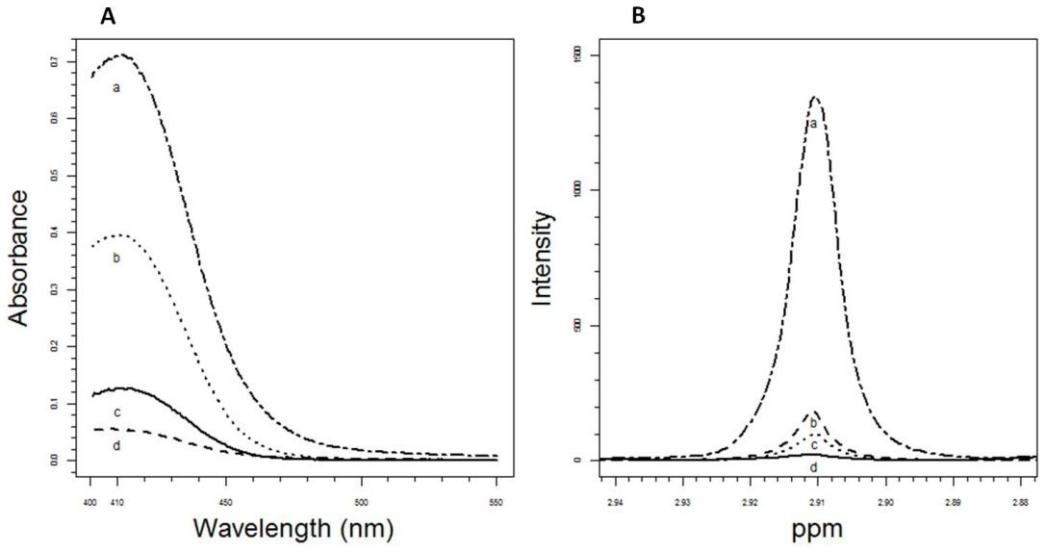

Fig. 4.5: detection of TMA-N by A) UV method and B) ^{1}H-NMR method; a) no spiked sample, b) sample spiked with 200 µl of the standard solution c) sample spiked with 400 µl of the standard solution d) sample spiked with 600 µl of the standard solution(1mg/ml TMA-N)

Table 4.2: Recovery of TMA-N during extraction and Accuracy of two methods in spiked samples

European seabass (*Dicentrarchus labrax*)												
TMA-N[a] (µg/ml)	**Recovery[b](%)**		**Bias[c](%)**		**RSD[d](%)**		**RSDH[e](%)**		**Horrat[f]**		**Accuracy[g]**	
	UV	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**
20(0.002)	100.9(1.1)	99.4(2.3)	0.9	-0.6	5.0	5.5	9.7	9.7	0.5	0.6	5.6	5.6
40(0.003)	98.9(0.9)	100.3(0.7)	-1.1	0.3	4.9	5.3	8.7	8.7	0.6	0.6	5.4	5.6
60(0.003)	100.3(0.5)	100.5(0.5)	0.3	0.5	4.9	5.2	8.2	8.2	0.7	0.6	5.5	5.5

Table 4.3: Recovery of TMA-N during extraction and Accuracy of two methods in spiked samples

Atlantic meckerel (*Scomber scombrus*)												
TMA-N[a] (µg/ml)	**Recovery[b](%)**		**Bias[c](%)**		**RSD[d](%)**		**RSDH[e](%)**		**Horrat[f]**		**Accuracy[g]**	
	UV	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**
20(0.007)	96.6(3.3)	98.1(2.8)	-3.5	-1.9	5.3	5.6	9.7	9.7	0.5	0.6	6.4	5.9
40(0.007)	98.8(1.7)	101.1(2.0)	1.2	1.1	5.5	5.7	8.7	8.7	0.6	0.7	5.6	5.8
60(0.005)	99.8(0.8)	99.3(1.4)	0.2	0.7	5.7	5.4	8.2	8.2	0.7	0.7	5.6	5.5

[a]Mean of 6 measurements per point, obtained for 3 different trimethylamine standard solutions.
[b]Recovery = [(C1-C2)/C3]* 100, where C1 is the analyte concentration, measured after the addition of standard solution; C2 is the analyte concentration, measured before the addition of standard solution; C3 is the concentration of added standard solution.
[c](mc-nc) x 100/nc, where mc is the measured concentration and nc is the nominal concentration.
[d]Relative standard deviation.
[e]Acceptable relative standard deviation according to Horwitz's formula for intralaboratory studies.
[f]Horrat=RSD measured/RSD Horwitz predicted. [g]Accuracy =$(p2 + b2)^{½}$ where p is precision (expressed as relative standard deviation, RSD) and b is the bias.

Before repeatability evaluation, the analysis of the variance (ANOVA) method is used to determine the statistical significance of the fixed effects: analyst spectrometer, sample preparation and ^{1}H-NMR spectrum processing. Generally a good processing step determines the precision of integration and consequently the accuracy of quantification. Its depends upon the noise level of the spectrum, the line shape, the quality of shimming and phasing, the baseline and drift corrections (Pauli, Jaki & Lankin, 2004).

For a significance level α=0.05, the variance component is not statistically significant if its p-value is equal to or greater than 0.05 (Maniara et al., 1998).

The data show that the variance component is not statistically significant for analyst (p_{UV}=0.345; p_{NMR}=0.885), for acquisition-instrumental (p_{UV}=0.345; p_{NMR}=0.123), for sample preparation (p_{UV}=0.062; p_{NMR}=0.123), and for ^{1}H-NMR processing (p_{NMR}= 0.875).

The repeatability of both analytical methods, expressed by RSD, is determined using 12 fish samples of Red mullet (n=6) and Bogue fish (n=6), with TMA-N concentration in a range between 0.1mg/100 g and 37mg/100g. The values of RSD reported in table 4.4 demonstrate the precision of both analytical methods.

Table 4.4: TMA-N content found in samples of Red mullet and Bogue fish

Red mullet (*Mullus barbatus*)								
# Fish sample	**TMA-N[a] mg/100g**		**RSD(%)**		**RSDH(%)**		**Horrat**	
	UV	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**
1	0.14(0.04)	0.16(0.02)	6.9	7.4	10.1	10.3	0.7	0.7
2	0.29(0.1)	0.27(0.02)	3.4	4.3	9.2	9.2	0.4	0.5
3	3.80(0.5)	2.86(0.2)	6.1	3.3	6.1	6.2	1.0	0.5
4	18.87(3.6)	11.95(2.4)	3.6	3.8	4.8	5.2	0.7	0.7
5	26.3(3.9)	19.26(1.2)	4.0	1.2	4.7	4.8	0.9	0.3
6	37.54(1.8)	25.11(1.1)	2.2	2.0	4.4	4.7	0.5	0.4

Bogue fish (*Boos boops*)								
# Fish sample	**TMA-N[a] mg/100g**		**RSD(%)**		**RSDH(%)**		**Horrat**	
	UV	**NMR**	**UV**	**NMR**	**UV**	**NMR**	**UV**	**NMR**
1	0.45(0.16)	0.33(0.05)	9.2	6.6	8.7	9.2	1.0	0.7
2	1.02(0.07)	0.66(0.07)	3.2	4.7	7.6	8.0	0.4	0.6
3	6.6(0.05)	6.8(0.4)	1.3	2.7	5.7	5.7	0.2	0.5
4	16.7(1.3)	11.4(0.9)	3.4	3.4	5.0	5.3	0.7	0.6
5	17.7(3.6)	12.8(1.9)	2.7	3.0	5.2	5.2	0.5	0.6
6	21.5(4.3)	20.03(4.3)	3.9	4.7	4.7	5.1	0.8	0.9

4.3.3 Limits of Detection (LOD) and Quantitation (LOQ)

The colorimetric analysis LOD and LOQ are calculated in relation to the IUPAC method.

The latter defined the LOD in terms of concentration (C_L) and the signal (X_L) generated by a solution of concentration C_L. They define the value of X_L in terms of the mean blank signal ($\overline{X_B}$) and the standard deviation (S_B) of these blank measurements as

$$X_L = \overline{X}_B + k * S_B$$

where k is a numerical factor chosen in accordance with the confidence level desired (Long & Winefordner, 1983). Long and Winefordner further link C_L to X_L as follows:

$$C_L = (X_L - \overline{X}_B) / \ m$$

where m is defined as 'analytical sensitivity' and expressed as the slope of the calibration curve line obtained from the linear regression analysis. By substituting the value of X_L from the first equation into the second, Long and Winefordner define C_L as:

$$C_L = (k * S_B) / \ m$$

Long and Winefordner along with several other authors agree on a value of k =3, which allows a confidence level of 99.86% if the values of X_B follow a normal distribution, and 89% if the values of X_B do not follow a normal distribution (Long et al., 1983).

The last equation calculates also the LOQ value when k =10. In this work, according to the last equation and on the base of the calibration curve of figure 4.2, a LOD of 0.3 mg/100g and a LOQ of 0.9 mg/100g are obtained. Only concentrations above LOD can be determined with this method. In fact, in table 4.3 most of the data with RSD (%) >5% are samples with a content of TMA-N

minor of the limit of detection (LOD) and also of quantitation (LOQ). In ^{1}H-NMR spectroscopy, the LOD and LOQ values depend upon acquisition parameters, mainly on the number of scans (NS) that influence the Signal/Noise ratio (S/N) and the receiver gain (RG) (Maniara et al., 1998). The LOD and LOQ values, using samples of fish at T0, in which TMA-N content is equal to 0.11 ± 0.029 mg/100g, are calculated by using the following equations

$$LOD = 3C/(S/N) \text{ and } LOQ = 10C/(S/N)$$

Where C is the TMA-N concentration and S/N is Signal/Noise ratio

With NS= 128 and RG=6 a LOD of 0.1mg/100g and a LOQ of 0.4 mg/100g are obtained.

4.3.4 Comparison of UV and ^{1}H-NMR methods and specificity

The R^2 of TMA-N values calculated by ^{1}H-NMR and UV method is greater than 0.9 (Figure 4.6), showing an excellent positive relationship between these two methods.

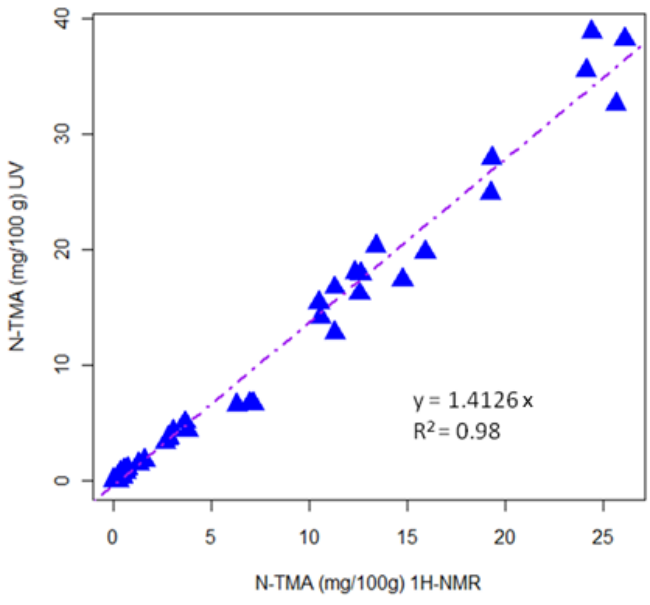

Figure 4.6: correlation between TMA-N content evaluated by ^{1}H-NMR and UV

Although there is a good correlation and precise data's found, in some cases, the concentrations TMA-N evaluated by UV method are higher than the ones

obtained from ^{1}H-NMR of about 41%. These are probably due to the reaction of TMA with picric acid not being specific, including certain quantities of other non-volatile compounds, such as putrescine, cadaverine, tyramine and histamine. Dyer and Mounsey (Dyer, 1945) shown that many amines, formed during bacterial breakdown of protein in the spoilage of fish, react with the picric acid reagent. Also Gill and Thompson (Gill & Thompson, 1984) report that colorimetric TMA-N data obtained using the Dyer procedure are consistently 35% higher than the results obtained by HPLC.

4.4 EVALUATION OF K-INDEX BY HPLC METHOD AND HR ^{1}H-NMR

4.4.1 Identification of nucleotides' peaks by HPLC and ^{1}H-NMR

The HPLC determination of nucleotides peaks from fish samples is done by comparing their chromatograms with the one obtained from a nucleotides standard solution (Fig. 4.7).

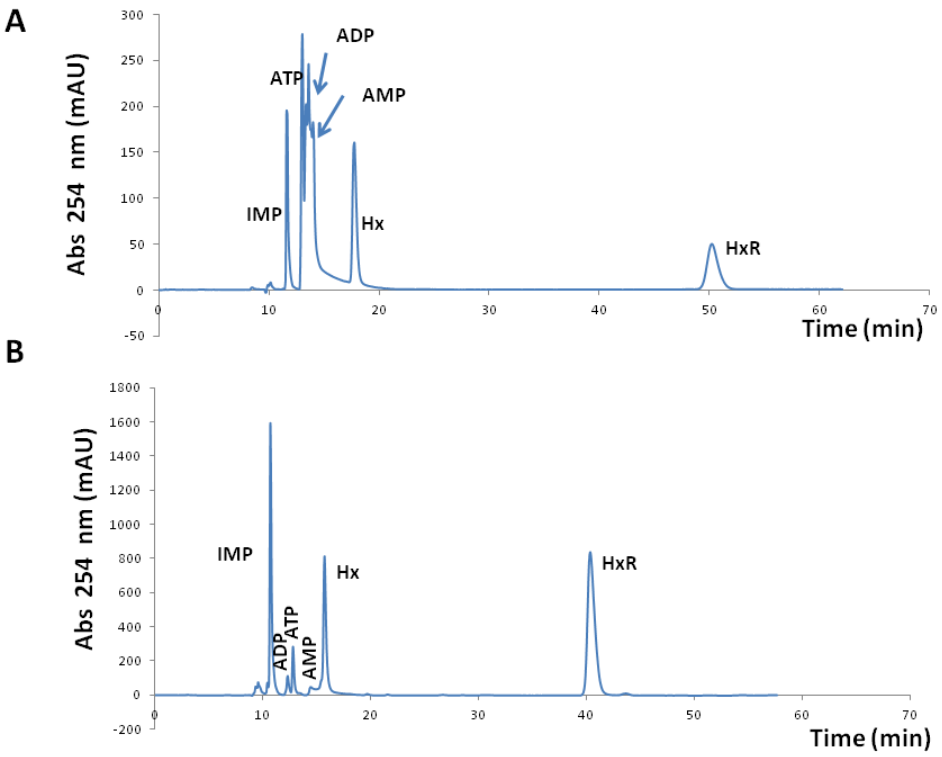

Figure 4.7: (A) nucleotides standard solution chromatogram. Operating conditions: Column Beckman C18; flow rate, 1.0 ml/min; mobile phase, 0.04 M potassium dihydrogen orthophosphate and 0.06 M dipotassium hydrogen orthophosphate, (B) chromatogram of nucleotides from Red mullet sample

The RSD value < 2% calculated for the elution time demonstrate the good reproducibility of HPLC method.

To determine the K-Index, each nucleotide's peaks area is integrated. The variation of K-Index during the spoilage is reported in table 4.5.

According to ^{1}H-NMR, nucleotides assignment is performed by comparison with previous published data (Ciampa, Picone, Laghi, Nikzad & Capozzi, 2012; Picone et al., 2011; Savorani et al., 2010) and represented in figure 4.8.

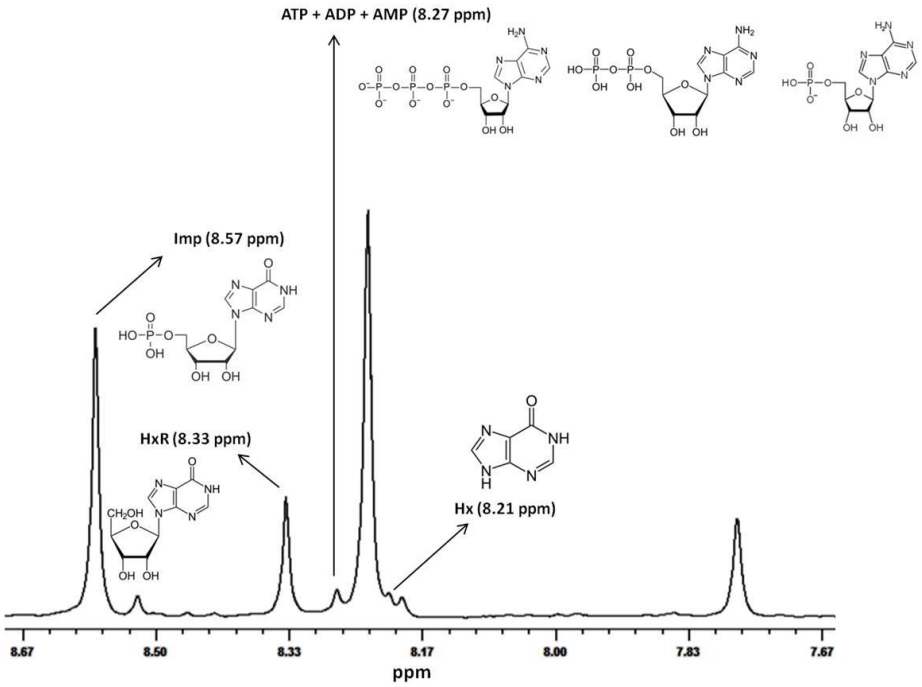

Figure 4.8: ^{1}H NMR (400.13 MHz) spectrum Red mullet; ATP, ADP, AMP contribute to the formation of peak at 8.27 ppm, IMP, HxR and Hx appear as a singlets respectively at 8.57 ppm, 8.33 ppm and 8.21 ppm.

The signals from hypoxanthine, inosine and inosine monophosphate are all well separated, and the area of the peaks can be determined by integration. Instead those assigned to ATP, ADP and AMP are overlapped with each other and can therefore not be integrated separately. When estimating the K-Index, there is no need for separate quantification of these compounds since phosphorylated compounds all contribute to its calculation and the total amounts of ADP and ATP and AMP can be determined as a sum.

The repeatability of these two analytical procedures in determining K-Index value is calculated using 10 samples, 5 Red mullet samples and 5 Bogue fish samples. RSD values are reported in table 4.5.

Table 4.5: K-Index values found in Red mullet and Bogue fish samples

Red mullet (*Mullus barbatus*)								
# Fish sample	K-index Value (%)		RSD(%)		RSDH(%)		Horrat	
	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR
1	32.9(2.9)	31.2(1.0)	1.1	1.5	1.6	1.6	0.68	0.95
2	53.1(1.1)	50.0(2.3)	0.05	0.5	1.5	1.5	0.34	0.034
3	69.3(1.2)	69.3(1.5)	0.7	1.0	1.4	1.4	0.48	0.74
4	83.5(1.2)	83.2(0.7)	0.4	0.4	1.4	1.4	0.32	0.30
5	88.6(1.1)	92.7(2.0)	0.35	1.0	1.4	1.4	0.26	0.75

Bogue fish (*Boops boops*)								
# Fish sample	K-index Value (%)		RSD(%)		RSDH(%)		Horrat	
	HPLC	NMR	HPLC	NMR	HPLC	NMR	HPLC	NMR
1	23.3(1.9)	21.4(1.2)	2.34	0.43	1.7	1.7	1.4	0.25
2	36.7(1.4)	35.5(1.3)	0.90	0.77	1.5	1.5	0.60	0.51
3	43.4(2.99	42.1(2.8)	1.9	1.3	1.5	1.5	1.3	0.87
4	66.1(2.5)	65.7(3.4)	1.5	1.6	1.4	1.4	1.07	1.14
5	68.8(1.69	74.4(1.9)	1.1	0.4	1.4	1.4	0.75	0.29

As shown in table 4.5, only in some cases the values of K-Index by HPLC show a RSD >1; this is probably due to a rapid *post-mortem* dephosphorylation and deamination of adenine nucleotides (ATP, ADP, AMP) throughout inosine monophosphate (IMP) by the autolytic process. Consequently their detection even after 24 hours *post-mortem* is characterized from a lower accuracy than that found for IMP, HxR and Hx.

4.4.2 Comparison of HPLC and ^{1}H-NMR methods

Between the two analytical instruments, according to their methods (section 4.2.4 and 4.25), ^{1}H-NMR has the advantage of using an internal standard respect to HPLC. In fact, the latter employs standard solutions before starting a measure of an unknown sample.

The correlations between the K-index obtained by ^{1}H-NMR and HPLC are characterized from a high value of R^2 (0.97) as shown in figure 4.9.

The initial levels of ATP and of the level of its breakdown compounds are subjected to large inter and intra species differences. In particular the ATP level in the fish flesh depends on capture conditions, time after catching, sexual maturity, water temperature and storage conditions (Luong, Male, Masson & Nguyen, 1992). In addition to these conditions, the degradation of ATP to AMP

and /or IMP is very quick and consequently more difficult to detect during the fish spoilage.

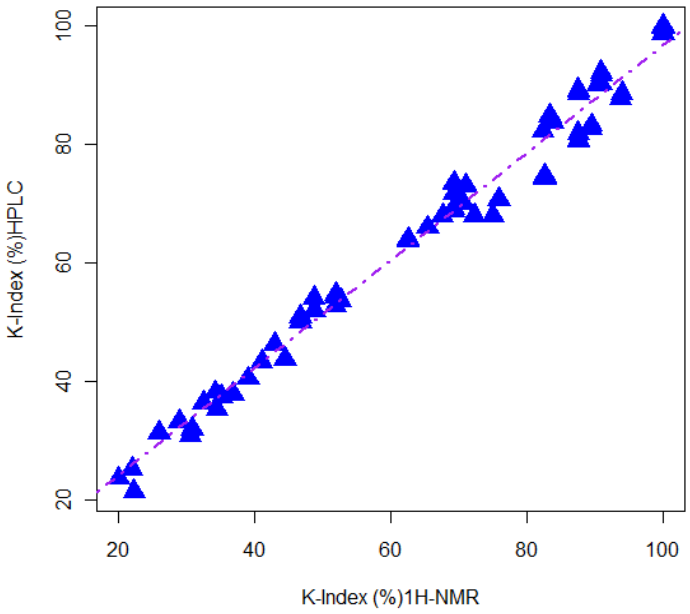

Figure 4.9: correlation between HPLC and ^{1}H-NMR in determining K-Index values

4.5 CONCLUSIONS

The comparison reveals a similar degree of accuracy of the data obtained from conventional analytical methods and HR ^{1}H-NMR. In particular, the ^{1}H-NMR has allowed both to observe a greater specificity in the measurement of TMA-N and a shorter acquisition time in the K-Index determination.

The spectroscopic method used in this work for identifying some metabolites in the evaluation of quality and freshness of the fish can be seen as a further study that increases the chance of nuclear magnetic resonance to officialise its analytic methods as a standard conventional method in the seafood field. In fact, currently there is an increasing request for proper analytical methods capable to give a complete picture of fish metabolism and to assess the nutritional quality of the product. These methods should be based on the analysis of a variety of metabolites and not only on a few specific classes of compounds.

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CHAPTER 5

Kinetics of wild and farmed fish's metabolites during different temperature storage by HR ^{1}H-NMR

5.1 INTRODUCTION

Fish is one of the most perishable foods. The muscle tissue of fish undergoes to a faster spoilage than mammalian muscles. The high water content and free amino acid content and the lower content of connective tissue as compared to other flesh foods lead to a more rapid spoilage of fish. Immediately after death, several biochemical and enzymatic changes are triggered in seafood muscles, especially with improper handling. Microbial spoilage of fish can be caused by the activities of enzymes and microorganisms, resulting in its unacceptability for human consumption (Pedrosa-Menabrito & Regenstein, 1988).

Moreover, each microbial growth during storage will depended on the preservation conditions, and in particular those of transportation.

In particular during transportation, the ambient temperature plays a crucial role in altering the stability of a product; the temperature and handling practices are the most important factors in determining the shelf life of all species of fish. If the fish product is handled carefully, the temperature at which it is held controls its useful life. Temperature will control the rate of bacterial spoilage and enzyme breakdown. An indisputable fact is that the higher the temperature the faster fish spoil. The immediate fish chilling after catch and storing the fish at 0 °C through proper icing will reduce the rate of spoilage.

Ice storage is a relatively short-term method of preservation with storage lives varying between a few days to four weeks. Lima dos Santos (1981) (Santos, 1981) reviews the literature on ice storage studies of fish and attempts to draw conclusions about the storage lives of fish putting these into different broad categories. These reviews confirm the existence of wide variations in storage lives between species and even within the same species under different conditions.

In this part of the research two different temperatures (4 °C and 0 °C) of storage may affect the quality and freshness of fish samples. In particular, metabolic fingerprinting approach is applied to the study of the metabolic changes that occur in fish spoilage. The use of the ^{1}H-NMR spectroscopy is useful to monitor changes of compounds classes, classifying and discriminating them according to their developmental stage, detecting possible biomarkers.

In fact, it should be stressed that NMR spectroscopy must be considered as a "non-targeted" technique able to quantify and to evaluate kinetics for unselected compounds, even a posteriori, e.g., after a pool of spectra is recorded on a population of samples and the multivariate analysis points out some interesting features in the molecular profile, that may be suitable for the development of new indicators related to quality and to freshness (Ciampa, Picone, Laghi, Nikzad & Capozzi, 2012).

In addition, the "freshness" is a result of a variety of definite properties that the fish possesses (Bremner & Sakaguchi, 2000). These properties, for practical purposes, could be designated as "freshness" indicators, that can be measured by various means, and obtained by ^{1}H-NMR with a single measure.

In this study, several compounds are quantified in aqueous extract muscle coming from different species of fish, forming during spoilage.

In particular, non-protein nitrogen compounds are monitored as TMAO that by reduction produces TMA-N, ATP that degrades into HxR and Hx and several acidic and basic free amino acids (FAAs).

5.2 MATERIAL AND METHODS

After verifing the correlation of ^{1}H-NMR with the standard fish quality determination methods (Chapter 4), fish samples aree used to evaluate the kinetics of freshness index metabolites during different temperature storage, 4°C and under ice (UI)

5.2.1 Biological material

Different types of fish are chosen in this study:

a) "standardized" species and commercially known in Italy such as Red mullet, Atlantic mackerel, European seabass and Gilthead seabream;

b) species such as Bogue belonging to the families of fish commonly called "poor fish" and typically known in the South of Italy and in different Mediterranean countries.

The wild fish species, Red mullet and Bogue fish (1st arrival), are analyzed using a 400.13 MHz spectrometer, while Bogue fish (2nd arrival) and Atlantic mackerel together with two farmed fish species (reared under different conditions),

Gilthead seabream and European seabass, are analyzed using a 600.13 MHz spectrometer (Fig. 5.1).

