A Feasibility Study on Non-destructive Classification of Frozen Atlantic salmon (Salmo salar) Fillets Based on Temperature History at the Logistics using NIR spectroscopy

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ABSTRACT: Temperature fluctuation commonly occurs in the cold chain leading to complete or partial thawing and re-freezing of frozen products resulting in a multi-frozen product. Such oscillation of temperature could cause significant quality reduction compared to single frozen products. This study was designed to differentiate frozen Atlantic salmon fillets based on the level of temperature fluctuation. Near-infrared spectroscopy (NIRS) coupled with chemometrics was used to classify the frozen fillets stored at no fluctuation (NF), low fluctuation (LF), high fluctuation (HF) and very high fluctuation (VF) temperature. Using spectral profiles obtained at both frozen and thawed states, fillets were classified based on the level of temperature fluctuation by partial least squares discriminant analysis (PLS-DA). The thawed samples showed better classification accuracy (71 %) than frozen samples (66 %) in a four-class model. Considering the small variation within the first two (NF, LF) and the last two (HF, VF) groups, a two-class classification model was developed using thawed samples, and the obtained model correctly classified the two groups (NF, LF) and (HF, VF) with 100 % classification accuracy. Protein and water-related changes were found important to distinguish the fillets. Based on the finding, the four-class prediction model is found insufficient to be used for nondestructive determination of temperature history of frozen fillets. However, the two-class prediction model with further external validation can be applied to determine the level of temperature fluctuation particularly using fillets scanned at thawed state.

Practical Application: NIR spectroscopy can be used to evaluate the degree of temperature fluctuation and thus related quality loss throughout the logistics of frozen Atlantic salmon

53 fillets. Researchers, food control authorities and the retail industry could be the primary

54 beneficiaries of this research output. -PAGE BREAK-

1. Introduction

Fish and fishery products are extremely susceptible to deterioration. Hence, an extension of shelf life is compulsory to avail fish for consumers in various parts of the world. To extend the shelf life of these products, cold chain has been serving as an excellent choice for more than a century. Cold chain is a temperature-controlled supply chain, using freezing and refrigeration technologies to maintain the products in a specific temperature range during production, storage, transportation, sales and consumption. However, it is not always practical to maintain a defined range of temperature at all steps of the cold chain, leading to temperature fluctuations (Mercier et al., 2017). Temperature fluctuation (TF) often occurs in cold chain, resulting in complete or partial thawing and re-freezing of frozen products (Gutierrez et al., 2017; Syamaladevi et al., 2011). Considerable losses in textural quality, nutritional value, and functional properties of various agro-food products have been reported due to temperature fluctuation. Even if the degree of fluctuation is too low to cause complete thawing, significant differences were observed in the size of ice crystals in frozen products, which might consequently lead to loss of quality attributes (Gutierrez et al., 2017; Stiles et al., 2013; Syamaladevi et al., 2011).

Several studies indicated the occurrence of significant temperature abuse during cold transport of fish and fish products. In a study on the air and sea transportation of fish, temperatures higher than 0 °C ± 1 °C were recorded (Martinsdottir et al., 2010). The temperature abuse was observed in 35% and 18% of the transportation time, respectively for air and sea transportations. In another study, temperature fluctuation above 5 °C was recorded for 17% and 36% of total time during air and sea transportation respectively (Mai et al., 2012). Besides

during transportation, temperature increase up to 10 °C was reported while truck loading and unloading operations (Mercier et al., 2017).

Several aspects of fish quality are known to degrade as a result of cold chain breakage throughout the logistics. Research on the impact of fluctuating temperature and constant temperature on lipid composition of fish fillet showed high free fatty acid and peroxide values for the fluctuating temperature regimes, while the lowest free fatty acids and peroxide value were observed on the super freezing (Gormley, 2019). In other studies, increased freeze-thaw cycles showed membrane disintegration, disruption of muscle cells, and an increase in exudation of fluid (Benjakul & Bauer, 2000; R. Gormley et al., 2002; Hamm, 1979; Rayeni, 2016). Furthermore, reduction in water holding capacity, reduction in protein solubility, and loss of salt soluble protein were reported (Benjakul & Bauer, 2000). Therefore, multiple freezing-thawing cycles caused by temperature abuses demonstrated detrimental effects on muscle tissues via damage to cell membranes and organelles leading to significant effects on quality attributes of fish muscle.

