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Innovative sensors to rapidly and non-destructively determine fish freshness (C017)

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Innovative sensors to rapidly and non-destructively determine fish freshness

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2009

Freshness is recognized as a main element of fish quality. The direct key functions of storage time and temperature have a significant influence on fish freshness. Therefore, objective assessment has been applied to find a reliable method to determine the fish freshness. Sensory methods such as Quality Index Method (QIM) or Torry assessment are commonly used; however, these techniques rely on skilled assessors and scoring can drift without regular re-training. As a result, a number of instrumental methods have been studied to evaluate fish freshness. Early studies of Nilsen *et al.* (2002) used a near infrared (NIR) spectroscopic technique applied to specific regions of cod fillets. In this study, imaging methods have been used to study several sections of cod to assess which have the greatest potential for discrimination of changes related to storage time. The appearance and changes in the NIR reflectance spectra of whole fish, fillets and gills during storage on ice have been measured with several instruments to assess their suitability for objective freshness evaluation.

Farmed cod were delivered to Campden BRI packed in ice. Samples were periodically re-iced over the length of the study and were scored in a chill store operating at 1-2^{o}C. Five fish per day were assessed over a period of 2 to 17 days after harvest. Instrumental assessments were made for whole fish, fillets and gills. Torry assessment was carried out on whole fish and cooked fillets.

The visual characteristics were measured with a digital colour imaging system calibrated to provide accurate CIELAB colour measurements and reliable assessment of changes in appearance. The images were analysed to measure the colour for three regions: eyes, fillets and gills. Regression analysis was applied to determine any significant effect of storage time on colour changes. Significant effects on gill filament colour were seen for up to 9 days in storage, represented by an increase in hue angle (change from red to yellow hue) and an increase in lightness (L^*). Fish eyes became cloudy and fillets became yellower with storage time, revealed by a rapid increase in L^* (lightness) of eyes up to day 7 on ice and an increase in b^* values of fillets. Of the colour measurements, it is considered that gills gave the best result of assessing freshness. Accurate determination of age of the fish at the earlier stages of shelf-life could be an important improvement over alternative analytical methods such as Total Volatile Base Nitrogen (TVB-N) which is known to be less accurate in this time-frame (Castro *et al*., 2006).

Near infrared (NIR) spectroscopy of bulk samples has been widely used in the evaluation of food quality. Recent developments in NIR hyperspectral imaging allows the method to be applied to assess the spatial distribution of food composition (Millar *et al.*, 2008), and to make measurements of selected regions of food samples. Both methods rely on the same principal technology but the main difference is that, in the imaging technique, entire spectra are collected for each pixel within the image. NIR measurements of several parts of the fish made with bulk and hyperspectral imaging instruments were compared with storage time and Torry scores for raw and cooked cod. For NIR spectroscopy as for colour measurements, gill filaments gave the best model with storage time. A comparison was made with the results from the previous study of Nilsen *et al.* (2002) for bulk spectroscopy measurements of fillets in the visible region. The result in our study gave a standard error of cross validation (SECV) of 2.25 days, improving on the majority of the calibrations reported by Nilsen *et al.* (2002). Results for gills using the visible region also showed good performance with an SECV of 1.72 days. For hyperspectral NIR, measurements for different parts of the fillet (head, middle and tail end) were compared to determine which gave the greatest potential for measuring freshness. The best result (SECV = 1.66 days) was achieved for the head end of the fillet, using the full NIR wavelength range.

In most cases, assessments were made over the full range of storage time studied (2-17 days on ice). The main changes occurred in the early stages (up to 9 days) and it is possible that improved calibration performance could be achieved if assessments over this duration only are required.

Contents

1 Introduction

It is believed that a suitable system for objective analysis of fish freshness would improve the ability to market fish on value and to monitor and manage the freshness of fish in the supply chain to reduce waste.

The rapid decline in fish freshness with storage time and temperature has been studied with the aim of developing suitable technologies for its evaluation. The most common methods are based on defined sensory characteristics such as the Quality Index Method (QIM) and Torry scheme. These sensory methods rely on skilled and experienced assessors. This is why instrumental methods could be of value. A number of studies have focused on the use of spectroscopic approaches, particularly those based in the visible and near visible/NIR regions. This is understandable as spectra from these regions include information based on both chemical and physical changes that samples undergo during storage, and which can be expected, therefore, to contain relevant information for assessment of fish freshness.

Near-infrared (NIR) spectroscopy is a rapid, typically non-destructive method widely used for food analysis. The method is based on measurement of the interaction of electromagnetic radiation with samples over a waveband between 700nm and 2500nm, allowing spectra to be recorded in reflection, transmission and transflection modes. The technique responds to molecular bonds such as C-H, O-H and N-H, with the vibration modes of these bonds each absorbing infrared radiation in different ways. Chemometric methods enable calibrations to be developed for physical and chemical properties of interest, based on comparison of the spectra with the measured properties of a set of reference samples.

A previous study by Nilsen *et al.* (2002) applied visible/NIR spectroscopy to both cod and salmon fillets and undertook an evaluation of the optimum measurement site. This work demonstrated the feasibility of predicting fish storage time by this route. Another study by Bøknæs *et al.* (2002) used a similar measurement approach to look at cod mince and also showed potential for the evaluation of freshness. Given the potential loss of information about the physical structure of fish muscle as a result of mincing, it is not thought that this represents the optimum route for future analysis. Further work by Nilsen and Esaiassen (2005) showed that QIM scores could be predicted for cod using visible spectroscopy.

Previous studies have shown the potential of NIR and colour measurements for assessment of fish freshness. However, the commercial application of these does not appear to have been realised. Therefore this project aims to evaluate these approaches further to identify the most effective approaches for further evaluation and commercial development. Work has been carried out using cod (*Gadus morhua*). Several NIR and visible measurements have been compared with days on ice and sensory assessments to assess their potential for assessment of freshness.

2 Objectives

The overall aim of this study was to evaluate the potential of colour measurements and near infrared (NIR) spectroscopy as reliable methods of determining fish freshness in commercial applications to enable the suitability of measurements for various parts of the fish to be assessed. Colour measurements were made with a calibrated colour imaging system and NIR measurements were made with a hyperspectral imaging system, and with a conventional bulk system. The following specific objectives were addressed:

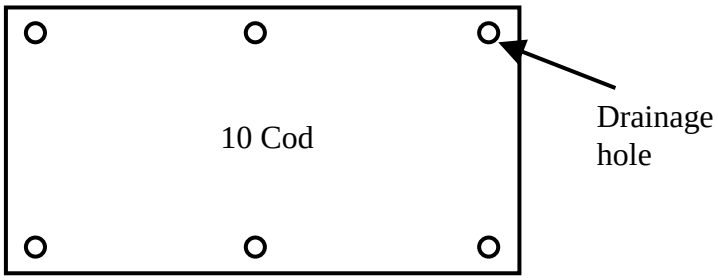
- Develop demonstrated calibrations for fish freshness as determined by storage time (days on ice) using NIR spectroscopy.
- Determine the potential of reliably assessing fish freshness using imaging techniques.

3 Methods

3.1 Sample storage and preparation

One hundred farmed cod (*Gadus morhua*) were sourced from CodFarmers ASA, Oslo, Norway and stored on ice in a chiller with a controlled temperature of 1-2^{o}C over the full experiment period. Ten cod were packed in ice in each of 10 boxes (100 cod in total) and delivered on the next day after harvest. Ice was refilled every day to ensure cod were placed on ice and covered by ice to maintain a low temperature. Six drainage holes (see Figure 1) were made in each box to prevent the accumulation of water.

Figure 1 – Positions of drainage holes of each box

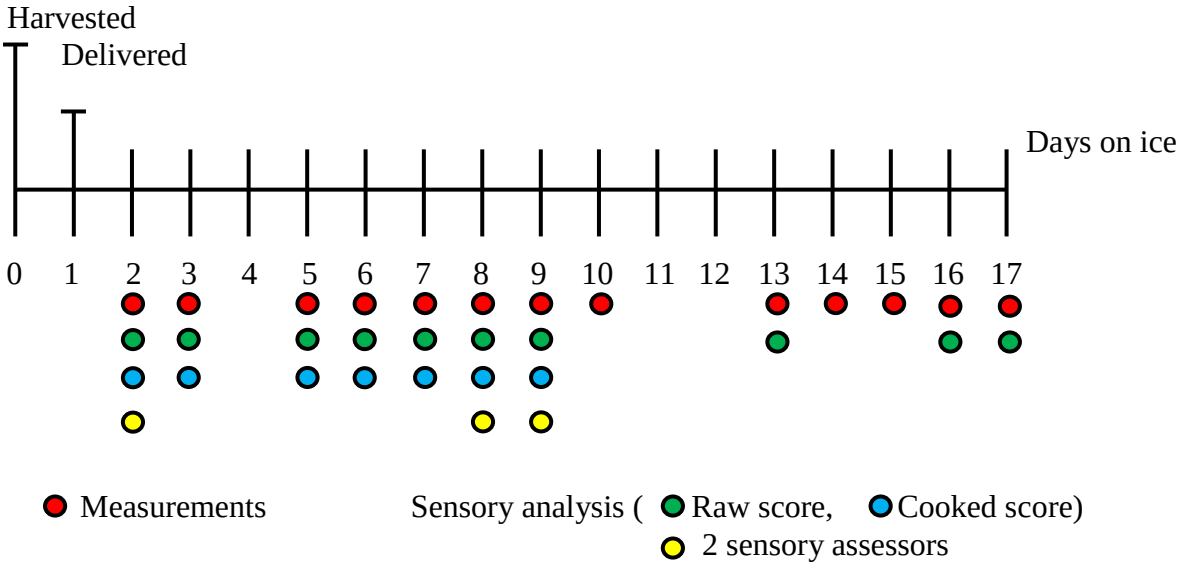


On each day of analysis (2 to 17 days on ice), 5 cod were taken at random from separate boxes for analysis. Sixty five fish were analysed in total. Figure 2 shows the timeline of the sensory assessment and measurements of cod on a scale of days on ice; note that cod were stored on ice after harvest and during delivery, indicated as Days 0 and 1 on ice. Cod selected for analysis on each assessment day were also placed on ice after removal from the chiller until ready for measurements, and between measurements.

For analysis, samples were first subjected to sensory assessment by one or two trained assessors. Whole cod were then imaged using a DigiEye imaging system to record the external visual characteristics and were scanned by a hyperspectral NIR imaging system to analyse the surface properties. The total length of each fish was measured and recorded.

After imaging the whole fish, they were filleted and skinned by trained operators and the gills were then removed and separated individually. The samples were retained on ice until ready for further measurements. For each fish, both fillets and 8 individual gills were presented together on a tray and imaged using the DigiEye and hyperspectral NIR imaging systems. Both sides of each fillet were imaged.

Figure 2 – Time line of sensory assessment and measurements of cod



After imaging, a portion of each fillet was cut from the loin (15cm long × 4.5cm wide × 1cm thick), wrapped in cling film and packed into a rectangular cell for bulk NIR scanning. Gill filaments were removed from the head, wrapped in cling film and packed into a ring cup for scanning. For the period up to 9 days on ice, further portions of the fillets were then cooked for further sensory analysis.

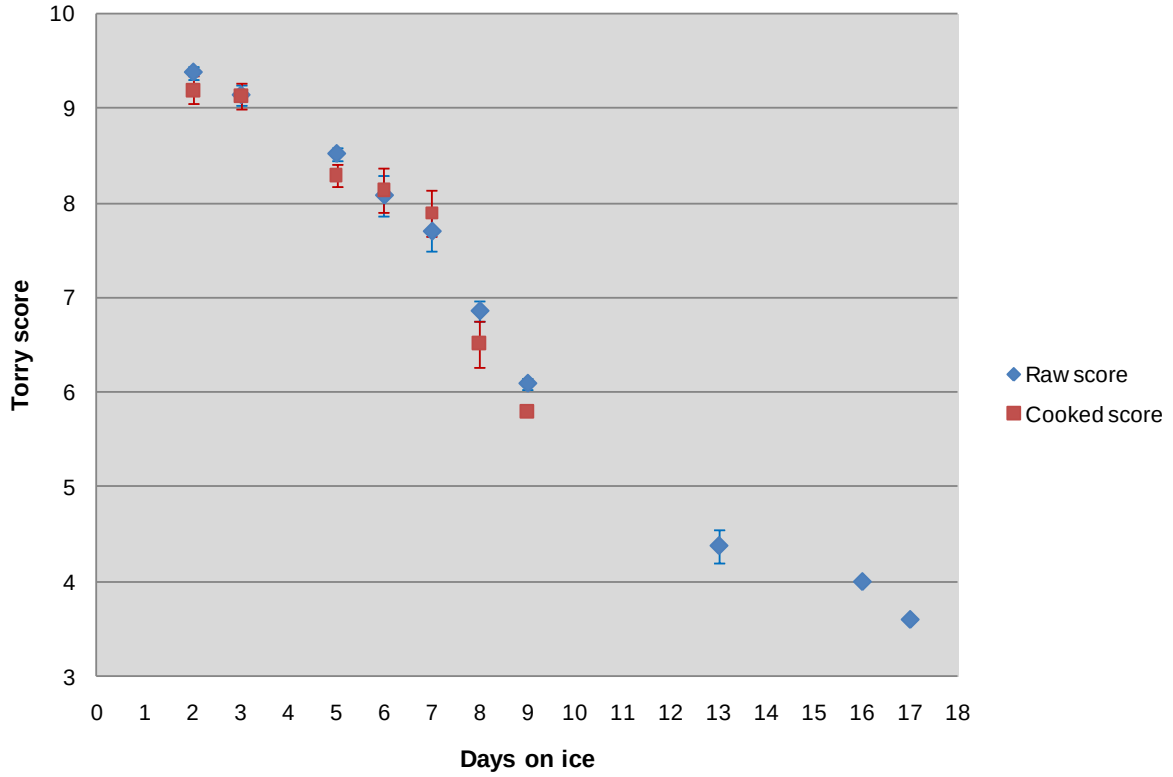
3.2 Length measurement

According to Florida Fish and Wildlife Conservation Commission regulations, the total length of fish is the maximum length of the fish, with the mouth closed and the tail fin pinched together. The total length of cod was measured by pushing the cod's head up against a vertical surface with the mouth closed and the cod lying along a ruler, and then pinching the tail fin closed. In this study, 65 cod were used during experimental periods (13 non-consecutive days) having a length of 53.61(mean) ± 2.56cm (standard deviation).

