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Rapid and Simultaneous Determination of Nutritional Constituents of United States Grown Oats Using Near Infrared Reflectance Spectroscopy (NIRS)

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RAPID AND SIMULTANEOUS DETERMINATION OF NUTRITIONAL CONSTITUENTS OF UNITED STATES GROWN OATS USING NEAR INFRARED REFLECTANCE SPECTROSCOPY (NIRS)

BY

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This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

˚C	Degree Centigrade
µm	Micrometer
1-VR	Variance Ratio
AACC	American Association of Cereal Chemists
AACCI	American Association of Cereal Chemists International
ANN	Artificial Neural Network
AOCS	American Oil Chemists Society
EFSA	European Food Safety Authority
FDA	Food and Drug Administration
Gm	Gram
GOPOD	Glucose Oxidase Peroxidase
LDL	Low Density Lipoprotein
Mg	Milligram
mM	Milli-molar
MSC	Multiplicative Scatter Correction
NIRS	Near Infrared Reflectance Spectroscopy
Nm	Nanometer
PCA	Principal Component Analysis
PCR	Principal Component Regression
PLS	Partial Least Square
RPD	Residual Predictive Deviation
RSQ	Coefficient of Determination of Calibration

RSQ_{val}	Coefficient of Determination of Validation
SD	Standard Deviation
SEC	Standard Error of Calibration
SECV	Standard Error of Cross Validation
SEP	Standard Error of Prediction
SNV	Standard Normal Variate
SSC	Soluble Solid Content
SVR	Support Vector Regression
TDF	Total Dietary Fiber
USA	United State of America
β- glucan	Beta-glucan

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ABSTRACT

RAPID AND SIMULTANEOUS DETERMINATION OF NUTRITIONAL CONSTITUENTS OF UNITED STATES GROWN OATS USING NEAR INFRARED REFLECTANCE SPECTROSCOPY (NIRS)

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Oats is a unique cereal owing to its nutritional and health benefits. Near Infrared Reflectance Spectroscopy (NIRS) is an efficient tool for monitoring the quality of cereal foods. NIRS holds potential for non-destructive multicomponent oat analyses with advantage of large sample throughput, speed, and reduced cost.

The purpose of this study was to develop predictive calibration models for estimating beta-glucan, protein, and oil content of US oat cultivars using NIRS and validated AACCI reference methods. A rapid, non-destructive (whole oat groat), and secondary NIRS method was developed to estimate beta-glucan, protein, and oil content based on the standard reference analyses procedures approved by AACCI.

Samples were collected from the 2014 to 2016 crop years from various locations in the United States (South Dakota, North Dakota, Minnesota, Washington, Iowa, and Wisconsin) representing a large geographical region and diverse genetic range (N=500). Predictive calibration equations were developed based on Modified Partial Least Square (MPLS) regression technique. Reference analyses were done by the following standard methods approved by AACCI and AOCS (AACCI method 32-

23.01 for beta-glucan, AACCI method 46-30.01 for crude protein, AOCS Am 5-04 for oil content and AACCI method 44-15.02 for moisture content).

Calibration for the estimation of beta-glucan content for ground oat groats yielded coefficient of determination (RSQ), standard error of calibration (SEC), standard error of cross validation (SECV) and one minus variance ratio (1-VR) ratio of 0.94, 0.16, 0.22 and 0.88, respectively. Whole oat groats beta glucan calibrations showed excellent RSQ, SEC, SECV and 1-VR of 0.93, 0.18, 0.23 and 0.89, respectively. Protein calibration for ground oat groats showed RSQ, 1-VR, SEC and SECV values of 0.93, 0.93, 0.61 and 0.64, respectively. For protein calibrations of whole oat groats, RSQ, SEC, SECV and 1-VR values of 0.92, 0.70, 0.80 and 0.89, respectively were obtained. Calibration from ground oat groats for oil content estimation yielded higher RSQ and 1-VR values of 0.93 and 0.92 and lower SEC and SECV values of 0.23 and 0.26, respectively. Oil content calibration with whole oat groats, RSQ, SEC, SECV and 1-VR values were 0.90, 0.27, 0.30 and 0.88, respectively.