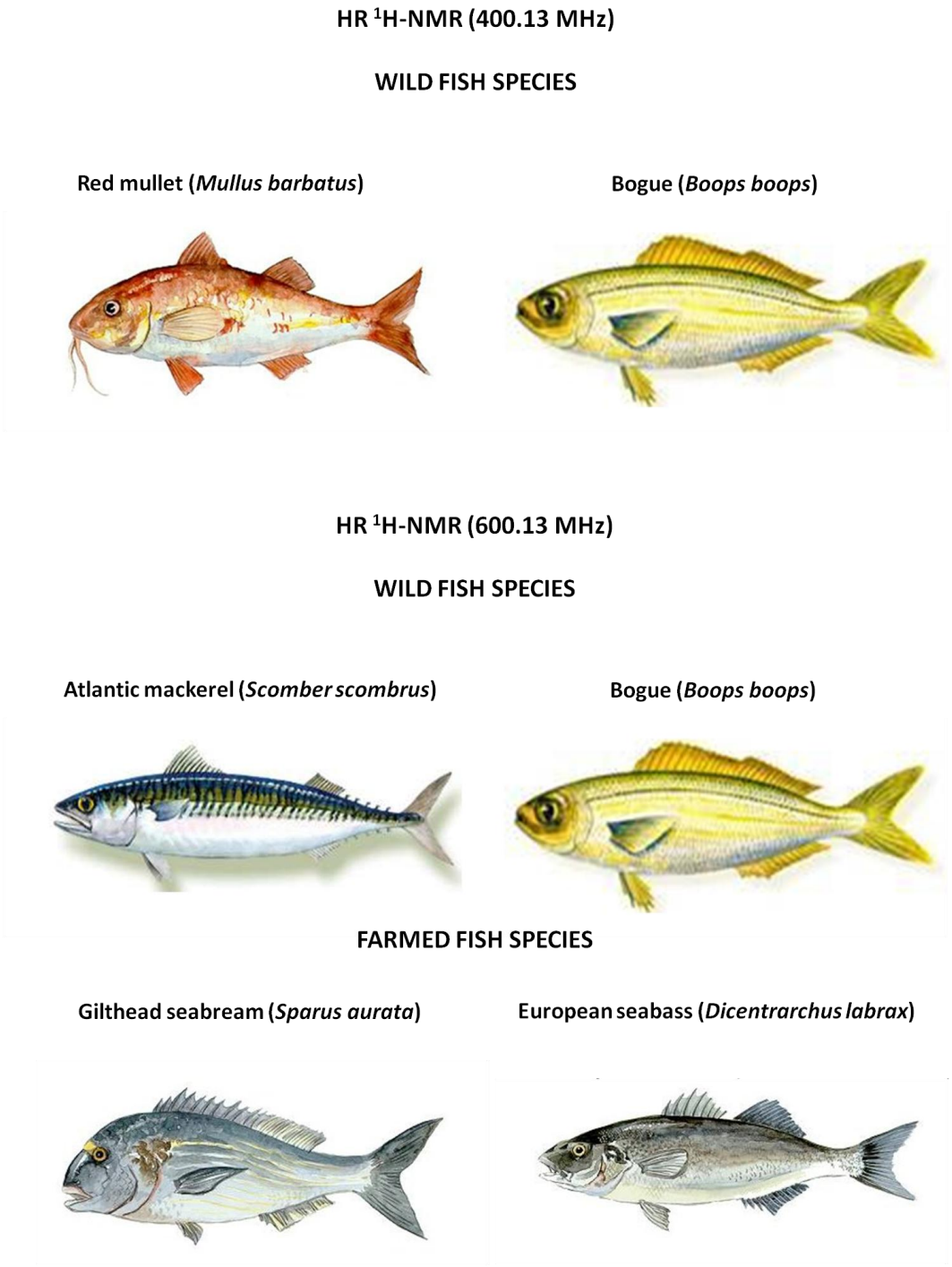

Figure 5.1: fish species used to evaluate metabolites kinetics during different time and temperature storage

5.2.2 Experimental design

Red mullet, Bogue and Atlantic fish are sampled during 11-15 days at 4 °C and under ice, as shown in figure 5.2 and Gilthead seabream and European seabass in figure 5.3.

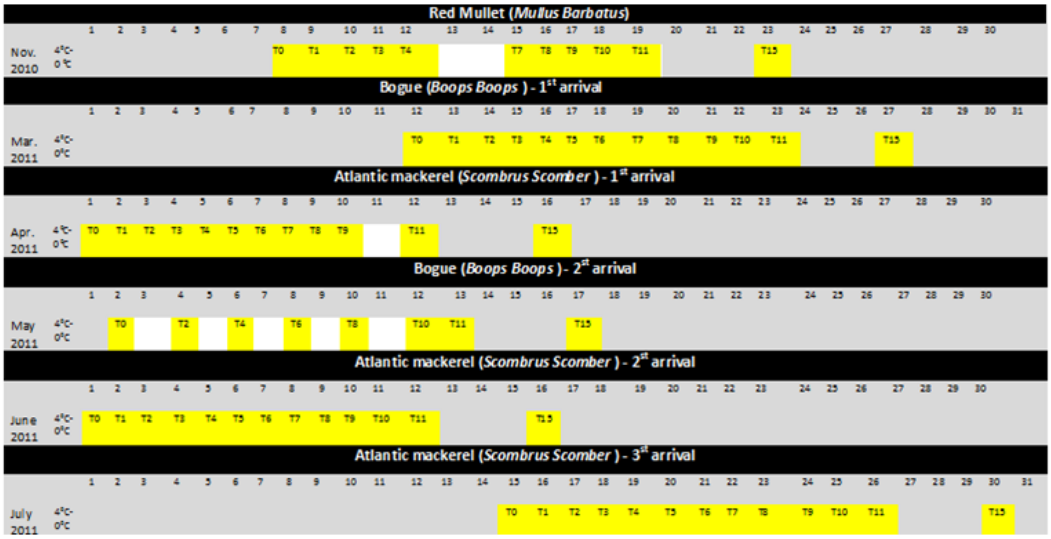

Figure 5.2: Red mullet, Bogue and Atlantic mackerel fish sampling performed during 11 days of storage at 4°C and 15 days UI (0°C)

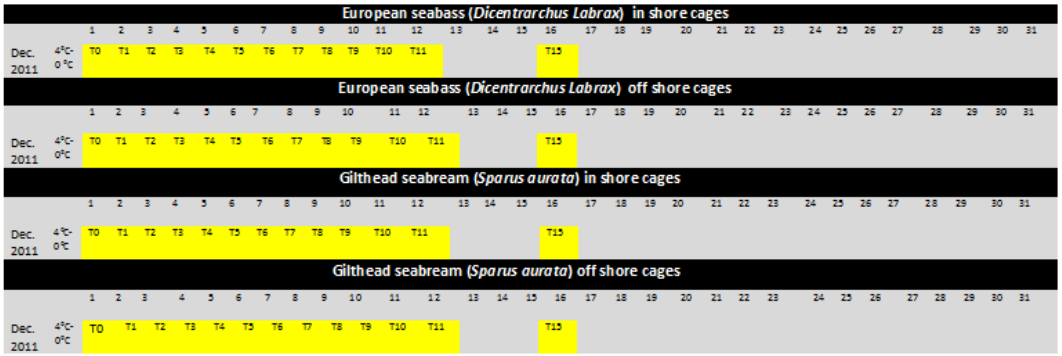

Figure 5.3: European seabass ("in shore" and "off shore" cages) and Gilthead seabream ("in shore" and "off shore" cages) fish sampling performed during 11 days of storage at 4°C and 15 days UI (0°C)

5.2.3 Sample preparation for HR ^{1}H-NMR data acquisition

All samples are prepared as described in par. 4.2.5. In particular, spectra coming from samples analyzed with the 600 MHz spectrometer, are recorded at 298 K with a Bruker AVANCE instrument operating at a frequency of 600.13 MHz, equipped with an autosampler with 60 holders. Each spectrum is acquired using 32K data points over a 7211.54 Hz spectral width and summing up 256 transients. A recycle delay of 5 s and a 90° pulse of 11.4 µs are set up. Acquisition time (2.27 s) and recycle delay are adjusted to be 5 times longer than the T1 of the protons under investigation, which has been considered to be not longer than 1.4 s. The saturation of residual water signal is achieved by irradiating it during the recycle delay at δ equal to 4.703 ppm. Each spectrum is processed with MestReC 4.9.8.0 (Mestreab Research SL, Spain) by manually adjusting phase

and base-line and applying a line broadening factor of 0.5 Hz. The peaks are assigned by comparing their chemical shift and multiplicity with the literature (Mannina et al., 2008; Picone, Balling Engelsen, Savorani, Testi, Badiani & Capozzi, 2011; Savorani, Picone, Badiani, Fagioli, Capozzi & Engelsen, 2010). When signals due to different protons of the same molecule are identified, both are employed for the quantification.

5.3 RESULTS AND DISCUSSION

5.3.1 Molecular profiling of fish aqueous extracts by 1D HR ^{1}H-NMR analysis

A typical ^{1}H-NMR spectrum obtained from fish muscle extract is shown in Figure 5.4.

Three groups of peaks can be identified. The peaks with the highest intensity, accounting for 30% of the total spectra area, pertain to TMAO, TMA-N, creatine and phosphocreatine. The region between 8.16 and 8.60 ppm is characterized by the presence of signals employed in the calculation of K-Index. The remaining signals can be mainly ascribed to some aminoacids and, to a minor extent, to organic acids and short chain fatty acids. Assigned metabolites are listed in Table 5.1.

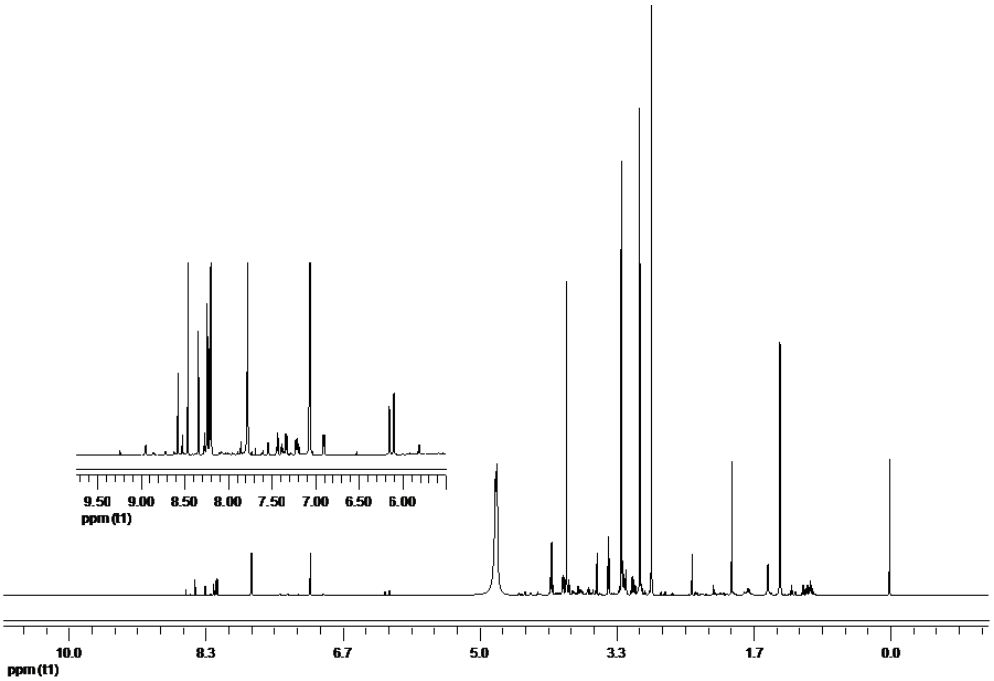

Fig. 5.4: a typical ^{1}H-NMR spectrum of fish recorded with a spectrometer operating at 600.13 MHz

Table 5.1: metabolites identified in ^{1}H-NMR spectrum, recorded with 600 MHz spectrometer, of Bogue muscle extract

Compound	Assignment	^{1}H (ppm)	Multiplicity
Isoleucine (Ile)	δ-CH_3	0.94	t
Leucine (Leu)	δ'-CH_3	0.96	d
Valine (Val)	γ-CH_3- γ'-CH_3	1.00-1.05	d
Ethanol	CH_3	1.19	t
Lactate (La)	β-CH_3	1.33	d
Alanine (Ala)	β-CH_3	1.49	d
Acetate	CH_3	1.93	s
Methionine (Met)	S-CH_3	2.14	s
Succinate	α, β-CH_2	2.41	s
Trimethylamine (TMA-N)	N-CH_3	2.90	s
Creatine/Phosphocreatine	N-CH_3 and N=C	3.04	s
Oxide Trimethylamine (TMAO)	N-CH_3	3.27	s
Taurin (Tau)	N-CH_2	3.42	t
Glycine (Gly)	α-CH	3.56	s
Glutamate (Glu)	α CH	3.75	t
Creatine/Phosphocreatine	N-CH_2	3.94	s
Serine (Ser)	β-CH	3.98	dd
α-Glucose (α-GLC)	CH-1	5.24	d
Inosine (HxR)	CH-1', ribosio	6.10	d
Inosine 5'-monophosphate (IMP)	CH-1', ribosio	6.14	d
Tyrosine (Tyr)	C3, 5H, ring	6.88	d
Histidine (His)	C2H ring/C4H ring	7.06/7.77	s
Tryptophan (Trp)	C5H ring	7.19	t
Phenylalanine (Phe)	CH-2,6	7.32	m
Hypoxanthine (Hx)	CH-8	8.19	s
Hypoxanthine (Hx)	CH-2	8.21	s
Inosine (HxR)	CH-8	8.233	s
Inosine 5'- monophosphate (IMP)	CH-8	8.236	s
Adenosine5' triphosphate (ATP)	CH-8	8.27	s
Adenosine5'- diphosphate (ADP)			
Adenosine 5'-monophosphate (AMP)			
Inosine (HxR)	CH_2, ring	8.33	s
Formate (Fo)	CH	8.46	s
Inosine 5'- monophosphate (IMP)	CH_2, ring	8.57	s

5.3.2 TMAO and TMA-N content in fish muscle during different time and temperature storage

TMAO and TMA-N are characterized in a generic ^{1}H-NMR spectrum by a singlet signal at 3.28 ppm and 2.89 ppm respectively. For this reason, the well resolved signals allow to easily use the NMR for monitoring the evolution of these two compounds during time.

As results by NMR analysis, immediately after the catch (T0 point) the initial quantity of TMAO in the various species of fish varies between 4 and 30 mg/100g, while the TMA-N concentrations are between 0.01 and 0.1mg/100g. These variations depend on several aspects: 1) fish species composition, 2) the sampling period and type of rearing in case of farmed fish. (Figure 5.5).

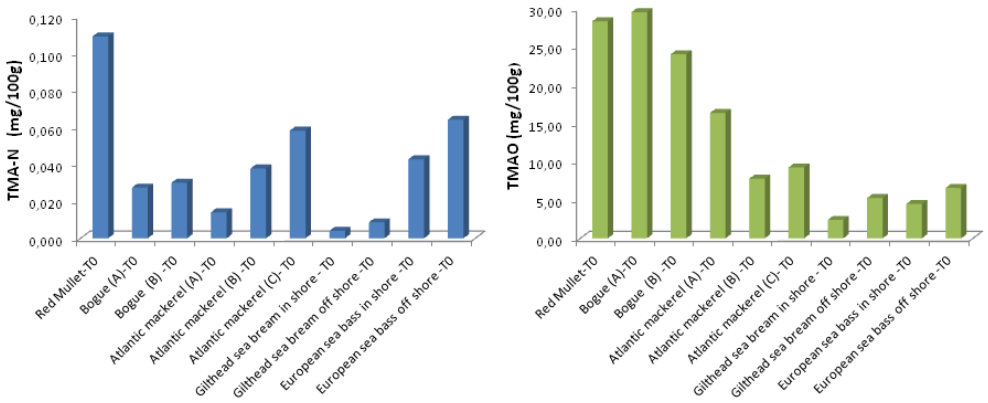

Figure 5.5: concentrations of TMA-N and TMAO (mg/100g) at T0 in different species of fish

Figure 5.6 shows the evolution of TMA-N during time, for both storage temperatures. According to wild fish samples at 4°C, the formation of TMA-N starts after three days from the catch (T3), while more days are required for the same samples stored UI. In particular, studies of TMA-N formation in stored fish after capture show an exponential increase in concentration, perhaps following a dwell period when the TMA-N does not increase (Howgate, 2010).

The exponential phase can be mathematically modeled by the following equation:

$$C_t = e^{kt}$$

where C_t is concentration at time t, k is the rate coefficient, and e is the base of natural logarithms. This expression gives a value of 1 at t = 0, and if C_t is expected to be 0 at t=0 then 1 needs be subtracted from the right-hand side or added to the left-hand (Howgate, 2010). The expression then becomes, with the former option:

$$C_t = e^{kt} - 1 + a$$

where a is a coefficient representing the concentration at t = 0.

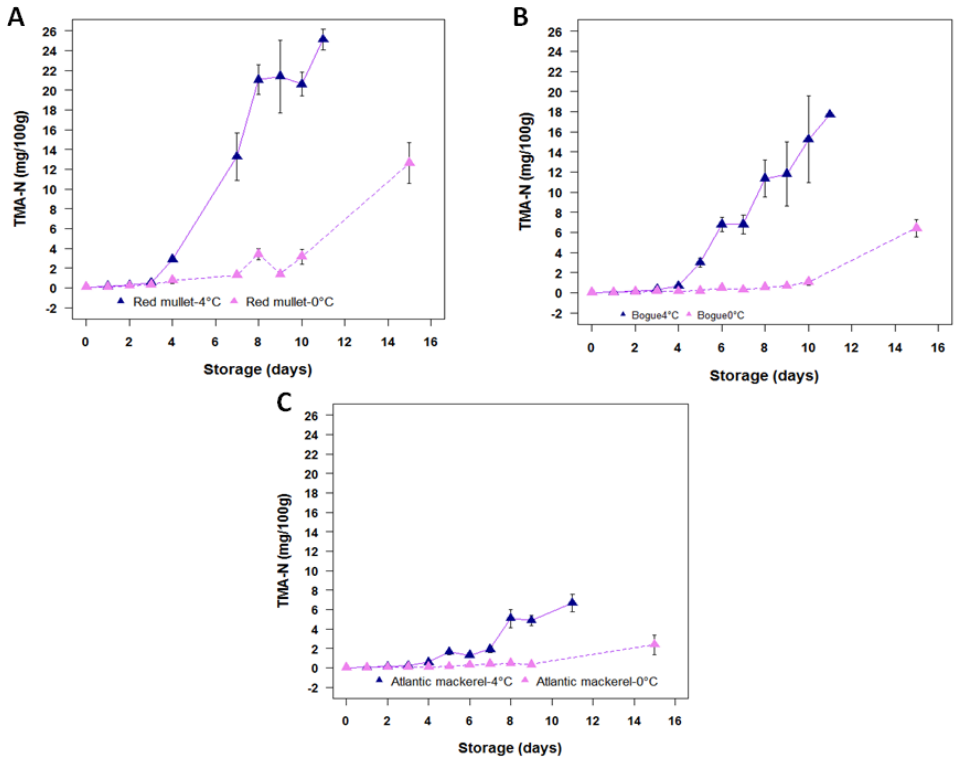

Figure 5.6: TMA-N concentrations (mg/100g) for Red Mullet (A), Bogue (B) , Atlantic mackerel (C) during the storage at 4 °C and 0 °C. For clarity only the evolution of TMA-N in fish of 1st arrival is reported

Table 5.2 shows the coefficient rate (k) expressed as a function of time (days) calculated after 4, 7 and 11 days for samples stored at 4°C and after 4, 7 and 15 stored at 0°C days.

Red mullet samples stored at 4 °C show much higher quantity of TMA-N after 11 days than Atlantic mackerel samples stored under the same conditions. This significant difference is explainable by their composition: a significant role is represented by the differences of the lipid/protein/water ratio during the degradation development: the higher this ratio, the slower the degradation of the fish (e.g., in the Atlantic mackerel).

In samples stored at 0 °C, the degradation is reduced as confirmed by the coefficient rate value that is smaller.

This variation has a clear cut and is caused by the slower conversion of TMAO into TMA-N. In fact, at 0 °C this conversion is replaced from a slow enzymatic reaction which leads to the formation of Dimethylamine (DMA) and Formaldehyde (Huss, 1995).

Table 5.2: TMA-N concentrations and corresponding coefficient rate (k) for Red mullet, Bogue and Atlantic mackerel at T0, T4, T7, T11 and T15

	TMA-N (mg/100g)	TMA-N (mg/100g)	k(t) ($days^{-1}$)	k(t) ($days^{-1}$)
Red mullet	**4°C**	**UI**	**4°C**	**UI**
T0	0.11 ± 0.03	0.11 ± 0.03	0.00	0.00
T4	2.856 ± 0.20	0.756 ± 0.30	0.33	0.12
T7	13.26 ± 2.36	1.285 ± 0.14	0.38	0.11
T11 (4°C) and T15 (0°C)	24.85 ± 1.07	12.62 ± 2.09	0.30	0.17
Bogue (1st arrival)				
T0	0.027 ± 0.0009	0.027 ± 0.0009	0.00	0.00
T4	0.657 ± 0.072	0.141 ± 0.030	0.12	0.03
T7	6.78 ± 0.679	0.304 ± 0.061	0.29	0.03
T11 (4°C) and T15 (0°C)	17.68 ± 4.31	6.46 ± 0.92	0.27	0.13
Bogue (2nd arrival)				
T0	0.039 ± 0.0025	0.039 ± 0.0025	0.00	0.00
T4	0.4299 ± 0.072	0.132 ± 0.025	0.08	0.02
T7	3.227 ± 0.434	0.200 ± 0.033	0.20	0.02
T11 (4°C) and T15 (0°C)	13.150 ± 1.12	4.611 ± 0.22	0.24	0.11
Atlantic mackerel (1st arrival)				
T0	0.014 ± 0.0080	0.014± 0.0080	0.00	0.00
T4	0.564 ± 0.23	0.091± 0.010	0.11	0.02
T7	1.918 ± 0.317	0.383 ± 0.269	0.15	0.04
T11 (4°C) and T15 (0°C)	6.67 ± 0.93	2.38 ± 1.01	0.19	0.08
Atlantic mackerel (2nd arrival)				
T0	0.037 ± 0.0070	0.037±0.0070	0.00	0.00
T4	0.186 ± 0.0159	0.133 ± 0.011	0.03	0.02
T7	1.002 ±0.127	0.1549 ± 0.024	0.10	0.02
T11 (4°C) and T15 (0°C)	3.492 ± 0.918	1.97 ± 0.103	0.14	0.07
Atlantic mackerel (3rd arrival)				
T0	0.0582 ± 0.012	0.0582 ± 0.012	0.00	0.00
T4	0.756 ± 0.011	0.539 ± 0.041	0.13	0.10
T7	2.192 ± 0.0946	0.257 ± 0.014	0.16	0.03
T11 (4°C) and T15 (0°C)	5.034 ± 0.21	1.148 ± 0.012	0.16	0.05

The DMA amount produced depends on the fish species, the temperature and time storage. Consequently, in some cases, it is considered an index of "spoilage" for certain frozen fish products.

The formaldehyde which can be produced together with the DMA is not toxic, but may interact with amino acid residues, with amino-terminal groups and low molecular weight compounds, causing the denaturation and the "cross-linking" of proteins (Venugopal & Shahidi, 1996).

For farmed fish samples (Gilthead seabream and European seabass), reared in two different conditions (“in shore” and “off shore” cages), different results are obtained (Fig. 5.7).

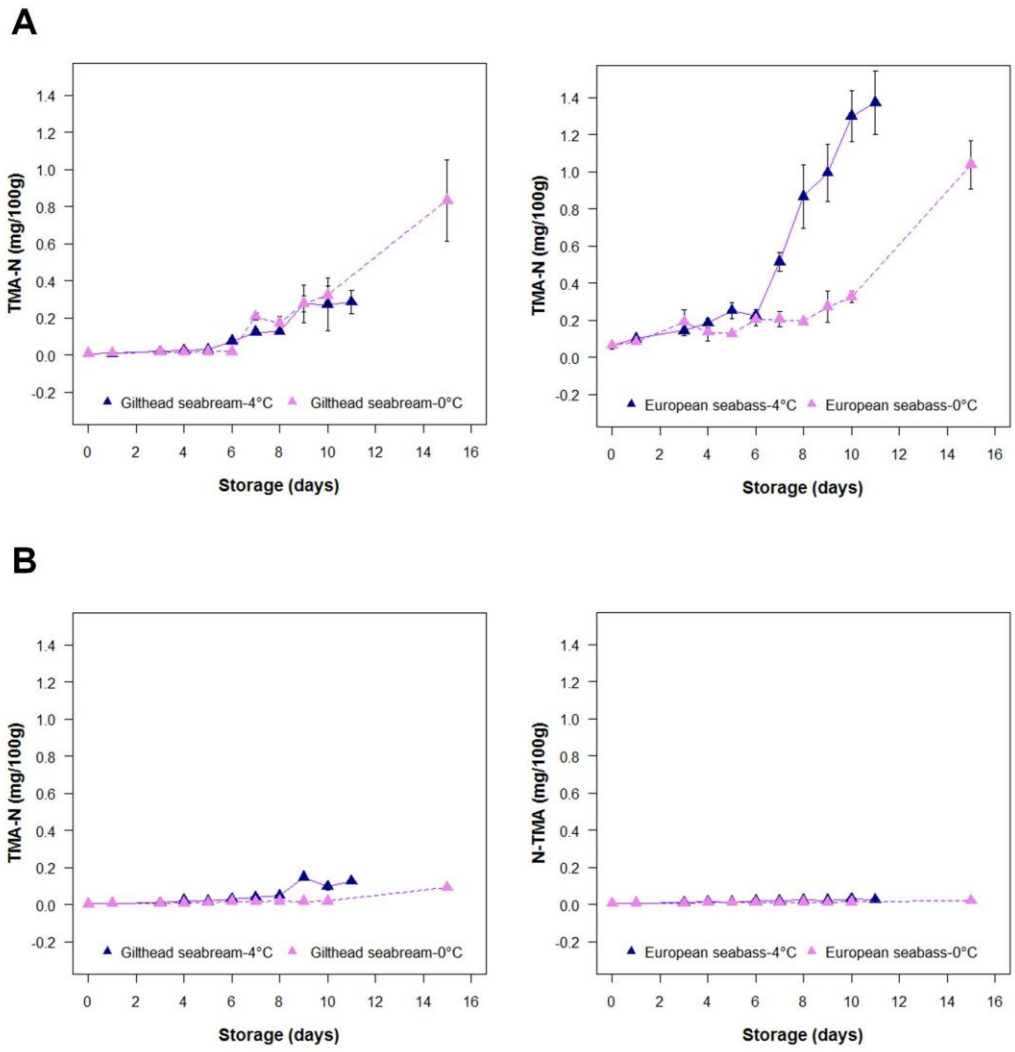

Figure 5.7: TMA-N concentrations for Gilthead seabream and European seabass during storage at 4 °C and 0 °C in different aquaculture systems: A) "off shore" cage and B) "in shore" cage

The lower level of TMA-N in these species is considered by the authors to be a reflection of the difference in the epidermal bacterial flora compared with that present in wild typologies. During all time storage, TMA-N concentration remains very low: 1mg/100g was reached after 10–11 days of storage at 4°C only for fish reared in "off shore" cages. In particular, for Gilthead seabream and European seabass an environmental factor such as water temperature can influence the metabolism of bacteria and therefore also the microbial flora of the fish (Haard, 1992; Kyrana & Lougovois, 2002). In fact, in cold water fish, the main spoilage organism is *Shewanella putrefaciens* which has considerable ability to produce TMA-N. In warmer waters, *Pseudomonas fragi* can be the dominant bacterial spoilage organism. This species does not produce TMA-N, so that spoilage can occur with little or no TMA-N production (Gram & Dalgaard, 2002; Gram & Huss,

1996). In table 5.3, TMA-N concentrations and the coefficient rate (k) for both species are reported. In "off shore" cages there are extremely low values of k, respect to wild fish.

Table 5.3: TMA-N concentrations and corresponding coefficient rate (k) for Gilthead seabream and European seabass, reared in two different aquaculture condition, at T0, T4, T7, T11 and T15

	TMA-N (mg/100g)	TMA-N (mg/100g)	k(t) (days $^{-1}$)	k(t) (days^{-1})
Gilthead seabream "in shore" cage	**4°C**	**UC**	**4°C**	**UI**
T0	0.0052 ± 0.00074	0.0052 ± 0.00074	0.000	0.000
T4	0.022 ± 0.0037	0.0096 ± 0.00047	0.004	0.001
T7	0.039 ± 0.0011	0.0167 ± 0.0021	0.005	0.002
T11 (4 °C) and T15 (0 °C)	0.127 ± 0.0072	0.092 ± 0.0146	0.010	0.006
Gilthead seabream "off shore" cage				
T0	0.0089 ± 0.0018	0.0089 ± 0.0018	0.000	0.000
T4	0.025 ± 0.01025	0.016 ± 0.0018	0.004	0.002
T7	0.123 ± 0.00854	0.208 ± 0.01781	0.015	0.026
T11 (4 °C) and T15 (0 °C)	0.286 ± 0.062	0.833 ± 0.22101	0.022	0.040
European seabass "in shore" cage				
T0	0.0065 ± 0.0007	0.0065 ± 0.0007	0.000	0.000
T4	0.0149 ±0.0053	0.0103 ± 0.0008	0.002	0.001
T7	0.0195 ± 0.0034	0.0103 ± 0.0031	0.002	0.001
T11 (4 °C) and T15 (0 °C)	0.0254 ± 0.0058	0.0208 ± 0.0011	0.002	0.001
European seabass "off shore" cage				
T0	0.064 ± 0.0192	0.064 ± 0.019	0.000	0.000
T4	0.185 ± 0.022	0.135 ± 0.045	0.029	0.017
T7	0.514 ± 0.052	0.206 ± 0.041	0.053	0.019
T11 (4 °C) and T15 (0 °C)	1.374 ± 0.170	1.040 ± 0.130	0.076	0.045

The low concentrations of TMA-N of farmed fish could be also attributed to the low initial concentrations of TMAO. According to Kyrana et al. (Kyrana et al., 2002), low values of TMAO are probably related to the composition of the feed for cultured fish, which may modify the role of TMAO in osmotic regulation. This role is probably taken over by free amino acids and other non-protein nitrogen compounds.

