Development of near infra-red method to detect and control microbial spoilage.

Shane M Powell

Project 2009/766

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Table of Contents

Non technical summary

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Objectives:

1. To show that a simple inexpensive method, near-infra-red spectrophotometry can be used to detect spoilage organisms on fresh salmon.

2. To develop a model for the relationship between the NIR spectra of fresh fish and the numbers of bacteria present after nine days of storage.

Outcomes achieved to date

This honours project has shown that there is a relationship between the NIR spectra of fresh salmon and the numbers of bacteria present nine days later after storage at 4°C. The mathematical model that predicts the numbers of bacteria still requires further work before it can be considered robust. However the method has potential to be developed further as a tool for predicting microbial spoilage of fresh fish products.

Spoilage of fresh fish products by the action of bacteria is one of the main causes of the short shelf-life of these products. A range of bacteria are responsible for this and are referred to collectively as “spoilage bacteria”. Currently methods to detect both spoilage of the product and the presence of number of bacteria are time-consuming, for example requiring 24-hour incubation periods, or require

specialised labour such as tasting panels. Near infra-red spectroscopy (NIR) is widely used in the food industry to monitor factors such as fat and moisture content in a range of foods. Although it has been used to distinguish different types of bacteria and, in a few cases, to quantify the number of bacteria in different materials, there is a lack of information on the ability of the method to quantify bacteria directly on food products. The aim of this project was to determine whether NIR had the potential to be used as a method to detect and predict microbial spoilage of fresh fish products.

NIR was easily able to distinguish between fresh Atlantic salmon fillets and those stored for nine days at 4°C indicating that NIR can detect spoilage. Partial least squares regression prediction models for the number of total bacteria and the number of *Enterobacteriaceae* present were developed. These models used the NIR spectra collected when the fish was fresh to predict the number of bacteria that would be present nine days later. The calibration equations for both models were good (R^2=0.94; RMSE= 0.17 log CFU/g and R^2=0.95; RMSE=0.12 log CFU/g, for *Enterobacteriaceae* and total aerobic plate counts respectively). However the error of the validation curves was larger (R^2=0.64; RMSE= 0.41 log CFU g^{-1} and R^2=0.64; RMSE=0.32 log CFU g^{-1}, for *Enterobacteriaceae* and total aerobic plate counts respectively) although still acceptable considering the number of samples used in constructing the model. There are many factors (protein and fat content of the salmon itself for example) that contribute to the differences in the NIR spectra that are unrelated to the numbers of bacteria. Hence for any model to be useful it needs to include as many of these variables as possible.

In conclusion, the results of this project show that NIR has potential to be a useful method for detecting and predicting bacterial levels on fish but much more work is required to develop a suitably robust model.

Keywords: Near infra red spectroscopy, microbial spoilage, Atlantic salmon

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The development of near infrared spectroscopic method to detect microbial spoilage communities in the Atlantic salmon (*Salmo salar*).

Photo: Nthabiseng Tito, 2010

Submitted in fulfilment of the requirements for the degree of Bachelor of Science with honours,

Nthabiseng Beta Tito

University of Tasmania, Hobart

November 2010

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma and, to the best of my knowledge, contains no copy or paraphrase or material published or written by any other person, except where due reference is made in the text of this thesis.

(Signed)

Nthabiseng Beta Tito
University of Tasmania, Hobart

November 2010

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Table of contents

List of Figures

List of tables

Literature Review

Abstract

The potential of using a near infrared spectroscopy (NIR) method to detect as well as predict microbial spoilage on Atlantic salmon was investigated. Principal component analysis results showed clear separation between the fresh salmon fillets and those stored for nine days at 4°C, indicating that NIR spectra could detect spoilage. Partial least regression (PLS) prediction models for microbial loads on the fish fillets were established for both *Enterobacteriaceae* and total aerobic counts on day 9. Calibration equations for both counts are good with R^2=0.94; RMSE= 0.17 log CFU/g and R^2=0.95; RMSE=0.12 log CFU/g for predicting log *Enterobacteriaceae* and log total aerobic counts respectively. These results indicate that with more samples NIR can be used to detect spoilage as well as predict microbial loads on Atlantic salmon (*Salmo salar*).

Introduction

Freshness is one of the most important quality attributes for fish products. It is affected by many factors including temperature and time of storage. Fish is usually stored in ice and chilled conditions to increase its shelf life. The shelf life of the fish varies depending on storage conditions. Fish spoilage is characterised by changes in aroma, taste, texture and appearance (Lin *et al* 2006). Fish stored in ice for a week is acceptable for two weeks. Fish stored under chilled conditions is much shorter. The fish that is modified atmosphere packaged lasts the longest time.

This review focuses on fish spoilage. The influence of origin, processing and storage conditions on the spoilage of fish will be looked into. There are many methods used for detecting spoilage on fish. These include microbial enumeration methods, volatile compound analysis, measurement of lipid oxidation, nucleotide and amine metabolite assays texture measurement and sensory evaluation (Bremner and Sakaguchi 2000),however these methods are limited because they are usually labour intensive, time consuming, invasive and unattractive for routine analysis (Lin *et al*. 2006). Therefore, there is need for a method that is objective, robust, non intrusive (Nilsen *et al* 2002) and rapid to ensure safe and high quality fish products on the market. NIR spectroscopy meets the requirements for the new method (Suthulik *et al*. 2008) and has been used on chicken, cabbage and on rainbow trout (Suthulik *et al*. 2008; Alexandrakis *et al*. 2008)).

A few of the methods used for monitoring spoilage will be described and advantages and limitations of each will be discussed.

General food spoilage

Food spoilage is any change that renders a food quality unacceptable to consumers (Sivertsvik *et al.* 2002). Food spoilage is a complex process and large amounts of food are lost because of microbial spoilage (Gram *et al*. 2002). Different foods have different microbiology. This is because different foods have different biochemical compositions (Table 1).

Table 1 Different type of foods and their spoilage based on biochemistry (Adams and Moss 2008)

Food	Process	Products	Effects
Pectin (fruits and vegetables)	Pectinolysis	Methanol, uronic acid	Loss of fruit and vegetable structure
proteins	Proteolysis deamination	Amino acids, amines Ammonia, indole, H_2S	Bitterness,souring,sliminess Bad odours
Carbohydrates (starchy foods)	Hydrolysis fermentations	Organic acids, CO_2 Mixed alcohols	Souring, acidification
lipids	Hydrolysis, Fatty acid degradation	Glycerol and mixed fatty acids	Rancidity bitterness

The bacteria involved in spoiling fruits and vegetables are usually those responsible for breaking down pectin in a process called pectinolysis. Pectin is found in the middle lamella of plant cells. Pectinolytic bacteria include the gram negative genera *Pectobacterium, Pseudomonas* and *Xanthomonas* (Adams and Moss 2008). Fish and other muscle foods undergo the process of breaking down proteins by proteolysis and deamination.

The biochemical products of spoilage of different food have been used to determine the quality of fish. Total volatile base nitrogen (TVBN), trimethylamine nitrogen (TMA-N), thiobarbituric acid (TBA) and free fatty acids (FFA) are examples of biochemical analysis used to predict the extent of spoilage. TVBN and TMA-N are useful for assessing bacterial spoilage whilst TBA and FFA measure the extent of lipid oxidation (Buransompob *et al.* 2003; Subramanian 2007).

Spoilage of fish

When compared to other muscle foods and milk, fish spoils the fastest even under refrigeration (Adams and Moss 2008). The spoilage of fish is due to a combination of microbiological, chemical and physical activities on the fish flesh (Abbas *et al.* 2009). The initial quality deterioration of fish is mainly caused by autolytic (self degrading) changes and is not related to the activity of the microorganisms (Gram and Huss 1996). This is possible because when the fish die, the enzymes secreted into the gut degrade the fish. However, the final stages of fish quality loss, changes in the texture of the fish flesh and production of off-odours and off-flavours are primarily due to microbial activity. Figure (1) which was adopted from Abbas *et al* (2009) is a graphic presentation of the four phases.

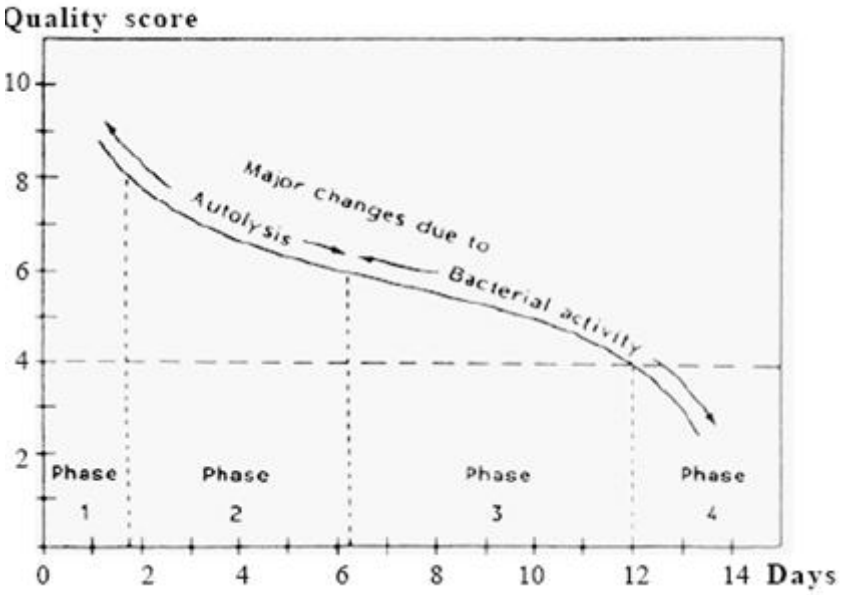

Figure 1 Different phases in the causes of fish spoilage (from Abbas *et al* 2009)

In phase 1 there is a small loss of the characteristic odour and flavour. A considerable loss of sweet sea weedy flavour of the fish happens in phase 2. In phase 3 there are strong offensive flavours and odours. In phase 4 the fish is spoiled and undesirable to eat.

Chemical spoilage

The lipid content of fish muscle ranges from as low as less than one percent to as high as 20 percent depending on the species, season, environment and age (Tarr 1954). Fish species have attracted a great amount of attention as a source of omega 3 fatty acids in the

human diet and people commonly refer to fish as a brain food. However, these fatty acids are polyunsaturated and very reactive and contribute significantly to the chemical spoilage of fish.

Lipid oxidation involves the formation of fatty acids radicals and hydro peroxides (Haard and Bracho 1995). There are two types of lipid oxidation, the enzyme catalysed oxidation and self catalysed oxidation. Auto oxidation, also referred to as non enzymatic oxidation, is promoted by metal ions like iron and cobalt and other haeme compounds (Doe 1998). The rate of auto oxidation increases logarithmically with the degree of unsaturation of the lipid (Doe 1998).

There are two known enzyme catalysed lipid oxidation reactions in fishery products. There is the lipoxygenase and the NADH-cytochrome b5 reductase. The lipoxygenases are responsible for adding oxygen at the specific sites on the specific fatty acid. The reductase is mainly involved with the reduction of ironIII to ironII (Haard and Bracho 1995). The reductase reaction requires ATP, ironIII and NADH but the lipoxygenase reaction does not. The product of lipid oxidation is hydroperoxides. The hydro-peroxides are then broken down to aldehydes and ketones. The rancid off-flavours are from the ketones and aldehydes (Bataringaya 2007).

Lipid oxidation can be a determining factor on the expansion or limitation of the commercial market for particular fish species. Lipid oxidation is affected by storage temperature, amongst other things, as shown in figure 2 (Alvarez 1982). The figure shows the increase in the lipid breakdown products and that the rate of lipid oxidation increases with temperature.

Mugil cephalus (the mullet) for instance, has a limited market for two reasons one of which is poor stability during storage due to its high lipid content. (Alvarez 1982; Deng *et al.* 1976) found out that lipid content of salted minced mullet significantly dropped from 18.53% to 9.66% when stored at room temperature for a month.

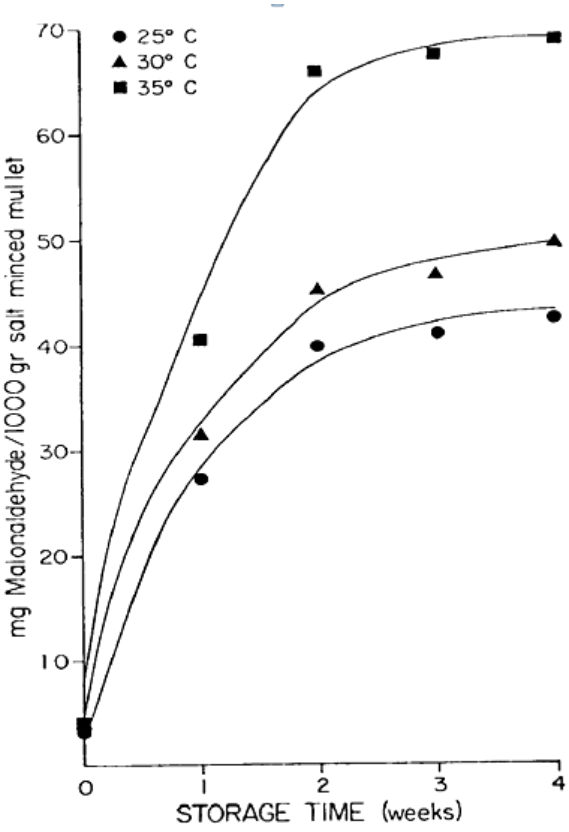

Figure 2 The effect of temperature on lipid oxidation (Alvarez, 1982)

Microbial spoilage of fish

The muscle of live fish is considered to be sterile, but microorganisms are found in the gut, gills and on the skin of live and newly caught fish (Bataringaya 2007). The fish‟s host defence mechanisms, which prevent invasion of the tissues by bacteria cease to function after death (Fraser and Sumar 1998). The protection mechanisms include the secretion of antimicrobial compounds such as lysozyme and antibodies (Rahman 2007).