Higher RSQ and 1-VR and lower SEC and SECV values provide evidence supporting the accuracy and precision of calibration models developed for beta-glucan, protein, and oil content estimation of oats. The study shows that NIRS is an efficient technology for oat quality measurement for large throughput breeding programs and in oat processing.

CHAPTER 1. INTRODUCTION

An increasing awareness by consumers on the importance of nutritious food for human health has drawn the attention of food processing industries on importance of healthy cereal grain like oat. Oats contain heart healthy soluble dietary fiber also called as β-glucan. This soluble fiber has become a key focus in the context of human health since it has been proven that it exhibits hypocholesterolemic effects (Anderson et al. 1984; Kirby et al. 1981). Compared to other cereals, oats have high protein content with many essential amino acids. The protein efficiency ratio and digestibility of oat protein are also high (Lockhart and Hurt 1986). Fat content in oats is equally important as it contains high amount of essential fatty acids compared to other cereals and is important from the nutritional point of view.

Over past three decades, Near Infrared Reflectance (NIR) spectroscopy has proven to be an efficient tool in monitoring and controlling the quality of a wide range of products. Quality parameters can be detected in the production line and can be compared with the real products processing environment and possible failures can be assessed early with this technology. NIR technology does not need extensive sample preparation and the analysis is simple and time-saving compared to chemical and enzymatic analytical methods. It can be used as an online (where the sample is diverted from the main streamline, measured by NIRS and returned to the process again), inline (sample is not diverted from main process stream and NIRS sensor is attached to the window of blender and takes spectra measurement in a continuous manner), offline (sample can be analyzed away from the process streamline) and at-line (sample is removed and analyzed by NIR close to the

processing stream) detection technique and can be used to measure several constituents of products at the same time. Through this technique, there will be permanent monitoring and the quality of product can be assessed. The first online application of NIR technology was used for the determination of various nutritional constituents like protein, fat, and moisture contents in ground beef by installing NIR instrument at the outlet of a meat grinder (Isaksson et al. 1996). NIR technologies offer the possibility of non-destructive, high-throughput sampling with low marginal cost per sample. They rely, however, on the regular and skillful performance of wet chemistry analyses (chemometrics) for calibration and validation if they are to generate accurate and precise output All of these properties make the NIR technique widely accepted as quantification tools in food science and crop quality (Huang et al. 2008; Krishnan et al. 1994a).

NIR spectroscopy is based on the absorption of electromagnetic radiation in the wavelength range of 780-2500 nm. The spectra that are obtained through scanning of the samples comprised of broad bands that arises from overlapping absorptions, and these correspond to overtones and vibrational modes of C-H, O-H and N-H chemical bonds (Huang et al. 2008; Osborne 2006). Because of this, it is a very practical system of measurement in organic and biological compounds. There are different types of NIR measurement modes with different applications that depend on the absorption, transmission, or reflection of the radiation. The best fitting modes also depend upon the type of products and the applications. Multiple variable calibration techniques such as principal component analysis (PCA) and partial least square (PLS) are often used to relate the spectra to the chemical information.

Today, NIRS technology is used by the grain and feed industries as a tool for rapid and simultaneous measurement of various quality constituents of raw as well as processed products. Very limited research work has been done on the calibration of NIRS for β-glucan, protein, and oil content in oats. Since NIRS is being a non-destructive and rapid screening technique, it helps in the quality determination of oats with reduced time, cost, and effort. The hypotheses tested in this study are as follows:

a. There are no statistical differences between NIRS methods and reference methods for measurement of β-glucan, protein, and oil content in oats.
b. There are statistical genetic and environmental effects that influence the variability of β-glucan, protein, and oil content of oat cultivars.

The objective of this research work was to analyze the nutritional constituents of oat cultivars grown mainly in the United States and to use the data to calibrate Near Infrared Reflectance Spectroscopy (NIRS). Thus, the objectives of this study are:

- To develop and validate ground oat groat and whole oat groat NIR spectroscopy calibration equations for the evaluation of β-glucan, protein, and crude fat content in United States grown oats and
- To study the variability in β-glucan, protein, and fat content in oat cultivars grown in South Dakota.

