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NIR SPECTROSCOPY FOR NON-DESTRUCTIVE QUALITY EVALUATION OF FISH

by
Beatriz Alves da Rocha

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Thesis presented to *Escola Superior de Biotecnologia* of the *Universidade Católica Portuguesa* to fulfill the requirements of Master of Science degree in Food Engineering

by
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“You don't choose your family. They are God's gift to you, as you are to them.”

Desmond Tutu

Resumo

A frescura do peixe é considerada um dos parâmetros mais importantes na caracterização da qualidade dos produtos aquáticos. No entanto, esta é inevitavelmente perdida devido ao processo de autólise que, por sua vez, desencadeia o crescimento de microrganismos e, consequentemente, a perda progressiva de qualidade. Este fenómeno é visível através das alterações das características sensoriais tais como a aparência, odor, paladar e textura assim como alterações químicas, bioquímicas e microbiológicas.

O preço de mercado do peixe depende da previsão exata da frescura e do tempo de vida útil. Prever o tempo de armazenamento no gelo define-se como o número de dias que o peixe está armazenado no gelo e é possível utilizar este valor para estimar o tempo de vida útil remanescente. O método do índex de qualidade (QIM) é atualmente o método mais completo e direto para descrever a frescura do peixe. No entanto, é um método lento e subjetivo e que não é exequível para aplicações em grande escala.

A espectroscopia na zona do infravermelho próximo (NIR) provou ser um método rápido e não destrutivo para avaliação dos constituintes do peixe (água, proteína, gordura, …) assim como exibiu bons erros de previsão do tempo de armazenamento. O objetivo desta investigação é testar a possibilidade de utilizar esta técnica para prever de forma não destrutiva níveis de frescura em amostras de solha em Flandres, Bélgica.

No estudo preliminar, análise espectral assistida pela técnica PLSDA foi utilizada em amostras de solha (n=10) sujeitas a diferentes tempos de armazenamento e os resultados indicaram que a espectroscopia na zona do infravermelho próximo tem um grande potencial para a discriminação não destrutiva da frescura da solha. Na etapa seguinte, o estudo principal, realizado no modo de reflectância difusa, em amostras de solha (n=90) classificadas pelo sistema de pontuação QIM na ILVO (Flandres, Bélgica) em conjunto com a técnica PLS, culminou em dois modelos aperfeiçoados para previsão da frescura expressada em dias de armazenamento em gelo (obtidos pela conversão dos pontos QIM): um para as medições na parte da pele escura, com parâmetros de 1.82, 2.22 e 0.804 para RMSECV, RMSEP e R^2_p no intervalo de comprimento de onda entre os 1400 e os 1580 nm; e um segundo para as medições na parte da pele branca, com parâmetros de 2.36, 2.59 e 0.677 para RMSECV, RMSEP e R^2_p utilizando a extensão completa de comprimentos de onda estudados de 940 a 1700 nm.

Palavras-chave: Solha, Espectroscopia de infravermelho próximo, Método do índex de qualidade, Frescura, Qualidade.

Abstract

Fish freshness is regarded as one major parameter for seafood quality. However, it is lost inevitably in practice after catching and fish death, owing to the natural autolysis process which, in turn, trigger the growth of microorganisms and, consequently, the progressive loss of food characteristics and quality. This phenomenon is perceptible by changes in the sensory characteristics such as appearance, odour, taste and texture of fresh fish as well as in chemical, biochemical and microbiological changes.

Fish market prices is highly depended on accurately predict its freshness and shelf-life. Predicted storage time in ice is defined as the number of days that the fish has been stored in ice and it is possible to use these results to estimate the remaining shelf life. Quality Index Method (QIM) is currently the most wholesome and straightforward method of describing freshness. However, it is time consuming and subjective and it is not always suitable for large-scale applications.

NIR spectroscopy has been proven to be a rapidly and non-destructive method for evaluating fish components (moisture, protein, fat, …) as well as it has shown good predictions errors associated with fish storage time prediction. The purpose of this research is to test the possibility of using NIR spectroscopy for non-destructively predicting freshness levels of plaice fish in Flanders, Belgium.

In the preliminary study, spectroscopic measurements were performed on tested plaice samples (n=10) subjected to different storage times assisted with Partial Least Squares Discriminant Analysis (PLSDA) indicated that NIR spectroscopy had great potential for non-destructive plaice freshness discrimination. In the next step, the main study employing NIR diffuse reflectance measurements for plaice samples (n=90) graded using commercial QIM scoring method at ILVO (Flanders, Belgium) together with Partial Least Squares (PLS) regression culminated on two different calibration models for predicting freshness expressed as storage days in ice (converted from the graded QIM scores): one for dark skin measurements with prediction performances of 1.82, 2.22 and 0.804 for RMSECV, RMSEP and R^2_p, respectively, using the selected wavelength range of 1400 to 1580 nm; and one for white skin measurements with those parameters of 2.356, 2.59 and 0.677 for RMSECV, RMSEP and R^2_p, respectively, using the full wavelength range studied of 940 to 1700 nm.

Keywords: Plaice; NIR spectroscopy, QIM, Freshness, Quality

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Contents

I. Introduction

Quality analysis and evaluation of fish and other seafood products is a significant subject of study (Cheng & Sun, 2014) due to its microorganism vulnerability and perishability (Cheng *et al.*, 2013) but also due to its important role in human diet (Liu *et al.*, 2013).

Fish quality is not a straightforward concept and involves a whole range of factors which vary accordingly to the markets in which the fish is traded. Quality factors include the expected physical attributes such as species, size and product type, as well as subtler elements such as seasonal condition, the effects of capture method, and ethical factors, such as assurances of the fisheries sustainability and the protection of marine mammals. However, besides these factors, fish freshness is considered as the most important attribute of fish quality (Luten *et al.*, 2003).

In several occasions, fish is priced accordingly to its freshness which is usually based on sensory analysis. Although it is one of the most important attributes related to fish quality, it is not a single attribute itself and it can be measured by different analytical methods (sensory, chemical, physical and bacteriological analysis) (Chebet, 2010).

Traditional methods and techniques for measuring fish quality are tedious, laborious, expensive, time-consuming, destructive, and often need trained personnel and produce waste (analysed products, chemicals …), which makes them unsuitable for on-line detection and/or large-scale operations. (Cheng & Sun, 2014). Further information on these techniques can be found in Appendix 1. Additionally, traditional microbiological and chemical methods are not advisable to be employed in the early stages of fish deterioration because they are more sensitive in the latter phases. Therefore, highly sensitive, non-destructive, inexpensive, precise and rapid methods are required to be developed for fish freshness assessment (Dowlati *et al.*, 2013). In that sense, a new platform technology, spectroscopy technology using the Visible (VIS) and Near infrared (NIR) wavelength range has been studied as a non-invasive tool for quality assessment of aquatic products.

1. Fish constituents

1.1 Composition

It is well known that fish products comprise an important part of balanced and nutritional diets specially owing to its constituents, such as high-quality and digestible animal proteins, vitamins, minerals and valuable fatty acids (Cheng & Sun, 2014). The main chemical components of fish are water, protein and lipids which make up about 98% of the total mass.

Other components such as carbohydrates, vitamins and minerals while in minor quantity, play a significant role in the biochemical dynamics of live fish (Venugopal & Shahidi, 1996).

The chemical composition of fish varies accordingly with the species, age, sex, size, feeding habits, geographic location, season, microbiological load and methods of catching (Huss, 1995).

1.1.1 Water

The major constituent of fish is water, accounting for about 66-81% of the weight of a fresh fish fillet (Liu *et al.*, 2013). Some species are described to have water content between the extremes of 30 and 90% (Murray & Burt, 1983).

The percentage of water is related with the percentage of lipids since normally both constitute around 80% of the fillet (Huss, 1995).

1.1.2 Lipids

In terms of lipid content, it is possible to discriminate three categories: lean, medium fatty and fatty species (Venugopal & Shahidi, 1996). The first group, lean or low-fat species, includes fish species that store lipids only in the liver, like cod and hake (Huss, 1995), and contain less than 2% (w/w) lipid (Venugopal & Shahidi, 1996). The second group, medium or moderate fat species, consists of fish which store lipids in limited parts of their body tissues or in lower quantities than fatty species (Huss, 1995) and present lipid contents between 2-5% (Venugopal & Shahidi, 1996). Finally, the third group, corresponds to fish storing lipids in fat cells distributed in other body tissues, which is the case of herring and mackerel (Huss, 1995) that comprehends more than 5% of fat (Venugopal & Shahidi, 1996).

Marine fish lipid includes triacylglycerols, phospholipids, sterols, wax esters, some glyceryl esters, glycolipids, sulfolipids and hydrocarbons. Triacylglycerols presents more variation than others and phospholipids show less (Venugopal & Shahidi, 1996).

It is known that fish is highly rich in unsaturated fatty acids, like tetradecane acid, palmitoleic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Rodriguez-Casado *et al*., 2007).

1.1.1 Proteins

Protein of fish muscle is rich in essential amino acids, has a high biological value and can be easily digested (Rehbein & Oehlenschläger, 2009). There are three major classes of fish

proteins: structural proteins which represent 70 to 80% of the total protein content; sarcoplasmic proteins which embodies 25 to 30% of the proteins; and finally, connective tissue proteins, constituting approximately 3% of the protein in teleostei and about 10% in elasmobranchii (Huss, 1995).

The first group, that includes actin, myosin, tropomyosin and actomyosin, is responsible for the structure and the rheological characteristics of the muscle tissue, usually denoted as tenderness/ toughness and coarseness/ fibrousness (Dunajski, 1980).

Most of the sarcoplasmic proteins are enzymes which participates in the cell metabolism. It was suggested to use sarcoplasmic proteins as a method for differentiating fresh from frozen fish. This proposal was based on the assumption that the composition of the sarcoplasmic protein fraction changed when the organelles were broken upon freezing which sometimes is not the case (Huss, 1995).

The connective tissue proteins form a supporting network through the whole fish muscle that is evenly distributed and plays a crucial role in textural properties (Kiessling *et al.*, 2006).

1.1.2 Nitrogen compounds from non-protein nature

These class can be defined as water soluble, low molecular weight, nitrogen-containing compounds of non-protein nature. The principal elements in this fraction are: volatile bases (for example, ammonia), trimethylamine oxide (TMAO), creatine, free amino-acids, nucleotides and purine bases (Huss, 1995).

The most important constituent of the amines is TMAO, which upon reduction contributes to fishy odours (Venugopal & Shahidi, 1996). This component is found in all marine fish species in quantities ranging from 1 to 5% of the muscle tissue (dry weight) but is virtually absent from freshwater species (Huss, 1995).

1.1.3 Vitamins and minerals

The vitamin contents in fish can vary considerably with species, age, size, sex, season, diet, state of health and geographic location. In fish farmed by aquaculture, the contents of vitamins reflect the composition of the corresponding components in the fish feed and, therefore, vitamin content will be different from wild fish (Rehbein & Oehlenschläger, 2009).

Fishes are rich in fat-soluble vitamins (A, D, E and K) and also in vitamin B (riboflavin - vitamin B_2, niacin – vitamin B_3, pantothenic acid – vitamin B_5, vitamin B_6, biotin – vitamin B_7, folic acid – vitamin B_9 and vitamin B_{12}) (Rehbein & Oehlenschläger, 2009).

Concerning minerals contents, fish tissue is regarded as a valuable source of calcium and phosphorus but also of iron, copper and selenium. Iodine can likewise be found in saltwater fish, contrary to sodium which content is very low in fish (Huss, 1995). The regular intake of selenium and iodine through food for human is of great importance because population in many European countries is not sufficiently supplied with these elements and the insufficient intake can lead to goitre and other diseases (Rehbein & Oehlenschläger, 2009).

1.1.4 Carbohydrates

Carbohydrates content in fish muscle is very low and it is usually present in striated muscle in the form of glycogen (Huss, 1995).

2. *Post mortem* alterations in fish

Immediately after death, many biochemical reactions start in the fish, which are of utmost importance for quality and shelf life of seafood products. These reactions depend on several different factors: fish species, physiological conditions of the fish, the environmental influences to which the living fish had been exposed, as well as, catching and harvesting methods, killing procedures and slaughtering performance (Rehbein & Oehlenschläger, 2009).

Even during the catching process, the concentration of ammonium ions in the fish increases and glycogen storages are reduced. Afterwards, when the fish is death, anaerobic glycolysis continues, leading to increasing the concentration of L-lactate with a simultaneous decrease in the pH value. The concentration of creatine phosphate and adenosine triphosphate (ATP) decreases, and when the concentration of ATP is no longer sufficient to remove the connection between thick (myosin) and thin (actin) filaments of the myofibrils, the onset of *rigor mortis* starts (Rehbein & Oehlenschläger, 2009). By this time, the fish texture becomes harder, a phenomenon that can last for a day or two (Luten *et al.*, 2003).

Fishes' freezing directly after catch standstills most of the enzymatic reactions. However, during thawing, glycolysis, proteolysis, lipolysis and other enzymatic reactions continue and may result in quality losses (Rehbein & Oehlenschläger, 2009).

The resolution of *rigor mortis* causes the muscle relaxation and the texture smoothing (Luten *et al.*, 2003) due to the action of endogenous proteases. Although the meat became smoother it will never reach the same elasticity as before *rigor*. Several proteolytic systems are involved in the degradation of the structural proteins of fish muscle including acid cathepsins

located in lysosomes, alkaline proteinases, proteasomes (multi-catalytic proteinase complexes), calpains, aminopeptidases, collagenases and elastases (Rehbein & Oehlenschläger, 2009).

During storage in melting ice or at higher temperatures in the refrigerator, enzymes are gradually released such as mitochondrial enzymes which are involves in ATP degradation and the increase of calcium ions (Ca^{2+}) in the sarcoplasm (Rehbein & Oehlenschläger, 2009).

As the muscle tissue of living marine fish is sterile, the quality of fish stored in melting ice is initially mainly influenced by autolytic reactions. Later (±10 days after catch), bacterial reduction of trimethylamine oxide (TMAO) to trimethylamine (TMA) starts, a process that dominates during further storage of the fish or fillet (Rehbein & Oehlenschläger, 2009).

Immediately after the catch, the muscle tissue of fish is free from bacteria except for the gills, skin and intestines. The bacteria penetrate the fillet mainly through the gills and body cavity, accompanied by changes in the composition of the bacterial flora and leading to the formation of biogenic amines (Rehbein & Oehlenschläger, 2009).

As lipids contained in fish are not stable during storage, lipolysis and lipid oxidation may occur even in frozen fish, at a lower rate. These processes led to the development of unpleasant flavours and tastes due to the carbonyl compounds and short-chain carbonic acids formed. Moreover, the binding of free fatty acids to fish muscle proteins results in texture deterioration (Rehbein & Oehlenschläger, 2009).

2.1 Sensorial alterations

Fish flesh deteriorates rapidly, even at refrigeration temperatures, and produces off-odours and off-flavours, which have been described by many researchers. Spoiled odours and flavours, which are developed as spoilage proceeds, lead to seafood rejection by the consumers. (Pearson & Dutson, 1994).

The first sensory changes of fish during storage are concerned with appearance and texture. The characteristic taste of the species is normally developed in the first couple days during storage in ice (Huss, 1995).

According to Huss (1995) there is a characteristic pattern of the deterioration of fish stored in ice and is divided into the following four phases (Figure 1):

- Phase 1 (approximately 2 days) where the fish is very fresh and has a sweet, seaweedy and delicate taste;
- Phase 2 (approximately 4 days) where there is a loss of the characteristic odour and taste. The flesh becomes neutral but has no off-flavours and the texture is still pleasant;

- Phase 3 (approximately 6 days) when appears some sign of spoilage and a range of volatile, unpleasant-smelling substances is produced depending on the fish species and type of spoilage (aerobic, anaerobic). At the beginning of the phase the off-flavour may be slightly sour, fruity and slightly bitter, especially in fatty fish. In the end, sickly sweet, cabbage-like, ammoniacal, sulphurous and rancid smells develop. The texture becomes either soft and watery or tough and dry.
- Phase 4 when the fish can be characterized as spoiled and putrid.

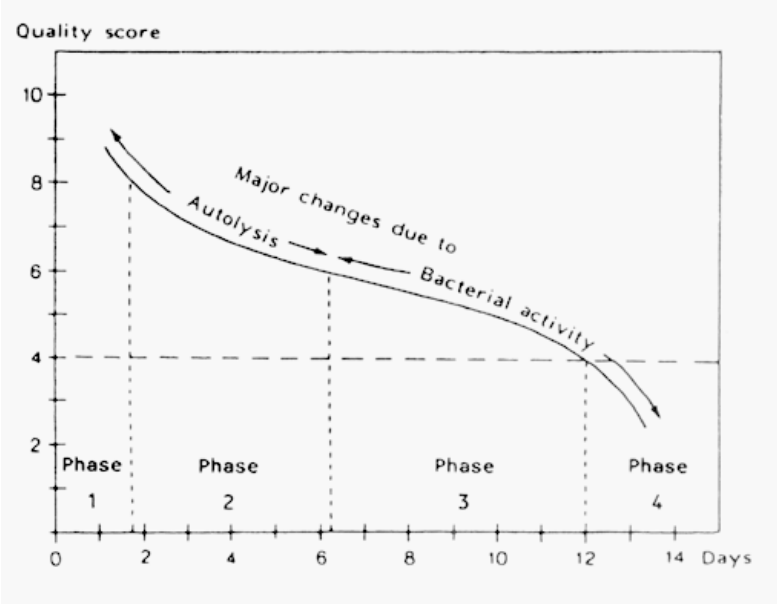


Figure 1 - Changes in the eating quality of iced (0ºC) cod (Huss, 1995).

According to Council Regulation (EC) No 2406/96 of 26 November 1996 laying down common marketing standards for certain fishery products there are three categories of freshness: Extra; A category and B category. In Extra category, fish must be free of pressure marks, injuries, blemishes and bad discoloration. In A category, fishes are required to be free of blemishes and bad discoloration but slight pressure marks and superficial injuries are still tolerated. In the last category, it is still required the absence of blemishes and bad discoloration but then again small proportion of fishes with more serious pressure marks and superficial injuries are endured. Fish presented in market is not admitted to have dull pigmentation or in a more advance stay of decay.

QIM Eurofish have estimated the shelf life of some fish species stored in ice and it fluctuates between 6 up to 20 days – Annex 1 (Martinsdóttir *et al.*, 2001).

2.2 Physic and chemical alterations

After death, fish flesh experiences *post mortem* changes due to a certain number of biochemical and physicochemical reactions like anaerobic glycolysis, pH changes, degradation of ATP, *rigor mortis*, autolysis and spoilage (Cheng *et al.*, 2015).

2.2.1 pH

Post mortem glycolysis results in the accumulation of lactic acid which in turn lowers the pH of the muscle. The amount of lactic acid produced is related to the amount of stored carbohydrate (glycogen) in the living tissue. In general, fish muscle contains a relatively low level of glycogen and thus a smaller amount of lactic acid is generated after death. Also, the nutritional status of the fish and the amount of stress and exercise encountered before death will have a dramatic effect on the levels of stored glycogen and consequently on the ultimate *post mortem* pH (Huss, 1995).

The natural pH of live fish is just above 7.0, typically about 7.3, but in most species, falls till the range of 6.0-6.8 (Rehbein & Oehlenschläger, 2009).

The *post mortem* reduction in the pH of fish muscle influences the physical properties of the muscle. As the pH drops, the net surface charge on the muscle proteins is reduced, causing them to partially denature and lose some of their water-holding capacity. Loss of water has a detrimental effect on the texture of fish flesh and it has been shown that there is an inverse association between muscle toughness and pH, unacceptable levels of toughness (and water-loss on cooking) occurring at lower pH levels (Huss, 1995).

After one week in ice storage, an increase in the pH value is verified due to the production of alkaline bacterial metabolites in spoiling fish which coincide with the increase in Total Volatile Basic Nitrogen (TVBN) (Abbas *et al.*, 2008).

2.2.2 Nucleotide catabolism

Nucleotides are the 5'-phosphate esters of nucleosides. The most important nucleotide in all living organisms is adenosine 5'-triphosphate (ATP), which consist of the nucleoside adenosine linked to three phosphate groups. ATP functions as the universal carrier of energy, transferring energy from chemical bonds to endergonic reactions within the cell (Rehbein & Oehlenschläger, 2009).

Although the fishing operation causes the death of the animal within minutes, the muscles continue to metabolise while ATP is present (Pearson & Dutson, 1994). In the *post*

mortem process, the decomposition of ATP generate some metabolites: ADP – adenosine diphosphate, AMP – adenosine monophosphate, IMP – inosine monophosphate, HxR - inosine, Hx – hypoxanthine, Xa – xanthine and uric acid (Cheng *et al.*, 2015).

The autolysis of ATP in the muscle begins soon after death and is enhanced by high temperature, rough handling, *pre-mortem* struggling and failure to bleed. ATP is rapidly degraded to IMP by endogenous enzymes and further breakdown to HxR and Hx (Özogul *et al.*, 2005a). The sequence of nucleotide degradation in teleost fish muscle is as follows: ATP → ADP → AMP → IMP →HxR → Hx (Wills *et al.*, 2004). The breakdown of IMP to Hx is slow and it is caused by both autolytic and microbial enzymes (Chebet, 2010).

The presence of IMP and HxR is associated with the desirable sweet flavour in fresh fish but the presence of Hx usually characterizes the fish flavour as undesirable and bitter (Chebet, 2010). The measurement of ATP and its breakdown concentrations is therefore believed to be considered as an indicator of fish freshness changes (Özogul *et al.*, 2005a).

2.2.3 Volatile compounds

Volatile compounds (related to nitrogen, amine, ammonia, alcohols, sulphur-containing compounds, and others) are one of the vital parameters of fish freshness determination and they are the result of microbial activity and endogenous enzymes decompositions (Cheng *et al.*, 2015). These compounds can be divided into three categories according to the origin of volatile compounds during fish storage: 1) fresh fish odour, mainly related to C_6 – C_9 alcohols and carbonyl compounds; 2) microbial spoilage odour, related to ammonia, trimethylamine (TMA), hydrogen sulphide and methyl mercaptan; and 3) lipid oxidation odour, mainly related to hexaldehyde and 2,4,7-decatrienal (Olafsdottir *et al*., 2004).

Fish odours are complex and each species has a characteristic aroma for which the concentration of the determined compounds and their odour thresholds are important factors. These compounds are derived from polyunsaturated fatty acids that are susceptible to autoxidation during prolonged storage (Rehbein & Oehlenschläger, 2009).

Ammonia and TMA are the predominantly basic compounds present in spoiling fish and are responsible for the strong ammoniacal and characteristic fishy odour (Huss, 1995). Extensive microbiological investigations have shown that TMA is formed by reduction of trimethylamine oxide (TMAO) by some species in the bacteriological flora of spoiling fish (Rehbein & Oehlenschläger, 2009). The TMAO reduction is mainly associated with the typical bacteria of the marine environment (*Alteromonas, Photobacterium, Vibrio* and *S. putrefaciens*),

but is also carried out by *Aeromonas* and intestinal bacteria of the *Enterobacteriaceae* (Huss, 1995).

Volatile sulphur compounds are common components of spoiling fish and most bacteria identified as specific spoilage bacteria produce one or several volatile sulphides. *S. putrefaciens* and some *Vibrionaceae* produce H_2S from the sulphur containing in amino acid l-cyteine. Methylmercaptan (CH_3SH) and dimethyl sulphide ($(CH_3)_2S$) are both formed from another sulphur containing amino acid, methionine (Huss, 1995). The volatile sulphur compounds are very foul-smelling and can be detected even at ppb levels, so even minimal quantities have a considerable effect on quality (Huss, 1995).

2.2.4 Biogenic amines

Biogenic amines are basic nitrogenous compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. Biogenic amines are organic bases of low molecular mass and according to the chemical structure can either be aliphatic (putrescine, cadaverine, spermine, spermidine), aromatic (tyramine, β-phenylethylamine) or heterocyclic (histamine, tryptamine) (Rehbein & Oehlenschläger, 2009).

Most of the biogenic amines present in fish are the result of the action of exogenous enzymes released by microorganisms associated with the seafood products. Histamine, putrescine, cadaverine, tyramine, tryptamine, β-phenylethylamine, spermine and spermidine are considered the most relevant biogenic amines in foods, although the last three are not the end products of bacterial decomposition in fishery products (Rehbein & Oehlenschläger, 2009).

Endogenous decarboxylase enzymes naturally occurring in fish or shellfish tissue may also contribute to the production of biogenic amines but this pathway is insignificant compared with exogenous production (Rehbein & Oehlenschläger, 2009).

