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Classification of Astaxanthin Colouration of Salmonid Fish using Spectral Imaging and Tricolour Measurement

IMM-Technical Report-2012-08

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Abstract

The goal of this study was to investigate if it is possible to differentiate between rainbow trout (*Oncorhynchus mykiss*) having been fed with natural or synthetic astaxanthin. Three different techniques were used for visual inspection of the surface colour of the fish meat: multi-spectral image capturing, tricolour CIELAB measurement, and manual SalmoFan inspection. Furthermore it was tested whether the best predictions come from measurements of the steak or the fillet of the fish. Methods used for classification were linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), and sparse linear discriminant analysis (SLDA).

1 Introduction

The colour of salmonid fish is one of the most important quality parameters for customers [1–3]. Consumers associate increased level of red in salmonid fishes with superior quality, and colour is the first quality parameter inspected by the customer [4]. Therefore, it is of outermost importance for the industry to understand the effect of breed conditions and processing on the colour development in salmonid fish fillets.

Astaxanthin has a high antioxidant activity, is essential for reproduction, growth and survival, and important for the development of colour in salmonid fish [5]. The primary use of astaxanthin within aquaculture is as a feed additive to ensure that farmed salmon and trout have similar appearance to their wild counterparts [6]. For this purpose, fish feed pellets are coated with fish oil with added astaxanthin in order for the fish to get the red meat pigmentation. Synthetic astaxanthin is more easily available and costs slightly less than natural astaxanthin and is therefore used more often in the industry. However, there is a demand for natural astaxanthin for the organic salmonid fish market where natural astaxanthin is mandatory.

Several studies has investigated how different processing conditions influences the colour in the fillets [7–10]. Some studies investigating the effect of astaxanthin source (natural versus synthetic) on astaxanthin concentration in the muscle and physical performance criteria such as growth [11, 12], and to distinguish between natural and synthetic astaxanthin chemically in fish [13] can be found, whereas literature about the effect of astaxanthin source on meat colour to our knowledge is almost non existing.

The aim of this study was to investigate if natural and synthetic astaxanthin give different fish meat colour. The goal was to be able to differentiate between fish having been fed with natural and synthetic astaxanthin by using machine vision techniques. This is important since the organic salmonid fish market has to use natural astaxanthin in the feed. Furthermore, it was tested whether the best predictions were obtained from vision analysis of the steak or the fillet of the fish.

The colour of salmonid fish fillets has previously been inspected by several methods such as tricolour measurements [9, 10, 14], spectroscopy [15–18] and visible imaging [9, 10, 19, 20]. Recently, Dissing et al. (2011) [21] predicted natural astaxanthin concentration level in salmonid fish fillets by multi-spectral images.

The fish colour in this study was measured using three different systems: multi-spectral imaging, CIELAB point measure, and SalmonFan visual judgement.

2 Materials and Methods

2.1 Fish

A total of 45 rainbow trout (*Oncorhynchus mykiss*) were used in the study. The fish were bread in indoor tanks holding 15° Celsius and fed with EcoLife Pearl 4.5 mm fish feed pellets (BioMar A/S, Brande, Denmark). The fish were segregated intro three holding tanks, with 15 fish in each tank, for the feeding trial:

- Control: Fish fed with feed using no additional astaxanthin.
- Natural: Fish fed with feed coated with 25 ppm of natural astaxanthin.

- Synthetic: Fish fed with feed coated with 25 ppm of synthetic astaxanthin.

Each fish was uniquely marked by a micro chip. This gave the opportunity to relate all information on individual level. All fish up to the experiment was fed with non pigmented feed.

Diets were prepared exclusively for this study by a commercial feed company (BioMar A/S, Brande, Denmark). The basic pellet, EcoLife Pearl, was used in all diets. All pellets where coated with fish oil containing either 25 ppm synthetic astaxanthin (BASF SE, Germany), 25 ppm natural astaxanthin [22], or no astaxanthin added (control). The fish oil used all originated from the same batch.

When slaughtered, all 45 fish where weighed and the fork length measured. Each fish was cleaned and de-headed before cut into both a steak and fillet, see Figures 1, 2, and 3. Two biopsies, left and right, were done for each steak, see Figures 3, and 4.

After cutting, the samples were placed in plastic petri dishes (90 mm diameter) and stored on ice in Styrofoam boxes. After 30 minutes of storage the samples were measured first by multi-spectral image analysis, then Minolta measurements where conducted before evaluation with the SalmoFan Lineal. Finally, each sample was minced and subsequently frozen at $-40°$ C. After 14 days of storage the astaxanthin concentration was determined by chemical determination.

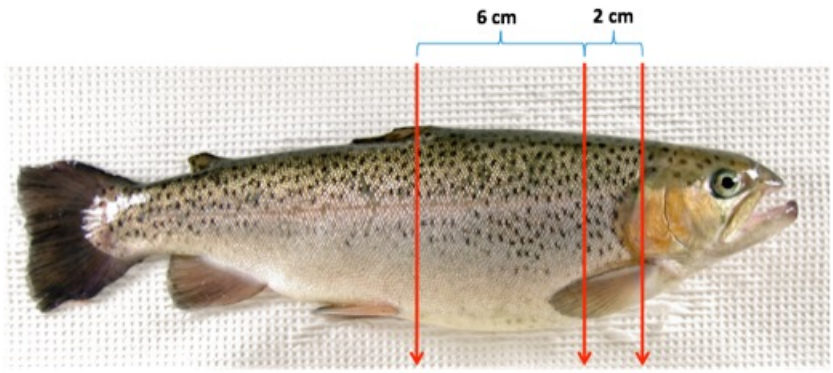

Figure 1: Overview of where the rainbow trout is cut for steak and fillet.

2.2 Methods

2.2.1 Chemical determination of astaxanthin content

Astaxanthin of the minced fillets or biopsies was determined in duplicate from the lipid extracts of the fish meat using an Agilent 1100 series high pressure liquid chromatography (HPLC) (Agilent Technologies, Palo Alto, CA), equipped with a UV diode array detector. The fillet or biopsy sample was minced, and 10

Figure 2: Example of a rainbow trout fillet.