Specific spoilage organisms

It has been determined that there is no correlation between the total numbers of bacteria (Huss et al. 1974; Gram and Huss 1996) and spoilage. However, specific spoilage organisms (SSOs) are described as the bacteria that are responsible for the production of the off-odours and off-flavours associated with fish spoilage and the numbers of these are expected to be correlated with spoilage (Gram and Huss 1996).

SSOs are typically initially found in low numbers and different SSOs are found in different seafood (Gram and Dalgaard 2002).Table 2 shows the SSO on fish of different origin and storage conditions.

Table 2 SSO on fish from different origins and storage conditions (Gram and Huss, 1996)

atmosphere	SSO of fresh, chilled fish			
	Temperate waters		Tropical waters	
	Marine	fresh	Marine	fresh
Aerobic	*Shewanella putrefaciens*	*Pseudomonas* spp	*S. putrefaciens*	*Pseudomonas* spp
	Pseudomonas spp		*Pseudomonas* spp	
vacuum	*S. putrefaciens*	Gram positive bacteria	LAB	LAB
	Photobacterium phosphoreum	Lactic acid bacteria (LAB)		
CO_2		LAB	LAB/ TMAO reducing bacteria	LAB/ TMAO reducing bacteria
	P.phosphoreum			

Tropical versus temperate fish spoilage

Fish are cold blooded and therefore the bacterial flora on their skin, gills and intestines are adapted to their specific environments (Sivertsvik 2002). The microflora from the temperate waters is dominated by psychrotrophic, aerobic or facultative anaerobic Gram negative, rod shaped bacteria (Fraser and Sumar 1998). Examples include the genera *Acinebacter, Flavobacterium, Moraxella, Shewanella, Pseudomonas, Vibrionaceae* and *Aeromnadacae*. However, the microflora of fish from tropical waters is dominated by gram-positive organisms such as *Bacillus, Micrococcus*, and coryneforms (Shewan 1977; Fraser and Sumar 1998).

Temperate fish spoil faster than tropical fish when both are stored in ice. Shewan (1977) suggested that it could be because fish from cold waters have a higher proportion of psychrotrophs than mesophiles when compared to fish from warmer waters. Psychrotrophs are cold tolerant bacteria able to grow at 0^0C with an optimum growth temperature of 25^0C. Psychrophiles have a maximum temperature of 20^0C and optimum growth temperature of less than 15^0C (Abbas *et al* 2009). Mesophiles however, are better adapted for growth at higher temperatures (Disney 1976; Al Bulushi *et al*. 2008), growing best in temperatures between 10 and 50^0C. Studies carried out by Summer in Australia and Maguo-Orejana in the Philippines showed the effect of temperature of origin on the shelf life of fish when

stored in ice, as shown in table 3 (Reilly 1985). *Mugil cephalus*, commonly known as the mullet, was used in Australia and tilapia was used in the Philippines. This table shows that *M. cephalus* from warm water (21-23^{0}C) had a longer shelf life (26 days) than the same species from cold water (8-9^{0}C). *Liza subviridis*, the greenback mullet from even warmer waters had a shelf-life as high as 29 days when stored in ice.

Table 3 Shelf lives of mullets from waters of different temperatures (Reilly 1985)

Species of fish	Temperature of water	Shelf life in days
M.	9	20
cephalus	8	21
	14	24
	23	26
	21	26
	26	29
L.subviridis	26	29
	26	28

Marine versus freshwater fish spoilage

Marine and freshwater fish have different microflora compositions as some bacteria are adapted to high salt concentration whilst others are not. Some bacteria require sodium to grow and these are typical of marine waters. Examples of such bacteria are *Photobacterium* and *Shewanella putrefaciens*. These two genera specifically require sodium so when culturing them in the laboratory, sodium cannot be replaced by potassium in the medium used. *Aeromonas* species are normally found in freshwater fish and are responsible for spoilage in freshwater fish species (Sivertsvik *et al*. 2002). Fish taken from lakes and rivers had *Aeromonas* and *Enterobacteriaceae* as the most prevalent bacteria. *Vibrios* were only isolated on fish from marine waters. Euryhaline bacteria such as *Halomonas elongata* are able to tolerate a wide range of sodium chloride concentrations (Hart and Vreeland 1988) and are therefore the most important in the spoilage of marine fish (Adams and Moss 2008). Atlantic salmon is a good target to study the effect of salt concentration on the microbiology of fish because it can be found in marine, estuarine and freshwaters (Cahill 1990).

Factors influencing the rate of spoilage

The demand for fresh and high quality fish is high. Retailers are constantly looking for ways of extending shelf lives of perishable food to minimise profit loss due to spoilage. The rate of microbial spoilage varies according to the species, area of catch and method of catch, processing, storage temperature and conditions (Subramanian 2007).

Handling

Harvesting by trawling, net, hook or line results in death of the fish. After the fish dies energy supply for normal body maintenance stops, antimicrobials are no longer produced, and therefore the microflora invade the flesh of the fish (Rahman 2007).

Hattula *et al*.(1995) studied the effect of three catching methods; trawling, gillnetting and poundnetting on the quality of Baltic herring. They found out that more fish died if trawling time was increased and that rigor mortis occurred more quickly in fish caught by gillnetting. Following capture, the fish must be protected from spoilage during transport to the processing plant. This includes rapid chilling or freezing and eviscerating the fish on board. Gutting the fish removes a significant amount of potential spoilage bacteria. However, cutting opens the flesh for bacterial attack.

Fish that have been excessively handled exhibit quicker spoilage (Abbas *et al*. 2009). Reckless harvesting and handling may lower the quality of fish. In order to reduce physical damage, harvesting equipment and containers must be checked for sharp edges regularly. This is because sharp edges on the container may result in bruising and cuts and therefore providing easy invasion of bacteria into the fish flesh. Other mishandling practices are overloading of fish and packing them on top of each other (Rahman 2007). Processing may also include cooking. Subramanian (2007) concluded that processing like cooking and freezing showed a significant reduction in the bacterial load of cuttlefish.

Packaging

As with many foods, the microbial spoilage of fish is also influenced by storage conditions and packaging (Weber *et al.* 2002). The gaseous composition of the atmosphere influences the dominance of different microorganisms. Aerobic organisms will dominate where there is oxygen but facultative anaerobes will grow where there is no oxygen. The spoilage organisms of fish changes from one dominated by aerobic psychrotrophs to one dominated

by facultative anaerobes in refrigerated modified atmosphere packed fish (Gittleson 1990). *Pseudomonas* species and *Shewanella putrefaciens* are associated with spoilage of refrigerated and aerobically stored fish (Koutsoumanis and Nychas 1999).

Modified atmosphere packaging (MAP) is the replacement of air with one gas or a mixture of gases. The technique has been around since the late 1920s but was only commercially introduced in the 1970s. Using MAP increases the shelf life and makes it possible to get high quality products to consumers (Phillips 1996). Some examples are shown in Table 4. MAP increases the shelf life of all chilled products and there is also little or no need for use of chemical preservatives. For meat the shelf life in air can be extended from one- two weeks to 21 weeks in a modified atmosphere. MAP also increases the shelf life of fish products but not as extensively as meat. However, using MAP is disadvantageous in that it has to be coupled with freezing/ chilling. Improper handling and extended storage might result in survival of *Listeria* or *Salmonella* spp or the production of toxins by *Clostridium botulinum* (Amanatidou *et al.* 2000).

Microbial growth of aerobic bacteria like *Pseudomonas* and *Shewanella* are inhibited in MAP. Limiting exposure to oxygen also decreases the rate of oxidation(Wierda *et al.* 2006). Carbon dioxide in modified atmosphere interferes with the metabolism of bacterial cells because it causes a reduction in pH (Sankar *et al.* 2008). However, some bacteria are able to grow under these conditions. For example, *Photobacterium phosphoreum* has been reported to be the main spoilage organism in MAP fish (Reynisson *et al.* 2009).

Table 4 The effect of packaging on the shelf life of chilled fish and meat products (Dalgaard 1995)

Type of product	Storage temperature (^{0}C)	Shelf life (weeks)		
		air	Vacuum packed	Modified atmosphere packed
Meat: beef,pork, poultry	1.0-4.4	1-3	1-12	3-21
Lean fish: cod, pollock, rock fish, trevally	0.0-4.0	1-2	1-2	1-3
Fatty fish: herring, trout, salmon	0.0-4.0	1-2	1-2	1-3
Shellfish: crabs, scampi, scallops	0.0-4.0	½-2	-	½-3
Warmwater fish: sheepshead, tilapia, swordfish	2.0-4.0	½-2	-	2-4

It can be concluded that the spoilage of fish is affected by a network of factors. Therefore to achieve the desired quality of fish all the factors should be taken into account.

Methods of detecting spoilage

It is important for industry to be able to evaluate the stage of freshness of any given fish and be able to estimate how much of storage life remains (Lerke and Farber 1969). Microbial spoilage of fish affects the texture, taste, odour, colour of the fish. The spoilage processes are a combination of physical, chemical, biochemical and microbiological interactions that are species-specific and environmentally determined. The methods that are used to detect spoilage make most of these changes. The traditional methods for assessing the extent of spoilage of fish include sensory evaluation, trimethylamine determination and bacterial counts.

The key difficulty with detecting fish freshness and spoilage is that many of the methods used are destructive, slow to get results, labour intensive and time consuming (Limbo *et al.* 2009). For example when using plate counts, media needs to be autoclaved, poured on plates, serial dilutions have to be made and then spread plating, and incubating for at least 24 hours and then colonies are counted.

The other limitation of traditional methods is that they are subjective and require highly trained people. For instance, sensory analysis requires a trained panel. Several authors have commented that there is a need for rapid, automated and objective tools for process monitoring and quality assurance of perishable food products (Nicolay 2006;Alexandrakis *et al*. 2008).

Microbial and microbial metabolites methods

The culturing and plating method is the oldest microbial detection technique and remains a standard method (Lazcka *et al.* 2007). Aerobic plate counts also known as total plate counts can give a general indication of the microbiological quality of a food but cannot distinguish between natural flora of a food, spoilage microbes or pathogenic microorganisms. A high plate count may indicate poor handling and storage. The standard plate count is used in Food Standards Australia New Zealand. The standards are reviewed and changed appropriately (Winger 2003). It is logical to assess spoilage by counting the bacteria on the fish since bacteria are the primary cause of fish spoilage (Lerke and Farber 1969). Total plate counts have been used to assess freshness and estimate the remaining shelf life. Different selective media are used to detect particular bacteria.

Sensory analysis

Sensory assessment includes using one or more of the five senses to evaluate the freshness of fish. The parameters used include changes in odour, appearance, texture and taste. Each parameter is represented in a scale of numbers using a demerit score system. These scores are then added to a total to give an index. This is where the concept of quality index method (QIM) comes from (Bremner and Sakaguchi 2000). The examiners need to be trained by experienced judges and training is required for each different product. Sensory assessment of raw fish can be non-destructive; however, that of cooked fish involves destructive sampling.

An advantage of sensory analysis is that it reflects whether the consumers would prefer the product. This is beneficial to both the industry and the consumer. However, sensory analysis is unappealing for routine analysis, because it relies on highly trained panels to lessen subjectivity and it is also expensive (Koutsoumanis and Nychas 1999). The expense associated with sensory analysis is the labour because it requires many people. This method is also time-consuming (Wierda *et al* 2006). The other drawback with sensory analysis is that the trained panels are not always accessible along the different stages in the fish industry from catching fish to the fish reaching the consumers (Macagnano *et al.* 2005).

Using biosensors

Biosensors are analytical devices that consist of a biological material, for example, microorganisms or enzymes, and a physicochemical transducer. There are many different kinds of biosensors and they are classified according to their transduction methods. Examples include optical, electrochemical and thermometric and magnetic biosensors (Lazcka *et al*. 2007).

Yano *et al*. (1996) developed a direct sensing method for monitoring meat quality. This was made possible by employing a potential-step chronoamperometry. It is an electrochemical method in which a step potential is applied and the current is measured as a function of time as shown in Figure 3.

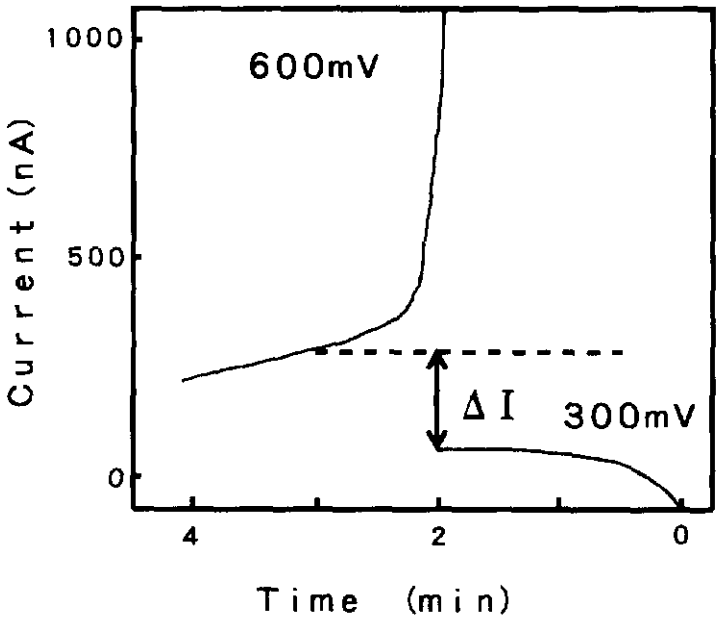

Figure 3 A typical chronoamperometric (current versus time) curve (Yano *et al*. 1996)

The sensor was made up of Ag/AgCl electrode and a platinum electrode where the enzymes (putrescine oxidase or xanthine oxidase) were immobilised. To remove interference from uric acid in the meat sample, a Nafion membrane was used. In order to strengthen the surface of the enzyme electrode a polycarbonate membrane was placed over the Nafion membrane (Figure 4). This particular biosensor detected putrescine and hypoxanthine. Putrescine has been confirmed to be a good chemical indicator of bacterial spoilage and hypoxanthine is a breakdown product of ATP (Gram and Huss 1996). The conclusion from using the developed method, was that with modifications it could be useful for monitoring meat quality (Yano *et al.* 1996).