Also another factor could be considered regarding farmed fish that are well fed and thus they have high glycogen reserves at the time of death, leading in this way to a low pH value of the flesh in *post-mortem*. Castell and Snow (Castell, Greenough, Rodgers & MacFablane, 1958) and Boskou and Debevere (Boskou & Debevere, 1998) show that the growth rate of *Pseudomonas putrefaciens* decreases as pH decreases and is insignificant below pH 6.0, though post-

mortem pH's of wild and farmed fish overlap. However the general tendency of the pH in farmed fish is lower than in wild ones. It could be a factor in the formation rate of TMA-N in farmed fish that is lower than that of wild-caught fish (Howgate, 2010). The small increase in TMA-N during the storage period precludes the usefulness of this compound as a freshness indicator in farmed fish.

5.3.3 ATP and its breakdown products

ATP is the main source of energy in the autolytic reactions controlled by endogenous enzymes present in the muscle tissue as well as those leaking from the gut. Its decrease, and consequently the K-Index value increase during the fish spoilage, is a species-specific freshness index (par. 1.3.3).

For wild species, Bogue is the only fish that showed a K-Index <15% immediately after the catch. For Atlantic mackerel, different samplings between spring and summer are performed; this leds to a seasonal effect on the K-Index value (Fig. 5.8) due to a higher autolytic activity during summer as reported in literature (Dingle & Hines, 1971; Grigorakis, Taylor & Alexis, 2003). K-Index values calculated for each species are listed in table 5.4.

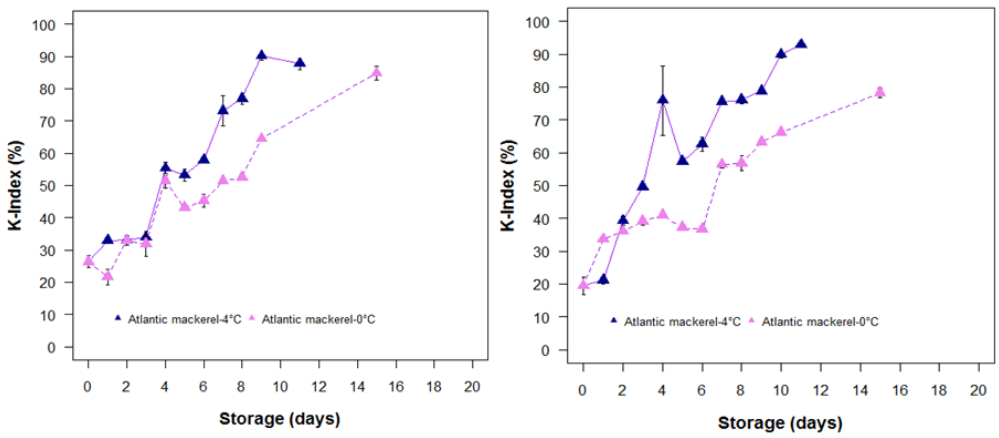

Figure 5.8: K-Index values for Atlantic mackerel samples at different times and temperatures storage and evaluated in different seasons: spring and summer

Table 5.4: K-Index values evaluated for wild fish samples

	K-Index (%) (4 °C)	K-Index (%) (0 °C)
Red mullet		
T0	27.87±1.9	27.87±1.9
T4	69.28±1.5	44.78±3.3
T8	89.74±1.3	70.41±1.9
T11 (4 °C) and T15 (0 °C)	92.67±1.5	85.99±1.8
Bogue (1° Arrival)		
T0	10.42±1.5	27.87±1.9
T4	37.17±2.6	35.06±7.8
T8	60.64±1.8	38.89±2.4
T11 (4 °C) and T15 (0 °C)	79.45±5.9	69.44±2.4
Bogue (2° Arrival)		
T0	20.79±2.8	27.87±1.9
T4	44.56±8.7	27.25±1.6
T8	62.11±7.7	39.76±1.9
T11 and T15 (0 °C)	76.54±1.5	57.85±0.5
Atlantic mackerel (1° Arrival)		
T0	26.33±1.9	27.87±1.9
T4	55.25±2.1	51.49±2.4
T8	76.91±1.7	52.46±0.7
T11 (4 °C) and T15 (0 °C)	87.65±1.8	84.79±2.3
Atlantic mackerel (2° Arrival)		
T0	20.27±0.3	27.87±1.9
T4	50.05±0.3	47.08±2.3
T8	68.72±1.1	49.17±1.2
T11 (4 °C) and T15 (0 °C)	74.86±4.3	78.94±2.4
Atlantic mackerel (3° Arrival)		
T0	19.46±2.7	27.87±1.9
T4	75.87±1.6	40.92±0.6
T8	75.98±1.1	56.77±2.2
T11 (4 °C) and T15 (0 °C)	92.74±0.8	78.21±1.4

Red mullet and 3rd arrival Atlantic mackerel samples stored at 4 °C show higher K-Index values, if compared to the other samples, starting from the 4th day of storage. Inside at 0 °C, significant differences are recorded only after 7 days from the catch. This is probably because in the first days there is only the contribution of enzymatic spoilage, while at a later time, the formation of inosine and hypoxanthine is mainly due to bacterial spoilage (Saito, Arai & Matsuyoshi, 1959).

In farmed fish, lower K-Index values with respect to wild's ones demonstrate a slower spoilage; Gilthead seabream samples, for example, reach the maximum K-Index value, equal to 47%, only after 11 days of storage at 4°C (Figure 5.9 and Table 5.5) (after the same time Atlantic mackerel showed a K-Index of about 87%).

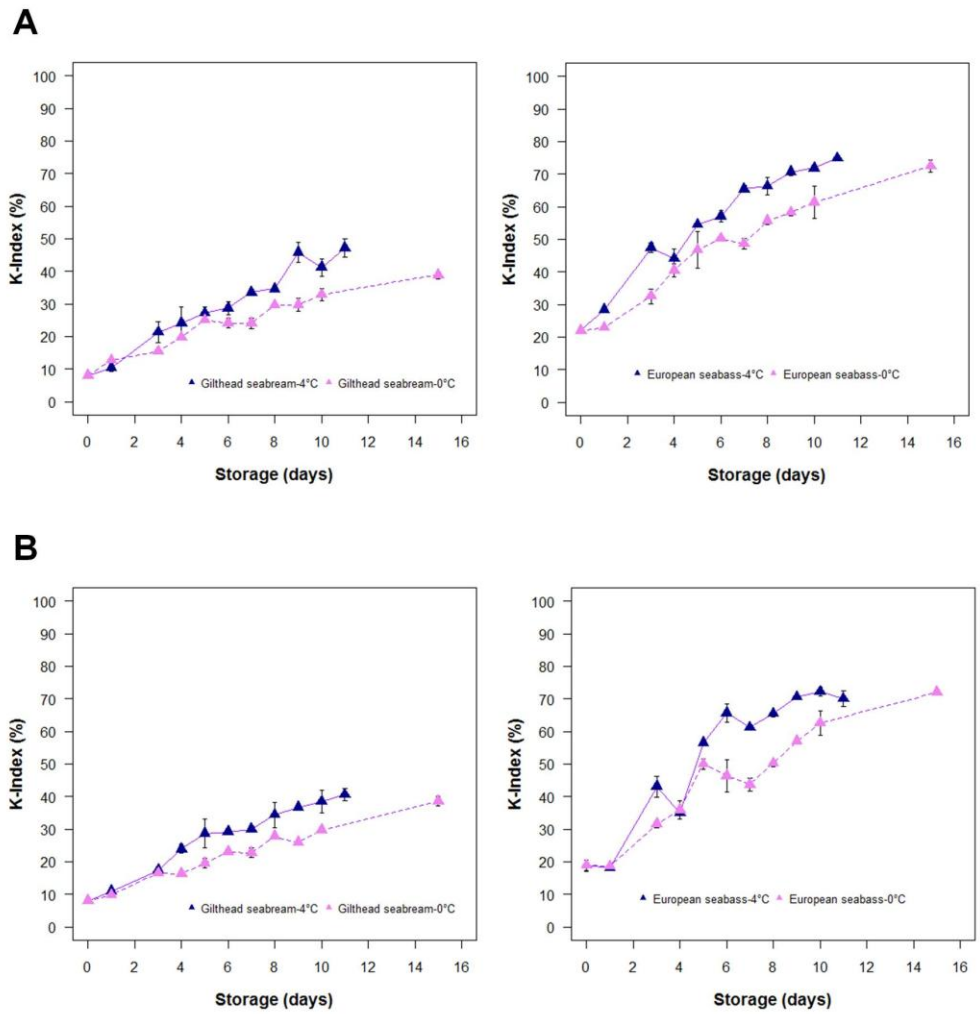

Figure 5.9: K-Index value for Gilthead seabream and European seabass during storage at 4 °C and 0 °C in different aquaculture systems: A) "off shore" cages and B) "in shore" cages

Table 5.4: K-Index values evaluated for farmed fish samples

	K-Index (%) (4 °C)	**K-Index (%) (0 °C)**
Gilthead seabream "in shore" cage		
T0	7.98 ± 0.35	7.98 ± 0.35
T4	23.90 ± 1.33	16.32 ± 0.22
T8	34.42 ± 3.88	27.79 ± 0.94
T11 (4 °C) and T15 (0 °C)	40.57 ± 1.92	38.52 ± 1.52
Gilthead seabream "off shore" cage		
T0	8.00 ± 0.90	8.00 ± 0.90
T4	24.08 ± 5.07	19.71 ± 0.59
T8	34.59 ± 0.73	29.59 ± 0.36
T11 (4 °C) and T15 (0 °C)	47.17 ± 2.9	38.88 ± 1.11
European seabass "in shore" cage		
T0	18.86 ± 1.70	18.86 ± 1.70
T4	34.95 ± 0.56	35.93 ± 2.81
T8	65.44 ± 1.03	50.22 ± 1.11
T11 (4 °C) and T15 (0 °C)	70.05 ± 2.39	71.97 ± 0.28
European seabass "off shore" cage		
T0	22.00 ± 0.70	22.00 ± 0.70
T4	44.13 ± 2.77	40.48 ± 2.11
T8	66.27 ± 2.57	55.70 ± 1.15
T11 (4 °C) and T15 (0 °C)	74.82 ± 0.21	72.46 ± 1.90

5.3.4 Changing in the free amino acids composition

The concentration of acidic and basic free amino acids is generally found to change during storage, respectively. These concentration changes are slow during the first days, as a consequence of protein autolysis, and at higher rates afterward, resulting from microbial development. Differences in the amino acids concentration trends are found to be related to the different storage temperatures from day 4 onwards. As an example, the variations of different amino acids in the second Bogue' sampling (Ciampa et al., 2012) during storage under ice and at 4°C are considered. The amino acids could be divided into three groups according to the trend of their concentration (Figures 5.10 and 5.11). The first group is represented by taurine only, whose concentration does not significantly change during storage. This is not unexpected, since this amino acid is not employed by organisms as a protein constituent. Thus it is not released during the lytic processes going on during autolysis reactions or bacterial development. Indeed, results in accordance with this observation can be found in the literature for several kinds of fish and fish preparations, stored from -20 °C (Jiang et al., 1985) to 25 °C (Je et al., 2005).

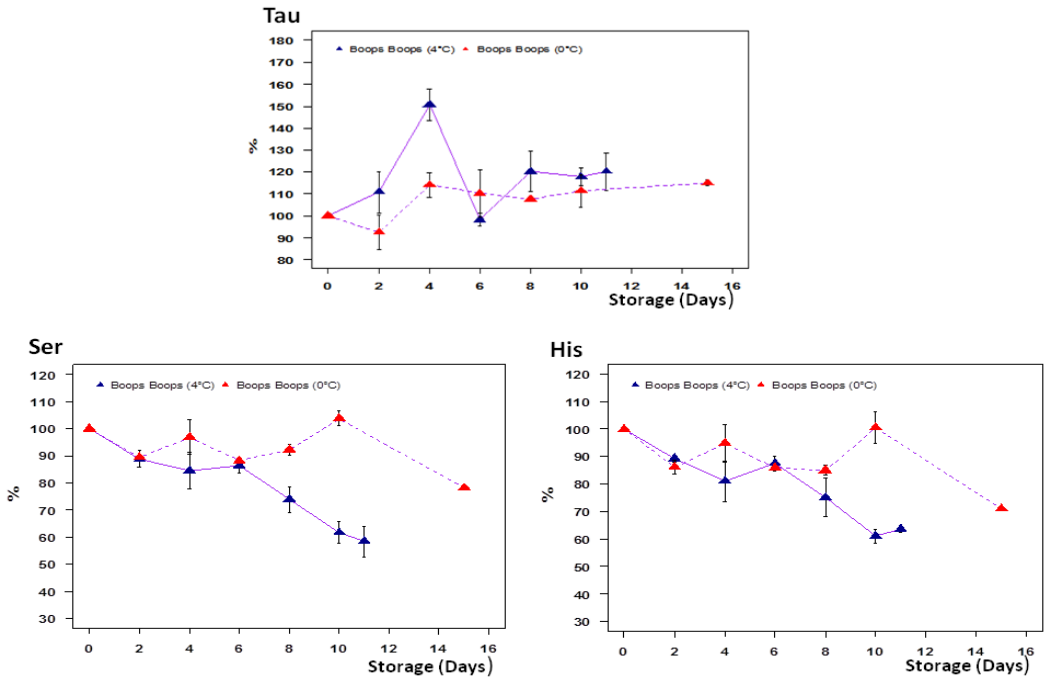

Figure 5.10: concentration changes relative to fresh samples of taurine, serine and histidine during storage at 4°C (blue symbols) and on ice (red symbols) in the Bogue

The second and third groups comprise the amino acids whose concentration increase and decrease, respectively, as a consequence of storage at 4 °C.

The former group is composed of alanine, phenylalanine, tryptophan, glycine, methionine and isoleucine-leucine-valine, while the latter group is made up of histidine and serine only.

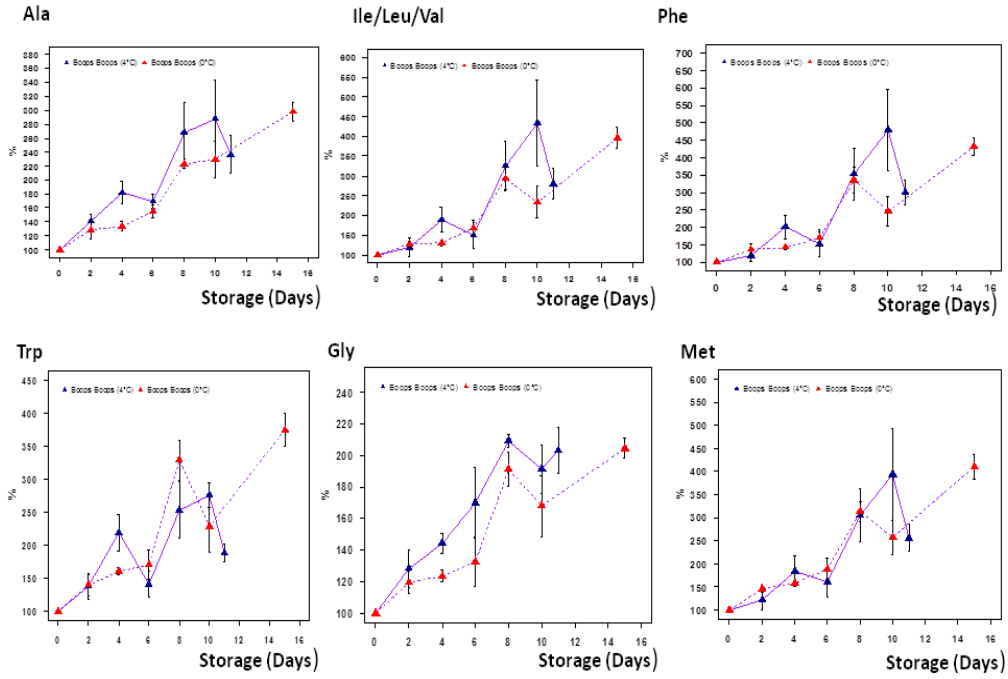

Figure 5.11: concentration changes relative to fresh samples of alanine, isoleucine-leucine-valine, phenylalanine, tryptophan, glycine, methionine, during storage at 4°C (blue symbols) and on ice (red symbols)

The decrease in concentration of the basic amino acids (serine and histidine) shows that their solubilisation from muscle proteins is slower than their transformation into byproducts, namely biogenic amines through decarboxylation. Such finding, together with the parallel increase of the acidic amino acids, is well documented at ambient temperature for a variety of fish and transformed products based on them (Ababouch, Afilal, Benabdeljelil & Busta, 1991; Özden, 2005).

Kiesvaara (Kiesvaara, 1975) set up specific quality indices for salted herrings based on such knowledge. Moreover, at room temperature, there is a general agreement about the decrease of methionine during storage (Love, 1980). The data collected in the present study seems to confirm these findings. Decreasing serine and increasing methionine appear to be more similar to those observed in

the literature for frozen samples. As an example, Jiang et al. (Jiang & Lee, 1985) find decreasing concentrations of serine in frozen samples of mackerel, amberfish, mullet and carp during storage, and at the same time an increasing concentration of methionine.

The fact that at both temperatures the tested methionine does not decrease is interesting from a consumer perspective, as the catabolism of this amino acid is known to be primarily responsible for methylmercaptan and dimethylsulphide formation, molecules strongly related to the development of off-flavors (Herbert & Shewan, 1975).

By focusing on the relationship between amino acid concentrations and storage time, it is possible to divide the observed molecules into two categories. The concentration of alanine, tryptophan, glycine and methionine are observed constantly to change during storage. In contrast, the other group of molecules seems to be characterized by a slow change until day 4 and by a higher change rate afterwards. In this respect, the concentration of some of the latter molecules, in particular histidine and serine, change at similar rates in the two storage methods until day 4, and at markedly different rates afterwards. Such a two-phase observation could be rationalized considering that the enzymes leading to autolysis are known to be poorly influenced by temperature, being still active even at temperatures as low as −17 °C (Mukundan, Antony & Nair, 1986). Therefore, the slow rate change until day 4 can be considered mainly due to autolysis, the fast rate change occurring afterwards due to bacterial development. Indeed, the concentration of some of the mentioned molecules seem to reproduce a bacterial development curve (Gram et al., 1996) characterized by: (i) a lag phase in which the fish can be considered as fresh.

During this phase, the concentration of some amino acids undergoes fluctuations appreciated by the consumer in specific cases. Free glycine, for example, is known to be important for the individual taste of different fish species (Grigorakis et al., 2003); (ii) an exponential multiplication phase, typically characterized by development of off-flavors; and (iii) a stationary phase during which the concentration of some free amino acids start to decrease.

5.4 CONCLUSIONS

The content of TMA, as well as the k-Index and the concentrations of amino acids are quality indexes in the determination of fish freshness, but according to the results so far obtained they are significant only for one metabolite and/or pathway taken into examination. For example, in the case of wild fish, the TMA-N content indicates that the Atlantic mackerel as the longest lasting species. On the contrary, considering the k-Index, Bogue is the less perishable. For farmed fish the TMA-N is a weak freshness indicator because these fish form it in very small quantities after 11 and 15 days of storage respectively at 4 °C and 0 °C.

Consequently, when confronting this problem, the attention has been shifted to the whole molecular profile, obtained through the ^{1}H-NMR spectrum.

This metabonomic approach will allow the identification of other metabolites, closely related with the storage and fish freshness. In fact, from the ^{1}H-NMR spectrum various information on a wide class of compounds such as amino acids, nucleotides, organic acids, osmoregulators, carbohydrates, amines, vitamins, etc. can be obtained through a one-shot analysis.

The relative advantage will be to acquire, in addition to the conventional indices of freshness, the molecular fingerprint, characteristic and distinctive of all metabolites present in fish extract. It could be used for the next generation indexes that, as seen in the next chapter, will be based on the multivariate data analysis. The combination of NMR spectroscopy together with the chemometric technique for data analysis become an important tool to identify unexpected differences in the metabolic profile.

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CHAPTER 6

Multivariate analysis of HR ^{1}H-NMR data spectra of wild and farmed fish samples

6.1 INTRODUCTION

Metabolomics can be defined as a new emerging omic science in systems biology that is aimed to decipher the metabolic profile in complex systems through the combination of data-rich analytical techniques (NMR, MS) and multivariate data analysis. As described in Chapter 2, NMR is one of the techniques that meets those requirements. Although NMR method development has mainly been driven toward the enhancement of qualitative information for general structure elucidation, the quantitative aspect has also been recognized since the early days of NMR (Pauli, Jaki & Lankin, 2004). Moreover, in the last decade a number of techniques have been devised to develop NMR spectroscopy as a fingerprinting tool for the quality assessment of crude plant materials, agri-food products, biofluids, tissues, and cell extracts.

Multivariate or pattern recognition techniques such as the well-described PCA (par. 2.2) are important tools for the analysis of data obtained by NMR.

Recently, NMR in combination with PCA has been applied in food science to evaluate the molecular profile of food stuff and also to evaluate the effect of external factor on food quality (Picone, 2009). The potentiality of ^{1}H-NMR in combination with chemometrics has been also applied in seafood research (par. 2.3) for both the molecular characterization of fish and seafood (Ciampa, Picone, Laghi, Nikzad & Capozzi, 2012) and also for the evaluation of the effect of aquaculture system on fish metabonome (Picone, Balling Engelsen, Savorani, Testi, Badiani & Capozzi, 2011; Savorani, Picone, Badiani, Fagioli, Capozzi & Engelsen, 2010a). Lot of research works on the lipid profile (seafood lipidomics) have also been published (Fiori, Solana, Tosi, Manfrini, Strim & Guella, 2012; Mannina et al., 2008; Vidal, Manzanos, Goicoechea & Guillén, 2012; Yan et al., 2012).

On the other hand, in the literature there are not detailed studies on the freshness indices determination through metabonomic approaches. In this study, Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) are used to separate fish samples among groups and to identify the biochemical compounds describing the evolution of their metabolic profile, during the storage at 4°C and UI.

6.2 NMR SPECTROSCOPY AND SPECTRA DATA PRE-TREATMENT

The ^{1}H-NMR spectra are recorded as described in par 4.2.5 for Red mullet and Bogue fish and in par 5.2.3 for Bogue, Atlantic mackerel, Gilthead seabream and European seabass.

The data are acquired under an automatic procedure, requiring about 30 minutes per sample. Free induction decays (FID) are Fourier transformed, with the MestReC Software, by performing an exponential multiplication with a 0.5 Hz line broadening. The TSP signal at 0.00 ppm is taken as chemical shift reference for all spectra. Phase and multipoint manual baseline corrections are performed manually for each FID and spectral data points are reduced by deleting the edge parts of the spectra, containing any signal above the noise, and by cutting off the solvents' signals (water). The resulting spectra are saved as ASCII file for the sub sequent statistical analysis, by using R language commands.

As noted in par 2.2.2, NMR spectral data often need to be preprocessed in several ways in order to conform to the prerequisites for chemometrics data analysis. Thus, prior to multivariate analysis, data underwent pre-statistical improvement, aiming at minimizing unwanted sources of variation due to slightly different instrumental conditions, imperfect baseline and phase corrections, and sample preparation artefacts. Such operations include first the choice of a pH independent signal (peak) to which refer all spectra (**alignment**), than the **normalization**, which mostly minimizes the differences due to dilution errors during samples preparation, as well as to small differences in the tuning conditions of the spectrometer.

After the referring of all the spectra to TSP signal, several shifts in peak positions are still observed, especially for those metabolites hardly dependent on the pH differences.

Interval correlation optimized shifting, *i*Coshift (Picone et al., 2011; Savorani, Tomasi & Engelsen, 2010b) is applied on those intervals in which signals are not aligned, obtaining a considerable reduction of shifting.

6.3 MULTIVARIATE DATA ANALYSIS (MvDA)

6.3.1 Fish samples analyzed at 400.13 MHz

After the application of the *i*Coshift tool, the PCA has been first applied on the whole spectral dataset (encompassing samples at 4°C and UI), acquired with 400.13 MHz spectrometer, in order to evaluate the evolution of the metabolome (freshness change) during time and at two different storage's temperature. Before that, data are mean centered with no scaling procedure.

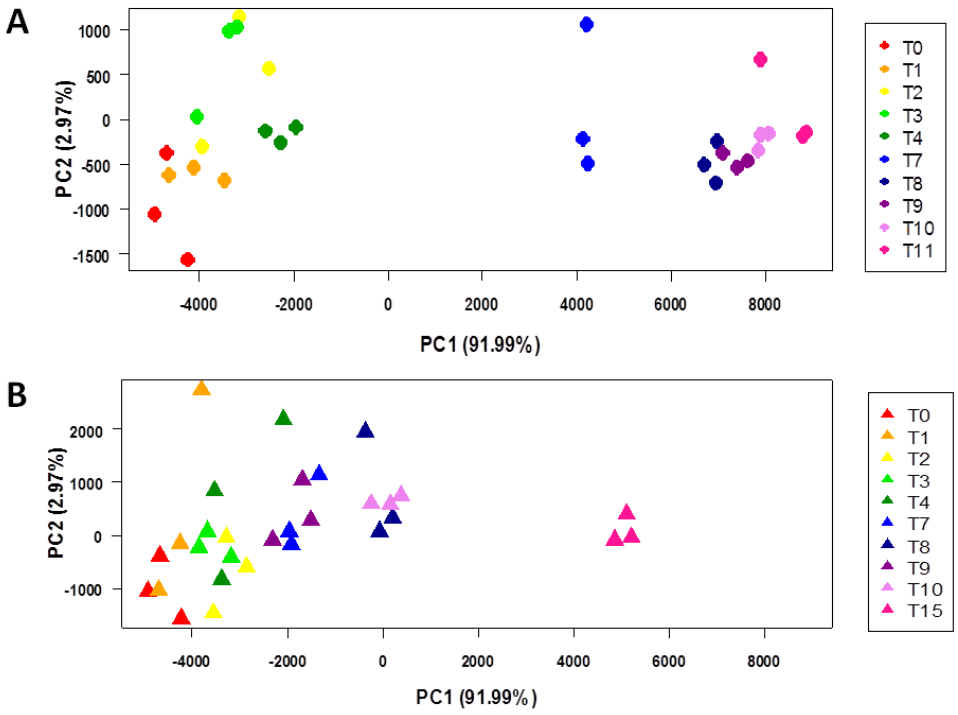

Figure 6.1: PCA scores plot of *i*Coshifted data set consisting of 66 ^{1}H-NMR Red mullet samples spectra; A) samples at 4°C and B) samples at 0°C (UI) (PCA was performed by using R program)

A PC analysis exhibits a significant ability to separate samples according to time storage with an explained variance of 91.9% along PC1 in the whole spectral data set analysis for both temperature, but above all to give an overall view of freshness evolution.

PC1 is the component along which the molecular composition of samples evolves during storage time. It is evident the effect of storage temperature: in fact, while the samples at 4 °C are widely spread along PC1, samples at 0°C are much more gathered in the negative side of PC1, except for samples at T15.

To better understand the nature of the variance between samples, the following plot of the PC1 loadings (Figure 6.2) highlights the contribution and even more indicate the weight of each variable in discriminating the samples with respect to storage time.

The examination of PC1 **loadings**, explained in Figure 6.2, allows the identification of the metabolites responsible the separation. A high loading indicates a strong contribution of the original NMR signal to the investigated PC.

