Master Thesis in Applied Biochemistry for Engineers

Comparison between Calibrations of Fish Oil and AkoFeed Examined with Near-Infrared Light for Use in Animal Feed

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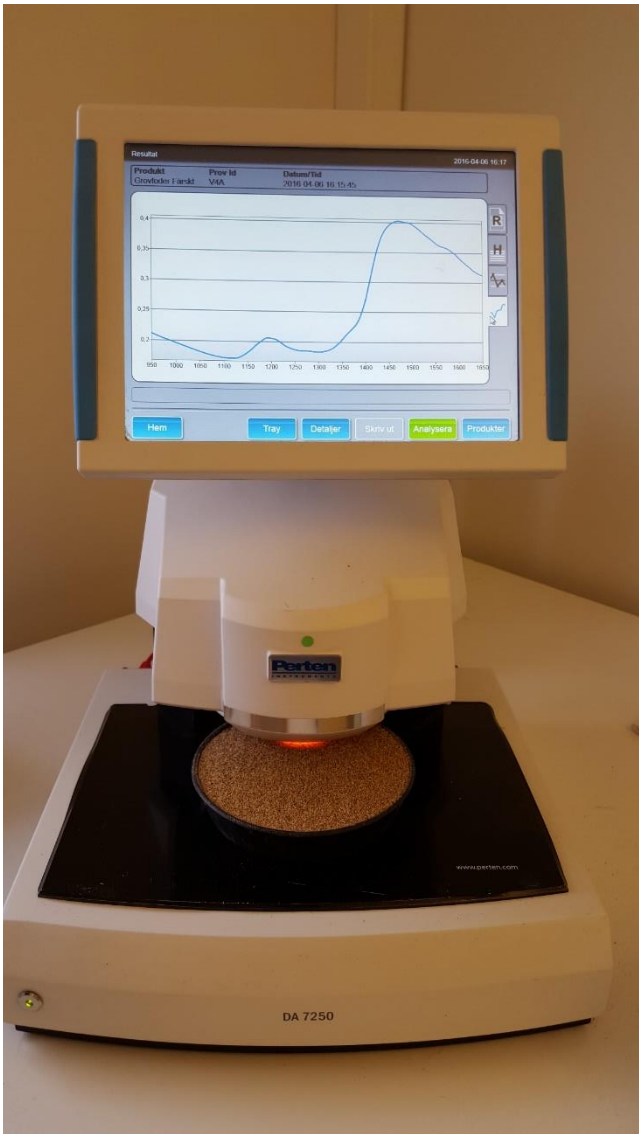

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För att skörda framgång

Abstract

The aim with this master thesis was to investigate if a certain sow feed requires different calibrations in *near-infrared analysis*, due to if the sow feed have fish oil or AkoFeed as fat source.

To investigate this, a basic sow feed mix was prepared and divided in 60 jars with 250 g in each. In 30 of the jars 0 to 15 g fish oil were added and in the other 30 jars 0 to 21 g AkoFeed were added. 2/3 of the jars were used for calibration and 1/3 were used as external validation sets. Then, all 60 samples were analyzed in NIR and spectra from 950 nm to 1650 nm were obtained. Additionally, the content of the nutrients water, protein, ash, fat and crude fiber were analyzed in the laboratory for some of the samples and calculated for the rest of the samples.

The calibration method PLS was used and three calibrations were made for the samples containing fish oil, AkoFeed and a combination of both. To investigate if there were any difference between the calibrations, they were validated by comparisons of the error values R^2 and RMSEP. However, the calibration of the fish oil indicated to be better than the calibrations of AkoFeed and the combination. Therefore, better predicted values of unknown samples of the sow feed containing fish oil would be obtained if the calibrations are separate, while it does not matter for the predicted values of the sow feed containing AkoFeed.

Key Words: Fish oil, AkoFeed Standard, Sow Feed, SNV, NIR, Calibration, PLS, Validation, RMSEP, R^2

Sammanfattning

Syftet med denna master uppsats var att undersöka om ett visst suggfoder kräver olika kalibreringar vid *nära infraröd analys*, beroende på om suggfodret innehåller fiskolja eller AkoFeed som fettkälla.

För att undersöka detta gjordes en basfodermix som delades upp på 60 burkar med 250 g basfodermix i varje burk. I 30 av burkarna tillsattes fiskolja med ett intervall på 0 till 15 g och i de andra 30 burkarna tillsattes 0- till 21 g AkoFeed. För kalibreringarna användes 2/3 av burkarna och för valideringen användes ett externt testset med 1/3 av burkarna. Alla 60 proverna analyserades i NIR och spektra från 950 nm till 1650 nm erhölls. Därefter analyserades näringsinnehållet för vatten, protein, aska, fett och växttråd i ett laboratorium för några av proverna för att sedan näringsinnehållet i resten av proverna skulle kunna beräknas.

Tre kalibreringar gjordes för proverna innehållande fiskolja, AkoFeed samt för kombination av dem båda, genom att använda kalibreringsmetoden PLS. För att undersöka om där fanns någon skillnad mellan kalibreringarna, validerades dem genom att felvärdena R^2 and RMSEP beräknades och jämfördes. Kalibreringen för fiskoljan antydde på att vara bättre än kalibreringarna för AkoFeed och kombinationen. Därför skulle troligtvis bättre predikterade värden för okända prover kunna erhållas för suggfoder som innehåller fiskolja om kalibreringarna görs separata, medan resultatet av de predikterade värdena inte skiljer sig märkbart för suggfoder innehållande AkoFeed, oavsett om kalibreringarna är separata eller kombinerade.

Nyckelord: Fiskolja, AkoFeed Standard, Suggfoder, SNV, NIR, Kalibrering, PLS, Validering, RMSEP, R^2

Preface

During the courses in biochemistry and analytic chemistry at Lunds Tekniska Högskola, I have discovered the charm with measuring nutrients by using different wavelengths in analytical instruments. Together with my animal interest that I have had since I was a kid, I have found how interesting it is to work with animal feed analysis.

This master thesis project was performed during the spring and summer of 2016 at the department Applied Biochemistry at Lund University and at the company Svenska Foder AB. The practical work took place at Optilab in Lidköping, which is the laboratory of Svenska Foder AB.

I want to thank Svenska Foder AB for the opportunity to do my master thesis at the company. I also want to thank my supervisor Magnus Semmelhofer at the company for supporting the master thesis and for organizing an apartment to me when I lived in Lidköping. Also, I want to thank the manager Kerstin Ramberg and the laboratory manager of Optilab Ulf Thorpert for great knowledge, support and for guiding me through this master thesis.

Finally, I want to thank my supervisor Johan Bonde Svensson and my examiner Leif Bülow at Applied Biochemistry for your support and supervision during the entire master thesis.

Table of contents

1. Introduction

1.1 Analysis of fish oil and AkoFeed Standard in animal feed

The quality of animal feed in the agriculture community is directly related to the health and quality of the animals. To ensure the quality of the animal feed it is important to control the quality of both the raw material in the production and the final product of animal feed. To obtain the nutrients in the animal feed, they have to be analyzed. To avoid the classical methods in the laboratory, which are both expensive and time consuming, predicted nutrient values can be obtained by using near-infrared (NIR) light. (Ren, 2009)

Near-infrared light contains wavelengths between 780 nm to 2500 nm, which is longer wavelengths than the visible light of human (Impublications, 2016). The advantages with NIR are especially that it is rapid, the feed is not destroyed and the analyzed samples can then be used to future analysis (Ren, 2009). However, if the NIR is to be able to give precisely predicted nutrient values, it has to be calibrated with similarly known feed samples (Ren, 2009).

It is important that the animals get all the nutrients they need in the feed to live and grow (Isberg, 2011). One of the most necessary nutrients are the fatty acids (Isberg, 2011). Usually, a kind of fat called AkoFeed Standard is used in animal products (AAK, 2016). AkoFeed is a vegetable liquid fat containing fatty acids from palm, rapeseed, sunflower, soy and shea oil (AAK, 2016). However, for lactating sows, it has been demonstrated that fish oil as fat source in the feed has several positive properties (Eastwood, 2012). For example, it has been shown that more of the piglets are born alive and fish oil improve pre-weaning survival, the piglets get a better immune system and they grow better (Eastwood, 2012).

Svenska Foder AB is a company producing and selling feed for a wide range of different kind of animals with different kind of requirements (Svenska Foder, 2016). Svenska Foder AB usually used AkoFeed Standard as fat source in most feed types. However, they used fish oil (instead of AkoFeed) in some of the feed aimed at sows.

Everything that are produced in the manufacturing will be analyzed on a NIR machine before the feed will be disclosed to the customer. As said previously, for a NIR machine to be able to analyze samples and give reliable nutrient values, it needs to be calibrated. A problem Svenska Foder AB has had, is that the analyzing of sow feed containing AkoFeed have worked well on the NIR machine but analysis of sow feed containing fish oil have not. When the sow feed containing fish oil thereafter are analysis in the laboratory, it has been shown that the nutrient values not differ from the expected values. It means that the NIR machine give incorrect information, which give the consequence that the staff not take the incorrect nutrient values seriously.

This problem has probably occurred due to the calibration on the NIR is constructed with samples containing AkoFeed and probably just a few number of samples containing fish oil. Speculation begun to grow about if samples containing fish oil or AkoFeed should be divided in two different calibrations on the NIR.

1.1.1 The aim

The aim with this master thesis is to investigate if different kinds of fat (limited to fish oil and AkoFeed) in sow feed influences on the calibration when using NIR. I.e. if it does matter if sow feed containing fish oil or AkoFeed have been constructed in the same calibration or if it is substantially better to have two separate calibrations. To investigate the problem, two identical sow feeds would be produced, in one of them would fish oil be added and in the other one would AkoFeed be added. Thereafter, both of them would be analyzed in the laboratory to obtain the nutrient value for water, protein, ash, fat and crude fiber, and on the NIR *DA 7250* to obtain the spectra. Then, Matlab *R2015b* would be used to make three different calibrations: two separate and one combined. The calibrations would then be validated to investigate how well they determine new predicted values on unknown sow feed samples.

1.1.2 Limitations

To be able to perform this master thesis during a reasonable time period, several limitations were required. Five usual nutrients of sow feed were chosen to be analyzed: content of water, protein, ash, fat and crude fiber. Of these nutrients, only a few number of samples were analyzed in the laboratory. Since the recipe were known, the nutrients of the rest of the samples were calculated.

One recipe of sow feed, that is usually used as sow feed in Svenska Foder AB, was used as the base mixture. Consequently, all the samples get the same content except the amount and kind of added fat. The reason to why the content of nutrients was chosen to be the same in all the samples was to be sure that eventual differences in the calibrations would be depending on the kind of added fat and nothing else.

To make the validations statistical right, it needs at least 20 samples according to ISO 12099:2010(E) (ISO12099:2010(E), 2010). Due to the limited of time, only ten samples were used to validate the two separate calibrations. Additionally, since the base mixture is identical for all the samples, the variation of samples in the calibrations are very small.

1.2 Nutrients in animal feed

A carefully estimated amount of nutrients in animal feed are necessary if the animals should be able to live a balanced and healthy life (Algae for feed, 2016). This is especially important for pregnant and lactating animals (Isberg, 2011). Depending on which species the animal belongs to and thus what digestive system the animals have, different nutrients are necessary to be able to live a qualitative life (Algae for feed, 2016). Consequently, depending on the requirement of nutrients for different kind of animals, the feed consists different raw materials and additives and are made as in either pellets or crumbles (Algae for feed, 2016).

The nutrients in the animal feed have to satisfy the fundamental needs in the animal body, such as gaining energy, a correct balance of water, the function of nerves and muscles and to build and maintain the body (Algae for feed, 2016). To determine the amount of the different nutrients in the animal feed, laboratory analysis is usually used (UGA Extension, 2016). Some of the most essential nutrients are carbohydrate, fat, fiber, protein, vitamins and minerals (Algae for feed, 2016). In this master thesis protein, ash, fat and crude fiber has been chosen to be analyzed, since these nutrients were deemed to involve most of the essential nutrients in sow feed. Additionally, water content was analyzed since this was necessary to be able to calculate the content of the analyzed nutrients in the feed (UGA Extension, 2016). With water content means the loss of weight that arise by drying of a sample (UGA Extension, 2016).

The nutrients protein, ash and crude fiber are all essential for the function of the animal organs (Algae for feed, 2016). Proteins have important roles such as building tissues of the body, form enzymes and hormones and are also needed for metabolism (Algae for feed, 2016). The content of protein is usually measured in a laboratory by determining of the amount of nitrogen in the samples (UGA Extension, 2016). Since proteins in feed, that originate from leaf and stem tissue, generally consists of 16 % nitrogen, the amount of nitrogen is multiplied with a factor of 6.25 to get the correct amount of protein (UGA Extension, 2016). However, it is necessary to be careful. If some kind of feed contains a larger amount of urea or ammonia the results of amount of protein can be misleading when using this analysis technology (UGA Extension, 2016). Ash is the inorganic minerals in the feed and is the solid residue after combustion of the samples at 550 °C (UGA Extension, 2016). Inorganic minerals include for instance: Calcium, Phosphorus, Magnesium, Sulphur, Sodium, Potassium, Iron and Iodine, which represents almost four percent of the animal body (UGA Extension, 2016). Crude fiber is the residues after washing with acid, base and acetone, following by subtracting the amount of ash. Crude fiber consists mainly of cellulose and lignin, which are indigestible carbohydrates (Algae for feed, 2016).

Fat is the part of a sample that can be extracted out during an extraction process with petroleum ether. The main functions of fat are as energy source, a carrier of fat-soluble vitamins and its insulating properties (Algae for feed, 2016). In addition to the amount of fat from common raw materials, there are several kind of fat that can be added in animal feed. (UGA Extension, 2016). In this master thesis, it has been focused on two kinds of fat sources: fish oil and AkoFeed in sow feed, which is presented in Figure 1. Notably, fish oil consists of the two fatty acids: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are believed to be responsible for many of the demonstrated health effects in sows (Eastwood, 2012). The fish oil that has been used in this master thesis, comes from

Figure 1. Two jars consisting of fish oil to the left and AkoFeed Standard to the right.

the manufacturing of omega-3. It is raw fish oil that has been exposed to several refining step. The AkoFeed is, as mention before, a commonly used fat for animal products, which is a mixture of several vegetable liquids fats (AAK, 2016).

Hence, a basic description of the laboratory measurements of the content of water, protein, ash, fat and crude fiber used in this master thesis follows. The descriptions of measurement of the nutrients are referred to five SOP (one for each measurement) from Svenska Foder AB, when nothing else is specified.

To measure the content of water in the samples, the samples are dried in an oven at 103 °C for four hours. Followed by cooling in a desiccator and thereafter the samples are weighed.