Considering the detrimental role of temperature fluctuation during frozen fish logistics, finding a method to predict the temperature fluctuation history of the frozen product is worthful. Several methods have been tested to evaluate quality related changes in fish. Enzymatic, chemical, microbiological and sensory analysis are among the applicable and useful analytical methods. These methods have been applied to differentiate fish based on freeze-thaw cycles (Davis, 1982; Fernández-Segovia et al., 2012; Howell et al., 1996; Kim et al., 1987, 1987; Li et al., 2018; Nott et al., 1999). Recent analytical techniques that use metabolomics have also allowed the successful classification of fresh and frozen/thawed fish samples (Leduc et al., 2012;

Massaro et al., 2021; Stella et al., 2022). However, these methods are destructive, time-consuming, expensive and require expert knowledge, which limits their practical application. Since recent decades, traditional methods of fish quality analysis have been replaced by spectroscopic-based techniques due to the advantages of fast and non-invasive analysis, simultaneous determination of numerous quality parameters, and applicability for online measurements (Duflos et al., 2002; Karoui et al., 2017; Hassoun, 2021). Among the spectroscopic techniques, hyperspectral imaging, nuclear magnetic resonance and near-infrared spectroscopy have been investigated to distinguish between fresh and frozen-thawed fish (Hassoun et al., 2020). NIR spectroscopy is a well-established method in both research and food industrial applications (Wang et al., 2018). It relies on measuring the absorption of photons in the near-infrared range by the vibrational modes of C-H, O-H, and N-H bonds (Prieto et al., 2017). Measurement of the electromagnetic radiation absorbed by those molecular bonds gives a unique spectral fingerprint that contains information related to the physical and chemical properties of a sample. Several researchers have applied Vis/NIR spectroscopy-based non-destructive techniques to distinguish between fresh and frozen-thawed fish (Fasolato et al., 2012; Sivertsen et al., 2011; Uddin & Okazaki, 2004; Wang et al., 2018). Since temperature fluctuation often occurs in the frozen fish supply chain, analytical methods capable of distinguishing fillets based on the level of temperature fluctuation are required. However, existing studies in this regard have considered complete freeze-thaw cycles, which are not common in the frozen fish supply chain (Fasolato et al., 2012; Sivertsen et al., 2011; Uddin & Okazaki, 2004; Wang et al., 2018). It is hypothesized here that near-infrared spectroscopy, which showed successful classification between fresh and frozen-thawed fish could also be

used to distinguish frozen fillets which have been subjected to minor temperature fluctuations. Therefore, this study was designed to evaluate the potential of near-infrared spectroscopy for the classification of frozen Atlantic salmon (Salmo salar) fillets subjected to minor temperature fluctuations.

2. Materials and Methods

2.1 Samples

Frozen Atlantic salmon (Salmo salar) fillets supplied by a local retailer (Colruyt Group) were used for the experiment. The fillets were generally rectangular with approximate sizes of 10 to 12 cm in length, 4 to 5 cm in width, and 2 to 3 cm in height. The fillet samples were individually vacuum-packed and supplied avoiding the possibility of temperature fluctuation. The samples were transferred into a freezer set at -18 °C for 24 hours until the start of the storage experiment.

2.2 Frozen Storage Experiment

The storage experiment mimicked four purposely selected levels of temperature fluctuation that represent actual conditions in frozen Atlantic salmon logistics. The storage conditions simulated under the scope of this study included four conditions that represent temperature fluctuation during loading, transportation, loading and transportation, and no fluctuation (ideal) conditions. The experiment involved four groups of 20 fillets in which group one, no fluctuation (NF) samples were stored at -18 °C for 18 days representing an ideal condition. Samples in the second group, low fluctuation (LF) represented a bad loading condition. Samples in this group were stored at 18 °C for 3 hours at the beginning of the storage experiment, resulting in partial thawing on the surface. After 3 hours, the samples were transferred to a freezer set at -18 °C

until the 18th day of the experiment. Samples in the third group, high fluctuation (HF), represented a bad transportation condition and were stored at -12 °C for 8 hours followed by -18 °C for 16 hours every 24 hours, till the end of the experiment period. The last group, very high fluctuation (VF), represented a condition in which both the loading and transportation are bad. Fillets in this group were stored at 18 °C for 3 hours at the start of the storage experiment. Then, the fillets were stored at -12 °C for 8 hours followed by -18 °C for 16 hours every 24 hours for 18 days.

2.3 Reference Analysis (drip loss, pH and space between muscle fibres)

Drip loss was measured by calculating the weight difference before and after the storage experiment. The final weight of the samples was taken after draining the fillet thawed overnight at 4 °C.

$$Drip\ loss\ (\%) = (Initial\ weight - Final\ weight) \div (Initial\ weight) \times 100$$

Measurement of pH was performed using a benchtop pH meter (Hanna Instruments, USA) by directly inserting the electrode into the fillet (Thorarinsdottir et al., 2002). A duplicate measurement was done on each fillet. Determining the space between muscle fibres was performed using sub-samples sectioned at 20 µm thickness using a microtome-cryostat (Microm HM 560 Cryostat, Thermo Fisher Scientific, Waltham, MA, USA) in an optimum cutting temperature environment, where the temperature of the medium was maintained at -27 °C. An inverted light microscope (VWR international, USA) equipped with Visicam 5.0 digital camera (VWR, Belgium) was used to acquire microscopic images. The images were recorded and processed using ImageJ software (National Institutes of Health, USA). The gap between muscle fibres was calculated using the inbuilt tool in the software.