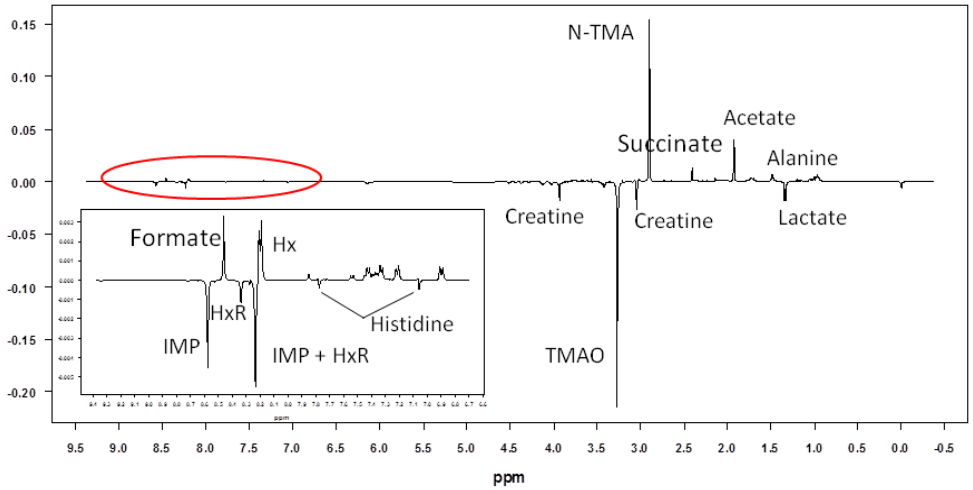

Figure 6.2: PC1 loading plots for Red mullet samples at 4°C

The most intense signals belong to TMA-N and TMAO's. The increasing absolute intensity of the TMA-N signal pushes the samples, in the score plot, towards higher values of PC1. The opposite occurs with the TMAO signal, for which lower values of PC1 corresponding to an increasing area of the peak. The source of variation is not confined only in TMAO and in TMA-N peaks. Rather, the whole metabolite's profile is subjected to change. In fact, also nucleotides, some amino acids and organic acids such as formate, lactate and acetate, play an important role both in the evolution of the freshness degree and in formation of an agreeable odor or not (Bramstedt, 1962).

A similar result is obtained from Bogue samples, as shown in the PC scores plot in figure 6.3 and by the PC1 loadings plot in figure 6.4.

It is worth noting here that the first principal component in PC plot is able to give a good and complete evaluation of freshness evolution during time storage, explaining 90% of the total variance. For this reason, PC scores are able to

condensate, with only one parameter, the whole different composition of fish due to time and temperature storage.

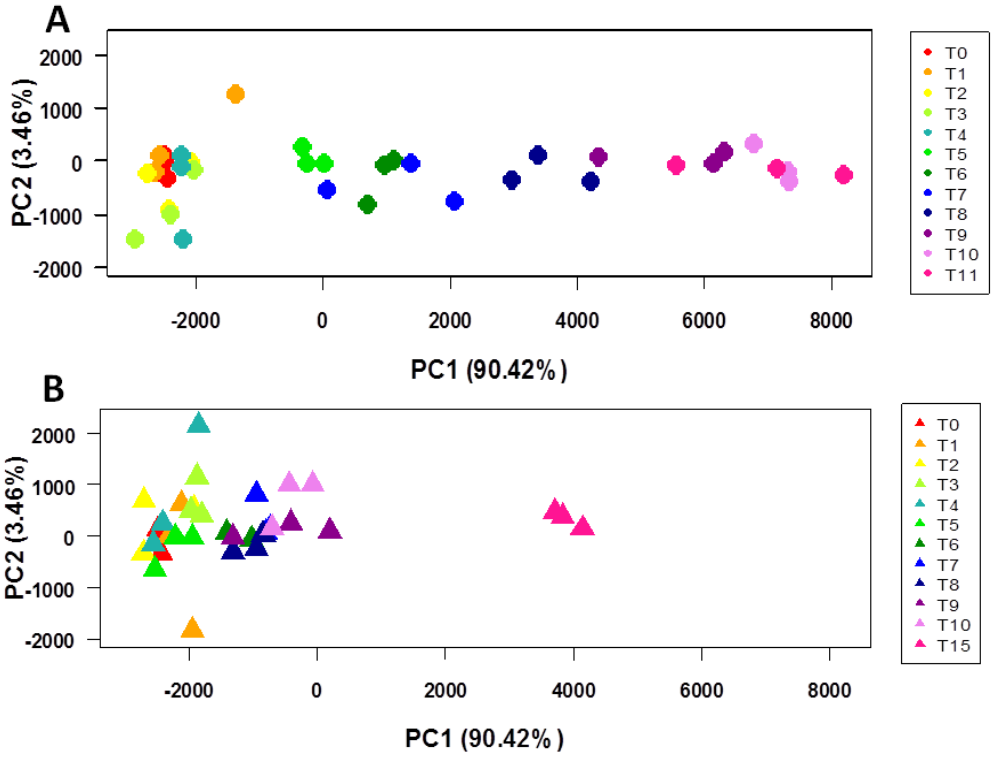

Figure 6.3: PCA scores plot of *i*Coshifted data set consisting of 66 ^{1}H-NMR Bogue samples spectra; A) samples at 4°C and B) samples at 0°C (UI) (PCA was performed by using R program)

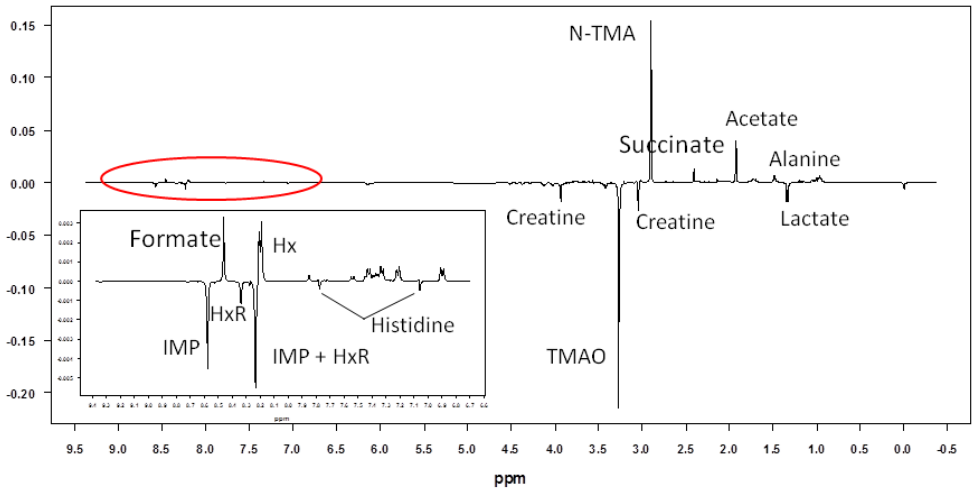

Figure 6.4: PC1 loading plots for Bogue samples at 4°C

6.3.2 Fish samples analyzed at 600.13 MHz

As well as previous data, samples also acquired at 600 MHz undergo a MvDA. In particular, Bogue fish samples, belonging to a 2nd shipping, show a modification

in metabolic profile, during storage, similar to that observed for the 1st shipping samples analyzed at 400 MHz (PC plot is not shown). This PC values equivalence (Tab. 6.1) in Bogue samples explains that there are no particular effects, on metabonomic changing, due to catching time (1st shipping in March and the 2nd in April), but above all that no instrumental effects change the results.

Table 6.1: PC1 and PC2 scores for Bogue samples acquired at 400.13 and 600.13 MHz

	PC1 (%) 4°C	PC1 (%) 0°C	PC2 (%) 4°C	PC2 (%) 0°C
		400.13 MHz		
Bogue – 1st Arrival	90.42	90.42	3.46	3.46
	PC1 4°C	**PC1 0°C**	**PC2 4°C**	**PC2 0°C**
		600.13 MHz		
Bogue – 2nd Arrival	90.08	90.08	3.93	3.93

A different result is obtained from Atlantic mackerel samples, as shown in the PC plot of figure 6.5, related to 1st shipping samples.

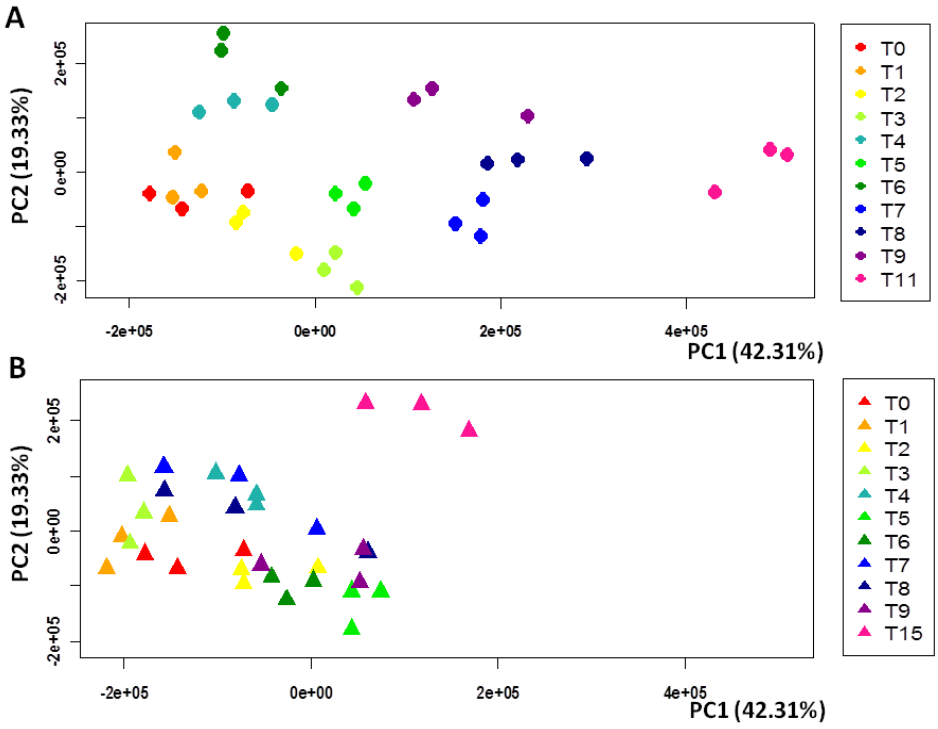

Figure 6.5: PCA scores plot of *i*Coshifted data set consisting of 60 ^{1}H-NMR Atlantic mackerel samples spectra; A) samples at 4°C and B) samples at 0°C (UI) (PCA was performed by using R program)

In fact, in this case, PC1 explains for 42.31% of the total variance and together with PC2 for the 61.64% of the total variance. This phenomenon can be explained with a slower loss of freshness during time storage.

This decrease could be ascribed to the presence of higher lipid content if compared to the Bogue samples. Although it is a quite high fat containing species and therefore more prone to rancidity, its water-soluble metabolites tend to degrade with more difficulty over time and to have less discriminating effects on fish spoilage (Shenouda, 1980). In particular, when oxidative rancidity progresses, changes in fish lipids may be related to changes in protein during storage. One possible mechanism of reaction between oxidized lipids and proteins occurs through stable oxidation products such as malonaldehyde, propanal, and hexanal (Shenouda, 1980), which covalently react with specific functional groups on protein side chains, including the –SH group of cysteine, and the N-terminal amino group of aspartic acid, tyrosine, methionine, and arginine (Konosu, 1979). Such interactions increase the hydrophobicity of proteins, making them less water soluble. According to Sikorski et al. (Sikorski, Olley, Kostuch & Olcott, 1976), when the hydrophobic sites of free fatty acid (FFA) interact with protein molecules, the protein molecules become surrounded with a more hydrophobic environment, which subsequently results in a decrease in protein extractability and hydrolyzation. This interaction may occur through hydrophilic and hydrophobic forces (Sikorski et al., 1976). In this situation, there are conditions less favorable for bacterial development because soluble proteins and the low molecular weight compounds yielded from the fish body during autolysis, after rigor mortis, are less available for bacterial growth. The same value of PC1 and PC2 are obtained also analyzing data from Atlantic mackerel samples caught at later time (2nd and 3rd shipping), as summarized in table 6.2.

Table 6.2: PC1 and PC2 explained variance for Atlantic mackerel samples

	PC1 (%) 4°C	PC1 (%) 0°C	PC2 (%) 4°C	PC2 (%) 0°C
Atlantic mackerel 1st Arrival	42.31	42.31	19.33	19.33
Atlantic mackerel 2nd Arrival	49.42	49.42	19.80	19.80
Atlantic mackerel 3rd Arrival	50.51	50.51	19.88	19.88

6.3.3 PC1 as an index for molecular quality measurement

Once again is better to underline that ^{1}H-NMR spectrum can give an overview of the whole metabolites present in the sample with one shot, thus making it possible to evaluate the effect of storage, rearing conditions and time on the whole molecular profile at the same time. However, the number of information coming from a spectrum need to be condensed in order to find out a unique parameter able to describe the loss of quality and the evolution of freshness, during time and due to external factors. The MvDA, as demonstrated in the previous paragraph, allows having a unique score able to summarize all of the hundreds of parameters, thus giving a measure of the molecular quality of fish.

As PC1 is the component describing the evolution of freshness in time, it can be taken to represent the summarizing parameter "index 1", as represented in the following equation:

$$PC1_{molecularquality} = \alpha * par_1 + \beta par_2 + \gamma par_3 + ... + \omega par_{22000}$$

where

$$PC1 = Index_1 = K - Index + TMA - N + His + FAA + FFA$$

Plotting the PC1 value vs. time storage at both temperatures for each species evaluated in this thesis, the trajectory is related to evolution of fish freshness and is obtained, as represented in figure 6.6 for Bogue samples, in figure 6.7 for Red mullet and in figure 6.8 for Atlantic mackerel.

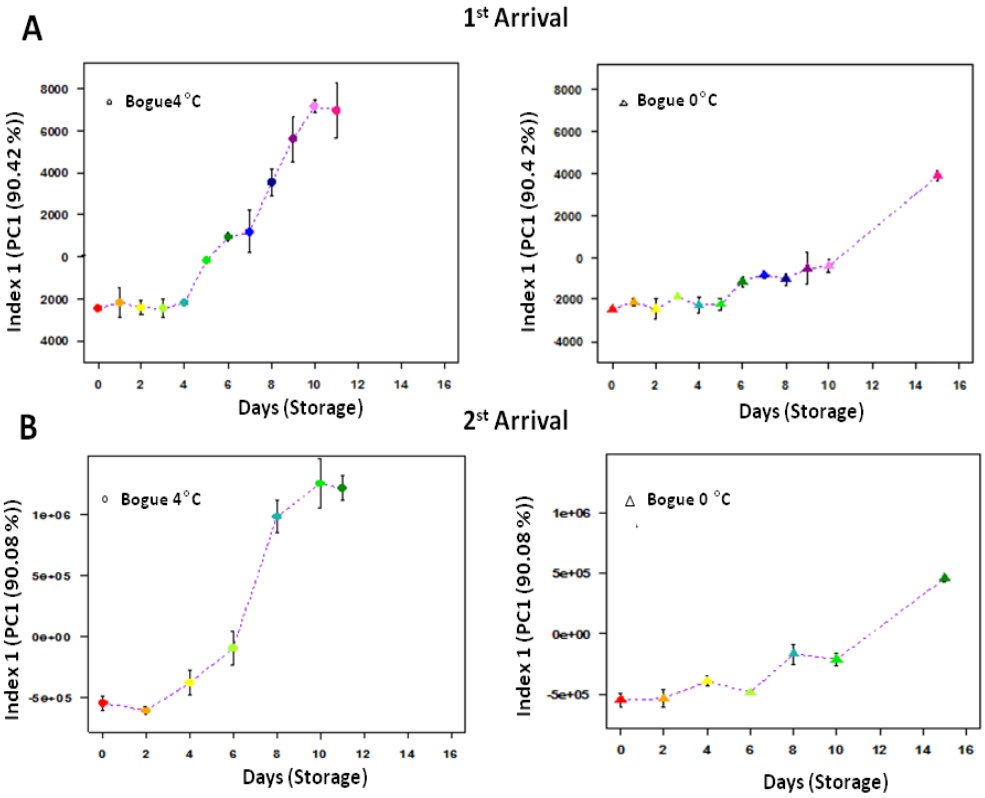

Figure 6.6: evolution of fish freshness valuated reporting PC1 values vs. time for Bogue fish samples at both storage temperatures.

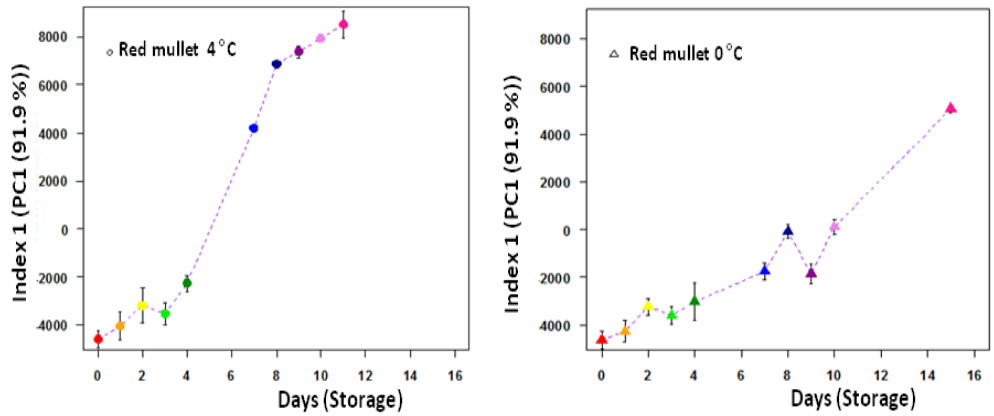

Figure 6.7: evolution of fish freshness valuated reporting PC1 values vs. time for Red mullet samples at both storage temperatures.

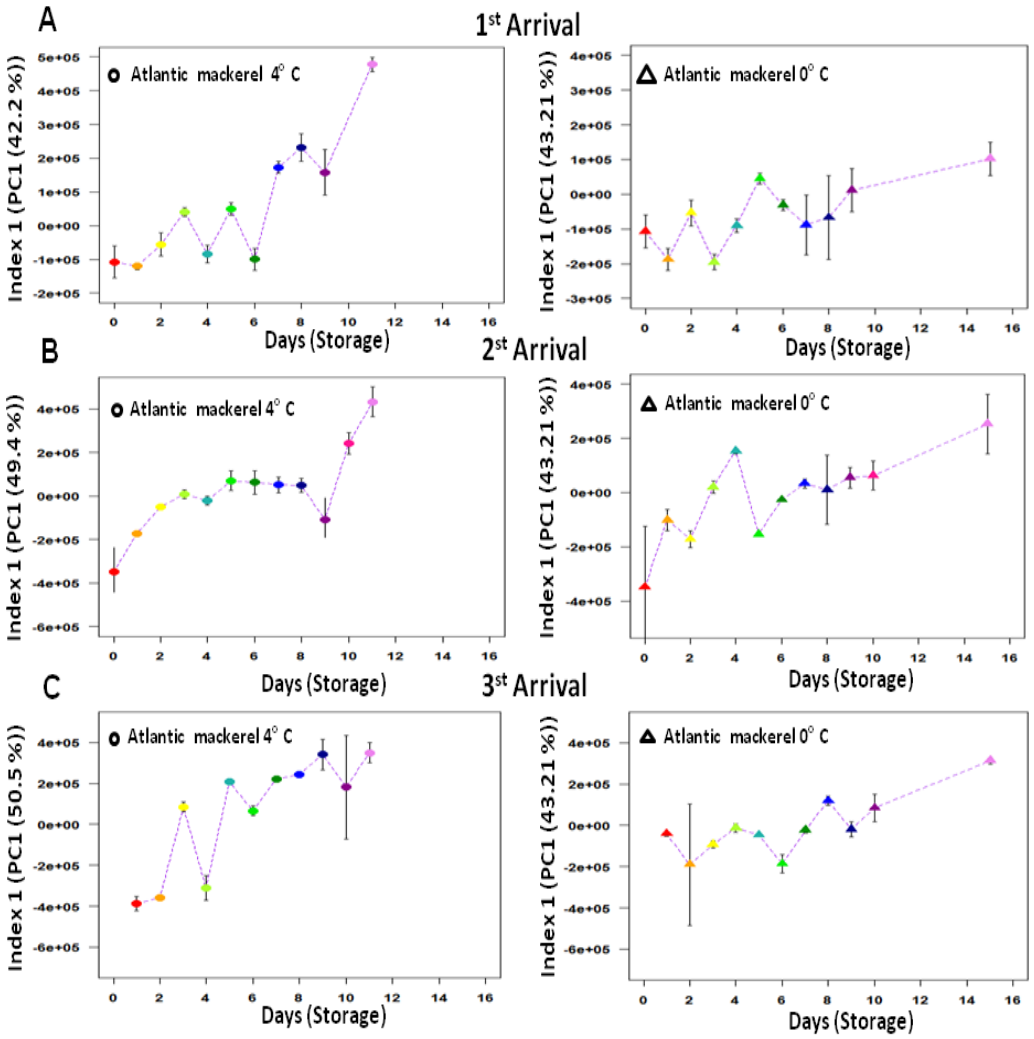

Figure 6.8: evolution of fish freshness valuated reporting PC1 values vs. time for Atlantic mackerel samples at both storage temperatures.

6.3.4 Effect of seasonal variation on Atlantic mackerel and Bogue fish samples

Seasonal variation in the metabolic rate of fish has been largely described in literature (Chipps, Clapp & Wahl, 2000; Laroche et al., 2012; Obermüller, Morley, Barnes & Peck, 2010), above all according to lipids as they are the predominant source of energy for fish and are stored in fat depots in different parts of the body regions (Özyurt, Kuley, Etyemez & Özoğul, 2012; Weil, Lefèvre & Bugeon, 2012). Season variation may affect the whole molecular profile of fish modifying in this way its nutritional and organoleptic characteristics. As a consequence, this may also affect the duration of the integrity status of freshness.

Applying a MvDA on all samples, grouped only on the base of temperature storage, shows that seasonality is much more discriminating than the differences due to species (Fig. 6.9 and 6.10) along PC1.

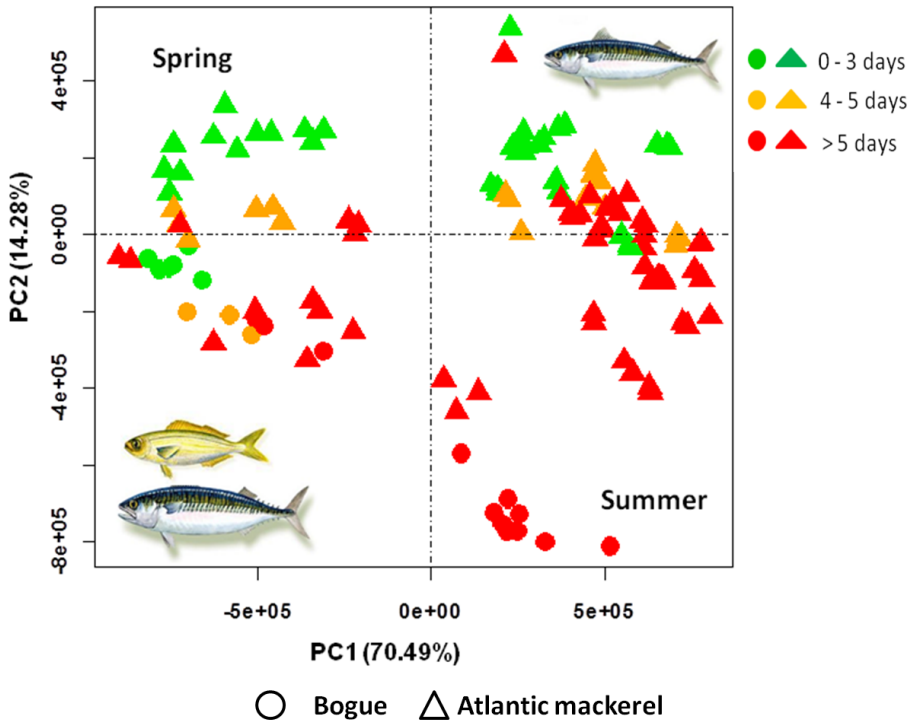

Figure 6.9: PC plot for fish samples at 4°C.

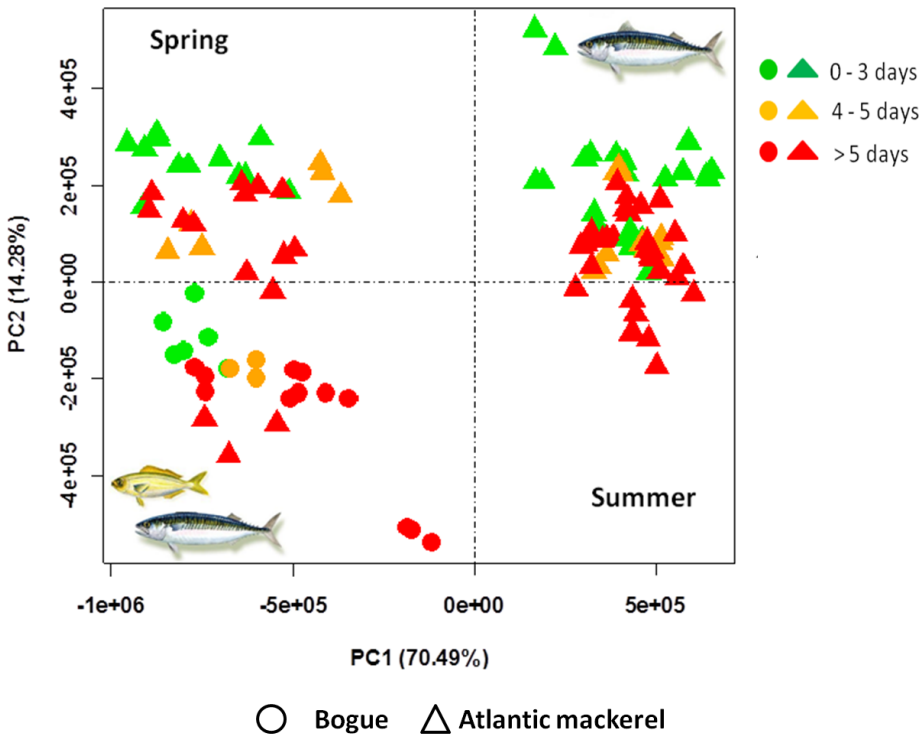

Figure 6.10: PC plot for fish samples at 0°C.

Spring samples are characterized by the negative value of PC1 for Atlantic mackerel and Bogue fish; on the contrary, the positive value of the same PC characterizes Atlantic mackerel samples caught during summer.

On the other hand, PC2, that accounts for the 14% of the total variance, ascribes importance to the time effect and then to the loss of freshness. According to this last aspect, samples are spread along PC2 from positive values, in the first days of storage, to negative values for later days of storage. This means that there is a loss of freshness along PC2. Moreover, this variable is also responsible for the differentiation of samples according to the species.

In summary, PC1 is an index of seasonality, while PC2 is the freshness index and it is species specific. The investigation of loadings, points out that TMAO is the metabolite responsible for the separation of samples according to the season catch.

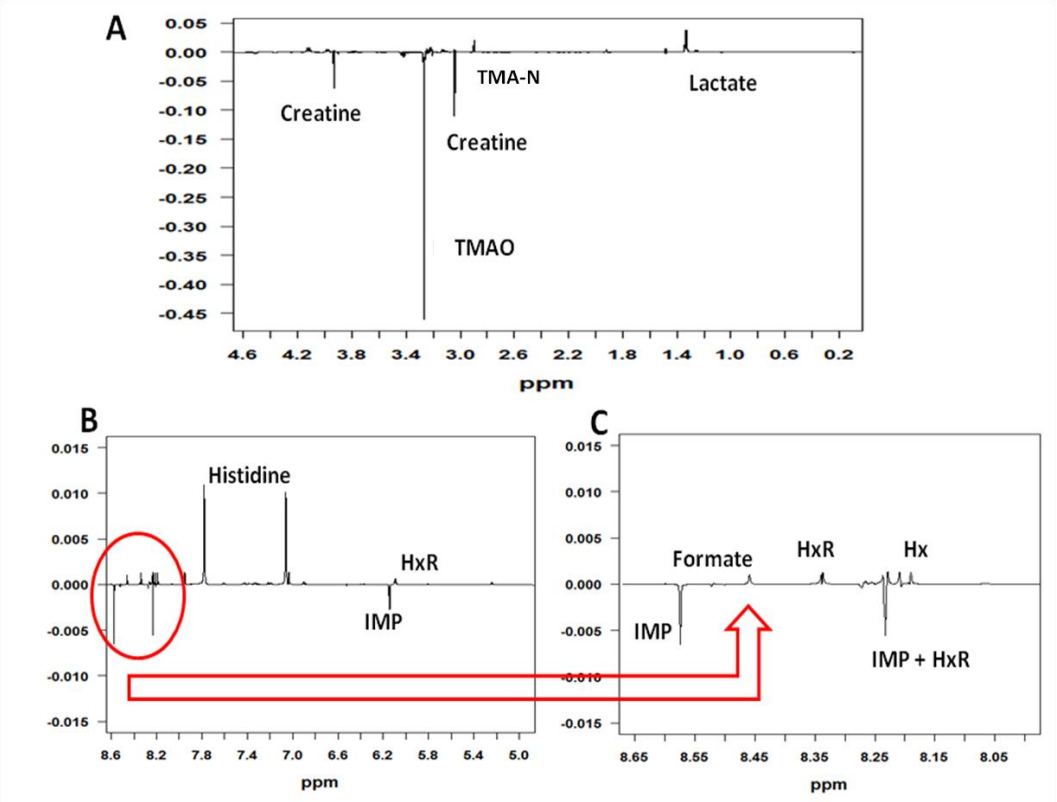

Figure 6.11: loadings plot of PC1 from 0.2-4.6 ppm (A), from 5.0-8.6 ppm and (B) from 8.0-8.6 ppm (C) at 4 °C and 0 °C

TMAO is strictly related to the season because it is an important osmoregulator. There are several groups of organic osmolytes that exhibit different properties (Yancey, Clark, Hand, Bowlus & Somero, 1982), but TMAO is one of the most common found in marine fish species.