The protein content was measured by using Dumas method. The ground sample was pelleted in nitrogen free foil with a pellet press and put into an oven at 900 °C. The pellet press was used to press out air from the sample to avoid contamination of nitrogen. Oxygen gas is added in the oven to allow for a quick combustion of the sample. This combustion is performed in the primary part of the oven, while sulfur dioxide (released by the combustion) is absorbed by calcium oxide in the secondary part of the oven. This is a necessary process to prevent water steam from reacting with the sulfur dioxide and form sulfuric acid. The rest of the gases pass a dust trap of quartz wool where larger particles from the combustion stays. Then the gas residuals travel further to a cool trap where substances with higher condensation temperatures are condensed to liquid. The substances that still are in a gas phase are collected in a four-liter jar of ballast where the gases are mixed to a homogenous mixture. Then, three milliliter of the gas are further transferred by helium to an oven at 750 °C where oxygen is absorbed and NO_x is reduced to nitrogen gas. Finally, carbon dioxide is absorbed by Lecosorb (consisting of Sodium Hydroxide on silicate carrier) and water steam is absorbed by anhydrone (consisting of the drying agents: Magnesium Perchlorate). The amount of nitrogen gas is analyzed by using a thermal conductivity detector.

Determination of ash content in the samples are made by combustion of the samples in an oven at 550 °C overnight. In the morning, the residue of the samples is taken out from the oven and is cooled in a desiccator before they are weighed.

Measurement of fat content in animal feed is made by hydrolysis followed by extraction. First, a small amount of the sample is weighed together with celite, whose function it is to prevent the sample from clogging the filter. In the hydrolyze step, the sample is boiled in hydrochloric acid to make the fats available for extraction both chemically and mechanically. Thus, the hydrochloric acid breaks up fatty acids, lipid-carbohydrate bonds and disrupts cell walls to make the lipids available for the next step: extraction by added petroleum ether (Medallion Labs, 2016). Before extraction glasschrot is added to the extraction beakers to provide more rotation of the petroleum. The petroleum ether dissolves fats, oils and other fat soluble substances, which are extracted out of the filter. The petroleum ether is recycled by capture after evaporation. Finally, the amount of fat is determined by drying the extraction beakers, which thereafter are weighed.

To determine the amount of crude fiber in a sample, the sample is boiled in sulfuric acid and thereafter in potassium hydroxide. The sulfuric acid is used for the extraction of starch and sugars, the potassium hydroxide is used for the removal of protein (Möller, 2014). In the end, acetone is added to remove residues of fat. What remains are crude fiber and ash. The crude fiber is removed by combustion it in 550 °C and the difference in weight could be calculated.

1.3 Understanding NIR technology

The NIR energy range was discovered in the 19th century by the astronomer and musician William Herschel. Sir Herschel wanted to find out which of the visible colors from the white sunlight that caused increased temperature in substances which become exposed to it. He finds out that the red light, which wavelengths are in the end of the interval of visible light, cause the increase in temperature. To investigate if it only was the red light that influences the increased temperature, sir Herschel tested to move the thermometer even further away and found that the temperature still increased. The near-infrared light was discovered. (Pasquini, 2003)

Then, it took until the 1980s before the research of NIR expanded since then NIR has been very useful for quantitative analysis, especially in the area of agriculture (Impublications, 2016). The agriculture was among the earliest to intensively use NIR spectroscopy (Pasquini, 2003). Some of the attractiveness with NIR are the quick answers and the little or no sample preparation needed before analysis (Impublications, 2016). One other advantage is the large amount of nutrient values that can be obtained from one NIR spectrum (Impublications, 2016). A disadvantage with using NIR technology is that it is necessary to perform a calibration, which requires a large number of samples with known nutrient content (Impublications, 2016). Except the usage of NIR in the agriculture area, NIR is used for food products, in the fuel industry, in the environmental sector and a large number of other areas (Impublications, 2016).

Infrared light has the wavelength interval above the visible light, from 780 nm to 1 mm, while the near-infrared light only is a small part of the infrared light, between 780 nm and 2500 nm (ICNIRP, 2016). These wavelength intervals are presented in the electromagnetic spectrum in Figure 2. NIR is very useful in quantitative analysis since the reflection light can detect vibrational overtones and combinations of several organic compounds (Impublications, 2016).

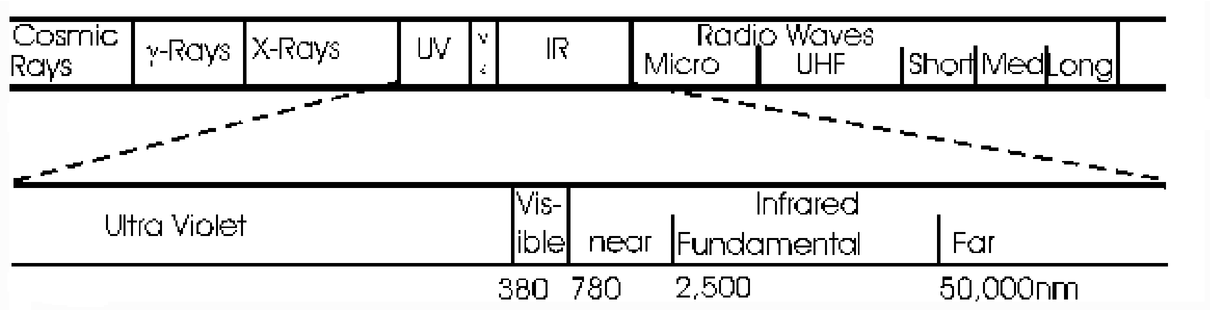


Figure 2. The interval of visible light is between 380 nm and 780 nm while near-infrared light is over 780 nm up to 2500 nm. (Impublications, 2016)

In NIR analysis the quantitative value of the nutrients is analyzed by reflected energy. The reflected energy is obtained when the sample is exposed to near-infrared light and absorbs the energy, which in turn causes vibrations between atoms such as O-H, C-H, N-H bonds in the molecules. These vibrations consist of stretches and bends in the bonds between the atoms, which are analyzable. Since different kind of bonds give different kind of vibrations, due to the strength of the chemical bonds, which make the vibration of each molecule unique. A molecule that consist only two atoms, can just emit vibration as stretching, which may depend on the stiff double bonds. If the molecule consists of three or more atoms, the bonds can emit vibrations as both stretching and bending, which generates a lot of different vibrations. (Impublications, 2016)

At an ambient temperature, molecules are located in their fundamental energy level of vibration. This energy level increase when more energy, such as wavelengths of light, is added. However, only a few wavelengths in the NIR interval can be absorbed of a specific molecule, which moves the molecule to new energy level. These energy levels are just a few in each molecules and are called overtones. Thus, overtones consist of a limited amount of energy levels where a molecule can exist, which emits several sequences by different wavelengths. Therefore, for one molecule several overtones can be observed in the spectrum. (Pasquini, 2003)

Additionally, in a spectrum sequences can be obtained from a more complex phenomenon called combinations. Together with overtones these combinations cause the vibrations in molecules. Combinations roughly means that molecules can absorb wavelengths in the NIR interval and share the energy between several parts of the molecules and in this way emits additional sequences. (Impublications, 2016)

In one sample containing a lot of nutrients, all these reflection energies give a spectrum which is difficult to interpret. This partly depends on over-lapping of several narrow peaks that generates spectra containing broad peaks. Additionally, the reflection depends on the physical texture of the sample, such as particle size and other physical parameters that can cause changes in the spectrum. Consequently, a spectrum is a mixture of chemical and physical reflection. There are peaks generated by overtones and combinations obtained from usual organic bonds that are easy to distinguish, these peaks are presented in Figure 3. (Impublications, 2016)

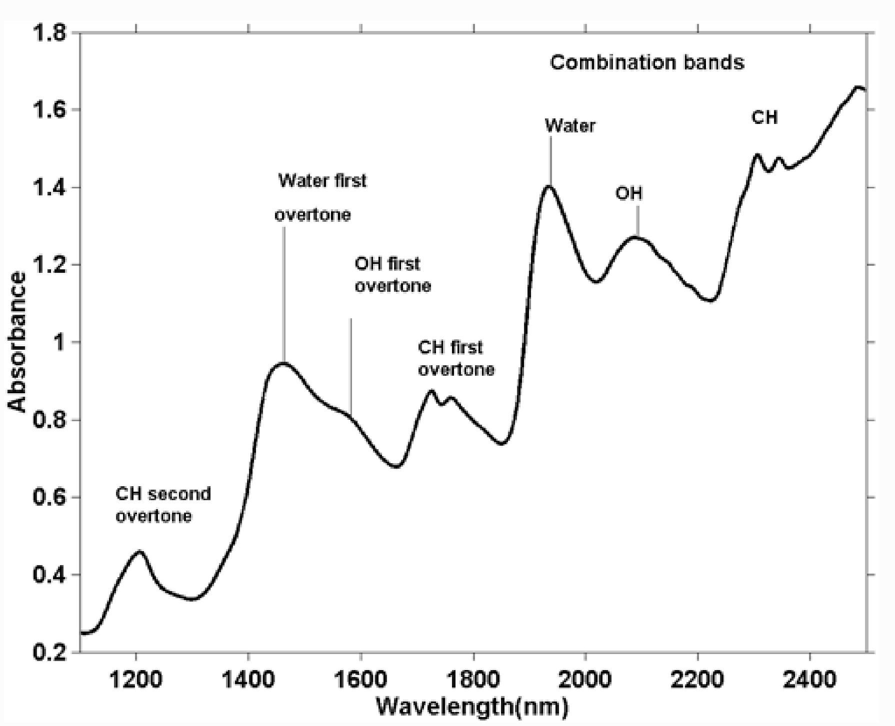


Figure 3. The main area of absorption of wavelengths in the near-infrared interval. *DA 7250* that was used in the laboratory work has an interval of 950 nm to 1650 nm. In this interval would the second overtone of C-H (1200 nm), the first overtone of water (1450 nm) and the first overtone of O-H (1600 nm) be founded. (Impublications, 2016)

In this master thesis a NIR machine called *DA 7250* was used, manufactured by Perten AB. NIR *DA 7250* has a wavelength interval between 950 nm and 1650 nm. According to Figure 3, the spectra in this master thesis are assumed to contain the second overtone of C-H (1200 nm) and the first overtones of water (1450 nm) and O-H (1600 nm).

1.4 Calibration and validation

According to Harris D.C. 2010, calibration is defined as *"Calibration is the process of measuring the actual quantity that correspond to an indicated quantity on the scale of an instrument"* (Harris, 2010). This means that several number of representative samples, with known contents of nutrients, is needed to create a calibration. If these samples, that should consist a wide range of the content of nutrients, obtains different responses from the analytical instrument, whereof a mathematical relationship can be established, a calibration model is possible to make. (FOSS, 2016)

Several, both linear and nonlinear, chemo metric techniques can be used to form a calibration model. With a linear model means that there is a linear relationship between the reference values and the obtained spectra. For instance, two commonly used linear regression models are Principal Component Analysis (PCA) and Partial Least Square (PLS). PLS was used for NIR calibration for the first time in 1982 and in agriculture in 1991. The largest advantage with PCA and PLS is their ability to reduce complex models to fewer components containing more relevant information. However, the limitation with using a PCA model is its incapacity to predict new unknown values. Two commonly used nonlinear calibration models are Artificial neutral networks (ANNs) and Least-Squares Support Vector Machine Regression (LS-SVM). Just as the models suggest, they can construct nonlinear relationship between reference values and obtained spectra. Nonlinear models are more complex than linear models. (Cao, 2013)

To find out if a constructed calibration model has created a mathematic relationship that is good enough, it needs to be validated. This has been commonly used to calculate some error values, for instance Coefficient of determination (R^2), Root mean squares error of prediction (RMSEP) and Root mean squares error of cross-validation (RMSECV). R^2 give an indication of how well the calibration is without predicting new samples (Olbjer, 2000). RMSEP and RMSECV give an indication about the possibility to predict unknown samples in the future (Burns, 2008)

In this master thesis PLS regression has been chosen in order to create calibration models of the nutrient values and the spectra, obtained from laboratory analysis and NIR *DA 7250*, respectively. The reasons are the ordinariness of the method and its ability to predict new values in unknown samples. These calibrations were validated by calculating of the error values R^2 and RMSEP, in order to find out how well the calibration works both with and without predicted new samples.

2. Material and methods

2.1 A short summary

The practical part of this master thesis was started with preparation of a base mixture of animal feed. This well mixed animal feed was divided into 60 jars with 250 g in each. In 30 of the jars fish oil was added with an interval 0-15 g and in the other 30 jars AkoFeed was added with an interval of 0-21 g. 2/3 of the samples would be used for calibration (C) and 1/3 would be used for validation (V).

All 60 samples were measured in NIR *DA 7250* and spectra from 950 to 1650 nm were obtained. Additionally, the content of water, protein, ash, fat and crude fiber were measured in some of the samples. Thereafter were statistical t-tests made on the measured values in order to ensure if any significant difference existed and the content of nutrients in all the samples could be calculated.

When all 300 values were calculated, three PLS-calibrations for each nutrient were made in Matlab *R2015b*: one with fish oil, one with AkoFeed and one with a combination of both. To investigate if there were any difference between the calibrations, RMSEP and R^2 were calculated and compared.

A more accurate description of the performance is presented below.

2.2 Laboratory work

2.2.1 Preparation of the samples

Materials

Analytical scale with a precision from 0.1 mg to 100 mg
Automatic pipettes, 1 mL and 10 mL
Laboratory Mill 3303
Domestic mixer

Performance

The raw materials that would be used to prepare a base mixture for all the samples were obtained from one of the feed manufactory. The recipe, that is present in Table 1, comes from a well-used animal feed for sows. Note that the amount of the ingredients ends up in around 97 %, which depends on the lack of fish oil (3.0257 %). In total, almost twenty kilograms of these ingredients were weighed and mixture in a big bowl, see Figure 4. Thereafter, everything was well ground in a mill and mixed to a homogenous mixture in a bowl.

Figure 4. Weighing and mixing of the base ingredients.

Table 1. The raw materials that were used to prepare the base mixture. Note that the amount of the ingredients ends up in smaller than 100 %. It depends on the lack of fish oil (3.0257 %) that not is included in the base mixture.