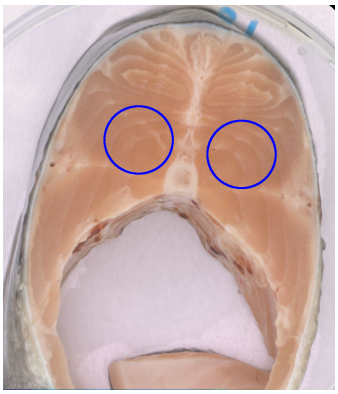

Figure 3: Example of a rainbow trout steak, with the places for the biopsies marked by blue circles.

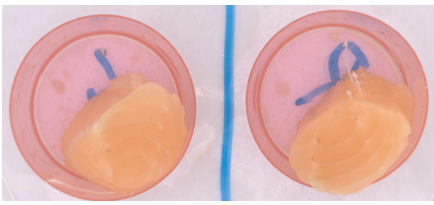

Figure 4: Example of left and right biopsies from the steak in Figure 3.

Figure 5: SalmoFan Lineal with pigmentation gradient from 20 to 34.

g in duplicates was used for extraction using chloroform and methanol according to the modified protocol of Bligh and Dyer [23]. A fraction of the lipid extract was evaporated under nitrogen and re-dissolved in 2mL of n-heptane before injection. The astaxanthin content was determined after injection of an aliquot (50 μL) of the n-heptane fraction onto a LiChrosorb Si60-5 column (100 mm $\times$ 3 mm, 5 μm) equipped with a Chromsep Silica (S2) guard column (10 mm $\times$ 2 mm; Chrompack, Middelburg, The Netherlands) and eluted with a flow of 1.3 mL/min using n-heptane/acetone (86:14, v/v) and detection at 470 nm. Concentrations of astaxanthin were calculated using authentic standards from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.2.2 SalmoFan

A SalmoFan Lineal (DSM Nutritional Products Ltd, Basel, Switzerland) pigmentation chart was used for manual inspection by three people individually. The SalmoFan has a colour gradient scale numbered 20 to 34, see Figure 5. The SalmoFan Lineal was visually compared to the fish meat and the closest match in colour intensity was decided manually. The SalmoFan Lineal is commonly used for colour quality inspection in the salmonid fish industry.

2.2.3 Tricolour Device

Tricolour point measurements were furnished using a hand-held Minolta Chroma Meter II-CR200 (Minolta Co. Ltd, Japan). The Minolta colorimeter provides controlled illumination of the sample and is commonly used for measuring the average colour of a food sample area. CIELAB values from the Chroma Meter's surface reflection measurements were used.

The CIELAB (L^*, a^*, b^*) colour space is perceptually uniform and specified by the International Commission on Illumination (CIE). L^* closely matches the lightness perceived by human vision, while a^* represents red and green, and b^* represents yellow and blue.

The CIE L^*, a^*, b^* values were determined at two locations on the fillet sample (see Figure 11) and in the centre of each biopsy.

2.2.4 Spectral Imaging

The equipment used for image acquisition is a camera and lighting system called VideometerLab (Videometer A/S, Hørsholm, Denmark) which supports a multi-spectral resolution of 20 wavelengths. These are distributed over the ultra-violet

A (UVA), visible and first near infra-red (NIR) region: 385, 430, 450, 470, 505, 565, 590, 630, 645, 660, 700, 850, 870, 890, 910, 920, 940, 950, 970, 1050 nm.

This system uses a Point Grey Scorpion SCOR-20SOM grey-scale camera and the objects of interest are placed inside an integrating sphere (Ulbricht sphere) with uniform diffuse lighting from light sources placed around the rim of the sphere. All light sources are light emitting diodes (LED) except for 1050 nm which is a diffused laser diode.

The curvature of the sphere and its white matte coating ensures a uniform diffuse light so that specular effects are avoided and likewise minimising the amount of shadows. The device is calibrated radiometrically with a following light and exposure calibration. The system is also geometrically calibrated to ensure pixel correspondence for all spectral bands [24].

The image resolution is 1280 $\times$ 960 pixels. Each file contains 20 images, one for each spectral band. In this situation one pixel represents approximately 0.072 $\times$ 0.072 millimetres. The Scorpion camera has a 12 bit analogue to digital converter (ADC), and the system used 8 bit data output from the camera. The correction for calibration gives reflectance intensity output of 32 bit precision.

The performance of the VideometerLab has previously been validated for similar surface chemistry applications [21, 25–35].

2.3 Data Acquisition

The fish fillets and biopsies were placed in petri dishes (plastic, diameter of 9 cm) and thereafter inspected using the VideometerLab, the Minolta Chroma Meter (CIELAB), and the SalmoFan Lineal. In total 45 fillet measurements were captured. For the steak, 45 CIELAB and SalmoFan Lineal measurements were performed. Moreover, for the steak biopsies (left and right) 45 multi-spectral images were captured. The measurement order of all samples was randomised for all measurement systems used in this study.

Standard red-green-blue (sRGB) colour image representations of the VideometerLab images for this paper were done by multi-spectral colour-mapping using penalised least square regression described in Dissing et al. (2010) [36].

2.4 Data Analysis

2.4.1 Pre-processing

Each multi-spectral image was normalised using standard normal variate (SNV) for each pixel. This means that the mean was subtracted from every pixel, and divided by the standard deviation of the pixel values [37]. This pre-processing was done in order to reduce the effect of difference in astaxanthin concentration levels between the three different groups since the scope of the study is to investigate if there is a colour difference between fish natural versus synthetic astaxanthin.

The region of interest (ROI) in each fillet image was segmented using the first factor of the maximum autocorrelation factor (MAF) method [38]. The

images of the biopsies were segmented manually.

After SNV the mean value of all pixels in the regions of interest was used as samples, resulting in 45 samples. The mean of left and right biopsy was used. Furthermore, nine different percentiles (1, 5, 10, 25, 50, 75, 90, 95, 99) were calculated of the SNV normalised pixels from the VideometerLab images, resulting in a total of 180 variables. With 45 samples and 180 variables this results in an ill-posed problem.

2.4.2 Model Selection and Validation

For validation and parameter calibration of the statistical models the leave-one-out cross-validation (LOOCV) method was used, were each sample is used as validation once. For LOOCV the error rate is almost unbiased for the true (expected) prediction error, but could have high variance since the training sets are so similar to each other [39].

Because of the low number of samples in the study the bootstrap re-sampling method was used for validation in some cases. In this way it was tested how the prediction generalises for different subsets of samples.