2.4 NIRS Measurement

Diffuse reflectance measurements in the 800 to 2500 nm range were performed using a Bruker optics multipurpose analyzer (MPA; Bruker Optics, Germany) by placing the fillets on the entry port of the integrating sphere. Spectral acquisition and control of the instrument were performed using OPUS software (v. 6.5, Bruker Optics, Ettlingen, Germany). For each measurement, the spectrum was averaged over 32 scans.

All fillet samples were scanned at the end of the 18-day storage experiment in their frozen condition. Scanning of fillets at thawed condition was done on 16 fillets from each group after 24 hours of thawing at 4 °C. The packaging material was removed and the surface of fillet samples was directly scanned at two different positions by placing the fillet on the device.

2.5 Data Analysis Tool

Statistical analyses of the reference quality measurements were performed using JMP software (JMP, Version 13; SAS Institute Inc.). Spectral analysis was performed using PLS Toolbox (Eigenvector Research, Inc., version 8.6).

2.6 Development of Classification Model

2.6.1 Principal Component Analysis (PCA)

Principal component analysis (PCA) was used to determine the main characteristics of the spectra and highlight the relations among the absorbance values at different wavelengths. Potential spectral outliers were detected by studying score plots using Hotelling's T^2 and Q residuals statistic.

2.6.2 Spectral Data Preprocessing

In order to improve the informativeness of the spectral data, several data preprocessing techniques were tested either individually or in combination with each other. Standard normal variate (SNV), detrend (DT) and Savitzky-Golay (second derivative) were among the selected data pretreatments based on the classification performance. For details on preprocessing methods the reader is referred to (Martens & Stark, 1991; Thennadil & Martin, 2005; Saeys et al., 2019).

2.6.3 Partial Least Squares Discriminant Analysis (PLS-DA)

The whole data matrix was split into a calibration set (70%) and a validation set (30%) using the Kennard-Stone method. Venetian blinds cross-validation with 10 splits was applied to the calibration data to optimize the number of latent variables during PLS-DA model development. During the development of the model, the calibration data (Y) represented the class membership using ones and zeros and then paired with the training matrix (X) (Barker & Rayens, 2003; Saeys et al., 2019). The point at which the lowest number of false positives and false negatives were achieved was selected as a threshold. Once the calibration model was trained, independent validation data were used to test the model and a classification output was generated.

2.6.4 Performance Evaluation

The overall performance of the individual models, in combination with the various pre-processing techniques, was validated by calculating the classification accuracy (Equation 3.1), false-positive rate (Equation 3.2), and false-negative rate (Equation 3.3). The efficacy of the overall model was described by the classification accuracy. A false positive occurred when a negative response (incorrect class) was incorrectly classified as a positive response (correct

class) and a false negative was when a positive response (correct class) was incorrectly classified as a negative response (incorrect class). Consequently, the false-positive rate describes the occurrence of misclassifications of an incorrect class in the model as a correct class, while the false negative rate describes the occurrence of misclassifications of a correct class to another incorrect class in the model (Payne, 2019):-

Classification accuracy (%) =(((TP+TN))/(TP+TN+FP+FN)) × 100% Equation 3.1

False positive rate (%) =(((FP))/(TP+TN+FP+FN)) × 100% Equation 3.2

False negative rate (%) =(((FN))/(TP+TN+FP+FN)) × 100% Equation 3.3

Misclassification rate (%) =(((FP+FN))/(TP+TN+FP+FN)) × 100% Equation 3.4

Where;

True Positives (TP) = Positive response correctly classified as a positive response; True Negatives (TN) = Negative response correctly classified as a negative response; False Positives (FP) = Negative response incorrectly classified as a positive response; False Negative (FN) = Positive response incorrectly classified as a negative response.

3. Results and Discussion

3.1 Effect of Temperature Fluctuation on Quality of Frozen Atlantic salmon

Table 3.1, reproduced from another part of a similar study, indicates changes in quality related parameters (drip loss, pH and space between muscle fibres) of fillets stored at varying levels of temperature fluctuation (Bezuayehu, 2021). All quality attributes of fillets stored at the highest level of temperature fluctuation (VF) were significantly affected ($p<0.05$) compared to fillets stored at the lowest level of temperature fluctuation (NF).

Table 3. 1 Mean ± standard deviation values of reference quality parameters: drip loss (%), pH and space between fibers (%).