Raw materials	Amount (%)	Measured amount (g)
Calcium carbonate	9.49210	1898.40
Monocalcium phosphate	1.42450	284.900
Rape seed meal	25.5900	5118.00
Wheat bran	5.00000	1000.00
DDGS	3.61000	722.000
Sodium chloride	2.07800	415.600
L-lysine sulphate	1.52060	304.120
Soy bean meal	45.7017	9140.30
Premixture of vitamins and trace elements	2.32000	464.000
L-threonine	0.164700	32.9400
DL-methionine	0.0727000	14.5400
Total	**96.9743**	**19394.8**

15 kg of the mixture was divided into 60 jars with 250 g mixture in each. In half of the samples (30 pieces) fish oil was added and in the other half (30 pieces) AkoFeed was added, see Figure 5. 20 samples with fish oil and 20 samples with AkoFeed were intended to be used in the calibration step in Matlab. The rest of the samples (ten samples of each) were intended to be used in the validation step in Matlab. The samples were named as follows: C1F-C20F and C1A-C20A means the calibration samples with fish oil and AkoFeed, respectively, V1F-V10F and V1A-V10A means the validation samples. The amount of added fish oil and AkoFeed is presented in Table 2. In the fish oil samples 0 to 15 g fish oil were added and in the AkoFeed samples 0 to 21 g AkoFeed were added. These intervals were chosen in order to cover the intervals that Svenska Foder AB has in all their different kind of animal feed. In C1F-C20F the

interval between the added fish oil in the samples was approximately 0.80 g, in V1F-V10F the interval was around 1.6 g and in C1A-C20A and V1A-V10A the intervals were around 1.1 g and 2.2 g, respectively. The intervals of added fat were chosen to avoid same fat content in the calibration samples and validation samples. In this master thesis, the purpose is to investigate how well the calibration models would be to predicted new unknown samples that probably not are identical with the calibration samples. Therefore, the calibration samples and the validation samples should not be identical.

Figure 5. Fish oil and AkoFeed were added in the samples.

The added fish oil and AkoFeed were mixed in the samples by a domestic mixer to make the fat evenly distributed without lumps. To avoid evaporation, the samples were stored with lids in a cool room at around 15 degrees, ready for future analysis with the NIR and for content of water, protein, ash, fat and crude fiber.

Table 2. 30 samples containing fish oil (F) and 30 samples containing AkoFeed (A). 2/3 of the samples will be used for calibration (C) and 1/3 of the samples for validation (V).

Samples containing fish oil	**Amount added fish oil (g)**	**Samples containing AkoFeed**	**Amount added AkoFeed (g)**
C1F	0.25000	C1A	0.25400
C2F	1.0050	C2A	1.2710
C3F	1.7800	C3A	2.2770
C4F	2.5640	C4A	3.3120
C5F	3.3140	C5A	4.3360
C6F	4.0590	C6A	5.3730
C7F	5.8910	C7A	6.4450
C8F	5.6390	C8A	7.4920
C9F	6.4130	C9A	8.5390
C10F	7.2080	C10A	9.6040
C11F	7.9950	C11A	10.692
C12F	8.8030	C12A	11.799
C13F	9.6430	C13A	12.935
C14F	10.418	C14A	14.028
C15F	11.216	C15A	15.134
C16F	12.085	C16A	16.247
C17F	12.878	C17A	17.393
C18F	13.719	C18A	18.545
C19F	14.540	C19A	19.714
C20F	15.391	C20A	20.852
V1F	0.75100	V1A	0.77300
V2F	2.2710	V2A	2.7730
V3F	3.8390	V3A	4.8840
V4F	5.3790	V4A	6.9520
V5F	6.9570	V5A	9.0690
V6F	8.5680	V6A	11.248
V7F	10.139	V7A	13.451
V8F	11.774	V8A	15.709
V9F	13.449	V9A	17.965
V10F	15.111	V10A	20.273

2.2.2 Measurement of spectra on NIR

Materials

NIR: *DA 7250* from Perten AB, with spectra interval from 950 nm to 1650 nm.

Performance

To measure the spectra of all the samples, a NIR machine was used. One by one, the samples were added to an associated plastic cup with a diameter of 15 cm and a flat surface was obtained by using a ruler, see Figure 6. Thereafter, the plastic cup with sample was put into the NIR machine and the spectrum was measured. This procedure was performed twice times for each sample.

All spectra were obtained by conversion to an excel document that contained one spectrum for each sample at every fifth wavelength (950 nm, 955 nm, 960 nm etc). This gives a matrix with a size of:

$$[141 \quad 60]$$

Figure 6. The samples were added to a plastic cup and by using a ruler a flat surface was obtained.

2.2.3 Measurement of the water content

Materials

Analytical scale with a precision of 10 mg
Drying bowl (d = 80 mm) with associated lids
Oven at 103 °C
Desiccator

Performance

The water content was measured in seven different samples. Three of the samples contained no amount of added fat, two of the samples contained different amount of fish oil (C10F and C20F) and the remaining two samples containing different amount of AkoFeed (C10A and C20A). The samples that contained fish oil and AkoFeed were measured in duplicates. The samples are represented in Figure 7.

Figure 7. Measurement of the water content in seven different samples. The three samples upper to the left containing no added fat and the rest of the samples containing fish oil (two from C10F and two from C20F) and AkoFeed (two from C10A and two from C20A).

Around ten grams of each sample were weighed in drying bowls. Both the weight of the drying bowls ($W_{1(water)}$) and the amount of added samples ($W_{2(water)}$) were noted by zeroing the analytical balance between the measurements. The samples were put in the oven at 103 °C for four hours. Thereafter, the samples were taken out and lids were added before they were placed in a desiccator in one hour. When the samples were cool the lids were taken off and the samples were weighed ($W_{3(water)}$).

The amount of the water content in the samples were calculated by following equation:

$$\%\, water\ content = 100 - \frac{W_{3(water)} - W_{1(water)}}{W_{2(water)}} * 100 \qquad \text{(Equation 1)}$$

2.2.4 Measurement of the protein content

Materials
Analytical scale with a precision of 0.1 mg
Pellet press
Protein analyzer *LECO F-P 528*
Foil: nitrogen free

Chemicals
Oxygen gas
Helium gas
EDTA

Performance
Eleven samples were measured in the protein analyzer to calculate the amount of protein in all of the samples. Three samples that not contained any added amount of fat, four samples containing fish oil (C10F, C20F, V5F and V10F) and four samples containing AkoFeed (C10A, C15A, C20A and V4A). In this measurement duplicates were used for all the samples and mean values were calculated.

To measure the protein content in the samples the Protein analyzer *LECO F-P 528* has to be calibrated. The pressures of oxygen gas and helium gas were turned on. Thereafter ten blanks were analyzed followed by one slush sample and six EDTA (around 200 mg of each). At least four blanks with values near 1.0 were chosen to blank correction and at least three EDTA values near 59.8 % protein (a nitrogen content of 9.57 %) were chosen for drift correction. A seventh EDTA was analyzed to control the calibration together with two control samples. The control sample was wheat middlings with a known protein concentration. When the seventh EDTA and the two control samples were approved the analyzing of the samples could begin.

Around 200 mg of each sample was weigh in a foil and the weight of the samples were noted in the computer. The foils were folded to packages with the sample on the inside and the packages were pressed to small pellets with a pellet press. The pellets were placed in a dosage apparatus that can be seen to the left in Figure 8. The pellets were burned in the protein analyzer, one by one, to determine the amount of protein in the samples. After approximately 20 samples had been analyzed two control samples were analyzed again to control the calibration.

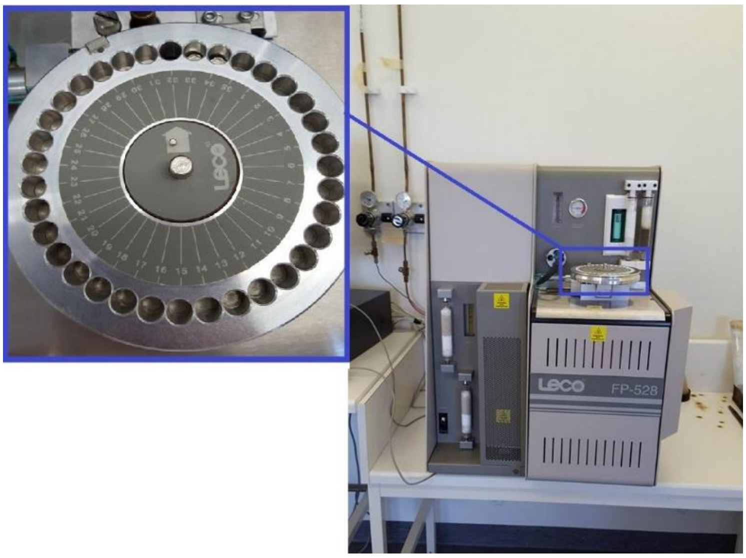


Figure 8. The protein analyzer LECO F-P 528 can be seen to the right in the figure and the dosage apparatus can be seen to the left.

2.2.5 Measurement of the ash content

Materials
Analytical scale with a precision of 0.1 mg
Ash cup made of porcelain
Oven at 550 °C
Desiccator

Performance
To calculate the ash content in the bowls with added fish oil and AkoFeed, 13 samples were measured in total. Three samples with no added fat, six samples with added fish oil (C5F, C10F, C15F, C20F, V5F and V10F), and four samples containing AkoFeed (C10A, C15A, C20A and V4A). The samples that contained fish oil and AkoFeed were measured in duplicates. The reason to measure more samples of the samples containing fish oil was an extra control due to that the duplicated samples gave unequal results. Some of the samples can be seen in Figure 9.

Figure 9. Samples ready to be burned in 550 °C overnight to then measured the ash content.

First, the weight of the ash cups was noted as $W_{1(ash)}$ and after zeroing the analytical balance around five grams of each samples were weighed in different ash cups and noted as $W_{2(ash)}$. In the evening the ash cups with samples were placed in the oven at 550 °C overnight. In the morning, the samples were taken out and placed in a desiccator for one hour. Then the cooling samples were weighed and the weights were noted as $W_{3(ash)}$.

The amount of the ash content in the samples were calculated by the following equation:

$$\%\, ash\ content = \frac{W_{3(ash)} - W_{1(ash)}}{W_{2(ash)}} * 100 \qquad \text{(Equation 2)}$$

2.2.6 Measurement of the fat content

Materials
Analytical scale with a precision of 0.1 mg
Oven at 60 °C and 103 °C
Desiccator
Extraction beaker
Hydrotec 8000, made by FOSS
Soxtec Avanti 2050 Auto System, made by FOSS
Cotton
Glasschrot (d = 5 mm)
Hydrocap sample capsule
Hydrocap with hook
Magnetic ring

Chemicals
Celite 566
HCl with a concentration of 3 M
Petroleum ether with boiling point: 40-60 °C

Performance
The content of fat was measured for eleven samples with a duplicate of each. Three samples containing no added fat, four samples containing fish oil (C10F, C20F, V5F and V10F) and four samples containing AkoFeed (C10A, C15A, C20A and V4A). Hydrolysis was performed in the first step and end extraction in the second step.

Hydrolysis: Around one gram celite was added into each hydrocap sample capsule. The analytical balance was zeroed and around 1.5 g of the samples were weighed in capsules and the weight were noted as $W_{1(fat)}$. Six capsules were placed on each sample holder (hydrocap with hook), two holders were placed in the boiler and the boiler was in turn placed in the *Hydrotec 8000*. The lid was closed, a new filter of fumes was added and the position of the float was controlled (need free movement). This construction can be seen to the left in Figure 10. A hydrolysis program was chosen with following settings:

- Boiling intensity: 50 %
- Boiling time: 1 hour
- 18 rinses, which give an end pH of 7.0
- Total time: 2 hours and 20 minutes

When the hydrolysis was finished, the lid was opened and the boiler was taken out. Sample remains on the wall of the twelve capsules were wiped down by cotton. The cotton pieces were put on the top of the samples in the capsules. The capsules were dried in an oven at 60 °C overnight.

End extraction: Six extraction beakers were provided with eight glasschrot in each and dried in the oven at 103 °C for 45 minutes followed by cooling in a desiccator for 45 minutes. The extraction beakers were weighed and the weights were noted as $W_{2(fat)}$. The capsules were taken out from the oven and the sample cakes in the bottom of the capsules were pressed until they were broken. A thin layer of cotton was placed like a lid on the top of each capsules. Magnetic rings were placed on the capsules and the capsules were placed in the *Soxtec Avanti 2050 Auto System*. 80 mL petroleum ether was added into the extraction beakers, which were then placed in a holder under the capsules in the *Soxtec Avanti 2050 Auto System*. The arrangement can be seen to the right in Figure 10. An end extraction program was chosen with following settings:

- Boiling time: 20 minutes
- Rinses: 40 minutes
- Total time: 1 hour

Figure 10. *Hydrotec 8000* can be seen to the left (hydrolysis) and *Soxtec Avanti 2050 Auto System* can be seen to the right (end extraction).

When the program had finished and all the petroleum ether was gone from the extraction beakers, the extraction beakers were dried in an oven at 103 °C at 45 minutes followed by cooling in a desiccator in 45 minutes. Finally, the extraction beakers were weighed and noted as $W_{3(fat)}$. The end extraction was performed twice due to just six samples could be extracted at the same time.

The amount of the fat content in the samples were calculated by following equation:

$$\%\, fat\ content = \frac{W_{3(fat)} - W_{2(fat)}}{W_{1(fat)}} * 100 \qquad \text{(Equation 3)}$$

2.2.7 Measurement of the crude fiber content

Materials

Cyclone Mill with 0.5 mm sieve
Analytical scale with a precision of 0.1 mg
Oven at 103 °C and 550 °C
Desiccator
Glass filter crucible
Tecator Fibertec 2010 Heat Extractor, made by FOSS
Tecator Fibertec 1021 Cold Extractor, made by FOSS

Chemicals

H_2SO_4 with a concentration of 0.128 M
KOH with a concentration of 0.223 M
n-octanol
Celite
Acetone

Performance

Since this measurement consists of several steps and take a long time to perform, just five samples without any duplicates were measured. One sample containing no added fat, two samples containing fish oil (C10F and C20F) and two samples containing AkoFeed (C10A and C20A). The samples were better divided into a finer grain size by a cyclone mill before the analysis was started. Since the water content has been changed by grinding, five new measurements of water were necessary. These measurements were performed at the same way as has been described in part 2.2.3. Then, in the first step heat extraction was performed, followed by cold extraction and measurement of the ash content.

Tecator Fibertec 2010 Heat Extractor was prepared by heating the boiler with 100 mL water for 5 minutes. Additionally, both reagents (H_2SO_4 and KOH) were heated to 80 °C before using them. Around 1.0 g of the five samples were weighed in five glass filter crucibles and the weights were noted as $W_{1(\text{crude fiber})}$. In the crucibles around 1.0 g celite aso was added in each sample. The five crucibles together with a control sample (containing wheat middlings) were placed in a holder in the heat extractor (under the boilers). See the construction in Figure 11. 150 mL H_2SO_4 and two drops n-octanol were added into the boilers and the boiling time was 30 minutes. When sample boiled up and got stuck on the walls it was rinsed down by more reagent.

One vacuum valve in the bottom of each crucible was opened and the reagent was filtered out from the boilers. Frequently, a sample cake was obtained in the bottom of the crucible, which made it hard to filter out all of the reagents. It could be solved by closing the vacuum valve and quickly open an air valve in the bottom to mix around in the boiler and then open the valve for the vacuum again. When all of the reagent was removed from the boilers water was added to rinse the boilers and was removed by vacuum. The boilers were rinsed with water three times in total. Then, whole this process was repeated the same way with KOH.

Figure 11. *Tecator Fibertec 2010 Heat Extractor* containing six crucibles that are placed under the boilers. At the bottom of the heat extractor the vacuum valve/air valve can be seen.