3.3 Sensory characteristics

Quality assessment of the cod was carried out using Torry schemes for raw and cooked cod by at least 1 trained assessor. Another trained assessor from Seafish was involved in the sensory assessment on Days 2, 8 and 9. The scores (see Figure 3) were used as reference values in addition to the actual storage times for development of NIR calibrations for freshness.

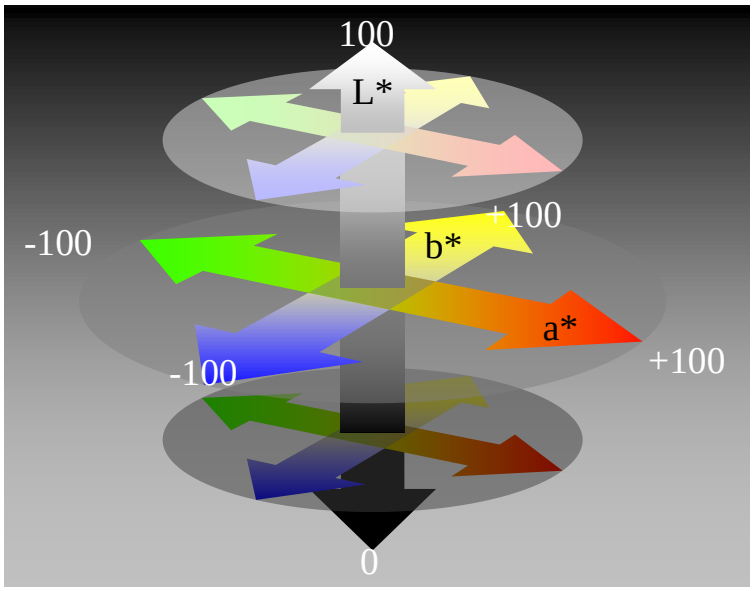
Figure 3 – Mean Torry scores for raw and cooked cod with days on ice



3.4 Colour imaging and measurement

3.4.1 DigiEye imaging system

Figure 4 – CIELAB space



Colour images were taken with a DigiEye digital imaging system (DigiEye plc, Enderby, Leicestershire). This system incorporates an imaging cabinet with diffuse D65 illumination, enabling images to be taken under controlled conditions. The system was calibrated daily

against the CIELAB colour space using a test card with 240 patches of known colour. The CIELAB system (CIE, 1986) describes colours by three values, *L**, *a** and *b** (Figure 4).

*L** represents the lightness of a sample, on a scale of 0 to 100, where 0 is black and 100 is white.

*a** represents variation from green to red on a scale of -100 to +100.

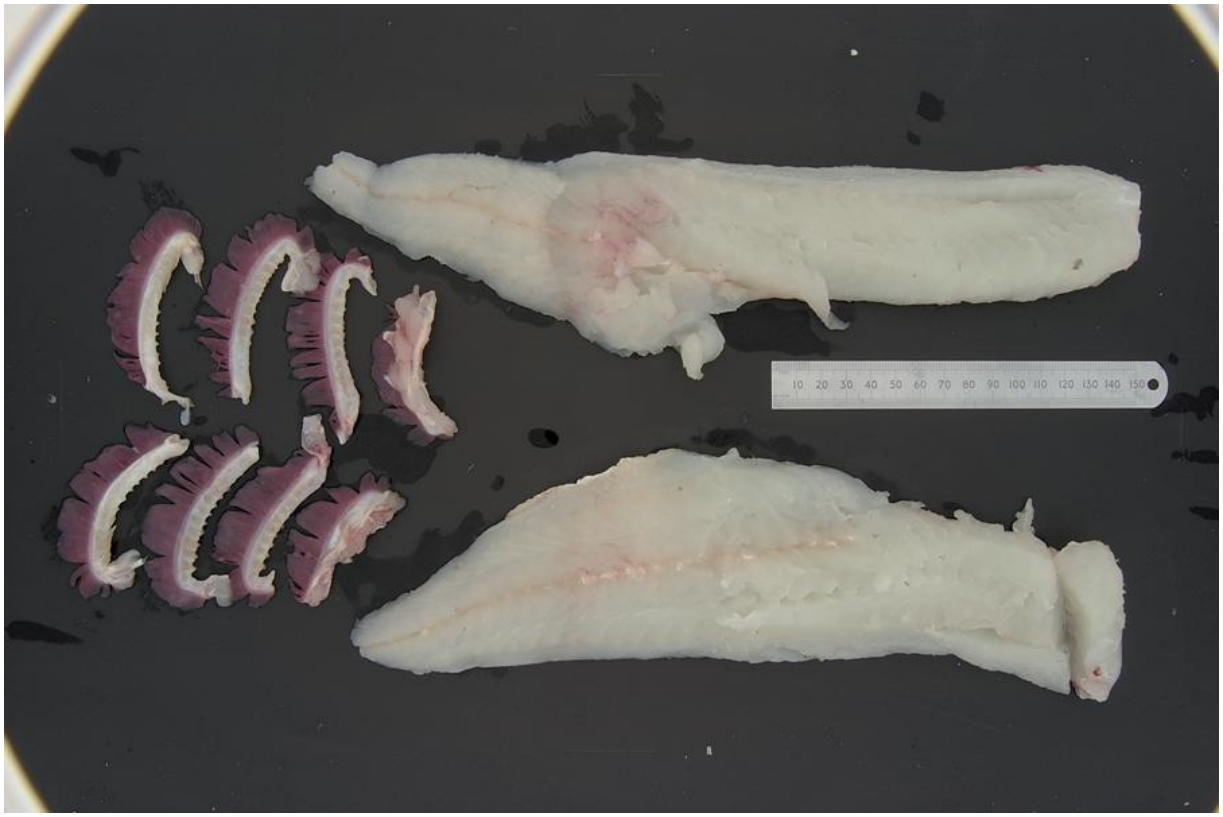
*b** represents variation from blue to yellow on a scale of -100 to +100.

Images were stored and analysed in CIELAB colour space. For the purposes of reproduction in this report, the CIELAB format images were converted to an RGB format using an Adobe RGB profile.

Images were taken of whole fish, fillets (separate images of both surfaces) and gills. All images were taken at a magnification of 0.166mm/pixel. Whole fish were presented on a blue tray (Figure 5) to provide contrast, enabling the fish to be discriminated from the background (with the exception of those on the first day of analysis, for which a black tray was used). The fish were too long for the field of view and two images were therefore taken, one of the head end and one of the tail, which were joined together digitally for presentation. Fillets and gills were presented together on a black tray (Figure 6).

Figure 5 – Whole cod presented on a blue tray for imaging

Figure 6 – Fillets and gills presented on tray for imaging



3.4.2 Data analysis

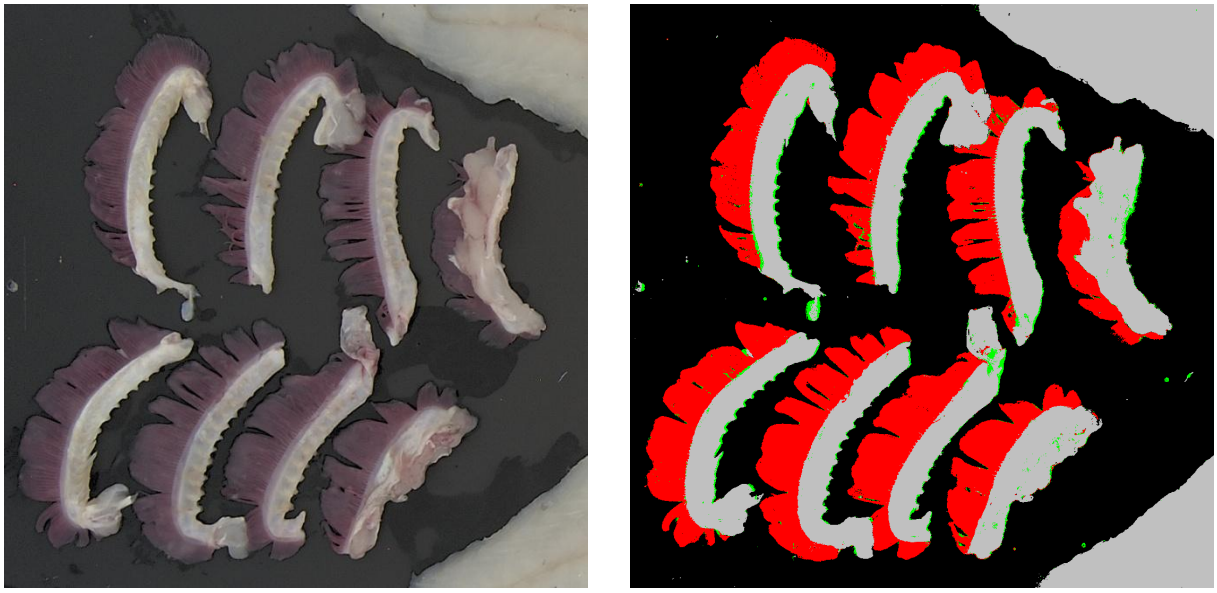
Images were analysed to measure the average colour of the fillets, the gills and the pupils of the eyes. It was considered that variations in natural skin pigmentation were too great for colour measurements of skin to be relevant for freshness. Measurements of fillets and eyes were made using the DigiEye software. The region of interest was selected manually. For measurements of whole fillets, a 'magic wand' tool was used to select contiguous pixels of similar colour. Measurements were also made for a rectangular region of the thickest part of the fillet corresponding approximately to the region used for bulk NIR analysis. For measurements of eyes, the region was selected using a circle tool centred on a manually selected position. The mean colour of the pixels in each selected region of interest was calculated by the software.

Measurements of gill filament colour were made using software developed by Campden BRI. The colour distribution in each image was analysed and used to classify each pixel as dark background (corresponding to the black tray), the red gill filaments, white (including the cartilage part of the gills and the flesh of the fillets), or other regions. An example of the classification is shown in Figure 7 for a region of one of the images.

Foreground regions with areas greater than 10,000 pixels (=275mm^2) and with red regions comprising at least 10% of their area were identified as gills. For each gill, the mean colour of the red region was calculated.

Figure 7 – Segmentation of a colour image containing gills

(a) Original image (b) Classified regions



3.5 NIR spectroscopy

NIR spectroscopy relates to the region of the electromagnetic spectrum starting at ~700nm and ending at ~2500nm. Within this, two regions may be identified, the near visible which corresponds to 700-1100nm and which is generally characterised by the use of instruments which operate on the principle of transmittance of energy, and 1100-2500nm, typically studied by using reflectance spectrometry. This technique has been widely used in food and agricultural industries due to the relatively strong penetrating power of the energy over these wavelengths, thus allowing for minimal sample penetration. The technique responds to molecular bonds demonstrating a strong dipole such as C-H, O-H and N-H with the vibration modes of these bonds each absorbing infrared radiation in different ways.

In addition to the chemical components which absorb infrared energy, the physical characteristics of a sample such as particle size also play a role in the final spectrum observed, and need to be taken into account in the calibration development.

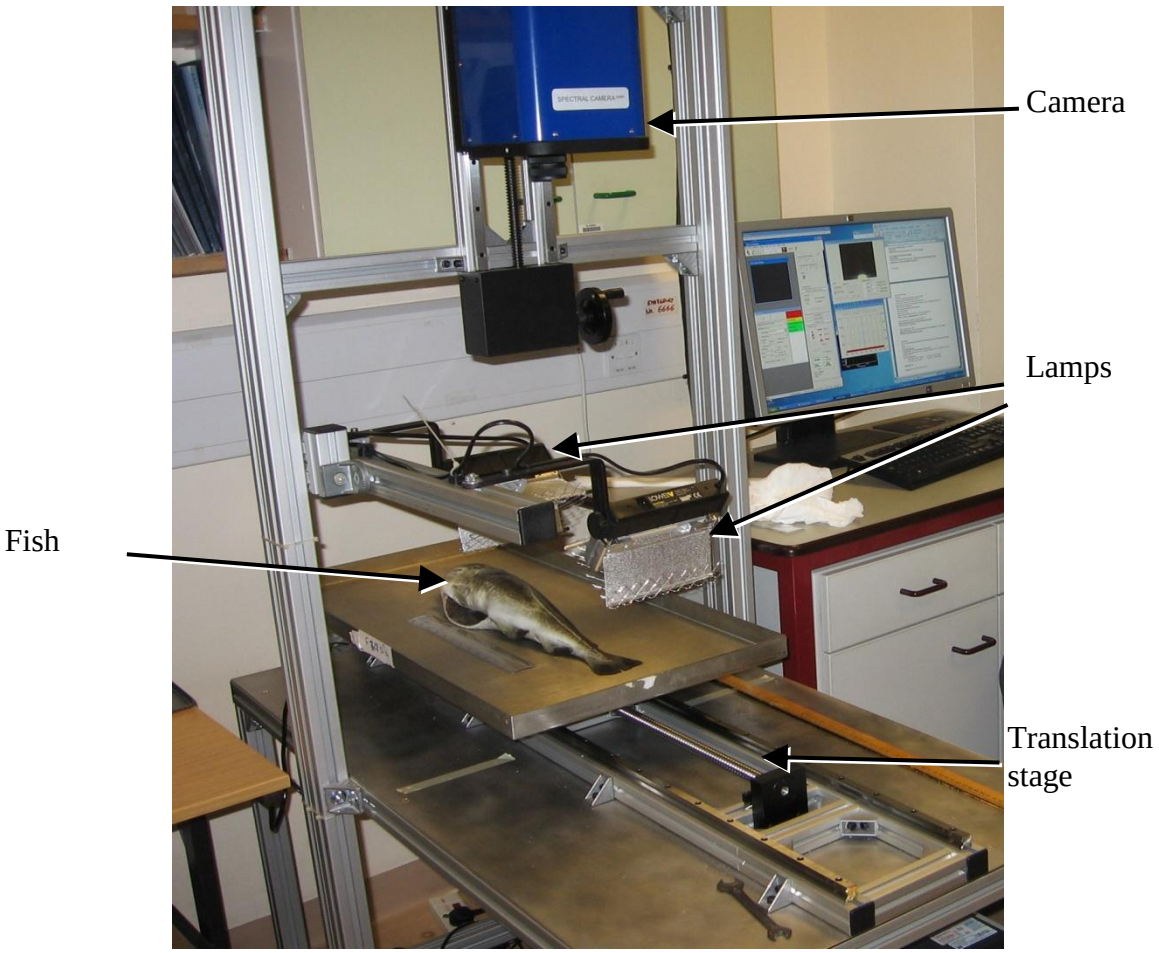
3.5.1 Hyperspectral NIR imaging system

Scanning was taken using an NIR hyperspectral imaging system (Figure 8) supplied by Gilden Photonics Ltd., Glasgow, U.K. The system produces images for which an NIR spectrum with a wavelength range of 892 to 2495nm is available for each pixel, providing information on the distribution of composition and structure across the surface of a sample.