2.2.5 Protein and free amino acids

Proteins account for almost 20% of fish and are an important nutritional component of fish flesh. However, proteins are also susceptible to decomposition by unsound handling and processing and by microbial and enzymatic activities. Therefore, after fish have been caugth, it is normally stored in low temperature or in ice to slow down microbial growth and proliferation and to reduce the enzyme activities and, subsequently, maintaining its freshness. Inevitably, the structure and fishes' properties undergo some changes during storage, thus impacting the taste, flavour, nutritional and commercial values (Cheng *et al.*, 2015).

Many proteases have been isolated from fish muscle and the effects of proteolytic breakdown are often related to extensive softening of the tissue. The low molecular weight peptides and free amino acids produced by the autolysis of proteins not only lower the commercial acceptability of pelagics, but in some cases, autolysis has been shown to accelerate the growth of spoilage bacteria by providing a superior growth environment for such organisms (Rehbein & Oehlenschläger, 2009).

Several proteolytic systems, consisting of enzymes and inhibitors, are involved in the degradation of structural proteins of fish muscle: acid cathepsins located in lysosomes, alkaline proteinases, proteasomes, calpains, aminopeptidases, collagenases and elastases. Autolytic enzymes are activated due to the pH drop and the increase of calcium ions in the sarcoplasm, causing an increase in tenderness of the muscle (Rehbein & Oehlenschläger, 2009).

2.2.6 Lipids

As stated before, fish is rich in unsaturated fatty acids, including tetradecane acid, palmitoleic acid, eicosapentaenoic acid and docosahexaenoic acid, which are susceptible to oxidation (Rodriguez-Casado *et al*, 2007).

There are two major distinct reactions in fish lipids for quality deterioration: oxidation and hydrolysis. They both result in the production of diverse substances some with unpleasant (rancid) taste and smell and others which may contribute to texture changes by binding to fish muscle proteins (Huss, 1995).

On the other hand, lipid oxidation can be divided into three types: autoxidation, photo oxidation and enzymatic oxidation. Autoxidation takes place when the unsaturated fatty acids are exposed to oxygen and proceeds through an autocatalytic chain reaction. Free radicals are formed when hydrogen ions are extracted from the fatty acids (Nollet, 2012). Successively, free radicals react with oxygen to produce fatty acids peroxides. The fatty acid peroxides are free radicals which can attack another lipid molecule, resulting in a peroxide and a new free radical. The primary product of lipid oxidation is the fatty acid hydroperoxide, measured as peroxide value (PV) (Özogul *et al.*, 2005b). Peroxides are not stable compounds and they break down into low molecular weight substances such as aldehydes, ketones and carboxylic acid groups originating smell, texture, colour and nutritional changes of fish (Trocino *et al.*, 2012).

Fatty fish, such as sardine, are particularly sensitive to oxidation during storage, giving rise to rancidity due to the fat content and the presence of highly unsaturated fatty acids. The smaller the fish size and higher the surface area/volume ratio, the faster the deterioration

(Rodriguez-Casado *et al*, 2007). Therefore, lipid oxidation can be used to evaluate fish freshness (Cheng *et al.*, 2015). PV and thiobarbituric values (TBA) are the major chemical indices of oxidative rancidity. TBA consists mainly of malondialdehyde as a representative of aldehydes (Özogul *et al.*, 2005b).

The lipid hydrolysis occurs by the action of endogenous enzymes, especially digestive enzymes and, therefore, this phenomenon in more profound in gutted fish. The formed compounds, the free amino acids, do not affect directly sensorial properties although could confer a little soap flavour (Huss, 1995).

2.3 Microbiological alterations

Foods of muscle origin, as in the case of fish, are sensitive to contamination and provide the growth of microorganisms involved in spoilage and foodborne illness. In unprocessed products, microorganisms multiply rapidly, particularly at non-refrigeration temperatures, resulting in quality losses and/or public health problems (Pearson & Dutson, 1994).

Fish contain microorganisms in the skin, gills and intestines and the extent of this bacterial contamination depends on fish quality and the sanitation prevailing during fishing, processing and storage. Usually, contamination is higher on fish from warmer waters and from those originating in areas of untreated human waste disposal. Likewise, the place of origin is a relevant factor in the initial type of contamination present on fresh seafood. Cold water fish are generally contaminated with gram-negative psychrotophs (e.g. *Pseudomonas, Moraxella, Shewanella, Acinetobacter, Flavobacterium, Aeromonas, Cytophaga* and *Vibrio*) while fish from the tropics are mostly contaminated with Gram-positive mesophiles (e.g. *Micrococcus* and *Bacillus* spp.) (Pearson & Dutson, 1994).

According to Huss (1995) the proportion of microorganisms on the surface and gills/guts of fish is 10^2-10^7 cfu (colony forming units)/cm^2 and between 10^3-10^9 cfu/g respectively. It may seem multitudinous but the strong defensive mechanism of live fish, prevents fish spoilage. Only a part of spoilage microflora participates in the spoilage process, the microorganisms include in this fraction are known as a specific spoilage organism (SSO). The qualitative ability to produce off-flavour (spoilage potential) and the quantitative ability to produce spoilage metabolites (spoilage activity) are essential in the identification of a SSO (Gram & Dalgaard, 2002).

Following fishes' death, microorganisms or enzymes are free to invade or diffuse into the flesh where they react with the complex mixture of natural substances present (Özogul *et al.*, 2005a).

In overall, the growth of specific bacteria and the accumulation of their metabolic products constitute the major spoilage changes in fish during storage. Essentially, freshly caught fish are usually characterised sensorially by "fresh fish flavours" (sweet, seaweedy). Later, during storage, it is reached a period where the odours and flavours are described as neutral or non-specific. This is the first indications of off-flavours that will progressively become more pronounced and lastly turn the fish unacceptable for consumption (Chebet, 2010).

3. NIR spectroscopy

3.1 Visible and near infrared light

In 1873, James Clerk Maxwell proposed that visible light consists of electromagnetic waves. Electromagnetic radiation is the emission and transmission of energy in the form of electromagnetic waves. Figure 2 shows various types of electromagnetic radiation, which differ from one another in wavelength and frequency (Chang, 2009).

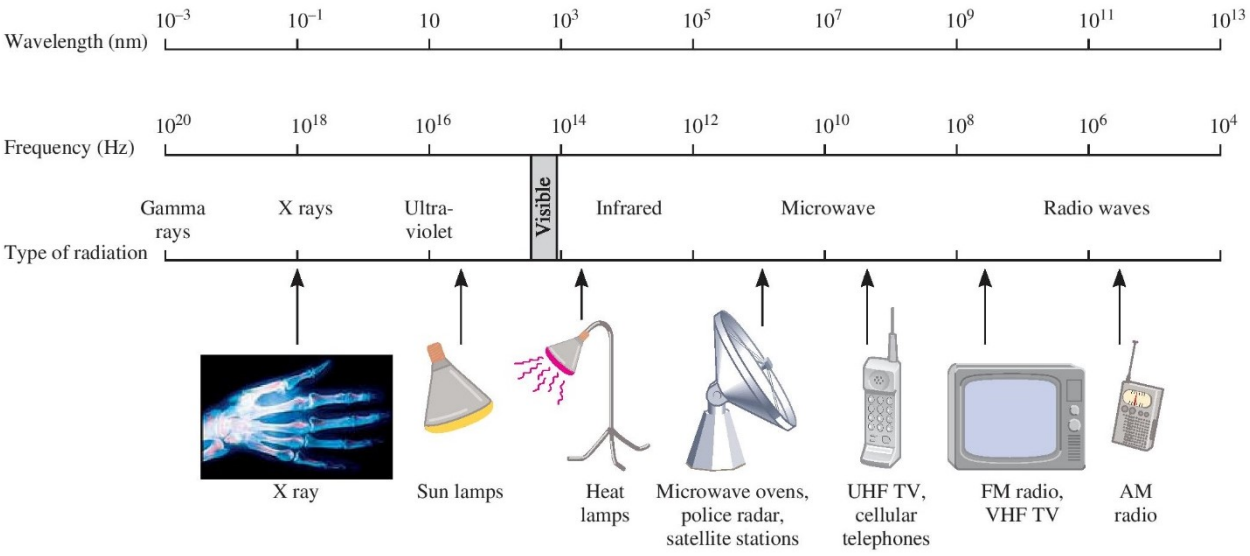


Figure 2 - Types of electromagnetic radiation (Chang, 2009).

Optical radiation covers the wavelength range of 100 nm -1000 µm of the electromagnetic spectrum. It is subdivided into the ultraviolet (UV) region from 100 to 380 nm,

the visible (VIS) light ranging from 380 to 780 nm, and the infrared (IR) radiation of wavelengths above 780 nm (Porep *et al.*, 2015).

The near-infrared (NIR) window defines the range of wavelengths from 780 - 2526 nm according to America Society for Testing and Materials (ASTM) and can be divided in two regions: short wave near infrared spectral region (SW-NIR) of 780-1100 nm and long wave near infrared region (LW-NIR) of 1100-2526 nm.

3.2 Interactions between light and biological materials

The rationale for the development of a spectroscopy-based measurement system as a tool for non-destructive food quality analysis is based on the physical understanding of the interaction of light photons with the molecular structure of food samples (Sun, 2010). The most common interactions between NIR light and biological samples are absorption and scattering.

Different parts of the electromagnetic spectrum have very different effects upon interaction with matter. Spectroscopy is the study of electromagnetic radiation and matter's interaction involving radiation absorption, emission or scattering (Pérez-Juste & Faza, 2015).

3.2.1 Light absorption

A beam of light (one wavelength) passing through a medium containing absorbing molecules transfers its energy to the molecules as it proceeds, and thus decreases progressively in intensity. This absorption process is described by the Beer-Lambert law which states that absorbance or optical density of the sample (A) equals the molar extinction coefficient or molar absorption coefficient (ε) times the concentration of the sample (C) (Parson, 2007). An important corollary of the Beer-Lambert law is that the absorbance of a mixture of noninteracting molecules is just the sum of the absorbances of the individual components (Parson, 2007).

NIR radiation absorption bands are related with *overtones* and *combinations* of fundamental vibrations. *Overtones* occur when there are transitions between energy levels that differ by two or more vibrational quantum number units (Siesler *et al.*, 2002). In other words, a molecular vibration absorption takes place when it is excited by a radiation with a specific frequency or its multiples. On the other hand, the phenomenon known as *combination* describes the situation when several group vibration absorptions happen at the same time and that unexpected absorptions bands can appear as well as overlapping of different absorptions resulting in broader peaks (Saeys, 2006).

Overtones and *combinations* of fundamental vibration contains feature information from the chemical bonds (such as O-H, N-H, C-H and C-O, etc.) of organic molecules (Cheng *et al.*, 2013). Since all biological substances contain numerous molecular bonds of C–H, O–H, and N–H, the exposure of this sample to NIR radiation results in a complex spectrum that contains qualitative and quantitative information about the physical and chemical properties of that sample (Sun, 2010).

3.2.2 Light scattering

Scattering of light is common in NIR spectroscopy due to internal heterogeneities in refractive index of biological samples. This refractive index heterogeneity results in multiple changes in light propagating directions when penetrating biological samples, known as scattering phenomena. Shorter wavelengths are scattered much more than the longer ones (Rayleigh scattering) (Flammer *et al.*, 2013). Different materials and even different states of the same material have different refractive index and these parameters are crucial to determine the angle of deviation of the incident light beam that moves from one medium to the other. (Saeys, 2006)

The light that come in contact with the matter is dispersed into a wide range of angles (Siesler *et al.*, 2002). Light that is scattered by more than a certain angle with respect to the incident beam misses the detector and is registered as an apparent absorbance (Parson, 2007).

3.3 Principles of NIR spectroscopy

Near Infrared (NIR) spectroscopy is a molecule-vibration measurement technique which offers a straightforward, rapid, and cost-effective alternative to traditional analytical methods for food quality evaluation (Sivertsen *et al.*, 2011). Spectroscopic methods provide internal information of the biological sample to be analysed using physical characteristics of the interaction between electromagnetic radiation and the sample material (Sun, 2010).

NIR Spectra can be recorded in diffuse reflection, transmission, and transflection modes and can provide complex information (compositional and structural) of the measured samples thanks to the relation of the vibrational behaviour of molecular bonds such as C-H, O-H and N-H of the sample composition (Liu *et al.*, 2013). The SW-NIR light (780-1100 nm) can penetrate relatively deep into biological materials thanks to the lower absorption property at this region, which makes NIR a good spectral region for performing non-destructive measurements on thick or bulky biological samples (ElMasry & Nakauchi, 2016; Liu *et al.*, 2013).

In diffuse reflection, both the light source and the detector are placed at the same side relative to the sample. Detectors combined with an integrating sphere or mounted under an angle of 45ºC are usually used to collect signals (Saeys, 2006).

Diffuse transmission mode is characterized by the measurement of the light that passes straight (collimated transmittance) as well as the light that has been scattered (diffuse transmittance) through the sample. In this mode, the sample is positioned between the light source and the detector (Saeys, 2006).

The final mode, transflectance, is a combination of the two previously mentioned. The sample, detectors and light source are arranged like in the case of diffuse reflection. Then a highly reflective material is placed in contact with the sample on the other side of the illumination. The light goes through the sample after being partially absorbed, scattered and changed. After reaching the far side of the sample it is sent back through the sample to the illumination side thanks to the highly reflective material and then being again absorbed, scattered and changed. The detector will measure this diffuse transflected light coming out of the sample. This mode is ideal for situations where the other approaches do not obtain enough signal (Saeys, 2006).

3.4 Seafood applications

Preliminary studies and implementations of NIR technology for at-line, on-line, in-line and off-line applications have been reported in order to facilitate food quality control, traceability, authentication, and production optimization (Weeranantanaphan *et al.*, 2011).

Many studies based on NIR spectroscopy has confirmed its ability to predict the main chemical components such as water, protein, fat and moisture in fish (Khodabux *et al.*, 2007; Liu *et al.*, 2013; Huang *et al.*, 2003; ElMasry & Wold, 2008; Folkestad *et al.*, 2008; Herrero, 2008). It is also proven to provide useful information on quality parameters such as freshness and detection of nematodes (Rehbein & Oehlenschläger, 2009). Visible/near infrared (VIS/NIR) spectroscopy have been also positively evaluated for differentiation between thawed and frozen red sea bream (Uddin *et al.*, 2005) and horse mackerel (Uddin & Okazaki, 2004); for freshness assessment of cod (Chau *et al.*, 2009; Nilsen *et al.*, 2002; Sivertsen *et al.*, 2011); for QIM (Nilsen and Esaiassen, 2005) and volatile compounds quantification (Armenta *et al.*, 2006; Armenta *et al.,* 2006).

Recently, near infrared hyperspectral imaging system was studied for measuring colour distribution in salmon fillets. The results showed that both computer vision and hyperspectral

imaging had potential in fish colour measurement in a rapid and non-invasive way (Wu et al., 2012b; Dowlati *et al.,* 2013). He *et al*. (2012, 2014) also proved that Vis-NIR hyperspectral imaging technique can rapidly and non-destructively predict pH value and its spatial distribution with good accuracy in salmon fillets.

Meanwhile, VIS/NIR spectroscopy was applied in texture analysis of farmed Atlantic salmon and the results confirmed that this technique offered fair predictions of Kramer shear force (Coppes et al., 2002). However, measuring texture in whole fish is still challenging due to inhomogeneous structure (density of muscle fibre, contents of fat and collagen) and difficulty in preparation of standard size of fish samples.

Lin *et al*. (2006) showed that NIR spectroscopy could correctly classify rainbow trout samples as fresh or spoiled after several days of storage at 4ºC as well as produce models to quantify the number of bacteria present. In the field of foreign contaminant detection, new methods have been developed to overcome the disadvantages of human vision inspection such as multispectral imaging in the VIS/NIR region for automatic detection of parasites (Wold *et al.*, 2001).

3.5 NIR spectroscopy – Multivariate evaluation

Although being suitable for off-, in- and on-line applications for quality control and process monitoring (potential to be used both as a small handheld device and as an online instrument for non-contact measurements on a conveyer belt during production) (Sivertsen *et al.*, 2011). NIR is based on indirect measurements and almost impossible to interpret with unaided eye and therefore, it requires calibration with mathematical and statistical tools (chemometrics) to extract analytical information (Porep *et al.*, 2015).

NIR bands are usually broad and severely overlapped resulting in strong multicollinearity which means further analysis of the spectrum is needed in order to extract analytical information (Porep *et al.*, 2015). To overcome this phenomenon, pre-treatment approaches such as noise reduction (e.g. Savitzky-Golay smoothing method), baseline correction (e.g. derivatives methods, Multiplicative Scatter Correction – MSC, Orthogonal Signal Correction – OSC, Standard Normal Variate – SNV), centering, normalisation and resolution enhancement (for overlapped and hidden bands) are required (Porep *et al.*, 2015).

The objective of multivariate data analysis is to take advantage of the correlation structure by substituting patterns of measurements for the single values. A sample is then characterised by an amount of each member of a usually small set of such patterns, and the

difference between samples expressed as the difference in amounts (Rehbein & Oehlenschläger, 2009).

Discriminant analyses are applied as multivariate classifications for qualitative determinations where it is possible to classify unknown samples into groups (Porep *et al.*, 2015). The most used techniques are Principal Component Analysis (PCA), Partial Least Squares (PLS), Partial Least Squares – Discriminant Analysis (PLSDA) and Soft Independent Modelling of Class Analogies (SIMCA) (Rehbein & Oehlenschläger, 2009).

PCA is used as an explorative technique where the data subject to analysis is decomposed into few uncorrelated components/variables that define the majority of the dataset differences as well as the random measurement error (Saeys, 2006). The objective of this method is to reduce noise and, consequently, reduce the complexity of the systems and minimize the effects of the measurements errors (Gemperline, 2006).

The most common multivariate technique used for quantification is PLS and its principal aim is to find the latent variables that describe the variance in the data and also reache maximum correlation between predicted variables Y and predictor variables X. As it happens in PCA, PLS can reduce the problem dimensionality significantly (Roffel & Betlem, 2006).

The aim of PLSDA is to find a model that separates classes according to the X-observations. PLSDA is used when a maximum separation between classes is needed. In this approach, the number of classes must not be too high, working better within 2-4 classes differentiation. Additionally, Discriminant Analysis also does not work when the classes are not homogenous and spread along the X-axis (Erikson *et al.*, 2013).

The calibration model performances can be evaluated by analysing some indicators: coefficients of determination (R^2), root mean square errors in calibration (R^2_C, RMSEC); in cross-validation (R^2_{CV}, RMSECV) and in prediction (R^2_p, RMSEP) respectively, and residual predictive deviation (RPD). A reasonable, comparable model should have higher values of R^2_C, R^2_{CV}, R^2_P and RPD and lower values of RMSEC, RMSECV and RMSEP as well as small difference between them (Cheng & Sun, 2014).

4. Research Objectives

The aim of this study is to test the possibility to evaluate the freshness of plaice fish that can be widely accepted by both consumers and companies using NIR spectroscopy as a non-invasive, objective, and rapid method in combination with multivariate statistical models. The successfulness of this research would provide a basis for industrial exploitation of multi-sensor techniques for real time application for fish quality assessment.

II. Preliminary study

1. Materials and Methods

1.1 Sample preparation

Ten freshly caught European plaice fishes (*Pleuronectes platessa*) were killed and transported on ice from Institute for Agricultural and Fisheries Research (ILVO) to the Biophotonics Lab, MeBioS Division, KU Leuven, Belgium. Upon arrival, all the fishes were taken out of the ice, cleaned and covered tightly with PE plastic film. They were then equilibrated to room temperature (19ºC) and were considered as the samples at 0 hours of storage for spectral measurements.

1.2 Spectral acquisition

Near-infrared spectra were acquired for the fish samples in diffuse reflectance mode with a MPA FT-NIR spectrophotometer (Figure 3) operating from 800 to 2700 nm (Bruker Optics, Germany) at 0, 4, 24 and 28 hours of storage. During spectral measurements, standard white reference was measured regularly every 30 minutes and dark noise correction was automatically implemented by the instrument before acquiring any sample spectrum.

Figure 3- MPA FT-NIR spectrophotometer.

At each designed storage time, spectra of a fish were acquired at five locations (Figure 4) on the sample surface on the dark skin side and also five locations on the white skin side with the white skin removed using a sharp knife and a peeler. Then the fish sample were covered tightly in PE plastic film and the same measurement procedure was also implemented through the film for that designed storage time. Each acquired spectrum by the instrument at one location on the fish sample was the averaged spectrum obtained from 32 consecutive scans at that location. All the sample spectra were collected at room temperature. After the spectra

acquisition for each designed storage time, the samples were stored at room temperature and covered tightly in plastic film to minimize moisture loss until the next measurements.

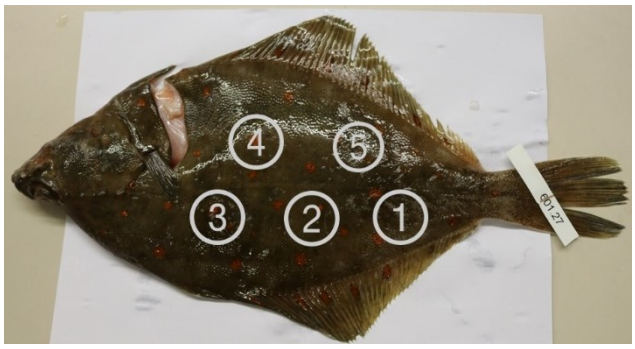


Figure 4- Illustration of the five measured locations on the dark skin of a plaice.

1.3 Data analysis

The spectral data obtained were divided accordingly to the designed sample preparation they belonged in: 1-skin; 2-muscle (skin removed); 3-skin with plastic foil; or 4-muscle (skin removed) with plastic foil. The spectral data for each sample preparation contained 200 reflectance spectra corresponding to the 4 different storage times.

Multivariate statistical models were constructed using the spectral data for classification of fish freshness levels. Two different strategies were performed:

- First strategy: classifying the fishes into two classes: fresh class, defined as the fish samples subjected to 0 hours of storage and unfresh class, containing the other samples (after 4, 24 and 28 hours of storage);
- Second strategy: classifying the fishes into four classes, each class consists of the fish samples subjected to one designed storage time (0, 4, 24, and 28 hours).

1.3.1 Data arrangement for calibration and validation of the multivariate statistical models

It is crucial assessing the prediction performances of the calibration models on future samples which were not included in model construction. Therefore, the sample spectra were divided into a calibration set for building the multivariate statistical models and a separate validation set that was not included in building these models. If the whole spectral data were used for model calibration, the obtained prediction performance on those sample spectra would be overoptimistic for future unknown samples (Porep *et al.*, 2015).

Regarding the first strategy (2 classes), 50 spectra at 0h of storage were considered as fresh fish and the other 150 spectra (4h, 24h, 28h) as unfresh fish. The spectra at 0h of storage were orderly divided into a set containing 38 spectra for model calibration and a set containing 12 spectra for model validation. At storage times 4h, 24h and 28h, 12, 13, and 13 spectra were selected in order, respectively, for model calibration; and the remaining spectra were used for model validation. This resulted in a calibration set containing 38 fresh fish spectra and 38 stored fish spectra from the whole spectral data. The validation set, therefore, contained 12 fresh fish spectra and 112 stored fish spectra.

For the second strategy (4 classes), the spectra at each time of storage (0h, 4h, 24h, 28h) were orderly divided into 35 spectra for model calibration and 15 spectra for validation, respectively. This resulted in a calibration set containing 140 spectra (35 at each storage time) and a validation set containing 60 spectra (15 at each storage time).

1.3.2 Development of the calibration models

Non-destructive fish freshness classification in this preliminary study was carried out using multivariate models such as Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). Spectral data handling and model construction and validation were conducted in Matlab (version R2013a, The MathWorks, MA, Natick, USA) and PLS toolbox (version 8.1, Eigenvector Research, Manson, USA).

PCA was firstly used to evaluate separability of the sample spectra into classes or its grouping characteristics, and then PLS-DA was applied to classify the sample spectra in both calibration and validation sets into the defined classes. Before model calibration, different pre-processing algorithms were also implemented on the sample spectra to minimize or remove irrelevant variations in the input spectra.

Contiguous blocks cross-validation and the percentage of misclassification points were used for optimizing the complexity (number of latent variables) of the constructed multivariate statistical models during model calibration procedure.

1.3.3 Selection of important wavelengths

The selection of specific wavelength ranges which are important for the model prediction can quicken the prediction operation in the future in the industry and reduce the sensor costs. A new model can be obtained using only the feature wavelengths selected instead of all the spectral range (Kamruzzaman *et al.*, 2012). In this study, the wavelength selection

was conducted using i-PLSDA method. Then the newly obtained models using these selected wavelengths were then compared to the previously built models using the full range regarding to their prediction performances.