After heat extraction came the cold extraction. The crucibles were placed in *Tecator Fibertec 1021 Cold Extractor* and 25 mL acetone was added in each samples and removed by vacuum. This process was repeated three times.

The samples were dried in room temperature for 30 minutes followed by drying in an oven at 130 °C for two hours. Thereafter, they were cooled in a desiccator and their weight were noted as $W_{2(\text{crude fiber})}$.

Finally, the ash content was measured according to the description in part 2.2.5 and the weights were noted as $W_{3(\text{crude fiber})}$.

The amount of the crude fiber content in the samples were calculated by following equation:

$$\%\, crude\ fiber\ content = \frac{W_{2(crude\,fiber)} - W_{3(crude\,fiber)} - 0.0022}{W_{1(crude\,fiber)}} * 100 * \frac{t_{s1}}{t_{s2}} \qquad \text{(Equation 4)}$$

where t_{s1} is the content of water in samples grinded by Laboratory Mill 3303 (unit: weight percent of dry matter), t_{s2} is the content of water in samples grinding by Cyclone Mill (unit: weight percent of dry matter) and 0.0022 compensates for residue of added celite.

2.3 Calculation of the nutrients in all samples

When the content of the five nutrients in some of the samples had been measured, the values would be used to calculate the content in all the 60 jars. The reason to just measure a limited selection of samples was the time limitation and that it would have contributed to unnecessary costs. Since the ingredients in the base mixture are known and the added fish oil and AkoFeed contain a negligible amount of the nutrients according to their corresponding certificates. Thus, it was assumed that the same amounts of the content of water, protein, ash and crude fiber were obtained in each sample (in the unit: g).

Of each nutrient (except crude fiber) the content was measured in three samples containing no added fat together with some samples containing fish oil and AkoFeed. To determine if the mean values from the samples containing no added fat were representative for all the samples, without any significant difference between them and the measured samples containing fish oil and AkoFeed, statistical t-tests were calculated. For crude fiber, where just one sample containing no added fat was analyzed, the other four samples (containing fish oil and AkoFeed) were used to perform the t-test.

The t-tests were calculated by following equation:

$$I = \bar{x} \pm t_{\alpha/2}(n-1) * d(\bar{X}) \qquad \text{(Equation 5)}$$

where α/2 is the quantile, n is the number of measured samples and d($\bar{x}$) is the mean error of an estimator and is calculated according:

$$d(\bar{X}) = \frac{s}{\sqrt{n}} \qquad \text{(Equation 6)}$$

where s is the estimator of the square root of the standard deviation (σ):

$$s = \sum \frac{(x_i - \bar{x})^2}{n-1} \qquad \text{(Equation 7)}$$

where x_i is the measured value and $\bar{x}$ is the mean value of the samples containing no added fat. (Olbjer, 2000)

To calculate the interval I, a confidence interval of 95 % was used. According to the Table 3 in Appendix 8.1, $t_{\alpha/2}(n-1)$ were determined as $t_{0.025}(2)$ = 4.30 for water, protein and ash, and $t_{0.025}(3)$ = 3.18 for crude fiber. When it was calculated for each nutrient, curves of normal distribution were drawn and the measured samples (containing fish oil and AkoFeed) were marked on the corresponding curves. In the case of crude fiber, the measured sample containing no added fat was marked in the curve.

Thereafter, the nutrient contents in all 60 jars could be calculated to then be used for calibration and validation.

In the calculation of the fat content in the samples containing fish oil and AkoFeed, linear regression was used to create two equations based on the measured samples. Then, these two equations were used to calculate the content of fat in all the 60 jars.

2.4 Calibration and validation in Matlab *R2015b*

When the contents of water, protein, ash, fat and crude fiber were calculated, several matrices were formed. The calibrations were performed with three calibration sets (fish oil, AkoFeed and the combination) containing all the “C”-samples and the validations were performed by three validation sets containing all the “V”-samples. Thus the spectra were divided in six parts: C1F-C20F, C1A-C20A, C1F-C20F + C1A-C20A, V1F-V10F, V1A-V10A and V1F-V10F + V1A-V10A. The nutrients were divided in the same way, one matrix for each of them.

2.4.1 Pretreatment of spectra and data

Before calibration by PLS method (Partial Least Squares), both the spectra and the data needed to be pretreated. To reduce the physical differences between the samples, that have been obtained due to the different amount of added fat, a baseline correction of the spectra was performed by the pretreatment method Standard Normal Variate (SNV) (Genkawa, 2015). The data were centered by reduce the values with the mean value of each data matrix and thereby create a center around zero.

2.4.2 Calibration and validation of data

15 different calibrations were created in Matlab: three calibrations (fish oil, AkoFeed and the combination) of each of the five nutrients (content of water, protein, ash, fat and crude fiber). PLS, that was used for all the calibrations, is a commonly used multivariate regression models that was using in Matlab by the *Plsregress*-function. (Gómez-Carracedo, 2007)

Since the aim of the calibrations is to be able to predict new samples as good as possible, the number of components (also called latent values) in the calibrations were chosen to give the lowest prediction errors (Gómez-Carracedo, 2007)

To validate the calibration and the possibility to predict unknown samples in the future, the error values R^2 (Coefficient of determination) and RMSEP (Root mean squares error of prediction) were calculated. R^2 was calculated by following equation:

$$R^2 = \frac{\sum_{i=1}^{n}(C_i - C_i^*)^2}{\sum_{i=1}^{n}(C_i - \bar{C})^2} \qquad \text{(Equation 8)}$$

where C_i is measured values of the calibration samples (C-samples) in the laboratory, C_i^* is predicted calibration values (calculated with *plsregress*) and $\bar{C}$ is the mean value which is zero due to the center pretreating of data (Olbjer, 2000).

R^2 give an indication of how much the calibration describes the variation of the data (C-samples) (Olbjer, 2000). If R^2 is 1.0 it means that 100 % of the data are described by the calibration. If R^2 instead is as low as 50-70 % the correlation of the data is weak and if $R^2 < 50$ % it begin to approach non correlation. (Dang, 2015)

RMSEP gives an indication of how good the calibration will be to predict unknown samples in the future. This error value should be as low as possible and is calculated by use of an independent validation set (V-samples). RMSEP was calculated by following equation:

$$RMSEP = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(V_i - V_i^*)^2} \qquad \text{(Equation 9)}$$

where V_i is measured values of the validation samples (V-samples) in the laboratory, V_i^* is predicted validation values and n is the number of measured samples.

Additionally, to get an overview of how well the predicted values in the calibrations and validations become, two figures of each nutrient were plotted that contains the reference values versus the predicted values.

3. Results

3.1 Calculation of the nutrients in all the samples

The results of the content of water, protein, ash, fat and crude fiber in the analyzed samples is presented in Table 4-8 in Appendix 8.2.

To make the curves of normal distribution by using t-tests (Equations 5-7), the values of the three samples containing no added fat were used for content of water, protein and ash. The measured samples containing added fat are marked on the corresponding curves. For calculation of normal distribution of crude fiber content, the measured values of C10F, C20F, C10A and C20A were used instead. Here is the measured sample containing no added fat marked in the curve. The four curves of normal distribution with the marked values are presented in Figure 12.

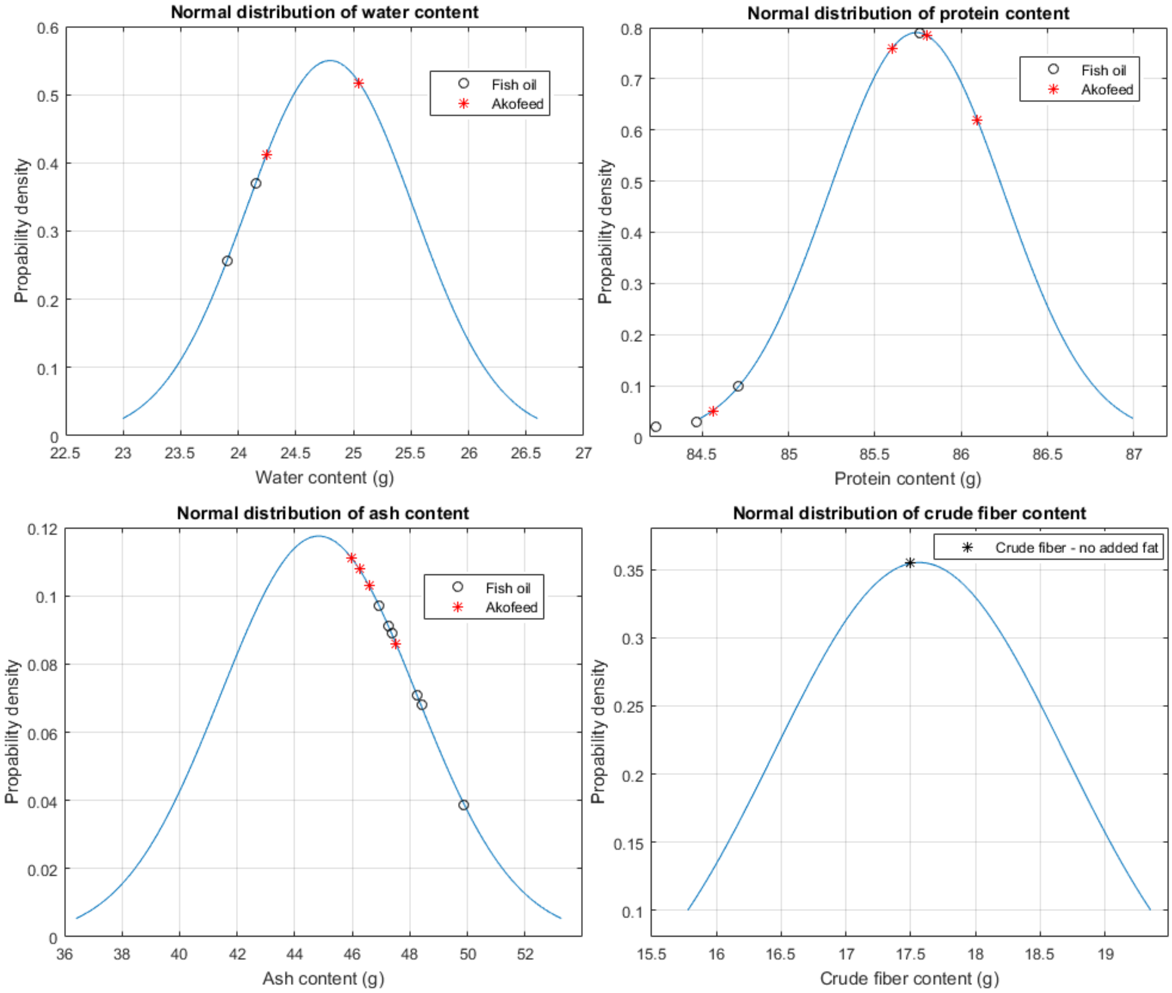


Figure 12. Curves of normal distribution made by t-test with 95 % confidence interval. The normal distributions of content of water, protein and ash were made with three samples containing no added fat. The marked point in the curves are the analyzed samples containing added fat. The normal distributions of content of crude fiber was made by C10F, C20F, C10A and C20A. Here is the marked point the analyzed sample containing no added fat.

To be able to calculate the content of the five nutrients in all the samples, one constant value of each nutrient was chosen from the normal distribution curves above. In the curve for water content, all the points were within the normal distribution range. Therefore, it was considered that the mean value of the samples containing no added fat was representative for all the samples: 24.8 g. In the curve of normal distribution for protein the measured values were divided in something that can be perceived as two groups. Also here the mean value of the samples containing no added fat was chosen: 85.7 g, even though one of the measured value was just outside of the range. Since all the measured values of ash were displaced at the right in the curve of normal distribution, a mean value of all the measured values was chosen: 46.8 g. In the curve of normal distribution for crude fiber the only measured sample was in the middle of the range and therefore this value was chosen: 17.5 g.

Two equations were made by linear regression (see Figure 13) to describe the content of fat in the fish oil samples and in the AkoFeed samples.

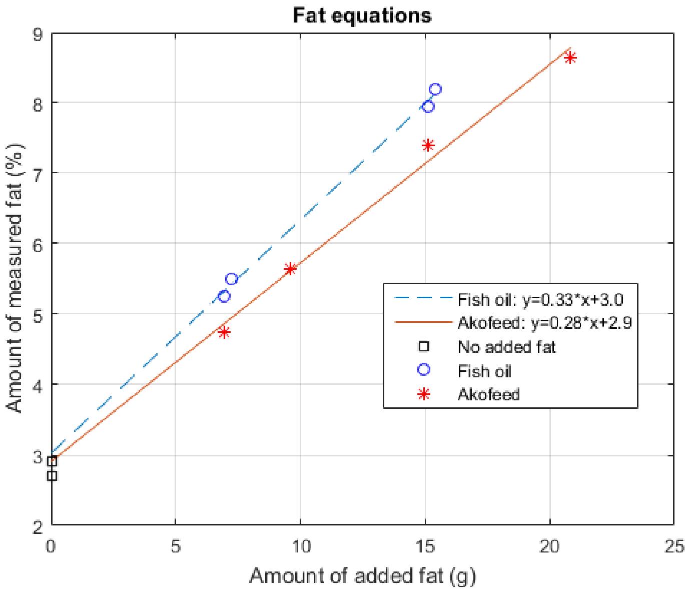


Figure 13. Four measure fat content values of each fat type (fish oil and AkoFeed) were used to created two different linear equations by using linear regression in Matlab.

The equation of fish oil is:

$$Y_{fish\ oil} = 0.33 * x_{fish\ oil} + 3.0 \qquad \text{(Equation 10)}$$

The equation of AkoFeed is:

$$Y_{akofeed} = 0.28 * x_{akofeed} + 2.9 \qquad \text{(Equation 11)}$$

where Y is the total amount of fat in the sample (%) and x is the amount of added fish oil or AkoFeed in the sample (g).

To calculate the amount of nutrients in the samples, Equations 1-4 and 10-11 were used. The calculated amount of content of water, protein, ash, fat and crude fiber is presented in Table 9 and 10 in Appendix 8.3

3.2 Pretreatment of spectra

To reduce the physical differences between the samples, the pretreatment method SNV was used on the spectra. The raw spectra are presented to the left in Figure 14 and the SNV-pretreated spectra are presented to the right.