The system includes a SWIR spectral camera (Specim Ltd., Oulu, Finland) containing a cooled 320×256 pixel HgCdTe detector and a spectrograph. This is mounted above a motorised translation stage, operating in a 'pushbroom' configuration. For these experiments, the camera was fitted with a Specim 22.5mm lens. This provided a field of view with a measured width of 298mm at the level of the sample tray and 267mm at a height of 80mm above the tray, roughly corresponding to the top of the whole cod samples. For an intermediate value of 280mm, this corresponds to a pixel size of 280mm / 320 pixels =

0.875mm. Samples were scanned by moving them under the camera on a motorised translation stage. Scans were taken at a camera frequency of 37 frames/s and the translation stage was driven at a speed of 32.4mm/s to provide the same vertical and horizontal resolution. The stage was moved through a distance of 600mm, sufficient to scan the full length of the fish.

Figure 8 – Hyperspectral NIR imaging system



Whole fish were presented on a metal tray as shown in Figure 8. Fillets and gills were presented on a black painted tray as also used for colour imaging of fillets and gills (see Figure 6). Whole fish and fillets were gently patted with paper towels before scanning to remove excess water, and any adhering ice was also removed. Samples were scanned immediately after presentation to minimise any heating of them by the lamps.

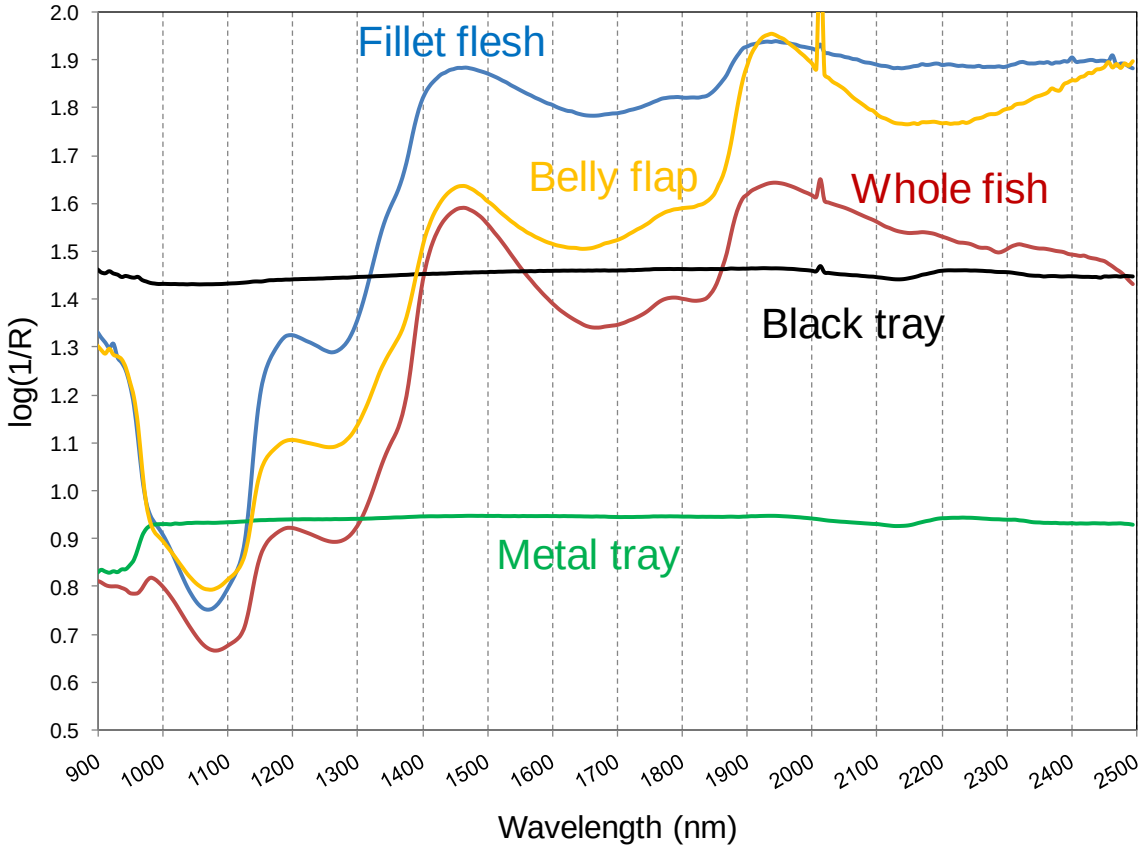
Scans were taken using Spectral Cube software (Specim) and raw data were saved as data cubes. At the end of each scan, the camera shutter was automatically closed and additional frames were recorded to determine the black level for each scan, which was later subtracted from the raw data during analysis. For each day's analysis, a scan was also made of a flat block of white PTFE material with a flat reflectance spectrum in the NIR region. This was used to normalise measurements of samples to calculate reflectance values. Exposure times were chosen to optimise the signal strength for each type of sample, while minimising

saturation in the regions of interest. An exposure time of 4ms was used for whole fish, 6ms for gills and fillets and 1ms for the white reference sample.

3.5.2 Data analysis

Data were analysed using ENVI 4.4 software (ITT Visual Information Systems, Boulder, CO, USA). This was used to perform the dark level and normalisation calculations and to convert the reflectance values to log (1/*R*) absorbance units. Figure 9 shows examples of mean spectra for a whole fish, the flesh and belly flap regions of a fillet and for typical regions of the metal tray on which whole fish were presented and the black tray on which fillets were presented.

Figure 9 – Example spectra from the NIR imaging system



Further analyses were carried out to identify regions of interest and to calculate mean spectra for these. For whole fish, images were analysed to identify the region corresponding to the sample. It can be seen from Figure 9 that the sample spectra had larger gradients than those of the tray, particularly over the region between 1081 and 1944nm. The sample was therefore identified according to the criterion:

$$\log (1/R_{1944\text{nm}}) - \log (1/R_{1081\text{nm}}) > 0.4 \qquad (1)$$

The mean spectrum for the whole of each fish was calculated. For fillets, images containing gills and fillets (skin side down) were analysed. As can be seen from the example spectrum in Figure 9, the black tray had a lower reflectance (higher log (1/*R*)) at short wavelengths than the fillets. The samples were therefore identified as regions for which:

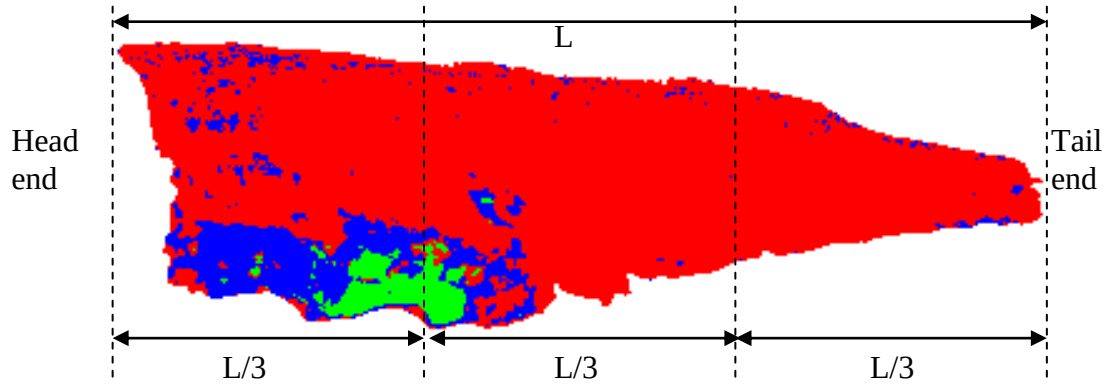
$$\log (1/R_{1069nm}) < 1 \quad (2)$$

The fillets were identified as the two largest such regions in each image. Separate measurements were made for each fillet, for regions as identified in Figure 10. Regions in which the raw data were saturated, typically corresponding to specular reflections, were excluded (indicated in green in the figure) and regions of belly flap were also excluded (indicated in blue), identified by the criterion:

$$\log (1/R_{1932nm}) - \log (1/R_{1617nm}) \geq 0.4 \quad (3)$$

As can be seen from the figure, this criterion also resulted in the elimination of a small amount of flesh. The remaining regions (indicated in red) were considered to represent flesh and were included in the analysis. Each fillet image was divided into head, middle and tail portions of equal length measured parallel to the scan direction, as indicated by dashed lines in the figure. Mean spectra were calculated for the flesh of the whole fillet and for each of these regions.

Figure 10 - Regions of fillet classified from an NIR hyperspectral image. Regions labelled in red were included for analysis.



3.6 Bulk NIR spectroscopy

A Foss NIRSystems 6500 scanning monochromator spectrometer (Foss NIRSystems Inc., Silver Spring, MD) equipped with a sample transport mechanism was used to collect sample spectra. Scans were taken between the visible region and infrared region, over the range 400-2498nm with the instrument bandwidth of 8nm.

Measurements were made of fillets and gill filaments. A section of each fillet 15cm long × 4.5cm wide × 1cm thick was cut from the loin of the fillet (Figure 11), wrapped in cling film and packed into a rectangular cell for scanning. Gill filaments were cut off, wrapped in cling film and packed into a small ring cup for scanning. The instrument was set to average 32 scans of each sample on each measurement.

Figure 11 – Section of fillet used for NIR scan

3.6.1 Calibrations

Spectra were collected from the NIRSystems 6500 using ISI software (Infrasoft International, LLC, Port Matilda, PA) and mean spectra from selected regions of hyperspectral NIR images were calculated using IDL software. WINISI software was used to analyse both datasets and to generate calibrations. In some cases, calibration coefficients were exported and applied to hyperspectral datacubes using IDL to calculate values for each spatial pixel.

Prior to calibration development, spectra were treated using different functions i.e. Standard Normal Variate (SNV) and De-trending (DT) to reduce the effects of variation in physical structure and pathlength between different samples (Barnes *et al.*, 1993). The spectra were also converted to different orders of derivative - i.e. first and second derivatives to accentuate subtle differences. The NIR calibrations were then developed using partial least squares (PLS) regression in which samples from the calibration set were successively removed and predicted using remaining samples. This was used to aid in selection of the most appropriate factors to be included as well as to give a more robust determination of error. To avoid the risk of over-fitting, usual guidance is to use a maximum of (*n*-10)/10 factors for *n* samples. Therefore, for the fillets (127 samples), a maximum of 10 factors were used, and for the gills (64 samples) a maximum of 5 factors were used. The standard error of cross validation (SECV) is based on the combined performance of the predictions of each of the groups removed in succession and as such gives a more realistic assessment of future calibration performance (Millar and Hall, 2004).

4 Results and discussion

4.1 Visual characteristics of cod with storage time

The visual characteristics of the whole cod for selected storage time are shown in Figure 12.

There was considerable variation in pigmentation between different cod and it was found that some black spots were present on the surface of the cod skin and eyes (Figure 13). It is speculated that the black spots could possibly be due to parasitic infection (Lysne *et al*., 1994), but this was not confirmed.

Figure 14 shows the closer views of eyes on every three days on ice. Eyes became white and cloudy over the period of storage time. Note that the angle of the head was not carefully controlled, as can be seen in Figure 12, and the eyes were therefore presented at various angles, which may have caused variability in their measured colour. Improved measurements of variations in eye colour may therefore be feasible with more precise alignment to a measurement system.

Figure 15 shows examples of the fillets for every three days on ice, selected to be representative of the measured colour changes. It is known that fresh fillets are bluish and become yellowish with time. Fillets for Day 2 were perhaps more yellow than would be expected, despite being of excellent quality and we can only speculate that this may be a feature of farmed cod. The b^* value represents variation from blue-to-yellow according to CIELAB colour space, and assessments of fillet colour variation therefore focussed on this value (see Figure 18).

Examples of the gills at 3 day intervals are shown in Figure 16. The colour changes of gill filaments with time were described by changes in hue angles (red to yellow) and lightness (L^*) (Figure 22). Note that the gill filaments were sufficiently thin and translucent to be affected by the colour of the substrate against which they are measured. For these experiments, a black tray was used throughout.