CHAPTER 2. LITERATURE REVIEW

Oats

Historical Perspective:

Throughout the western world, cereal crops have always played a role in human nutrition and thus directly or indirectly, have been involved in the socio-cultural development of those communities. Oat is one of the oldest cereal crops known to the mankind. Archaeological records reveal that oats were not in existence in northern Europe till the later prehistoric period. It was introduced together with rye as a weed contaminant in wheat and barley in early Greece from Asia Minor (South-western part of Asia). The oldest cultivated oats were found in Switzerland originating in the Bronze Age. As a cultivated crop, oats were present significantly at the end of prehistory. The Roman invasion of Britain during 43 A.D. brought the cultivation of oats to highland and lowland zones. Greeks referred to the crop as *vromos* or *bromos* and the Romans call them *avena* (Lásztity 1998). During the Trajan and Hadrian ruling period, oats were exported from northern Europe to Britain to sustain the Roman cavalry. This might have stimulated the production of this crop (Raftis 1974). Oats were first brought to North America by Spanish, English, and North European people (Coffman 1977). Climate change was also another compelling factor which encouraged the cultivation of oats favored by moist climate and decline in soil pH and this caused the marginalization of barley and wheat cultivation. From the late eighteenth century, a range of varieties and sub varieties of oats were developed and selected to suit local environmental conditions. In the nineteenth century, oat production expanded in response to the growing emphasis

on livestock production which provided the foundation for the activities of breeders and geneticists during the early decades of the present century (Welch 2012).

Importance of Oat crop

Oat is a low input cereal crop that is mostly grown in the great plains of United States and many other cooler regions of the world (Doehlert 2002). Oats are mainly grown for forage, animal feed and human consumption. It was important mostly for human consumption in most of Northern Europe but later used as animal feed, especially a choice for horses. In many parts of the world, oats are grown for grains as well as for fodder and forage, straw for bedding, hay, haylage, silage, and chaff (Stevens et al. 2004). Oat has a potential of generating threefold green fodder and about double the number of animals per item area can be fed compared to other traditional fodder crops (Hussain et al. 1993). Oats are important constituents of valuable feed for all classes of livestock such as horses, cows, poultry, young and breeding animals. Oats are rich in vitamin B_1, fats, protein, and minerals such as phosphorus and Iron (Stanton 1953). Thus, it contributes to the considerable need for fodder in terms of quality and nutrition (Dost 2001). Oats have been a part of the cuisine of the people living in cooler regions of the world. However, there is now a rising interest due to scientific evidence about the importance of oats in view of its high dietary value (Small 1999; Welch 2012). The increasing demand of milk and meat increase the need for more efficient means of their production through improved pastures and grain production. The dual purpose of grains owing to having proteins and vitamins besides carbohydrates have only increased the biological value of oats compared to wheat, maize, and barley.

Dietary importance of oats for human beings

Among all the cereals, oat is distinct because of its multifunctional characteristics and nutritional status. Recent scientific studies have provided evidence that oats contain various components of importance to human nutrition. It has a good source of dietary fiber specifically, β-glucan. Oats and its byproducts are proven to be helpful against diabetes and heart related diseases (Labeling 1997; Webster 2011). Oat bran is also considered as a good source of vitamin B-complex, protein, fatty acids, minerals, and dietary fiber β-glucans (Butt et al. 2008; Suttie and Reynolds 2004). Oat bran is also associated with the reduction of serum low density lipoprotein cholesterol in human beings (Anderson et al. 1984; Kirby et al. 1981). This has prompted the industrial preparation of oat bran and the incorporation of oat bran into the human diet. Compared to other cereals, oat possesses high amounts of well-balanced proteins and could be a choice to those communities of the world where the diet is mainly plant-based (Peterson 1992). Oat also contains high amount of oil compared to other cereals which is about 3 to 12 % on a dry weight basis (Saastamoinen et al. 1989). Besides this, oat also contains tocopherols (20-30 μg/100g of grain) (Nechaev and Sanbler 1975) and water-soluble vitamins such as folate and biotin (Lásztity 1998). Oat bran is about 50% of oat groats and has at least 16% of total dietary fiber (TDF) on a moisture free basis and of which 33% is soluble (Williams et al. 1991; Wood et al. 1991).