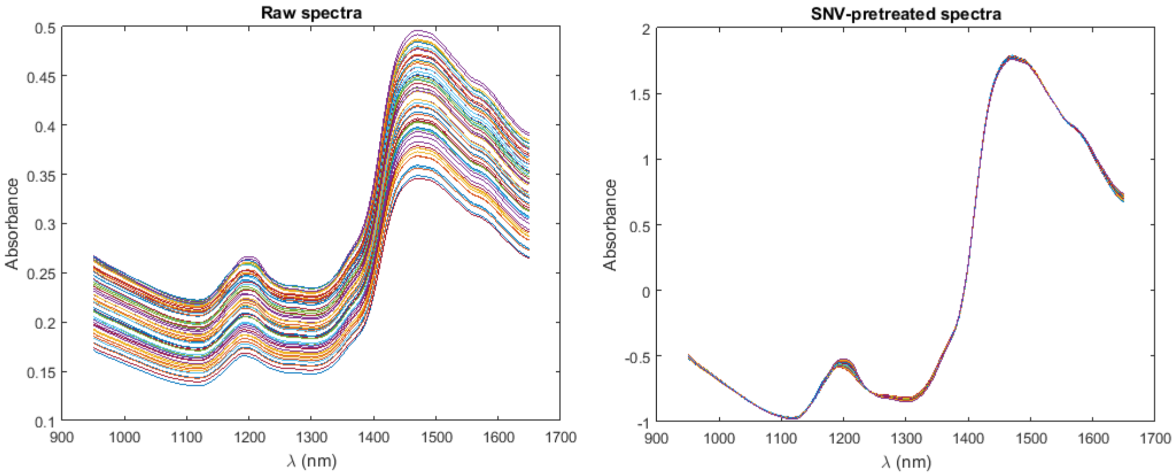


Figure 14. The raw spectra are presented to the left and the SNV-pretreated spectra to the right.

In the raw spectra the samples containing lower amount of added fat are obtained further down in the spectra while samples containing more fat are obtained further up. Probably, it depends on the texture difference of the samples. Samples that contains no added fat or have low amount of added fat were more dry and dusty, compared with samples containing more added fat that have a moister texture.

In the figure of the SNV-pretreated spectra the absorbance is more compressed. However, at the wavelengths around 1200 nm the lines are wider than at the other wavelengths. Two clear peaks and one weaker peak can also be seen in the figure at wavelengths 1200 nm, 1450 nm and 1600 nm, respectively.

3.3 Calibration and validation

3.3.1 Water content

The number of components that gave the lowest RMSEP were used in the different calibrations. The degree of explanation of the components for the three different calibrations of the water content is presented in Figure 15. Both the components of the fish oil and AkoFeed increases fast and steady while the number of components of the combination increases more slowly and unsteadily.

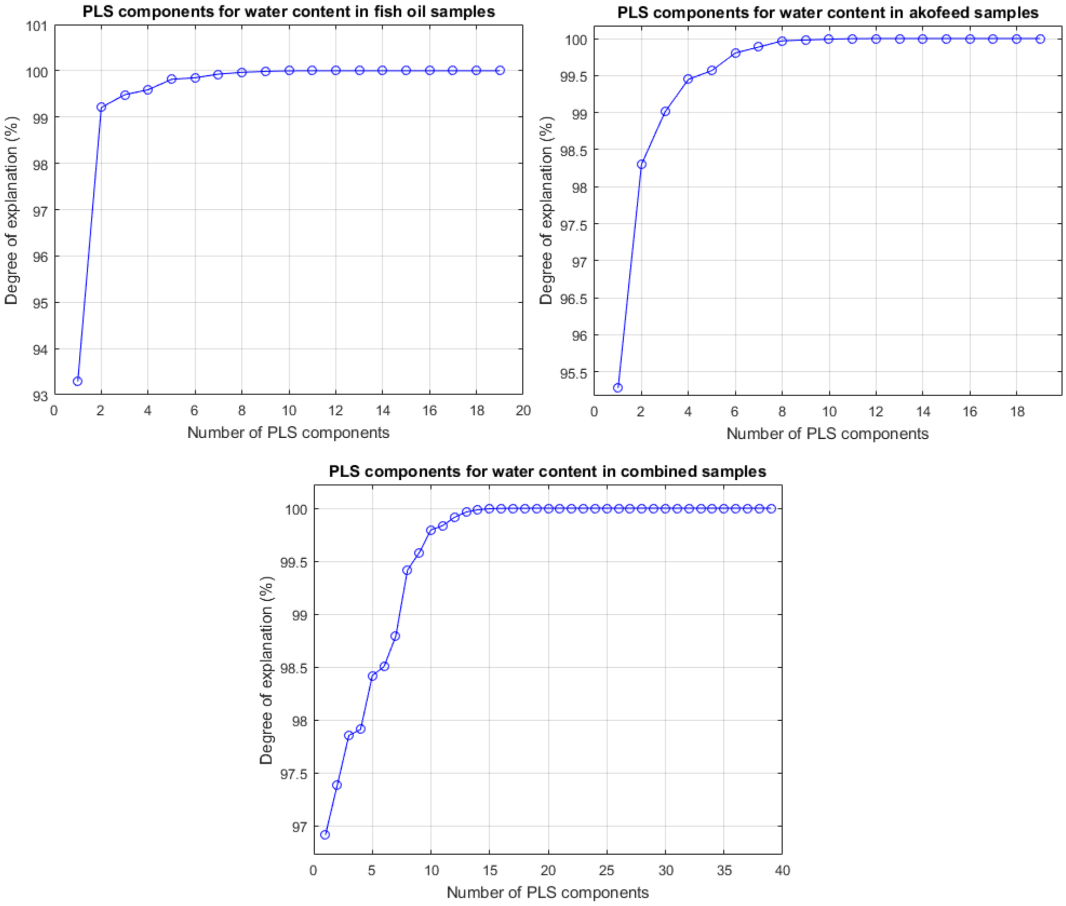


Figure 15. The degree of explanation of the components in the three different calibrations of water content. In the right corner the components of fish oil are presented, to the left the components of AkoFeed are presented and at the bottom the components of combination are presented.

When the number of components were chosen the error values R^2 and RMSEP could be calculated by using Equations 8 and 9, respectively. These values are presented in Table 11 together with the chosen number of components of each calibration.

Table 11. The calculated error values RMSEP and R^2 of the three calibrations of the water content together with the chosen number of components.

Water content	**RMSEP**	**R^2**	**Number of components**
Fish oil	0.0051	0.9985	6
AkoFeed	0.0262	0.9945	4
Combination	0.0247	0.9738	2

It can be seen in the Table 11 above that RMSEP has the lowest value of the fish oil together with the highest R^2 value (that is equal with the AkoFeed). Both the RMSEP of AkoFeed and the combination are a bit higher while the combination obtained the lowest R^2 value.

In Figure 16 it can be seen how well the predicted values from the calibrations match the calculated C-values and how well the predicted values from the external validation sets match the calculated V-values. For perfect calibration and validation, the points will be on a line with a gradient k=1. However, in the calibration curve is there one point that could be an outlier: one combination point that is unusually far from the curve. What this may depend on is mentioned in the discussion section.

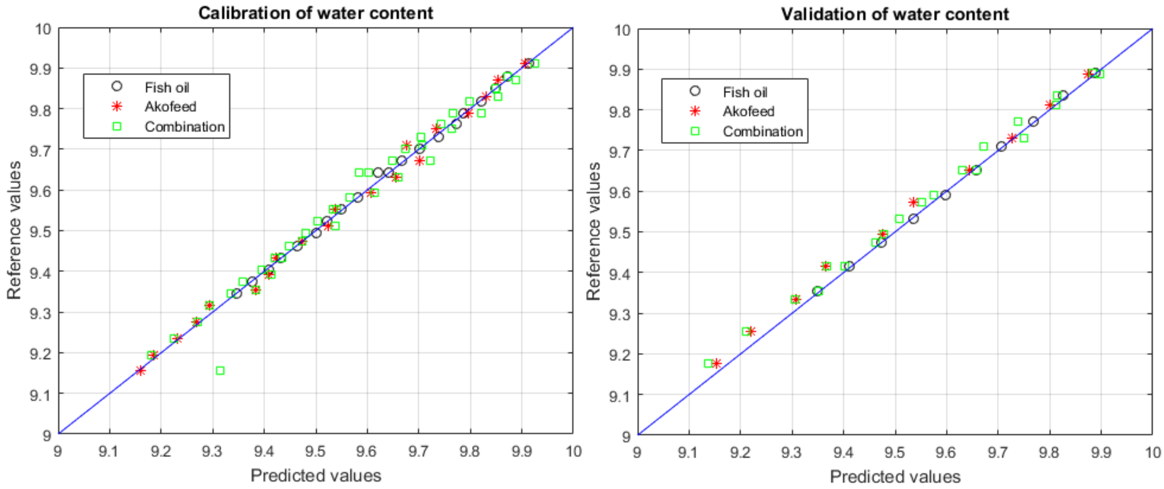


Figure 16. The left figure shows how well the predicted values from the calibrations match the calculated C-values (from Table 9 and 10 in Appendix 8.3) and the right figure shows how well the predicted values from the external validation sets match the calculated V-values (also from Table 9 and 10 in Appendix 8.3). For perfect calibration and validation will the points be on the linear curve with k=1.

3.3.2 Protein content

The degree of explanation of the components for the three different calibrations of the protein content is presented in Figure 17. Also here, both the components of the fish oil and AkoFeed increases fast while the number of components of the combination increases more slowly.

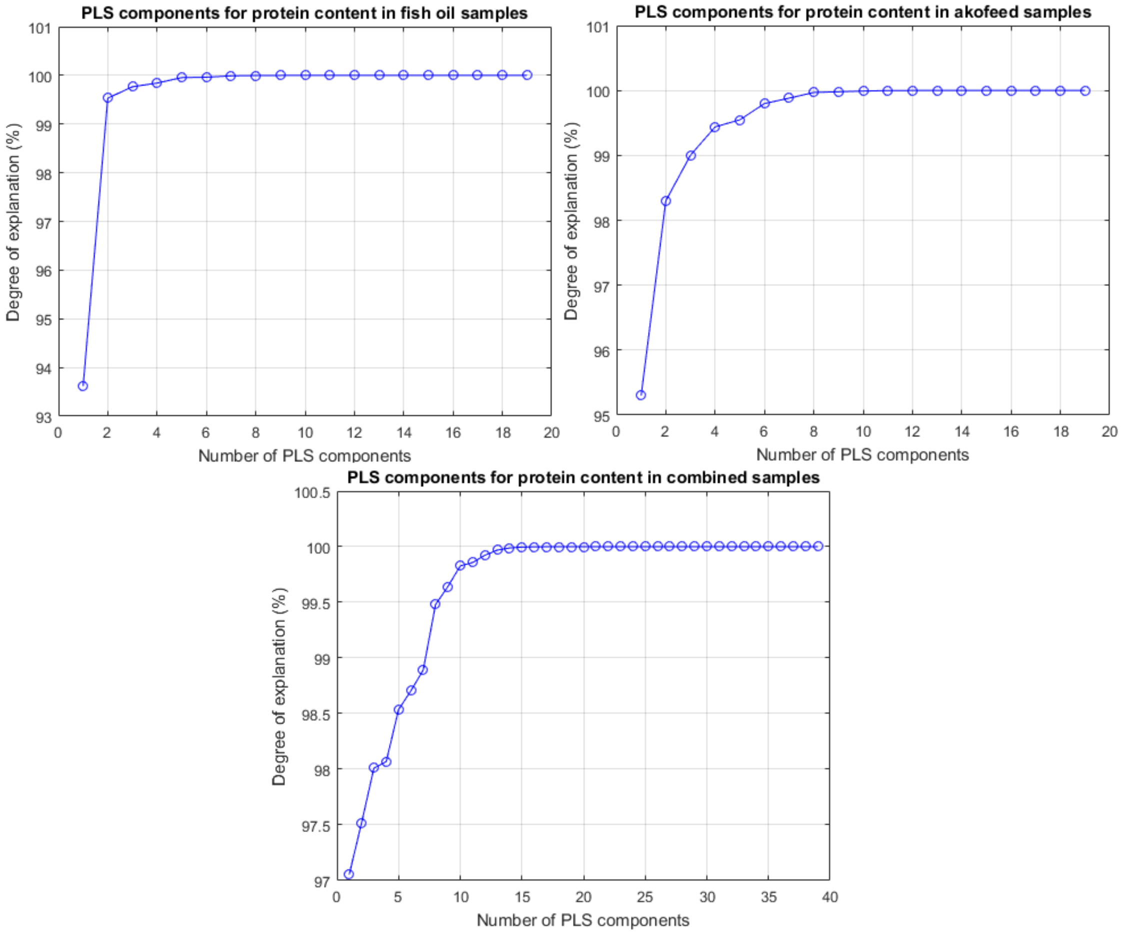


Figure 17. The degree of explanation of the components in the three different calibrations of protein content. In the right corner the components of fish oil are presented, to the left the components of AkoFeed are presented and at the bottom the components of combination are presented.

When the number of components were chosen the error values R^2 and RMSEP could be calculated by using Equations 8 and 9. These values are presented in Table 12 together with the chosen number of components of each calibration.

Table 12. The calculated error values RMSEP and R^2 of the three calibrations of the protein content together with the chosen number of components.

Protein content	**RMSEP**	**R^2**	**Number of components**
Fish oil	0.0235	0.9996	6
AkoFeed	0.0910	0.9944	4
Combination	0.0863	0.9752	2

The fish oil calibration has the lowest RMSEP value and a high R^2 value that is almost equal with the R^2 value of AkoFeed. Both the RMSEP of AkoFeed and the combination are a bit higher while the combination obtained the lowest R^2 value.

In Figure 18 can it be seen how well the predicted values from the calibrations match the calculated C-values and how well the predicted values from the external validation sets match the calculated V-values. Also, in the calibration curve for the combination there is one point that could be an outlier.