A training set of 30 samples and test set of 15 samples were defined, randomly selected so that the training set has 10 samples from each group, and the test set has 5 samples from each group. When not using LOOCV or bootstrap, the statistical models were assessed using this test and training set. The samples in the training and test sets are shown in Tables 1 and 2.

Table 1: Training set

Natural	Synthetic	Control
6	22	32
3	16	40
11	30	34
7	28	35
14	17	45
8	29	33
5	21	38
15	25	42
1	27	41
2	26	37

2.4.3 Discriminant Analysis

Statistical discriminant analysis was made in order to separate fish fed with added natural astaxanthin from synthetic astaxanthin. Methods used were linear discriminant analysis (LDA), quadratic discriminant analysis (QDA) [40], and sparse linear discriminant analysis (SLDA) [41].

Table 2: Test set

Natural	Synthetic	Control
4	19	31
13	23	43
9	18	44
10	24	36
12	20	39

SLDA was used to regularise the ill-posed problem and select the most important variables for discriminating between the groups. SLDA is using the elastic net (EN) for variable selection [42]. The EN tends to select variables that are correlated with each other. EN needs two calibration parameters: the λ_1 steers the L_1 norm for determining the number of non-zero components, and λ_2 controls the L_2 (Euclidean) norm for the regularisation. The two model parameters, the number of selected variables and λ_2, were chosen using LOOCV on 10 samples from each group, and the chosen model was then validated on 5 samples from each group.

The λ_1 parameter is steering the selection of variables and was calculated so that the number of selected variables was varied from 1 to 10. The λ_2 parameter was varied with 12 logarithmic steps from 10^{-7} to 10. The data were normalised for each calculation of the SLDA. If more than one combination of number of selected variables and λ_2 was found to give the best calibration result, then the lowest number of selected variables and the highest value of λ_2 was used, giving the least complex model.

Since the number of samples is relatively small, this procedure was then wrapped in a bootstrap of 50 iterations in order to see how stable the model was. For comparing purposes the same randomised indices for calibration and validation sets used in the bootstrap were the same for both fillet and biopsy. In this way the same fish were used for calibration and validation sets for both fillet and biopsy.

The SLDA algorithm calculates sparse discriminant components that give the best classification of the groups. The number of components is one less than the number of groups. These components are linear combinations of the selected variables.

Further more, another method for evaluating spectral bands was done by performing LDA classification on band combinations (subsets). One band at a time was tested, along with all exclusive combinations of up to six bands in an extensive test for the lowest classification error. LOOCV was used for model selection.

In order to compare LDA with subsets and SLDA we used Wilk's Λ which in principle consists of the ratio of the within group variation ($\mathbf{W}$) and the total variation ($\mathbf{T}$), i.e. the within group plus the between group variation, see Equation 1. A value of Wilk's Λ which is close to zero indicates that the groups are well separated. The band combination with the lowest value of Wilk's Λ

was chosen.

$$\Lambda = \frac{\det(\mathbf{W})}{\det(\mathbf{T})} \ . \tag{1}$$

Hotelling's T^2 test was used in order to see if the two group means of natural and synthetic astaxanthin were significantly different [43].

All image analyses and statistics were carried out using Matlab 7.9 (The Mathworks Inc., Natick, MA, USA).

3 Results

The experimental results are presented in this section, divided into three parts. Firstly, an overview of the experiment is presented. Secondly, the classification of astaxanthin type using tricolour measurement and SalmoFan inspection is reported. Thirdly, the classification of astaxanthin type using spectral imaging is shown.

3.1 Experiment Overview

The fish were weighed in the beginning and end of the feeding time period, the increase in weight can be seen in Figure 6. This shows that some fish ate much of the feed and some fish did not eat much, which also would relate to the amount of astaxanthin they have assimilated.

In the end of the experiment, after 14 days of frozen storage, the chemical content of astaxanthin was determined using HPLC analysis, see Figure 7. It can be seen that the average astaxanthin content is different between the three groups. Especially between the natural and synthetic astaxanthin group there is a large difference in average astaxanthin concentration. Here we can confirm the large variation of astaxanthin content between the fish as implied by the weight differences.

3.2 Tricolour and SalmoFan

The fish meat was analysed using a CIELAB detector, which was compared with using an ordinary SalmoFan sensor panel.

The CIELAB values can be seen in scatter plots in Figure 8. It shows that a^* and b^* show a structure for the three groups, while the groups does not seem to be separated with regards to L^* values. This means that the colour information is more important than the lightness with respect to separating natural and synthetic astaxanthin.

Mean results from the SalmoFan sensor panel can be seen in Figure 8 where a clear grouping of the three groups can be seen, especially for the biopsy measurements.

Classification of the three groups was done using LDA and QDA. Because of the relatively few samples the classification was repeated by doing a bootstrap

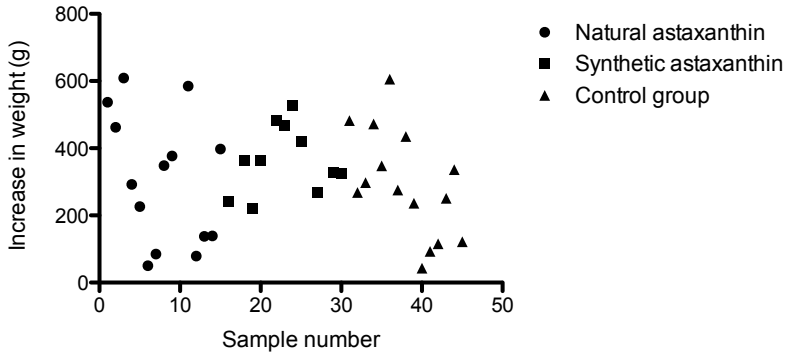

Figure 6: The increase of weight of the fish during the experiment.

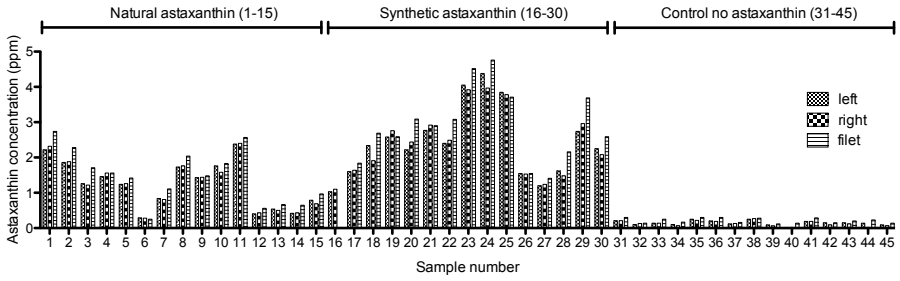

Figure 7: The astaxanthin concentration in ppm in the fillet, as well as right and left biopsy, measured by HPLC.