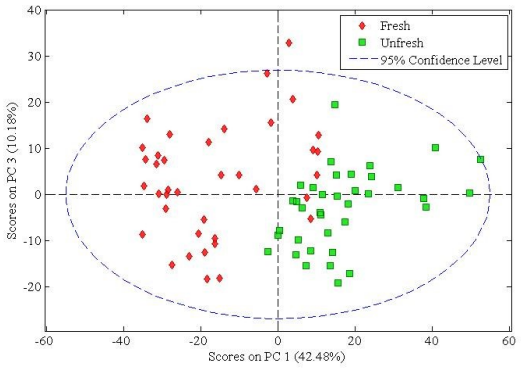
A complete flow chart of the procedure described can be found in Appendix 2.1.

2. Results and Discussion

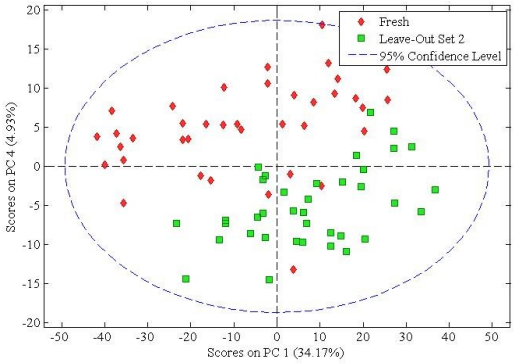
2.1 First strategy: 2-class discrimination

The NIR spectra acquired on the dark skin side and on the muscle, both without and with plastic foil are shown in Appendix 2.3. Firstly, PCA was carried out on the spectral data using different pre-processing techniques and combination of them (Mean center, derivatives, smoothing, MSC, autoscale) until the best model was found for each sample preparation. The best model was selected through the visualization of the grouping characteristics. The PC score plots of the selected PCA models for each of the four configurations are shown in Figure 5. These score plots show a clear grouping between fresh (red points) and stored samples (green points), defined as unfresh, for each case. These results suggest that NIR can be used as a freshness indicator for plaice.

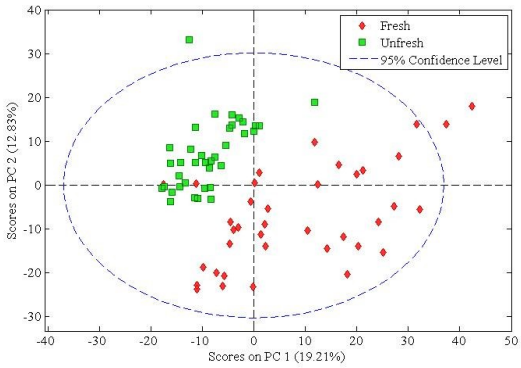
1) Dark skin



2) Muscle (white skin removed)



3) Dark skin with plastic foil



4) Muscle (white skin removed) with plastic foil

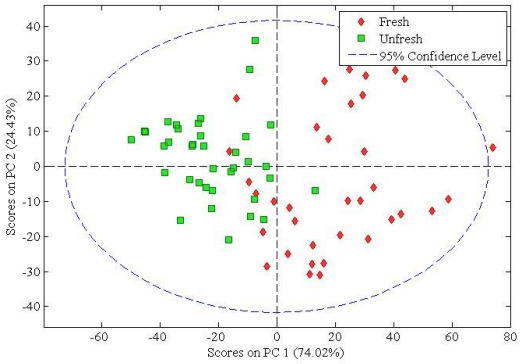


Figure 5 - PC scores plots for fresh (0h) and unfresh (4h, 24h and 28h) for the four types of sample preparations.

In the next step, PLS-DA models using contiguous block cross validation procedure and different pre-processing methods was performed until reaching the best model. The best models were selected based on model's characteristics as lowest number of latent variables and high percentage of corrected prediction values for both classes of freshness. Subsequently, i-PLS-DA was implemented for important wavelength selection, which aimed at maintaining the prediction performance even if the wavelength range is reduced. The table below summarizes the best results obtained for each of the four sample preparations with the full wavelength range used and the best selected wavelength range in model calibration and validation.

Table 1 - Summary on the PLS-DA results for 2-class discrimination of all four sample separations.

Sample preparation	Pre-processing methods[1]	Points excluded	Full wavelength range		Best selected wavelength range using i-PLSDA		
			No. Latent Variables	Sensitivity Specificity Accuracy (%)[2]	No. Latent Variables	Wavelength range (nm)	Sensitivity Specificity Accuracy (%)[2]
Dark Skin	MSC + D1 + AS	1	6	100 99.1 99.2	2	[1063:1271]	100 100 100
Muscle	D1 + AS	1	6	100 98.2 98.4	6	[801:2782]	100 98.2 98.4
Dark Skin + Plastic foil	D1 + AS	1	5	100 98.2 98.4	2	[1063:1271]	100 100 100
Muscle + Plastic foil	SM + AS	2	9	100 93.8 94.4	5	[914:1701]	100 94.6 95.2

[1] MSC – Multiplicative Scatter Correction; D1 – First Derivative (Savitzky-Golay); AS – Autoscale; SM – Smoothing (Savitzky-Golay).

[2] Misclassified points: Appendix 2.5

The specificity, sensitivity and accuracy parameters were determined in order to assess the performance of the classification models on the prediction of the validation set. The following formulas were used for calculating the above parameters.

$$\text{Sensitivity} = \frac{\text{Number of correctly classified fresh spectra}}{\text{Total number of fresh spectra}} \quad \textit{II.1}$$

$$\text{Specificity} = \frac{\text{Number of correctly classified unfresh sepctra}}{\text{Total number of unfresh spectra}} \quad \textit{II.2}$$

$$\text{Accuracy} = \frac{\text{Number of correctly classified spectra}}{\text{Total number of spectra}} \quad \textit{II.3}$$

During the calibration, outliers were identified and removed. If not removed, outliers can have a significantly larger and detrimental effect on the model, leading to misleading statistics. Outliers were identified by: 1- an atypical spectrum resulting in a large spectral residual, 2- an extreme leverage, resulting in a large distance to the model center and 3- a large residual in the dependent variable, or a combination of these (Kamruzzaman *et al.*, 2012).

From Table 1, it is clearly observed that the selected PLS-DA models could provide good classification on freshness for the fish samples in this research as it is perceptible through the high values of sensitivity, specificity and accuracy provided from all the classification models.

The PLS-DA models of the dark skin (without and with plastic foil) of plaices showed equal accuracy percentages of 100% using the reduced wavelength range of 1063:1271 nm, which means that it would be possible to correctly predict all samples. By reducing the wavelength range it was possible to increase the performance parameters of specificity and accuracy while reducing the model complexity.

The results obtained from the muscle side presented sensitivity and specificity percentages of 100 and 98.2 for without plastic foil and 100 and 93.8 for with plastic foil. Although in the last case, with a reduction of the wavelength range to 914:1701 nm, improvements in the evaluated parameters were verified, this outcome did not happen for the muscle side without plastic foil where the best model includes all the wavelength range measured. Moreover, by covering the plastic foil, the prediction performance of the model

slightly deteriorated as observed for the cases of dark skin vs dark skin + plastic foil and muscle vs muscle + plastic foil.

Given that the relevant changes in fish muscle are due to the microbial growth which has its relevant activity in approximately 10 days after the catch (considering that the fish is stored in ice) (Rehbein & Oehlenschläger, 2009), in the early stages of storage the changes in muscle freshness must be less noteworthy than the those in the skin side which naturally had higher initial microbial load. Thus, the obtained higher misclassification percentages for muscle as compared to those for the dark skin side could be easily explained in this preliminary study in which the fish samples were stored at much higher temperature (ambient temperature).

The higher misclassification for muscle with plastic foil as compared to without plastic foil could be obviously explained due to the fact that by introducing another material layer with completely different optical properties, the recorded diffuse reflectance spectra for the case of plastic foil contained also spectral properties of the plastic foil besides those of the muscle, which added more irrelevant variations in the input spectra which deteriorate the model prediction performance. As an illustration, the absorption spectra of the used plastic foil are recorded in Appendix 2.2.

Further investigation on the obtained results indicated that the misclassification points of the models of muscle without and with plastic foil mainly happened in the storage group of 4 hours, which again confirmed the aforementioned reasoning of increasing difficulty to discriminate in the early stages of storage for the flesh (skin removed).

The regression coefficient plots displaying the contribution of each wavelength to the selected PLSDA calibration model are shown in Figure 6.

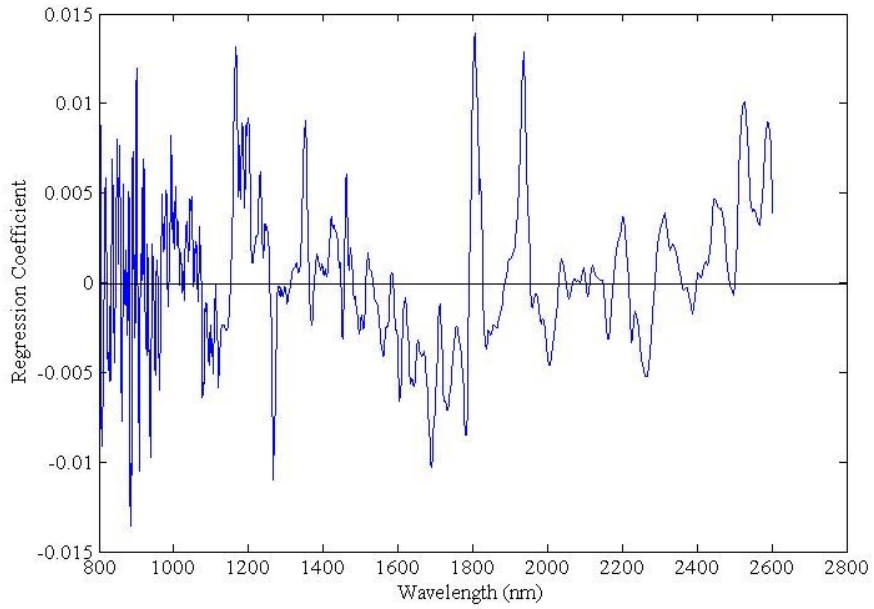
In the 2-class discrimination, the regression coefficient plots exhibit important wavelengths. In the dark skin model, the important wavelength ranges, corresponding to the higher values of regression coefficient, were approximately around 1150, 1250, 1700, 1800, 1850 and 1950 nm. The first three wavelengths (1150, 1250 and 1700 nm) and 1850 nm are related with C-H vibration (Workman & Weyer, 2012). Absorption in the wavelength range between 1680 and 1760 nm (1700 nm) is currently related with C-H bands of fatty acids (Liu *et al.*, 2013). As stated before, fish is rich in unsaturated fatty acids which are oxidized over time contributing to fish odours (Rehbein & Oehlenschläger, 2009).

The peaks at 1800 and 1950 nm are connected with O-H from water vibration and acids and esters vibration, respectively. Although water is one of the principal components in the fish constitution, it does not provide useful information on fish freshness loss over time and

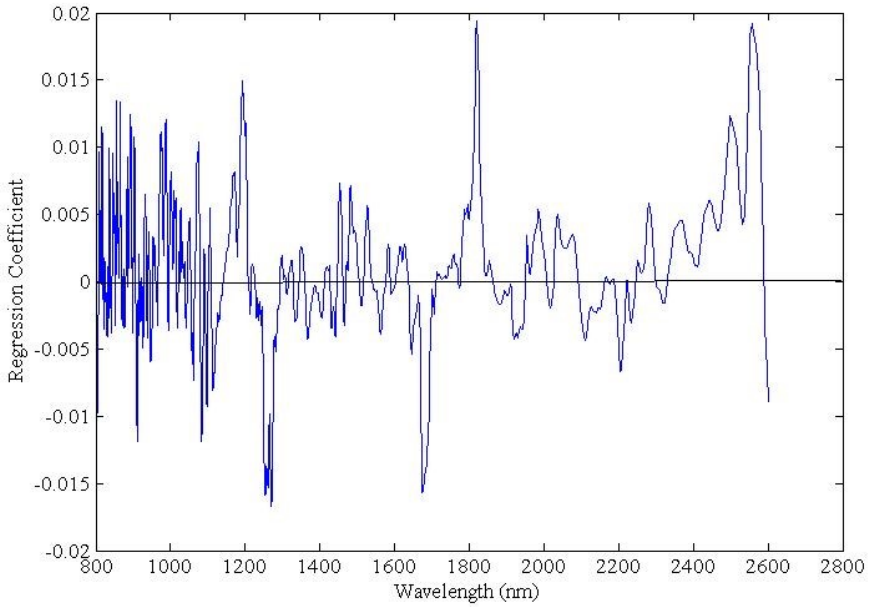
consequently, on the discrimination process. However, esters or at least 5'-phosphate esters of nucleosides as ATP and its degradation, are an important phase in fish deterioration (Rehbein & Oehlenschläger, 2009).

According to the region of 1063-1271 nm, that has been selected by i-PLSDA as most important range, is mainly related with the absorption of O-H, C-H (aromatic), C=O and C-H interaction (Workman & Weyer, 2012), is well appropriate for predicting freshness. O-H bond absorption is related to alcohols and water molecules and, as described previously in this document, alcohols formation is the result of microbial activity and endogenous enzyme decompositions (Cheng *et al.*, 2015) in the same way as aromatic compounds and carbonyl compounds (C=O), and therefore related to *post mortem* alterations in fish.

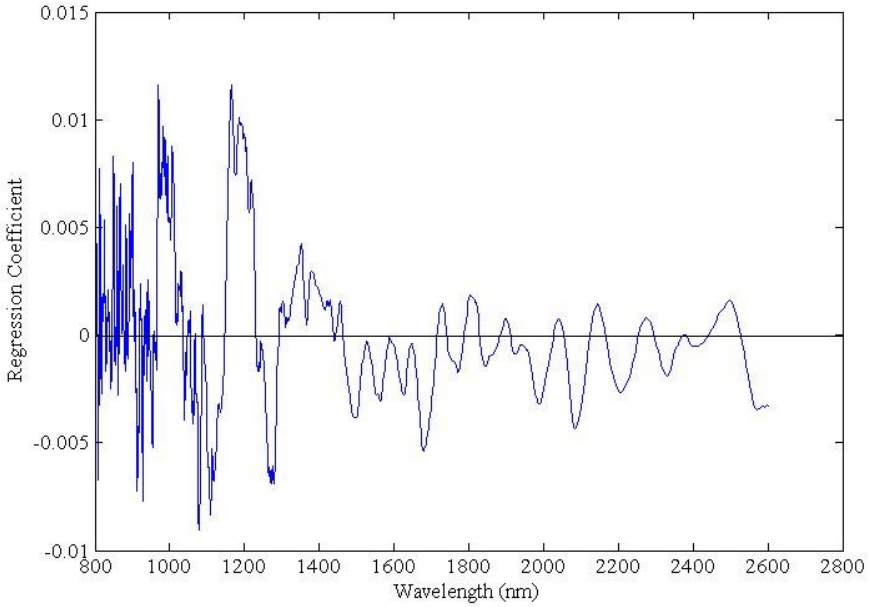
1) Dark skin



2) Muscle



3) Dark Skin + Plastic foil



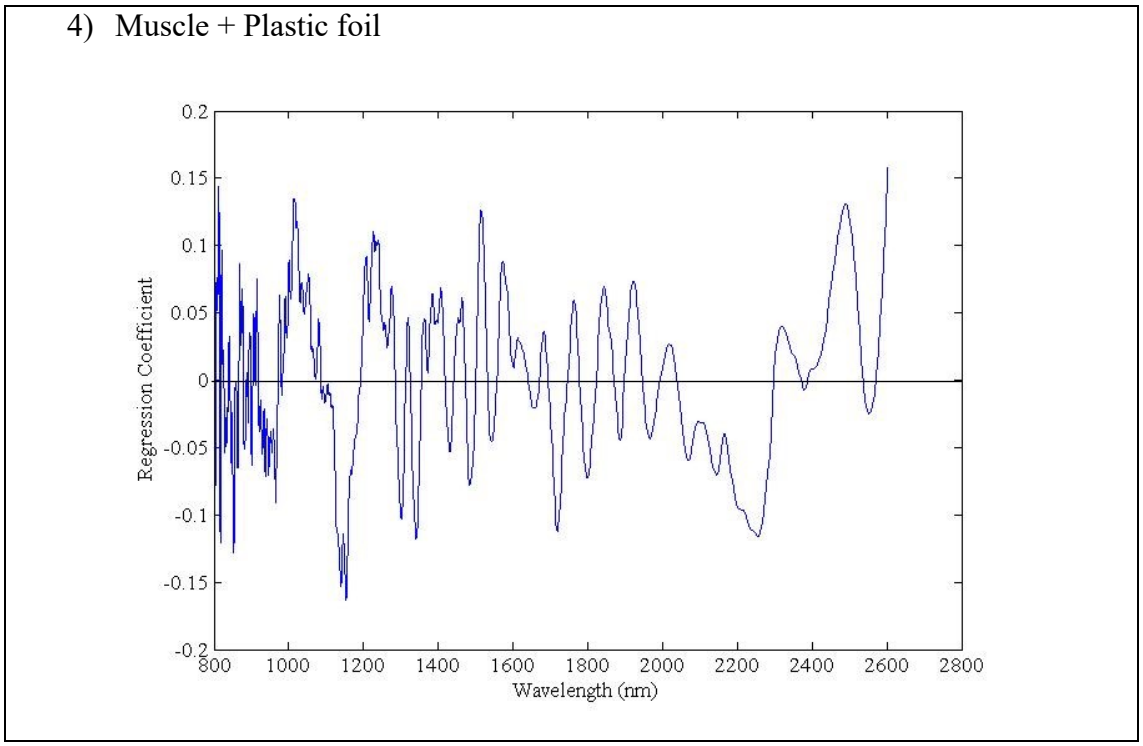


Figure 6- Regression coefficients of the PLSDA model for the four sample preparations (2-class discrimination).

In the muscle case, the important wavelength regions were approximately around 1200, 1250, 1700 and 1800 nm, corresponding to the absorption of C-H bond (1150-1250 nm) and to C-H bond of aromatic compounds (1650 nm) (Workman & Weyer, 2012) that are formed just as fish rot (Cheng *et al.*, 2015).

The regression coefficient plot for the third case (dark skin with plastic foil) presents approximately six important wavelength regions at around 1100, 1200, 1300 and 1700 nm. The wavelengths peaks in the beginning and the end of the range were not taken deeply into consideration due to higher noise effects present in the input spectra. Similar to the first case (dark skin), the regions before 1063 nm and after 1271 nm were considered by the i-PLSDA program to not contribute or contribute in fewer amount to the model prediction process. The peaks included in the previous region are related with C-H bond and C-H bond associated with aromatic compounds (Workman & Weyer, 2012). These results are similar to those obtained in the first case which is expected since the only difference is the covering with plastic foil.

In the last case (muscle with plastic foil), protruding wavelengths were found in the regions of approximately 1150, 1200, 1300, 1500, 1700 and 2250 nm. The wavelength region, which has more important information for the correct classification between classes of fresh and unfresh plaice, is 914-1701 nm according to i-PLSDA. Within this range, all the peaks are mainly associated with O-H from water or alcohol, N-H amine, C-H methyl or ether associated

as R-O-CH_3 (Workman & Weyer, 2012) and C-H bonds of fatty acids (Liu *et al.*, 2013). All the compounds described are related with fish quality degradation over time (Cheng *et al.*, 2015). The peak at 2250 nm it is not include in the region provided by i-PLSDA software probably because it is subject to noise effects but it can be associated with protein, specifically N-H bonds absorption (Liu *et al.*, 2013).

When comparing results with and without plastic foil it is possible to see that excepted for the peak at 2250 nm in the last case, the models built with measurements with plastic foil do not present peaks after the wavelength of 1800 nm (approximately) while the other peaks are very similar. This situation is better perceptive in the next figure where 10 samples of dark skin at 4h with and without plastic foil are plotted. The graphic clearly show more variability after 1800 nm for plastic foil samples. This is probably due to the plastic absorption capability as possible to check in Appendix 2.2 and also more challenge for the NIR light in this range, which inherits low penetration depth for biological media, to penetrate the fish tissue through the plastic layer.

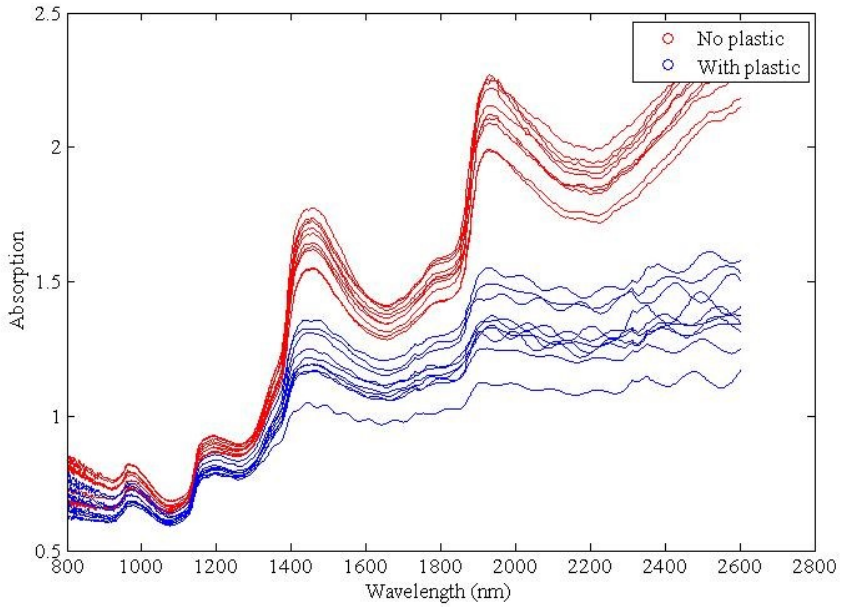


Figure 7 - Spectra of dark skin samples points at 4h with and withou plastic foil.

2.2 Second strategy: 4-class discrimination

The NIR spectra acquired on the dark skin side and on the muscle, both without and with plastic foil are shown in Appendix 2.4. Similarly to the 2-class discrimination, PCA was first carried out on the spectral data using different pre-processing techniques and combination of them (Mean center, derivatives, smoothing, MSC, autoscale) until the best model was found

for each sample preparation. The best model was selected through the visualization of the grouping characteristics. The PC score plots of the selected PCA models for each of the four sample preparations are shown in Figure 8.

These score plots show a clear grouping between samples at 0h (red points) and stored samples (4, 24, and 28h; other colours), defined as unfresh. Groups subjected to storage are not well separated. These results suggest that NIR spectra can be used as a freshness indicator for plaice but that other multivariate methods are needed for assigning or classifying fish samples subjected to different storage times.

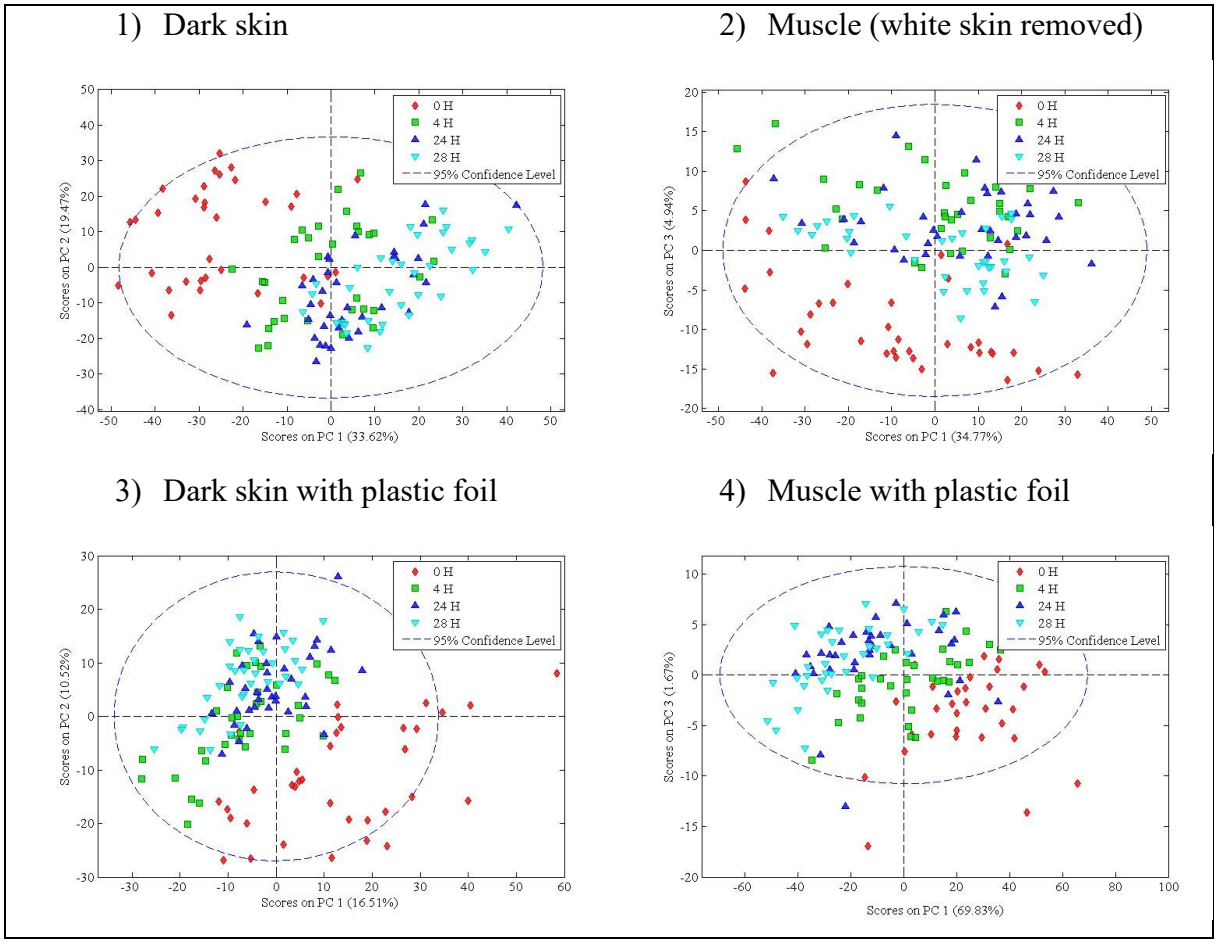


Figure 8 - PC scores plots for fresh (0h) and unfresh (4h, 24h and 28h) for the four types of sample preparations.