In marine fish, the osmotic pressure of the blood varies with the osmotic pressure of the environment. Osmoregulation in these animals is cellular: when the osmotic pressure of the blood increases, the concentration of organic substances in the cells, to which the cell membrane is slightly permeable increases by the same amount. As a result, the salt concentration and water content of the cell do not change, and the osmotic pressure is equalized by the accumulation of osmotically active substances. A decrease in the osmotic pressures of the blood and environment decreases the concentration of organic substances in the cells. Thus, cellular osmoregulation provides for the limited adaptation of marine fish to fluctuations of osmotic pressure in the environment.

For example, low temperatures and increased hydrostatic pressure are environmental conditions that impose additional osmotic stress upon fish cells by destabilizing cellular proteins and membranes. Destabilization results in protein unfolding and the concomitant loss or reduction of functions such as enzyme activity. Fish cells accumulate organic osmolytes to counteract the action of the destabilizing forces.

In this case, the high levels of TMAO, higher in cold water fish and lower in warmer water fish (Raymond, 1998), could therefore be necessary for two purposes: increasing serum osmolarities and protecting proteins from the denaturation.

It has been suggested that the extra strength of folding caused by the presence of TMAO is brought about by TMAO organizing the solvent molecules in such a way that the intraprotein hydrogen bonds are strengthened and this prevents proteins unfolding (Bennion & Daggett, 2004) (Figure 6.12).

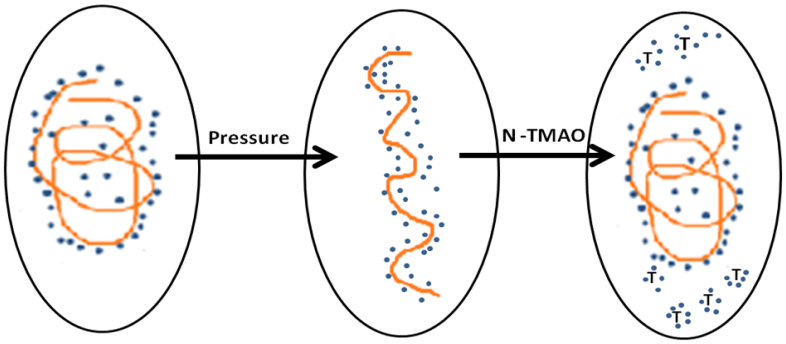

Figure 6.12: Effect of pressure on protein three-dimensional structure and effect of TMAO within a cell (oval). Protein (red line) three-dimensional structure is compromised by increased pressure. The effect is counteracted by the addition of TMAO (T). Dots represent solvents molecules

Finally, PC2 results to be really important considering the evolution of freshness during the time of storage and to differentiate samples according to the species. The loadings plot of this component (Figure 6.13), that account for the 14.28% of the total variance, once again confirms that fish quality, above all fish freshness, is a condition that involves the whole metabolome. Then, external factors induce deep changes in all metabolites. Moreover, in this specific case, it is also clear how freshness fragility depends on fish species. In fact, Bogue samples, which are much more spread along PC2, result to be more subjected to spoilage if compared to Atlantic mackerel.

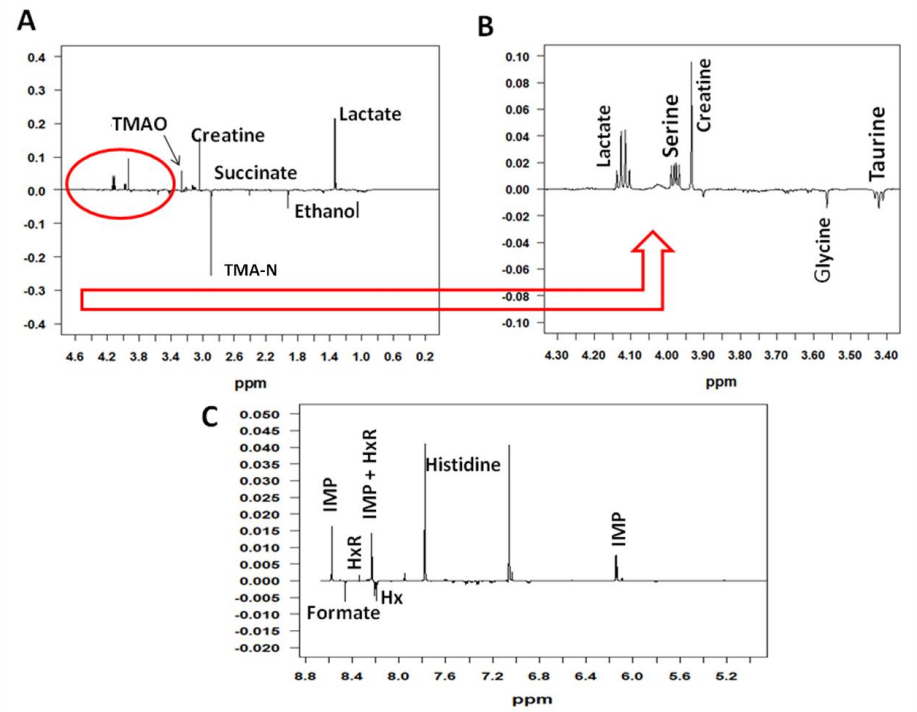

Figure 6.13: loadings plot of PC2 from 0.2-4.6 ppm (A), from 3.40-4.30 ppm and (B) from 6.0-8.8 ppm (C) at 4 °C and 0 °C

6.3.5 MvDA as tool for determinate index of freshness in farmed fish

A preliminary study by Savorani et al (Savorani et al., 2010a) and Picone et al (Picone et al., 2011) have demonstrated how rearing conditions can be a factor able to induce changes on metabolic profiling of fish.

In this study it has been demonstrated how these changes affected the freshness condition.

Using the MvDA, in particular PCA, on spectra data emerges that rearing condition deeply affects the spoilage of fish. The PC plot in figure 6.14 points out different freshness trajectories for "off shore" and "in shore" cages samples along PC1.

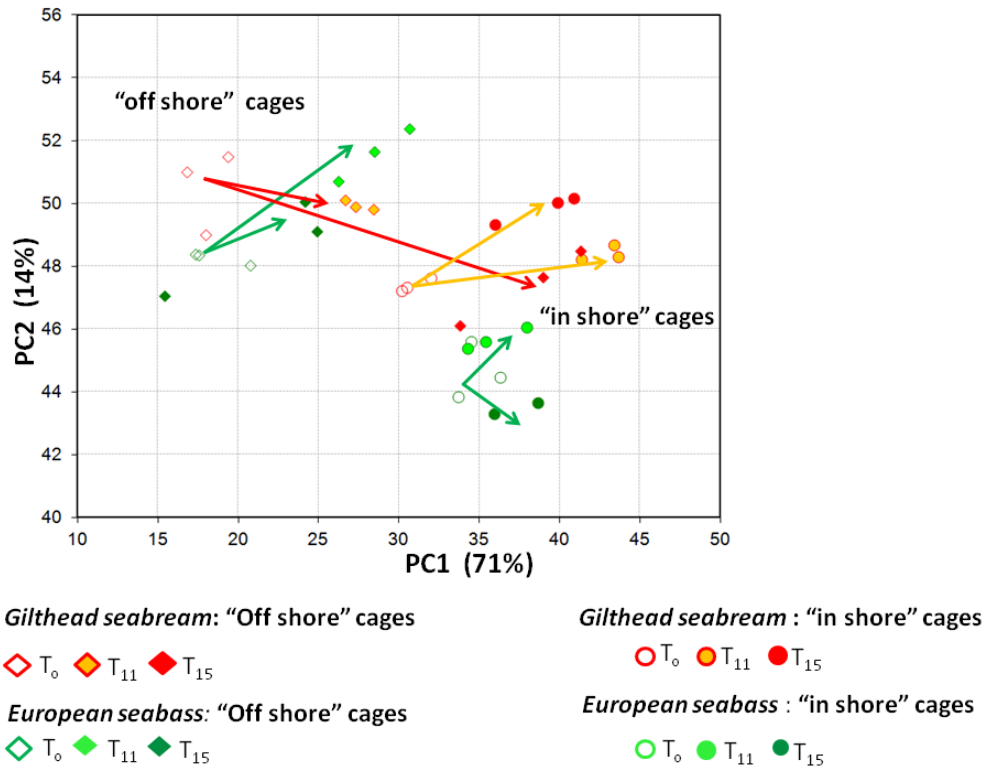

Figure 6.14: PCA scores plot of Gilthead seabream and European seabass samples stored at 4°C and 0°C.

In addition, along PC1 it is possible to observe as well that the storage has a different effect on the spoilage of the two species. In particular, the conservation under ice (0 °C) after 15 days from the catch, would seem to have more positive effects on the European seabass than on the Gilthead seabream. This means that

the components of the two muscle types, red and white respectively, will not undergo the same chemical changes during storage in the refrigeration condition at 0 °C. It is reported that the TVB-N value is not stable during storage at low temperatures and could be changed according to species, processing methods, and storage temperature (Tokur, Ozkütük, Atici, Ozyurt & Ozyurt, 2006).

Also physicochemical changes, after 15 days, due to extended storage at 0°C causes the denaturation of protein as well as the cell disruption in the two species, but the degree of changes is dependent upon species (Benjakul, Visessanguan, Thongkaew & Tanaka, 2003).

PC2 indeed is related to the kind of rearing and to fish species and its variance (14%) demonstrates that many compounds contribute to the spontaneous grouping of fish samples according to their aquaculture system.

In this analysis only samples at T0, T11 and T15 undergo PC analysis because it is observed that, unlike wild fish, there are not significant changes in the molecular profile during the intermediate days at 0 °C.

These results confirm that the farmed fish have additional ways to influence product quality and consequently the freshness. These ways include control of physiological factors such as biological age and growth rate; control of environmental factors such as water temperature, pressure, flow and chemistry and control of dietary factors, such as feeding cycle, starvation, overfeeding, and the presence or absence of specific dietary components.

For example, microbial spoilage can be better controlled in cultured fish than in fish caught at sea because farmed animals fasted for several days will clear the intestinal tract and moreover, depuration of live fish in sanitized water will reduce bacterial contamination of the fish's surface.

Diet can also influence enzyme activities involved with energy metabolism. In fact, the rate of enzyme catalyzed reactions such as glycolysis, can influence the quality attributes of meat (Haard, 1992). For example, acclimation of flounder to a cold water temperature increases the amount of phosphofructokinase in the muscle, a key enzyme in the control of glycolysis) (Longerich & Feltham, 1978). Consequently, a different activity of enzymes that usually are involved in

autolysis processes, could be the cause of different spoilage in wild and farmed fish.

Then, to find a discriminant index of freshness both for wild fish and farmed ones, a PLS-DA is carried out. PLS-DA is able to highlight differences among already known groups providing a classification. Two models are constructed, one for fish with dark muscle (Atlantic mackerel, Bogue and European seabass) and another for those from white muscle (Gilthead seabream), because the first model is not suitable for the latter (Figure 6.15).

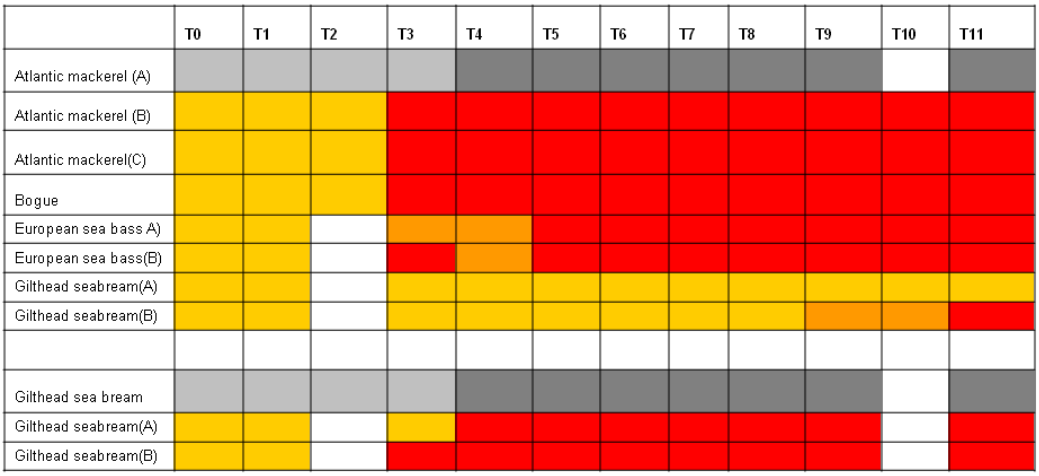

Figure 6.15: PLS-DA result. In yellow, fresh samples correctly predicted. In red misclassified samples and in orange ambiguous samples. The model has been built using only samples at 4°C. Atlantic mackerel (A, B, C): 1st, 2nd, 3rd Sampling. European sea bass (A, B): in-shore and off-shore cages; Gilthead sea bream (A, B): in-shore and off-shore cages.

The first model built on the Atlantic mackerel species classifies “fresh fish”, with a probability of 100%, samples such as Bogue up until the second day from the catch and with ambiguity the European sea bass samples farmed in off shore cages after three days of storage. For most groups, from the third day, the same fish samples were classified as no longer fresh.

The second model built exclusively for Gilthead seabream classifies “fresh fish”, with a probability of 100%, samples of fish reared in shore cages until three days from the catch but not those raised in off shore cages.

6.4 CONCLUSIONS

The results of this study point to the relevance the importance of the combination of several multivariate data analysis.

Interesting results by chemometric tools show that the rate of spoilage bacteria is different for wild and farmed fish.

For this reason, different freshness indexes have been identified by analysis multivariate, PCA and PLS-DA, which have reflected all those factors, from the physiological to the environmental, that could influence the chemical composition and edible qualities of fish.

These indexes are built up with the contribution of many compounds: from those that determine loss of freshness (TMA-N, HxR, Hx) and from those that detect the nutritional status of fish (vitamins, amino acids, etc.)

From this point of view, the NMR approach provides a great deal of information in one shot about the nutritional status of the fish taking into consideration both time and temperature effects.

The relative speed of analysis and the simple evaluation of the data suggest that ^{1}HNMR could be applied to a large number of fish species with high throughput.

In fact, in the future, the freshness indexes by NMR could be the means to validate methods for assessing the freshness of all species that until today not have the *reference official cards* for the evaluation of the "Quality Index Method".

Today, it is increasingly necessary to establish useable criteria for fish freshness and spoilage that are practical both for the fish industry and the consumer, reflecting their demand for fish freshness (Delbarre-Ladrat, Chéret, Taylor & Verrez-Bagnis, 2006; Delbarre-Ladrat, Verrez-Bagnis, Noël & Fleurence, 2004a; Delbarre-Ladrat, Verrez-Bagnis, Noël & Fleurence, 2004b).

In this thesis only the polar metabolites were taken into consideration evaluating fish freshness, but since there is a close ratio between protein, fat and water in the conservation of this food, thus easily degradable, that further studies on the lipid compounds would be very useful to identify in more detail the molecular fingerprint of this product.

In addition, further studies will be carried out through the use of homo-nuclear scalar NMR experiments for the assignment of unknown metabolites that could be of considerable importance in the evaluation of fish freshness.

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GENERAL CONCLUSION

“Fresh fish”

Conclusion

An objective approach establishing the shelf-life of a food product consists of observing during its conservation the change of some organoleptic properties which degrade or form. With regard to fish and seafood, most of the microbial, oxidative, nutritional and organoleptic indexes are conventionally obtained through long, expensive, highly specialized analyses sometimes using toxic or dangerous reagents (such as TMA-N determination.)

In this PhD thesis, a rapid method has been utilized to evaluate the evolution of the most important parameters relating to the freshness indexes of fish and seafood through the **metabonomics** approach. This method is based on the combination of chemometrics (multivariate data analysis) and HR ^{1}H-NMR, the latter giving a great deal of information on metabolic profiling **(metabolic fingerprint)** in a single, short period without using hazard reagents. This combination of methodologies has been applied on different fish species, ranging from wild to farmed, to find a unique, common parameter evaluating the modification of the fish's metabolome regarding freshness during storage. **PC1** values have been identified as a good indicator of the loss of freshness and, at the same time, the evolution of freshness in fish samples stored at 4 °C and UI (0 °C).

Thus, this parameter better defines the criteria evaluating fish and seafood freshness determining the duration in time and in relation to storage conditions. Therefore, effective practices preserve fish freshness for several days. The application of this information may overcome the consumer's natural diffidence based upon the principle: "Fish stinks after three days!"

APPENDIX

^{1}H NMR Spectra

Appendix A.1 Red Mullet (*Mullus Barbatus*) – T0 - spectrum ^{1}H-NMR

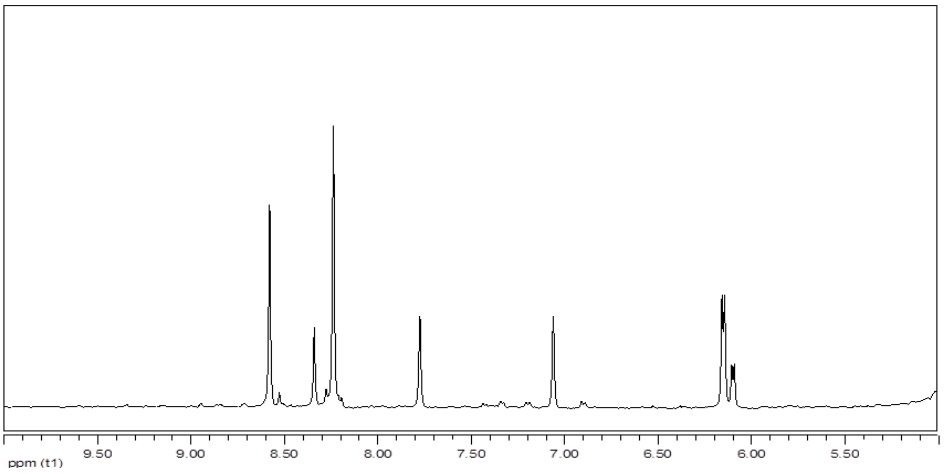

Figure Appendix A1: Red Mullet (*Mullus Barbatus*): Downfield Region (> 5 ppm)

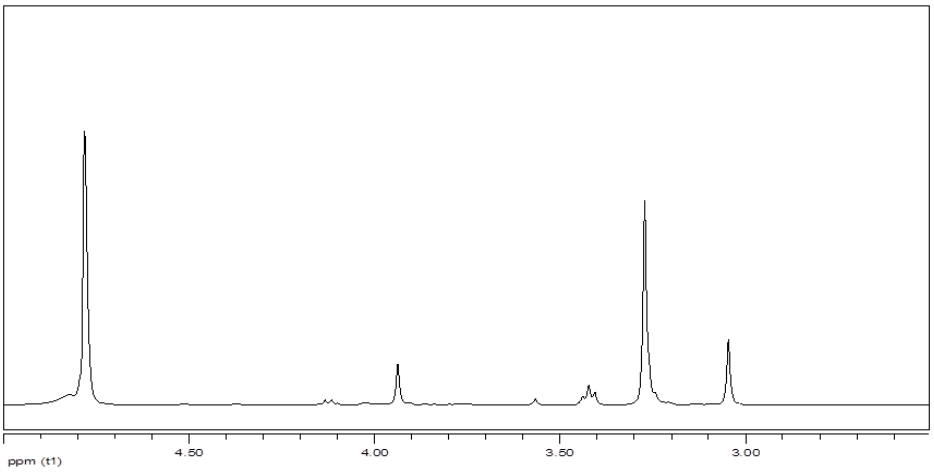

Figure Appendix A1: Red Mullet (*Mullus Barbatus*): Midfield Region (5 < ppm < 2.5)

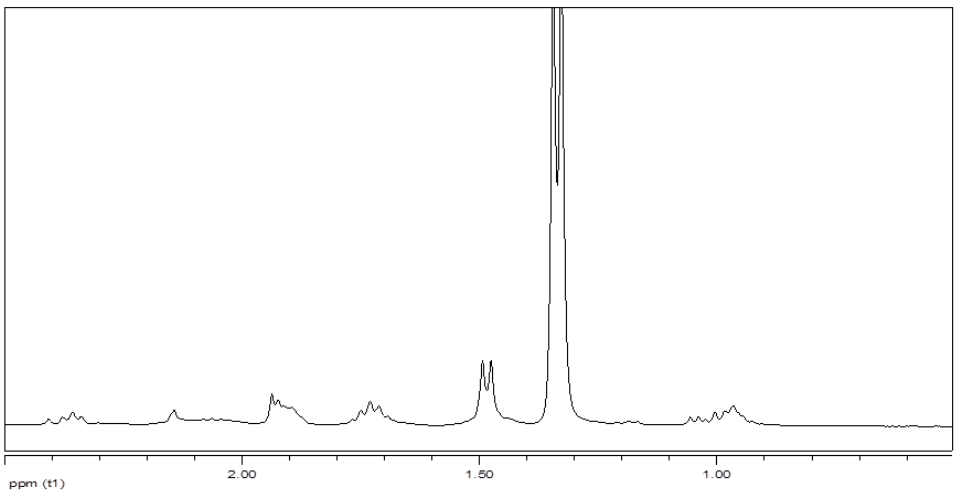

Figure Appendix A1: Red Mullet (*Mullus Barbatus*): Upfield Region (< 2.5 ppm)

Appendix A.2 Red Mullet (*Mullus Barbatus*) – T11 4 °C - spectrum ^{1}H-NMR

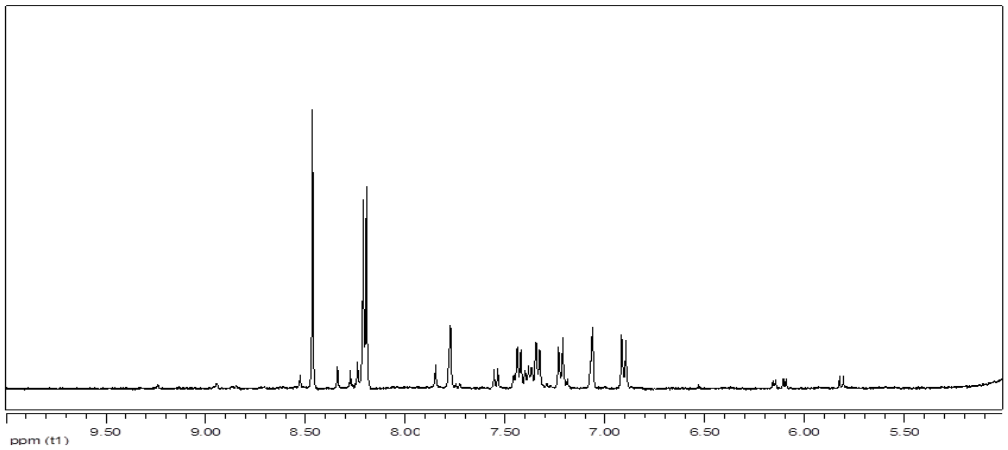

Figure Appendix A2: Red Mullet (*Mullus Barbatus*): Downfield Region (> 5 ppm)

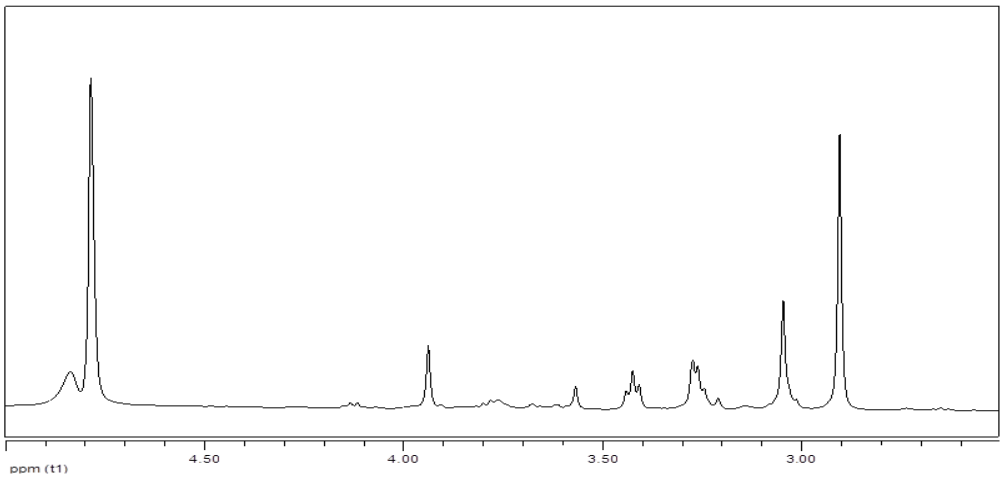

Figure Appendix A2: Red Mullet (*Mullus Barbatus*): Midfield Region (5 < ppm < 2.5)

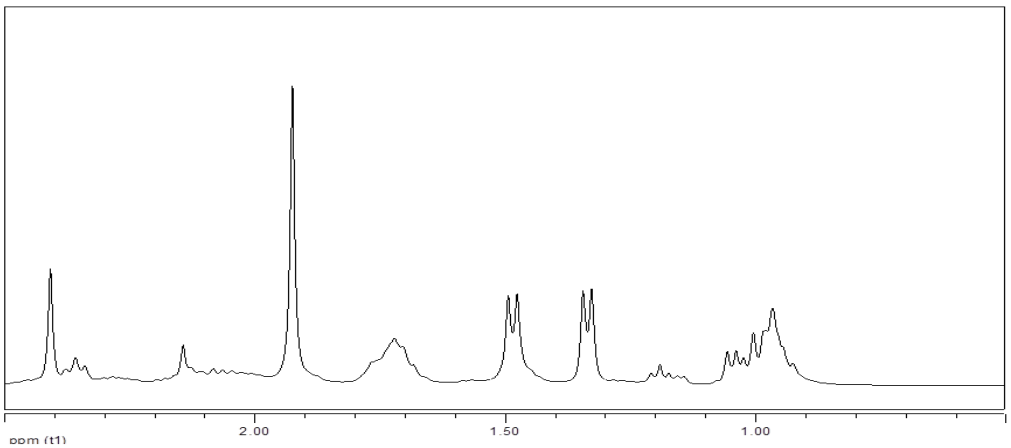

Figure Appendix A2: Red Mullet (*Mullus Barbatus*): Upfield Region (< 2.5 ppm)

Appendix A.3 Red Mullet (*Mullus Barbatus*) –T15 0 °C - spectrum ^{1}H-NMR

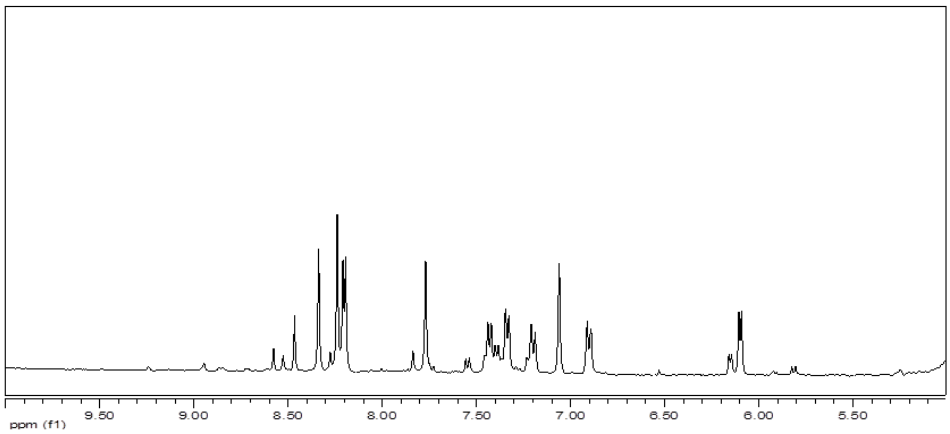

Figure Appendix A3: Red Mullet (*Mullus Barbatus*): Downfield Region (> 5 ppm)