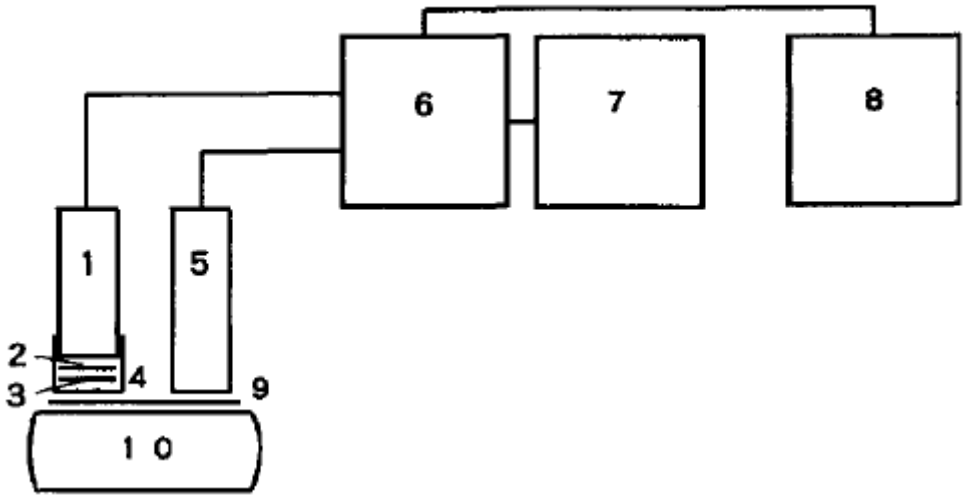

Figure 4 The schematic diagram of the direct sensing system for evaluating meat freshness: 1 platinum electrode; 2 immobilised enzyme; 3 Nafion membrane; 4 polycarbonate membrane; 5. Ag/AgCl reference electrode; 6. Potentiostat; 7. Function generator; 8. Recorder; 9. Filter paper; 10. Meat (Yano *et al*. 1996).

There is still need for development in the biosensors for them to be an attractive (pathogen) detecting method. They should be time effective, cost effective and capable of detecting microbes at same or lower concentrations than the traditional methods (Lazcka *et al*. 2007).

Colorimetry

Another way of estimating the number of bacteria is by colorimetry. The ability of bacteria to reduce tetrazolium salts has been used to measure the freshness of iced fish (Lerke and Farber 1969; Liston 1957). Triphenyl tetrazolium dye salts are colourless, ionised, water soluble and are able to pass through the cell wall. Upon entry into the bacterial cell, the salts are reduced and the red coloured insoluble product is formed. The colour intensity is

proportional to the number of bacteria present. The intensity of the colour of the solution is compared with the standard colour chart colour strip and the fish in question can be graded excellent, good, fair or poor. It is assumed that the more intense the colour, the poorer the quality of the fish as shown in figure 5. A bleaching agent is added to dark coloured fleshed fish like tuna before the colour of the product is compared to the standard colour strips.

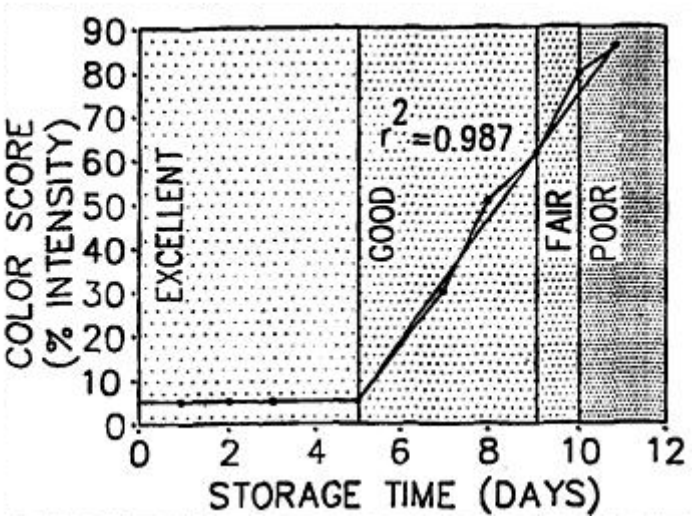

Figure 5 Colour score in % intensity in relation to storage time in days (Harewood 1998)

There are also developments in colorimeters. Cakli *et al*. (2006) reports using a spectral colour meter Spectro-pen (R), a colorimeter using visible spectral range at specific wavelength intervals. It also employs computer software for data analysis. Despite the developments in the colorimeter technique, there still are drawbacks. There is need for preparation of the samples which is time consuming and it can also be destructive if homogenates rather than whole fish or fillets are used (Cakli *et al.* 2006). In addition colorimetry involves the use of many chemicals which is undesirable because it can be a hazard to the workers and the environment.

Gas chromatography

Gas chromatography (GC) can be used to measure volatile compounds as indicators of fish freshness and spoilage (Boyd *et al.* 1998). The difficulty with GC is that it is restricted to the laboratory because it requires other infrastructure such as a gas supply. This is not ideal as the industry is looking for techniques that are rapid and portable.

Electronic nose

Electronic noses (e-nose) have been developed to mimic the human sense of smell and can be used to rapidly detect quality changes in fish (Olafsdottir *et al*. 2002). Sensor technologies on which electronic noses are based include metaloxide sensors (MOS), amperometric sensors, conducting polymers and metalloporphryns-coated quartz microbalance (QMB) sensors (Di Natale *et al.* 1996; Schweizer-Berberich *et al.* 1994). The composition of volatile compounds in fish contributing to the characteristic odours can be determined and related to quality. The e-noses are based on the selective detection of the main classes of volatile compounds that are characteristic of fish spoilage like amines, hydrogen sulphide, alcohols and aldehydes (Olafsdottir *et al*. 2006). Total volatile bases (TVB) analysis is traditionally used to evaluate fish freshness in industry. Olafsdottir *et al*. (2002) reported that e-nose measurements can be used to predict TVB values of raw capelin stored under different conditions.

The electronic nose is promising for applications to the food industry. Ólafsdóttir *et al.* (2002) found that traditional sensory method correlated well with electronic nose results. The different sensors used in the e-nose can be informative in which SSO is responsible for spoilage at different stages of fish spoilage. The growth of *Pseudomonas* spp. is detected by NH_3 and TMA measurements.

The formation of alcohols, aldehydes and esters (chemical changes) was suggested by the increased response in the CO sensor during the early storage of fish (Olafsdottir *et al.* 2006).The H_2S and sulphur dioxide sensors seem to be related to the hydrogen sulphide producers like *Shewanella putrefaciens* activity.

One of the disadvantages of using e-noses is that sampling is a critical step because it influences the selection of compounds detected by the sensors (Mille and Marquis 1999). However, Ólafsdóttir *et al.* (2002) claimed that rapid e-nose measurements required no sample preparation. However, humidity can be a problem when using metaloxide sensors and conducting polymers. When using the electrochemical sensors the electronic nose needs to be located in an environment free of gases and organic solvents. There is also need for cautious control of the temperature of samples during measurement (Ólafsdóttir *et al.* 2002).The sensitivity of e-nose drifts with time which makes them unsuitable for long- term use (Ólafsdóttir *et al.* 2002).

Polymerase chain reaction (PCR)

DNA- based methods are not influenced by environmental conditions of the bacterial cells and thus have an advantage over phenotypic based methods (van der Vossen and and Hofstra 1996). However, PCR- based detection methods are limited in that they cannot distinguish between viable and non-viable microorganisms (Scheu *et al.* 1998). In order to prevent detection of dead microorganisms, culture enrichment should be done before performing the PCR. When compared to other techniques like culturing and plating, PCR is less time consuming. The other advantage of PCR-based methods is that they can be coupled with other techniques such as most probable number (MPN) and fluorescence *in situ* hybridisation (FISH-PCR)(Tanaka 2010; Velusamya and Arshaka 2010; Williams 1992).

Spectroscopic methods

Many methods have been proposed to monitor and detect bacterial spoilage in literature, and some are already applied in industry (Ellis *et al.* 2002a) some of which were discussed above. The major limitation is that they are time-consuming, labour-intensive and mostly destructive. Spectroscopic methods however, are fast, relatively easy to run and often non destructive.

In modern food processing, detecting and monitoring procedures have to provide results in real-time so that any problems can be dealt with as soon as possible. This is because there is pressure in the food industry for food safety as unsafe food can make people sick and may be result in death (Lazcka *et al*. 2007).

Fourier Transform-Infrared spectroscopy (FTIR)

FTIR is a vibrational spectroscopic technique with a (4000-600cm^{-1}) wave number range (Lin *et al.* 2004). The ability of FTIR to measure vibrations of biochemical composition and structure provides a metabolic "fingerprint" (Alexandrakis *et al.* 2009). FTIR spectroscopy is a rapid, non-invasive technique that has potential for routine analysis in the food industry (Ellis *et al.* 2002b). FTIR spectroscopy coupled with principal component analysis has been used to discriminate between intact and injured *Listeria monocytogenes* (Lin *et al.* 2004). Chen and Irudayaraj (1998) rapidly determined the fat and protein in cheese samples using FTIR spectroscopy. For determining spoilage FTIR spectroscopy has

been tested on chicken (Alexandrakis *et al.* 2009; Ellis *et al.* 2002b). The down-side of FTIR is that it requires sample preparation.

Near infrared (NIR) spectroscopy

In the 1960s Karl Norris"s engineering skills led to instruments which could record NIR spectra and he also applied the multivariate treatment of the spectra to analyse the chemical composition in organic samples (Norris 1996; (Batten 1998)). As shown in figure 6 near infrared (NIR) spectroscopy is a type of vibrational spectroscopy that corresponds to a wavelength range of 750 to 2500nm (wave numbers: 13,300 to 4000cm^{-1}) (Berzaghi and Riovanto 2009). NIR radiation applied to a sample may be absorbed, transmitted or reflected. The molecules within a sample undergo wavelength specific transitions when IR energy is applied. NIR energy spectra give rise to overlapping overtones and combination vibrations of bonds in molecular functional groups such as O-H, N-H, C-H and S-H bonds. Molecular overtones and combinational vibrations of bonds characteristic of NIR produce very broad, complex and overlapping spectral outputs that can obscure specific information on chemical assignments. It is necessary to develop calibrations using more than one wavelength in order to interpret the information contained in an NIR spectrum. Transforming the spectra to the first or second derivative, or calculating the deviation of each spectrum from the mean spectra allows for more accurate calibrations (Batten 1998). Spectral data treatments increase the accuracy by reducing variation in spectra caused by, for example, moisture difference between the samples. Multivariate data analysis tools and calibration techniques such as principal component analysis are used to get the desired chemical information from any given spectral data (Alexandrakis *et al*. 2009).

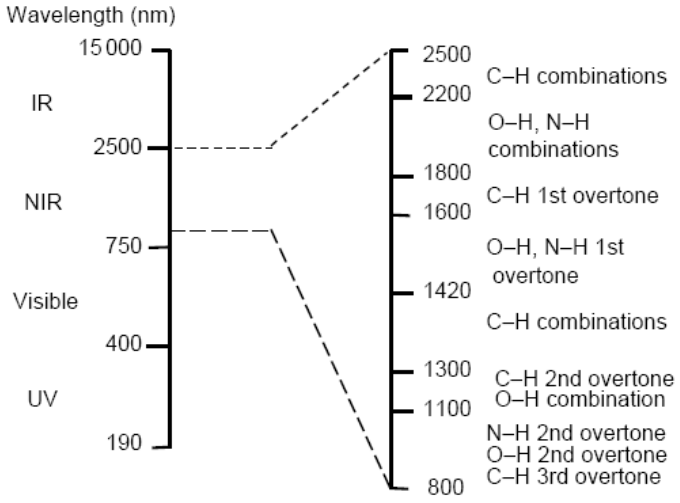

Figure 6 The principal types of NIR absorption bands and their locations (Osborne 1993).

NIR spectroscopy has been widely used in quality assessment of many agricultural and food products (Suthiluk *et al*. 2008). For example it has been used to evaluate the freshness of cod and salmon (Lin *et al.* 2006). It has also been used to detect and quantify the microbial spoilage of chicken (Alexandrakis *et al*. 2009). Suthiluk *et al*. (2008) tested the possibility of using NIR spectroscopy for evaluation of bacterial contamination on shredded cabbage. Alexandrakis *et al*. (2009) and Suthiluk *et al*. (2008)concluded that NIR spectroscopy has great potential as a rapid and non-destructive method for detecting spoilage. According to Alexandrakis *et al*. (2009), NIR had the potential to detect spoilage of chicken breast muscle if the microbial load (total aerobic plates) on the surface of the chicken breast fillets was greater than 6.75 $\log_{10}$ cfug^{-1}. The NIR prediction results failed to distinguish between day zero and day four samples but showed that successful discrimination can be achieved for longer storage times.

Advantages of NIR spectroscopy

NIR spectroscopy is gaining a lot of attention in analytical industry because of its ability to perform fast, accurate, non-destructive monitoring of changes in samples (Alexandrakis *et al*. 2008).

NIR provides information about the chemical composition as well as the structural and physical properties of biological materials (Alexandrakis *et al.* 2008).This ability to give information about many parameters of a sample is desirable when detecting spoilage of fish because fish spoilage is the result of multiple changes in the fish.

Other spectroscopic techniques such as ultraviolet-visible spectrophotometer require calibration against standards daily or a number of times a day followed by analysis of a relatively small batch of samples. In NIR spectroscopy however, once the instrument is calibrated it is used for days or months without recalibrating (Batten 1998) instead relying on post data collection recalibration. No need for recalibrating saves time.

Any analytical technique that reduces the use of chemicals is preferable. This is because chemicals can be hazardous to the health of the staff, the general community as well as a source of pollution to the environment. There are reports in the literature of NIR spectroscopy being adopted because it reduces exposure to many chemicals. For example, mercury which is a very dangerous chemical was used as a catalyst in determining the organic nitrogen content in plants and the procedure was replaced by the rapid NIR

spectroscopy(Williams 1992). In addition, colorimeters involve the use of chemicals. If chemicals are involved in NIR, it may be 70% ethanol to wipe the probe between samples to avoid cross contamination. Otherwise the samples do not need to be mixed with any chemical reagents.