Group	Drip loss (%)	pH	Space between muscle fibres (%)
	Average ± SD	Average ± SD	Average ± SD
NF	2.85 ± 0.43^{b}	6.12 ± 0.05^{a}	26.55 ± 8.77^{c}
LF	2.86 ± 0.42^{b}	6.10 ± 0.06^{a}	37.31 ± 6.47^{b}
HF	3.62 ± 0.51^{a}	6.08 ± 0.07^{ab}	50.37 ± 4.55^{a}
VF	3.81 ± 1.02^{a}	6.04 ± 0.06^{b}	52.12 ± 2.81^{a}

All values are mean ± SD for measured quality attributes. Values in the same column with different superscripts are significantly different ($p<0.05$)

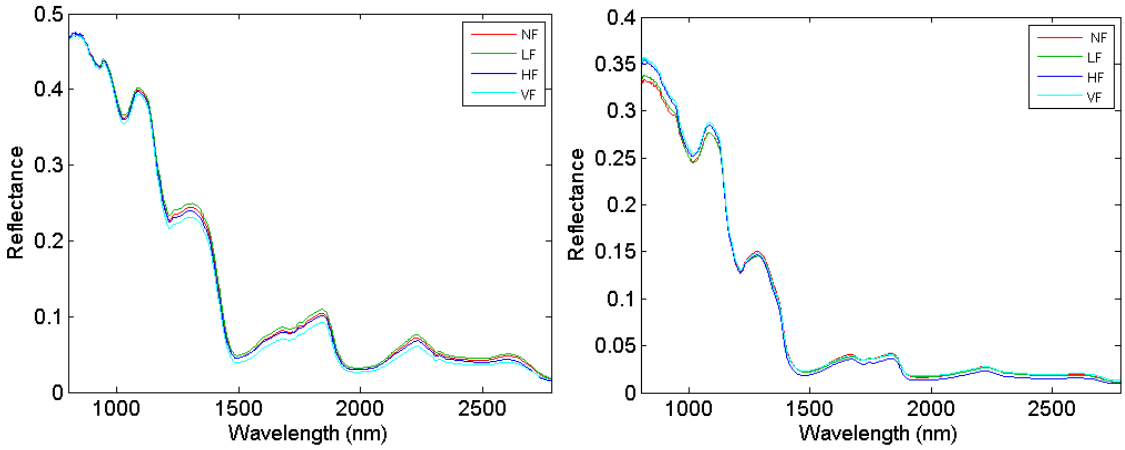
Drip loss and the space between muscle fibres increased with increasing levels of temperature fluctuation, while the pH of fillets decreased with increasing levels of temperature fluctuation. The relation between the obtained results from the three quality indicators confirmed the reduction of quality as a result of higher temperature fluctuation. Changes in drip loss and space between muscle fibres were more intense in HF and VF groups compared to LF and the control group. The difference within the two lowest temperature fluctuation groups (NF and LF), and the two highest temperature fluctuation groups (HF and VF) were insignificant for all quality attributes except for the space between muscle fibres, where NF and LF differ considerably. This can be attributed to the small variation in the levels of temperature fluctuation between the NF and LF, and HF and VF groups.

3.2. Spectral Interpretation

In Figure 3.1, the mean spectra of each group of fillet samples scanned at frozen (a) and thawed (b) conditions are illustrated. High overall reflectance values were observed in frozen fillets compared to thawed fillets. A similar result was reported on multi-frozen-thawed tilapia (Wang

et al., 2018). The high overall reflectance could be due to water migration which occurs during freezing or variation in the water status in frozen and thawed samples (Wang et al., 2018). In a study on the effect of a freeze-thaw cycle in Cuttlefish muscle, migration of water from the inside to the outside of fish muscle bundles was observed (Ying and Jing 2021). Scanning of the fillet at their frozen state was therefore easily influenced by the increased water content at the surface of the fillet muscle, thereby showing high overall reflectance. High absorption was observed around 980 nm, 1210 nm, 1450 nm and 1950 nm. The dominating absorption peak appearing at 980 nm is attributed to the water content (second overtone O-H stretching), while the peak at 1450 nm originated from the first overtones of O-H stretching. The combination tone O-H stretch forms the peak around 1950 nm (He et al., 2014b). This indicates that water is a major absorbing constituent affecting the spectral characteristics of salmon flesh in the NIR region because of the high-water content of the flesh (He et al., 2014b). The absorption peak around 1210 nm can be attributed to C-H stretching, which is related to the presence of fat (Fernández-Segovia et al., 2012; He et al., 2014a). From the overall spectra and all the dominant absorption peaks of samples scanned at a frozen state (Fig 3.1. a), it can be seen that sample groups exposed to significant temperature fluctuation (HF and VF) had slightly higher absorption than the other groups (NF and LF). Samples scanned at thawed state demonstrated a similar profile of increased absorbance with an increasing level of temperature fluctuation across the entire region except for the wavelengths where water absorption peaks arise (Fig. 3.1. b). On the contrary, the spectral profile at which water absorption peaks originate showed a decreased absorbance with an increasing level of temperature fluctuation.