PLS-DA with contiguous block cross validation procedure and different pre-processing methods in addition with wavelength selection using i-PLSDA, was subsequently performed until reaching the best model. The table below summarizes the best results obtained for each of the four configurations.

The accuracy and the misclassification percentage were determined in order to assess the performance of the classification models and the formulae used for calculation were the following:

$$\text{Accuracy} = \text{Number of correctly classified spectra/Total number of spectra} \quad \textit{II.4}$$

$$\text{Misclassification percentage} = \text{Number of fish incorrectly classified/Total number of fish} \quad \textit{II.5}$$

The misclassification percentage was determined taking into consideration the majority vote on the 5 points measured, that is that if 3 or more points of one fish are wrongly misclassified then the fish is considered wrongly classified, otherwise, if just one or two points on the 5 measured are not correctly classified then the fish is considered well classified.

For the 4-class discrimination, only samples from the last 3 fishes corresponding to 15 spectra in each designed storage time were included in the validation dataset (fish 8, 9 and 10). Considering the four storage times (0h, 4h, 24h and 28h) this results in 12 fishes (60 spectra) evaluated in the validation steps.

Only one outlier was identified and removed from the muscle measurements. If not removed from the cross-validation dataset it would contribute to a model calibration with more errors associated. Outliers were identified in the similar way as described in the 2-class discrimination, through analysis of spectrum and plots displaying distance to the model and residuals in the dependent variable (Kamruzzaman *et al.*, 2012).

It is also possible to see in Table 3, that the accuracy percentages for the case of muscle (without and with plastic foil) were higher than the ones obtained in the case of dark skin (without and with plastic foil). Additionally, and with exception for the case of dark skin with plastic foil, the other cases present the best results when using full wavelength range (801 to 2782 nm).

Table 2 - Summary on the PLS-DA results for 4-class analysis of all four sample preparations.

Sample preparation	Pre-processing methods[3]	Points excluded	Full wavelength range		Best selected wavelength range using i-PLSDA		
			No. Latent Variables	Accuracy Misclassification (%)[4]	No. Latent Variables	Wavelength range (nm)	Accuracy Misclassification (%)[4]
Dark Skin	D1+AS	-	4	70.00 33.33	4	[801:2782]	70.00 33.33
Muscle	D1+AS	1	4	90.00 0.00	4	[801:2782]	90.00 0.00
Dark Skin + Plastic foil	D1+AS	-	5	61.67 33.33	4	[801:1063]	70.00 33.33
Muscle + Plastic foil	D1+AS	-	5	83.33 0.00	5	[801:2782]	83.33 0.00

[3] D1 – First Derivative (Savitzky-Golay); AS – Autoscale.
[4] Misclassification points: Appendix 2.6

The PLS-DA best models for the dark skin side after wavelength selection with i-PLSDA provided the same accuracy percentage (70%) and misclassification percentage (33.33%) both for without or with plastic foil. For the dark skin with plastic foil this result was obtained with only a part of the full wavelength range, between 801 and 1063 nm.

As for data collected from the plaice muscle, better models were developed resulting in higher percentages of accuracy, 90.00% and 83.33% for data without and with plastic foil as compared to those of the dark skin. These models were based in the whole range of wavelength measured.

In contrast with the previous results, there is a significant difference between the accuracy and misclassification percentages of the data measure in the dark skin and in the muscle. Clearly, the models built with muscle samples provided better results: higher accuracy percentages and lower misclassification percentages. The results indicate that, although some points were erroneously classified, considering all of 5 measured points to classify a fish, none of the fishes were incorrectly classified (misclassification percentage equals to zero).

In the beginning of fish freshness decaying, autolytic processes are the main responsible for *rigor mortis* and afterwards, the autolysis of proteins and fats happen. This produces nutrients that allow bacterial proliferation which is the second phase of fish degradation (Li *et al.*, 2014). Fish skin is a less suitable surface for microbial growth than the flesh for most spoilage microflora and as a consequence, the spoilage process on the flesh side proceeds faster than on the skin side (Lin *et al.*, 2006). It has already been proven that NIR spectroscopy could be used for measuring protein, fat and moisture in fish (Khodabux *et al.*, 2007; Liu *et al.*, 2013; Huang *et al.*, 2003; ElMasry & Wold, 2008; Folkestad *et al.*, 2008; Herrero, 2008). Considering that protein and fat are degraded in the first stage of freshness loss that may explicate why the NIR models are able to correctly classified samples with short storage time.

Microbial activity is the main culprit behind the quality changes in fish during the last stages of spoilage (Hernández *et al.*, 2009). According to Cheng *et al.* (2013), NIR spectroscopy have been proven to be a useful and trustworthy technique for detecting and accurately quantifying microbial load in fish which could explain why it is easier to correctly classified spectra of later hours with the model created using the spectral data collected from the muscle side (where the microbial growth is predominant in later phases).

In this analysis, the points wrongly classified were mainly associated with the groups of 24 and 28 hours. This could be explained by the fact that the spectra of these two groups are highly overlapping probably because the fish samples were already heavily degraded after 24

hours at room temperature and consequently the differences in properties of the two groups are not substantial.

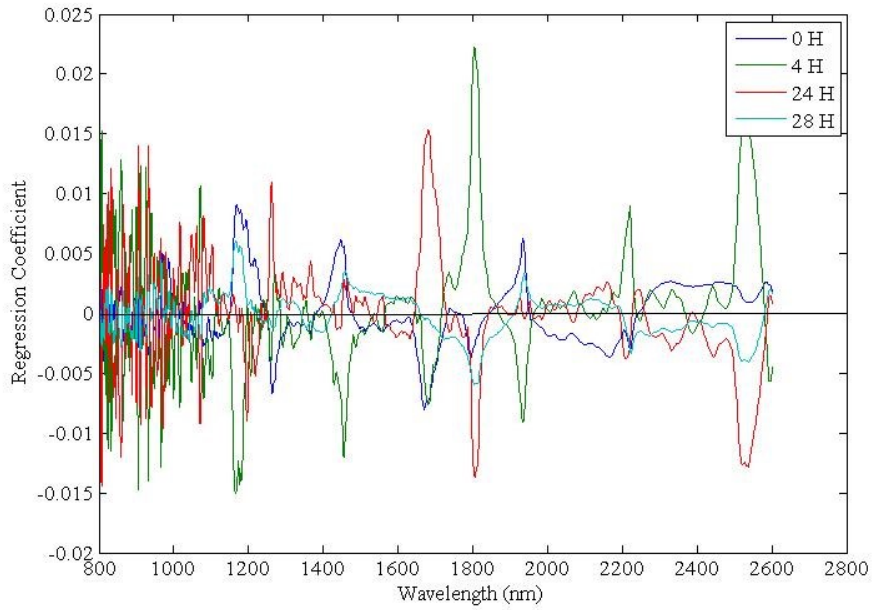
Regression coefficient plots of the PLSDA models are displayed in Figure 9. In each plot, each line shows the importance of individual wavelengths for classifying samples in each group (one storage time) against others.

In the first case (dark skin), the important wavelengths, having relatively higher regression coefficients, are in the regions of 1200, 1450, 1600-2000, 2200 and 2500 nm. These wavelength regions could be associated with C-H bond, O-H bond from water and other molecules, C-H bonds of aromatic compounds, S-H from sulphur compounds, C=O from carbonyl compounds and N-H bonds from protein (Workman & Weyer, 2012; Liu *et al.*, 2013). All the compounds containing the bonds previously mentioned are related with the formation of volatile compounds between others which are mainly the result of microorganism proliferation (Cheng *et al.*, 2015).

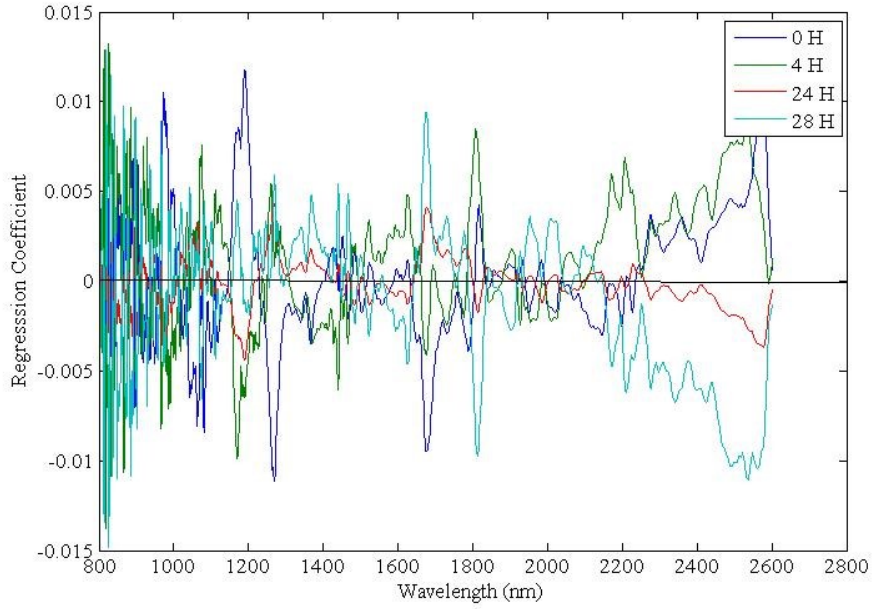
The analysis of the muscle, the wavelengths highlighted were in the regions of 1000-1300, 1600-1800 and 2500 nm. It is estimated that these wavelengths ranges are linked to the absorption of C-H bond from aromatic compounds, S-H bond from sulphur compounds and O-H combination from water (Workman & Weyer, 2012).

The regression coefficient plots for the third and four cases, both with plastic foil, do not show any pertinent wavelength which do not permit any basic interpretation of the composition of the sample throughout time. This situation could be related to the influences of the plastic foil since the only differences between the first and the third, the second and the fourth case, respectively, is the use of PE plastic film.

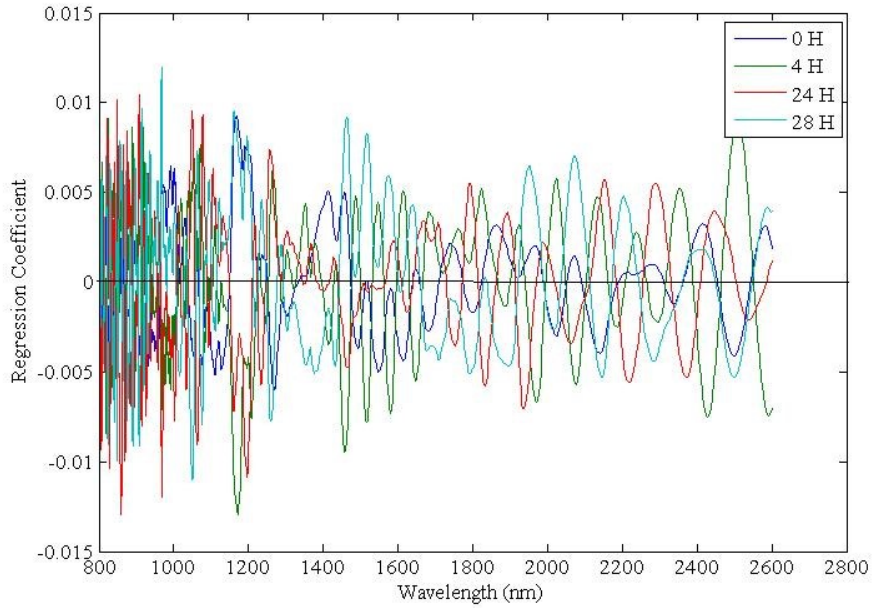
1) Dark skin



2) Muscle



3) Dark skin with plastic foil



4) Muscle with plastic foil

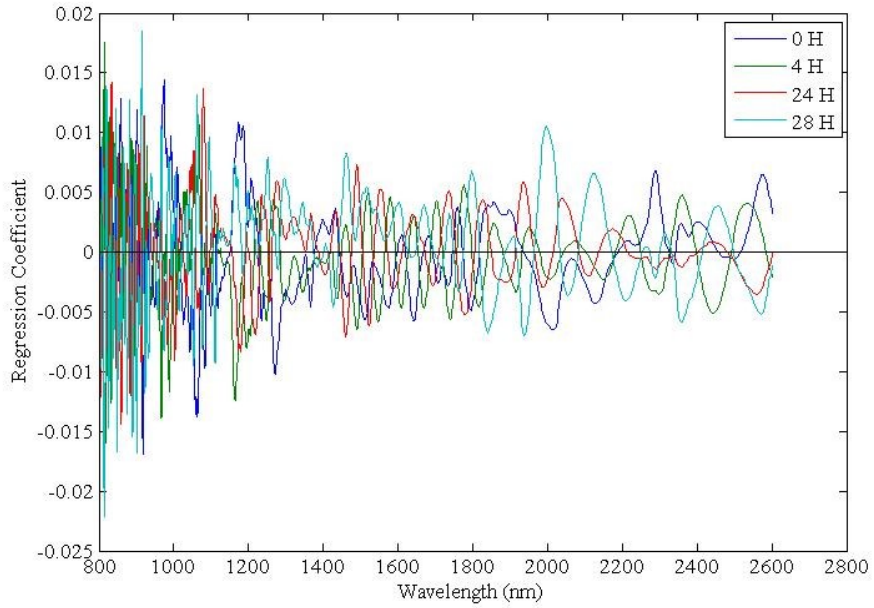


Figure 9- Regression coefficients of the PLSDA models for the four sample preparations (4-class discrimination).

3. Conclusions

Referring to the results obtained both in the first analysis (2-class classification) and in the second analysis (4-class classification), PLSDA models provided a good tool for correctly classifying plaice into the defined classes. This indicates that NIR spectroscopy has great potential for non-destructive plaice freshness evaluation and therefore will be used for the next measurements which will be elaborated on more number of samples subjected to commercial plaice quality grading method. As the majority of wavelength peaks selected by i-PLSDA were between the range of 1000 and 1800 nm in the next study a restriction of the NIR range will be use: 940 to 1700 nm, Short Wavelength – Infrared Region, aiming for cheaper sensor costs for future applications in the fish industry.

NIR spectroscopy offers obvious advantages with respect to costs, rapidity and mass-evaluation, comparing with traditional methods such as Quality Index Method (QIM). It should, however, be emphasized that this study was performed on a limited number of samples (10 fishes) which, therefore, does not allow to take concrete straightforward conclusions. In the next section, a much bigger experiment using higher number of plaice samples combined with commercial QIM plaice quality grading will be implemented.

III. Materials and Methods

1.1 Sample preparation

Ninety caught European plaice fishes (*Pleuronectes platessa*) were killed and transported carefully on ice to the Institute for Agricultural and Fisheries Research (ILVO). The fish samples were assigned into three different classes according to their sizes - Appendix 3.1. Then, they were evaluated using the Quality Index Method (QIM) by experts at ILVO. Afterwards, the fishes were transported carefully on ice to the Biophotonics Lab, MeBioS Division, KU Leuven, Belgium, where they were stored on ice in the fridge having temperature controlled at 1 ºC during all the subsequent experiments.

1.2 Spectral measurements

Shortwave infrared (SWIR) spectra of the fish samples were acquired using a Corona Fibre VISNIR spectrophotometer (Zeiss, Germany) (Figure 10, Left), operating from 940 to 1700 nm coupled with an external OMK head (OMK 500, Zeiss, Germany) (Figure 10, Right) for acquiring diffuse reflectance spectra for the fish samples. The OMK head has an integrated halogen light source illuminating light to the sample placed on a sample placement support at a distance of 1 cm from the OMK glass surface (non-contact measurement mode). Several optical fibres were circularly aligned surrounding the light source to collect the diffusely reflected light coming from the measured sample and guide it to the spectrometer for spectral acquisition. The measured area was a circle having the diameter of 6 cm.

Figure 10 – (Left) Measurement setup using the Corona Fibre VISNIR spectrophotometer (Zeiss, Germany); (Right) The OMK head used for diffuse reflectance spectra acquisition

Spectral measurements were implemented on all the fish samples after one day, two days, four days, seven days, nine days and eleven days of storage in the fridge (6 different

storage times). Before collecting the sample spectra, the dark reference was measure. The white reference was measured after fifteen measurements (approximately each half an hour). Both were used for automatically compensating for dark noises and variations in the intensity of the illumination to get the sample diffuse reflectance spectrum in each measurement.

At each storage time, the diffuse reflectance spectra for each fish was acquired on both sides (white skin (WS) and dark skin (DS)) at two different surface locations each side (Figure 11). Each spectrum at one location was the average of 64 continuous scans with a 40-ms exposure time for each scan. The sample spectra were collected at room temperature. At each storage time, 360 spectra were obtained.

Figure 11 – Illustration for 4 measured locations for a fish sample.

1.3 Data analysis

The spectra obtained were divided accordingly to the surface of the fish measured: dark skin (DS) or white skin (WS) to evaluate which of those has more potential for freshness evaluation.

The evaluated QIM scores for the fishes were reported in Appendix 3.2. These scores were then used to estimate the real storage days in ice of each fish using the following formula specifically applicable for plaice (Martinsdóttir *et al.*, 2001):

$$\text{Quality Index} = 1.28 \times \text{days in ice} + 0 \quad (R^2 = 0.89) \qquad III.1$$

QIM analysis is considered the foremost and straightforward method for measuring fish freshness in industry. Therefore, QIM is use nowadays as the reference technique for predicting fish shelf life.

According to the obtained values of storage days in ice, the spectral data of each side (dark skin and white skin) was reorganized according to its storage time (Appendix 3.3). In this research, the converted days in ice was used for all the analyses.

1.3.1 Data arrangement for calibration and validation of the multivariate statistical models

To assure good prediction performances of the constructed multivariate models on future samples, the spectral data was divided into a calibration set for building the multivariate models and a separate validation set which was not incorporated in building the models to test their prediction performances (Porep *et al.*, 2015). The ninety fishes were measured at 6 different storage times, 2 locations for each side: dark skin and white skin, which resulted in 1080 measurements per side. After rearranging the samples using the converted days in ice, each storage time had different number of spectra: 6, 6, 36, 108, 108, 96, 94, 52, 68, 72, 90, 86, 82, 90, 48, 24, 6, 6 and 2 spectra for 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 and 21 days in ice, respectively.

For each storage time, the spectra were divided into two groups: two thirds of the total number of the spectra went into the calibration set and the remaining one third were assigned into the validation set. This resulted in the calibration and validation sets which contained 720 and 360 spectra, respectively.

1.3.2 Development of the multivariate calibration models

Data analysis using Partial Least Squares (PLS) Regression was conducted using Matlab (version R2013a, The MathWorks, MA, Natick, USA) and PLS toolbox (version 8.1, Eigenvector Research, Manson, USA). Different pre-processing strategies were also implemented on the input spectra for searching the best calibration model. Three different data transformations applied for Y-values (storage days in ice) individually in order to obtain the best correlations between spectral data X and Y were investigated: no transformation, using log(Y) and using 1/Y.

The complexities of the calibration models or the selected number of latent variables was optimized by internally cross-validating the performances of those models in predicting the quality parameters of the samples in the calibration set. Two different cross validation methods were used in this research: in the first method, iteratively leaving one group corresponding to one designed storage time out for cross-validation; and in the second method,

one third of the number of spectra in each group corresponding to one storage time were left out for cross-validation. The optimized PLS models were the ones that minimized Root Mean Square Error of Cross-Validation (RMSECV) or did not significantly improve this value. The optimized model was then applied on the validation set for evaluation of its performance in predicting new samples. The model prediction accuracy was represented by Root Mean Square Error of Prediction (RMSEP).

The RMSECV and RMSEP are projections for the prediction error in cross validation and in the validation process and can be calculated using the following formula:

$$RMSECV\ (or\ RMSEP) = \sqrt{\sum_{i=1}^{N} \frac{(\hat{y}_i - y_i)^2}{N}} \qquad III.2$$

Where N is the number of samples in the test set, $\hat{y}_i$ is the predicted value and y_i is the measured value for the i^{th} sample (Saeys, 2006).

The best PLS model corresponding to each Y-value transformation was selected referring to several important parameters: number of latent variables, RMSECV, RMSEP and the coefficient of determination for predicting the validation dataset (R^2_p). Generally, good models provided low RMSECV, RMSEP values and small number of latent variables and high R^2_p.

1.3.3 Selection of important wavelengths

Similar to the preliminary study, i-PLS Variable Selection Interface was used for important wavelength selection. This wavelength selection aimed at reducing the number of measured wavelengths necessary to obtain similarly good prediction results and thus, reducing the sensor costs and operation time in future practical applications.

A complete flow chart of the procedure described can be found in Appendix 3.4.

IV. Results and Discussion

1. Prediction of storage time

1.1 Dark skin spectral data

Figure 12 presents the measured mean SWIR spectra of dark skin samples from 940 to 1700 nm with increasing days in ice. It is observed that over storage time the mean diffuse reflectance spectrum has a trend to go up, which means that as the plaice degrades there were changes in optical properties of the fish tissue (scattering and absorption). Absorption in the region of 940 to 1000 nm is related to O-H bond associated with water, alcohols and phenolic compounds (Workman & Weyer, 2012). The region 1100-1250 nm is mostly related with the second overtones of C-H bond (Siesler *et al.*, 2002) of aromatic compounds and also in C=O bond of carbonyl compounds (Workman & Weyer, 2012). All the compounds mentioned exist in large number of organic molecules containing in the fish tissue and in the products of fish degradation (Cheng *et al.*, 2015). A small peak between 1250 and 1350 nm is verified that is correlated with C-H bond absorption. The broad valley between 1400 to 1700 nm is concomitant with O-H bond absorption from phenolic, alcoholic and aromatic compounds as well as water; C-H bond absorption from aromatic compounds; N-H bond absorption from aromatic amines and proteins; and C=O bond absorption from carbonyl compounds (Workman & Weyer, 2012).

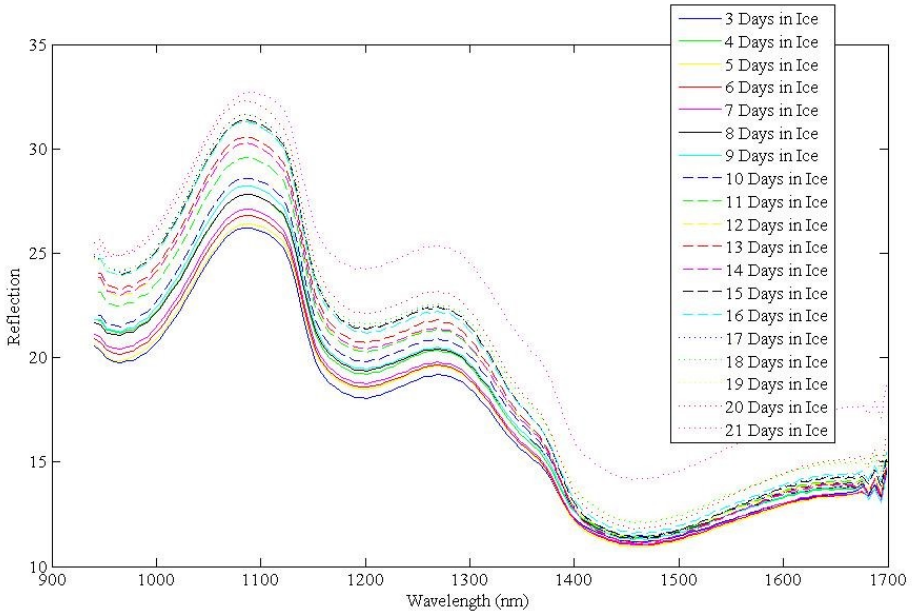


Figure 12 – Measured mean SWIR spectra of the fish samples on the dark skin side with increasing days in ice.