Figure 12 – Whole cod appearance with days on ice

Days on ice	0 5 10 15 20 cm
2	
5	
8	
10	
14	
16	

Figure 13 – Black spots appeared on the skin and eyes of cod

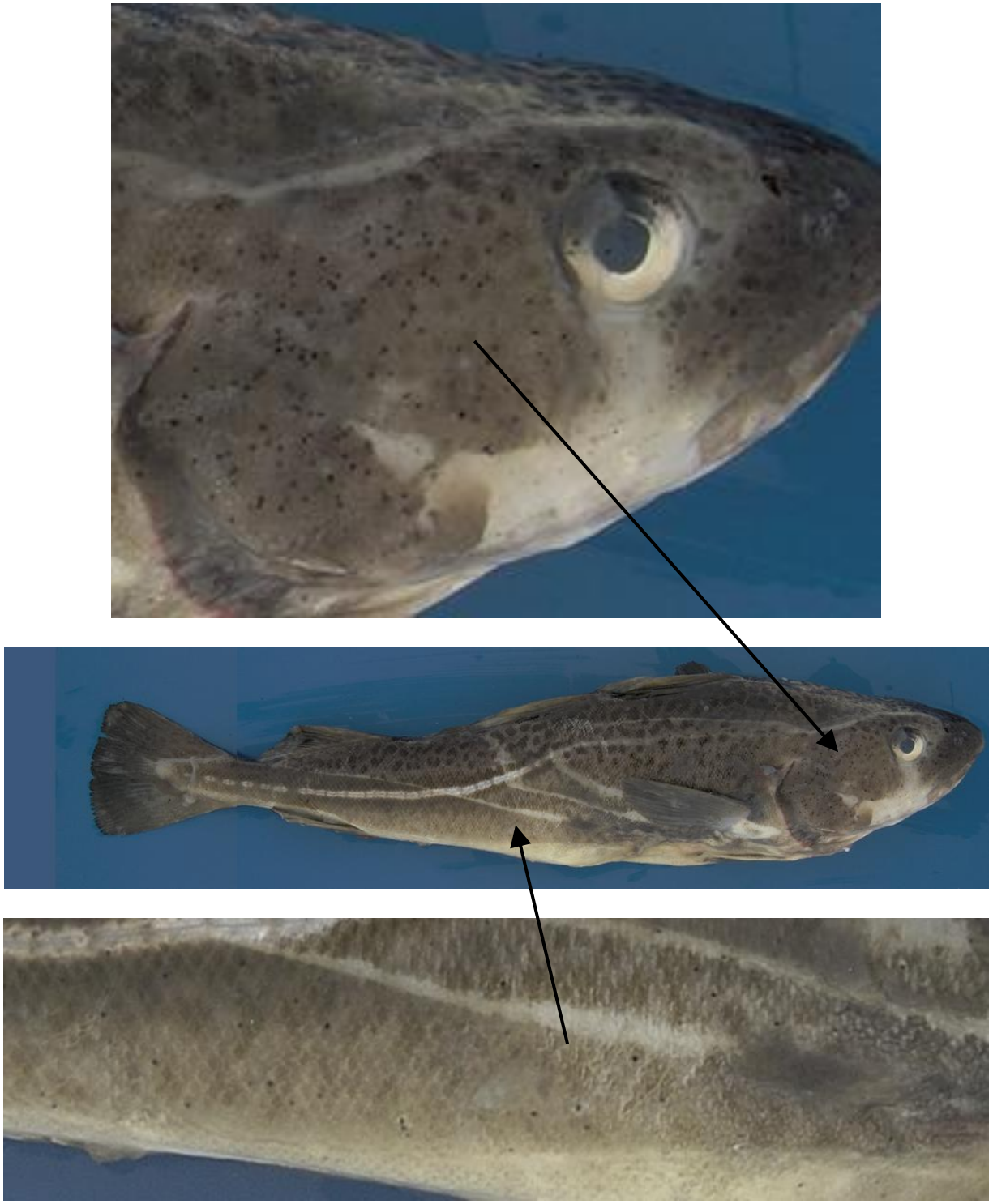
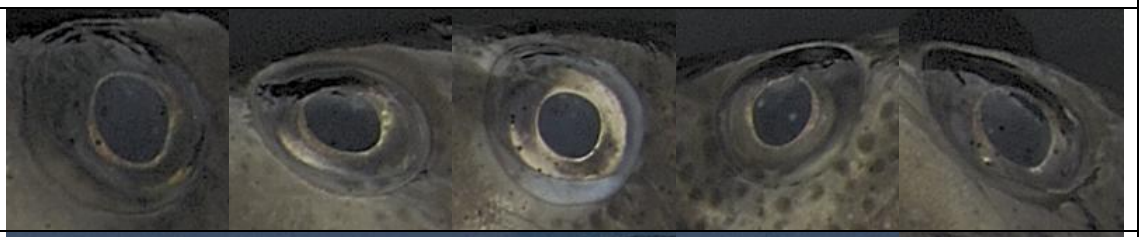
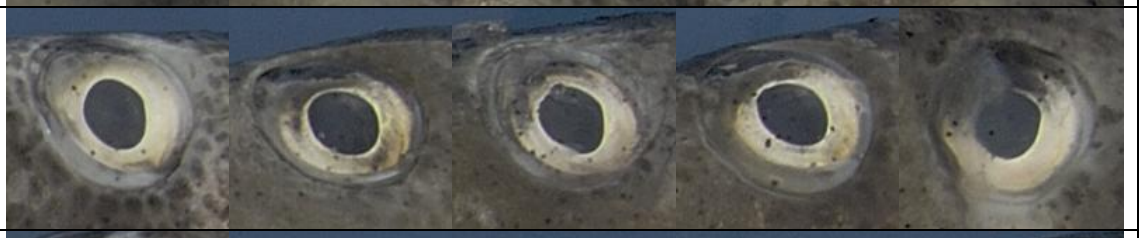


Figure 14 – Eye colour changes with days on ice

Days on ice	
2	
5	
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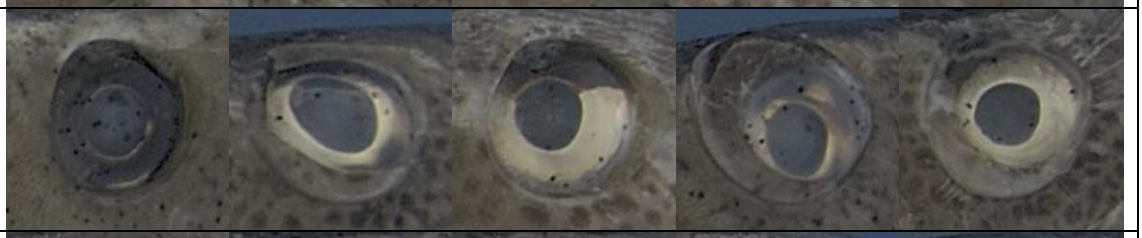
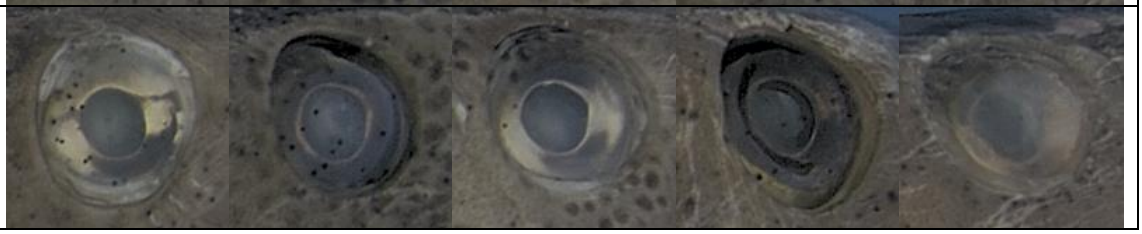
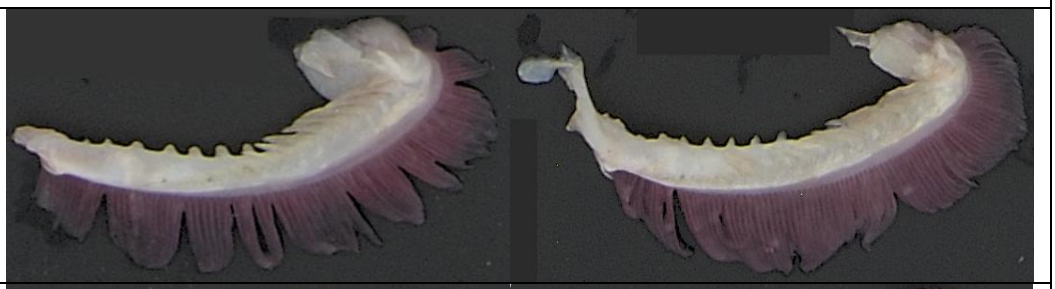
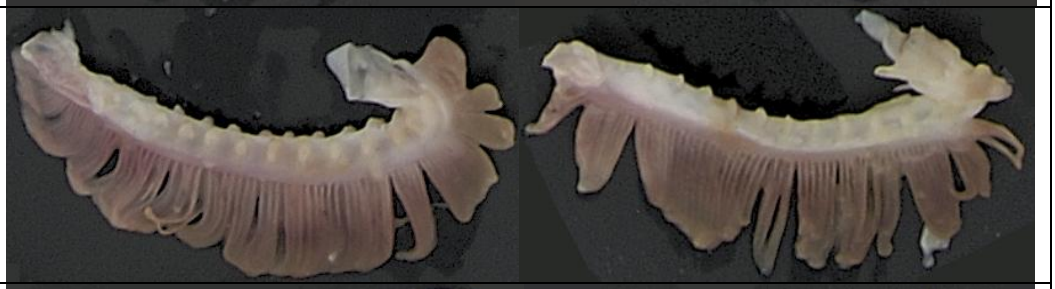
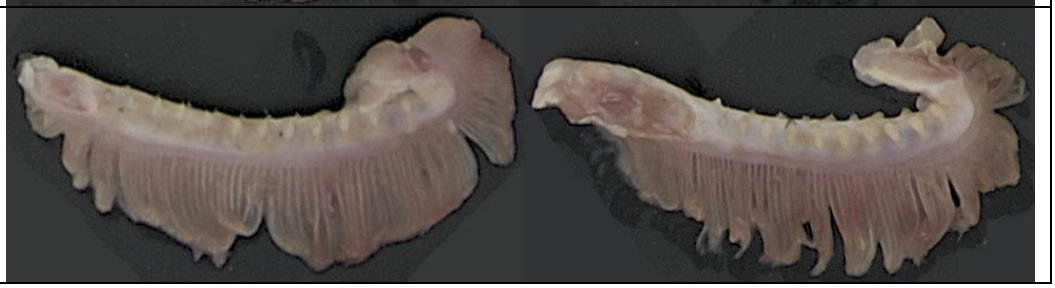
14	
17	

Figure 15 – Fillets with days on ice

Days on ice	
2	
5	
8	
10	
14	
17	

Figure 16 – Gills with days on ice

Days on ice	
2	
5	
8	
10	
14	
17	

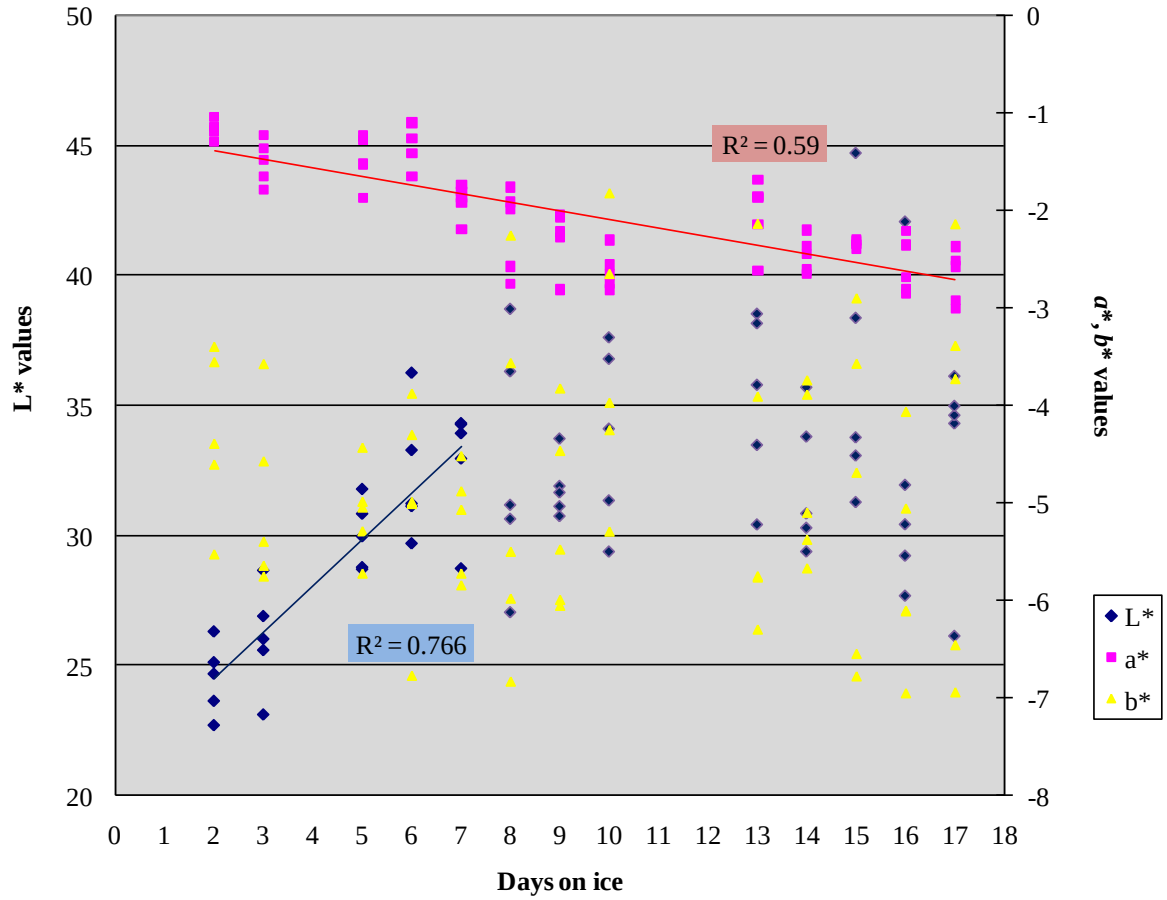
4.2 Measurement of colour changes

4.2.1 Eyes

The colorimetric analysis of eyes is shown in Figure 17 as a function of days on ice. The main change was an increase in *L** values over the initial storage period from about Day 2 to Day 8. This corresponds to an increase in lightness as the colour of the eyes changed rapidly from black to cloudy white during this period of storage, as can be seen in the images in Figure 14.

The measurements of *L** showed a correlation of $R^2 = 0.77$ with days on ice. A smaller effect was seen in *a** which slightly decreased (increased greenness) during storage and was significantly correlated ($P<0.05$) with days on ice ($R^2 = 0.59$ over the full assessment period).

Figure 17 – Effect of days on ice on CIELAB values for eyes



4.2.2 Fillets

As might be expected, the main colour change in fillets during storage was an increase in yellowness. This can be seen in the calibrated images in Figure 15. This colour change was described by an increase in the *b** measurement, as shown in Figure 18 ($R^2 = 0.51$).

During filleting, it was noticed that the first cut fillet was always the thicker one. Therefore, the factor of filleting techniques of the operators was considered. For the alignment of the samples as presented on the tray for colour measurement, the top fillet referred to the first cut

fillet which was always thicker than the bottom one (second cut fillet). ANOVA showed that there was a significant difference in *b** between the first cut and the second cut fillets ($P<0.05$). Therefore, the correlations of measurements for the first and second fillets with the storage time were separately calculated. Differences of *b** values between the first cut fillets and the second cut fillets are shown in Figure 19. The *b** values calculated from the first cut fillets gave the best correlation with days on ice ($R^2 = 0.58$).