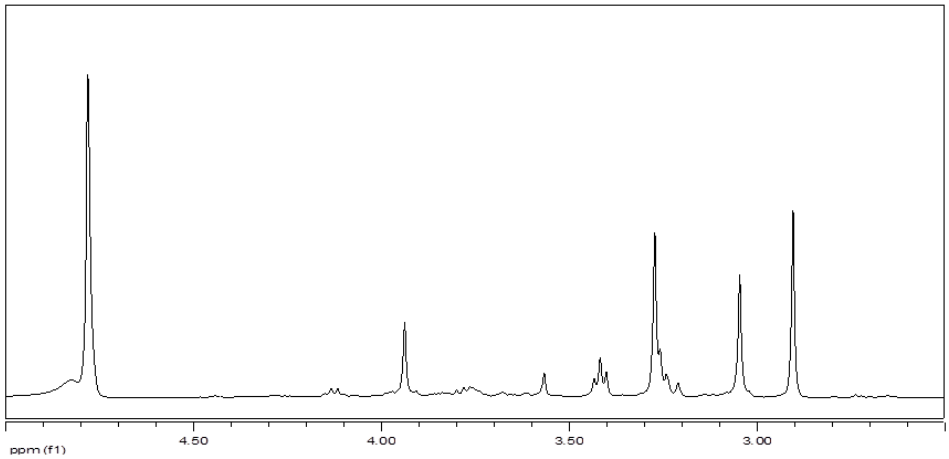

Figure Appendix A3: Red Mullet (*Mullus Barbatus*): Midfield Region (5 < ppm < 2.5)

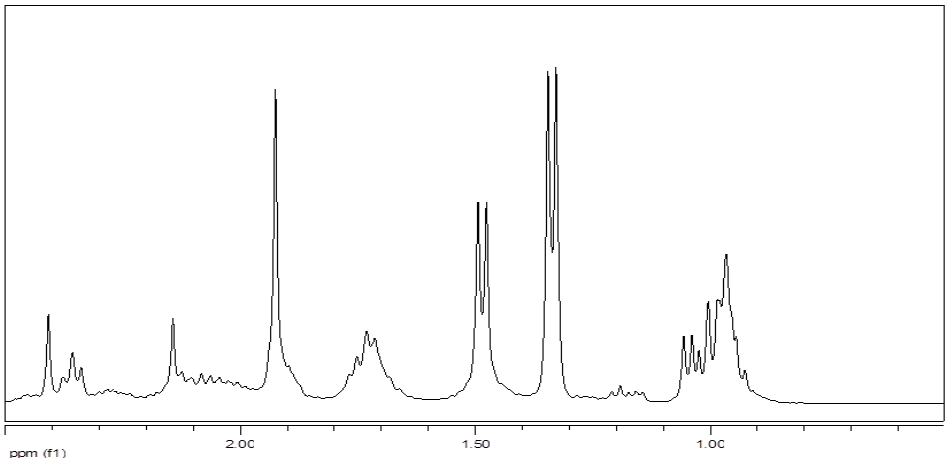

Figure Appendix A3: Red Mullet (*Mullus Barbatus*): Upfield Region (< 2.5 ppm)

Appendix A.4 Bogue (*Boops Boops*) – T0 - spectrum ^{1}H-NMR

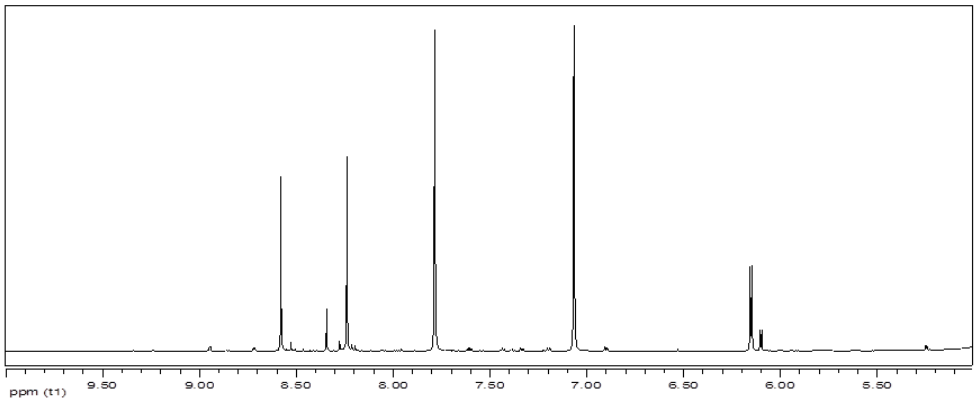

Figure Appendix A4: Bogue (*Boops Boops*) - Downfield Region (> 5 ppm)

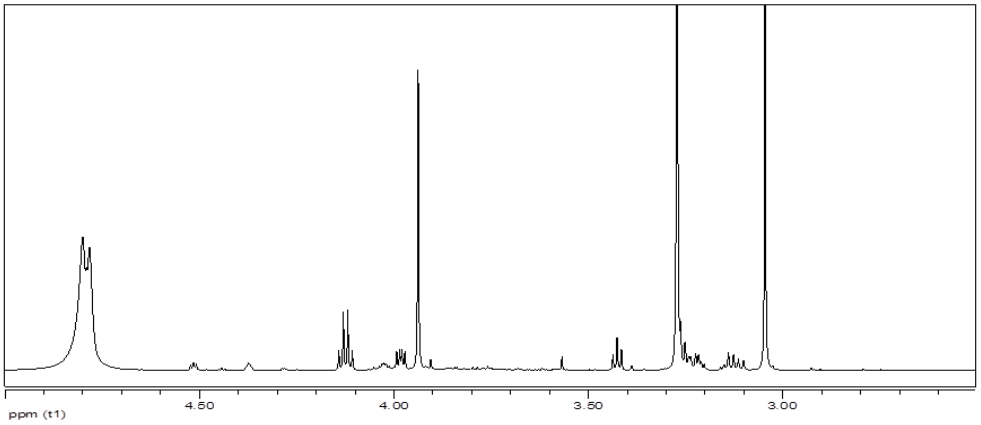

Figure Appendix A4: Bogue (*Boops Boops*) - Midfield Region (5 < ppm < 2.5)

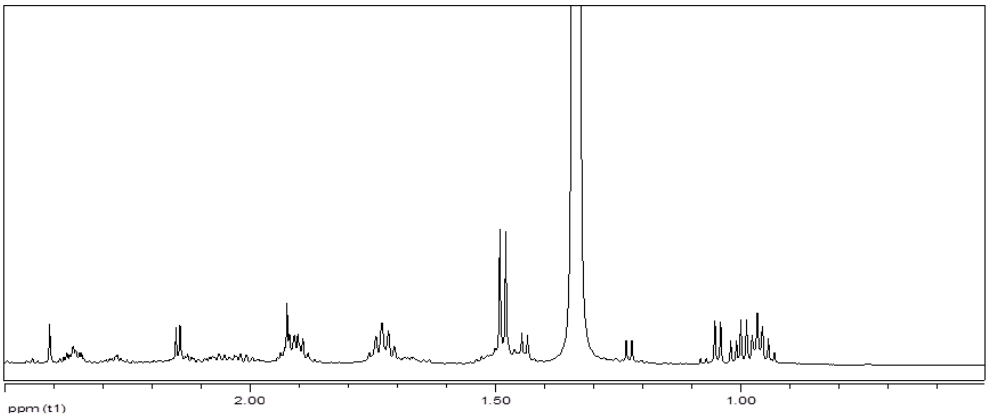

Figure Appendix A4: Bogue (*Boops Boops*) - Upfield Region (< 2.5 ppm)

Appendix A.5 Bogue (*Boops Boops*) – T11 - 4 °C - spectrum ^{1}H-NMR

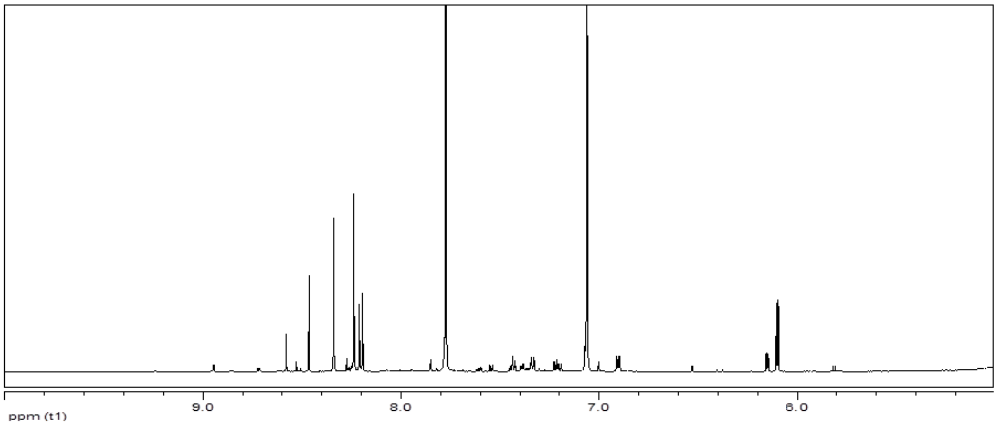

Figure Appendix A5: Bogue (*Boops Boops*) - Downfield Region (> 5 ppm)

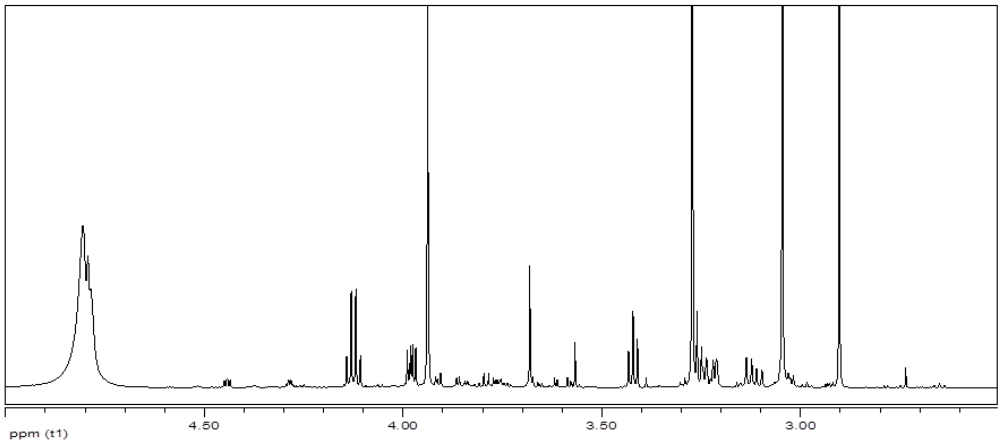

Figure Appendix A5: Bogue (*Boops Boops*) - Midfield Region (5 < ppm < 2.5)

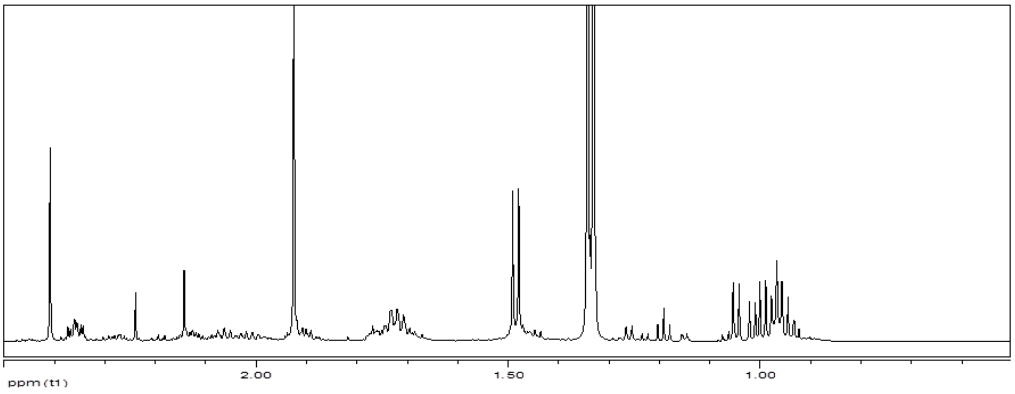

Figure Appendix A5: Bogue (*Boops Boops*) - Upfield Region (< 2.5 ppm)

Appendix A.6 Bogue (*Boops Boops*) – T15 - 0 °C - spectrum ^{1}H-NMR

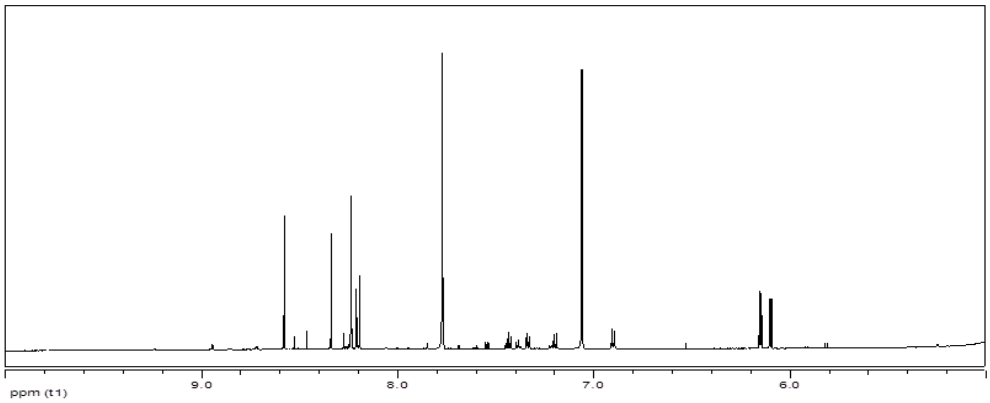

Figure Appendix A6: Bogue (*Boops Boops*) - Downfield Region (> 5 ppm)

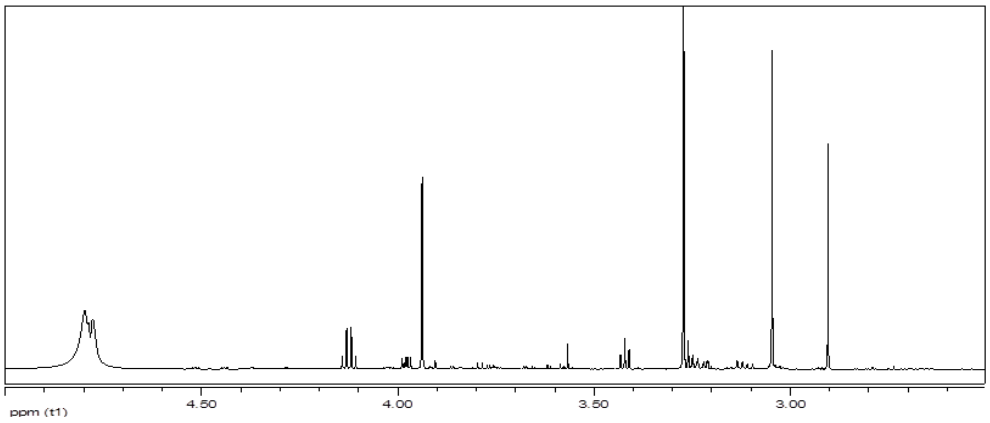

Figure Appendix A6: Bogue (*Boops Boops*) - Midfield Region (5 < ppm < 2.5)

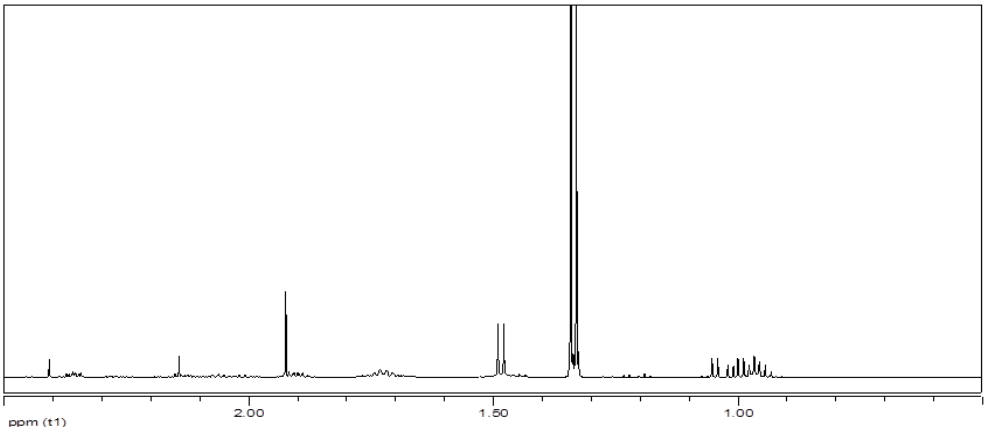

Figure Appendix A6: Bogue (*Boops Boops*) - Upfield Region (< 2.5 ppm)

Appendix A.7 Atlantic mackerel (*Scomber scombrus*) – T0 - spectrum ^{1}H-NMR

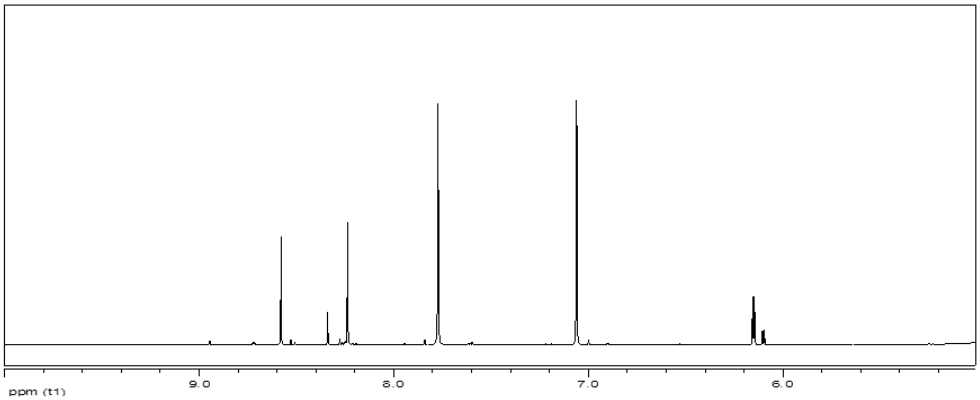

Figure Appendix A7: Atlantic mackerel (*Scomber scombrus*) Downfield Region (> 5 ppm)

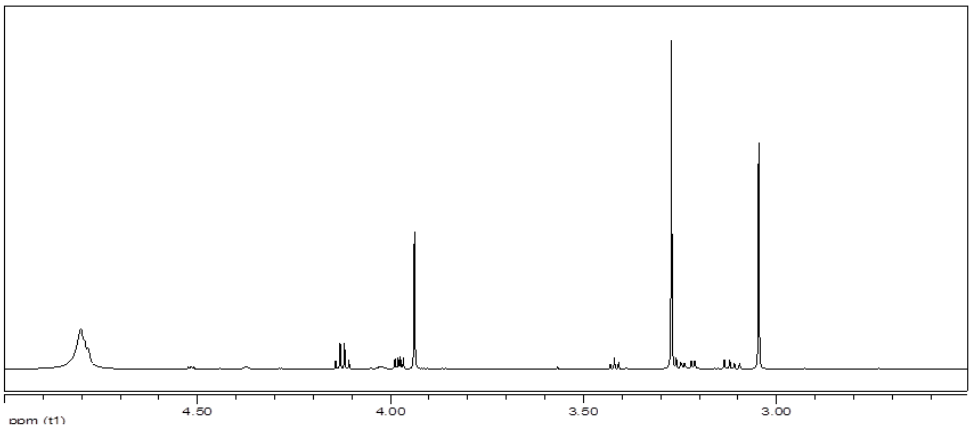

Figure Appendix A7: Atlantic mackerel (*Scomber scombrus*) - Midfield Region (5 < ppm < 2.5)

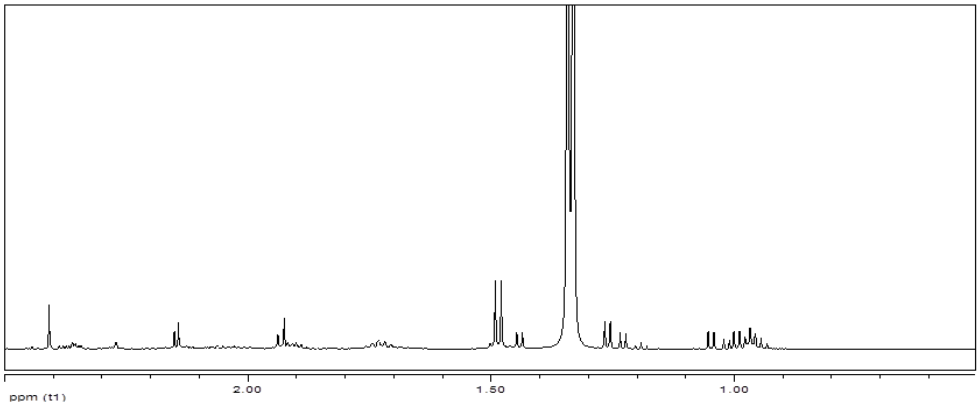

Figure Appendix A7: Atlantic mackerel (*Scomber scombrus*) - Upfield Region (< 2.5 ppm)

Appendix A.8 Atlantic mackerel (*Scomber scombrus*) – T11 4 °C - spectrum ^{1}H-NMR

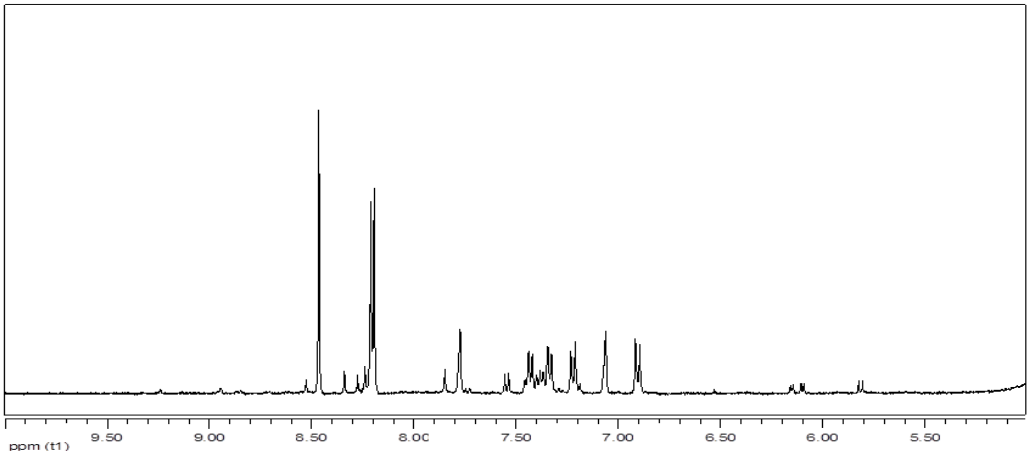

Figure Appendix A8: Atlantic mackerel (Scomber scombrus) Downfield Region (> 5 ppm)

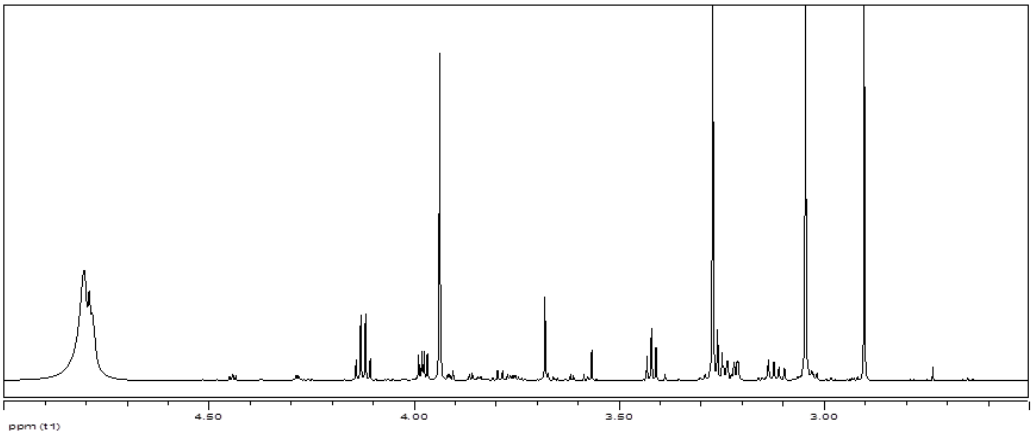

Figure Appendix A8: Atlantic mackerel (*Scomber scombrus*) - Midfield Region (5 < ppm < 2.5)

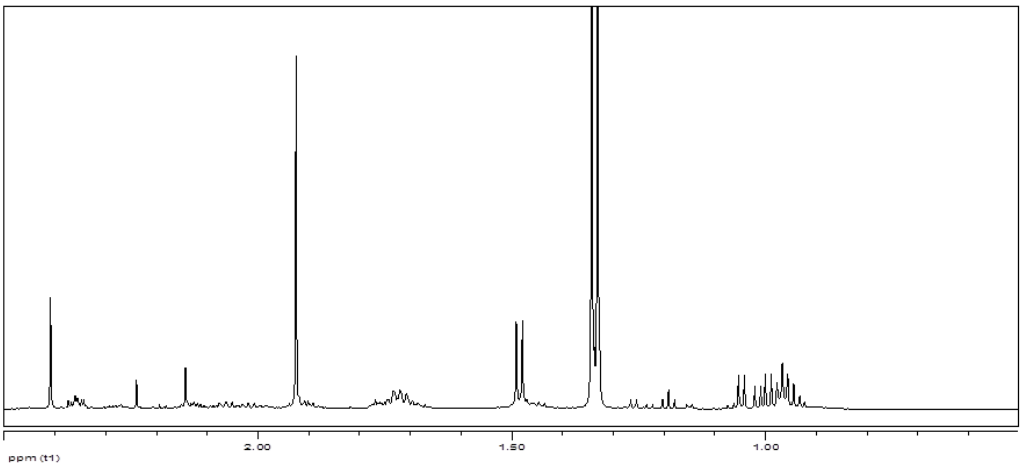

Figure Appendix A8: Atlantic mackerel (*Scomber scombrus*) - Upfield Region (< 2.5 ppm)

Appendix A.9 Atlantic mackerel (*Scomber scombrus*) – T15 0 °C - spectrum ^{1}H-NMR

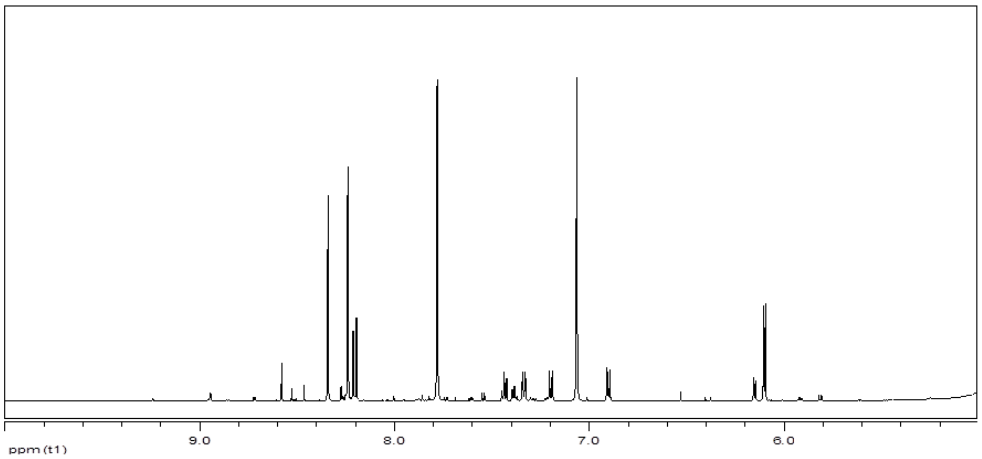

Figure Appendix A9: Atlantic mackerel (*Scomber scombrus*) Downfield Region (> 5 ppm)