The best selected PLS models after testing different pre-processing strategies in combination with transformation of Y-variables are reported in Table 6.

Table 3 – Selected PLS models to predict storage time using dark skin spectra.

Cross-Validation	**Y**	**Pre-processing[5]**	**Points excluded**	**Full wavelength range**		**Best selected wavelength range using i-PLS**		
				No. Latent Variables	**RMSECV RMSEP R^2_p (days in ice)**	**Wavelength range (nm)**	**No. of Latent Variables**	**RMSECV RMSEP R^2_p (days in ice)**
Custom 1	--	D2 + AS	1	8	2.017 2.269 0.745	940:1030, 1400:1580	10	1.8239 2.2226 0.804
Custom 2	Log	MSC + D2 + AS	--	8	2.4235 2.3323 0.677	940:1700	8	2.4235 2.3323 0.677

[5] MSC – Multiplicative Scatter Correction; D2 – Second Derivative (Savitzky-Golay); AS – Autoscale.

One outlier was removed on the first approach because it presented an uncharacteristic spectrum that resulted in higher distance to the model center and a huge residual in the dependent variable.

The first method of cross-validation, leave-one-group-out, is useful for assessing linearity relation between X spectral data and Y values of the calibration set. Therefore, due to the lower values of RMSECV, RMSEP and sufficiently higher value for R^2_p, linearity relation between X and Y it observed with the PLS calibration model. Notwithstanding the fact that this method usually constructs overly pessimist models, this has not been the case since the results obtain were better than those from the second cross-validation approach.

The second method, where one third of all the groups were used for the cross-validation, presented results to some extent slightly worse than the first cross-validation. Slightly better prediction performances obtained for the custom 1 as compared to custom 2 could possibly be explained by more number of samples included during model calibration for custom 1.

Prediction performance in the first approach was improved by selection of wavelength region by i-PLS software. Although there was an augmentation on the number of latent variables and, subsequently on the model complexity, the decrease of wavelength range used plays a contradictory effect. The overall result is an increase in the coefficient of determination of the validation dataset (R^2_p) and decrease in the RMSECV and RMSEP.

As it is also possible to see, in the second case, the best wavelength range corresponds perfectly to the all wavelength region used and therefore, there was no shortening that provided better prediction results.

The prediction errors, RMSECV and RMSEP, are however possibly relatively high when comparing to plaice shelf life of 13 days in ice. It would not be so crucial if it was not the case that the numbers obtained were a representation of the mean deviation of the total errors committed when predicting the days in ice of plaice samples. In other words, it means that there are errors of predicting with higher and lower values. Consequently, there could be plaice fishes which are classified as fresh when they may not be that fresh and vice versa. This is an extremely delicate topic because unfresh fish being classified as fresh are going to be sell to the consumer without fulfilling the criteria of freshness and fresh fish classified as unfresh when it may not be, will be going to the garbage, decreasing the profit of the company.

The industry had mentioned that the errors obtained should reduce if possible. Despite there are not any error criteria in literature nor by the industry, these values do not compare to some of the results obtained in the majority of other studies, for example: 1.04 days in ice for

cod freshness evaluation (Nilsen *et al.*, 2002), 1.20 days in ice for salmon freshness estimation (Nilsen *et al.*, 2002) and 1.60 days in ice for cod fillets freshness determination (Sivertsen *et al.*, 2011).

It should be noted that most published studies using NIR spectroscopy for fish freshness prediction are done on fish fillets, while in this work whole body with skin and not gutted plaice fish samples were used. Obviously, measuring the optical properties of the flesh through the intact skin as done in this work would have incorporated more unwanted variations and/or more challenges for the constructed multivariate models.

As cod as a similar shelf life as plaice (15 and 13 days in ice, respectively) in this work, comparison between plaice and cod studies will be of interest. Despite the better cod freshness results obtained by Nilsen *et al.* (2002), it must be also highlighted that it was obtained in the visible range of 400 to 700 nm in the muscle side. The measurements in the skin in another research, however, presented poorer results of 3.54 days in ice as an error of prediction. In the other cod research, Sivertsen *et al.* (2011) were able to determine cod fillets freshness with the prediction accuracy of 1.6 days in ice which is better than ours. However, it is noted that the wavelength range used in their work was definitely very different: 410 to 1010 nm, which is similar to the range used in the previously referred study by Nilsen *et al.* (2002), and also that it was not used the entire fish, only the fish fillet and on different fish rather than plaice.

Kimiya *et al.* (2013) obtained a prediction accuracy of 2.4 days using VIS/NIR spectroscopy (400–2500 nm) for predicting freshness expressed as storage days in ice of Atlantic salmon (*Salmo salar* L.) fillets, which is comparable to our result. When comparing this result with those obtained by Nilsen *et al.* (2002) it is possible to see a huge difference. Even though they both used salmon fillets as object of the study, Nilsen *et al.* (2002) managed to obtained better results by using again a smaller range of wavelength in the infrared region: 700 to 1100 nm. The reduction of wavelength range is not only preferred in terms of model complexity reduction but also in economical point of view since broader ranges in the further NIR region would lead to higher sensor costs for future implementation in practical applications.

Covering the visible range would positively reduce the sensor costs and possibly incorporate the colour changes of the fish during storage into the model for future sample prediction. It could work in the case of plaice since skin alterations are visible, going from bright, iridescent pigmentation to dull, mat and discoloured (Martinsdóttir *et al.*, 2001). However, the downsides are that there are usually large variations in colour for one type of fish

(coming from different origins, having eaten different feeds…) and that the colour changes in fish might not always directly correlate to fish freshness degradation, which could deteriorate the robustness of the constructed PLS models.

It must be mentioned that the values of days in ice were calculated through the QIM scores provided by ILVO. The mathematical formula used for the conversion of QIM scores into days in ice has a $R^2 = 0.89$ which means that before data processing there was already prediction error from the imprecisions in the predicted values for storage time in ice. Therefore, it should be considered that these results obtained are also not only due the accumulation of the inherent errors from spectroscopy analysis but also from the primary transformation of the dataset.

Plots of the measured versus predicted days in ice for the two selected PLS models in Table 6 are shown in Figure 13.

In Figure 13, the first model (figure on the top) shows a pattern of overestimating the number of days in ice in almost every storage time, more pronouncing at small values of days in ice. Whereas, on the figure below, the model is overrating in the beginning of the prediction and underestimating the number of days in ice in the final days of prediction.

In general, when days in ice increases, with both PLS models the predicted values also increase in general. This clearly confirmed the possibility of using NIR spectroscopy for plaice freshness prediction.

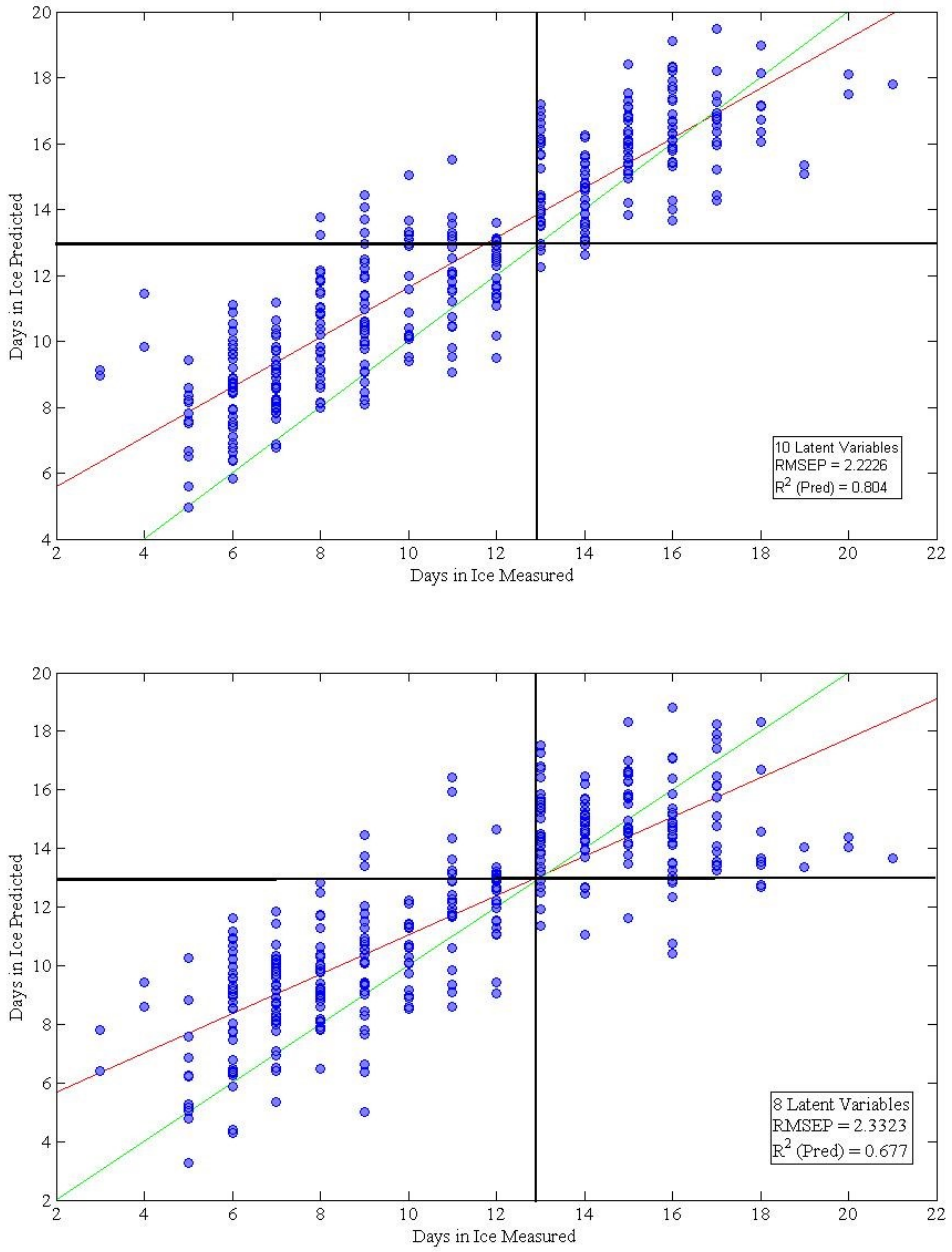


Figure 13- Predicted versus measured days in ice for first (top) and second (bottom) PLS models using the spectra of dark skin samples in the best wavelength range.

The regression coefficient plots of the two selected PLS models are displayed in Figure 14. In the first model, the relevant wavelengths are exhibit only for the important wavelength range set up before (940:1030 and 1400:1580 nm) while in the second model all wavelength range of the study is considerate vital (940.37:1698.9 nm). Higher regression coefficients correspond to the wavelength of approximately 1000, 1500 and 1570 nm for first model and 1000, 1520 and 1640 nm for the second. It is also noted that the selected wavelength ranges here cover the previous important wavelengths analysed in the preliminary study.

Phenolic compounds and aromatic compounds are associated with absorption in the wavelength of approximately 1000 nm (N-H 2nd overtone), while amines are typically absorbed between 1520-1570 nm (N-H 1st overtone) and near 1650 nm (C-H 1st overtone) (Workman & Weyer, 2012). Although the only information given by wavelength interpretation of those graphics is the functional group of compounds, the data provided converged with the knowledge of the compounds related to fish decaying (development of aromatic compounds, ammonia, between others) (Rehbein & Oehlenschläger, 2009).

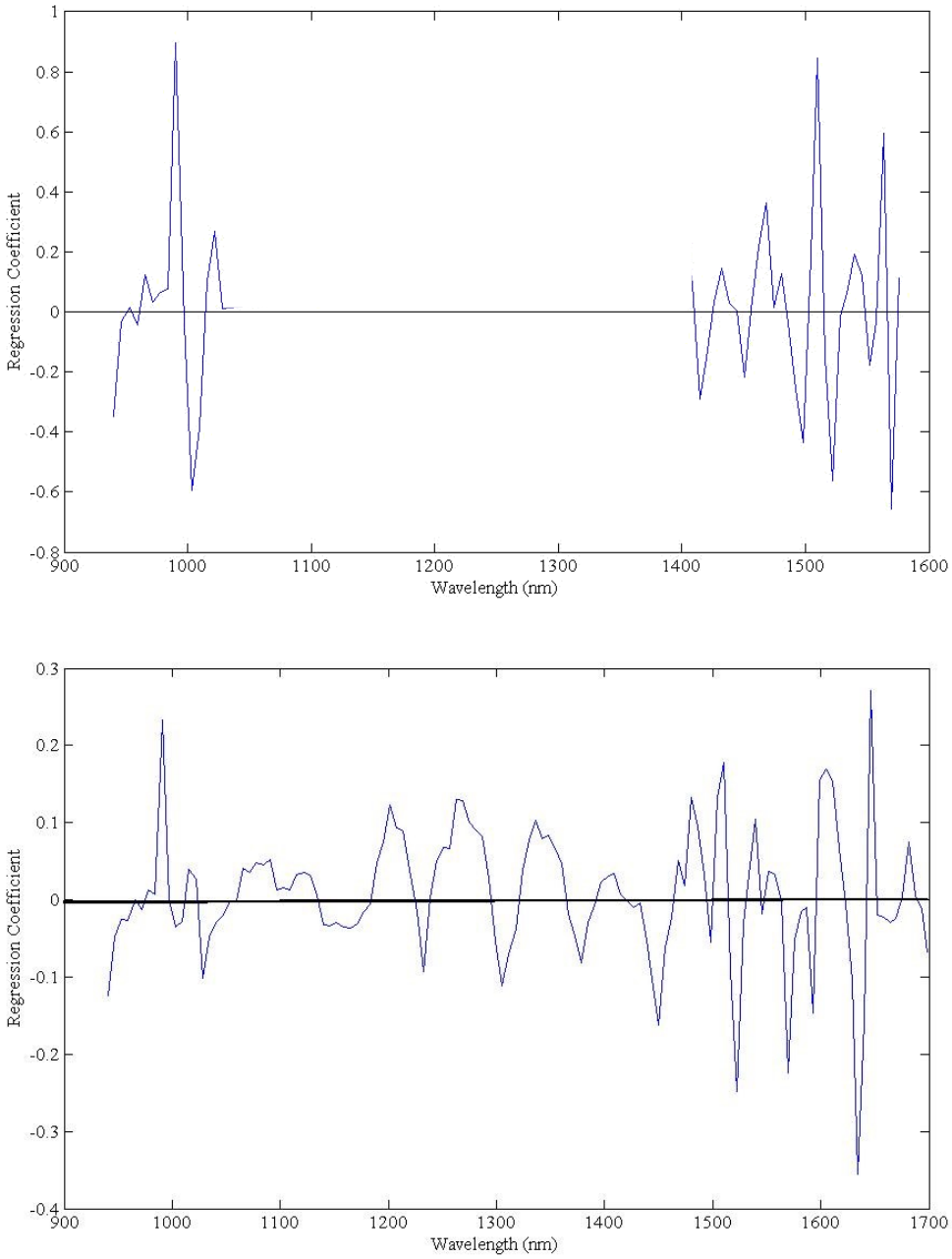


Figure 14 - The regression coefficients in the first (top) and second (bottom) PLS calibration models using the spectra of dark skin samples in the best selected wavelength ranges.

1.2 White skin spectral data

Figure 15 shows the evolution of SWIR spectra, ranging from 940 to 1700 nm, measured on white skin samples over storage time. There is generally a shift in reflectance spectra of the white skin when comparing with those of the dark skin. The valley from 940 to 1050 nm is related to O-H bonds absorption which is associate with alcohols and phenolic compounds (Workman & Weyer, 2012). A broad peak between 1150 and 1250 nm is correlated with C-H bond absorption and a valley between 1400 and 1500 nm is linked with C-H, O-H, N-H and C=O bonds from aromatic, phenolic, alcoholic and carbonyl compounds, aromatic amines and proteins (Workman & Weyer, 2012).

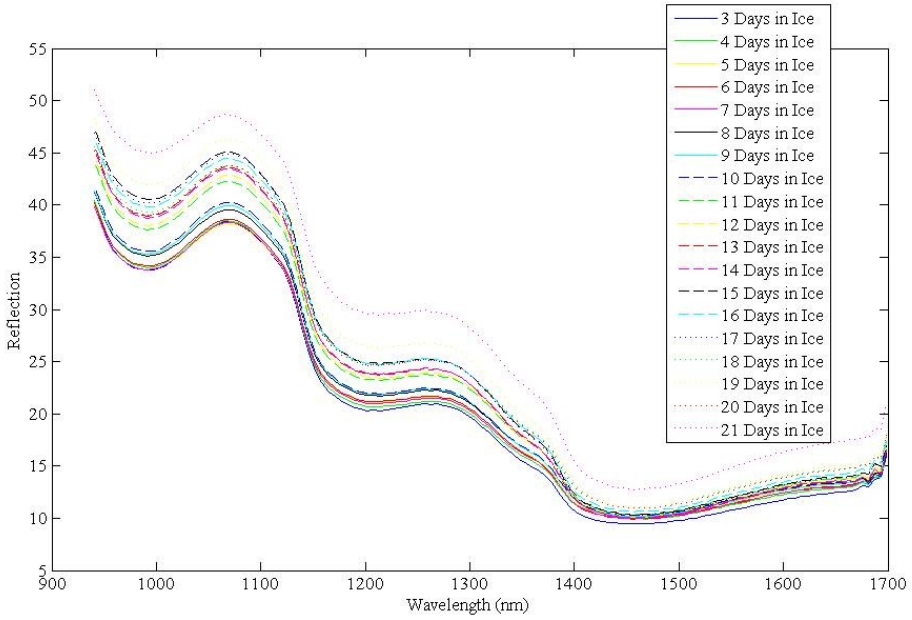


Figure 15 - Measured mean SWIR spectra of the fish samples on the white skin side with increasing days in ice.

The best models were selected, after testing all different pre-processing strategies and Y-variables transformations, based on lowest RMSECV, RMSEP and number of latent variables and higher R^2_p, and are display in Table 4.

Table 4- Selected PLS models to predict storage time using white skin sepctra..

Cross Validation	**Y**	**Pre-processing [6]**	**Points excluded**	**Full wavelength range**		**Best selected wavelength range using i-PLS**		
				No. Latent Variables	**RMSECV RMSEP R^2_p (days in ice)**	**Wavelength range (nm)**	**No. Latent Variables**	**RMSECV RMSEP R^2_p (days in ice)**
Custom 1	--	MSC + D2 + AS	--	7	2.3564 2.5933 0.677	940:1700	7	2.3564 2.5933 0.677
Custom 2	--	MSC + D2 + AS	--	8	2.3601 2.5307 0.699	940:1700	8	2.3601 2.5307 0.699

[6] MSC – Multiplicative Scatter Correction; D2 – Second Derivative (Savitzky-Golay); AS – Autoscale.

The prediction results in Table 4 are slightly worse than those for the dark skin side in Table 3. For both cross-validation strategies in Table 4, the best selected wavelength ranges correspond to the whole wavelength range measured: 940 to 1700 nm.

Although the first model has proven to have a slightly better result for RMSECV, the others parameters of RMSEP and R^2_p are slightly surpassing with the second one. However, the differences between this last two parameters are minimal (0.0626 days in ice for RMSEP and 0.022% for R^2_p). It is important to denote that the first model utilizes one less latent variable than the second one and thus, it has less complexity without discarding the necessity of accuracy and efficiency for freshness prediction.

Nevertheless, the obtained errors of 2.5 / 2.6 days in ice are considered high by the industry and also according to the commercial shelf life of 13 days in ice for plaice. Although errors criteria for freshness prediction on plaice fish are not described in literature, it is interesting to compare them to similar publishing studies performed in other fish species.

The results obtained in this work is marginally higher than those attained by Kimiya *et al.* (2013) regarding the prediction of storage days in ice of salmon fillets using VIS/NIR spectroscopy (2.4 days in ice); and remarkably higher than 1.04 days in ice for cod (Nilsen *et al.*, 2002); 1.20 days in ice for salmon (Nilsen *et al.*, 2002) and 1.6 days in ice for cod fillets (Sivertsen *et al.*, 2011). Most these studies were performed in the muscle side of the fish (skin removed) and using a wavelength range mostly including visible light.

Figure 16 below it is possible to see the plots of predicted days in ice versus the actual converted days in ice for the first and second models.

Considering the first model, on Figure 16 (top plot), it is observed that until the fourteenth day in ice, the estimations were overrated. The same circumstances were found in the dark skin models. After the 14^{th} day, underestimation for the predicted values is observed but, as previously discussed, it is not worth worrying because it is past the plaice commercial shelf-life (13 days in ice). In the bottom side of Figure 16, for the second model, before approximately the 15 days in ice, the red line is on the top of the green line which leads to the overestimation and after 15 days in ice, underestimation of prediction occurs. In general, and similarly to the case of dark skin side, when days in ice increases, with both PLS models the predicted values also increase. This also clearly confirmed the possibility of using NIR spectroscopy for plaice freshness prediction.

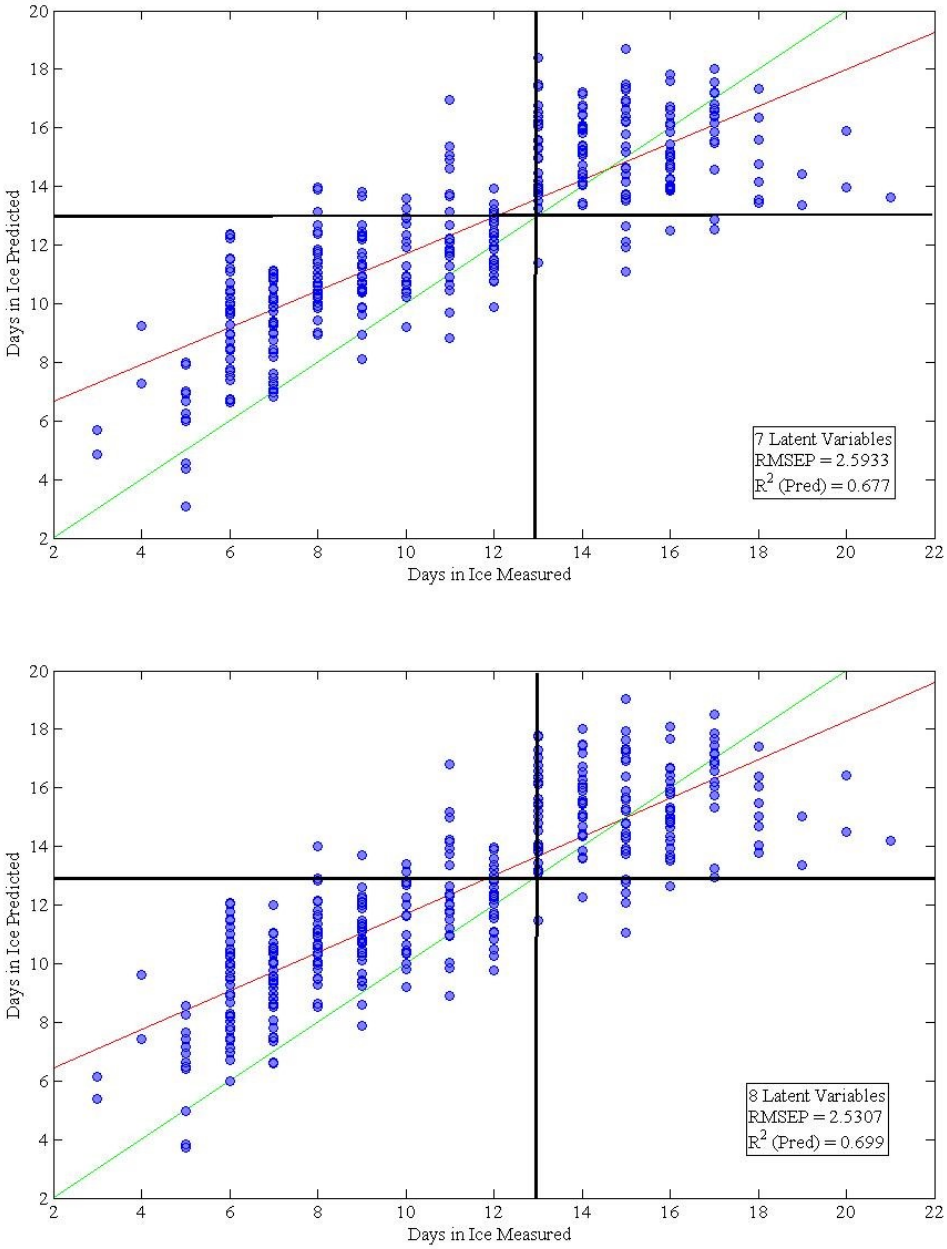


Figure 16 – Predicted versus measured days in ice for first (top) and second (bottom) PLS models using the spectra of white skin samples in the best wavelength range.