(a) (b)



271

272 **Figure 3.1.** Average reflectance spectra of samples exposed to different temperature conditions
273 measured at frozen state (a) and after thawing (b): No temperature fluctuation (NF), low
274 fluctuation (LF), high fluctuation (HF) and very high fluctuation (VF) groups of fillets

275 **3.3 Principal Component Analysis (PCA)**

276 The spectral profiles were analyzed using PCA to evaluate the possibility of rapidly separating
277 the different groups of samples. The general overview of the spectral groups was observed
278 from the two datasets (frozen and thawed), plotted on a multivariate coordinate space where
279 the dimensions were ordered according to decreasing explained variance (Fig. 3.2). The first
280 two principal components explained more than 94% of the total variation among the samples in
281 both frozen and thawed samples, indicating a good representation. The major peaks in the
282 loadings plot (Fig. 3.3) correspond to the second and first overtone O-H stretching (980 and
283 1450 nm, respectively), and the second overtone of C-H stretching (1210 nm).

284 (a) (b)

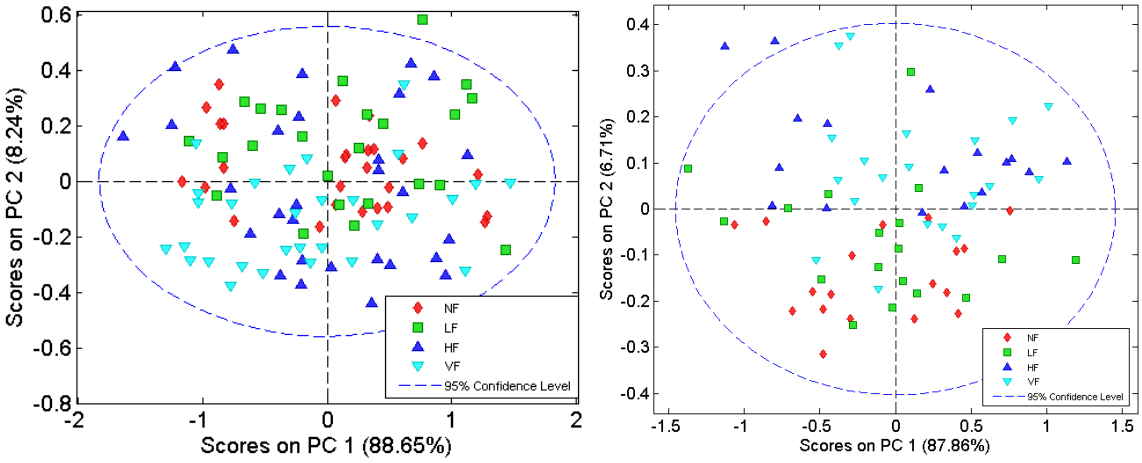


Figure 3.2. Principal component analysis (PCA) score plots for the mean centered spectra of fillets scanned at frozen state (a) and fillets scanned at thawed state (b).

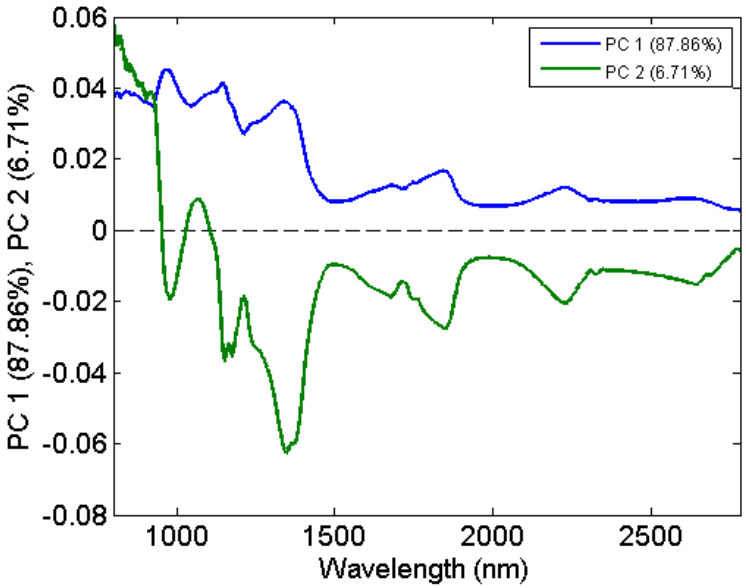


Figure 3.3. PCA loadings of the first two PCs for samples scanned at thawed state.

As observed in Fig. 3.2., particularly in thawed fillets (b), the four groups demonstrated grouping into two groups where NF and LF were closer to each other and HF was closer to the VF group. As observed in Fig. 3.2 (b), samples in thawed state showed better separation between the two groups compared to samples scanned in the frozen state (a). This could be

294 due to the pronounced interference by specular reflectance in samples in frozen state. In
295 addition, the difference in the state of water in the frozen and, the thawed fillets could affect
296 the spectral characteristics, thereby influencing the separation between the groups.
297 Furthermore, the variation in water content of fillets in the frozen state and thawed state may
298 also affect the spectral characteristics.