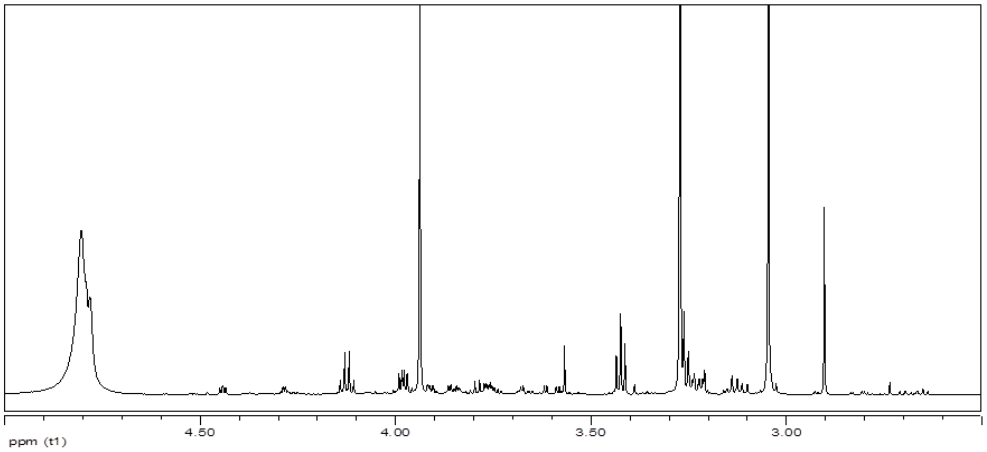

Figure Appendix A9: Atlantic mackerel (*Scomber scombrus*) - Midfield Region (5 < ppm < 2.5)

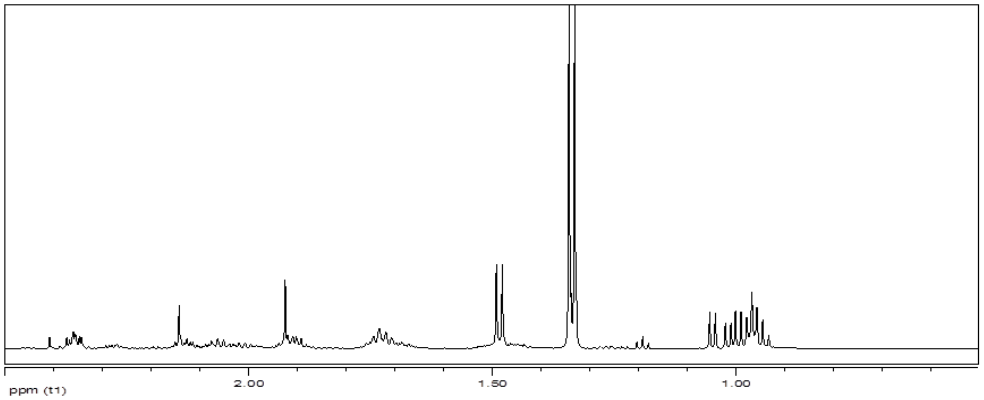

Figure Appendix A9: Atlantic mackerel (*Scomber scombrus*) - Upfield Region (< 2.5 ppm)

Appendix A.10 European seabass (*Dicentrarchus labrax*) "Off shore" cages – T0 - spectrum ^{1}H-NMR

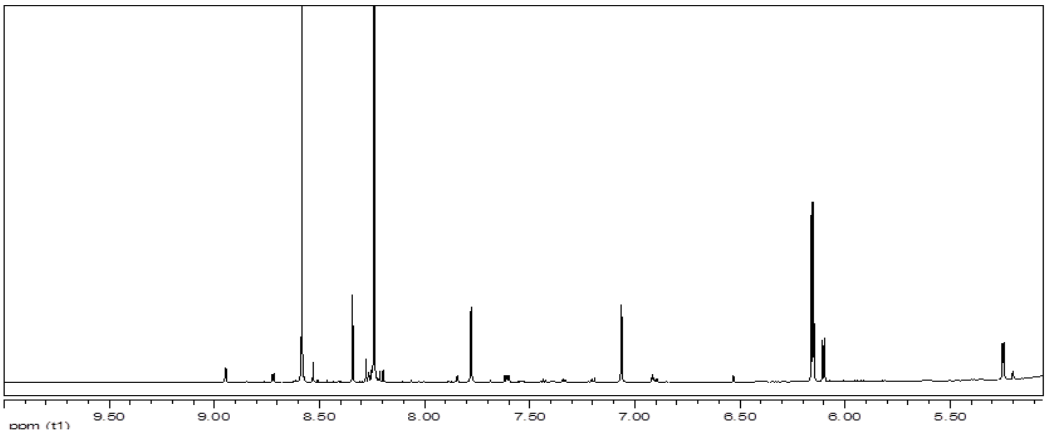

Figure Appendix A10: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Downfield Region (> 5 ppm)

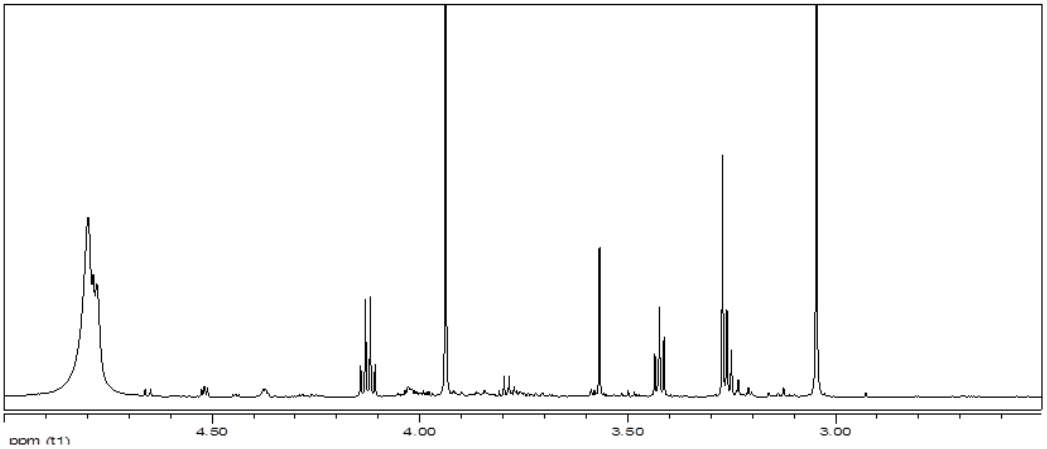

Figure Appendix A10: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

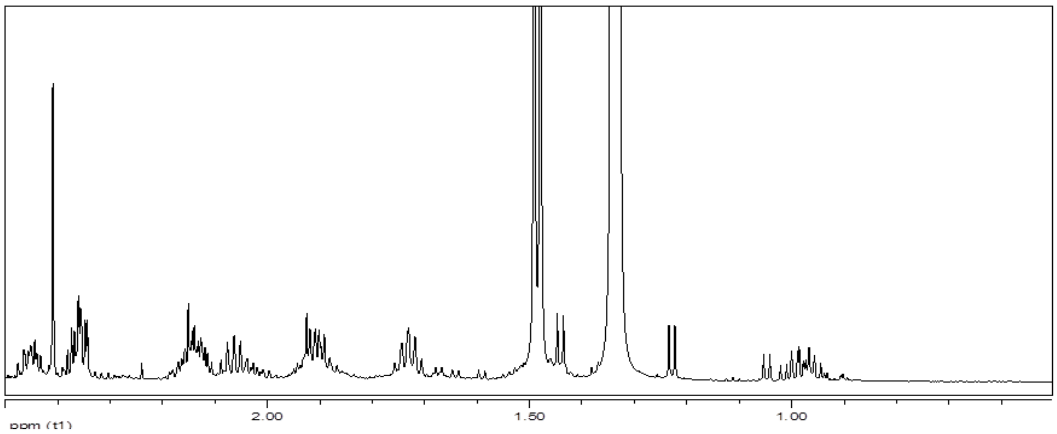

Figure Appendix A10: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.11 European seabass (*Dicentrarchus labrax*) "Off shore" cages – T11 4 °C - spectrum ^{1}H-NMR

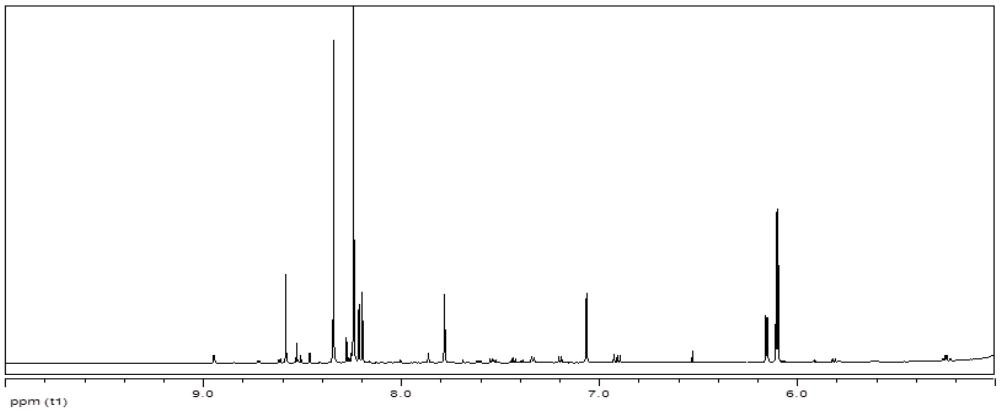

Figure Appendix A11: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Downfield Region (> 5 ppm)

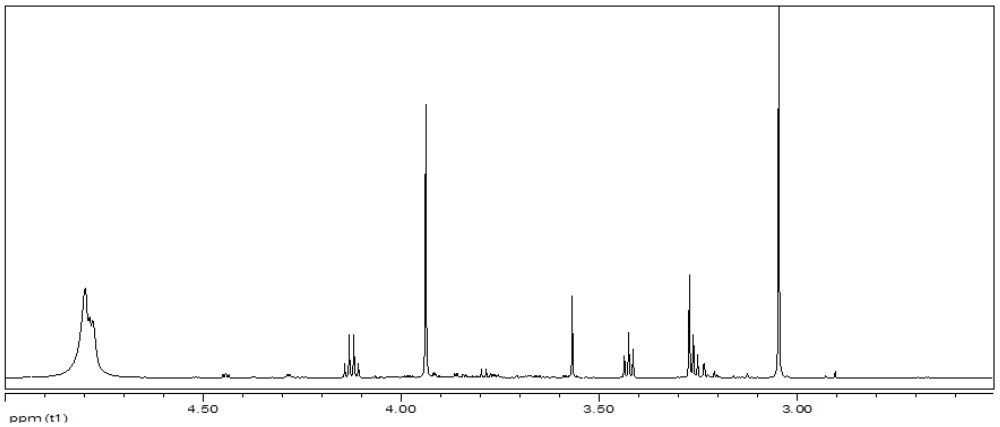

Figure Appendix A11: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

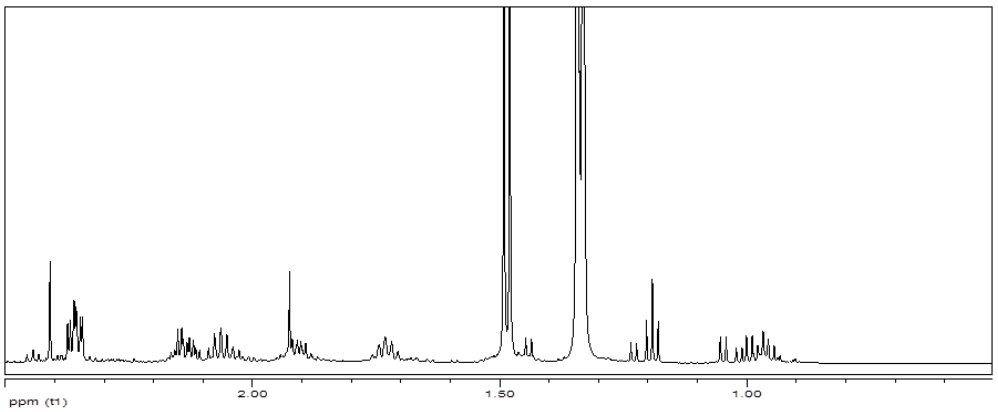

Figure Appendix A11: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.12 European seabass (*Dicentrarchus labrax*) "Off shore" cages – T15 0 °C - spectrum ^{1}H-NMR

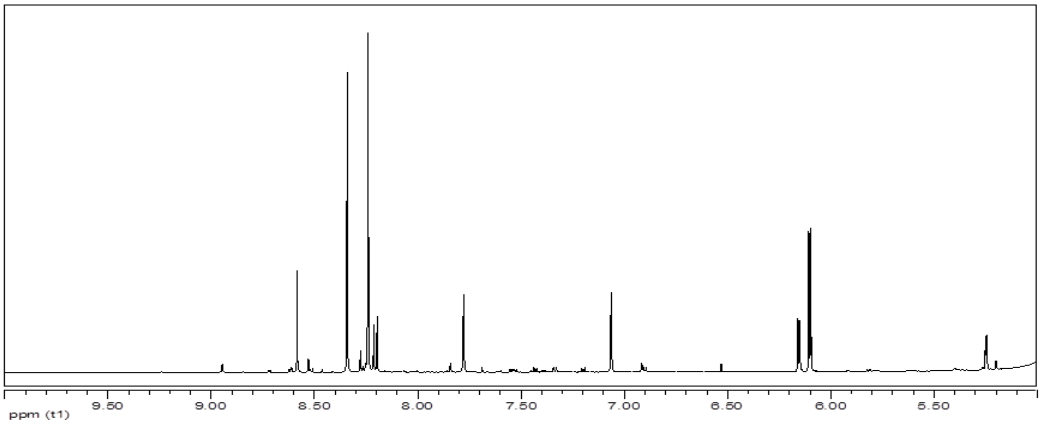

Figure Appendix A12: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Downfield Region (> 5 ppm)

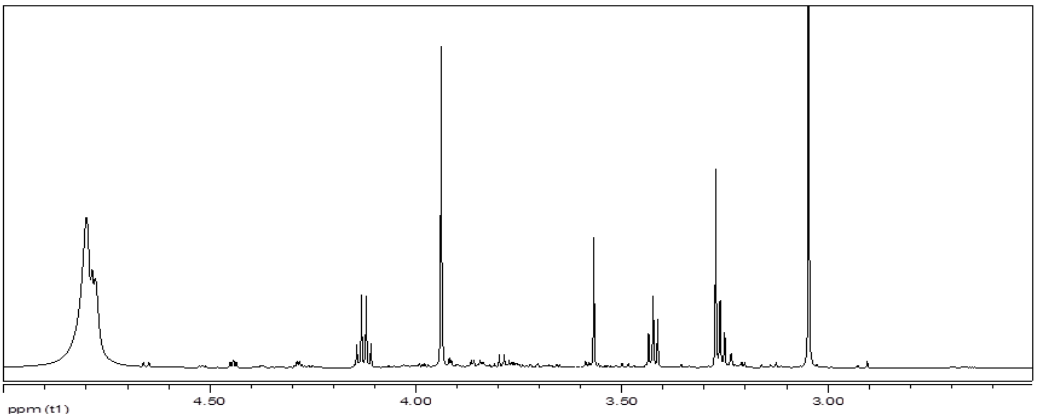

Figure Appendix A12: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

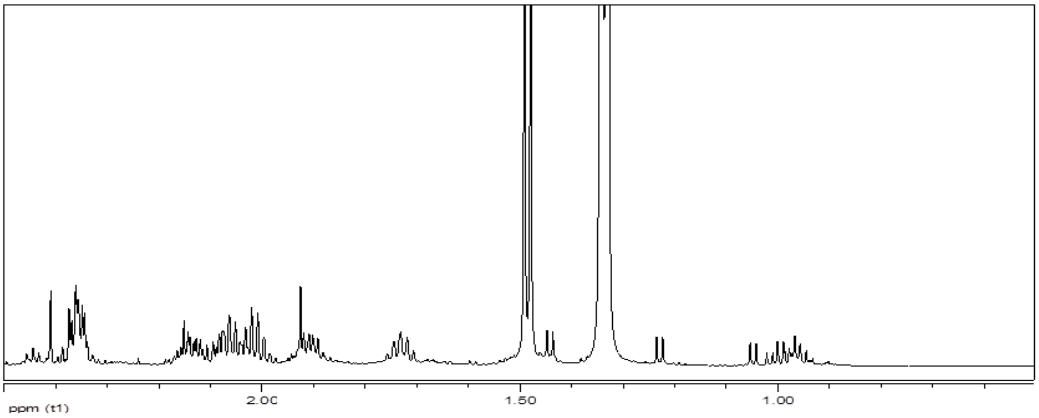

Figure Appendix A12: European seabass (*Dicentrarchus labrax*) "Off shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.13 European seabass (*Dicentrarchus labrax*) "In shore" cages – T0 - spectrum ^{1}H-NMR

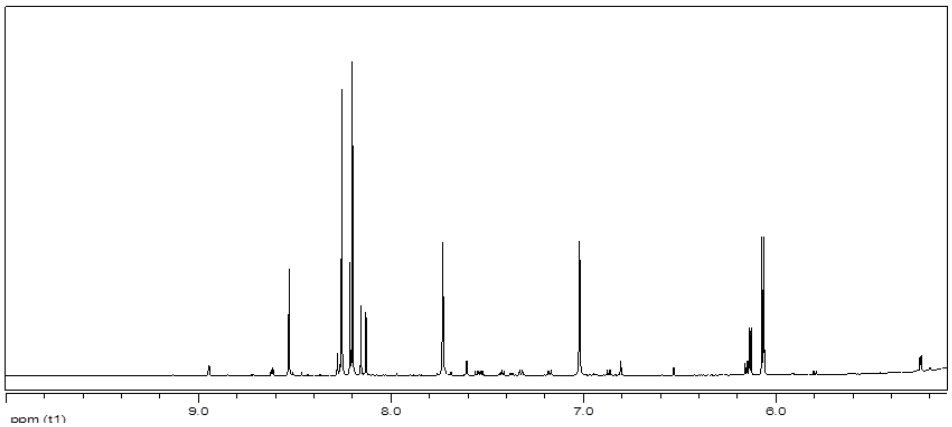

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Downfield Region (> 5 ppm)

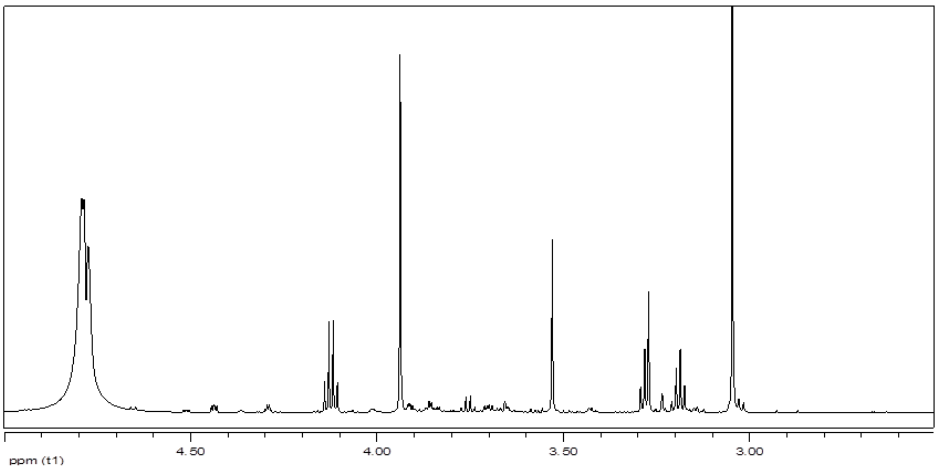

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Midfield Region (5 < ppm < 2.5)

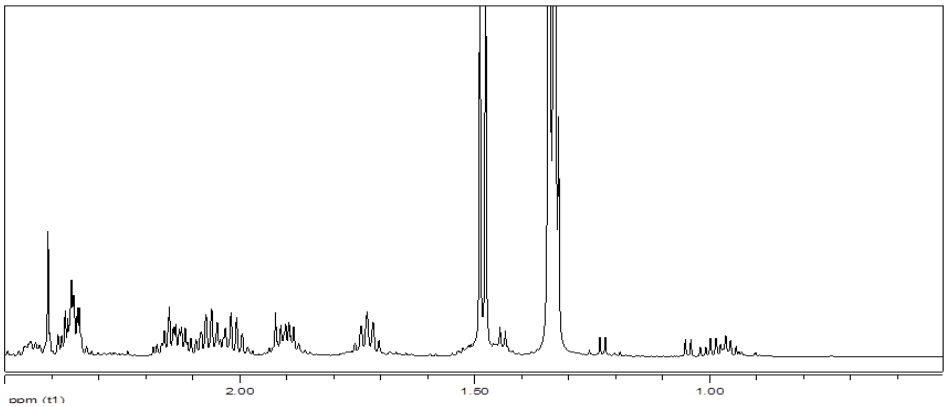

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.13 European seabass (*Dicentrarchus labrax*) "In shore" cages – T11 4 °C - spectrum ^{1}H-NMR

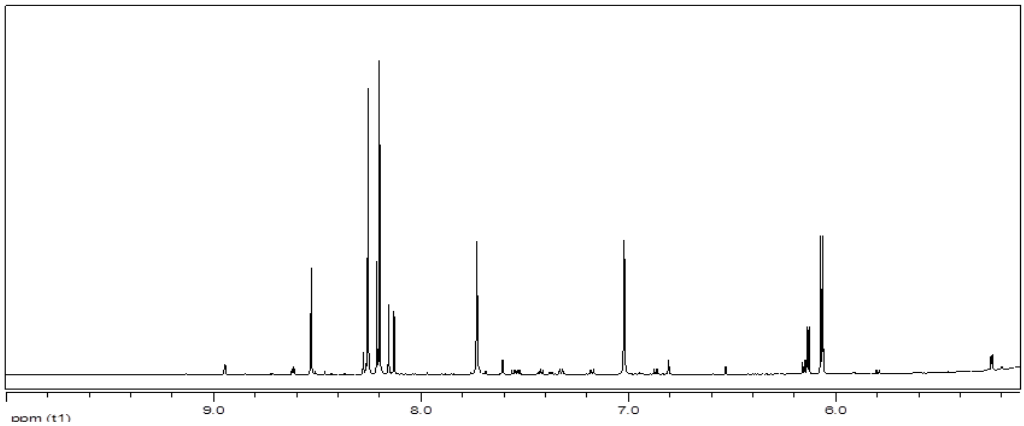

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Downfield Region (> 5 ppm)

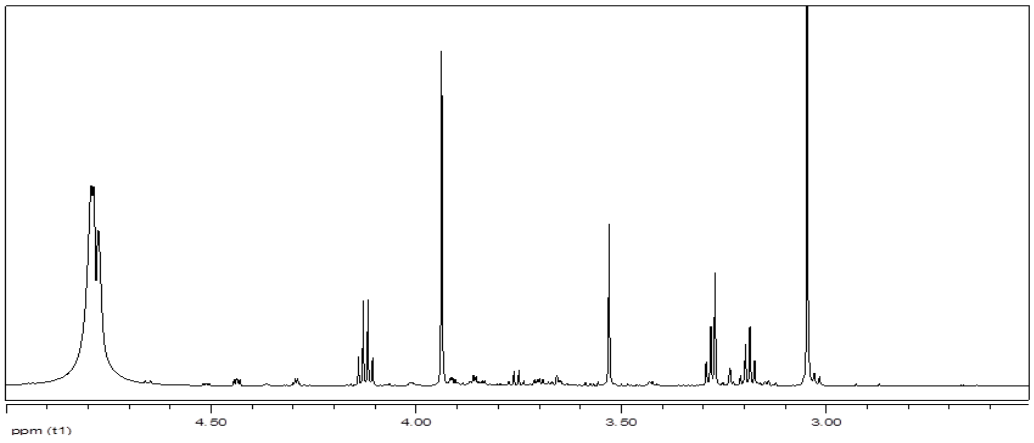

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Midfield Region (5 < ppm < 2.5)

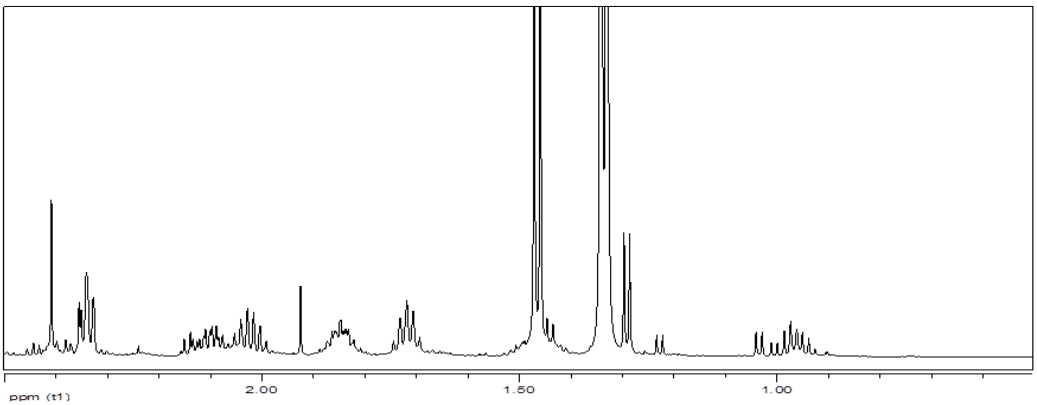

Figure Appendix A13: European seabass (*Dicentrarchus labrax*) "In shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.14 European seabass (*Dicentrarchus labrax*) "In shore" cages – T15 0 °C - spectrum ^{1}H-NMR

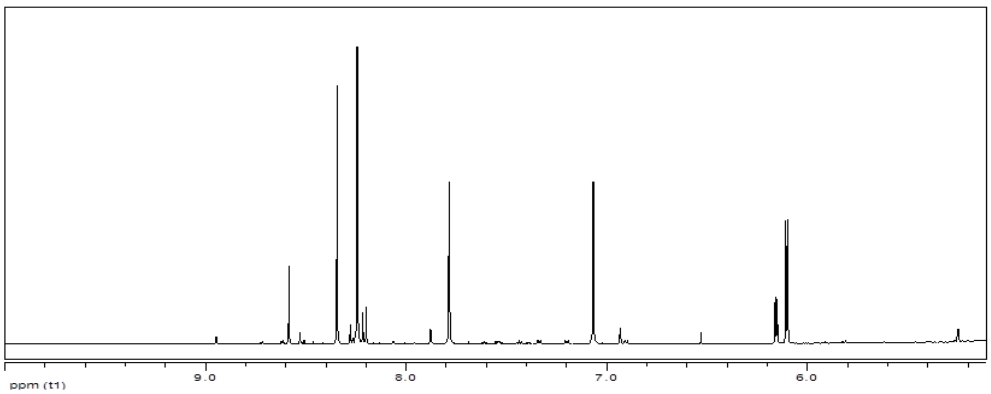

Figure Appendix A14: European seabass (*Dicentrarchus labrax*) "In shore" cages - Downfield Region (> 5 ppm)

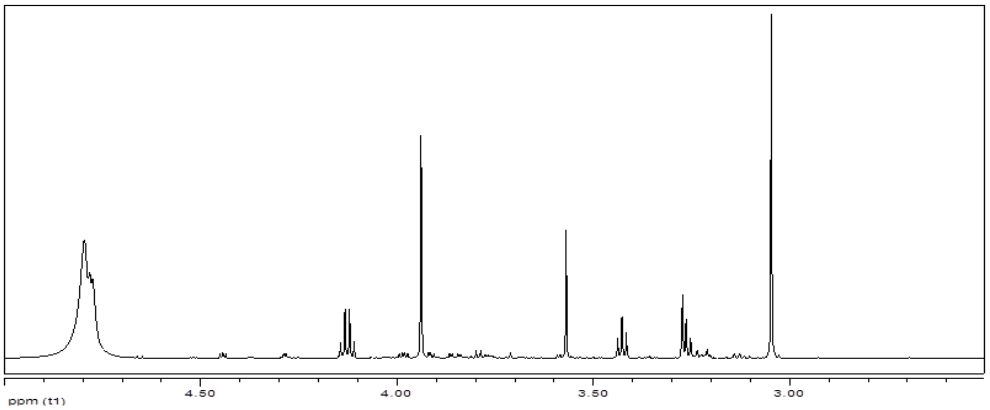

Figure Appendix A14: European seabass (*Dicentrarchus labrax*) "In shore" cages - Midfield Region (5 < ppm < 2.5)