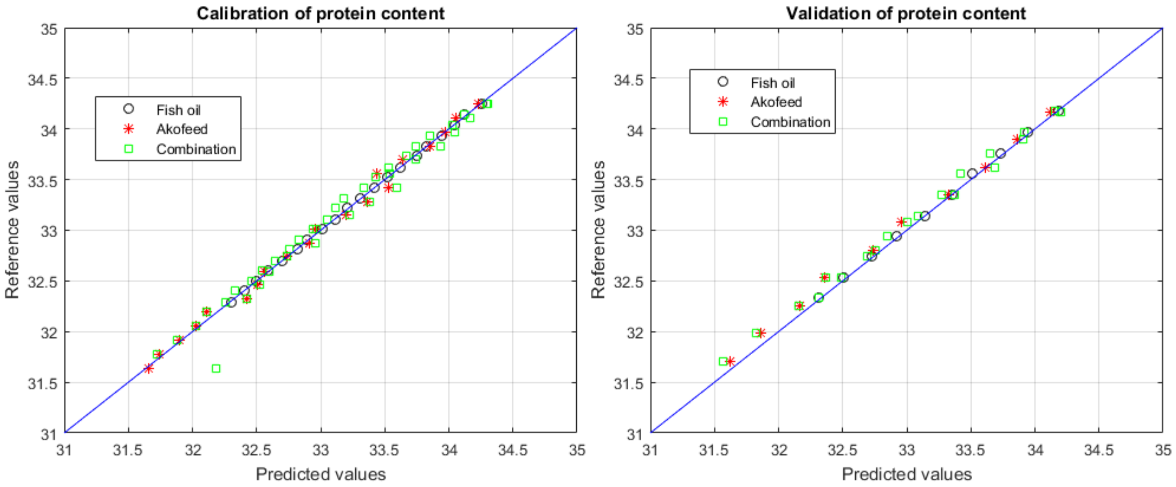
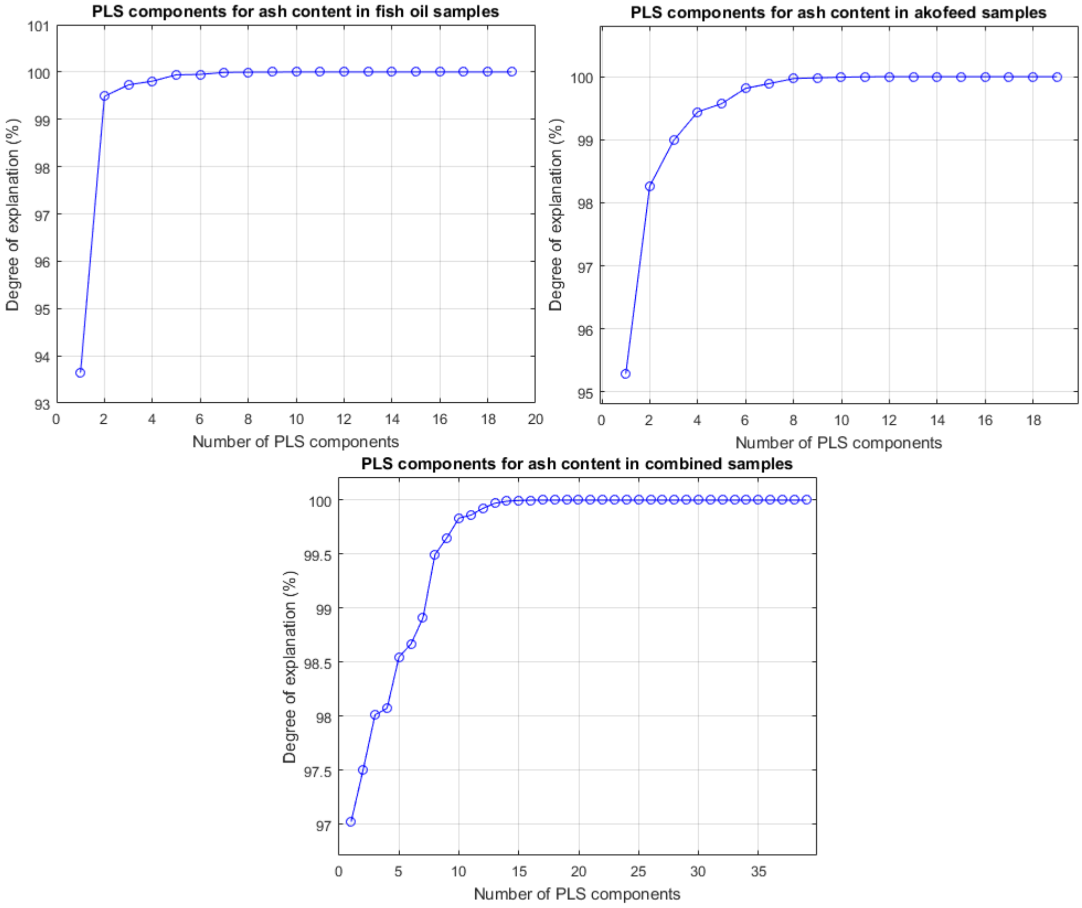


Figure 18. The left figure shows how well the predicted values from the calibrations of protein content match the calculated C-values and the right figure shows how well the predicted values from the external validation sets match the calculated V-values.

3.3.3 Ash content

The degree of explanation of the components for the three different calibrations of the ash content is presented in Figure 19. Also here, both the components of the fish oil and AkoFeed increases fast and steady while the number of components of the combination increases more slowly and unsteadily.


PLS components for ash content in fish oil samples

PLS components for ash content in akofeed samples

PLS components for ash content in combined samples

Figure 19. The degree of explanation of the components in the three different calibrations of ash content. In the right corner the components of fish oil are presented, to the left the components of AkoFeed are presented and at the bottom the components of combination are presented.

When the number of components were chosen the error values R^2 and RMSEP could be calculated by using Equations 8 and 9, respectively. These values are presented in Table 13 together with the chosen number of components of each calibration.

Table 13. The calculated error values RMSEP and R^2 of the three calibrations of the ash content together with the chosen number of components.

Ash content	RMSEP	R^2	Number of components
Fish oil	0.1393	1.000	8
AkoFeed	0.0484	0.9998	9
Combination	0.0473	0.9801	3

In this case, the fish oil calibration has the highest RMSEP value while the R^2 is higher than the R^2 value of the other two calibrations. The RMSEP of AkoFeed and the combination are lower compare to the fish oil.

In Figure 20 it can be seen how well the predicted values from the calibrations match the calculated C-values and how well the predicted values from the external validation sets match the calculated V-values. Also, in the calibration curve for the combination, there is one point that could be an outlier.

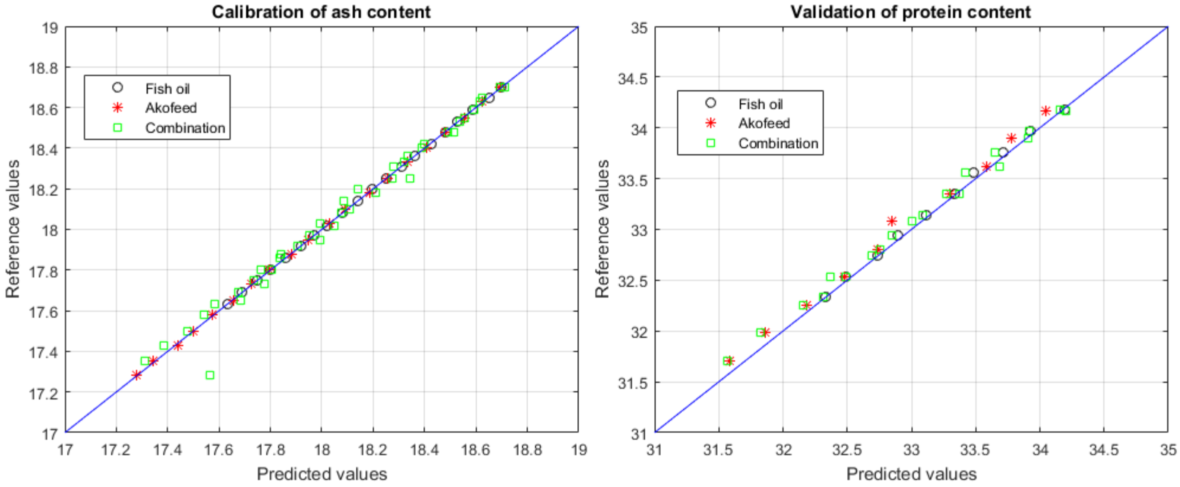


Figure 20. The left figure shows how well the predicted values from the calibrations of ash content match the calculated C-values and the right figure shows how well the predicted values from the external validation sets match the calculated V-values.

3.3.4 Fat content

The degree of explanation of the components for the three different calibrations of the fat content is presented in Figure 21. For all the three calibrations looks the curve of the components quite similar. The curve that presenting the fish oil increase faster than the other two curves at the same time as the three curves increases in an equally steady manner.

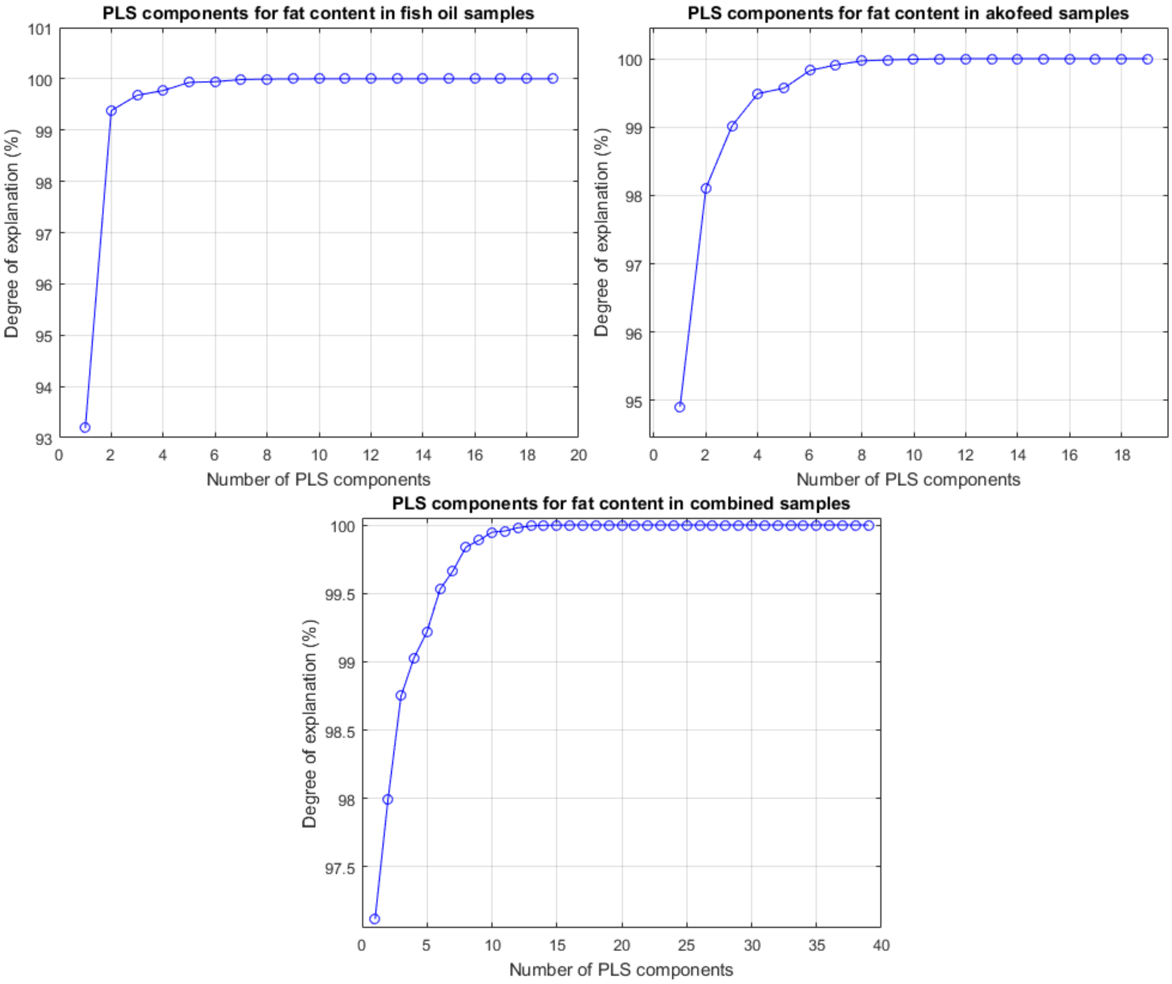


Figure 21. The degree of explanation of the components in the three different calibrations of fat content. In the right corner the components of fish oil are presented, to the left the components of AkoFeed are presented and at the bottom the components of combination are presented.

When the number of components were chosen the error values R^2 and RMSEP could be calculated by using Equations 8 and 9, respectively. These values are presented in Table 14 together with the chosen number of component of each calibration.

Table 14. The calculated error values RMSEP and R^2 of the three calibrations of the fat content together with the chosen number of components.

Fat content	**RMSEP**	**R^2**	**Number of components**
Fish oil	0.0581	0.9994	6
AkoFeed	0.1548	0.9998	9
Combination	0.1389	0.9966	7

The R^2 values are quite equal of the three calibrations while the fish oil obtains the lowest RMSEP value. The RMSEP values of AkoFeed and the combination are quite equal.

In Figure 22 it can be seen how well the predicted values from the calibrations match the calculated C-values and how well the predicted values from the external validation sets match the calculated V-values. In the calibration curve for fat content, no observable outlier could be seen at the left corner in the figure.

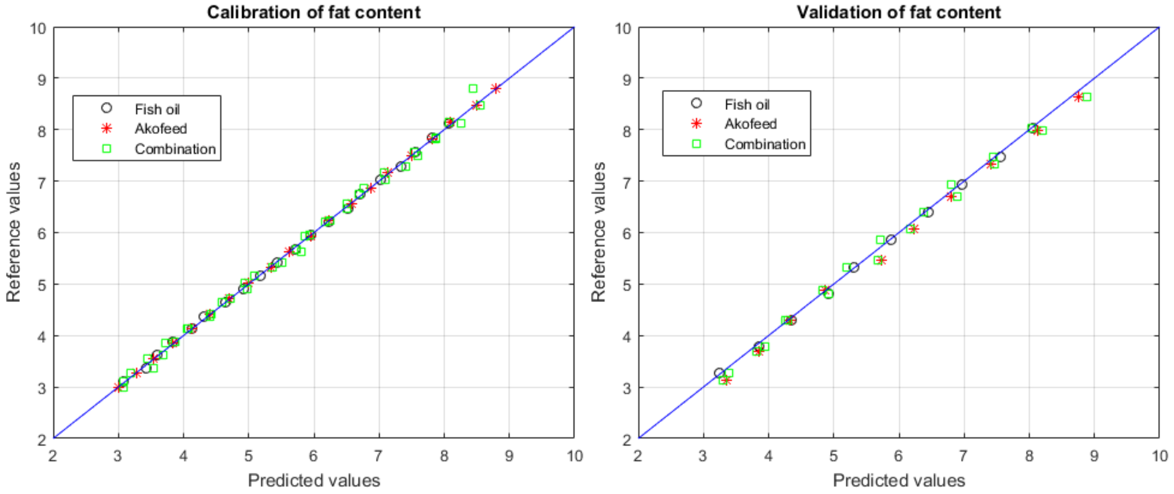


Figure 22. The left figure shows how well the predicted values from the calibrations of fat content match the calculated C-values and the right figure shows how well the predicted values from the external validation sets match the calculated V-values.

3.3.5 Crude fiber content

The degree of explanation of the components for the three different calibrations of the crude fiber content is presented in Figure 23. Both the components of the fish oil and AkoFeed increases fast and quite steady while the number of components of the combination increases more slowly and unsteadily.