Figure 17 shows the regression coefficient plots of the two PLS models. It is easily recognized that the important wavelengths for the two models are generally the same. Peaks can be found around 1300, 1520, 1560, 1630 and 1640 nm. Some of these are in compliance with the ones obtained in the case of dark skin side.

Amides and amines are related with absorption at 1520 and 1560 nm (N-H 1st overtone) (Workman & Weyer, 2012). Amines are one functional group with great representability in fishes' degradation: volatile compounds, trimethylamine, dimethylamine and biogenic amines (Rehbein & Oehlenschläger, 2009). The chemical information used by the PLS models show good agreement with chemical constituents existing during fish degradation as stated in theory.

Finally, it is concluded that predicting plaice storage days in ice could be based on spectral measurements either on the white skin or darks skin side and slightly better prediction performance was obtained when using the dark skin spectra.

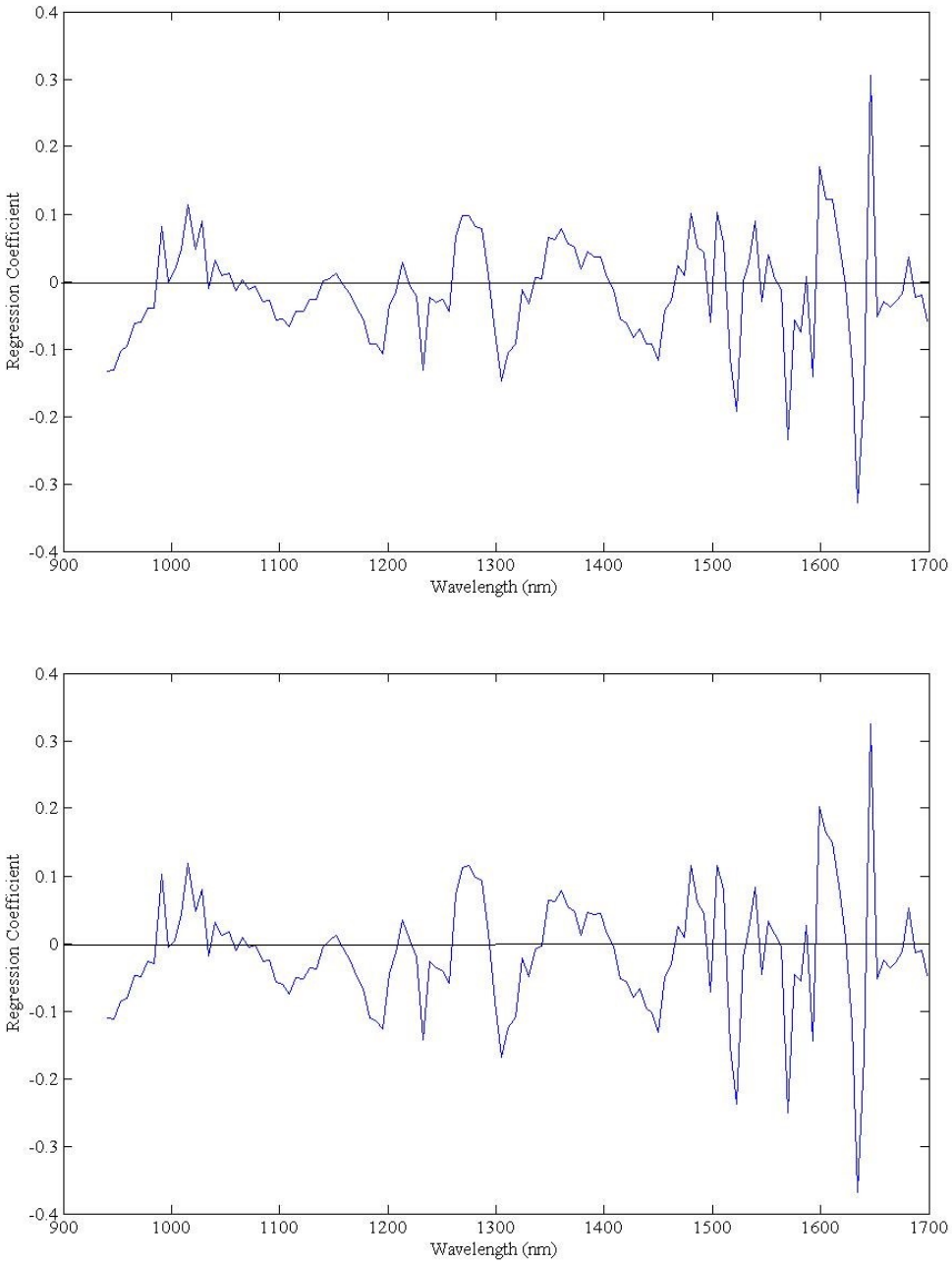


Figure 17- The regression coefficients of the first (left) and second (right) PLS calibration models using the spectra of white skin samples in the best selected wavelength ranges.

V. General Conclusions

NIR spectroscopy proved its capacity to hastily and in a non-destructive way to predict plaice freshness expressed as storage time in ice.

Good results were obtained in the preliminary study where accuracy between 95.2 and 100.0 % were found for 2 class discrimination (fresh and unfresh) and 70.0 and 83.3 % for 4 class discrimination (0h, 4h, 24h and 28h at room temperature). In the first case (2-class discrimination), better results were performed by the dark skin side measurements while in the second case (4-class discrimination), the muscle side measurements culminated in highest accuracy percentages and lowest misclassification percentages. The best models included the selected wavelength ranges of [1063:1271] nm and [801:2782] nm for the first and second case, respectively.

The followed main research was to evaluated NIR spectroscopy model's robustness in predicting plaice's storage time in ice converted from graded QIM scores using the wavelength range of 940 to 1700 nm. The best model for dark skin samples, using the whole wavelength region, culminated in errors of prediction of 2.017 and 2.269 for RMSECV and RMSEP; and 0.745 for R^2_p. However, buy reducing the wavelength range to two selected regions, 940 to 1030 nm and 1400 to 1580 nm, model parameters were improved, resulting in 1.8239, 2.2226 and 0.804 for RMSECV, RMSEP and R^2_p. White skin samples best model was obtained with the full wavelength range and lead to 2.3564, 2.5933 and 0.677 for RMSECV, RMSEP and R^2_p. All prediction errors determined were considered slightly higher than those obtained in other studies (Nilsen *et al.*, 2002) and also when compared relatively with the commercial storage time in ice of plaice (13 days in ice).

With respect to commercial use of this NIR technology, the fact that blood stains presence on the used plaice samples and the fish size variation did not affect the ability to predict days in ice is considered a strength. NIR spectroscopy is also recognised for being a fast inspection technique without the need of samples preparation.

VI. Future Work

Although the objectives of this research were attained, future research should be done in corresponding the selected wavelength range of each model to the specific deterioration process. This research could be performed by evaluating the correlation between the spectroscopy results with other measurement methods such as sensory evaluation and chemical/biochemical methods.

In upcoming work, it should be of upmost importance to consider other fish handling and storage parameters as well as seasonal or regional variations. Visible range of the electromagnetic spectrum should also be studied since this optical region access human sensory perception and would provide lower sensor costs. Since plaice skin evolves from bright and incandescent to dull and mat, visible light might register these changes and therefore, be effective and efficient in freshness determination.

Hyperspectral imaging using the NIR and visible ranges could also be further investigated for plaice freshness evaluation since this imaging technology has a big advantage of providing spatial distribution (at pixel level) for the quality attribute of interest on a certain fish sample. This could help to retain the parts that are still fresh and cut off the unfresh parts on a fish to save food materials and to reduce waste.

In the future, some further large-scale on-line studies in the fish industry conditions would be needed to verify the accuracy of NIR spectroscopy under real conditions. Nevertheless, NIR spectroscopy has already been proven to be suitable for freshness quality control for plaice fish.

VII. Annexes

Annex 1: Estimated shelf life of some fish species stored in ice

Table 5 - Estimated shelf life of some fish species stored in ice (Martinsdóttir et al., 2001).

Species	Estimated shell life in ice
Brill (*Rhombus laevis*)	14 days
Cod (*Godus morhua*)	15 days
Depp water shrimp (*Pandalus borealis*)	6 days
Farmed salmon (*Salmo salar*)	20 days
Fjord shrimp (*Pandalus borealis*)	6 days
Haddock (*Melanogrammus aeglefinus*)	15 days
Herring (*Clupea harengus*)	8 days
Peeled shrimp (*Pandalus borealis*)	6 days[7]
Plaice (*Pleuronectes platessa*)	13 days
Pollock (*Pollachius virens*)	18 days
Redfish (*Sebastes mentella/marinus*)	18 days
Sole (*Solea vulgaris*)	15 days
Turbot (*Scophtalmus maximus*)	13 days

[7] The storage life before peeling.

VIII. Appendix

Appendix 1: Quality evaluation of fish quality

Fresh fish is commonly understood as fish being caught/harvested and then chilled and stored for a short period before use (Sivertsen *et al.*, 2011b).

In the sense of providing fish products of high quality, several methods and techniques have been developed to evaluate fish freshness. These approaches may be based on biochemical and instrumental measurements, methods using refractive index or spoilage and freshness indices and techniques based on sensor technology such as colorimetric sensor array, semiconducting metal-oxide sensor array, gas sensor or electronic nose (Cheng *et al.*, 2015).

a) Sensory methods

Sensory methods are used to interpret food characteristics as they are perceived by the senses of sight, smell, taste related to odour, flavour and texture. It is considered a useful tool due to its wide range of applications in fish freshness evaluation (Cheng *et al.*, 2015).

Sensory testing can be both objective and subjective. The objective tests include discriminative (triangle test and forced choice, which indicate whether there is a difference between the samples) (Ólafsdóttir *et al.*, 1997), descriptive (profiling, quality index method and structured scaling) and affective tests (market test) (Huss, 1995). For the first two types of tests, a trained panel is used but affective tests are subjective consumer tests that are based on a measure of preference or acceptance (Ólafsdóttir *et al.*, 1997).

Flavour changes can be measured by difference tests such as triangle tests, paired comparison tests, ranking tests, between others as well as by instrumental means, gas chromatography, high-performance liquid chromatography or piezo electric crystals-mass balance measurement (Pearson & Dutson, 1994).

The most common methods of sensory evaluation include European Union (EU) scheme and Quality Index Method (QIM). QIM has been gradually replacing EU scheme (or EU freshness grading scheme) and it has become the reference method for freshness evaluation. (Cheng *et al.*, 2015).

In the United Kingdom, Torry Sensory Assessment scheme is also used. Although there are variations on the execution of these schemes, they all rely on using the physical characteristics of raw seafood to determine a score or rating indicating the freshness quality of the products. In Torry and QIM the score is used to estimate the “days in ice” of the seafood, although the QIM also allows to estimate the remaining shelf-life (Archer, 2010).

1. *Grading schemes: EU-scheme and Torry scheme*

Grading is the process of applying a categorical value to a lot or group of products. Generally, it involves a process of integration of perceptions by the grader. The grader is asked to give one overall rating of the combined effect of the presence of the positive attributes, the blend or balance of those attributes, the absence of negative characteristics and the comparison of the products being graded with some written or physical standard (Rehbein & Oehlenschläger, 2009).

Specific for seafood, the EU regulation "Council Regulation (EC) No 2406/96 of 26 November 1996, laying down common marketing standards for certain fishery products" has only one sensory method in place: the EU-scheme for fresh fish. This method is to be used at first point of sale and implies freshness and other quality items (parasites, pressure marks, injuries, blemishes and bad discolouration). There are different schemes depending on the fish species and it is supposed to be used by experts or by the competent authority (inspection body). The method is not suitable for predicting the shelf life, nor for statistical analyses of reliability and reproducibility (Rehbein & Oehlenschläger, 2009).

The Torry scale is the first detailed scheme developed for evaluating the freshness of cod and it is most frequently used on industry scale for evaluating the freshness of cooked fish. It is a descriptive 10-point scale that has been developed for lean, medium fat and fat fish species. Some results showed a high correlation between Quality Index Method scores and Torry scores (Rehbein & Oehlenschläger, 2009).

2. *Quality Index Method*

The Quality Index Method (QIM) was developed at the Tasmanian Food Research Unit (TFRU), Australia in the late 1970s and early 1980s with the purpose of overcoming the inherent limitations contained in the EU grading scheme. It has been adopted in different countries as an alternate sensory technique using specific descriptive attributes for selected species that are evaluated (Alasalvar *et al.*, 2011).

QIM has turned out to be the foremost reference method for quality assessment of fresh fish based upon an objective evaluation of the pertinent attributes of raw fish using a demerit points scoring system (0-3) that gives scores of zero for very fresh fish and increasingly larger total result as the fish deteriorates (Table 6) (Cheng *et al.*, 2015).

Table 6 - Quality Index Method (QIM) scheme for plaice (Archer, 2010).

Quality parameter		Descriptions	Point
Appearance	Skin (both dark and white side)	Fresh, bright, metallic, no discolouration	0
		Bright, but without shine	1
		Matt, rather dull, slight green/blue or purple discolouration	2
		Dull, green/blue, purple discolouration	3
	Mucus	Clear, not clotted	0
		Slightly clotted and milky	1
		Clotted and slightly yellow	2
		Yellow and clotted	3
Eyes	Form	Convex	0
		Convex but slightly sunken	1
		Flat or swollen (like a balloon)	2
		Flat, sunken in the middle	3
	Brightness	Clear, black shining pupil	0
		Rather matt, black pupil	1
		Matt, opaque pupil	2
		Milky, grey pupil	3
Gills	Odour	Fresh oil, seaweedy, metallic, peppery	0
		Neutral, oily, grassy, slightly musty	1
		Musty, bread, beer, malt, slightly rancid	2
		Rancid, sour, rotten, sulphurous	3
	Colour	Bright, light red	0
		Slightly discoloured, especially at the end of gill filaments	1
		Discoloured	2
		Yellowish, brown, grey	3
	Mucus	No mucus	0
		Clear	1
		Yellowish, slightly clotted	2
		Yellow, brown, clotted	3
Flesh, fillets	Colour	Fresh, translucent, bluish	0
		Waxy, milky	1
		Dull, slightly discoloured, yellowish	2
		Opaque, discoloured, yellow, brown	3
Quality Index			0-24

QIM reflects the different quality levels in a simple, reliable and documented way, in addition of being rapid, cheap to use, non-destructive and objective compared with other sensory methods (Rehbein & Oehlenschläger, 2009).

(b) Biochemical and chemical methods

Chemical measurements are mainly associated with chemical composition changes of fish and are indispensable for evaluating fish freshness (Cheng *et al.*, 2015). There are available various chemical methods for monitoring fish quality and safety which are mainly associated with moisture measurement, volatile compounds measurements, ATP decomposition, K value measurement (Cheng *et al.*, 2015), protein degradation measurements techniques, lipid oxidation and compositional changes of amino acids and fatty acids (Cheng & Sun, 2014).

1. Moisture measurement

Moisture content is a very important parameter for fish freshness evaluation because it is related to fat content and affects microbial growth (He *et al.*,2013) which can affect the muscle of fish and consequently the texture (Cheng *et al.*, 2015).

The moisture is unevenly distributed, with high shares in the thick loin parts and much lower in the thinner belly flaps and tail. The surface of fish is usually much drier than the interior and is normally covered by a layer of salt which can make it difficult to get good results. The water content is the most important criterion in the market: low percentage of moisture gives higher price per kg fish. (Wold *et al.*, 2006).

Traditional technique for measure the moisture content is oven-drying method, although some new methods have been tested: freeze-drying or lyophilisation, electronic moisture analyser and NIR spectroscopy (Khodabux *et al.*, 2007; He *et al.*, 2013).

2. Volatile Compounds Measurements

Odour is one of the main indicators that consumers use to assess fish freshness. The smell of fish changes rapidly according to the product's degree of freshness and it can be used as a quality indicator by the measurement of the key volatile compounds (Duflos *et al.*, 2010).

Analysis of volatile components in food is still a challenging process due to the presence of extremely low levels of volatile solutes in highly complex non-volatile matrices and thus, it is required isolation or sampling of volatiles prior to instrumental analysis (Alasalvar *et al.*, 2011).

Headspace methods for the analysis of volatile compounds involve the collection and concentration of the volatiles for subsequent chromatographic separation to identify and quantify the separated compounds. Extremely volatile, low molecular weight compounds can be analysed by static headspace methods. More efficient, dynamic headspace methods are necessary for collecting and concentrating less-volatile compounds such as those contributing to "fresh fish" and "oxidized" odours (Ólafsdóttir *et al.*, 1997). If the interested compounds are low-boiling then solvent extraction and evaporation are not appropriate methods because of its high percentage of lost recovered compounds (Pearson & Dutson, 1994).

After extraction, instrumental analysis of the components is required. There are new methods and techniques for detection that have been developed such as solid-phase micro-extraction headspace analysis (Triqui & Bouchriti, 2003), gas chromatography (GC) mass spectrometry (Duflos *et al.*, 2010), the electronic noise (EN) technique (Ólafsdottir *et al.*, 2004; Limbo *et al.*, 2009; Dini *et al.*, 2010; Ólafsdóttir *et al.*, 1997, 2004) and the computer screen photo-assisted technique (CSPT) based gas sensor array (Alimelli *et al*., 2007).

i. Amines – Total Volatile Basic Amines

Total volatile basic amines is one of the most widely used measurements of seafood quality. This term generally includes the measurement of trimethylamine (TMA) (produced by spoilage bacteria), dimethylamine (DMA) (produced by autolytic enzymes during frozen storage), ammonia (produced by the deamination of amino acids and nucleotides) and other volatile basic nitrogenous compounds associated with seafood spoilage (Huss, 1995). Many industries have used TVBN (total volatile basic nitrogen) and/or TMA as indicators of fish freshness (Cheng *et al.*, 2015).

The principle for measuring TVBN is very straightforward: a suspension of fish muscle or an extract of fish muscle is made alkaline and the free bases are distilled, usually at boiling point at atmospheric pressure, collected, and estimated using standardised acid or alkali. (Rehbein & Oehlenschläger, 2009). The reference method involves steam distillation of an extract deproteinised by perchloric acid (Castro *et al.*, 2006).

Fish exceeding certain limits of the TVBN value is considered unsuitable for human consumption. TVBN cannot identify the early stages of deterioration of freshness quality and is generally considered unreliable for the measurement of spoilage during the first 10 days of chilled storage of cod as well as other species but it can identify later stages of spoilage and it is routinely used as the standard method to determine spoilage of chilled, frozen, dried and

canned seafood (Özogul et al., 2005a). Özogul *et al*, (2005a) suggested that the acceptability limit of TVBN level could be about 10 mg TVBN per 100g flesh.

Additionally, an innovative method using ammonium ion-selective electrode (NH_4-ISE) to measure the signal changes of NH_4^+-ISE, due to the changes of the ammonia content of the fish were developed and the results were correlated well with the content of volatile amines (TVBN) in cod fillets (Heising *et al.*, 2012).

ii. Ammonia

Ammonia is formed by the bacterial degradation/deamination of proteins, peptides and amino acids. There are two convenient methods which are specific for identifying ammonia: the first involves the use of the enzyme glutamate dehydrogenase, NADH and alpha-ketoglutarate. The molar reduction of NH_3 in a fish extract yields one mole of glutamic acid and NAD which can be monitored conveniently by absorbance measurements at 340 nm. The second method uses a modification of the glutamate dehydrogenase procedure to determine the ammonia levels semi-quantitatively without the use of a spectrophotometer, but with a formazan dye, which changes colour (Huss, 1995).

iii. Trimethylamine (TMA)

TMA is an important smelly odour and can indicate the spoilage degree of fish (Cheng *et al.*, 2015). It is present in spoiling fish due to the bacterial reduction of trimethylamine oxide (TMAO) which is present in the living tissue of many marine fish species (Huss, 1995).

Analytical methods for TMA determination have been described – picrate method, specific ion electrode, high performance liquid chromatography (HPLC) and enzymatic flow injection. Unfortunately, these methods seem more suited to use in research laboratories and need further evaluation before possible use in commercial quality assurance (Rehbein & Oehlenschläger, 2009).

The picrate procedure, developed by Dyer, is described as follows: an aliquot of a trichloroacetic extract of fish muscle is taken, formaldehyde added, and the mixture made alkaline. The free bases (ammonia, dimethylamine and TMA) are extracted into toluene and reacted with picric acid to give a yellow-coloured picrate salt that can be measured in a spectrophotometer. Potassium hydroxide is used as the alkaliser due to the fact of being more efficient in suppressing interference from dimethylamine and in giving higher recoveries of TMA than the potassium carbonate used previously. The equipment requires is quite cheap and

the procedure can be performed by any competent laboratory assistant (Rehbein & Oehlenschläger, 2009).

iv. Dimethylamine (DMA)

Dimethylamine (DMA) is produced by autolytic enzymes during storage conditions. This compound is only formed in fish species that contain a specific enzyme, trimethylamine oxidase demethylase (TMAOase), which catalyses the cleavage of trimethylamine oxide (TMAO) into equimolar quantities of DMA and formaldehyde (FA) (Rehbein & Oehlenschläger, 2009).

DMA is usually measured as the dithiocarbamate by a procedure first applied to fish by Dyer and Mounsey (1945) and later modified to replace benzene as the solvent by chloroform, or by gas liquid chromatography (GLC) (Rehbein & Oehlenschläger, 2009).

v. Biogenic Amines

Biogenic amine determination is taken very seriously due to its potential toxicity as well as the possibility to using them as markers of food quality. Mietz and Karmas (1977) proposed a chemical quality index to establish the extent of decomposition in fresh tuna before canning. The relationship of five amines (histamine, putrescine, cadaverine, spermine and spermidine) in canned fish was quantified and calculate on a part per million basis in order to be used as an index of tuna decomposition:

$$Index = \frac{Histamine\ (ppm) + Putrecine(ppm) + Cadaverine(ppm)}{1 + Spermine\ (ppm) + Spermidine\ (ppm)} \qquad VIII.1$$

In general, the use of more than a single biogenic amine is advised to overcome the limitation of possible variability in the concentration of one amine, and has been considered a more appropriate quality indicator (Rehbein & Oehlenschläger, 2009).

There are several methods for histamine determination in fish products: amine oxidase-based flow biosensor, capillary zone electrophoresis (CE), colorimetric method with imidazole reacting p-phenyldiazonium sulfonate, DAO-based amperometric sensor, electrochemical biosensor, enzyme sensor array, enzyme-based screening test, flow injection determination with a histamine dehygrogenase-based sensor, fluorimetric method, HPLC-post column method, Ion chromatography-integrated pulsed amperometric detection, monoclonal anti-body based

ELISA, oxygen-sensor based method, thin-layer chromatography method, etc. (Rehbein & Oehlenschläger, 2009).

vi. Nucleotide Catabolites

While there is no single biochemical marker compound which may be used universally to indicate freshness or to predict future shelf-life, nucleotide degradation has been used as a biochemical indicator of fish freshness in several species (Wills *et al.*, 2004).

ATP alone cannot be used as a chemical index of fish freshness because it is so rapidly converted to inosine monophosphate (IMP). Concentrations of its intermediate degradation products rise and fall, making them unreliable indexes of freshness. Thus, attention has focused on inosine (HxR) and hypoxanthine (Hx), the terminal catabolites of ATP. HxR accumulates in some species of fish whereas Hx accumulates in others as terminal catabolites (Ólafsdóttir *et al.*, 1997). HxR and Hx concentrations in fish increased during storage and either of the two can be used as freshness indicators. However, the use of a single compound as freshness indicator is not advisable because many factors can affect nucleotide degradation such as the type of spoilage bacteria, mechanical handling of fish and the disappearance of the degradation products differs from one species to another (Nollet & Toldrá, 2010).

In that sense, and based on ATP decomposition, the quality indicator K value was introduced and it is defined as the ratio (%) of the total amount of HxR and Hx to that of ATP-related compounds, as described by the formula below:

$$\text{K value} = \frac{HxR+Hx}{ATP+ADP+AMP+IMP+HxR+Hx} \times 100 \qquad \textit{VIII.2}$$

A fresh fish will have a low K value. This parameter is dependent on: species, *post mortem* time, temperature storage conditions, handling conditions, method of killing, among others and therefore, it must be established a profile of K value versus time for each specie and its specific handling and storage conditions before K value can be used to evaluate freshness (Ólafsdóttir *et al.*, 1997).