The ability of NIR spectroscopy to record spectra for solid and liquid samples without prior manipulation is one of its very attractive features (Blanco and Villarroy 2002). This is in contrast with other methods like FTIR spectroscopy that are only suitable for liquid samples (Chen and Irudayaraj 1998). Consequently measuring the spectra of solid and non- homogeneous samples using FTIR spectroscopy involves blending and homogenising the samples (Biggs 1972). Non-destructive sampling is attractive to the food industry because more products can be tested without product loss.

Cozzolino *et al.* (2003) studied the feasibility of the use of visible and NIR spectroscopy to discriminate between wines of different origins. This was necessitated by the fact that other methods used required expensive and sophisticated analytical equipment. Examples given were high performance liquid chromatography, mass spectroscopy, gas-liquid chromatography and atomic absorption spectroscopy. The NIR spectroscopy reduced costs because it was less laborious. This is in comparison to the cost of using a panel of expensive judges to test the freshness of fish for example. The cost is both in paying them to do the job as well as in training them.

NIR spectroscopy can be performed outside of the laboratory facilities. Unlike microbial enumerating techniques, there is no need for specialist laboratory equipment such as a gas supply for the Bunsen burner, incubators, autoclaves and bacterial growth media.

There have been relatively rapid developments in NIR instruments and these have resulted in spectrophotometers that are flexible enough for use in different situations (Blanco and Villarroya 2002). In the food safety and quality industry the portability of instruments is of paramount importance in that it allows monitoring at different stages of the production line. This is in contrast to the sensory panel that cannot be available at every stage of the fish production. Even if the panel was to be available, the expense would negatively affect the profit returns of the company.

Disadvantages of using NIR spectroscopy

Though there are numerous advantages associated with using NIR spectroscopy it would be unrealistic to find absolutely nothing disadvantageous with this technique in detecting food spoilage. One outstanding limitation is the need for a reference method. The spectra obtained from the food samples need to be correlated with results from relevant traditional analyses (Batten 1998). In regard to detecting food spoilage, spectral data have been correlated to concentration of microbial metabolites and total aerobic plate counts (Alexandrakis *et al.* 2009). This limitation can only be overcome by developing a model for each specific food type and storing the data for future reference. However, at the moment the use of NIR spectroscopy is relatively new and the available data is limited.

NIR spectroscopy is a very sensitive analyser therefore for any valid model to be developed calibration is an important step. This involves taking into account all the variation present in the batches and to allow elimination of them and to leave only the one the researcher is interested in. Sample preparation must be identical each time, otherwise any difference in the preparation will introduce a new unnecessary variable.

Though the recording of spectra is easy and can be done by a layman, the model development step requires specialist knowledge. One has to be able to use statistical analysis and make decisions on what tools to use to get the desired information from the spectra. For example if the trends seen on the graphs are related to the real spectra or not.

Conclusions

Spoilage of fish is a complex process and includes chemical, physical and microbiological processes. Bacterial spoilage by SSOs is the most important type of spoilage. Detecting spoilage microbial communities provides the food industry with the opportunity to reduce economic losses by designing control measures. Many methods are available to assess fish spoilage however they are time-consuming, destructive and labour intensive. Near infrared spectroscopy has the potential to be an objective, robust, non-intrusive and rapid method to ensure high quality fish products on the market. The advantages of NIR make it attractive for routine analysis. However, there is still need for more research on using NIR to detect fish spoilage.

Research Paper

Development of near infrared spectroscopy method to detect and predict microbial spoilage on Atlantic salmon (Salmo salar).

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Running title: NIR and salmon spoilage

Abstract

The potential of using a near infrared spectroscopy (NIR) method to detect as well as predict microbial spoilage on Atlantic salmon was investigated. Principal component analysis results showed clear separation between the fresh salmon fillets and those stored for nine days at 4°C, indicating that NIR spectra could detect spoilage. Partial least squares regression (PLS) prediction models for microbial loads on the fish fillets were established for both *Enterobacteriaceae* and total aerobic counts on day 9. Calibration equations for both counts were good with R^2=0.94; RMSE= 0.17 log CFU/g and R^2=0.95; RMSE=0.12 log CFU/g for predicting log *Enterobacteriaceae* and log total aerobic counts respectively. These results indicate that with more samples NIR can be used to detect spoilage as well as predict microbial loads on Atlantic salmon (*Salmo salar*).

Keywords: NIR, microbial spoilage, Atlantic salmon

Introduction

Fish is an important source of nutrition for humans and eating fish at least twice a week is a well known recommendation from the health authorities. The Omega-3 fatty acids mainly found in fatty fish such as salmon are believed to improve the cardio vascular health (Sallam 2007). However, fish spoil faster than meat under the same storage conditions because of relatively high pH, high content of unsaturated fats and the growth of psychrotrophic microorganisms (Lin *et al*. 2006). Prediction or early detection of spoilage is not always easy since mechanisms underlying microbiological spoilage can be quite complex. Total microbial counts are a poor indicator of spoilage potential as many of the enumerated organisms may not grow in the food or will not responsible for spoilage (Adams and Moss 2008). Spoilage of chilled fish is due to the activity of psychrotrophic Gram-negative rods more specifically *Shewanella putrefaciens* and *Pseudomonas* spp (Chytiri *et al*. 2004).

Many methods have been proposed and used to detect fish spoilage (Lin *et al*. 2006) and these include microbial enumeration methods, volatile compound analysis, and measurement of lipid oxidation, nucleotide and amine metabolite assays and sensory

evaluation (Bremner and Sakaguchi 2000). However, these methods are limited in that they are invasive, destructive, time consuming, labour intensive and require highly trained panel and are therefore unattractive for industrial routine analysis. The industry is interested in a method that is objective, robust, non-intrusive (Nilsen *et al*. 2002) and rapid to ensure high quality fish products on the market as well as to minimise economic loss due to spoilage.

Near infrared spectroscopy (NIR) (750-2500nm wavelength) is an efficient and advanced tool for routine monitoring and control of process and product quality in the food processing industry. The use of this technique comes from its ability to perform fast, accurate and non destructive monitoring of changes in samples (Alexandrakis *et al*. 2008). Other advantages of using NIR include the minimal use of chemicals and generating of results in real time (Frake *et al*. 1998).

Several authors have applied NIR in evaluating food quality attributes such as fatty acid and protein content as well as freshness (Nilsen *et al*. 2002; Zhang *et al*. 1997; Woodcock *et al*. 2008). There has been an increase in interest of investigating the potential of using NIR on detecting food spoilage in the last decade. Suthiluk *et al*. (2008) investigated the possibility of NIR to measure the amount of bacterial contamination in shredded cabbage and reported that sufficiently accurate predictions could be obtained with the bias-corrected standard error of prediction of 0.46 log CFU /g and 0.44 log CFU/g. Alexandrakis *et al*. (2009) examined if NIR and fourier-transform-infrared could be used to detect onset of spoilage on chicken muscle meat and found that PCA and PLS-DA regression failed to completely discriminate between days 0 and 4 total viable counts. However, successful discrimination was achieved for longer storage times (between days 0 and 8 and days 0 and 14). Lin *et al*. 2006 concluded that short wavelength-NIR spectroscopy (600 to 1100nm) can be used to monitor the spoilage process in rainbow trout rapidly and non-destructively and that PCA analysis indicated that samples could be separated when the differences in the microbial levels is at least one to 2 log cycles. It was also suggested that NIR could be applied for the prediction of shelf life and microbial levels in seafood products (Lin *et al*. 2006).

The aim of this study was to examine the potential of near infrared spectroscopy combined with multivariate techniques to detect microbial spoilage as well as to predict microbial numbers on Atlantic salmon (*Salmo salar*) fillets stored at 4°C for nine days.

Materials and methods

Experimental design

Salmon fillets were purchased from the local supermarket on Monday and Wednesday mornings, days when salmon was delivered to the supermarket. Fillets were cut into half, and one half was analysed on the day of purchase (day 0) and the other half stored at 4°C for nine days (day 9). Nine batches (n=6 or 10) were analysed. Analyses were performed on the days of purchase and after nine days of storage at 4°C. This consisted of performing the NIR, aerobic plate counts (APC) and *Enterobacteriaceae* counts and swabbing the fish fillet surface with sterile swabs (COPAN). Swabs were frozen at -20°C until required for tRFLP analysis.

Microbiological analyses

Samples were weighed, placed in 200 mL of diluent ((0.1% LP 0037, Oxoid, Basingstoke, UK) and 0.9% sodium chloride) and homogenised for 60 seconds in a stomacher (Seward, London, UK) at room temperature. Serial dilutions were prepared, and duplicate aliquots (0.1mL) of the appropriate dilutions were spread onto MacConkey agar (CM 0115, Oxoid, Basingtoke, UK) for *Enterobacteriaceae* counts and Long and Hammer (Konstantinos and Nychas 1999) agar for total plate counts. Plates were then incubated for 24 hours at 25°C. Dominant colonies were isolated and stored in 30% glycerol (Sigma Ultra, Malaysia) and nutrient broth (CM0001, Basingstoke, UK) at -80°C for sequencing.

Identification of dominant isolates

Dominant isolates were identified by partially sequencing the 16SrRNA gene. A PCR product was generated by directly placing cells from pure colonies into a PCR reaction that contained Immomix (Bioline) and the primers 519F (CAGCMGCCGGTAATAC) and 1492R (TACGGYTACCTTGTTACGACTT). The thermal cycling program consisted of a 10 minute initial denaturation step at 95°C, followed by 35 cycles of 1 minute at 94°C; 1

min annealing at 55°C and 1 minute, with a final step of 10 minutes at 72°C. PCR products were sent to Macrogen (South Korea) for sequencing.

Microbial community analyses

MicroBead Solution from the UltraClean kit was added to the frozen swabs which were vortexed. DNA was then extracted from the frozen swabs using the UltraClean Microbial DNA kit (MoBio) following the manufacturer‟s directions.

Terminal restriction fragment polymorphism analysis was carried out on the 16S rRNA gene as described in (Powell *et al*. 2010). The 16S rRNA gene was amplified using Immomix mastermix by PCR with fluorescently labelled universal primers 10F (GAGTTTGATCCTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT). The thermal cycling program consisted of a 10 minute initial denaturation step at 95°C, followed by 35 cycles of 1 minute at 94°C; 1 min annealing at 55°C and 1 minute, with a final step of 10 minutes at 72°C. Two PCR reactions were combined and 5µL of the combined PCR product was then digested with 5U of each enzyme separately for 3 hours. The enzymes used were *HaeIII, HhaI* and *HinfI* (New England Biolabs).

Ten micro litres of the 1 in 10 diluted digests were purified by ethanol precipitation into a 96 –well plate. The purified digests were resuspended in 30 µL of CEQ sample loading solution (Beckman Coulter) with 0.25 µL of GenomeLab size standard 600 (Beckman Coulter).The digests were run on the Beckman CEQ Genetic Analyser. A list of fragment length and peak area was collated for each enzyme for each sample. Fragments outside the range of the size standard (60-600) were excluded as their size could not be accurately determined. Peaks with a height of less than 500 relative fluorescence units were also excluded. The peak height was then used to calculate the percentage peak height of each fragment contributed to the overall peak height for that sample. Fragments that made less than 1% of the total peak for a sample were assigned a value of zero; fragments with a peak over 1% were left as a percentage (Powell *et al*. 2010). Multivariate methods were used to analyse differences in the microbial communities using Primer6 package (Primer-E, Plymouth Laboratory). Non-metric multidimensional scaling ordination plots (MDS) were used to explore relationships between groups of samples based on the strength of the similarities, calculated with the Bray-Curtis between the day 0 and day 9 microbial communities. Analysis of Similarity (ANOSIM) in Primer6 was used to test the null

hypothesis that there was no difference between microbial communities on fresh fish (day 0) and old fish (day 9).

Near infrared analyses

FT-NIR measurements were taken on the pink flesh of the fish, and white bits were avoided as much as possible. The fillets were placed in tissue culture dishes, 145x 20mm (Greiner Bio-One GmbH, Germany). The spectra were collected using the fibre optic probe (with a plastic spacer about 2 mm thick between the probe and the fillet) on a Bruker MPA, collecting spectra over the wave number range of 12500 to 4000 cm^{-1}. A background measurement using 64 scans was performed prior to the sample analysis. The probe accumulated 16 scans per measurement with a resolution of 4 cm^{-1} (wavelength 800-2500 nm). For a single evaluation spectra were collected on eight random spots and averaged. Between samples, the fibre optic probe was cleaned with acetone and dried with Kimwipes.

Model development

Principle component analysis (PCA) was performed using Unscrambler X to investigate the potential of the NIR spectroscopy to differentiate between the salmon fillets on day and after nine days of storage. This was only tested on the first four batches. The first principal component is describes the most variance, with each subsequent component describing less variance. The principal components are independent of each other. PCA can be used to identify outliers which might need to be removed from the data range before the development of a PLS model.

PCA was also performed on all the day 0 spectra to test for any other sample groupings. Initially Opus Quant2 software was used to describe how the log bacterial counts on day 9 related to day 0 spectra. The same task was carried out using the Unscrambler X software because Unscrambler X allows for data interrogation compared to Opus Quant2. Spectra in the range 7500 to 10000 cm^{-1} were subjected to spectral pre-treatment using unit vector normalisation for log *Enterobacteriaceae* counts and area normalisation for log total bacteria counts.

Evaluation of model performance

The partial least squares regression model was validated using random cross validation with 20 segments on Unscrambler X software. The Opus Quant2 software used full cross validation to validate the PLS regression model. The model was evaluated by the root mean square error (RMSE), which measures the average deviation between observed and predicted values.