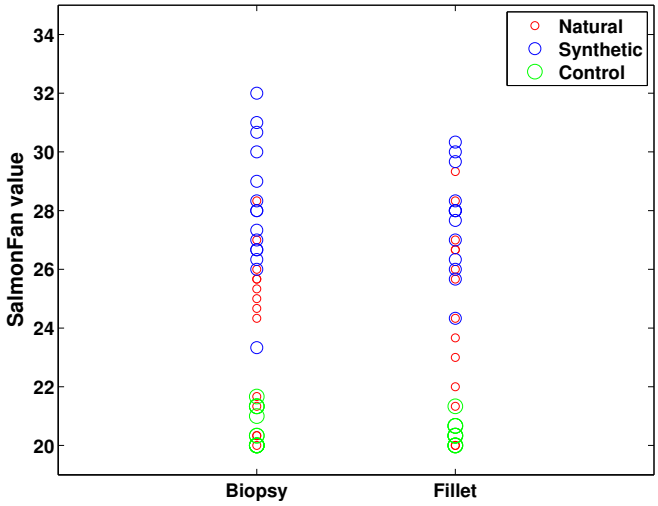

Figure 8: The SalmoFan Lineal mean values for biopsy and fillet.

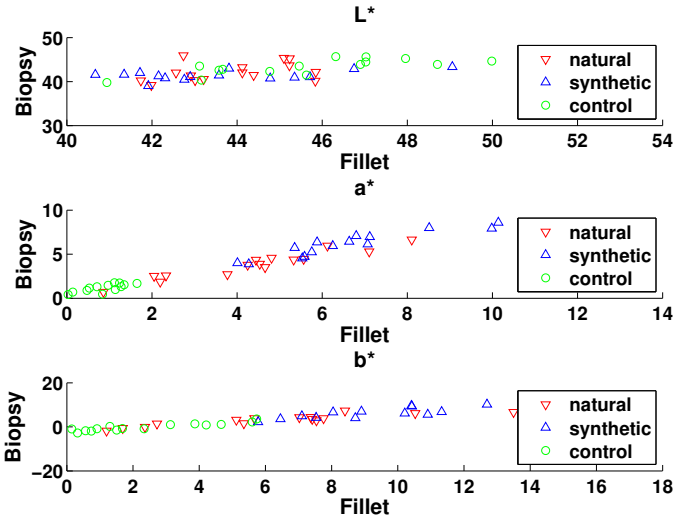

Figure 9: The CIELAB values in scatter plots for biopsy and fillet.

with randomly chosen sets for 100 iterations and calculating the mean of the classifications.

The reflection spectra of the SalmoFan individual pigmentation levels was analysed using the VideometerLab and the result can be seen in Figure 10.

The control group is classified by 92-99% by QDA. For LDA this group is classified by 99-100% for the SalmoFan data, and 96% for CIELAB.

For CIELAB the classification of natural and synthetic astaxanthin is in the range of 63-76%, while for the SalmoFan the corresponding classification is 38-82%.

Both LDA and QDA show generally better results for synthetic compared to natural astaxanthin classification for the SalmoFan data. The same tendency is not clearly seen for CIELAB values.

It shows that the classifications for steak are somewhat better than those for fillet on the CIELAB data.

Overall QDA showed about the same or better results than the LDA, therefore the QDA results are here presented and can be seen in Table 3.

To see if the length and weight influence the result we did the same bootstrap but on the residuals from a regression on length and weight. The results were similar to the ordinary bootstrap results but showed improvement for natural astaxanthin for the SalmoFan data, mostly on fillet but also for steak. For the CIELAB values QDA improved on the synthetic astaxanthin with this method, see Table 4 for the QDA results.

To see if the length, weight and astaxanthin concentration influence the result we did the same bootstrap but on the residuals from a regression on length, weight and astaxanthin concentration. The results were all (about twice as) worse than the ordinary bootstrap results (results not shown).

To see if the astaxanthin concentration alone influences the result we did the same bootstrap but on the residuals from a regression on the astaxanthin concentration. The results were all (about twice as) worse than the ordinary bootstrap results (results not shown).

(a) Confusion matrix for CIELAB steak on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.7220	0.2080	0.0700
Synthetic	0.2540	0.7440	0.0020
Control	0.0580	0.0140	0.9280

(b) Confusion matrix for CIELAB fillet on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.6520	0.2780	0.0700
Synthetic	0.3140	0.6860	0
Control	0.0420	0.0300	0.9280

(c) Confusion matrix for SalmoFan steak on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.4680	0.2240	0.3080
Synthetic	0.2180	0.7820	0
Control	0.0360	0	0.9920

(d) Confusion matrix for SalmoFan fillet on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.4820	0.3400	0.1780
Synthetic	0.1840	0.8160	0
Control	0.0520	0	0.9480

Table 3: Confusion matrices for QDA.

(a) Confusion matrix for CIELAB steak residual on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.7260	0.1280	0.1460
Synthetic	0.1880	0.8060	0.0060
Control	0.1000	0.0280	0.8720

(b) Confusion matrix for CIELAB fillet residual on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.6720	0.2340	0.0940
Synthetic	0.2580	0.7400	0.0020
Control	0.0720	0.0420	0.8860

(c) Confusion matrix for SalmoFan steak residual on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.5620	0.2040	0.2340
Synthetic	0.2180	0.7740	0.0080
Control	0.1080	0	0.9340

(d) Confusion matrix for SalmoFan fillet residual on a 100 QDA classification bootstrap

	Natural	Synthetic	Control
Natural	0.6420	0.2020	0.1560
Synthetic	0.1160	0.8840	0
Control	0.0740	0	0.9260

Table 4: Confusion matrices for QDA.