299 **3.4 Four Class Classification of Frozen Fillets**

300 Several pre-processing methods including combinations of standard normal variate (SNV),
301 detrend and Savitzky-Golay derivatives were tested to improve the classification performance.
302 The best classification for samples in frozen state was obtained with a model using four latent
303 variables based on spectra preprocessed with a Savitzky-Golay 2nd derivative (2nd order
304 polynomial, 11 points). The PLS-DA model achieved 79% classification accuracy with 21%
305 misclassification rate in calibration, 55% classification accuracy with 45% misclassification rate
306 in cross-validation and 66% classification accuracy and misclassification rate of 34% on the
307 validation dataset (Table 3.2). The PLS-DA model using samples scanned in frozen state yielded
308 poor classification performance with an overall accuracy of 66 %. In addition, the overall
309 classification accuracy was reduced from 79% on the calibration set to 66% on the validation set
310 samples. The accuracy of classification reported in this study was lower compared to a related
311 study on tilapia (Oreochromis) fillet which obtained more than 80% classification accuracy using
312 samples scanned in frozen state (Wang et al., 2018). The difference could be due to the fact
313 that in their study samples were completely thawed and frozen several times before analysis,
314 which may have resulted in a more pronounced effect on the quality attributes of the fillets

compared to the present experiment which tested temperature fluctuation without complete thawing of samples.

Table 3.2. An overview of accuracies of the PLS-DA models with various pre-processing techniques applied to distinguish samples scanned at frozen state based on the level of temperature fluctuation

Pre-processing	LV	Calibration		Cross-validation		Validation	
		CA (%)	MR (%)	CA (%)	MR (%)	CA (%)	MR (%)
SNV	5	68	32	59	41	59	41
SNV + DT	5	67	33	58	42	59	41
SNV + DT + $SGD_2(7)$	4	85	15	53	47	64	36
$SGD_2(11)$	4	79	21	55	45	66	34
$SGD_2(15)$	7	83	17	60	40	58	42

(SNV) Standard normal variate; (DT) Detrend; ($SGD_x(y)$) Savitzky-Golay, (x) derivative order (y) number of window points; (CA) Classification accuracy, (MR) misclassification rate

3.5 Four Class Classification of Thawed Samples

The spectral dataset collected from the thawed samples was pre-treated with several techniques to maximize the prediction performance of the PLS-DA model. SNV, detrend and Savitzky-Golay derivatives (second-order polynomial, 5, 7, 9 and 11 points) were applied to the absorbance spectra of the thawed samples. Table 3.3 summarizes the performance measures with the obtained results after a pre-processing using Log (1/R), SNV, detrend and Savitzky-Golay second derivative (second-order polynomial, 7 points). Accordingly, the model yielded a correct classification rate of 80 %, 67 % and 71 % respectively on the calibration, cross-validation and validation set samples.

Table 3.3. Performance measures used to assess the four-class PLS-DA model based on thawed samples after pre-processing using 1/R, SNV, detrend and Salvitzky-Golay (second derivative, second-order polynomial, 7 points)

	Class	TPR	FPR	TNR	FNR	CA (%)	MR (%)
Calibration	NF	0.78	0.13	0.87	0.22	82.16	17.84
	LF	0.55	0.09	0.90	0.44	72.97	27.03
	HF	0.89	0.11	0.88	0.11	88.68	11.32
	VF	0.56	0.05	0.94	0.44	75.35	24.65
Cross validation	NF	0.50	0.13	0.87	0.50	68.27	31.73
	LF	0.33	0.21	0.79	0.67	56.09	43.91
	HF	0.67	0.13	0.87	0.33	76.60	23.40
	VF	0.50	0.18	0.81	0.50	65.74	34.26
Validation	NF	0.78	0.15	0.85	0.22	81.20	18.80
	LF	0.33	0.08	0.92	0.67	62.82	37.18
	HF	0.89	0.23	0.77	0.11	82.94	17.06
	VF	0.25	0.11	0.89	0.75	56.94	43.06

True positive ratio (TPR), false positive ratio (FPR), true negative ratio (TNR), false negative ratio (FNR), classification accuracy (CA), misclassification rate (MR)

The PLS-DA score plot for the four groups of samples is given in (Fig. 3.4). In contrast to the model for samples scanned in frozen state, the PLS-DA score plot LV1 (58%) vs. LV2 (3.03%) for thawed samples demonstrates less overlap among the different classes, particularly between the first two classes (NF, LF) and the remaining two classes (HF, VF). As the score plot demonstrated, NF and LF were separated from HF and VF groups without any overlap. This could be due to the fact that the temperature fluctuations in the first two groups (NF and LF) did not cause a destructive change in the composition of fillets. In contrast, the HF and VF

groups might have undergone significant changes during frozen storage which may have resulted in a change in the spectral profile of the fillets.