Results

Microbiological analysis

The initial (day 0) mean total aerobic counts and *Enterobacteriaceae* counts were 3.79 log CFU/g and 3.46 log CFU/g respectively. The day 9 counts were 7.01 log CFU/g and 6.93 log CFU/g for total aerobic and *Enterobacteriaceae* respectively. The bacterial counts on the day of purchase were 4 log cycles lower than the counts on the fillets after nine days of storage at 4°C (Figure 7 and 8). Although the day 0 log bacteria counts are quite similar for all the batches, day 9 counts varied more. For example day 0 counts for batch 1 and batch 3 were 3.79 and 3.59 log CFU/g respectively but day 9 counts were 7.99 and 5.64 log CFU/g (Figure 7). Both log *Enterobacteriaceae* and total aerobic counts on salmon fillets at day 9 exhibited normal distribution.

Isolates with different colony morphologies were identified by partial sequencing of the 16S rRNA gene. At day 9 the culturable microflora of the fish fillets were dominated by *Pseudomonas* spp (Table 4).

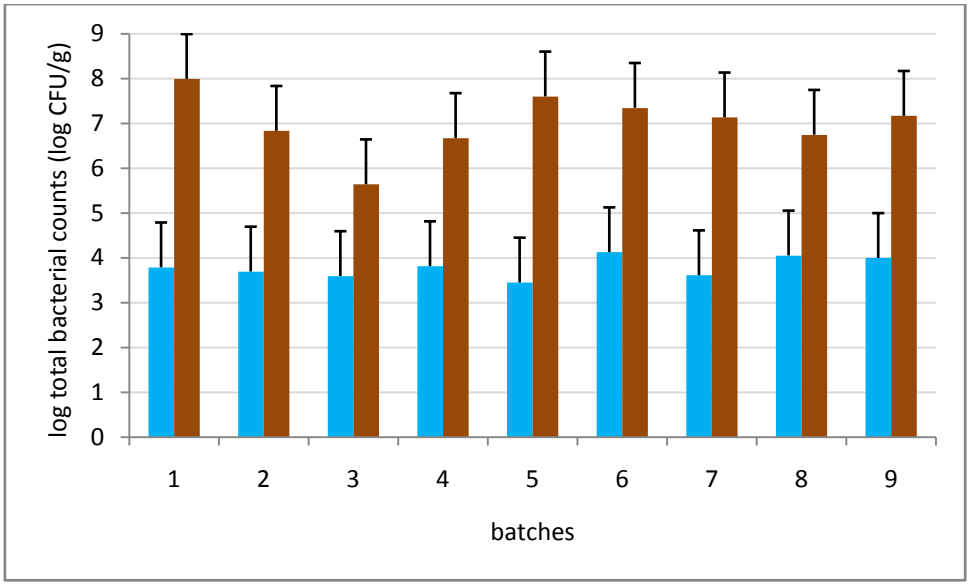

Figure 7 Graph of log total bacteria counts (log CFU/g) for each batch. Error bars represent standard deviation. Blue and brown represent log counts on day 0 and day 9 respectively.

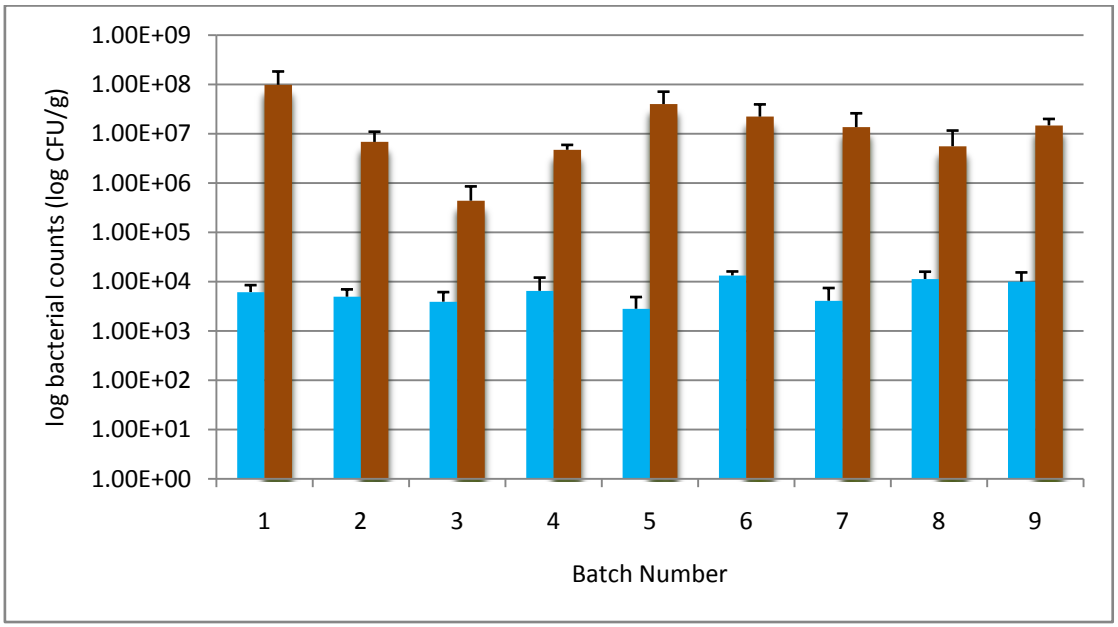

Figure 8 Graph of log *Enterobacteriaceae* counts (log CFU/g) for each batch. Error bars represent standard deviation. Blue and brown bars represent day 0 and day 9 respectively.

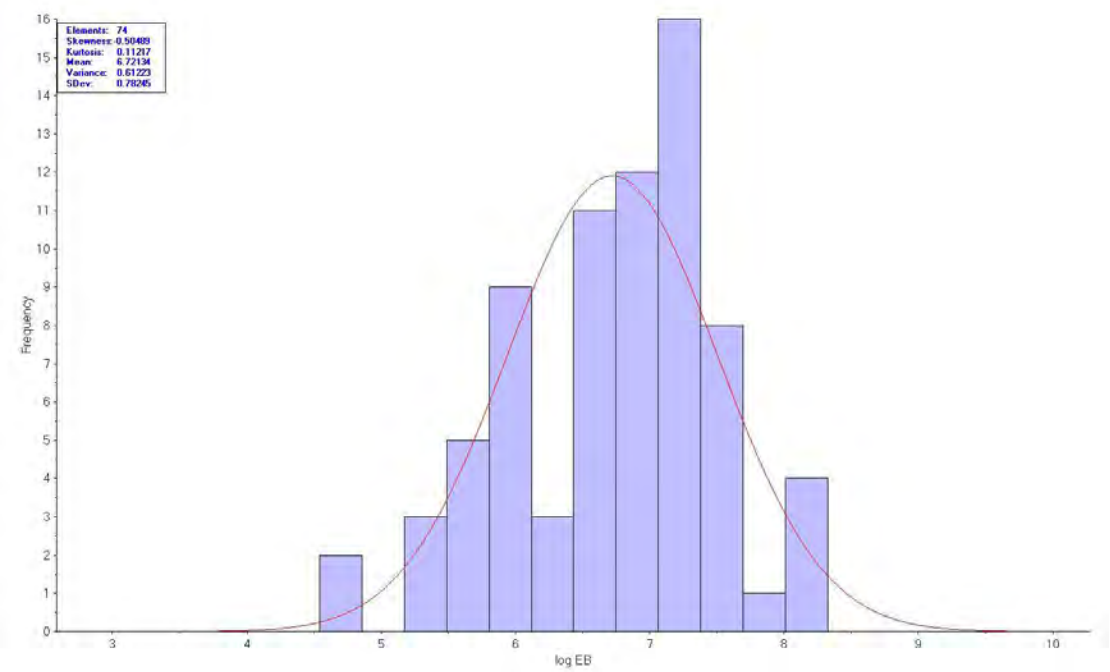

Figure 9 Distribution of log *Enterobacteriaceae* counts at day 9 exhibiting normal distribution

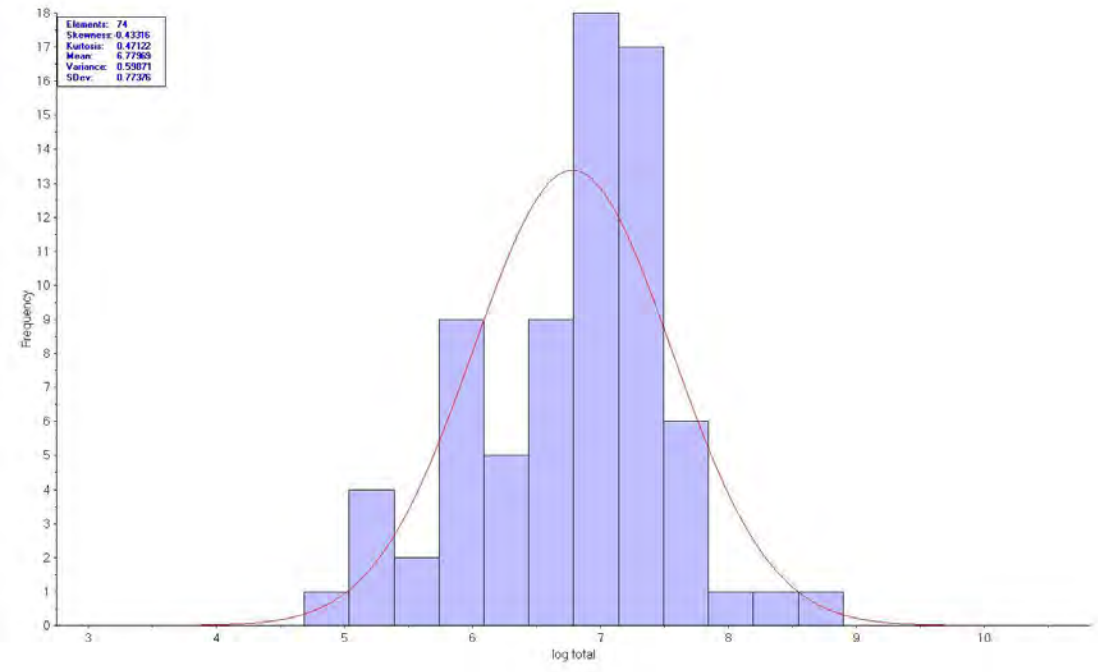

Figure 10 A histogram showing the distribution of log total bacteria counts at day 9

Table 5 Tentative identification of isolates based on sequencing of 16S rRNA gene from the day 9 samples.

Number of isolates	Accession number of nearest match	genus	species	Percentage similarity
9	AY450556	*Pseudomonas*	*putida*. L	97
3	DQ084459.1	*Pseudomonas*	*flurorescens.* FLM05-1	97
1	FJ950690.1	*Pseudomonas*	*psychrophilia* .d390	86
7	NR_028619	*Pseudomonas*	*psychrophilia*. E-3	97

The tRFLP analysis produced a „fingerprint" of the structure of the microbial community where different fragments are assumed to be representative of different species. The similarity between the microbial community on fresh and old salmon fillets is represented by the MDS plot (Figure 11). In this plot the closer the two points, the more similar they are. Day 0 (A) samples clustered into two groups whereas day 9 (B) samples formed one group with only two outliers. There is a significant difference (ANOSIM test; R=0.558, $p<0.05$) between microbial communities on day 0 and day 9 based on the 16S rRNA gene tRFLP data. In addition, there was a significant decrease in the number of fragments from 57 to 38 during the nine days of storage (student t-test; $p=0.01$).

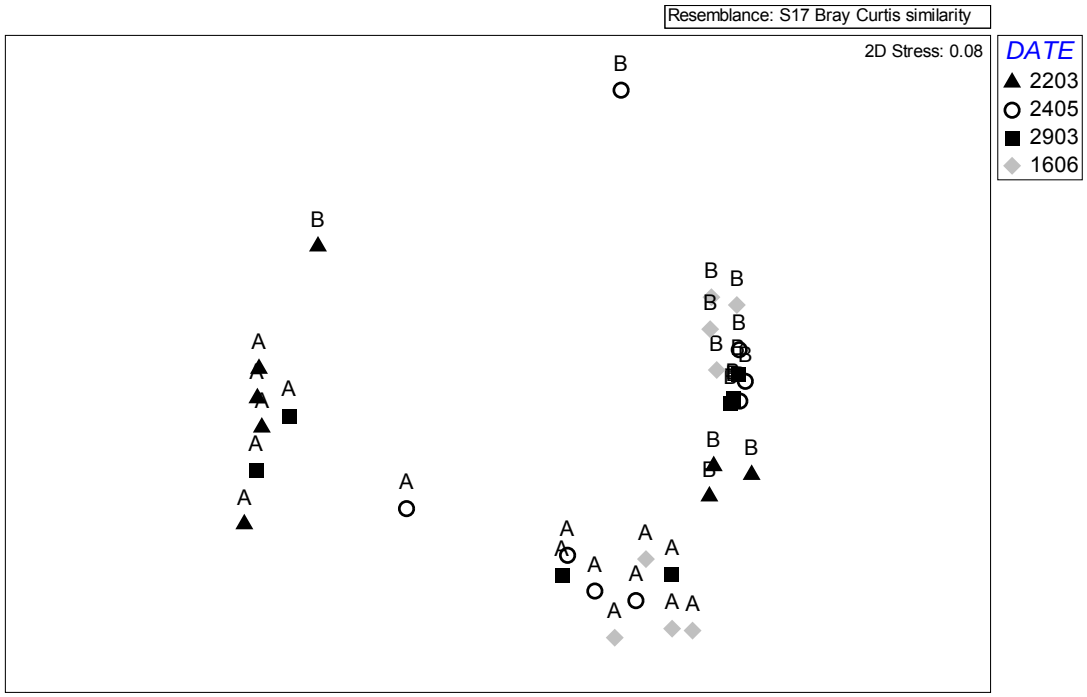

Figure 11 A MDS plot showing the similarity of microbial communities on salmon as measured by tRFLP using the 16S rRNA gene. A= day 0 and B=day 9.

Fourier Transform-Near infrared spectroscopy

Preliminary results

Spectra collected on the day 0 and day 9 fillets were subjected to multi scattering correction to remove most of the variation that came from differences in light scatter (Figure 12). PCA was then applied to the day 0 and day 9 spectra to examine if the NIR could distinguish between fresh and old fish. Figure 13 shows that fresh samples were differentiated from old samples using PC1 and PC2 which account for 70% and 26% of the explained variance.