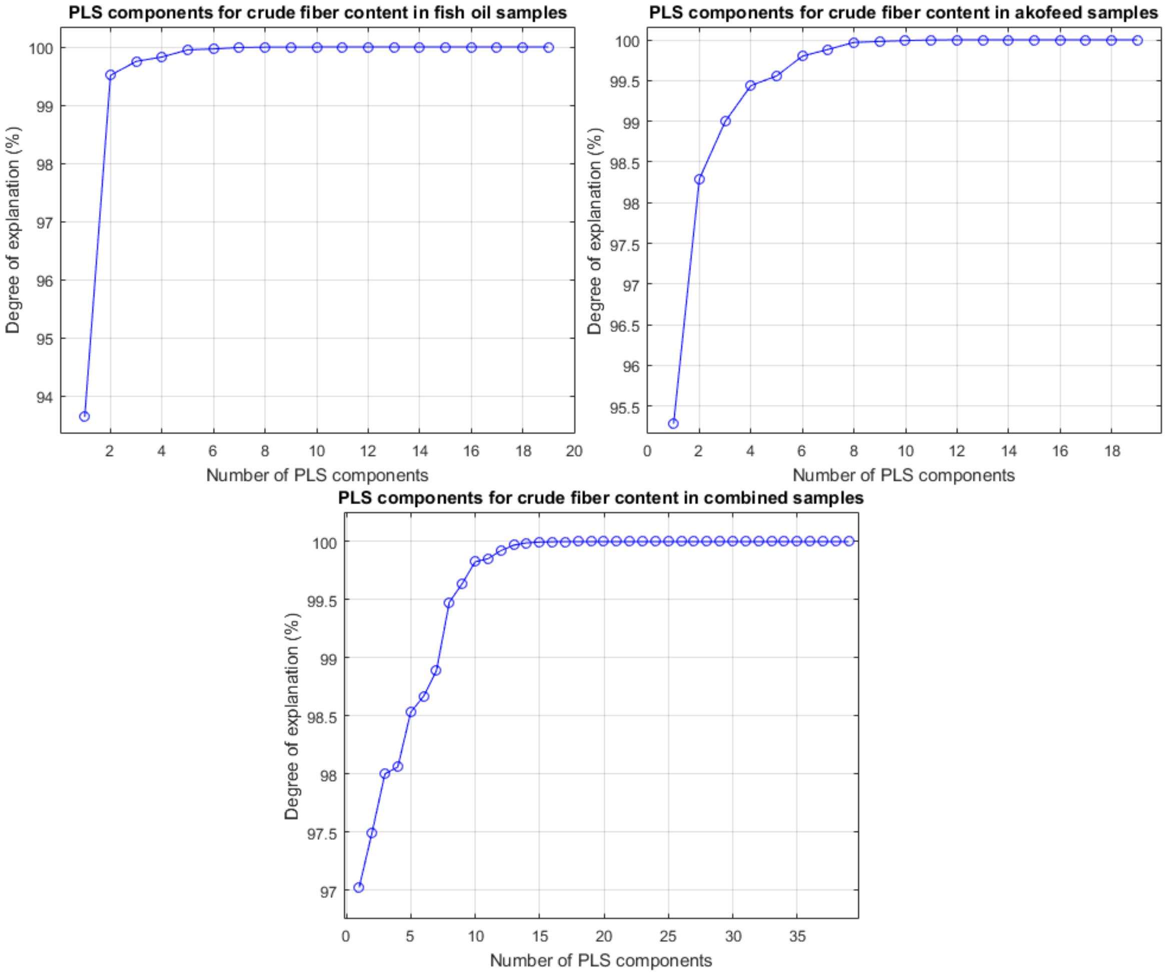


Figure 23. The degree of explanation of the components in the three different calibrations of crude fiber content. In the right corner the components of fish oil are presented, to the left the components of AkoFeed are presented and at the bottom the components of combination are presented.

When the number of components were chosen the error values R^2 and RMSEP could be calculated by using Equations 8 and 9, respectively. These values are presented in Table 15 together with the chosen number of components of each calibration.

Table 15. The calculated error values RMSEP and R^2 of the three calibrations of the crude fiber content together with the chosen number of components.

Crude fiber content	RMSEP	R^2	Number of components
Fish oil	0.0049	0.9997	6
AkoFeed	0.0185	0.9944	4
Combination	0.0175	0.9750	2

All the three RMSEP values are quite low. However, the RMSEP value of the fish oil is slightly lower than the other RMSEP values. The R^2 values are quite equal for the fish oil calibration and the AkoFeed calibration. The R^2 value of the combination is lower than the other.

In Figure 24 it can be seen how well the predicted values from the calibrations match the calculated C-values and how well the predicted values from the external validation sets match the calculated V-values. In the calibration curve for the combination there is one point that could be an outlier.

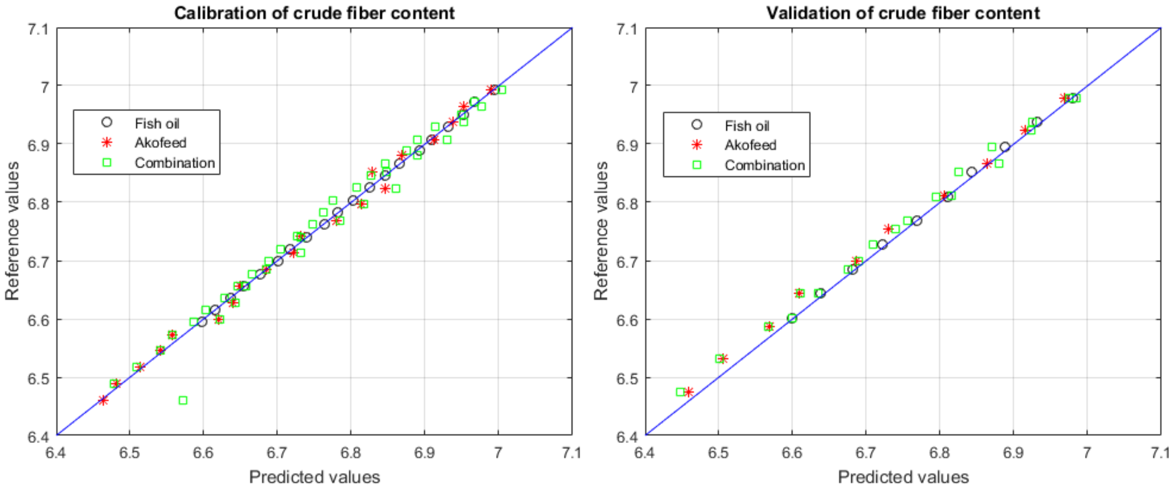


Figure 24. The left figure shows how well the predicted values from the calibrations of crude fiber content match the calculated C-values and the right figure shows how well the predicted values from the external validation sets match the calculated V-values.

4. Discussion

To use the reflection from near-infrared light for quantitative analysis is nowadays widely accepted (Burns, 2008). During the last few years the role of NIR spectroscopy for quantification of ingredients in animal feed has been well demonstrated (Burns, 2008). To get reliable results from the NIR, the calibration needs to be created by representative samples. (FOSS, 2016).

A company that produces animal feed, Svenska Foder AB, has for a long time had problem with the analysis of sow feed containing fish oil on the NIR machine. The suspicion has been that it depends on the lack of samples containing fish oil in the calibration. Instead, most of the compound feeds contains the vegetable liquid fat AkoFeed. This master thesis was created to investigate if this may be the reason for the problem with NIR analysis of samples containing fish oil. The result of this master thesis is interesting for Svenska Foder AB, since it is important for them to guarantee their customer correct nutritional contents in the animal feed.

In the beginning of the experiment, the nutrients in some of the samples were analyzed in a laboratory. If it not had been for the limitation of time, it would have been advantageous to measure the nutrients in several number of samples. Additionally, the results of the t-tests and the creation of the distribution curves would then be more statistically reliable. Further on, only three samples containing no added fat were used to create the distribution curves of water, protein and ash content. The mean value of the water content seems to be a representable value for the samples. The marked points in the normal distribution curve of the protein content seems to be divided in two groups. However, the two groups are not as so far away from each other as it looks like since the entire interval is tight and the difference may depend on the measurement uncertainty of the instrument. Additionally, the two groups seem not to have anything that separate them physically, such as the kind of added fat or the amount of added fat. For the normal distribution curve of the ash content, all the values seem to be on the right side of the curve. In the measurement of ash in the laboratory, the duplicated samples were perceived to have a large measurement uncertainty. Consequently, to measure more than only three samples containing no added fat would probably improve the results. To create the normal distribution curve of crude fiber, only five samples were analyzed in the laboratory. It gave a stable result even though the number of analyzed samples were smaller than desired.

The aim of creating a calibration for a NIR machine is that the predicted values will represent the analytical values which otherwise have been obtained by analysis in the laboratory. Thus, the NIR is constructed to compensate the time-consuming laboratory analysis. In this experiment, there was an evident difference between the content of fat in the measured samples containing added fish oil and AkoFeed, respectively. To get the values as similar as the laboratory analysis as possible, two different linear equations were created to describe the fat content for all the samples.

In the NIR spectra, three peaks can clearly be seen at 1200 nm, 1450 nm and 1600 nm. These peaks describing the second overtone of C-H bonds, the first overtone of water molecules and the first overtone of O-H bonds, respectively (Impublications, 2016), which also can be seen in the Figure 3 in the introduction chapter. In the pre-treated spectra, the peak at 1200 nm is wider than the other parts of the spectra. It may depend on the fact that the analyzed samples contained slightly different amount of fat and therefore consisted of different amount of C-H bonds.

To validate the calibration, the amount of components was chosen to obtain the lowest RMSEP values for each calibration. The reason for that is the purpose with the NIR: to be able to predict new values from unknown samples in the future. There is always a risk to over-fit the calibration by choosing to many components (Håkansson, 2015). An optimal model should only describe the part of the data that contains relevant information (Håkansson, 2015). If a too large model is created, the noise can be included and the calibration gets a less accurate ability to predict new samples (Håkansson, 2015).

In the evaluation of the calibrations, R^2 and RMSEP were calculated. To get a clear overview, a table with all these compiled values are presented in Appendix 8.4, Table 16. R^2 should be a high value to be a good calibration and RMSEP should be a low value to be good at predicting new values in the future. In this master thesis, the values of the R^2 were always a bit lower for the combination. This indicates a bit weaker correlation in the combined calibration compared with the separate calibrations of fish oil and AkoFeed. The values of RMSEP are lowest for the fish oil when measuring the content of water, protein, fat and crude fiber, compare with the AkoFeed and the combination which are quite equal. This indicates that samples containing fish oil get better predicted values for these nutrients if the calibrations are separated. However, RMSEP was higher for the ash in fish oil than for the AkoFeed and the combination. As mentioned before, the content of ash was perceived to have a large measurement uncertainty, which may has affected the results.

Noteworthy, the content in the samples is almost identical, which make it easy to obtain a more specific calibration limited for these samples. In a more realistic world, the nutrients in the samples (even if it is same kind of animal feed) varies depending on the season, the price on the raw material on the market etc. A calibration that is fits for that kind of variation probably becomes less specific and the RMSEP would perhaps be higher. Consequently, if there would have been more variation between these samples, it could have reduced the prediction accuracy of new samples (Cao, 2013). Another aspect that may influence on the calibrations is the error source that come from the measurement uncertainly in the laboratory measurements (Swedac, 2016). The size of the measurements depends on several factors, such as the human factor, the environmental conditions and which kind of method that was used (Swedac, 2016).

The calibration method that has been chosen in this master thesis is PLS, which is a linear regression method. Perhaps, the calibrations could have been improved by using a nonlinear calibration method, which not is unusual in calibration of animal feed. However, in the figures that present the predicted values from the calibrations versus the calculated C-values of water, protein, ash and crude fiber, there is a point that could be an outlier. This point could only be seen for the calibration of the combination. Since the outlier shows in more than one calibration, it means that it was something in the spectra that was wrong. The pre-treated spectra were investigated and one spectrum was clearly different, this is presented in Figure 25. A hypothesis is that it could have been caused by non-homogeneity in the sample. Since this spectrum is from an AkoFeed sample, it could have been able to affect both the R^2 and RMSEP negatively for the calibrations of AkoFeed and the combination.

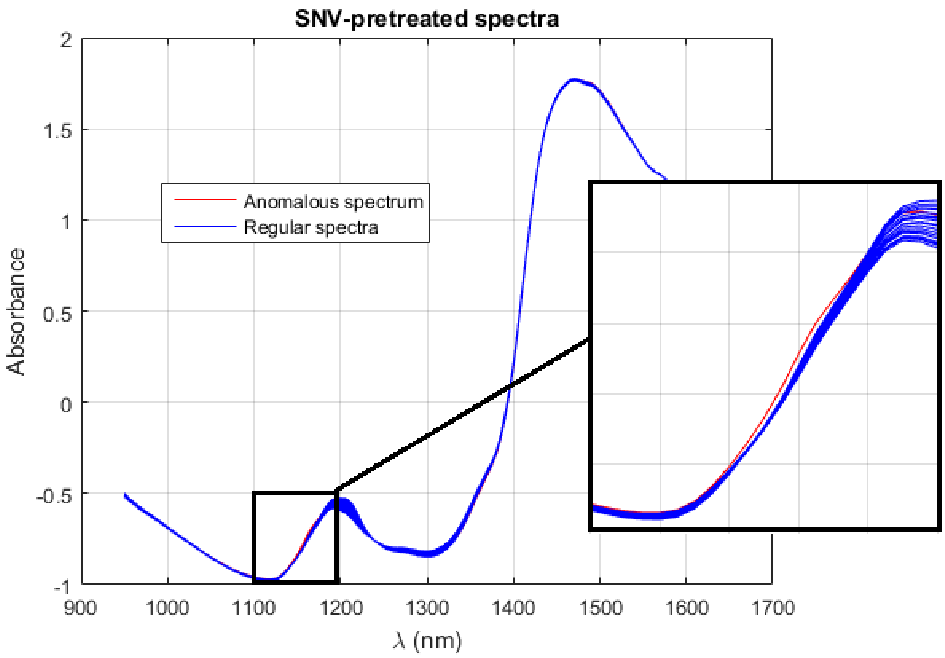


Figure 25. In the result section has an outlier been indicated in the calibration of the combination. The spectrum that belongs to an AkoFeed sample, which give the outlier point, was appeared clearly.

The aim, which was to investigate if different kinds of fat (limited to fish oil and AkoFeed) in sow feed influence the calibration, is considered to be achieved. There are small but essential differences in the calibration of fish oil compared to the calibrations of AkoFeed and the combination, where the separated calibration of fish oil is better to predict new samples for fish oil samples. However, to obtain a more reliable result, a larger study should be done, with several number of samples and a wider variation.

5. Conclusion

By comparing the ability to predict new values in unknown samples of three different calibrations for five different nutrients in sow feed, it could be observed that the calibrations of fish oil in general are more stable than the calibrations of AkoFeed and the combination. This applies for all the nutrients except the contents of ash, where the calibration of fish oil obtained the highest RMSEP value. The ability to predict new values for the AkoFeed and the combination were quite similar. However, the R^2 values were always a bit lower for the calibration of combination compared to the calibrations of fish oil and AkoFeed.

In the total, the calibrations of the fish oil were better than the calibrations of AkoFeed and the combination. Therefore, more accurate values of the sow feed containing fish oil would be predicted if the calibrations are separate, while it does not matter for the predicted values of the sow feed containing AkoFeed.