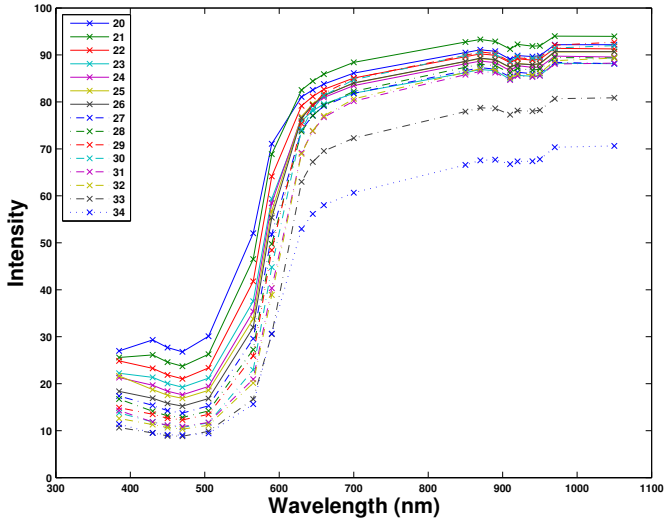

Figure 10: VideometerLab mean reflection spectra of the SalmoFan with pigmentation scale of 20-34.

3.3 Spectral Imaging

Multi-spectral images of trout fish meat were captured using the VideometerLab and segmented using CDA. An example of a segmented fillet image with the ROI visualised can be seen in Figure 11, and examples of the three different groups can be seen in Figure 12. All fish fillets can be seen in Figure 13, illustrating the group variation. The pixels in the ROI in each image were normalised using SNV and two different feature sets were extracted: mean spectra, and nine percentiles. The features were analysed using LDA, QDA, and SLDA in order to discriminate between fish meat from fish fed with synthetic astaxanthin, natural astaxanthin and no astaxanthin.

The mean sample spectra show a separation between the groups between 450 and 500 nm, see Figure 14. However, the separation is more distinct for the control group than between natural and synthetic astaxanthin.

Classification between all three groups of fish fillets and steak biopsies using LDA on the mean spectra shows that the control group is always correctly classified, both for fillet and biopsy, and both when using the train and test set as when using LOOCV, see Tables 5, 6 and 7. We therefore focus on the results and optimal variables used in order to classify between natural and synthetic astaxanthin.

Hotelling's T^2 test for separate means done on the mean spectra showed that natural and synthetic astaxanthin is not separated with good significance level. For the biopsy spectral data $p = 0.25$ and for fillet the two groups were not significantly different. However, according to Wilk's Λ the two groups should be better separated for the fillet images than for the biopsy images, as can be

Figure 11: Trout fillet image example. An sRGB representation of the multi-spectral VideometerLab image, with the segmented ROI visualised with a white outline.

Figure 12: Trout fillet images of the three different groups: Fish fed with feed using natural astaxanthin (left), synthetic astaxanthin (middle), and no additional astaxanthin (right). Here showing cropped sRGB representations of the multi-spectral VideometerLab images.

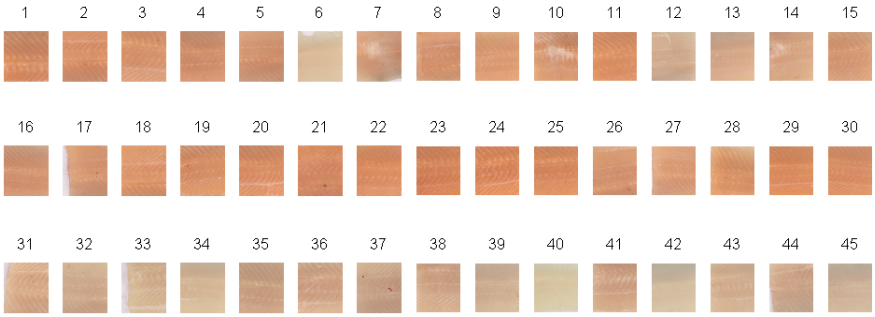

Figure 13: All trout fillets. Top row, sample 1-15: Fish fed with feed using natural astaxanthin. Middle row, sample 16-30: Fish fed with synthetic astaxanthin. Bottom row, sample 31-45: Fish fed with no additional astaxanthin (control group). Here showing cropped sRGB representations of the multi-spectral VideometerLab images.

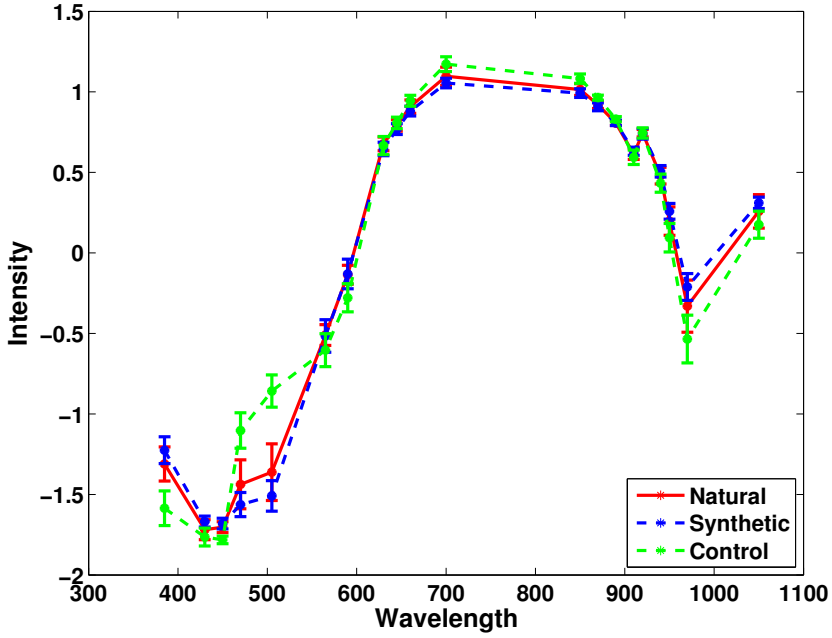

Figure 14: Mean spectra of 45 multi-spectral images of trout biopsy, with ±1 sample standard deviation for each spectral band.

seen in Table 8.

Table 5: Confusion matrix for LDA classification of all three groups using mean spectra of fillet images. Validated on the test set with 5 samples in each group.

Group	Natural	Synthetic	Control
Natural	3	2	0
Synthetic	3	2	0
Control	0	0	5

Table 6: Confusion matrix for LDA classification of all three groups using mean spectra of biopsy images. Validated on the test set with 5 samples in each group.

Group	Natural	Synthetic	Control
Natural	2	2	1
Synthetic	2	3	0
Control	0	0	5

Table 7: Classification between synthetic astaxanthin, natural astaxanthin and control group, using LDA on the mean spectra.