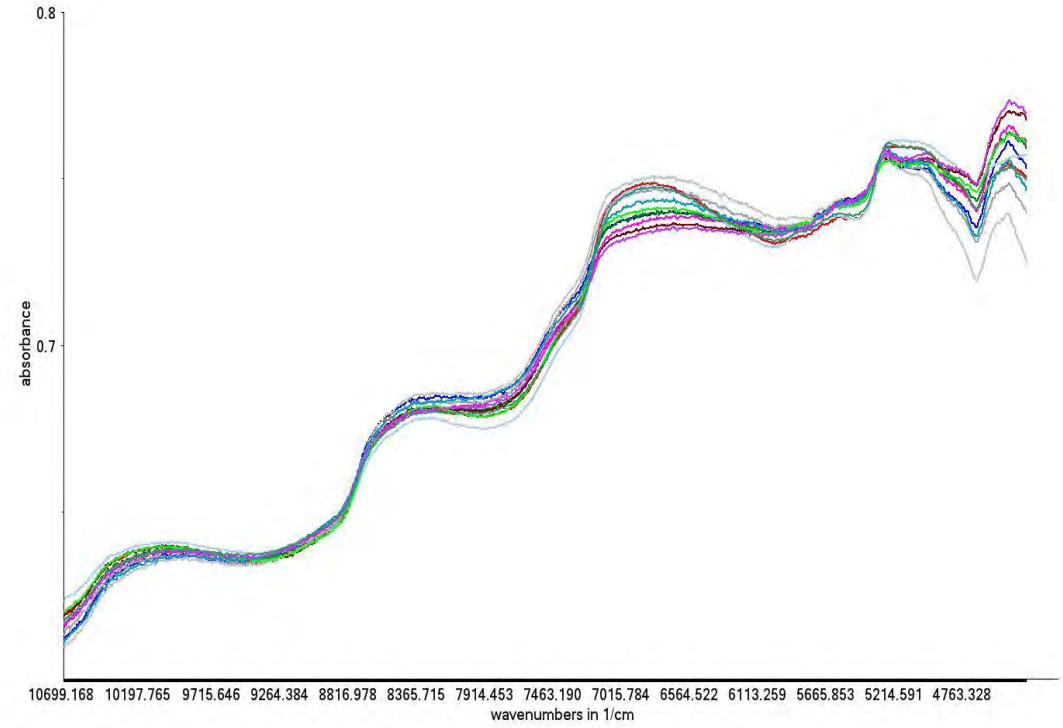

Figure 12 A plot of multi scattering corrected spectra from the first batch

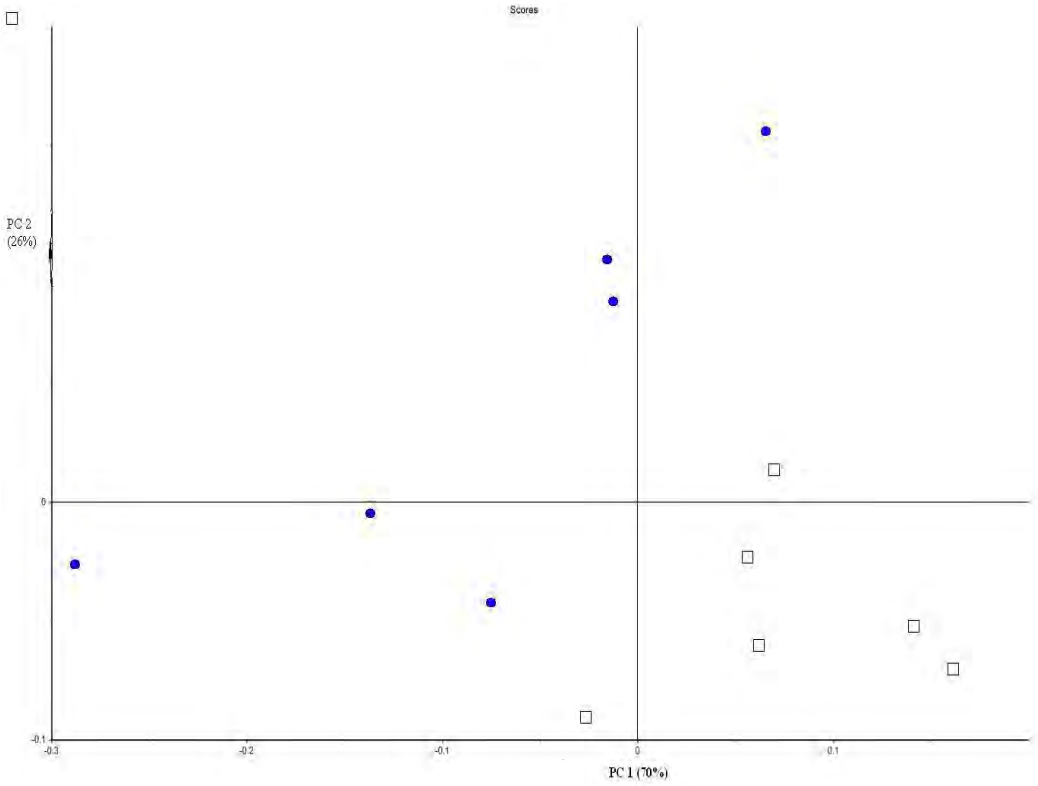

Figure 13 PCA of spectra from fresh and old salmon fillets of batch 1. The squares represent fresh fillets and blue circles represent old fish fillets.

Spectra

Typical FT-NIR spectral raw data from 10700 to 4350 cm^{-1} collected from fresh salmon fillets (day 0) are presented in (Figure 14). The main features on the spectra plots of fillets were the same.

The Opus Quant2 software was used to describe how the difference in log (bacteria counts) on day 9 related to the difference in day 0 spectra. The optimise function within Opus Quant2 software tested different regression models. The best fit was found on the wave number range 10000 to 7500 cm^{-1} which is a typical CH region. Opus Quant2 used Min-Max normalisation to obtain the best model. The best model is one that has the lowest error of prediction (RMSECV) and the highest R square value. The information from Opus Quant2 software was used with the Unscrambler X software. Different normalisation techniques were tried on the Unscrambler X software to come up with the best PLS model. Those included area, unit vector, minimum, maximum, and range and peak normalisations. The best PLS model for log total aerobic counts and log *Enterobacteriaceae* counts were achieved by using area normalisation and unit vector normalisation respectively (Figure 15 and 16).

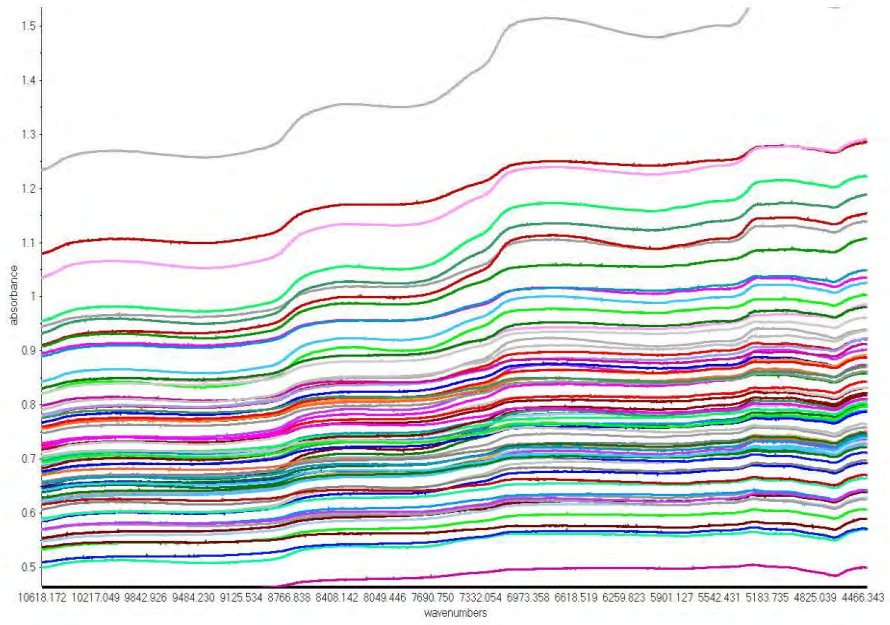

Figure 14 Typical FT-NIR spectra in the range 11000-4462 cm^{-1} collected on both day 0 and day 9 fish fillets.

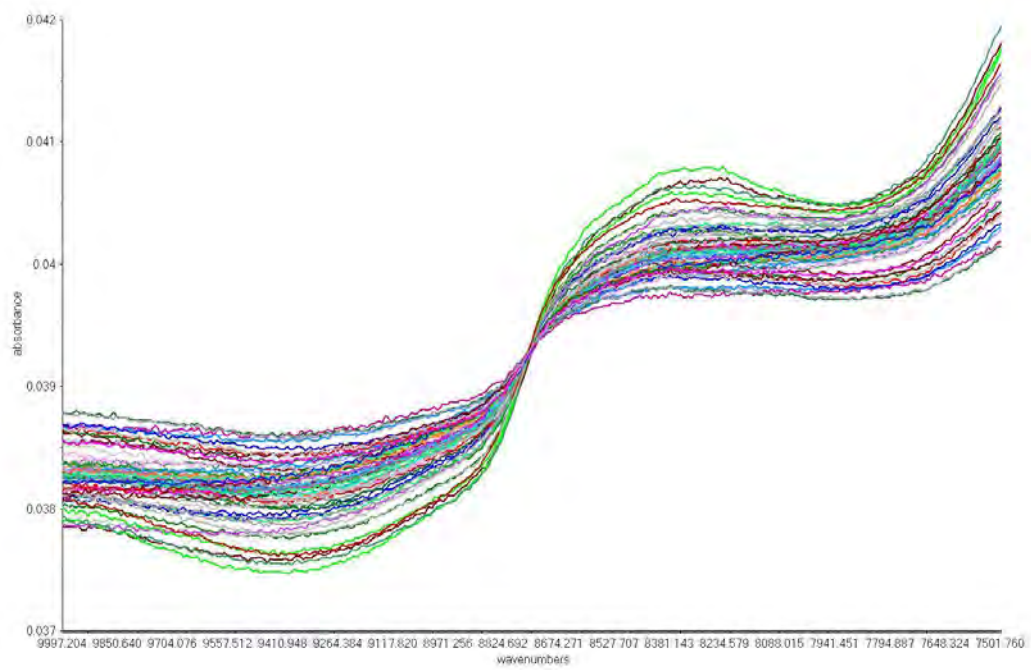

Figure 15 Spectral data wavenumber range 7500 to 10000 cm^{-1} after unit vector normalisation.

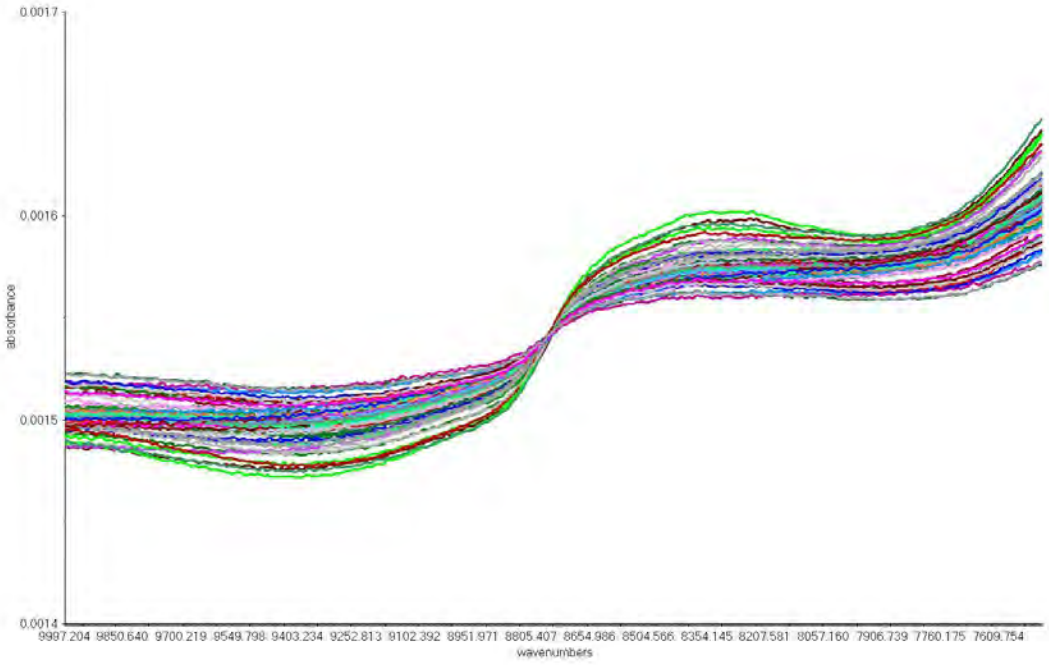

Figure 16 Spectral data wavenumber range 7500 to 10000 cm^{-1} after area normalisation

Principle component analysis of NIR spectra from day 0 fillets

Principal component analysis plots were used to identify outliers. The score plots following PCA of day 0 salmon fillets spectral data are shown in Figure 17. PC1 and PC2 accounted for 82% and 18% of the explained variance in spectra of all the salmon fillets respectively. Two of the batches grouped separately from the rest indicating that there is a batch effect (Figure 17).