Figure 18 – Effect of days on ice on CIELAB values for whole fillets

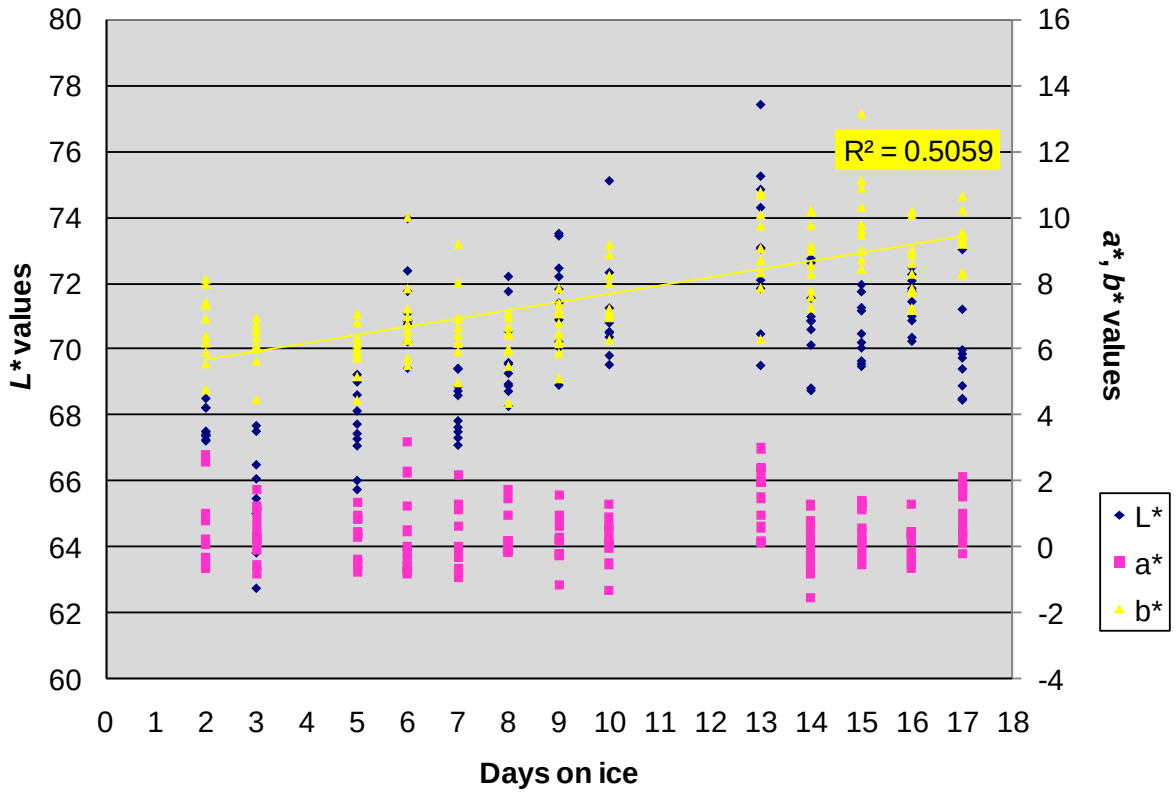
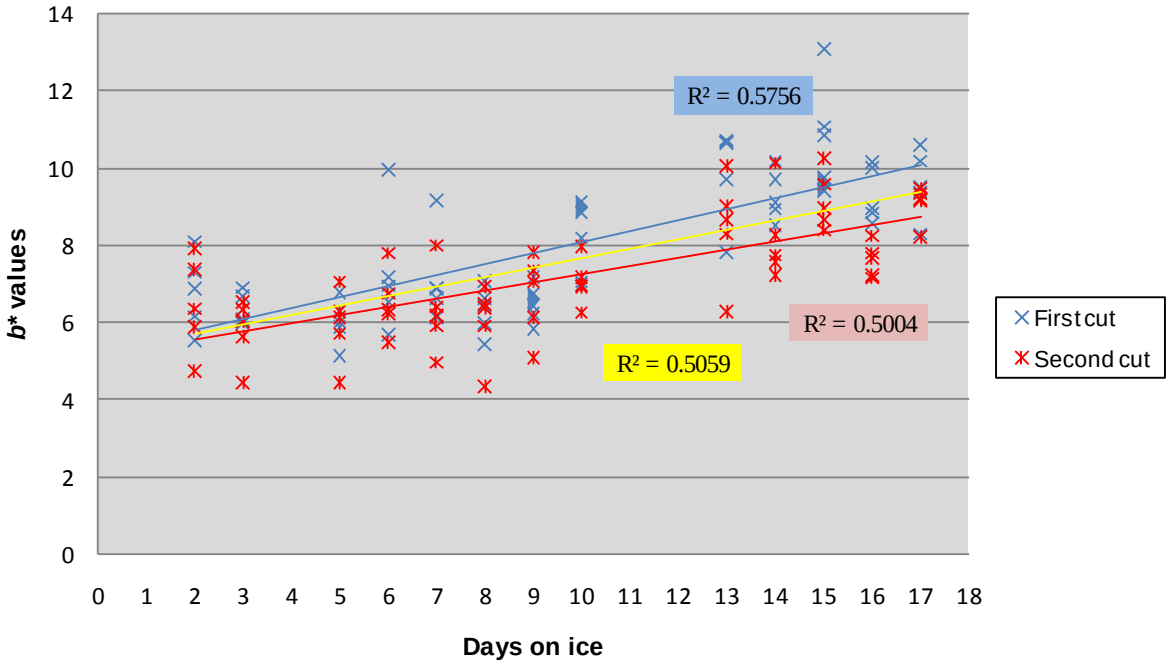


Figure 19 – Effect of days on ice on CIELAB *b values for whole fillets according to the order in which they were prepared: Top = 1st cut, typically thickest; Bottom = 2nd cut.**



The thickness of the fillets was thought to be one of the factors affecting the CIELAB values. The measurement of the thin parts of the fillet could be affected by the background colour. Therefore, further measurements were made for manually selected regions of each image corresponding to the thickest part of the fillet, which it was thought would be sufficiently thick for the colour to be minimally affected by the background. The results are shown in Figure 20. As for the whole fillet, a significant difference in *b** was seen for the first and second cut fillets, with a low R^2 value of 0.40.

As for measurements of the whole fillets, even when the thickest part of the fillet was selected, ANOVA showed that there was a significant difference in *b** between the first and second cut fillets ($P<0.05$). Therefore, the correlation of each side of the fillet with the storage time was calculated. Differences of *b** values between the first and second cut fillets are shown in Figure 21. Again, the *b** values calculated from the first cut fillets give the best correlation with days on ice ($R^2 = 0.53$).

Although there was a statistically significant difference between the *b** values for the first and second cut fillets, the effect was small in comparison with the overall effect of storage time on yellowness of either fillet and measurements of *b** therefore provide a useful basis for assessment of this colour change as a potential indicator of freshness.

Figure 20 – Effect of days on ice on CIELAB values for fillet sections

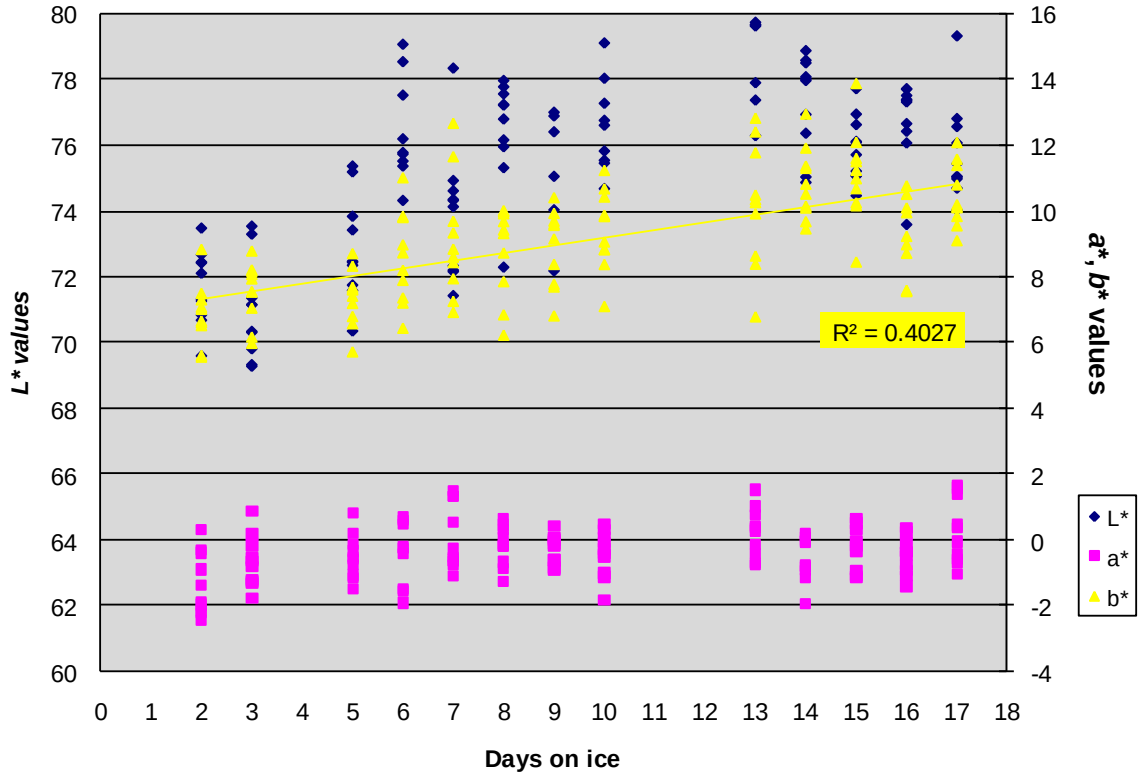
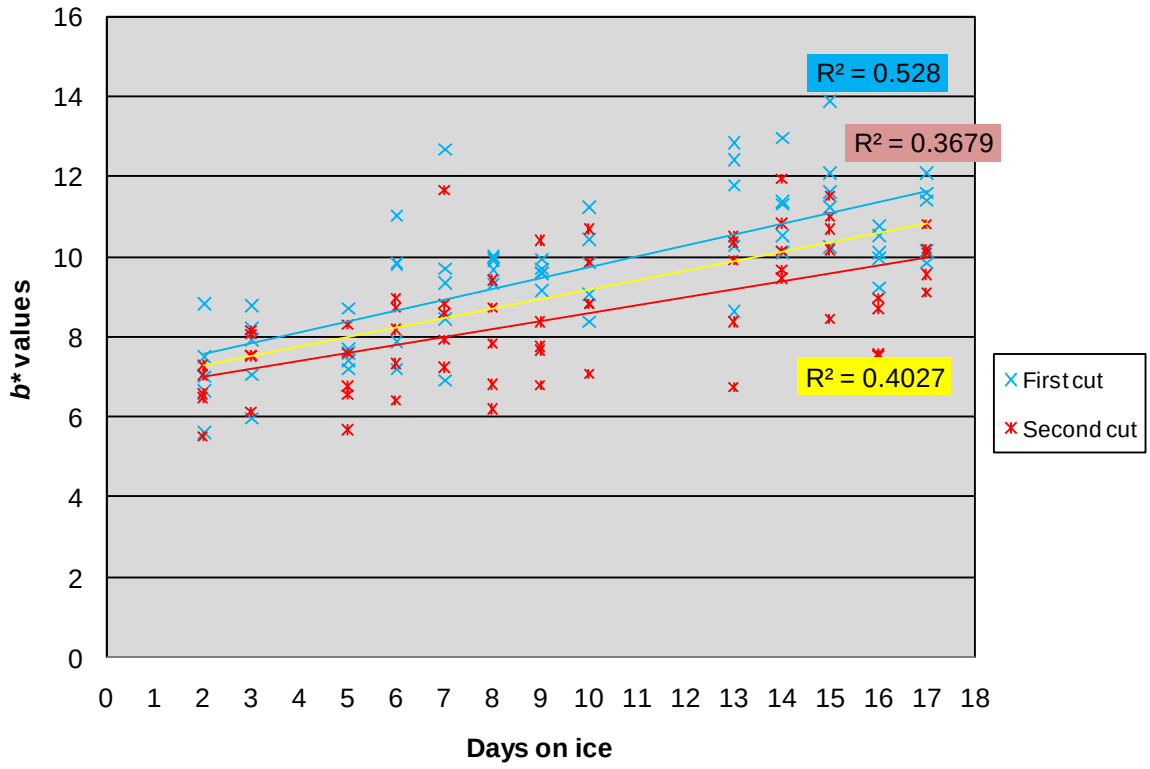


Figure 21 – Effect of days on ice on CIELAB b* values for the thickest region of fillets cut from different sides of the cod



4.2.3 Gills

Gill filaments showed a typical characteristic change from a deep red colour to a pale yellower colour with time. This was described by a decrease in *a** (redness), increase in *b** (yellowness) and increase in *L** (lightness). The variation in *a** and *b** was measured by a change in hue angle, which is the angle in the *a** and *b** plane measured anticlockwise from the positive *a** axis. This parameter is plotted against the *L** value in Figure 22 which provided useful information for discriminating the colours of gill filaments with days on ice.

The low *L** values and hue angle of gills for the first few days correspond to a lower reflectance (darker in colour) and redder colour, respectively. With storage time, gills became lighter and the red colour faded which can be seen in Figure 16. This is revealed by an increase in *L** values (R^2 = 0.55) and hue angle (R^2 = 0.77) as shown in Figure 23. It is considered that the measurement of gills could be a potential method of measuring the freshness of fish up to Day 9 on ice. After this time, changes were less consistent and, therefore, less useful for predicting storage time (Figure 22). Note that the gill filaments are thin so that measurements will depend on the background. For these measurements, a black tray was used to present the gills throughout. Accurate determination of fish age at the earlier stages of shelf-life could be an important improvement over alternative methods such as TVB-N which shows no indication of freshness or relevant results during the edible storage time of fish (Castro *et al.*, 2006).