Type	LDA CV error	LDA Test error
Fillet	0.2667	0.3333
Biopsy	0.3111	0.3333

Classification of natural and synthetic astaxanthin using LDA on mean spectra of fillet and steak biopsy show poor results with an error larger than 50%. However, SLDA on percentiles and LDA using a subset of 6 mean spectral bands show promising results. For SLDA using percentiles, the classification is between 70% and 82%, and for LDA using 6 mean spectral bands gives 90% correct classification on fillet, and 80% on steak biopsy. Wilk's Λ show that natural and synthetic astaxanthin is better separated in the fillet images than the steak biopsy images, irrespective of mean spectra or percentiles. Classification between synthetic and natural astaxanthin, using LDA and SLDA on the mean spectra can be seen in Table 8, and the results for using SLDA on the spectra percentiles can be seen in Table 9.

The results show that it is possible to classify the type of astaxanthin that has been fed to the trout, and the best results for classification between synthetic

Table 8: Classification between synthetic and natural astaxanthin, using LDA and SLDA on the mean spectra of fillet and biopsy respectively.

Type	LDA CV error	SLDA Val. error	SLDA Val. min.	SLDA Val. std.	LDA 6 bands CV error	Wilk's Λ
Fillet	0.6667	0.2440	0	0.1232	0.1000	0.7718
Biopsy	0.5333	0.3000	0	0.1512	0.2000	0.8333

Table 9: Classification between synthetic and natural astaxanthin, using SLDA on the percentile features. Ordinary LDA is not possible on ill-posed problems.

Type	LDA CV error	SLDA Val. error	SLDA Val. min.	SLDA Val. std.	LDA 6 bands CV error	Wilk's Λ
Fillet	-	0.2160	0	0.1376	-	0.7839
Biopsy	-	0.2540	0	0.1487	-	0.8375

Table 10: Top 5 variables selected by SLDA for classification between synthetic and natural astaxanthin using the mean spectra. Frequency (Freq.) is the number of times that feature was selected in the 50 iteration bootstrap, a kind of variable importance.

Type	Freq.	Wavelength (nm)
Fillet		
	28	385
	23	700
	22	1050
	18	565
	18	590
	17	505
Biopsy		
	31	385
	26	920
	21	565
	21	890
	20	430
	18	910
	18	1050

Table 11: Top 5 variables selected by SLDA for classification between synthetic and natural astaxanthin using the percentile features. Frequency (Freq.) is the number of times that feature was selected in the 50 iteration bootstrap, a kind of variable importance. Chosen band wavelength in nanometre and the percentile of that band.

Type	Frequency	Wavelength (nm)	Percentile
Fillet			
	17	700	99
	15	385	1
	9	1050	1
	8	590	25
	7	385	25
	7	630	99
Biopsy			
	17	385	1
	11	385	95
	11	890	1
	8	385	90
	8	630	99
	7	430	99
	7	920	90
	6	385	75
	6	385	99

and natural astaxanthin is achieved by SLDA on percentiles and LDA using a subset of 6 bands. It seems as fillet is better than biopsy for classifying between synthetic and natural astaxanthin.

The wavelength most often chosen in the bootstrap generally for all tests is the UVA band 385 nm. For fillet the band of 700 nm is also highly important. The most often selected variables in the form of mean of spectral bands are shown in Table 10. The most often selected variables in the form of percentiles of spectral bands can be seen in Table 11.

To summarise, the results show that the control group, which was not fed with astaxanthin, is quite easy to separate from the two astaxanthin groups, while it is more difficult to separate the natural and synthetic groups, as can be seen in Figure 15.

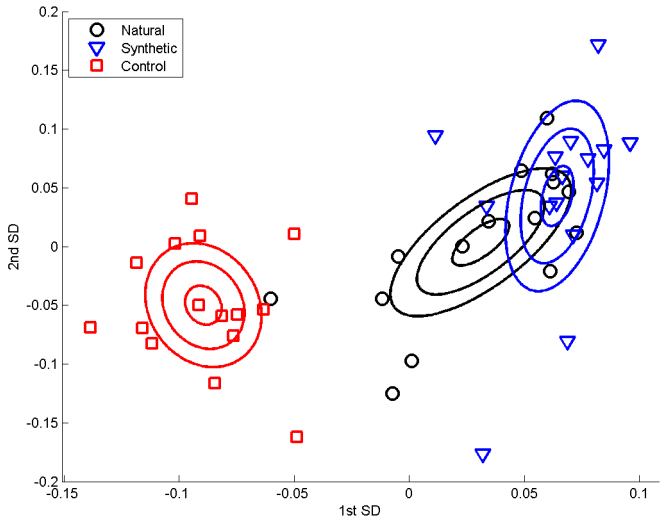

Figure 15: The biopsy of salmonid fish fed with natural astaxanthin, synthetic astaxanthin, and no astaxanthin (control group). The samples are plotted using the two sparse discriminant components from SLDA, and estimated normal distribution contours are visualised for each group.

4 Discussion

Previous studies of astaxanthin [44–46] found distinguished absorbance peaks of astaxanthin around 450 – 505 nm and secondary peaks around 500 – 600 nm for various solvents, as well as around 870 nm. The lowest maximum found in petroleum ether (467-470 nm) and highest in carbon disulphide (502-505 nm). However, the spectral response of astaxanthin in fish meat is different from that

of astaxanthin in oil due to how the astaxanthin is bind in the flesh. This means that the prediction model of astaxanthin in fish meat would be different from the prediction model for astaxanthin solved in oil.

In Dissing et al. (2011) [21] the concentration level of natural astaxanthin in fish fillet was highly correlated with the largest independent variance component in the multi-spectral image data. This means that the astaxanthin concentration is highly dependent on the overall image intensity.

With relatively few samples and large variation within the groups with regards to astaxanthin content this classification is challenging. Normalisation using SNV on each pixel was used in order to reduce the effect of different concentration level between the groups. However, it is hard to reduce the difference completely. We cannot exclude the cause of concentration level completely in the results. An apparent overlap of synthetic and natural astaxanthin groups can be seen in the presented scatter plots (see e.g. Figure 15), and it is possible that the classification is distinguishing the groups dependent on concentration level. However, when compensating for the concentration difference by using regression residuals in the classification the results were still not improved.