6. Future perspective

Due to the limitations in this master thesis, especially time-limitations, there are several factors that could be improved in a future project. For instance, only one recipe for one kind of sow feed, produced at the same day, was chosen. To get a more realistic result, the samples should include different kinds of sow feed, produced at different times in the season and consequently contain raw materials of different quality. Additionally, it could be possible to analyze more kinds of nutrients, such as starch and carbohydrate.

One other important part that can be developed is the number of samples that were used in the calibrations and the validations. To be able to get a reliable result, at least 20 samples should be used for validation and therefore in the calculation of RMSEP (ISO12099:2010(E), 2010). This number of samples were only achieved for the validations of the combinations, while only ten samples were used for the validations of fish oil and AkoFeed, respectively.

To get reference values to the calibrations and validations, only a few number of the samples was analyzed in the laboratory, due to time-limitations and costs. To get a reliable result, as many samples as possible should be analyzed in the laboratory and thereby avoid that the calibrations are based in calculated reference values. The purpose with creating a calibration on a NIR machine, is that the predicted values represents the analytical values. Therefore, the NIR and the laboratory values should be as equal as possible.

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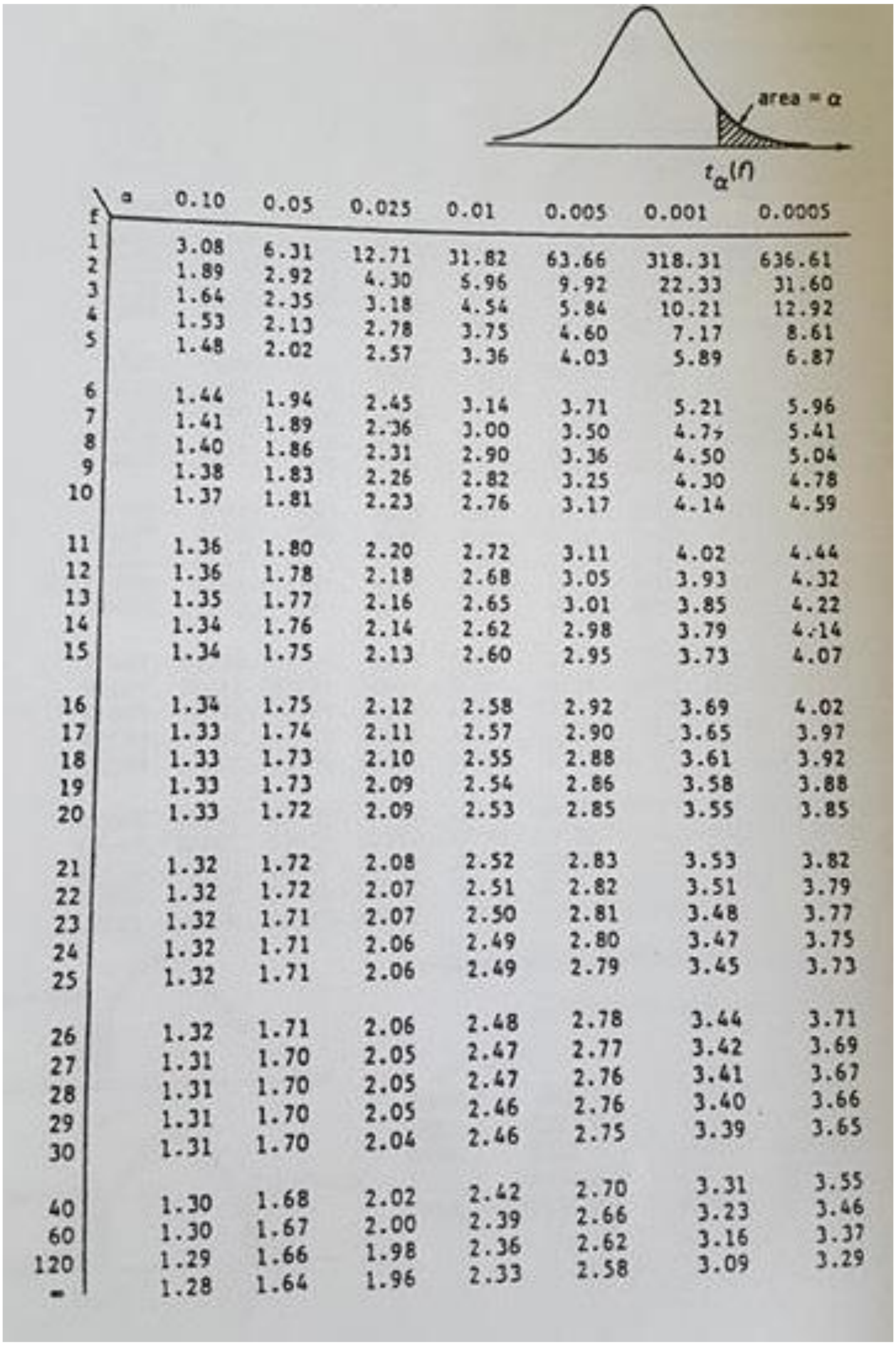
8. Appendix

8.1 T-test

Table 3. The quantile number (α/2) at t-test calculation (Olbjer, 2000). With a confidence interval of 95 %, $t_{\alpha/2}(n-1)$ were determined as $t_{0.025}(2)$ = 4.30 for water, protein and ash, and $t_{0.025}(3)$ = 3.18 for crude fiber.



f \ α	0.10	0.05	0.025	0.01	0.005	0.001	0.0005
1	3.08	6.31	12.71	31.82	63.66	318.31	636.61
2	1.89	2.92	4.30	5.96	9.92	22.33	31.60
3	1.64	2.35	3.18	4.54	5.84	10.21	12.92
4	1.53	2.13	2.78	3.75	4.60	7.17	8.61
5	1.48	2.02	2.57	3.36	4.03	5.89	6.87
6	1.44	1.94	2.45	3.14	3.71	5.21	5.96
7	1.41	1.89	2.36	3.00	3.50	4.7[illegible]	5.41
8	1.40	1.86	2.31	2.90	3.36	4.50	5.04
9	1.38	1.83	2.26	2.82	3.25	4.30	4.78
10	1.37	1.81	2.23	2.76	3.17	4.14	4.59
11	1.36	1.80	2.20	2.72	3.11	4.02	4.44
12	1.36	1.78	2.18	2.68	3.05	3.93	4.32
13	1.35	1.77	2.16	2.65	3.01	3.85	4.22
14	1.34	1.76	2.14	2.62	2.98	3.79	4.14
15	1.34	1.75	2.13	2.60	2.95	3.73	4.07
16	1.34	1.75	2.12	2.58	2.92	3.69	4.02
17	1.33	1.74	2.11	2.57	2.90	3.65	3.97
18	1.33	1.73	2.10	2.55	2.88	3.61	3.92
19	1.33	1.73	2.09	2.54	2.86	3.58	3.88
20	1.33	1.72	2.09	2.53	2.85	3.55	3.85
21	1.32	1.72	2.08	2.52	2.83	3.53	3.82
22	1.32	1.72	2.07	2.51	2.82	3.51	3.79
23	1.32	1.71	2.07	2.50	2.81	3.48	3.77
24	1.32	1.71	2.06	2.49	2.80	3.47	3.75
25	1.32	1.71	2.06	2.49	2.79	3.45	3.73
26	1.32	1.71	2.06	2.48	2.78	3.44	3.71
27	1.31	1.70	2.05	2.47	2.77	3.42	3.69
28	1.31	1.70	2.05	2.47	2.76	3.41	3.67
29	1.31	1.70	2.05	2.46	2.76	3.40	3.66
30	1.31	1.70	2.04	2.46	2.75	3.39	3.65
40	1.30	1.68	2.02	2.42	2.70	3.31	3.55
60	1.30	1.67	2.00	2.39	2.66	3.23	3.46
120	1.29	1.66	1.98	2.36	2.62	3.16	3.37
∞	1.28	1.64	1.96	2.33	2.58	3.09	3.29

8.2 Measurement of content of the nutrients in the samples

Table 4. Water content (in g), measured for seven different samples in 2.2.3. Duplicated samples were measured for C10F, C20F, C10A and C20A and mean values were calculated.

Sample	Amount of water (g)
Sample without any kind of fat 1	24.08
Sample without any kind of fat 2	24.78
Sample without any kind of fat 3	25.53
C10F	24.15
C20F	23.90
C10A	24.52
C20A	25.05

Table 5. Protein content (in g), measured for eleven different samples in 2.2.4. Duplicated samples were prepared and analyzed for all the samples and mean values were obtained.

Sample	Amount of protein (g)
Sample without any kind of fat 1	85.56
Sample without any kind of fat 2	86.31
Sample without any kind of fat 3	85.35
C10F	85.76
C20F	84.47
V5F	84.71
V10F	84.23
C10A	85.80
C15A	86.09
C20A	85.60
V4A	84.56

Table 6. Ash content (in g), measured for 13 different samples in 2.2.5. Duplicated samples were measured for all the samples except the three samples where no fat where added. For the duplicated samples were mean values calculated.

Sample	Amount of ash (g)
Sample without any kind of fat 1	42.75
Sample without any kind of fat 2	48.75
Sample without any kind of fat 3	43.00
C5F	47.37
C10F	46.94
C15F	49.89
C20F	48.41
V5F	47.28
V10F	48.25
C10A	47.51
C15A	46.26
C20A	46.59
V4A	45.99

Table 7. Fat content (in g), measured for 11 different samples in 2.2.6. Duplicated samples were measured for all the samples and mean values were calculated.

Sample	**Amount of fat (%)**
Sample without any kind of fat 1	2.90
Sample without any kind of fat 2	2.70
Sample without any kind of fat 3	2.70
C10F	5.50
C20F	8.20
V5F	5.25
V10F	7.95
C10A	5.65
C15A	7.40
C20A	8.65
V4A	4.75

Table 8. Crude fiber content (in g), measured for 5 different samples in 2.2.7.

Sample	**Amount of crude fiber (g)**
Sample without any kind of fat	17.50
C10F	16.20
C20F	17.51
C10A	18.95
C20A	17.60

8.3 Calculated content of the nutrients in the samples

Table 9. The calculated amount of content of water, protein, ash, fat and crude fiber in the samples containing added fish oil.

Sample	Water (%)	Protein (%)	Ash (%)	Fat (%)	Crude fiber (%)
C1F	9.910	34.25	18.70	3.109	6.993
C2F	9.880	34.14	18.65	3.359	6.972
C3F	9.850	34.04	18.59	3.616	6.951
C4F	9.819	33.93	18.53	3.876	6.929
C5F	9.790	33.83	18.48	4.124	6.908
C6F	9.762	33.73	18.42	4.371	6.888
C7F	9.730	33.62	18.36	4.647	6.866
C8F	9.701	33.52	18.31	4.894	6.846
C9F	9.672	33.42	18.25	5.151	6.825
C10F	9.642	33.32	18.20	5.414	6.804
C11F	9.240	33.22	18.14	5.675	6.783
C12F	9.583	33.11	18.08	5.942	6.762
C13F	9.552	33.01	18.02	6.220	6.740
C14F	9.523	32.91	17.97	6.477	6.720
C15F	9.494	32.81	17.92	6.741	6.699
C16F	9.463	32.70	17.86	7.029	6.677
C17F	9.434	32.60	17.80	7.292	6.657
C18F	9.404	32.50	17.75	7.570	6.635
C19F	9.375	32.40	17.69	7.842	6.615
C20F	9.375	32.29	17.63	8.124	6.594
V1F	9.890	34.75	18.66	3.275	6.979
V2F	9.835	33.97	18.55	3.779	6.937
V3F	9.770	33.76	18.44	4.298	6.894
V4F	9.711	33.56	18.33	4.808	6.853
V5F	9.651	33.35	18.21	5.330	6.810
V6F	9.591	33.14	18.10	5.864	6.768
V7F	9.533	32.94	17.99	6.385	6.727
V8F	9.474	32.74	17.88	6.926	6.685
V9F	9.414	32.53	17.76	7.481	6.643
V10F	9.355	32.33	17.65	8.031	6.601

Table 10. The calculated amount of content of water, protein, ash, fat and crude fiber in the samples containing AkoFeed.

Sample	Water (%)	Protein (%)	Ash (%)	Fat (%)	Crude fiber (%)
C1A	9.910	34.25	18.70	2.981	6.993
C2A	9.870	34.11	18.63	3.267	6.965
C3A	9.830	33.97	18.55	3.551	6.937
C4A	9.790	33.83	18.48	3.843	6.908
C5A	9.751	33.70	18.40	4.131	6.881
C6A	9.711	33.56	18.33	4.424	6.853
C7A	9.671	33.42	18.25	4.726	6.824
C8A	9.631	33.28	18.18	5.021	6.796
C9A	9.592	33.15	18.10	5.316	6.769
C10A	9.553	33.01	18.03	5.617	6.741
C11A	9.513	32.87	17.95	5.924	6.713
C12A	9.473	32.74	17.88	6.236	6.685
C13A	9.432	32.59	17.80	6.556	6.656
C14A	9.393	32.46	17.73	6.864	6.628
C15A	9.354	32.32	17.65	7.176	6.600
C16A	9.315	32.19	17.58	7.490	6.573
C17A	9.275	32.05	17.50	7.813	6.545
C18A	9.235	31.91	17.43	8.138	6.517
C19A	9.195	31.77	17.35	8.467	6.488
C20A	9.156	31.64	17.28	8.788	6.461
V1A	9.889	34.17	18.66	6.978	6.978
V2A	9.811	33.90	18.51	6.923	6.923
V3A	9.730	33.62	18.36	6.866	6.866
V4A	9.652	33.35	18.21	6.811	6.811
V5A	9.573	33.08	18.06	6.755	6.755
V6A	9.493	32.80	17.91	6.699	6.699
V7A	9.414	32.53	17.76	6.643	6.643
V8A	9.334	32.25	17.61	6.586	6.586
V9A	9.255	31.98	17.46	6.531	6.531
V10A	9.176	31.71	17.32	6.475	6.475

8.4 Overview of calculation RMSEP and R^2

Table 16. The calculated values of RMSEP and R^2 of the content of water, protein, ash, fat and crude fiber, to get an overview by comparison of these values.

	RMSEP	**R^2**	**Number of components**
Water content			
Fish oil	0.0051	0.9985	6
AkoFeed	0.0262	0.9945	4
Combination	0.0247	0.9738	2
Protein content			
Fish oil	0.0235	0.9996	6
AkoFeed	0.0910	0.9944	4
Combination	0.0863	0.9752	2
Ash content			
Fish oil	0.1393	1.000	8
AkoFeed	0.0484	0.9998	9
Combination	0.0473	0.9801	3
Fat content			
Fish oil	0.0581	0.9994	6
AkoFeed	0.1548	0.9998	9
Combination	0.1389	0.9966	7
Crude fiber content			
Fish oil	0.0049	0.9997	6
AkoFeed	0.0185	0.9944	4
Combination	0.0175	0.9750	2