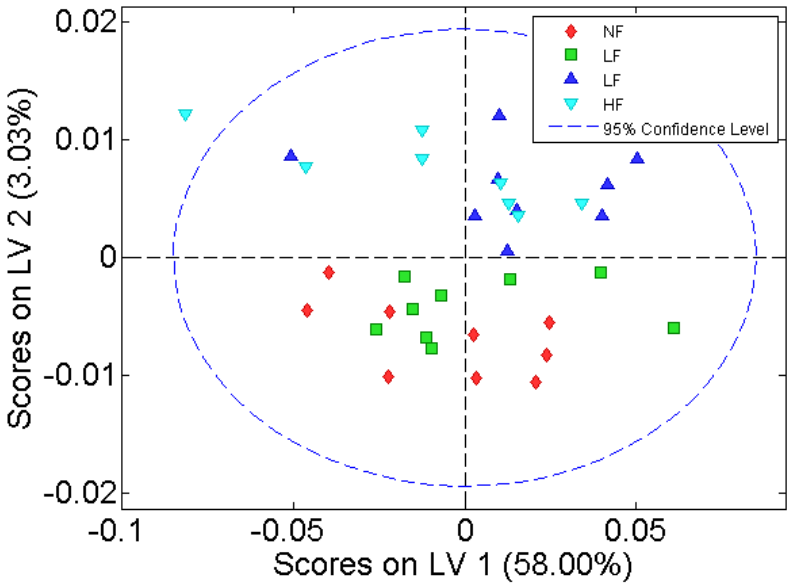


Figure 3.4. PLS-DA score plot for the thawed fillets based on spectra pre-processed with log (1/R), SNV, detrend and Savitzky-Golay second derivative (second-order polynomial, 7 points). Legend: No fluctuation (NF), low fluctuation (LF) high fluctuation (HF) and very high fluctuation (VF) groups of thawed fillets.

Compared to a classification result using spectra of fillets at their frozen state, the model based on thawed samples performed better and had a higher classification accuracy. This result is in agreement with the report of Shimamoto et al. (2001), who showed a more precise prediction for the fat content of mackerel using samples in thawed conditions suggesting a better observation of changes in fillet constituents in thawed state than at the frozen state. However, better classifications of once frozen-thawed from repeatedly frozen-thawed tilapia (Oreochromis) fillets were obtained under frozen conditions (Wang et al., 2018). This could be due to the fact that the previous researchers used fillets that were frozen in laboratory

conditions and had flat surfaces. However, commercially frozen samples that had irregular surfaces were used in this experiment. This could have led to measurement-related variations despite the effort to reduce those variations, such as repeated scanning at different positions of the fillet. Spectral measurements of samples in frozen state might have affected the accuracy of the collected spectral data due to the fact that fillets with irregular surfaces cannot be scanned properly compared to fillets with a flat surface. Additionally, the frost on the surface of fillets measured in frozen conditions might have affected the light scattering conditions compared to samples that were measured in the thawed state. In contrast, samples in thawed condition allowed relatively uniform scanning, which provided more accurate information about the composition-related changes that took place during frozen storage. One of the compositional changes could be a difference in water content which is brought by the effect of different levels of temperature fluctuation. In the samples scanned at thawed state, the HF and VF groups might contain lower moisture than NF and LF groups, thereby resulting in easy classification based on the difference in moisture content. Although measurement of moisture content was not performed, it can be derived from the increased drip loss value that the moisture content in HF and VF groups fillets was reduced compared to that of NF and LF groups.

Since the spectral features of the fillets in the first two groups and the other two groups were closer to each other, the classification of fillets into four different classes demonstrated insufficient performance (71 % of classification accuracy). From the reference analyses, it could be noted that variation in quality related changes (pH, drip loss, space between muscle fibres) within the first (NF, LF) and the last (HF, VF) two groups were found insignificant ($p>0.05$), while the difference between the first and the last two groups was found significant ($p<0.05$).

379 Moreover, the fact that temperature fluctuation is not totally avoidable in the cold chain, a
380 two-class classification model would provide a robust class prediction into the good (NF & LF)
381 that could represent ideally transported and/or stored fillets with a minimum level of
382 temperature fluctuation; and the bad (HF & VF) which represent potential temperature
383 fluctuation leading to a significant level of quality degradation.