Nevertheless, ATP, ADP and AMP disappear early *post-mortem*, generally within 1 day of storage in ice after death in all fish species and, consequently, a revised K value, often designed K' value is more often considered. K' value is defined as the ratio of HxR and Hx to the sum of IMP, HxR and Hx (Nollet & Toldrá, 2010).

The most common methodology used to evaluate ATP and its breakdown products is based on extraction of the nucleotides and derivatives in acid (perchloric and trichloroacetic acid), neutralization of the extracts and further separation by high performance liquid chromatography (HPLC) (Rehbein & Oehlenschläger, 2009). Other methods included ion mobility spectrometry technique (Wills *et al.*, 2004), nuclear magnetic resonance spectroscopy (NMR), high-performance capillary electrophoresis (HPCE), radioimmunoassay, thin-layer chromatography (TLC), reversed-phase high-performance liquid chromatography (RP-HPLC) with and without ion-pair, ion-exchange HPLC, ion chromatography (IC) and enzymatic assays (Nollet & Toldrá, 2010).

vii. Oxidative rancidity

Once the lipid oxidation initiated, the extent of lipid oxidation can be followed using either the reactants or the products. Measurements of oxygen consumption can be monitored with an oxygen electrode, whereas the loss of fatty acids and antioxidants can be measured using gas chromatography (GC) and high-performance liquid chromatography (HPLC) (Ólafsdóttir *et al.*, 1997). Free fat and the total fat can be measured using Soxhlet method and acid-hydrolysis method, respectively (Khodabux *et al.*, 2007).

Although several methods for measurement of hydroperoxides as lipid oxidation indices have been proposed, instability and diversity of such compounds in a complex food system hinder accurate and simple analysis. The widely accepted iodometric titration and the enzymatic assays have inherent problems with sensitivity, selectivity and interference with contaminations (Alasalvar *et al.*, 2011). The PV (peroxide value) is the most common measure of lipid hydroperoxides, also called primary lipid oxidation products (Ólafsdóttir *et al.*, 1997). Besides the titration method, several colorimetric methods can be used to determine this value, such as colorimetric ferric thiocyanate method or the International Dairy Federation (IDF) method. High-performance liquid chromatography (HPLC) can also be used to determine individual peroxides (Nollet & Toldrá, 2010).

Several studies have already related Raman spectroscopy to traditional methods to determine modifications in lipids of muscle foods, for example, Raman spectroscopy results and oxidation levels, determined by a traditional colorimetric method such as peroxide values, were related to lipids extracted from both mackerel and horse mackerel (Herrero, 2008).

To overcome the difficulties of sensibility and selectivity of traditional methods, the developed a flow injection analysis (FIA) system coupled with a fluorescence detection system

enables the determination of hydroperoxides in fish muscle at picomole levels during early stages of lipid oxidation (Alasalvar *et al.*, 2011).

Peroxides are unstable and rapidly metabolised into secondary oxidation products and thus, PV must be combined with secondary products determination such as thiobarbituric acid-reactive substances (TBARS) and anisidine value (AnV). The conjugation of the previously referred outcomes in the Totox value (Totox value = 2*PV + AnV) (Nollet & Toldrá, 2010).

Tertiary products, arising from interactions between oxidizing lipids and nitrogen-containing compounds, can be followed using fluorescence spectroscopy or, in later stages, by visual assessment or colorimetry (Ólafsdóttir *et al.*, 1997).

The thiobarbituric acid (TBA) value is considered as a helpful indicator for predicting the degree of lipid oxidation and it is usually expressed as mg melonaldehyde (MDA) per kg muscle (Cheng *et al*., 2015).

3. *Protein Changes*

It is possible to determined changes on the size of proteins by electrophoretic and chromatographic techniques although these techniques are unsuitable for industrial use (Ólafsdóttir *et al.*, 1997). Electrophoretic methods consist on: native isoelectric focusing (nIEF), urea IEF and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Rehbein & Oehlenschläger, 2009). SDS-PAGE technique has been studied for indicating protein changes, thus the freshness quality of fish but its measurement process is very tedious and the pH of the solution is difficult to control (Cheng *et al.*, 2015).

In the last decades, several attempts have been made to establish HPLC as an alternative to electrophoresis techniques due to its shorter time of sample preparation and possibility of automation (Rehbein & Oehlenschläger, 2009).

(c) Physical methods

1. *Colour*

Colour is one of the most significant physical property indicating fish freshness quality to consumers (Cheng & Sun, 2014) and results from the detection of light after it has interacted with an object (Luten *et al.*, 2003). For fish, skin and flesh discoloration is a very important problem since the skin and flesh colour must be very vivid (Nollet, 2012). The colours of eyes and gills are also parameters often used to estimate fish freshness. Normally they are analysed using the Quality Index Method but they can also be measure by machine vision although the

results obtained depend on the method used since both eyes and gills are very reflective (Genç *et al.*, 2016).

The measurement of colour can be carried out by visual inspection, using a trichromatic colorimeter or a spectrophotometer. This last one is the most accurate type of colour-measuring instrument (Pearson & Dutson, 1994). Spectrophotometer uses a light source to illuminate the object in study. The reflected light passes to a grating which breaks it into a spectrum that is then processed and transformed in the CIELAB values: L*, a* and b* (Rehbein & Oehlenschläger, 2009). In this system, L* denotes lightness on a 0–100 scale from black to white; a*: (+) red or (-) green; b: (+) yellow or (-) blue (Ólafsdóttir *et al.*, 2004).

1. Electrical Properties

Changes in fish freshness can also be determined by measuring the electrical properties of the fish muscle (Ólafsdóttir *et al.*, 1997). Measuring the electric properties of fish either transversal to the body axis or parallel with the body is a characteristic that can be measured by rapid, non-destructive and non-invasive methods and that can be carried out by untrained non-scientific personnel (Luten *et al.*, 2003).

Three different instruments are available to measure the changes in electrical properties: the Torrymeter, the Fishtester and the RT-Freshness Grader, which all show good correlations with sensory scores of fish freshness, when used within their applicable range of operation. The advantage of electrical testers is their immediate responses and their suitability for field use and for use by personnel without previous experience (Ólafsdóttir *et al.*, 1997).

2. pH and Eh

An important quality characteristic of seafood is pH value, which is closely related with protein, fat and other quality attributes. Commonly, pH is measured by using a pH meter by placing the electrodes either directly into the flesh or into suspension of fish flesh in distilled water (Huss, 1995). Nevertheless, this process is invasive and time-consuming which makes it unsuitable for evaluating large amounts of samples. Furthermore, pH measurements using pH meter does not allow visualize the pH distribution which is critical and necessary for the quality and safety inspection and control of fish products (He *et al.*, 2012).

Eh is a fish freshness measurement based on dielectric properties on fish tissue which shows the relationship between the occurrence of O_2 and microorganism measured by an electrometer. The redox or oxidation-reduction (O-R) potential (*Eh*) measures the potential

difference in a system generated by a coupled reaction in which one substance is oxidized and the other is reduced. Redox potential values measured depends on pH, each measurement of redox potential should be accompanied by a statement on pH (Susanto *et al.*, 2011).

3. Texture

The firmness of raw fish muscle is a critical parameter that determines the acceptability of the seafood products (Casas *et al.*, 2006). The texture of fish flesh depends on intrinsic biological factors related to muscle fibres, collagen (responsible for tensile strength) and myofibrils (myosin and actin) (Wu *et al*., 2012), as well as the acidity of the muscle which itself depends on the pre-slaughter stress or activity of the fish (Alasalvar *et al.*, 2011). After death and resolution of *rigor mortis* the autolytic spoilage disrupts the fibres and the muscle becomes softer and less elastic (Luten *et al.*, 2003).

There are several methods and techniques for measuring texture but they can be divided in two main approaches: organoleptic assessment and instrumental methods. The first approach uses trained taste panels that perform routine assessments of fish, which includes visual examination and hand touch with raw materials (Wu *et al*., 2012). The execution of the design described is time-consuming, laborious, tedious, inconsistent, requires skilled personnel (Ashton *et al.*, 2010) and is considered a subjective method as it relies in human inspection (Pearson & Dutson, 1994; Khoshtaghaza *et al.*, 2016).

In that sense, instrumental methods are preferred as their assessments may reduce variation among measurements due to human factors and are more precise (Casas *et al.*, 2006). Among textural attributes (hardness, adhesiveness, cohesiveness, springiness, chewiness, gumminess and fracture ability) (Nollet, 2012), hardness is the most important to consumer and, therefore, is decisive on the commercial value of the meat. (Nollet & Toldrá, 2010).

Besides existing diverse instrumental methods for texture analysis, not all the techniques can be applied to fish (Khoshtaghaza *et al.*, 2016). Moreover, there is no ideal texture measurement equipment or system. Khoshtaghaza *et al.* (2016) used the compression test method to analyse fish texture. This method is equivalent to the traditional and widely used method of finger-like compression test which is used for fish freshness detection. In this test, if the pressure region returns to its initial state, then the fish is fresh, otherwise, if the shape is changed, then the sample is not fresh.

Other possible instrumental texture methods are the Kramer test, Warner-Braztler (Peasron & Dutson, 1994), puncture, tensile and viscoelastic methods such as stress relaxation, creep and oscillatory measurements (Rehbein & Oehlenschläger, 2009).

One of the problems that restricts the routinely use of instrumental methods by the industry is its non-representability due to its impossibility to measure several samples and the fact that the measurements are not done to the whole fish. Besides this, instrumental methods are destructive, laborious, costly and require lengthy sample preparation (Wu *et al.*, 2012).

(b) Microbiological methods

Microbial deterioration process is one of the main contributors to the *post mortem* changes of fish, causing fish spoilage and therefore, affecting its shelf life (Cheng *et al.*, 2015). Microbial growth and metabolism result in the formation of amines, sulphides, alcohols, aldehydes, ketones and organic acids with unpleasant and unacceptable off-flavours, as well as some discolouration (Gram & Dalgaard, 2002).

Microbial methods can provide useful measures of fish freshness; however, the most promising results have been achieved with relatively slow detection methods such as plate count and other growth techniques that involve a period of incubation (Ólafsdóttir *et al.*, 1997).

1. Total Viable Counts

The total viable counts (TVC) is the traditional method used to assess the freshness of different kinds of aquatic products. Based on TVC index, most countries have established standards, guidelines and specifications of fish freshness under diverse storage conditions of temperature, time and atmosphere. This indicator is convenient for accurate detection of the degree of fish freshness and for predicting the remaining shelf life of fish (Cheng *et al.*, 2015).

Upon fishing, seafood is not exempted of microorganism, containing an initial value of TVC of approximately 10^2-10^4 CFU/g (Gram & Dalgaard, 2002).

TVC method is time-consuming and inconvenient both in terms of operation and collection of data. Several methods have been explored to improve the efficiency of the viable cell count procedure as the spiral plating method, the counting spiral-plated colonies, the autoplater, the isogrid system, the petrifilm system, the redigel system, the direct epifluorecent filter technique and the double-tube method (Pearson & Dutson, 1994). This led to the development of a faster alternative for inspection of microorganisms, the EN (electronic noise)

technique which have revealed to be able to classify in three freshness stages a sardine sample that were according to the results obtain with TVC values (Barbri *et al.*, 2009).

2. *Spoilage Bacteria*

Studies on bacteria that can produce smelly odours, like the common sulphide producer *Shewanella putrefaciens*, could be an indicator to determine the time to sensory rejection (Kyrana & Lougovois, 2002; Lougovois *et al.*, 2008). This microorganism was determined as the specific spoilage organism (SSO) of some chilled fresh fish and can be enumerated in iron-containing agar. Correlation coefficients as high as 0.97 were achieved when comparing log numbers of *S. putrefaciens* with the remaining shelf life of aerobically stored fish, as determined by sensory evaluation (Ólafsdóttir *et al.*, 1997).

Photobacterium phosphoreum was considered a SSO in some modified-atmosphere packed (MAP) fish and it can be specifically identified using a conductance technique which has proved to have a good correlation with the remaining shelf life of MAP cod fillets (Ólafsdóttir *et al.*, 1997).

3. *Pathogenic Bacteria*

Fish and fish products are known vehicles for transmission of foodborne diseases. Pathogenic bacteria associated with seafood can be categorised into three general groups: bacteria (indigenous bacteria) that belong to the natural microflora of fish (*Clostridium botulinum,* pathogenic *Vibrio* spp., *Aeromonas hydrophila*); enteric bacteria (non-indigenous bacteria) that are present due to faecal contamination (*Salmonella* spp., *Shigella* spp., pathogenic *Escherichia coli*, *Staphylococcus aureus*); and bacterial contamination during processing, storage or preparation for consumption (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfingens*) (Rehbein & Oehlenschläger, 2009).

The prevalence of pathogenic bacteria in samples can be very low and it is possible that there is a lack of proper selective microbiological media for their detection. Therefore, enrichment in liquid media is often needed. In such cases, serial dilution tests measuring the concentration of a target microbe in a sample with an estimate – the most probable-number (MPN) methods – can be used (Rehbein & Oehlenschläger, 2009).

Standards methods for recovering microorganism from foods may include enrichment culture, streaking out onto selective or differentiating media or direct plating onto these, and identification of colonies be morphological, biochemical and immunological tests. This

requires manual labour, is expensive and usually needs between 2 and 5 days. Furthermore, the results may be incorrect due to the influence of cultivation conditions or the fact that certain bacteria might not grow on the medium used (Rehbein & Oehlenschläger, 2009).

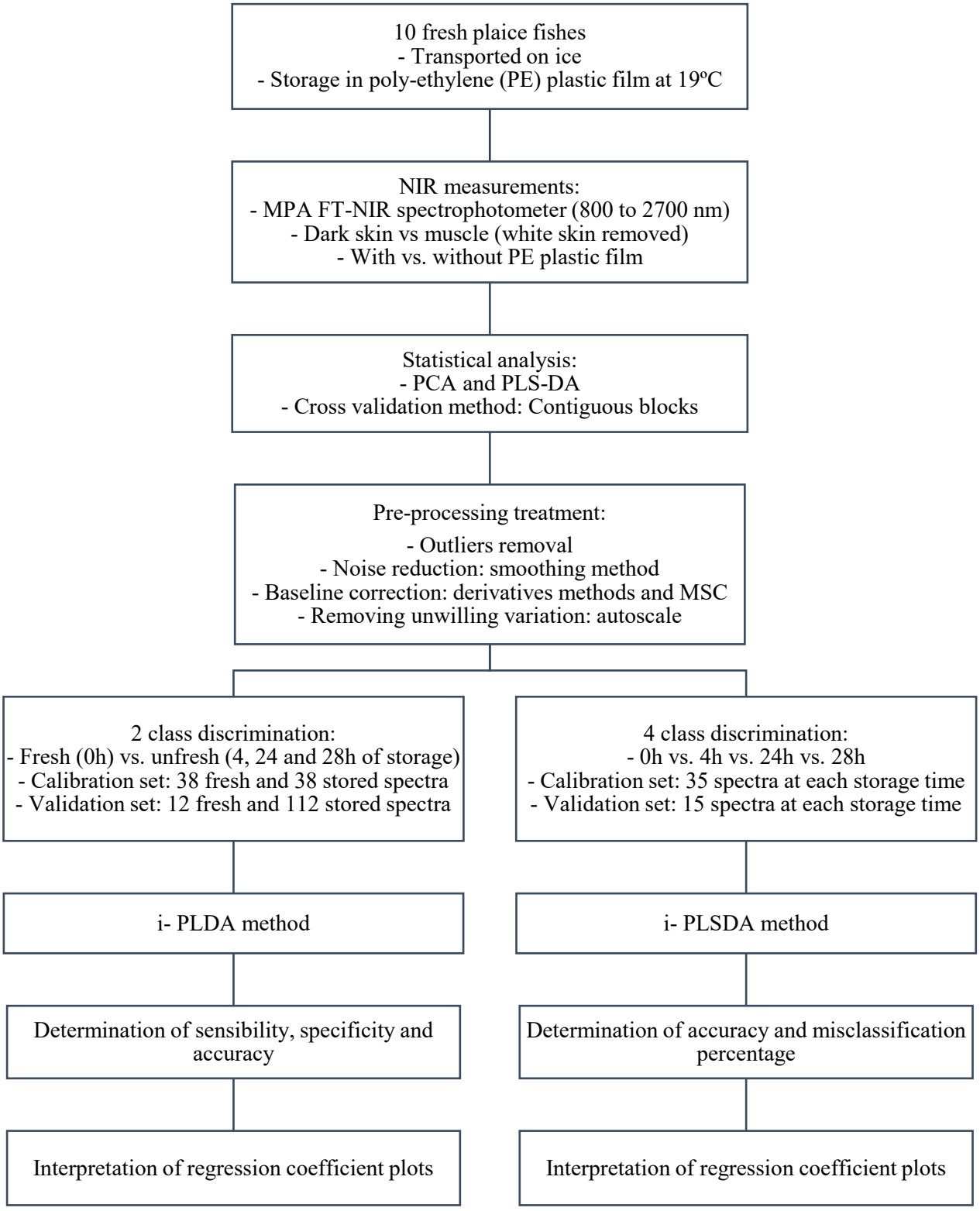
4. Foreign Contaminant Detection

Although human consumption of parasites in fish muscle is not considered a healthy issue if the fish is exposed to normal cooking temperatures, the finding of nematode infection in fish muscle will cause immediate consumer rejection of the product (Heia *et al.*, 2007).

In wild-caught marine fish, *Anisakis simplex* is among the most frequently occurring parasites and besides the considerable quality-reducing effect of *Anisakis* larvae with respect to seafood products. They are of direct human health concern, especially regarding the increasing interest in Asian-inspired seafood dishes based on undercooked, brined or marinated, or even raw fish meat (Werner *et al.*, 2010). Currently, there is no effective method available to detect parasites except manual vision inspection on candling tables. Still, manual vision inspection is inaccurate, time-consuming and laborious (Cheng *et al.*, 2015). The fillets are placed onto a white light table, and parasites embedded to a depth of 6 mm into the fillet can be spotted and removed manually, although the efficiency rate is only 60 to 70 % (Heia *et al*, 2007).

Appendix 2: Preliminary study

Appendix 2.1: Flow chart



Appendix 2.2: Plastic foil spectra

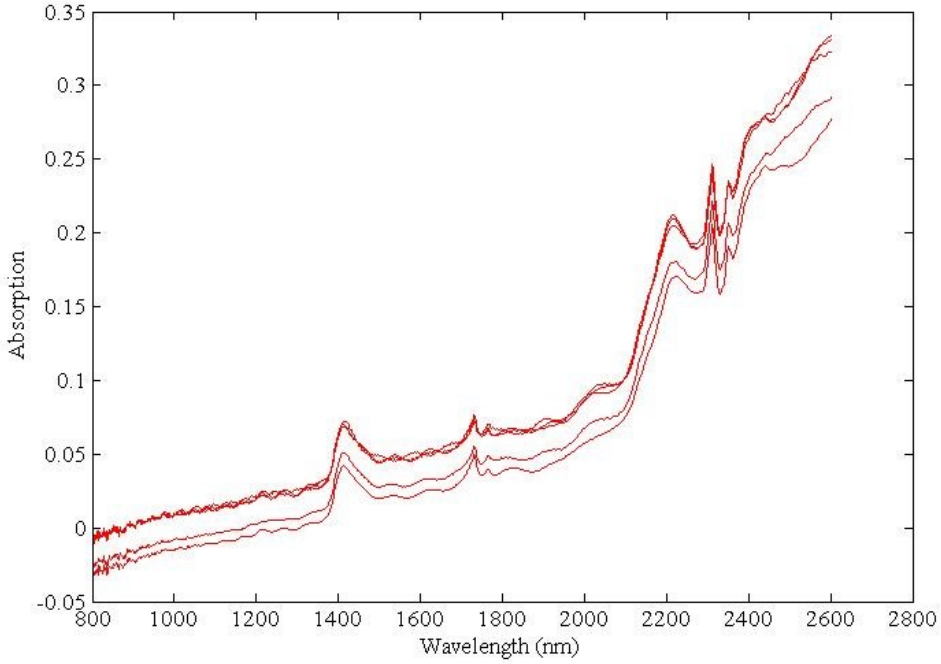


Figure 18 - Plastic foil spectra.

Appendix 2.3: Spectra plot: 2-Class discrimination

1) Dark Skin

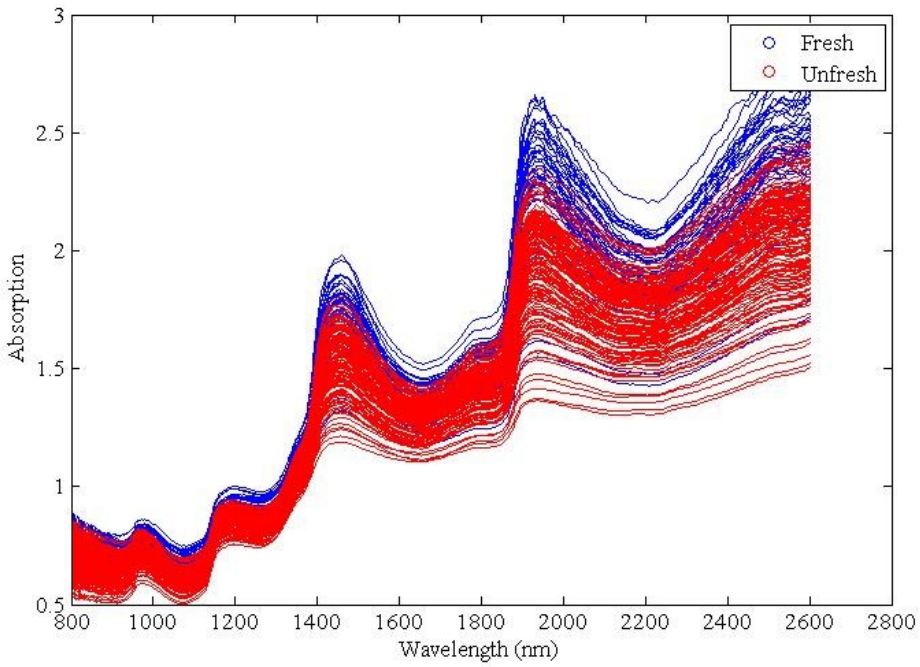


Figure 19 - Dark skin samples spectra in 2 class discrimination.

2) Muscle

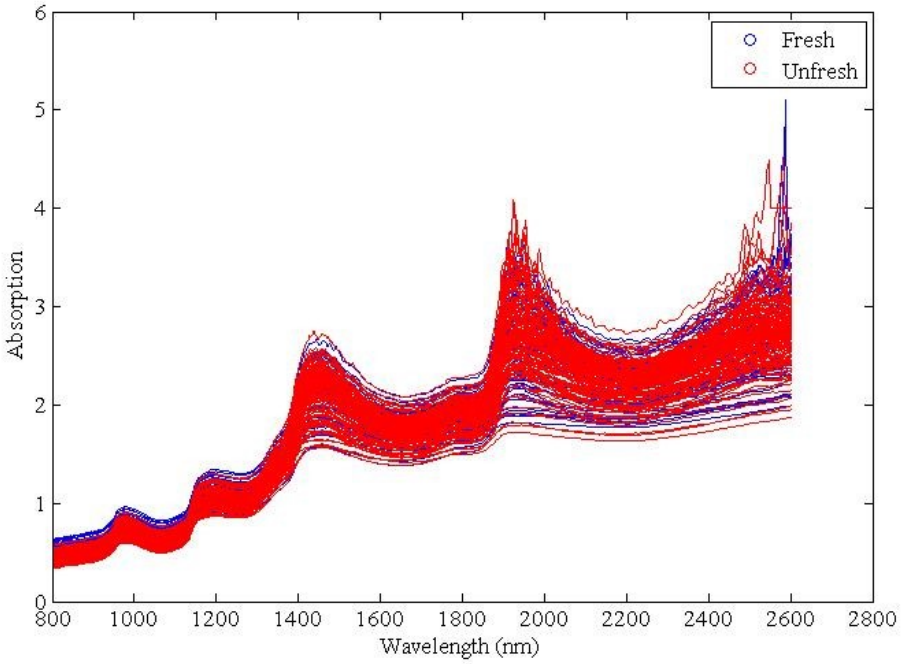


Figure 20 - Muscle samples spectra in 2 class discrimination.