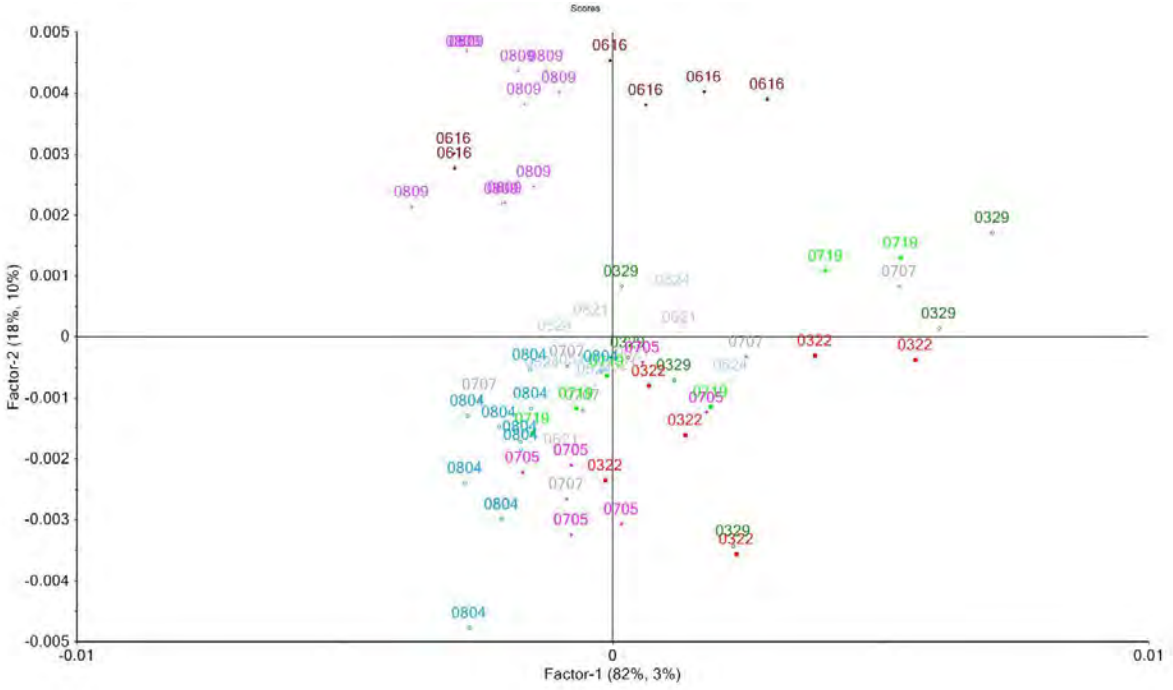

Figure 17 Score plot showing principal component analysis of near infrared data (different colours show different batches).

Partial least squares regression models

Results for the PLS models (7 and 8 factors) of predicting bacteria counts in salmon fillets are shown in table 6. Both Figures 18 and 19 show that there is a positive correlation between day 0 spectra and bacteria counts on day 9. The R^2 for the validation plots was 0.64 for both log *Enterobacrteriaceae* and log total aerobic counts (Figures 18 and 19). However, the error (RMSE) was slightly lower for total counts (0.32 log CFU/g) compared to the *Enterobacrteriaceae* counts (0.41 log CFU/g))

Table 6 Standard error of prediction (RMSE) for calibration and validation of the PLS regression models for predicting bacteria counts on day 9 using day 0 spectra

Bacteria counts	Calibration		validation	
	RMSE	R^2	RMSE	R^2
Log *Enterobacteriaceae*	0.17	0.94	0.41	0.64
Log total aerobic counts	0.12	0.95	0.32	0.64

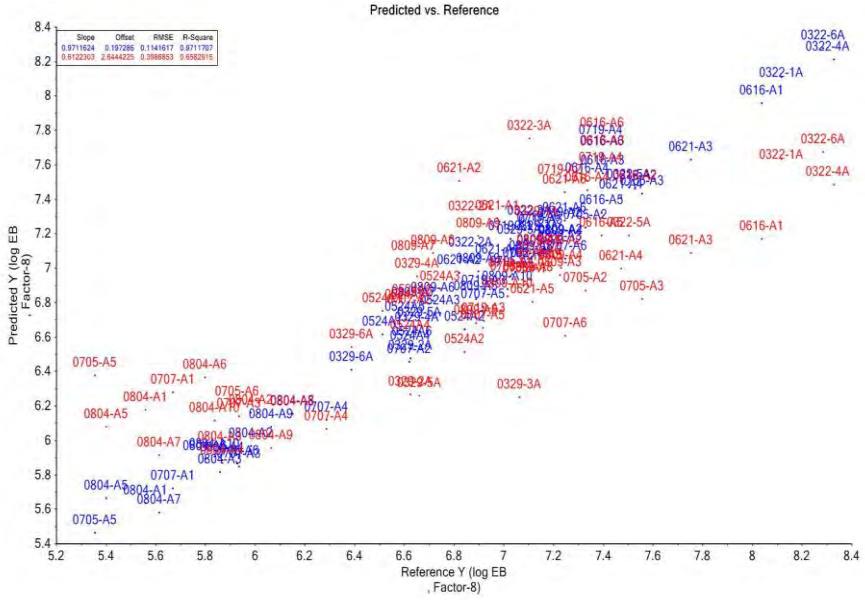

Figure 18 Correlation plots for actual log *Enterobacteriaceae* counts and values predicted from spectra using PLS regression modelling technique.Blue points show the calibration plot and the red points show the validation plot for log *Enterobacteriaceae*

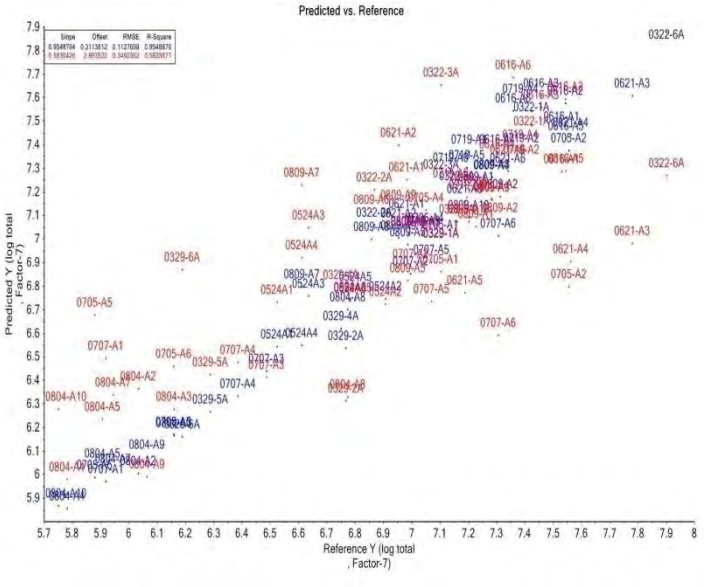

Figure 19 Correlation plots for actual log total aerobic counts and values predicted from spectra using PLS regression model. Blue points show the calibration plot and the red points show the validation plot.

Discussion

Microbiological analysis

Log total aerobic counts and log *Enterobacteriaceae* exhibit normal distribution as expected of natural populations. There is an increase in both total aerobic and enterobacteria counts over the nine days of storage at 4°C (Figure 7 and 8). This was expected because the bacteria were given time to utilise the highly nutritious fish fillets and multiply. The bacterial counts were performed on the fillets (3logCFU/g) and hence were lower than if they were performed on the gut tissue. Fish products with a microbial load of 7 to 8 log CFU/g are considered too spoilt for eating when using sensory analysis (Lin *et al*. 2006). Higher numbers on day 0 did not necessarily result the highest numbers on day 9. The counts on day 9 varied significantly between the batches. For example on (Figure 8) batch 4 is higher than batch 1 on day 0 but lower on day 9. There are several explanations why microbial spoilage may differ between the same type of product. These include geographical origin, storage conditions and the place in which the fish was harvested (Koutsoumanis and Nychas 1999). Minor changes in the processing and packaging may also cause striking differences in the microbiology of the fish product (Gram and Huss 1996).

tRFLP was used to measure if the microbial community structure was different between the fresh fillets and fillets stored at 4°C for nine days. This analysis showed (Figure 11) that there was a significant difference between the microbial communities on day 0 and day 9 (ANOSIM test R=0.558, $p<0.05$). In addition in Figure 11 it is apparent that the day 0 samples cluster in two groups. This means that different bacteria are present on different batches of fish at day 0. However, day 9 samples formed one group with only two outliers (Figure 11), this means that the microbial communities were similar to each other on day 9. The tRFLP analysis also shows that the microbial community structure was less diverse on day 9 samples than the day 0 samples. Number of fragments, which are assumed to be representative of the number of species, decreases during this time frame from 57 to 38. The student t-test showed that the two means of day 0 and day 9 samples were significantly different (student t-test; $p= 0.01$). It appears that the microbial communities are becoming dominated by particular bacteria and that some species that were initially present cannot be detected after nine days. Several interactions between bacteria which may end up with a particular group of bacteria dominating have been described in food ecosystems (Gram *et al*. 2002). These interactions include antagonistic and cooperative behaviour. Competition for nutrients is a major cause of these interactions and may select for bacteria that are capable of using the limiting nutrient (Leroi *et al*. 2006).

Dominant isolates were identified by partial sequencing of the 16S rRNA gene. All of the 20 isolates sequenced from the day 9 plating belonged to the genus *Pseudomonas*. This is not contrary to the tRFLP data which implied dominance by particular bacteria. Bacteria from the genus *Pseudomonas* are often present at spoilage of aerobically refrigerated flesh foods (Nychas and Tassou; Alexandrakis *et al*. 2009) because many species of *Pseudomonas* are psychrotrophs. Although *Shewanella putrefaciens* has been reported as a specific spoilage bacterium of marine temperate-water stored aerobically in ice it was not found in the 20 isolates that were sequenced. *Pseudomonas* species were expected to predominate over other bacteria because of their ability to produce siderophores and increase their iron-binding capacity on the iron limited salmon fillets (Gram and Dalgaard 2002).

Fourier Transform-Near infrared spectroscopy

Near infrared spectra are dependent on the chemical make-up on the surface being analysed. The spectra obtained in this work therefore are likely affected by the amount of

protein, fat and moisture present on the fish fillets as well as the bacteria and the metabolites they are producing. NIR spectra are also influenced by factors unrelated to the chemistry of fish for example measurement technique. For this reason it is crucial that sample preparation and measurement technique are as consistent as possible. Throughout this study the NIR measurements were taken on the inside of the fillet (as opposed to the skin side). Lin *et al*. (2006) have shown that the PLS regression equations differed between samples on which the NIR measurement was taken on the different sides of the fillet.

The limitation is that unlike FTIR, NIR is not capable of detecting bacterial cells on the fillet surface directly due to other interfering signals (Alexandrakis *et al*. 2008). Though the interest is on bacteria counts, using NIR is based on the assumption that the change in the entire chemical make-up of the fish fillets was due to metabolic activity of the microorganisms (Panagou *et al*. 2010).

The spectra in Figure 14 have the same spectral features. The vertical offset observed between the samples was also observed by Alexandrakis *et al*. (2009) and could have been due to different reasons. These include differences in light scatter caused by variations in the physical structure of the salmon fillet surfaces. There was also varied distance and angle of measurement between the fibre optic probe and the fillet. It was therefore important to normalise the spectra (Figure 15 and 16) to remove any variation that was unrelated to the biochemistry and microbiology of the fish fillets. The problem with the variation in the physical structure of the fillets is usually taken care of by homogenising samples before analysis, for instance Suthiluk *et al*. (2008) homogenised the cabbage before analysing their samples. However, homogenising the fish fillets will be destructive and that would not meet the requirement for a non-destructive method of analysis that the food industry requires. In this study in an effort to reduce the effect of variation in the physical structure of the fillet to influence spectra, spectra was collected from eight different six millimetre spots on each fillet. A two millimetre spacer between the surface of the fillet and the probe also helped with reducing the variation in the distance between the probe and the fillet.

Comparison of day 0 and day 9 spectra from the preliminary results

PCA was performed to investigate the potential of NIR to differentiate fresh (day 0) and old (day 9) salmon fillets for only one batch (Figure 13). The day 0 and 9 samples separated into two distinct groups as was expected. The separation of day 0 and day 9 samples is consistent with the microbiological analysis (Figure 11).This segregation could be attributed to the biochemical changes arising from proteolysis of fish muscle proteins after nine days of storage. Also on day 9, spoilage was evident with fillets discoloured, and covered with slime. A combination of these changes impacts on the NIR spectra (Alexandrakis *et al*. 2009). As the bacteria on the fish fillets increase rapidly during storage they secrete huge amounts of enzymes that break down proteins into peptides and amino acids and fats into fatty acids and glycerols (Fraser and Sumar 1998), hence big changes in the biochemistry of the fillets that result in different NIR spectra.

In addition the ability of the PCA to distinguish between day 0 and day 9 salmon fillets is consistent with what Alexandrakis *et al*. (2009) concluded that NIR has the potential to detect chicken spoilage if the microbial load on the surface of the chicken fillets is greater than 6.75 log CFU/g. The microbial load on the fish fillets after nine days of storage was on average 7.01 log CFU/g total aerobic counts and 6.93 log CFU/g for *Enterobacteriaceae* counts. These two values were higher than the threshold (6.75 log CFU/g) indicated by Alexandrakis *et al*. (2009).

Model development and validation

Principal component analysis was performed on the day 0 spectra of all batches and no outliers were found in the PCA plot (Figure 17). However, two batches were separated from the rest indicating batch effect. Removal of these two batches from the model development was not justified as it would result in the loss of a significant amount of information. The batch effect suggests that there is a variable, or variables influencing the spectra other than the variable of interest (log bacteria counts), such as minor variation during storage, differences in salmon flesh related to feeds and season. The best way of reducing this effect would be to analyse more batches.

Partial least square regression models were developed by using day 0 spectra (X matrix) and day 9 bacteria counts, both *Enterobacteriaceae* and total aerobic counts (Y-variables).

The model could be used to predict the bacteria counts on salmon fillets stored at 4°C nine days later using day 0 NIR measurements.

The two PLS models show a good correlation between the NIR spectra and the bacteria counts, using seven and eight factors for the model. For example the standard error of prediction (RMSE) for the *Enterobacteriaceae* model was 0.41 log CFU/g which is only marginally bigger than the standard error from the actual plate counts (0.34 log CFU/g). Consequently, the model could be improved by decreasing the standard error of the actual plate counts. This could be done by analysing triplicate plates instead of duplicate for each dilution. However, plate counts are inherently variable and it would be difficult to improve the error. Generally error within 20% of the mean for a plate count is considered acceptable (Sutton 2006).