384 **3.6 Two-Class Classification of Thawed Samples**

385 NF and LF group samples were modeled as one class while HF and VF group samples were
386 modeled as the other class after pre-processing the spectra using a previously selected
387 combination of spectral pre-treatment techniques: - SNV, detrend and Savitzky-Golay second
388 derivative (second-order polynomial, 7 points). This resulted in an overall classification
389 accuracy of 100% (calibration), 93.27% (cross-validation) and 100 % (validation), indicating the
390 effectiveness of the model to classify fillets based on the level of temperature fluctuation (Table
391 3.4).

392 **Table 3.4.** Overview of accuracies of two-class PLS-DA model using thawed samples

Pre-processing	**LV**	**Calibration**		**Cross-validation**		**Validation**	
		CA (%)	MR (%)	CA (%)	MR (%)	CA (%)	MR (%)
SNV, DT, SGD_2 (7)	3	100	0	93.2	6.7	100	0

(SNV) standard normal variate; (DT) detrend; (SGD_x(y)) Savitzky-Golay, (x) derivative order (y) number of window points; (CA) classification accuracy, (MR) misclassification rate

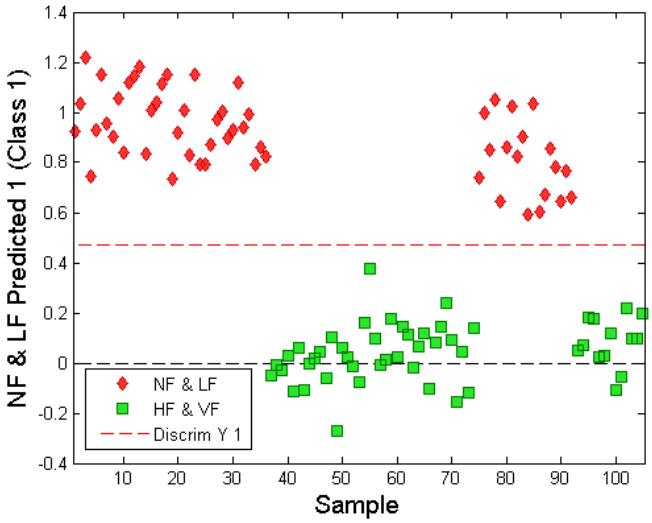


Figure 3.5. PLS-DA calibration and prediction score plot for spectra of thawed samples pre-processed with SNV, detrend and Savitzky-Golay second derivative (second-order polynomial, 7 points). Red indicates (NF & LF) group and green indicates (HF & VF) group. The plot indicates the calibration samples on the left side & the validation samples on the right side.

This model appeared to be the best performing in overall classification accuracies compared to the two models discussed above, and the two-class classification model using frozen samples, which yielded 83 % overall accuracy (Table 3.5). The PLS-DA prediction score plot (Fig. 3.5) demonstrates that all fillets from both (NF & LF) and (HF & VF) groups were correctly classified.

Table 3.5

4. Conclusions and Recommendations

Classification of fillets according to the level of temperature fluctuation was performed based on their NIR spectra acquired in frozen and thawed state. The four-class classifier based on the spectra of thawed fillets outperformed that on the spectra of frozen samples with a classification accuracy of 70.9% compared to 66%, respectively. The classification model failed to distinguish the fillets stored at no fluctuation (NF) from fillets stored at low fluctuation condition (LF), and fillets stored at high fluctuation (HF) from very high fluctuation (VF). An

alternative two-class classification model provided more efficient classification with 100% accuracy on the spectra of thawed samples subjected to a combination of SNV, detrend and Savitzky-Golay 2nd derivative (2nd order polynomial, 7 points) pre-processing. This two-class classification model based on spectra of thawed fillets can be used to determine the level of temperature fluctuation after further external validation involving a larger number of samples. Future research is recommended to obtain more insight into the changes in the optical properties of fish muscle. Particular focus should be given to the factors affecting the optical properties during storage under fluctuating temperatures because this would allow to further optimize the NIR-based prediction models.

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Conflicts of Interest

The authors declare that there are no known conflicts of competing interests.

Data Availability

Data will be shared on request

Nomenclature

CA	Classification Accuracy
CV	Cross-Validation

431	DT	Detrend
432	FP	False Positive
433	FN	False Negative
434	HF	High Fluctuation
435	KS	Kennard Stone
436	LF	Low Fluctuation
437	LV	Latent Variable
438	MR	Misclassification Rare
439	MSC	Multiplicative Scattering Correlation
440	NIR	Near Infrared
441	NIRS	Near Infrared Spectroscopy
442	NF	No Fluctuation
443	PC	Principal Component
444	PCA	Principal Component Analysis
445	PLS-DA	Partial Least Squares Discriminant Analysis
446	RMSE	Root Mean Square Error
447	RMSECV	Root Mean Square Error of Cross-Validation
448	RMSEP	Root Mean Square Error of Prediction
449	SG	Savitzky-Golay
450	SNV	Standard Normal Variate
451	TN	True Negative
452	TP	True Positive

VF Very high Fluctuation

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