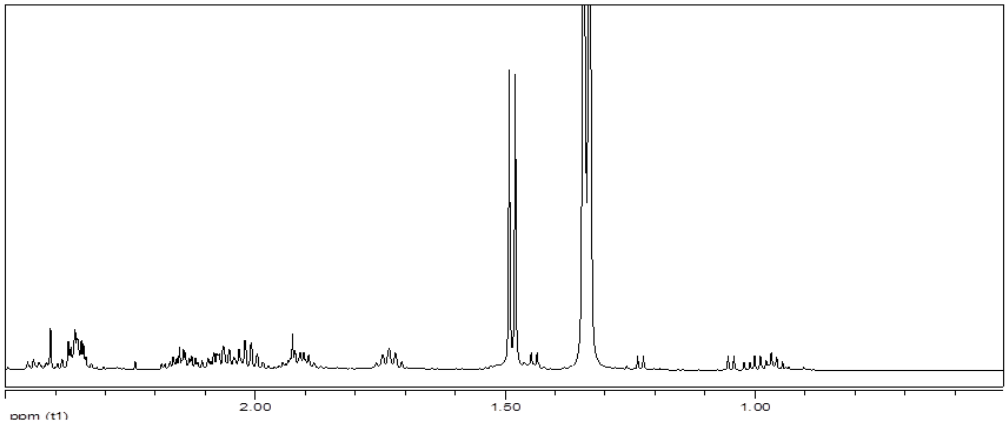

Figure Appendix A14: European seabass (*Dicentrarchus labrax*) "In shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.15 Gilthead seabream (*Sparus aurata*) "Off shore" cages – T0 - spectrum ^{1}H-NMR

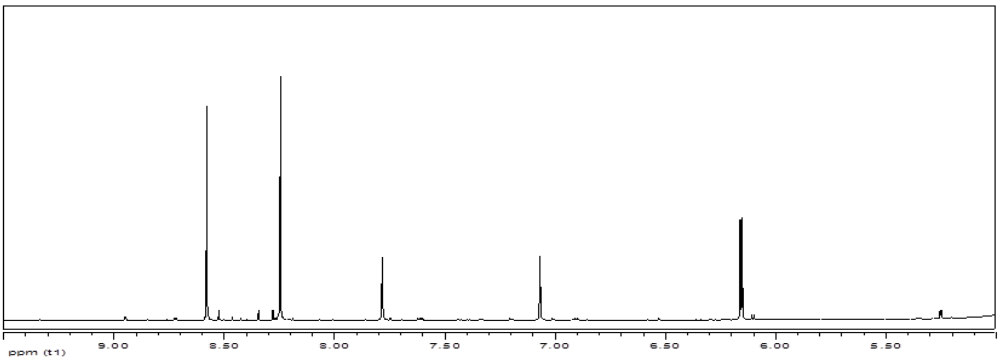

Figure Appendix A15: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Downfield Region (> 5 ppm)

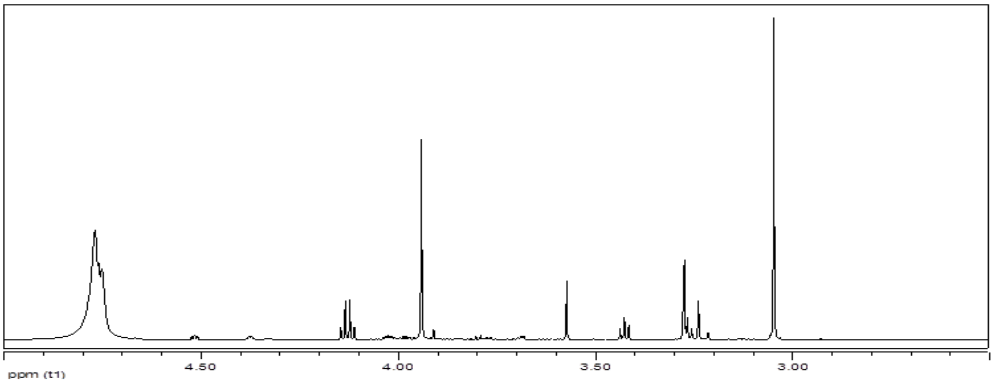

Figure Appendix A15: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

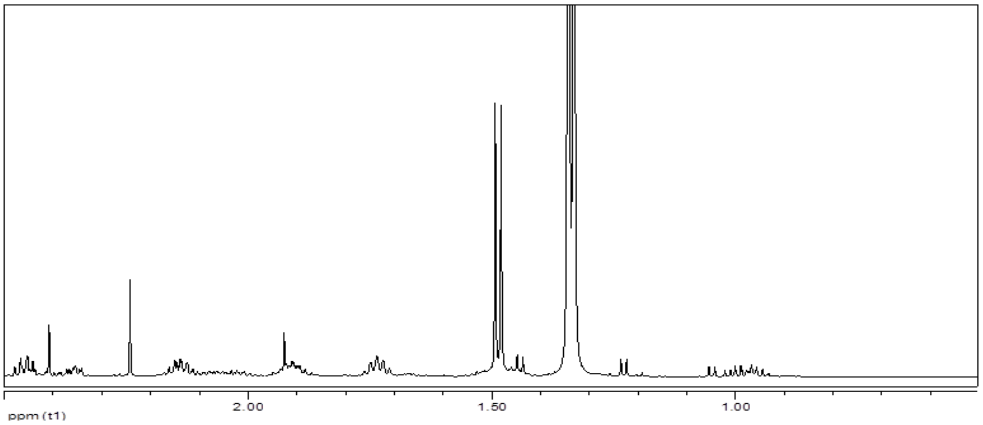

Figure Appendix A15: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.16 Gilthead seabream (*Sparus aurata*) "Off shore" cages – T11 4 °C - spectrum ^{1}H-NMR

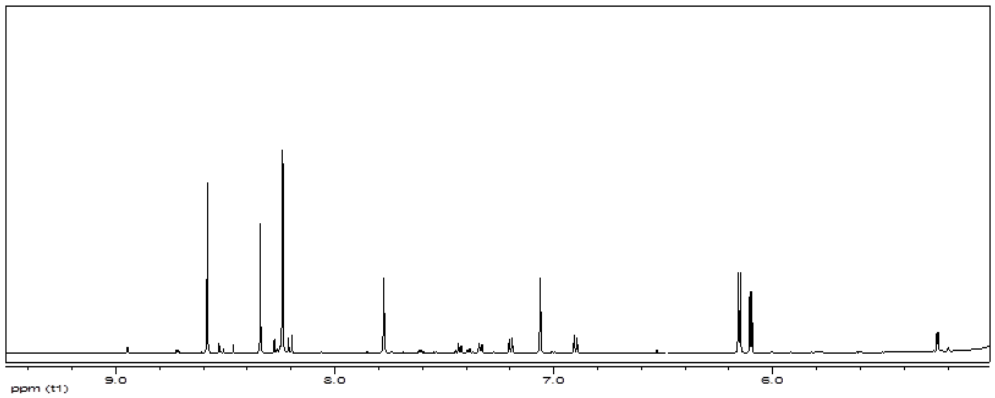

Figure Appendix A16: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Downfield Region (> 5 ppm)

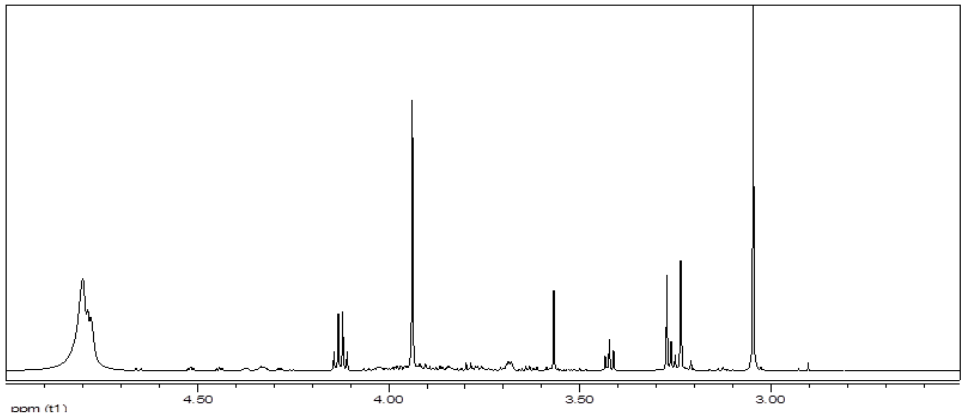

Figure Appendix A16: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

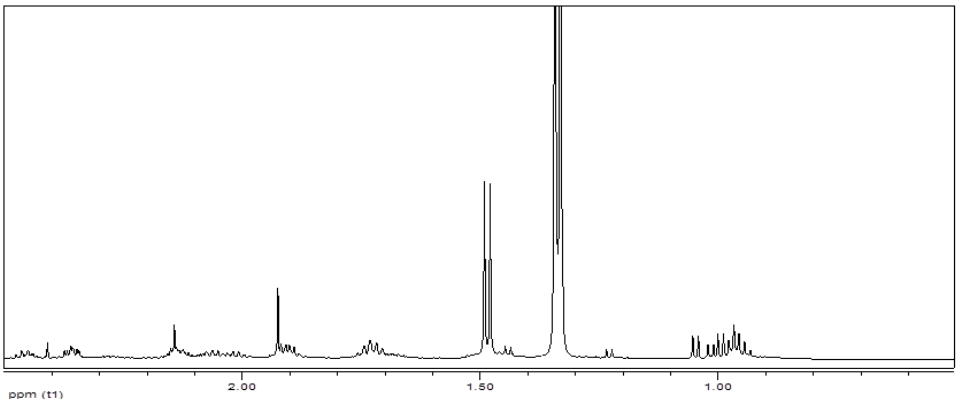

Figure Appendix A16: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Upfield Region (< 2.5 ppm)

Appendix A.17 Gilthead seabream (*Sparus aurata*) “Off shore” cages – T15 0 °C - spectrum ^{1}H-NMR

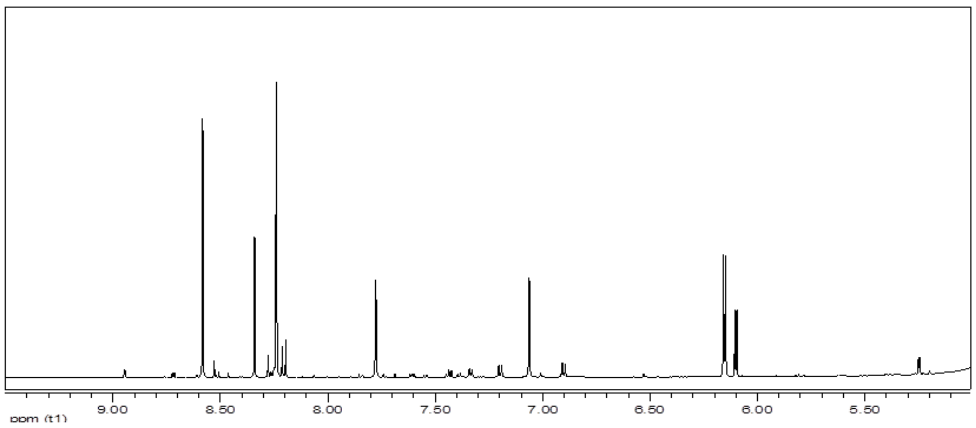

Figure Appendix A17: Gilthead seabream (*Sparus aurata*) “Off shore” cages - Downfield Region (> 5 ppm)

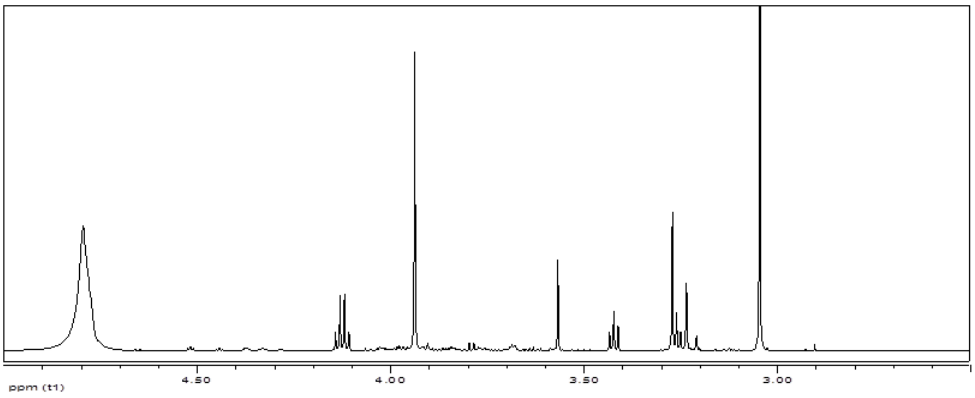

Figure Appendix A17: Gilthead seabream (*Sparus aurata*) “Off shore” cages - Midfield Region (5 < ppm < 2.5)

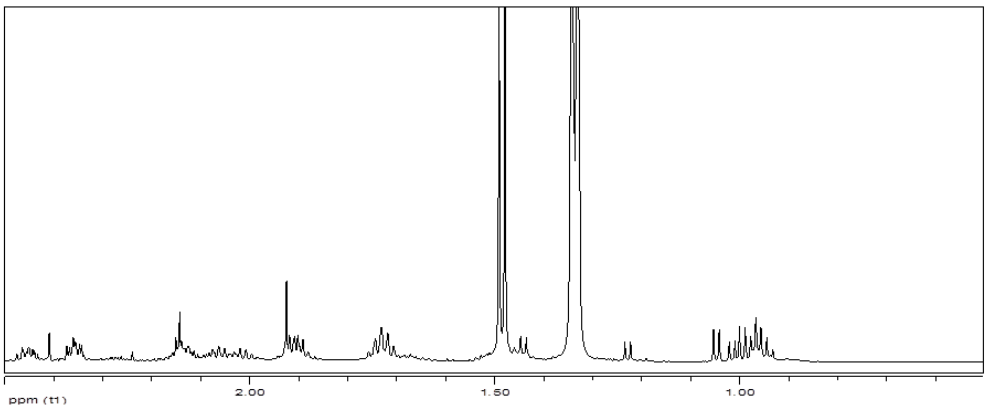

Figure Appendix A17: Gilthead seabream (*Sparus aurata*) “Off shore” cages - Upfield Region (< 2.5 ppm)

Appendix A.18 Gilthead seabream (*Sparus aurata*) "In shore" cages – T0 - spectrum ^{1}H-NMR

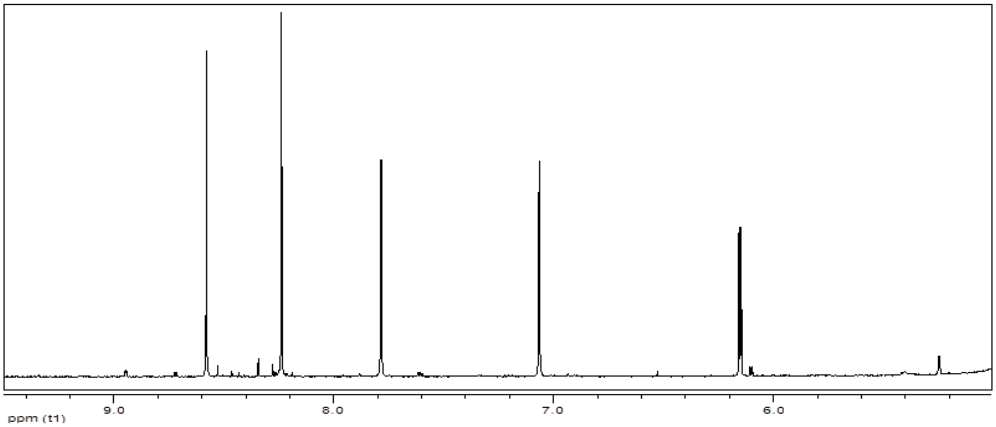

Figure Appendix A18: Gilthead seabream (*Sparus aurata*) "In shore" cages - Downfield Region (> 5 ppm)

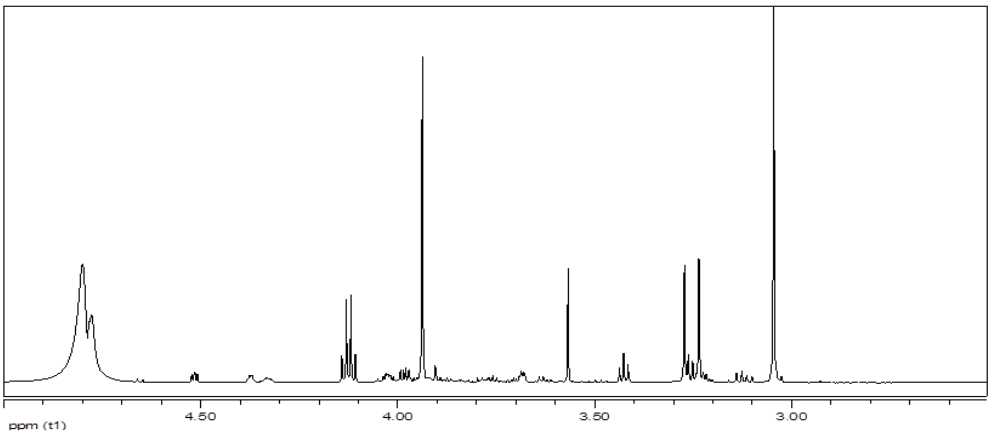

Figure Appendix A18: Gilthead seabream (*Sparus aurata*) "In shore" cages - Midfield Region (5 < ppm < 2.5)

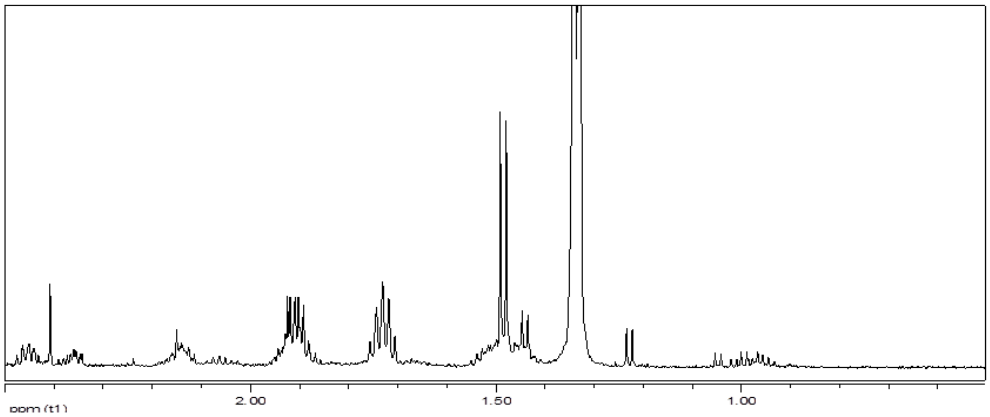

Figure Appendix A18: Gilthead seabream (*Sparus aurata*) "Off shore" cages - Midfield Region (5 < ppm < 2.5)

Appendix A.19 Gilthead seabream (Sparus aurata) “In shore” cages – T11 4 °C - spectrum ^{1}H-NMR

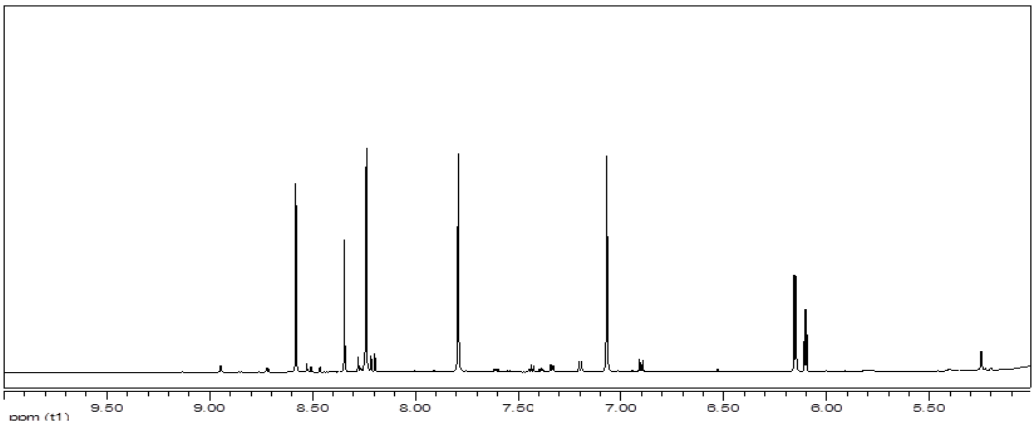

Figure Appendix A19: Gilthead seabream (*Sparus aurata*) “In shore” cages - Downfield Region (> 5 ppm)

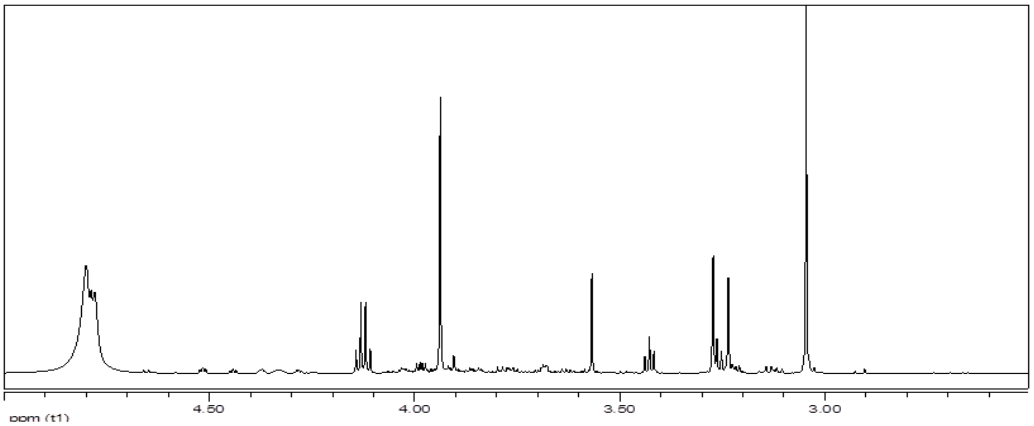

Figure Appendix A19: Gilthead seabream (*Sparus aurata*) “In shore” cages - Midfield Region (5 < ppm < 2.5)

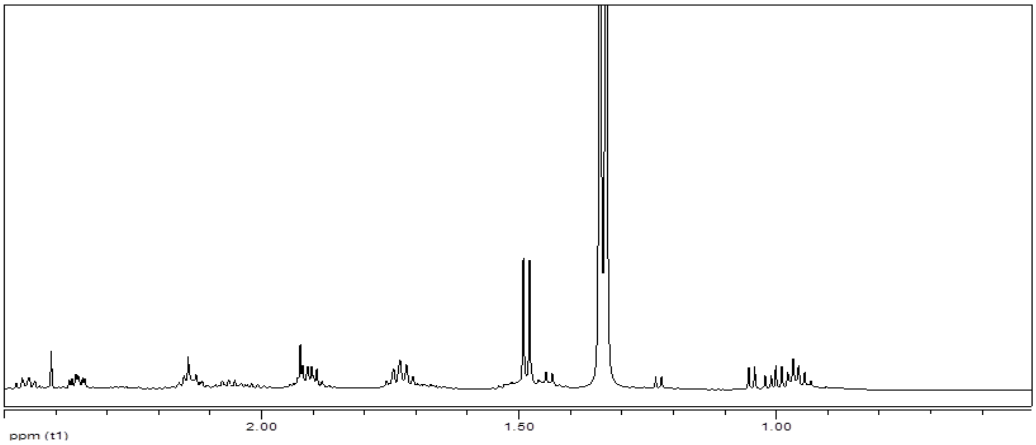

Figure Appendix A19: Gilthead seabream (*Sparus aurata*) “Off shore” cages - Midfield Region (5 < ppm < 2.5)

Appendix A.20 Gilthead seabream (*Sparus aurata*) “In shore” cages – T15 0 °C - spectrum ^{1}H-NMR

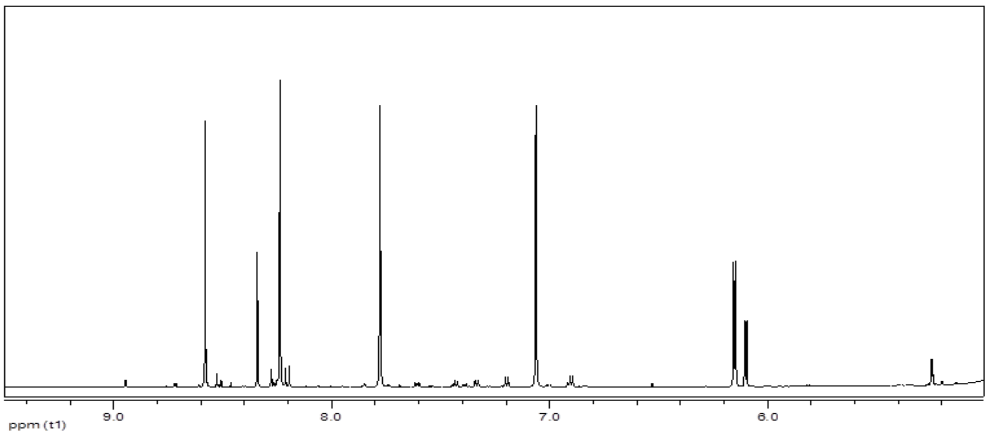

Figure Appendix A20: Gilthead seabream (*Sparus aurata*) “In shore” cages - Downfield Region (> 5 ppm)

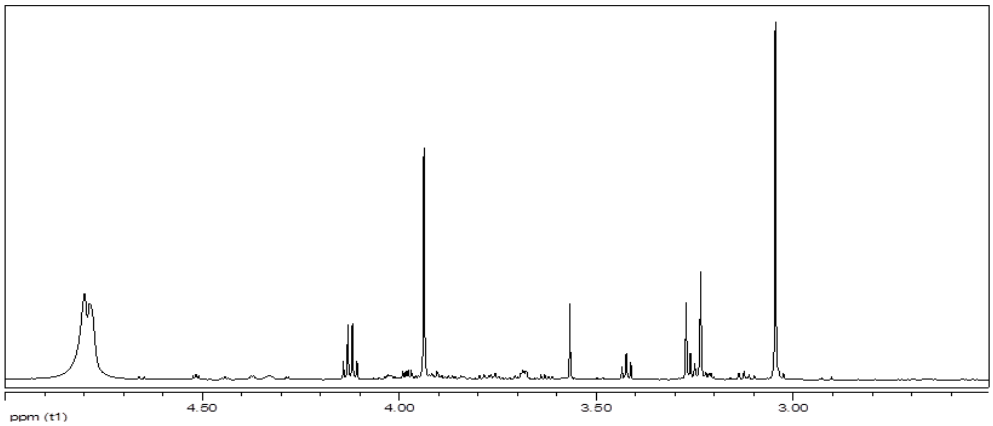

Figure Appendix A20: Gilthead seabream (*Sparus aurata*) “In shore” cages - Midfield Region (5 < ppm < 2.5)

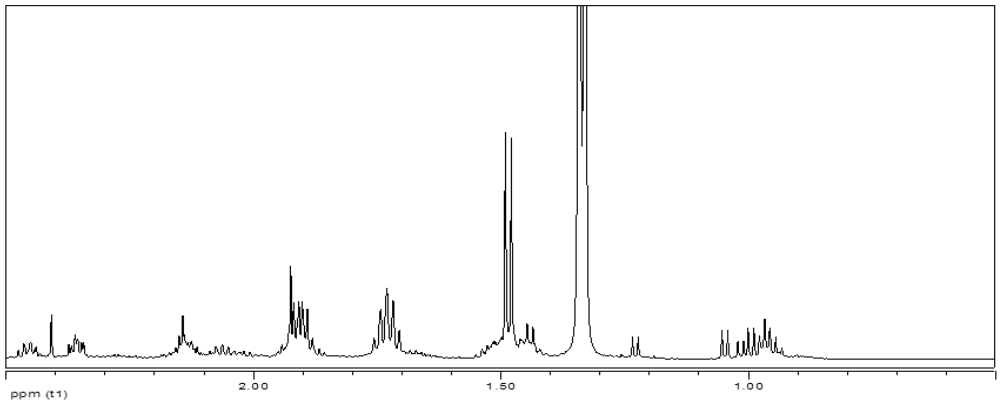

Figure Appendix A20: Gilthead seabream (*Sparus aurata*) “Off shore” cages - Midfield Region (5 < ppm < 2.5)

ACKNOWLEDGEMENTS

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