The relatively low R^2 for both models can also be explained by the tendency of both models to over-predict lower values and under-predict higher values. The over-prediction and under-prediction could also be explained from the normal distribution of both log *Enterobacteriaceae* and log total aerobic counts at day 9 (Figures 9 and 10). The normal distribution of the log bacteria counts was expected as the samples analysed were from a natural population. However, lack of data at the extreme ends resulted in the software unable to accurately predict very high and low values. As a result if more samples with extreme bacteria counts are included in a future model, the R^2 and consequently the PLS model will improve.

The values for RMSE and R^2 are much more favourable if considering the calibration data only (Table 6). However, as Wold *et al*. (2001) that any model needs to be validated before it can be applied it is important to consider the validation data. It is preferable to use a new independent validation set to validate the model but because there were not enough samples in this study cross validation was used instead (Wold *et al*. 2001). There was a significant discrepancy between the calibration and the validation data (Table 6). This could indicate that too many factors were used were used in developing the PLS model, a phenomenon referred to as over-fitting (Frake *et al*. 1998). Again, with more samples the discrepancy between the calibration and validation data should disappear.

Conclusions

In this study it was shown that there is a relationship between NIR spectra of fresh Atlantic salmon fillets and the aerobic plate counts (either total or enterobacteria) nine days later. However the model is not yet robust and in the future more samples should be analysed to improve the model.

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References

Adams MR, Moss MO (2008) Food microbiology (3rd edn) (The Royal Society of Chemistry, Thomas Graham house, Science park, Milton road, Cambridge, UK)

Abbas KA, Saleh AM, Mohamed A, Lasekan O (2009) The relationship between water activity and fish spoilage during cold storage: A review. *Journal of Food Agriculture & Environment* **7**, 86-90.

Alexandrakis D, Downey G, Scannell AGM (2008) Detection and identification of bacteria in an isolated system with near-infrared spectroscopy and multivariate analysis. *Journal of Agricultural and Food Chemistry* **56**, 3431-3437.

Alexandrakis D, Downey G, Scannell AGM (2009) Rapid Non-destructive Detection of Spoilage of Intact Chicken Breast Muscle Using Near-infrared and Fourier Transform Mid-infrared Spectroscopy and Multivariate Statistics
Food and Bioprocess Technology.

Alvarez RJ (1982) Effect of storage on the lipid content and rancidity development of salt-minced mullet. *Caribbean Journal of Science,* **18**, 71-74.

Amanatidou A, Schlüter O, Lemkau K, Gorris LGM, Smid EJ, Knorr D (2000) Effect of combined application of high pressure treatment and modified atmospheres on the shelf life of fresh Atlantic salmon. *Innovative Food Science & Emerging Technologies* **1**, 87-98.

Bataringaya A (2007) Analysis of quality deterioration at critical steps/poits in fish. The United Nations University.

Batten GD (1998) Plant analysis using near infrared reflectance spectroscopy: the potential and the limitations. *Australian Journal of Experimental Agriculture* **38***, 697–706*.

Berzaghi P, Riovanto R (2009) near infrared spectroscopy in animal science production: principles and application. *Italian Journal of Animal Science* **8**, 39-62.

Biggs DA (1972) Precision and accuracy of infrared milk analysis. *Journal of the Association of Official Analytical Chemists* **55**, 488-497.

Blanco M, Villarroya I (2002) NIR spectroscopy: a rapid-response analytical tool *TrAC Trends in Analytical Chemistry* **21**, 240-250.

Boyd LC, Nwosu VC, Young CL, MacMillian L (1998) Monitoring lipid oxidation and antioxidant effects of phospholipids by headspace gas chromatographic analyses of rancimat trapped volatiles. *Journal of Food Lipids* **5**, 269-282.

Bremner HA, Sakaguchi M (2000) A critical look at whether freshness can be determined. *Journal of Aquatic Food Product Technology* **9**, 5 - 25.

Buransompob A, Tang J, Mao R, Swanson BG (2003) Rancidity of walnuts and almonds affected by short time heat treatments for insect control. *Journal of Food Processing and Preservation* **27**, 445-464.

Cahill MM (1990) Bacterial flora of fishes: A review. *Microbial Ecology* **19**, 21-41.

Cakli S, Kilinc B, Cadun A., Dincer T, Tolasa S (2006) Effect of ungutting on microbiological, chemical and sensory properties of aquacultured sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) stored in ice. *Eur Food Res Technol* **222**, 719-726.

Chen M, Irudayaray (1998) Sampling technique for cheese analysis by FTIR Spectroscopy *Journal Of Food Science* **63**, 96-99.

Cozzolino D, Smyth HE, Gishen M (2003) Feasibility study on the use of visible and near-infrared spectroscopy together with chemometrics to discriminate between commercial white wines of different varietal origins. *Journal of Agricultural and Food Chemistry* **51**, 7703-7708.

Dalgaard P (1995) Modelling of microbial activity and prediction of shelf life for packed fresh fish. *International Journal of Food Microbiology* **26**, 305-317.

Deng JC, Orthoefer D, R.A.,, Watson M (1976) Lipids and fatty acids in mullet (Mugil cephalus): Seasonal and locational variations. *Journal of Food Science* **41**, 1479-1483.

Di Natale C, Davide FAM, D'Amico A, Nelli P, Groppelli S, Sberveglieri G (1996) An electronic nose for the recognition of the vineyard of a red wine. *Sensors and Actuators B: Chemical* **33**, 83-88.

Doe PE (Ed.) (1998) 'Fish drying and smoking:production and quality.' (CRC Press, Taylor and Francis Group: New York).

Ellis DI, Broadhurst D, Kell DB, Rowland JJ, Goodacre R (2002) Rapid and quantitative detection of the microbial spoilage of meat by Fourier transform infrared spectroscopy and machine learning. *Applied and Environmental Microbiology* **68**, 2822-2828.

Frake P, Gill I, Liscombe CN, Rudd DR, Waterhouse J, Jayasooriya UA (1998) Near-infrared mass median particle size determination of lactose monohydrate, evaluating several chemometric approaches. *The Analyst* **123**, 2043-2046.

Fraser O, Sumar S (1998) Compositional changes and spoilage in fish - an introduction. *Nutrition and Food Science* **98**, 275-279.

Gittleson R (1990) quantification of physico-chemical modes of deterioration in sous-vide processed salmon.

Gram L, Dalgaard P (2002) Fish spoilage bacteria - problems and solutions. *Current Opinion in Biotechnology* **13**, 262-266.

Haard NF, Bracho GE (1995) Identification of two matrix metalloproteinases in the skeletal muscle of pacific rockfish (Sebastes sp.). *Journal of Food Biochemistry* **19**, 299-319.

Harewood P, N,Branford, Conn (1998) Detection of fish spoilage by colorimetry. (GEM Biomedical, Inc., Hamden, Conn: United States of America).

Hart DJ, Vreeland RH (1988) Changes in the hydrophobic-hydrophilic cell surface character of Halomonas elongata in response to NaCl. *J. Bacteriol.* **170**, 132-135.

Hattula T, Luoma T, Kostiainen R, Poutanen J, Kallio M, Suuronen P (1995) Effects of catching method on different quality parameters of Baltic herring (Clupea harengus L.). *Fisheries Research* **23**, 209-221.

Leroi F, Joffraud J-J, Chevalier F, Cardinal M (2006) Study of the microbial ecology of cold-smoked salmon during storage at 8°C. *International Journal of Microbiology* **39**, 111-121.

Koutsoumanis K, Nychas G-JE (1999) Chemical and sensory changes associated with microbial flora of mediterranean boque (*Boops boops*) stored aerobically at 0,3,7, and 10^0C. *Applied and Environmental Microbiology* **65**, 698-706.

Lazcka O, Del Campo FJ, Munoz FX (2007) Pathogen detection: a perspective of traditional methods and biosensors. *Biosensors and Bioelectronics* **22**, 1205-1217.

Lerke P, Farber L (1969) Direct bacterial count as a rapid freshness test for fish fillets. *Applied and Environmental Microbiology* **17**, 197-201.

Limbo S, Sinelli N, Torri L, Riva M (2009) Freshness decay and shelf life predictive modelling of European sea bass (Dicentrarchus labrax) applying chemical methods and electronic nose. *LWT - Food Science and Technology* **42**, 977-984.

Lin M, Al-Holy M, Al-Qadiri H, Kang D-G, Cavinato AG, Huang Y, Rasco BA (2004) Discrimination of intact and injured Listeria monocytogenes by fourier transform infrared spectroscopy and principal component analysis *Journal of Agricultural and Food Chemistry* **52**, 5769-5772.

Lin M, Mousavi M, Cavinato AG, Rasco BA (2006) Rapid near infrared spectroscopic method for the detection of spoilage in rainbow trout (Oncorhychus mykiss) fillet. *Journal Of Food Science* **71**, 18-23.

Liston J (1957) The occurrence and distribution of bacterial types on flatfish. *Journal of Genetics and Microbiology* **16**, 205-216.

Macagnano A, Careche M, Herrero A, Paolesse R, Martinelli E, Pennazza G, Carmona P, D'Amico A, Di Natale C (2005) A model to predict fish quality from instrumental features. *Sensors and Actuators B-Chemical* **111**, 293-298.

Nicolay X (Ed.) (2006) 'Odors in the food industry.' (Springer + Business Media LLC: New York).

Olafsdottir G, Lauzon HL, Martinsdottir E, Kristbergsson K (2006) Influence of storage temperature on microbial spoilage characteristics of haddock fillets (Melanogrammus

aeglefinus) evaluated by multivariate quality prediction. *International Journal of Food Microbiology* **111**, 112-125.

Ólafsdóttir G, Lib X, Lauzona HL, Jónsdóttira R (2002) Precision and application of electronic nose for freshness monitoring of whole redfish (Sebastes marinus) stored in ice and modified atmosphere bulk storage *Journal of Aquatic Food Product Technology* **11**, 229-249.

Olafsdottir G, Nesvadba P*, et al.* (2004) Multisensor for fish quality determination. *Trends in Food Science & Technology* **15**, 86-93.

Panagou EZ, Mohareb FR, Argyri AA, Bessant CM, Nychas G-JE (2010) A comparison of artificial neural networks and partial least squares modelling for the rapid detection of the microbial spoilage of beef fillets based on fourier transform infrared spectral fingerprints. *Food Microbiology* doi: 10.1016/jfm.2010.05.014.

Phillips CA (1996) Review: modified atmosphere packaging and its effects on the microbiological quality and safety of produce. *International Journal of Food Science & Technology* **31**, 463-479.

Powell SM, Bowman JP, Fergusson SH, Snape I (2010) The importance of soil characteristics to the structure of alkane-degrading bacterial communities on sub-Antarctic Macquarie Island. *Soil Biology & Biochemistry* **42**, 2012-2021.

Rahman MS (Ed.) (2007) 'handbook of food preservation.' (CRC Press, Taylor and Francis Group: Boca raton).

Reynisson E, Lauzon HL, Magnusson H, Jonsdottir R, Olafsdottir G, Marteinsson V, Hreggviosson GO (2009) Bacterial composition and succession during storage of North-Atlantic cod (Gadus morhua) at superchilled temperatures. *Bmc Microbiology* **9**.

Sankar RCN, Lalitha KV, Jose L, Manju S, Gopal TKS (2008) Effect of packaging atmosphere on the microbial attributes of pearlspot (Etroplus suratensis Bloch) stored at 0-2 °C. *Food Microbiology* **25**, 518-528.

Scheu PM, Berghof K, Stahl U (1998) Detection of pathogenic and spoilage micro-organisms in food with the polymerase chain reaction *Food Microbiology* **15**, 13-31.

Schweizer-Berberich PM, Vaihinger S, Göpel W (1994) Characterisation of food freshness with sensor arrays. *Sensors and Actuators B: Chemical* **18**, 282-290.

Sivertsvik M, Jeksrud WK, Rosnes JT (2002) A review of modified atmosphere packaging of fish and fishery products - significance of microbial growth, activities and safety. *International Journal of Food Science and Technology* **37**, 107-127.

Subramanian TA (2007) Effect of processing on bacterial population of cuttlefish and crab and determination of bacterial spoilage and rancidity developing on frozen storage. *Journal of Food Processing and Preservation* **31**, 13-31.

Suthiluk P, Saranwong S, Kawano S, Numthuam S, Satake T (2008) Possiblity of using near infared spectroscopy for evaluation of bacterial contamination in shredded cabbage. *International Journal of Food Science and Technology* **43**, 160-165.

Tanaka Y, Takahashi, H., Kitazawa, N., Kimura, B (2010) Rapid estimation of microbial populations in fish samples by using terminal restriction fragment length polymorphism analysis of 16S rDNA. *Journal of Food Protection* **73**, 104-113.

Tarr HLA (1954) Microbiological deterioration of fish post mortem,its detection and control. *Bacteriological Reviews* **18**, 1-15.

van der Vossen JMBM, and Hofstra H (1996) DNA based typing, identification and detection systems for food spoilage microorganisms: development and implementation *International Journal of Food Microbiology* **33**, 35-49.

Velusamya V, Arshaka K, Korostynskaa, O, Oliwab, K. and Adley, C (2010) An overview of foodborne pathogen detection: In the perspective of biosensors **28**, 232-254

Weber CJ, Haugaard V, Festersen R, Bertelsen G (2002) Production and applications of biobased packaging materials for the food industry. *Food Additives and Contaminants* **19**, 172-177.

Wierda RL, Fletcher G, Xu L, Dufour J-P (2006) Analysis of volatile compounds as spoilage indicators in fresh king salmon (*Oncorhynchus tshawytscha*) during storage using SPME-GC-MS. *Journal of Agricultural and Food Chemistry* **54**, 8480-8490.

Williams PC (1992) The Phil Williams's episode. *NIR News* **3**, 3-4.

Winger R (2003) Australia New Zealand Food Standards Code. *Food Control* **14**, 355-355.

Wold S, Sjostrom M, Eriksson L (2001) PLS-regression: a basic tool of chemometrics. *Chemometrics and Intelligent Laboratory Systems* **58**, 109-130.

Yano Y, Yokoyama K, Tamiya E, Karube I (1996) Direct evaluation of meat spoilage and the progress of aging using biosensors. *Analytica Chimica Acta* **320**, 269-276.