3) Dark Skin with plastic foil

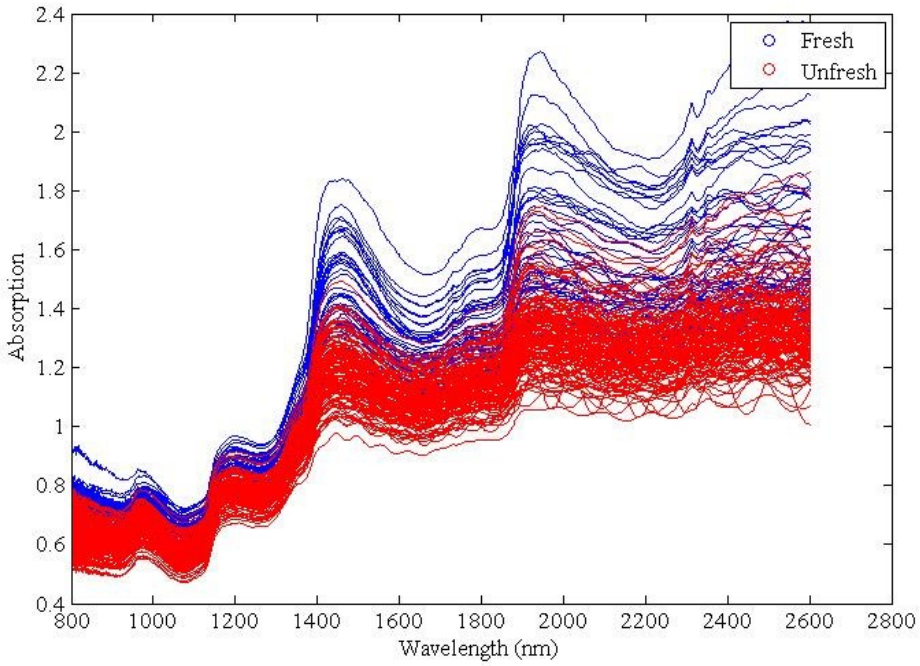


Figure 21 - Dark skin with plastic foil spectra in 2 class discrimination.

4) Muscle with plastic foil

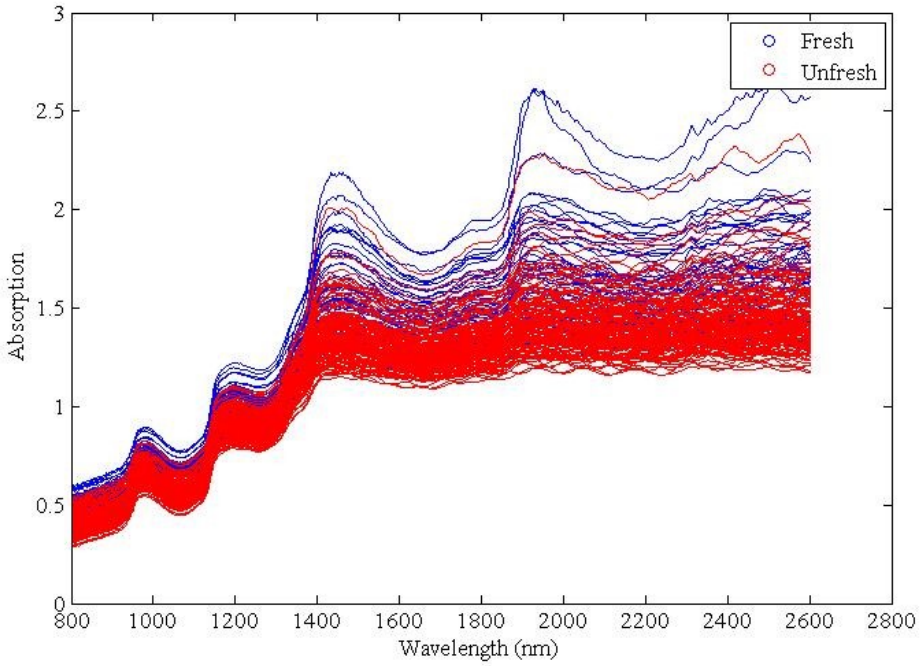


Figure 22 - Muscle with plastic foil spectra in 2 class discrimination.

Appendix 2.4: Spectra plot: 4-Class discrimination

1) Dark Skin

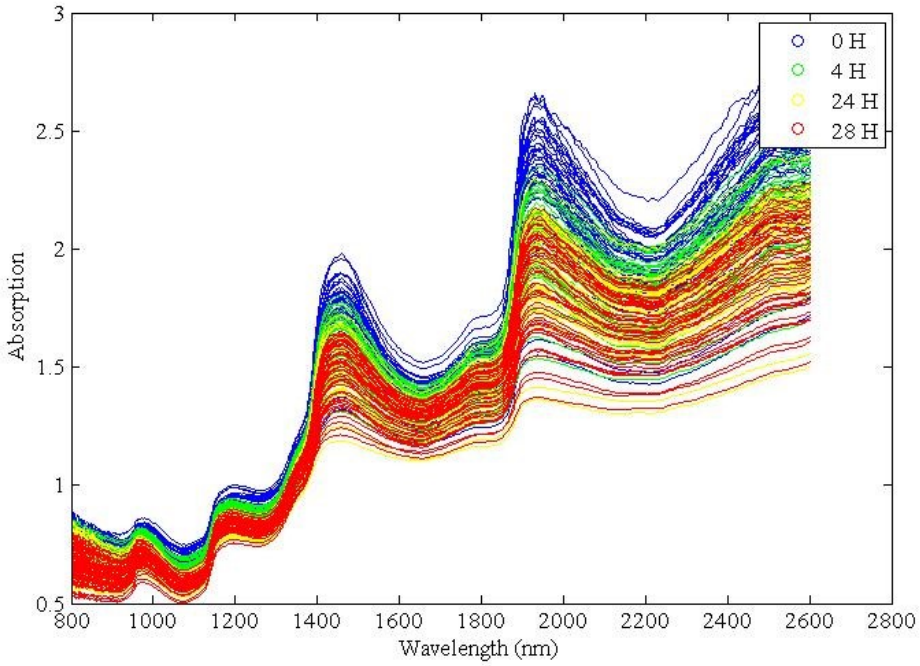


Figure 23 - Dark skin samples spectra in 4 class discrimination.

2) Muscle

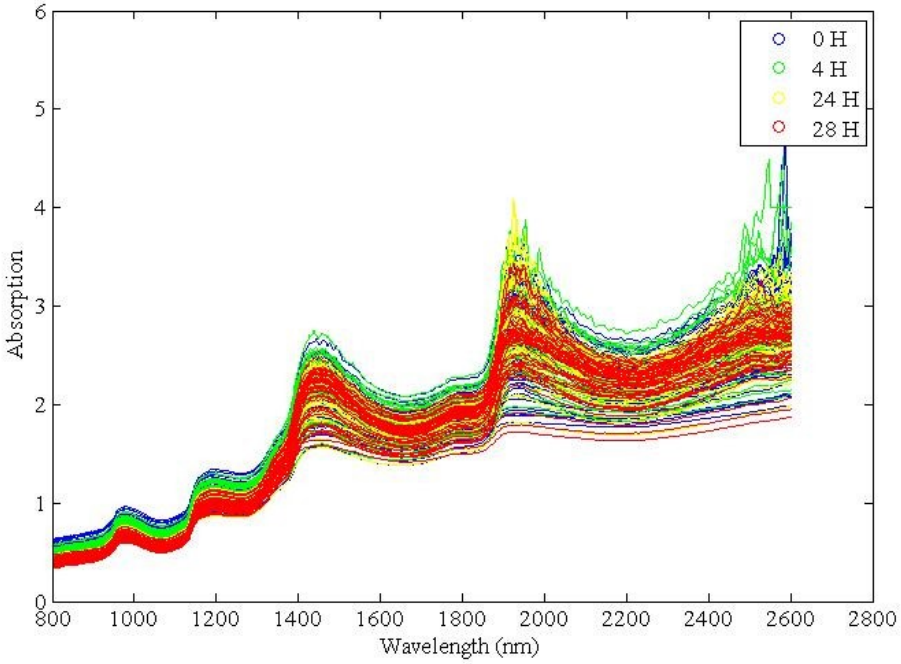


Figure 24 - Muscle samples spectra in 4 class discrimination.

3) Dark Skin with plastic foil

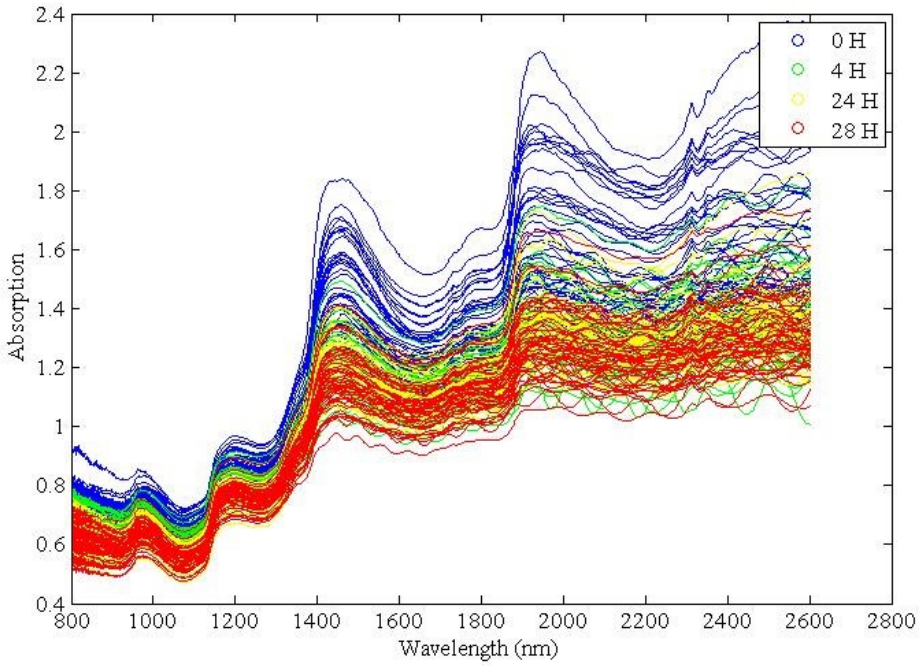


Figure 25 - Dark Skin with plastic foil samples spectra in 4 class discrimination.

4) Muscle with plastic foil

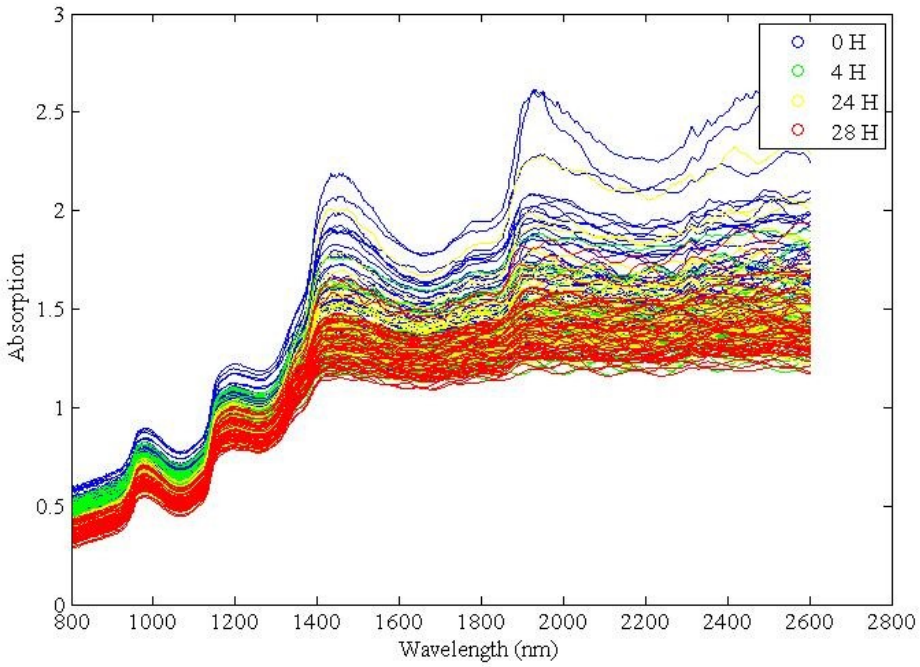


Figure 26 - Muscle with plastic foil samples spectra in 4 class discrimination.

Appendix 2.5: Misclassification points: 2-Class discrimination

1) Full wavelength range

a. Dark skin

Table 7 - Misclassification points in dark skin samples model for 2 class discrimination.

Point	Real Classification	Predicted Classification
19	Unfresh (4h)	Fresh (0h)

b. Muscle

Table 8 - Misclassification points in muscle samples model for 2 class discrimination.

Point	Real Classification	Predicted Classification
17	Unfresh (4h)	Fresh (0h)
28	Unfresh (4h)	Fresh (0h)

c. Dark Skin with plastic foil

Table 9 - Misclassification points in dark skin with plastic foil samples model for 2 class discrimination.

Point	Real Classification	Predicted Classification
30	Unfresh (4h)	Fresh (0h)
51	Unfresh (24h)	Fresh (0h)

d. Muscle with plastic foil

Table 10 - Misclassification points in muscle with plastic foil samples model for 2 class discrimination.

Point	Real Classification	Predicted Classification
16	Unfresh (4h)	Fresh (0h)
28	Unfresh (4h)	Fresh (0h)
36	Unfresh (4h)	Fresh (0h)
41	Unfresh (4h)	Fresh (0h)
42	Unfresh (4h)	Fresh (0h)
61	Unfresh (24h)	Fresh (0h)
109	Unfresh (28h)	Fresh (0h)

2) Selected wavelength range

a. Muscle

Table 11 - Misclassification points in dark skin samples model for 2 class discrimination using a selected wavelength.

Point	Real Classification	Predicted Classification
17	Unfresh (4h)	Fresh (0h)
28	Unfresh (4h)	Fresh (0h)

b. Muscle with plastic foil

Table 12 - Misclassification points in muscle samples model for 2 class discrimination using a selected wavelength.

Point	Real Classification	Predicted Classification
28	Unfresh (4h)	Fresh (0h)
29	Unfresh (4h)	Fresh (0h)
38	Unfresh (4h)	Fresh (0h)
41	Unfresh (4h)	Fresh (0h)
41	Unfresh (4h)	Fresh (0h)
59	Unfresh (24h)	Fresh (0h)

Appendix 2.6: Misclassification points: 4-Class discrimination

1) Full wavelength range

a. Dark Skin

Table 13 - Misclassification points in dark skin samples model for 4 class discrimination.

Point	Real Classification	Predicted Classification
25	4h	24h
31	24h	28h
33	24h	4h
36	24h	28h
38	24h	28h
40	24h	28h
41	24h	28h
47	28h	24h
48	28h	24h
49	28h	24h
50	28h	24h
51	28h	24h
52	28h	24h
54	28h	24h
55	28h	24h
58	28h	24h
59	28h	24h
60	28h	4h

b. Muscle

Table 14 - Misclassification points in muscle samples model for 4 class discrimination

Point	Real Classification	Predicted Classification
23	4h	24h
30	4h	28h
36	24h	28h
41	24h	4h
43	24h	28h
52	28h	24h

c. Dark Skin with plastic foil

Table 15 - Misclassification points in dark skin with plastic foil samples model for 4 class discrimination

Point	Real Classification	Predicted Classification
16	4h	24h
20	4h	24h
21	4h	24h
22	4h	24h
23	4h	24h
26	4h	24h
27	4h	24h
31	24h	28h
32	24h	28h
33	24h	4h
35	24h	4h
36	24h	28h
38	24h	28h
40	24h	4h
44	24h	4h
49	28h	24h
51	28h	4h
52	28h	24h
53	28h	24h
54	28h	24h
57	28h	24h
58	28h	24h

d. Muscle with plastic foil

Table 16 - Misclassification points in muscle with plastic foil samples model for 4 class discrimination

Point	Real Classification	Predicted Classification
16	4h	24h
28	4h	24h
30	4h	24h
36	24h	28h
37	24h	4h
42	24h	4h
44	24h	4h
46	28h	24h
48	28h	4h
55	28h	4h

2) Selected wavelength range

a. Dark skin

Table 17 - Misclassification points in dark skin samples model for 4 class discrimination using the selected wavelength.

Point	Real Classification	Predicted Classification
25	4h	24h
31	24h	28h
33	24h	4h
36	24h	28h
38	24h	28h
40	24h	28h
41	24h	28h
47	28h	24h
48	28h	24h
49	28h	24h
50	28h	24h
51	28h	24h
52	28h	24h
54	28h	24h
55	28h	24h
58	28h	24h
59	28h	24h
60	28h	4h

b. Muscle

Table 18 - Misclassification points in muscle samples model for 4 class discrimination using the selected wavelength.

Point	Real Classification	Predicted Classification
23	4h	24h
30	4h	28h
36	24h	28h
41	24h	0h
43	24h	28h
52	28h	24h

c. Skin with plastic foil

Table 19 - Misclassification points in dark skin with plastic foil samples model for 4 class discrimination using the selected wavelength.

Point	Real Classification	Predicted Classification
16	4h	28h
17	4h	28h
18	4h	28h
20	4h	24h
22	4h	28h
23	4h	24h
24	4h	28h
25	4h	24h
31	24h	28h
35	24h	28h
43	24h	4h
49	28h	24h
51	28h	4h
52	28h	24h
55	28h	24h
56	28h	4h
57	28h	24h
58	28h	24h

d. Muscle with plastic foil

Table 20 - Misclassification points in muscle with plastic foil samples model for 4 class discrimination using the selected wavelength.

Point	Real Classification	Predicted Classification
16	4h	24h
28	4h	24h
30	4h	24h
36	24h	28h
37	24h	4h
42	24h	4h
44	24h	4h
46	28h	24h
48	28h	4h
55	28h	4h

Appendix 3: Main study

Appendix 3.1: Plaice Classes

Figure 27 - Plaice class 301.

Figure 28 - Plaice class 401.

Figure 29 - Plaice class 601.

Appendix 3.2: QIM scores

Table 21- QIM scores for plaice samples class 601.

Fish number	QIM score Karen	QIM score Daphné
1	5	4
2	5	6
3	7	6
4	5	6
5	6	7
6	7	6
7	9	9
8	4	2
9	5	7
10	5	7
11	9	7
12	11	9
13	6	6
14	8	8
15	2	3
16	6	6
17	8	8
18	11	8
19	7	8
20	4	5
21	6	5
22	4	5
23	6	7
24	8	7
25	6	6
26	7	8
27	8	7
28	4	5
29	8	9
30	8	8

Table 22 - QIM scores for plaice samples class 401.

fish number	QIM score Karen	QIM score Daphné
1	7	7
2	4	7
3	7	7
4	5	9
5	3	6
6	7	8
7	6	7
8	7	8
9	12	9
10	7	11
11	5	6
12	4	7
13	5	7
14	6	6
15	6	8
16	5	8
17	13	10
18	6	7
19	4	6
20	6	6
21	5	9
22	7	8
23	5	8
24	8	8
25	8	8
26	7	7
27	7	7
28	6	8
29	9	7
30	5	7

Table 23 - QIM scores for plaice samples class 601.

fish number	QIM score Karen	QIM score Daphné
1	8	7
2	4	6
3	2	4
4	4	7
5	7	8
6	7	7
7	9	7
8	9	9
9	8	7
10	6	6
11	9	9
12	11	12
13	8	9
14	6	7
15	5	7
16	5	7
17	6	6
18	13	9
19	6	7
20	8	8
21	4	5
22	9	8
23	3	6
24	5	7
25	9	10
26	8	8
27	5	6
28	13	13
29	7	9
30	5	5

Appendix 3.3: Spectral data reorganization

Table 24 - Spectral data reorganization

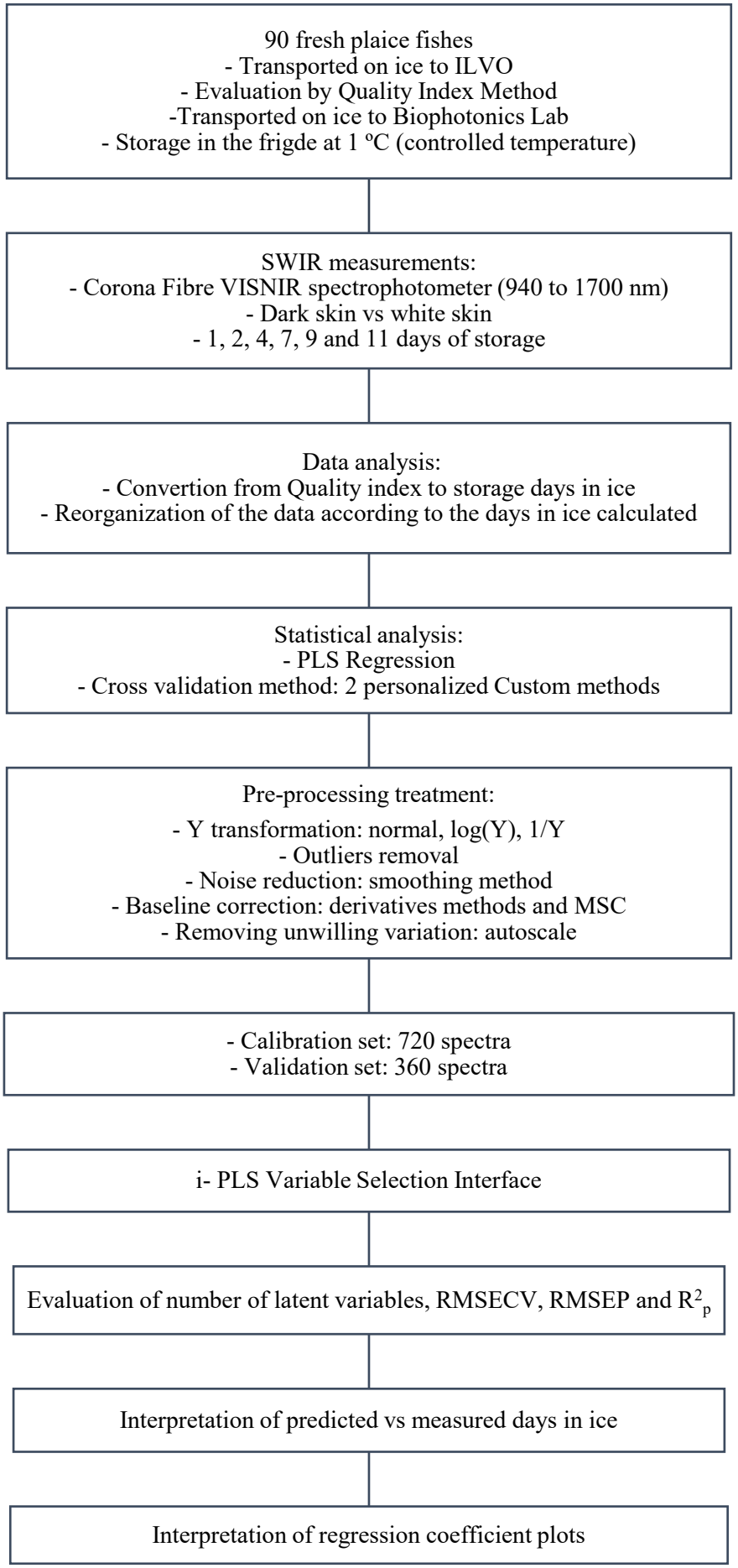
Days on ice	20/June			21/June			23/June			26/June			28/June			30/June		
	601	401	301	601	401	301	601	401	301	601	401	301	601	401	301	601	401	301
3	8, 15		3															
4				8, 15		3												
5	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30															
6	3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30	8, 15		3									

7	11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29	3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24												
8	7, 18, 29	10	8, 11, 13, 22, 25	11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30									
9	12	9		7, 18, 29	10	8, 11, 13, 22, 25	3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24	8, 15		3						

10		17	12, 18	12	9		11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29									
11			28		17	12, 18	7, 18, 29	10	8, 11, 13, 22, 25	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30	8, 15		3			
12						28	12	9		3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24						
13								17	12, 18	11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30	8, 15		3
14									28	7, 18, 29	10	8, 11, 13, 22, 25	3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24			

15										12	9		11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29	1, 2, 4, 20, 21, 22, 28	2, 5, 11, 12, 19	2, 4, 21, 23, 27, 30
16											17	12, 18	7, 18, 29	10	8, 11, 13, 22, 25	3, 5, 6, 9, 10, 13, 16, 23, 25	1, 3, 4, 7, 13, 14, 15, 16, 18, 20, 21, 23, 26, 27, 28, 30	6, 10, 14, 15, 16, 17, 19, 24
17												28	12	9		11, 14, 17, 19, 24, 26, 27, 30	6, 8, 22, 24, 25, 29	1, 5, 7, 9, 20, 26, 29
18														17	12, 18	7, 18, 29	10	8, 11, 13, 22, 25
19															28	12	9	
20																	17	12, 18
21																		28

Appendix 3.4: Flow chart



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