Furthermore, it has previously been shown that measuring the surface colour of a non-homogeneous object, such as meat, using a colorimeter such as the Minolta Chroma Meter usually gives erroneous colour results [9, 47]. Often a grey or purplish colour is reported, which is due to the light penetrating the sample and scattering inside the object, by the light coming from the colorimeter's illumination being close to the surface [47]. Colorimeters also only samples specific points on the surface. In comparison, imaging techniques usually have diffuse illumination and gives a more clear surface colour as a result. Imaging techniques therefore has the advantage for monitoring the entire surface of a non-uniform food sample capturing both shape and colour, including surface variations and producing a permanent picture reference.

5 Conclusions

The results show that it is easy to separate natural and synthetic astaxanthin from the control group using multi-spectral image analysis, tricolour analysis and SalmoFan analysis. However, it seems to be a more challenging task to separate natural astaxanthin and synthetic astaxanthin. Natural and synthetic astaxanthin show an overlap in spectral reflection, tricolour values, and SalmoFan values.

Using tricolour CIELAB measurements it shows that the classification of natural and synthetic astaxanthin is slightly better using the steak than the fillet.

For discriminating between fish fed with natural and synthetic astaxanthin the CIELAB measurements show better performance than the SalmoFan values.

Using spectral imaging, the results show that fillet is better than steak for classifying between synthetic and natural astaxanthin.

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References

[1] T. R. Gormley. A note on consumer preference of smoked salmon colour. *Irish Journal of Agricultural and Food Research*, 31(2):199–202, 1992.

[2] J. Ostrander, C. Martinsen, J. Liston, and J. McCullough. Sensory testing of pen-reared salmon and trout. *Journal of Food Science*, 41(2):386–390, 1976.

[3] S. Sigurgisladottir, C.C. Parrish, S.P. Lall, and R.G. Ackman. Effects of feeding natural tocopherols and astaxanthin on atlantic salmon (salmo salar) fillet quality. *Food Research International*, 27(1):23–32, 1994.

[4] Stewart Anderson. Salmon color and the consumer. In *Microbehavior and Macroresults: Proceedings of the Tenth Biennial Conference of the International Institute of Fisheries Economics and Trade Presentations*, July 2000.

[5] Artemis P. Simopoulos. Omega-3 fatty acids in health and disease and in growth and development. *American Journal of Clinical Nutrition*, 54:438–463, 1991.

[6] O.J. Torrisen, R.W. Hardy, and K.D. Shearer. Pigmentation of salmonids - carotenoid deposition and metabolism. *Reviews in Aquatic Sciences*, 1(2):209–225, 1989.

[7] U. Erikson and E. Misimi. Atlantic salmon skin and fillet color changes effected by perimortem handling stress, rigor mortis, and ice storage. *Journal of Food Science*, 73(2):C50–C59, 2008.

[8] Fanbin Kong, Juming Tang, B. Rasco, C. Crapo, and S. Smiley. Quality changes of salmon (oncorhynchus gorbuscha) muscle during thermal processing. *Journal of Food Science*, 72(2):S103–S111, 2007.

[9] Yavuz Yagiz, Murat O Balaban, Hordur G Kristinsson, Bruce A Welt, and Maurice R Marshall. Comparison of minolta colorimeter and machine vision system in measuring colour of irradiated atlantic salmon. *Journal of the Science of Food and Agriculture*, 89(4):728–730, 2009.

[10] Yavuz Yagiz, Hordur G. Kristinsson, Murat O. Balaban, Bruce A. Welt, Maria Ralat, and Maruice R. Marshall. Effect of high pressure processing

and cooking treatment on the quality of atlantic salmon. *Food Chemistry*, 116(4):828–835, 2009.

[11] B. Bjerkeng, M. Peisker, K. von Schwartzenberg, T. Ytrestoyl, and T. Asgard. Digestibility and muscle retention of astaxanthin in atlantic salmon, salmo salar, fed diets with the red yeast phaffia rhodozyma in comparison with synthetic formulated astaxanthin. *Aquaculture*, 269(1-4):476–489, 2007.

[12] John N. C. Whyte and Kathleen L. Sherry. Pigmentation and composition of flesh of atlantic salmon fed diets supplemented with the yeast phaffia rhodozyma. *North American Journal of Aquaculture*, 63(1):52–57, 2001.

[13] S. A. Turujman, W. G. Wamer, Rong Rong Wei, and R. H. Albert. Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. *Journal of AOAC International*, 80(3):622–632, 1997.

[14] R. Christiansen, G. Struksnaes, R. Estermann, and O. J. Torrissen. Assessment of flesh colour in atlantic salmon, salmo salar l. *Aquaculture Research*, 26(5):311–321, 1995.

[15] Claus Borggaard, Lars Bager Christensen, Knut Erik Gulbrandsen, and Allan J. Rasmussen. Method and apparatus for determining quality properties of fish, November 2003.

[16] Are Folkestad, Jens Petter Wold, Kjell-Arne Rørvik, Jon Tschudi, Karl Henrik Haugholt, Kari Kolstad, and Turid Mørkøre. Rapid and non-invasive measurements of fat and pigment concentrations in live and slaughtered atlantic salmon (salmo salar l.). *Aquaculture*, 280(1-4):129–135, 2008.

[17] Silje Ottestad, Oddvin Sørheim, Karsten Heia, Josefine Skaret, and Jens Petter Wold. Effects of storage atmosphere and heme state on the color and visible reflectance spectra of salmon (salmo salar) fillets. *Journal of Agricultural and Food Chemistry*, 59(14):7825–7831, 2011.

[18] Jens Petter Wold, Brian J. Marquardt, Brian K. Dable, Dave Robb, and Bjarne Hatlen. Rapid quantification of carotenoids and fat in atlantic salmon (salmo salar l.) by raman spectroscopy and chemometrics. *Applied Spectroscopy*, 58(4):395–403, 2004.

[19] Lars Helge Stien, Fredrik Manne, Kari Ruohonene, Antti Kause, Krisna Rungruangsak-Torrissen, and Anders Kiessling. Automated image analysis as a tool to quantify the colour and composition of rainbow trout (oncorhynchus mykiss w.) cutlets. *Aquaculture*, 261(2):695–705, 2006.

[20] E Misimi, J R Mathiassen, and U Erikson. Computer vision-based sorting of atlantic salmon (salmo salar) fillets according to their color level. *Journal of Food Science*, 72(1):S030, 2007.