Figure 22 – Hue and variation lightness for gill filaments over days on ice

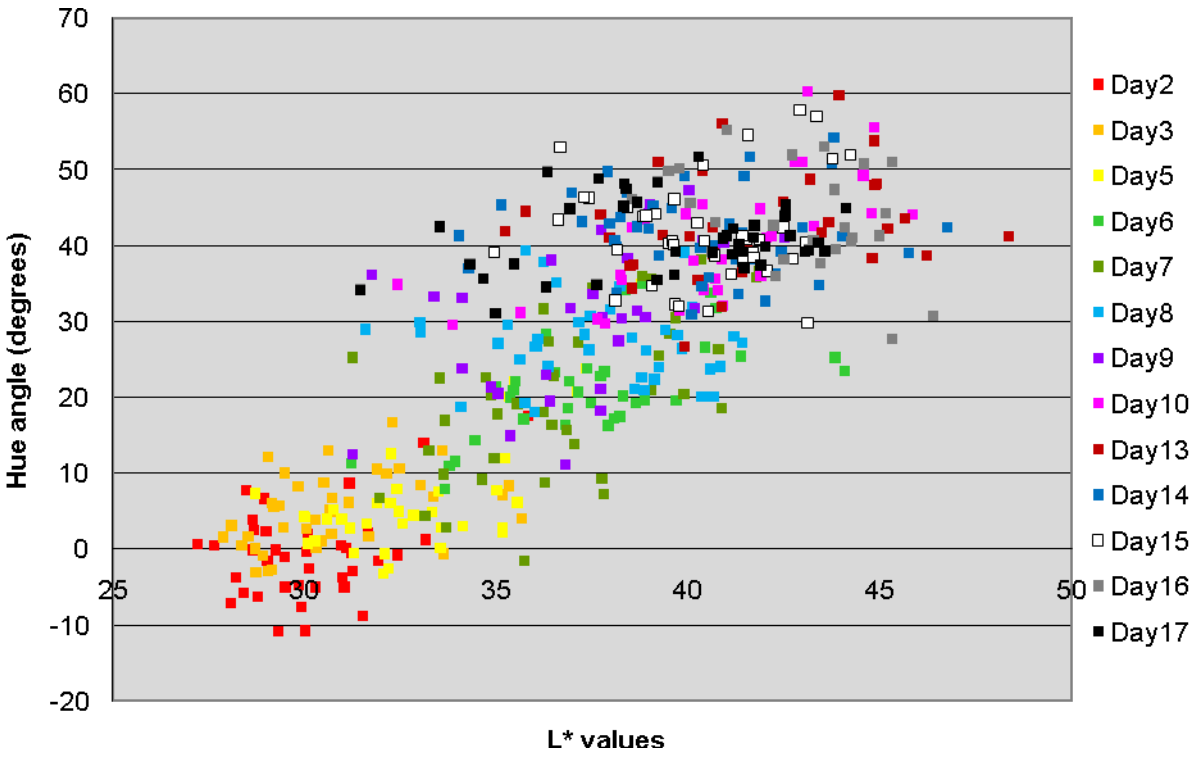
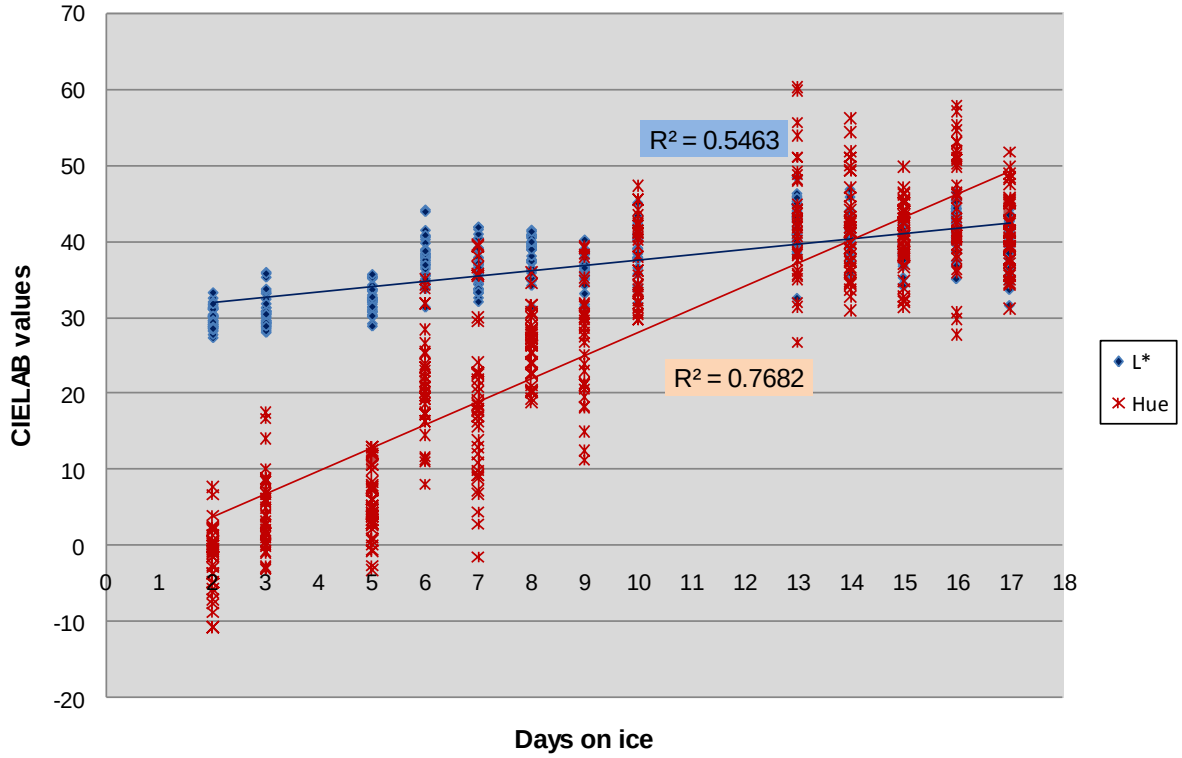


Figure 23 – Effect of days on ice on *L values and hue angle for gills**



4.3 NIR spectra

4.3.1 NIRSystems 6500

Spectra collected from the NIRSystems 6500 were pre-treated with SNV & DT and were converted into different orders of derivatives for further calibration development. Figure 24 shows the spectral differences between the fillets and gills on Day 2 and Day 17 on ice. With increasing storage time, peaks in the spectra from the fillet are less obvious and the same effect is shown in the lower region (400-1300nm) of the spectra from the gills. However, the peaks in a region beyond 1300nm of the spectra from the gills are noticeable with storage time. Figure 25 shows the spectral differences of gills on different days on ice showing considerable changes in peak B and peak E (see below).

There is a sharp doublet peak (A) followed with a minor peak (B) in the visible region (400-700nm) of fresh gills (Day 2 on ice). Peak B disappeared in the old gills as shown in Figure 25. These changes are consistent with a progressive oxidation of haem pigments having iron in the ferrous form (e.g. reduced myoglobin or haemoglobin) to an intermediate state where some of the haem pigments present have iron in the oxidised, ferric form (e.g. metmyoglobin or methaemoglobin) (Millar et al., 1996). This information is not obvious or noticeable in the spectra from the fillet, presumably due to the apparently low concentration of haem pigments in this part of the fish. However, sharper peaks (C) are shown in a region of 925-1300nm of the spectra from the fillet. Peak D at about 1440nm (associated with O-H groups) is less prevalent in the spectra of both the fresh and old fillet and the spectra beyond that wavelength are described as ‘noise’. This is probably due to surface characteristics such as gloss reducing the contribution of the energy absorbed by the fillet to the overall spectrum collected. This

also appears to the case for the spectra from fresh gills. A big difference is found in a higher region of the spectrum from the old gills showing a sharp peak D followed by peak E at about 1980nm (also due to O-H groups). Again, this is thought likely to be due to an increased gloss component from fresh gills than those having been stored for some time. A reduced range 408-1440nm was selected for analysis of the fillet spectra, excluding the upper wavelength region where more susceptibility to sample surface characteristics was observed. This region was also applied to the gills for comparison, although some information is given for old gills over a wider wavelength range.

PLS regression with full cross validation was used to determine any correlation between the spectral data for the fillet and gills and three reference measurements of freshness: storage time (days on ice) and the Torry scores for raw and cooked cod. Several calibration methods were assessed. Table 1 shows the best values of the parameters R^2 and SECV describing the fitness of the model. The overall results of the calibration parameters obtained with spectra from the gills are better than those from the fillets. However, some caution should be applied to this conclusion as fewer gill samples were available for calibration development than for the fillets.

In terms of days on ice, for the calculation of the complete spectrum (408-2492nm), the best model was obtained for the gills, giving an SECV of 1.88 days. This means that the model could correctly predict age to ± 1.88 days (68% of the time, ± 3.76 days 95% of the time) i.e. approaching 0.5 Torry points. The SECV for the correlation between fillets and days was 2.28 days which was reduced to 2.12 days for the restricted wavelength range. The best model was obtained for gills in the spectral range 408-1440nm which had a considerable improvement in the result with the SECV reduced from 1.88 to 1.63 days and R^2 increased from 0.87 to 0.92.

In a previous study of Nilsen *et al*. (2002), a NIRSystems 6500 instrument was used in the evaluation of cod freshness using a fibre optic probe which was located directly on the loin of the fillet. Fifty fish were used and 5 were selected at random and used for measurements on Days 0 – 5, 7, 9, 11 and 14. An average of 10 spectra from each fillet were collected and used for analysis. The model best correlated with storage time was obtained from the inside of the fillet over the visible region (400-700nm) giving a standard error of prediction (broadly comparable to SECV, as quoted here) of 1.04 days with R^2 of 0.97. For comparison, results for the data from this study using the visible region (400-700nm) are shown in Table 1. In our study, the best model was obtained from gills (SECV = 1.63 days, R^2 = 0.92). Note that the improved fit model by Nilsen *et al.* (2002) may have resulted from the use of a large number of factors (10) for the 50 fish assessed. This is greater than that used in this study where the number of factors included has been constrained to (*n*-10)/10. When other calibrations from the Nilsen study are reviewed, generally fewer factors are used and the prediction performance falls to levels slightly below those generated in the work reported here.

Comparing all of the results from both studies, it is considered that they show similar performance of approximately ±1.5 to 2 days, corresponding to approximate 0.5 Torry points for a trained assessor. It may be possible to achieve better models if assessments over the initial few days only are required, although this was not tested directly.

Figure 24 – Spectral differences between fillets and gills on Day 2 and Day 17 on ice

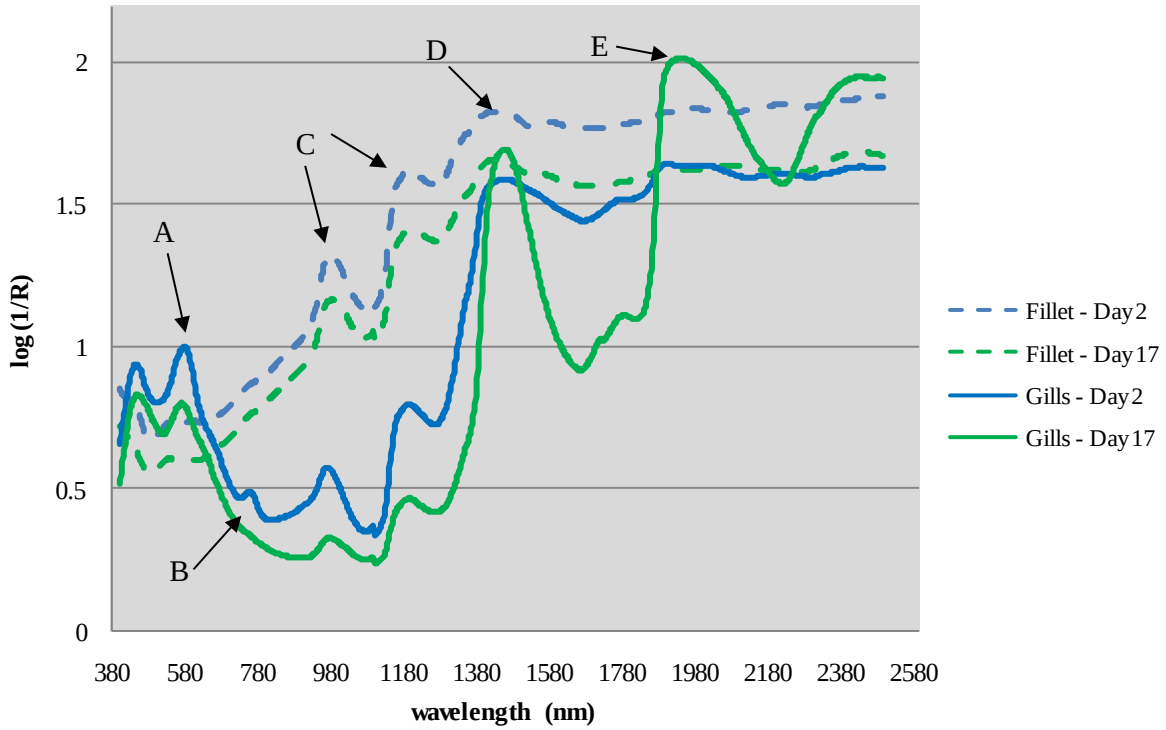


Figure 25 – Spectral difference of gills with days on ice

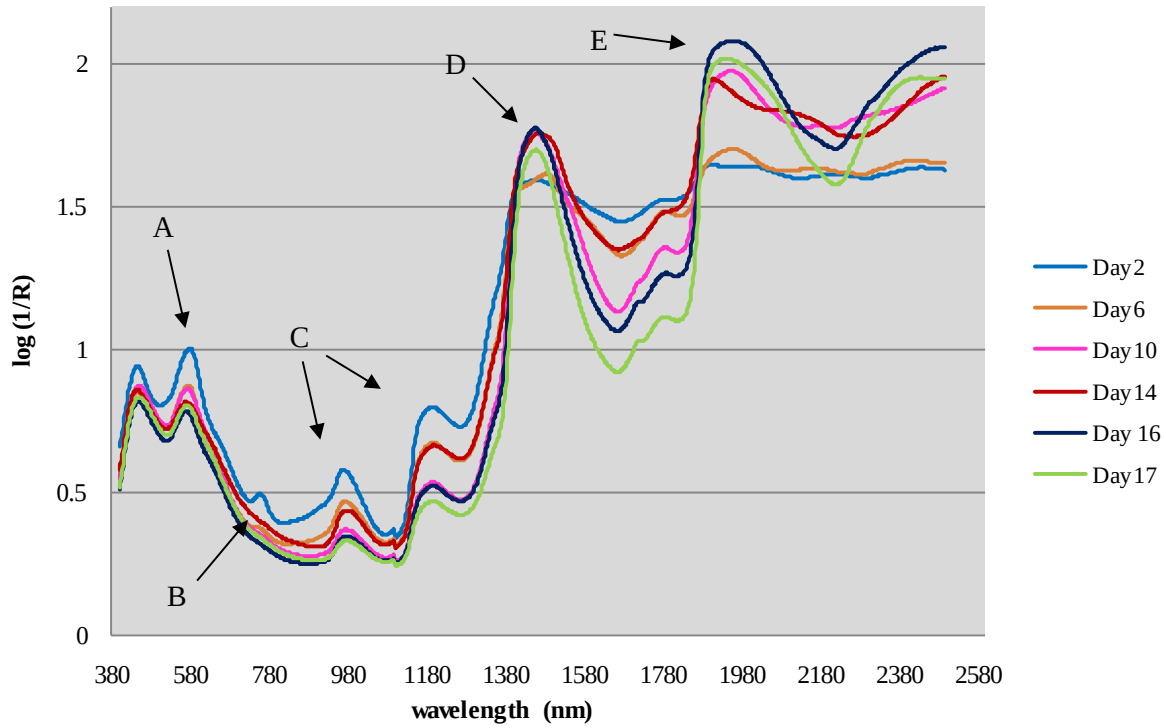


Table 1 – Parameters describing the fitness of modelling the correlation between measurements and the reference freshness measurements, in comparison with results from a previous study (* presents the best results obtained in visible region reported from Nilsen *et al.* (2002))

		Spectral range							
		408 – 2492nm		408 – 1440nm		400 – 700nm		400 – 700nm*	
	Factors	R^2	SECV	R^2	SECV	R^2	SECV	R^2	SEP
Fillet / days	10	0.78	2.42	0.88	2.12	0.81	2.25	0.97	1.04
Fillet / raw score	5	0.76	0.81	0.77	0.84	0.83	0.87		
Fillet / cooked score	5	0.74	0.60	0.64	0.65	0.62	0.61		
Gills / days	5	0.87	1.88	0.92	1.63	0.90	1.72		
Gills / raw score	2	0.92	0.57	0.89	0.69	0.88	0.59		
Gills / cooked score	2	0.91	0.34	0.72	0.49	0.89	0.31		

4.3.2 Hyperspectral NIR imaging system

The spectra recorded by this system are collected for each pixel over a range of about 900-2500nm. It was considered whether particular parts of the fillet would produce better calibration models for freshness. Spectral differences over the course of the experiments are shown in Figure 26 for the head, middle and tail parts of the fillet, with peaks labelled corresponding to the labels in Figure 24. In comparison with the fillet spectra collected from the NIRSystems 6500, the hyperspectral NIR system provided more information at longer wavelengths (>1440nm) e.g. peaks D and E which may improve models. However, the instrument did not include visible wavelength information. A reduced range of wavelength of 892-2094nm was used in the calculation for comparison to reduce the low signal at the end of the spectrum which had limited information possibly due to specular reflections caused by the glossy nature of the samples.

Table 2 shows the parameters of R^2 and SECV describing the fitness of the models based on different fillet sections against each of the three reference parameters. As for the results from the NIRSystems 6500 instrument, the overall results of the correlation between each fillet section and the cooked score gave the lowest SECV. This may be because cooked scores were only determined for the first 9 days, during which the changes in freshness are most apparent. For the calculation using the complete spectra (892-2495nm), the model best correlated with time was obtained from the head end of the fillet giving an SECV of 1.66 days. This may be due to the reduction in the thickness of the fillet from the head to the tail end that could possibly affect the spectra due to the background reflection. This may also explain why the better models were always obtained from the head part of the fillet. However, it is possible that there are also biological differences in the structure and composition along the length of the fillet and it is known that the sensory characteristics differ between the head and tail end of the fillet, because of which sensory assessments of cooked fillets in this study were all carried out for the middle part of the fillet. It appears that the head end of the fillet may be more sensitive to aging and is a better indicator of fish freshness. The results obtained from the

head part were not improved by the reduced wavelength range. It was therefore concluded that practical NIR assessments of fillet freshness should preferably be carried out for the head end of the fillet and using the full wavelength range. Figure 27 gives a scatter plot showing the measured reference values versus the predicted storage time for cod, based on the calibration achieved in this way.

Figure 26 – Spectral differences between head, middle and tail part of the fillet where peaks C, D and E are corresponding to the peaks C, D and E in Figure 24

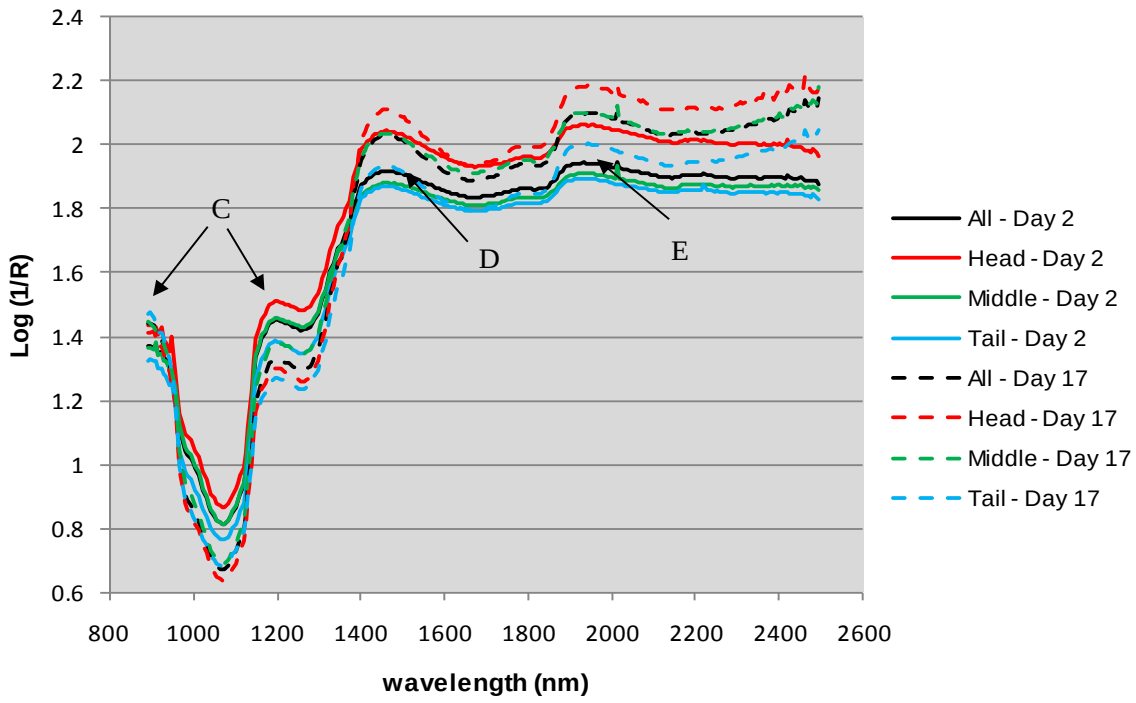
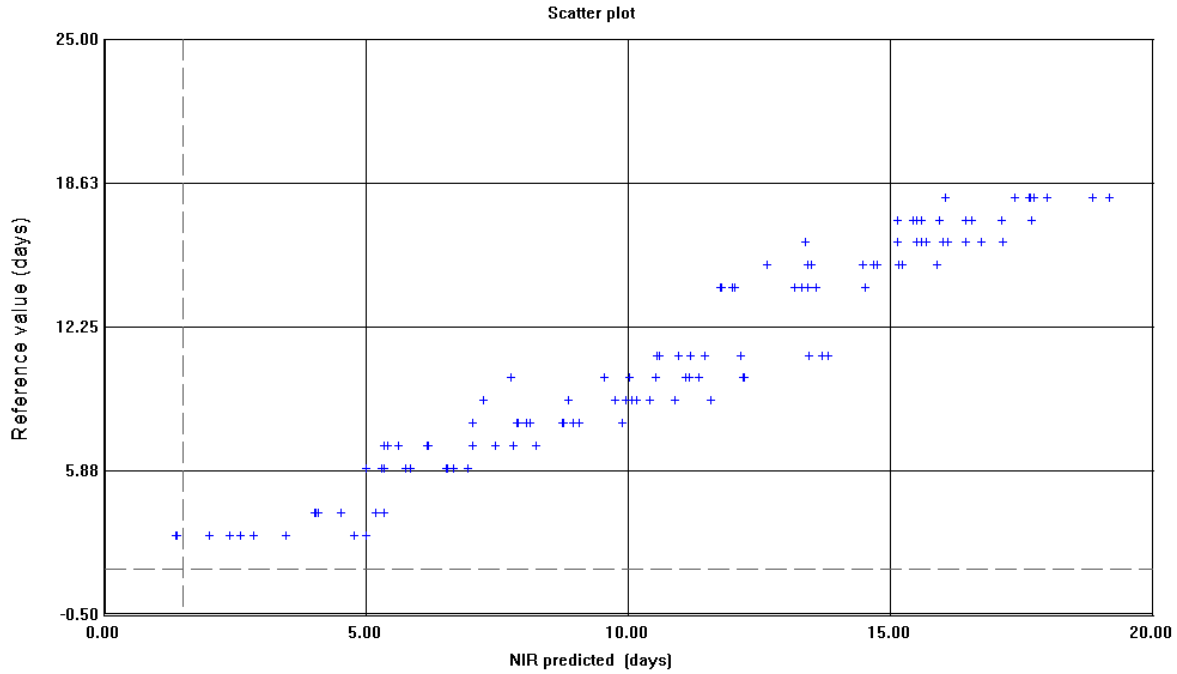


Table 2 – Parameters describing the fitness of modelling the correlation between measurements and the reference freshness data

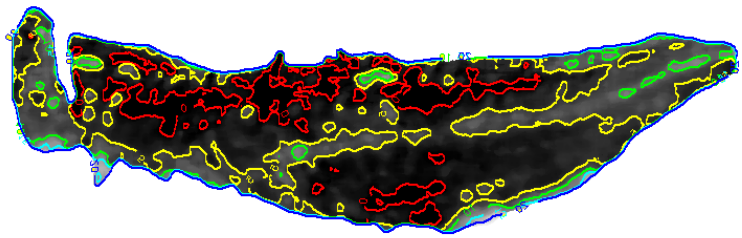
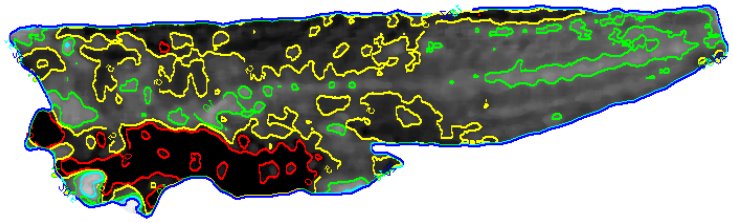
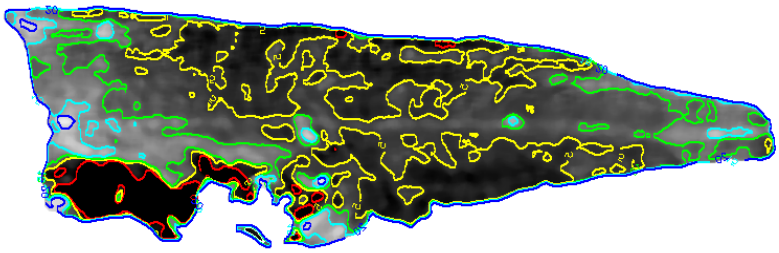
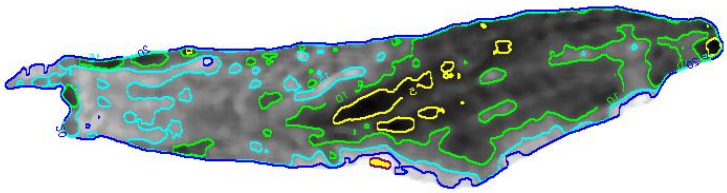
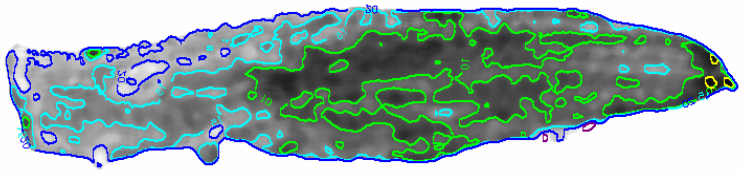
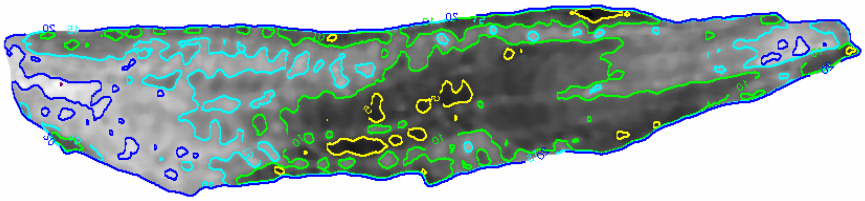
		Spectral range			
		892 – 2495nm		892 – 2094nm	
Correlation	Factors	R^2	SECV	R^2	SECV
All / days	10	0.91	1.82	0.91	1.73
Head / days		0.93	1.66	0.92	1.75
Middle / days		0.89	2.04	0.89	1.90
Tail / days		0.81	2.54	0.91	1.73
All / raw	5	0.88	0.81	0.84	0.80
Head / raw		0.92	0.69	0.91	0.65
Middle / raw		0.83	0.98	0.72	1.03
Tail / raw		0.76	1.13	0.72	1.17
All / cooked	5	0.77	0.57	0.80	0.57
Head / cooked		0.83	0.42	0.80	0.48
Middle / cooked		0.70	0.71	0.68	0.66
Tail / cooked		0.76	0.62	0.82	0.56

Figure 27 – Measured versus predicted storage time (days on ice) for the head end of the fillet over the complete wavelength range (892-2495nm) for the hyperspectral imaging system.



In Figure 28, contour plots of the predicted age of each part of the fillet are shown, based on the calibration generated from the head part of the fillet. The predicted age is shown as a grey scale with coloured contours at 5 day intervals. As would be expected, the predicted age at the head end shows a good correspondence with the actual age against which the calibration was developed, consistent with the results in Figure 27. However, the other parts of the fillet show different predicted ages, indicating that the spectra do not change in a consistent manner at all parts of the fillet and also indicating that care should be taken in applying calibrations developed for particular region of a fillet for measurement of samples from other or unknown locations.

Figure 28 – Contour plots of predicted days on ice for examples of fillets, based on a NIR calibration model developed for the head end (left in image).

Days on ice	0 5 10 15 20 25
2	
5	
8	
10	
14	
17	

4.3.2.1 Whole cod

The spectra of the whole cod were collected with the hyperspectral imaging system over a range of wavelengths about 900-2500 nm. Whole cod show a wide range of external structures, individual analysis of which was beyond the scope of this project. However, mean spectra for the whole fish showed evidence of an increase in reflectance (decrease in log (1/R) with age. The best correlation (R^2 = 0.59) of the average value for whole cod at a single waveband against days on ice was at 1164 nm (Figure 29). NIR images of the whole cod at this particular wavelength are shown in Figure 30 with log (1/*R*) values shown on a false colour scale. In Figure 29, the average absorbance of the whole cod with days on ice ranges from 0.7 to 0.95 which corresponds to the region between cyan and green on the chosen colour scale for Figure 30. Although many variations in reflectance can be seen across the body of each fish, the general trend of an increase in overall reflectance with storage time is clear, signified by a shift towards the blue end of the false colour scale.