[21] Bjørn S. Dissing, Michael E. Nielsen, Bjarne K. Ersbøll, and Stina Frosch. Multispectral imaging for determination of astaxanthin concentration in salmonids. *PLoS One*, 6(5):Article No.: e19032, 2011.

[22] M. E. Nielsen, H. Mikkelsen, L. B. Nielsen, and O. Joensen. By-product based production of natural astaxanthin (nax). *7th Joint meeting: 50th Annual Atlantic Fisheries Technology Conference and 29th Annual Seafood Science and Technology Society of the Americas*, 2005.

[23] EG Bligh and WJ Dyer. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8):911–917, 1959.

[24] Jørgen Folm-Hansen. *On chromatic and geometrical calibration.* PhD thesis, Technical University of Denmark, 1999.

[25] Line H. Clemmensen, Michael Hansen, and Bjarne K. Ersbøll. A comparison of dimension reduction methods with application to multi-spectral images of sand used in concrete. *Machine Vision and Applications*, 21:959–968, 2010.

[26] Line H. Clemmensen, Michael E. Hansen, Jens C. Frisvad, and Bjarne K. Ersbøll. A method for comparison of growth media in objective identification of penicillium based on multi-spectral imaging. *Journal of Microbiological Methods*, 69(2):249–255, 2007.

[27] Line Katrine Harder Clemmensen and Bjarne Kjær Ersbøll. Multispectral recordings and analysis of psoriasis lesions. *MICCAI 06 - Workshop on Biophotonics Imaging for Diagnostics and Treatment, October 6, 2006 proceedings, 9th MICCAI Conference*, 2006.

[28] Bjørn Skovlund Dissing, Bjarne Kjær Ersbøll, and Jens Adler-Nissen. *New vision technology for multidimensional quality monitoring of food processes.* PhD thesis, Technical University of Denmark (DTU), 2011.

[29] Bjørn Skovlund Dissing, Olga S Papadopoulou, Chrysoula Tassou, Bjarne Kjær Ersbøll, Jens Michael Carstensen, Efstathios Z Panagou, and George-John Nychas. Using multispectral imaging for spoilage detection of pork meat. *Food and Bioprocess Technology*, pages 1–12, 2012.

[30] David Delgado Gomez, Line Harder Clemmensen, Bjarne K. Ersbøll, and Jens Michael Carstensen. Precise acquisition and unsupervised segmentation of multi-spectral images. *Computer Vision and Image Understanding*, 106(2-3):183–193, 2007.

[31] Michael Edberg Hansen, Bjarne Kjær Ersbøll, Jens Michael Carstensen, and Allan Aasbjerg Nielsen. Estimation of critical parameters in concrete production using multispectral vision technology. *Lecture Notes in Computer Science*, pages 1228–1237, 2005.

[32] Martin Georg Ljungqvist, Bjarne Kjær Ersbøll, Michael Engelbrecht Nielsen, and Stina Frosch. Multispectral image analysis for astaxanthin coating classification. *Journal of Imaging Science and Technology*, 56(2):020403–1 – 020403–6, 2012.

[33] Martin Georg Ljungqvist, Stina Frosch, Michael Engelbrecht Nielsen, and Bjarne Kjær Ersbøll. Multispectral image analysis for robust prediction of astaxanthin coating. *Applied Spectroscopy*, 67(7), July 2013. Accepted for publication.

[34] Martin Georg Ljungqvist, Ken-ichi Kobayashi, Stina Frosch, Michael Engelbrecht Nielsen, Bjarne Kjær Ersbøll, and Shigeki Nakauchi. Near-infrared hyper-spectral image analysis of astaxanthin concentration in fish feed coating. *Proceedings of the IEEE International Conference on Imaging Systems and Techniques*, pages 136–141, July 2012.

[35] Martin Georg Ljungqvist, Otto Højager Nielsen, Stina Frosch, Michael Engelbrecht Nielsen, Line Harder Clemmensen, and Bjarne Kjær Ersbøll. Hyper-spectral imaging based on diffused laser light for prediction of astaxanthin coating concentration. *Machine Vision and Applications*, 2013. Accepted for publication.

[36] Bjørn S. Dissing, Jens M. Carstensen, and Rasmus Larsen. Multispectral colormapping using penalized least square regression. *Journal of Imaging Science and Technology*, 54(3):0304011–0304016, 2010.

[37] Asmund Rinnan, Frans van den Berg, and Søren Balling Engelsen. Review of the most common pre-processing techniques for near-infrared spectra. *TrAC Trends in Analytical Chemistry*, 28(10):1201–1222, 2009.

[38] Paul Switzer and A. A. Green. Min/max autocorrelation factors for multivariate spatial imagery. Technical Report 6, Stanford University, Department of Statistics, 1984.

[39] Trevor Hastie, Robert Tibshirani, and Jerome Friedman. *The Elements of Statistical Learning: Data Mining, Inference, and Prediction*. Springer, 2nd edition, February 2009.

[40] Bjarne K. Ersbøll and Knut Conradsen. *An Introduction to Statistics, vol. 2*. DTU Informatics, 7 edition, 2007.

[41] Line Clemmensen, Trevor Hastie, Daniela Witten, and Bjarne Ersbøll. Sparse discriminant analysis. *Technometrics*, 53(4):406–413, November 2011.

[42] H Zou and T Hastie. Regularization and variable selection via the elastic net. *Journal Of The Royal Statistical Society Series B - Statistical Methodology*, 67:301–320, 2005.

[43] Harold Hotelling. Relations between two sets of variates. *Biometrika Trust*, 28(3/4):321–377, December 1936.

[44] Sergiu Amarie, Ute Förster, Nina Gildenhoff, Andreas Dreuw, and Josef Wachtveitl. Excited state dynamics of the astaxanthin radical cation. *Chemical Physics*, 373(1-2):8–14, 2010.

[45] Manuel Buchwald and William P. Jencks. Optical properties of astaxanthin solutions and aggregates. *Biochemistry*, 7(2):834–843, 1968.

[46] Jian-Ping Yuan and Feng Chen. Identification of astaxanthin isomers in haematococcus lacustris by hplc-photodiode array detection. *Biotechnology Techniques*, 11(7):455–459, 1997.

[47] Antonio Girolami, Fabio Napolitano, Daniela Faraone, and Ada Braghieri. Measurement of meat color using a computer vision system. *Meat Science*, 93(1):111–118, 2013.