Figure 29 – Correlation of mean spectra with days on ice for whole cod at 1164 nm

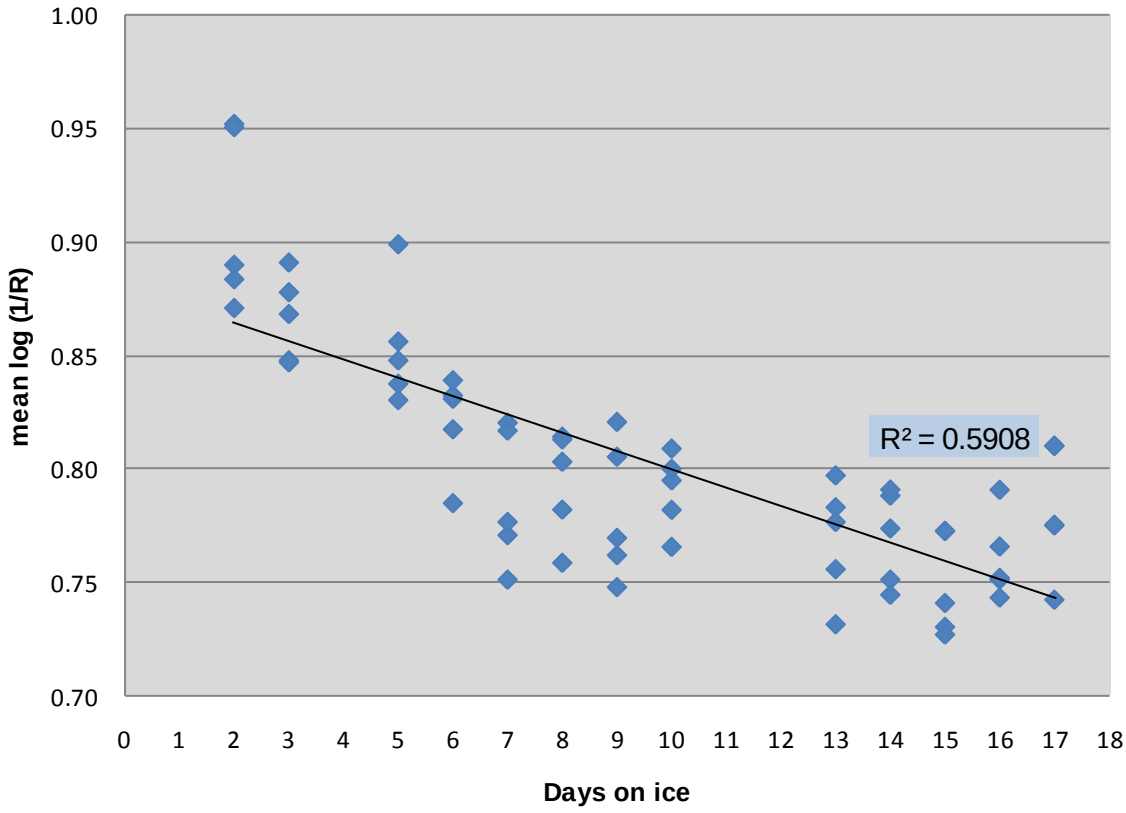
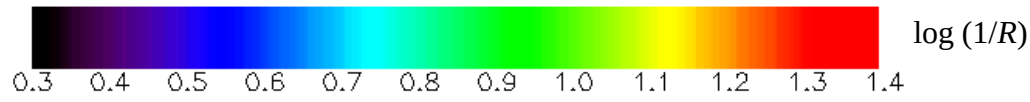
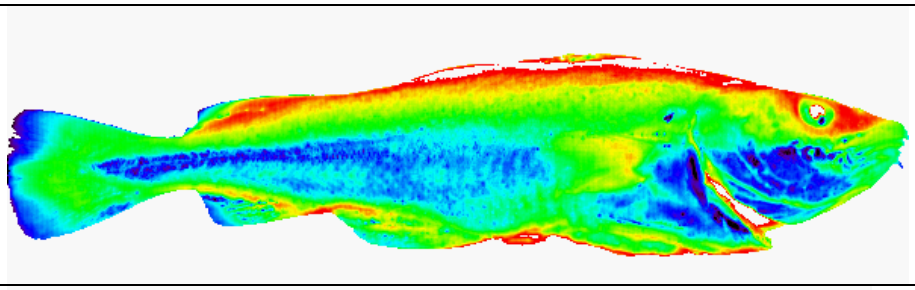
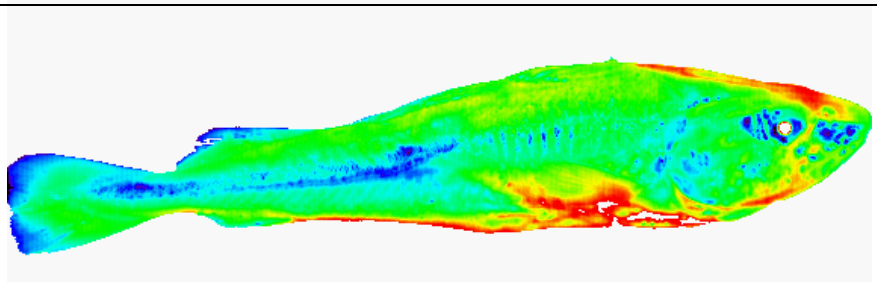
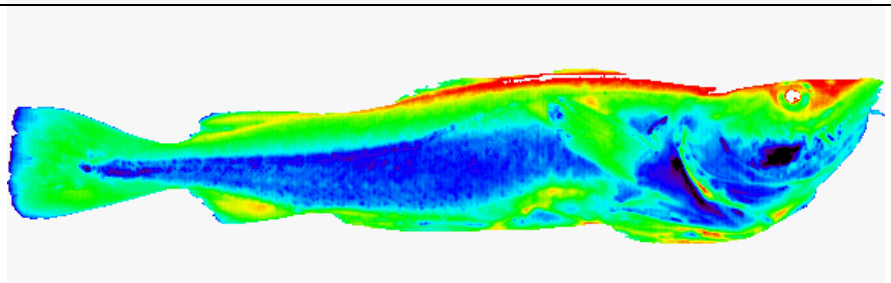
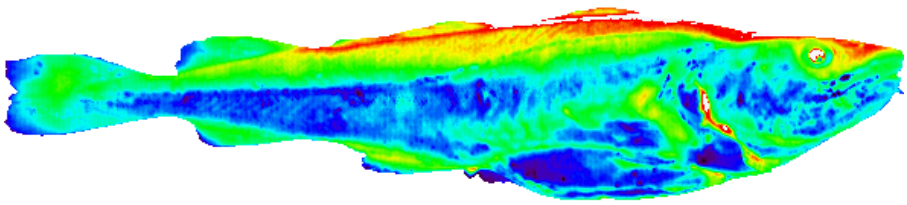
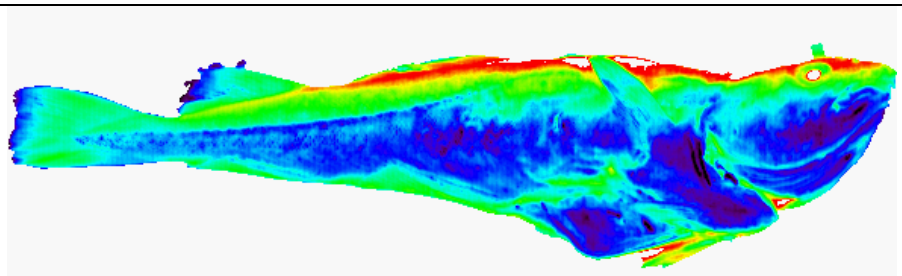
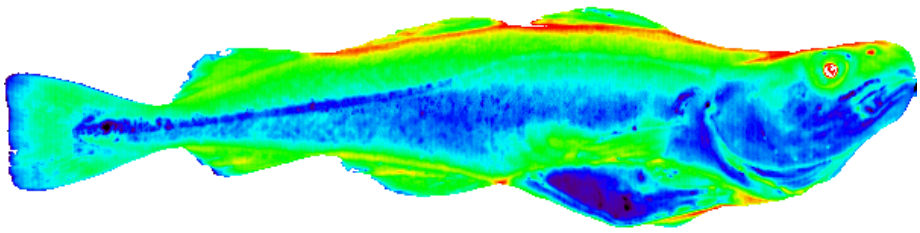


Figure 30 – NIR false colour images of the whole cod at 1164 nm

Days on ice	
2	
5	
8	
10	
14	
17	

5 Conclusions

- Assessment of freshness is an important issue in fish quality and the development of objective assessments is desirable. In this study, sensory assessments (Torry scheme) and several instrumental methods were used to assess their suitability for freshness evaluation.

- A DigiEye imaging system was used to take calibrated colour images and to make colour measurements of eyes, fillets and gills for cod stored on ice up to 17 days.

- Two NIR systems have also been used to make measurements of whole cod, fillets and gill filaments. Bulk NIR was used to analyse fillets and gill filaments, and a novel hyperspectral NIR imaging system was used to assess whole cod and several regions of fillets.

- The following table shows the performance of the best model for each instrument for each property with time:

	DigiEye imaging	**NIRSystems 6500**		**Hyperspectral NIR imaging**			
		408-1440nm		***892-2094nm***		***892-2495nm***	
	R^2	**R^2**	**SECV (days)**	**R^2**	**SECV (days)**	**R^2**	**SECV (days)**
Eyes	***Lightness (L*)*** 0.77 (up to Day 7)	-		-		-	
Whole fillet	***Yellowness (b*)*** First cut: 0.58 Second cut: 0.50 Combined: 0.51	-		0.91	1.73	-	
Fillet section	***Yellowness (b*)*** First cut: 0.53 Second cut: 0.37 Combined: 0.40	0.88	2.12	Middle: 0.89 Tail end: 0.91	1.90 1.73	Head end: 0.93	1.66
Gills	***Hue angle*** 0.77 ***Lightness (L*)*** 0.55	0.92	1.63	-		-	

- For the colour measurements, the results showed potential for use in determination of fish freshness. Objective measurements were identified that corresponded with colour changes expected from sensory experience. An increase in the reflectance of eyes with time was indicated by increased values of *L** up to about 7 days; increasing yellowness of fillets was indicated by an increase in *b**, and lightening and yellowing of gill filaments was indicated by increases in *L** and hue angle. It is concluded that an objective measurement of fish freshness can be carried out for eyes and gills, described by *L** and hue angle, respectively.

- NIR measurements of gill filaments and fillets showed better predictions of age than colour measurements. The overall best performance was obtained from gills (SECV = 1.63 days). The best model for fillets was obtained from the head end (SECV = 1.66 days). Therefore, it is recommended that the assessment of fish freshness should be carried out for the head end of the fillet.

- In comparison, the best model reported by Nilsen *et al*. was obtained from the inside of the fillets, with a standard error of prediction of 1.04 days. This performance is probably due to the larger number of factors per sample used in the calculation and models using similar numbers of factors to those in the current study gave poorer performance than those reported here. In the current study, the number of factors used was varied with sample number to avoid the risk of over-fitting the model.

- NIR measurements of whole cod showed a decrease in average absorbance and an increase in average reflectance with storage time. Although less detailed analysis was made of the data collected for whole cod than for fillets and gills, this shows that there is potential for information on freshness to be obtained by non-invasive measurements of external properties alone, with potential relevance for measurements early in the supply chain or for automated, on-line measurement applications.

- The work showed the potential of colour and NIR reflectance measurements for assessing freshness of cod with age, particularly over the earlier time frame (up to about 9 days) for which the performance of an alternative analytical method, TVB-N, is not recommended as a reliable indicator of fish freshness.

- Although this study has shown the potential of several methods for objective instrumental assessment of fish freshness, further work would be required to develop robust models and a practical instrument for commercial use. It is recommended that any such work should be done in collaboration with a suitable instrument manufacturer, following careful consideration of where in the supply chain the instrument would be used, the design requirements arising from this and the potential market for a successful commercial instrument. Therefore, although this study has shown the technical potential for instrumental measurement of fish freshness, some time would still be required to bring a commercial instrument to market.

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Appendix - overview of spectroscopic method and terms

This report relies on the application of spectroscopy to the measurement of aspects of fish quality. Spectroscopy covers a range of techniques which describe the interaction of electromagnetic radiation with matter. In this case, two regions have been used, the visible (energy which human beings can see, i.e. light) and near infrared (NIR) (a region which human beings can't see). The former allows changes in fish appearance to be determined instrumentally while the latter primarily allows instruments to be used to determine compositional differences. In addition, both the regions used respond spectroscopically to the physical structure of the samples analysed.

Individual spectra arising from foodstuffs generally and fish specifically in this case contain a lot of information but can be difficult to interpret. To overcome this potential problem, spectral data are typically converted to measurement units which are easier to use. In the visible region, the outputs used in this project have been based on Tristimulus colour measurement, a system designed to allow interpretation of results in a way which is similar to how human beings respond to colour stimuli. For the NIR data, calibrations have been developed to predict parameters of interest, such as measures of fish freshness, using the underpinning spectral data.

NIR calibrations are based on the use of linear regression where both multiplicative and additive constants are used to convert spectral data into units of interest. A broadly analogous situation is the comparison between temperature scales:

Temperature in degrees Fahrenheit = 32 + (1.8 × Temperature in degrees Celsius)

Where 32 is the additive constant and 1.8 is the multiplicative constant.

Having developed a calibration based on such an approach, there are a number of ways in which the performance of the calibration may be established. This is important as no calibration will show a perfect relationship between the spectral data and the units of interest. As a result, it is important to understand how good the prediction is to assess the calibration's potential for routine use. The following represents a glossary of the main terms used in this report related to NIR calibrations and their performance.

R^2 (squared correlation coefficient)

This is a measure of the goodness of fit between the true values and the predicted values. It represents the proportion of the total variation which is accounted for in the calibration. Larger values indicate better performance. In practice, values >0.8 indicate some value for screening purposes and values >0.9 indicate a useful degree of accuracy for routine analysis.

Standard error of calibration (SEC), standard error of prediction (SEP), standard error of cross validation)

These terms all estimate the error associated with a calibration's performance (relationship between true and predicted values). In each case, a lower value indicates better performance (less error or uncertainty). The term SEC relates to samples on which the calibration is based, SEP represents an estimate of performance for a separate validation set of samples while SECV uses samples in the calibration set to assess future predictive performance.

Partial least squares (PLS) regression

A technique which may be used to generate calibrations where all of the spectral data are used rather than a selection of specific wavelengths. It is generally more powerful and robust than methods based on individual wavelengths alone. The number of PLS factors included in a calibration is analogous to the number of multiplicative constants described above.