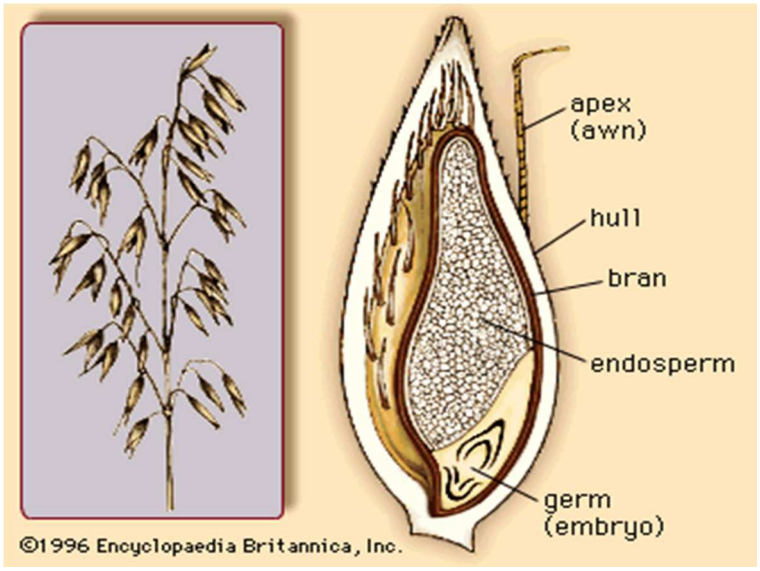

Figure 1. Structure of Whole Grain Oat (Image source: kellyspantry.blogspot.com)

β-glucan in oats

β-glucan is a viscous polysaccharide that is made of units of monosaccharides D-glucose and comes from the endosperm cell walls of the kernel (Figure 1. and Figure 2). About 20-30% of the weight of oat kernel consists of hulls that contains about 90% of total insoluble fiber. The main component of oat soluble fiber is a linear polysaccharide (1, 3), (1.4) β-D-glucan, also known as β-glucan. It is a large, linear homopolysaccharide mainly composed of β (1→3) linked cellotriosyl and cellotetraosyl units with few numbers of consecutive (1→4) linked units and is found mainly in oats and barley (Cui and Wood 2000). Since it is present in the endosperm cell walls, adjacent to the aleurone layer, it varies greatly among different cultivars of oats (Fulcher and Miller 1993) and is also affected by the growing location, storage conditions, and processing of the oat grain (Wang and Ellis 2014). Variation in β-glucan content by 2 to 3-fold among oat cultivars have been shown (Ajithkumar et al. 2005; Peterson et al. 1995). Various surveys have shown that the β-glucan content in oats ranges from 1.8-5.5% on a dry weight basis and many varieties occur in the range of 4.5 to 5.5% (Dvoncova et al. 2010; Saastamoinen et al. 1989) while some varieties have also been reported with β-glucan content of 7% (Decker et al. 2014).

This polysaccharide dissolves readily in water to provide highly viscous solutions and is the main component of oat gum (Wood and Webster 1986). The role of β-glucan in human nutrition and health is very important. Oat products as a source of soluble dietary fiber have recently become the key focus in the nutritional and medicinal communities. Rich in water soluble fiber, oat products are believed to have distinct hypocholesterolemic effects in human beings (Anderson et al. 1984). The knowledge of

relation between the components present in oats and human health has increased interest among producers, processors, and consumers in the last two decades and β-glucan is considered as a functional and bio-active compound (Cui and Wood 2000; Lazaridou et al. 2004). Studies have shown that β-glucan can lower serum total and LDL cholesterol in hypercholesterolemic people thereby reducing the risk of cardiovascular disease (Anderson et al. 1990; Herrera et al. 2016; Queenan et al. 2007; Wolever et al. 2010). β-glucans are also associated with the reduction of postprandial serum glucose levels in human beings as well as in animals (Bhatty 1999). Besides this, β-glucans are also used as thickening agents to change the texture in foods such as gravies, salad dressings, ice-cream (Peter 2011), and as fat mimetics in the manufacturing of low calorie foods (Lee et al. 2005). After the health claims approval by different food standard agencies such as the Food and Drug Administration (FDA) of United States of America (USA) (2002), European Food Safety Authority (EFSA) (Efsa Panel on Dietetic Products and Allergies 2011), Health Canada of Canada and Food standards Australia New Zealand of Australia and New Zealand, the demand of oat crop in food processing has increased. Hence, the consumption of oat has increased in recent year due to the presence of health enhancing nutritional constituents (Marquart et al. 2001).

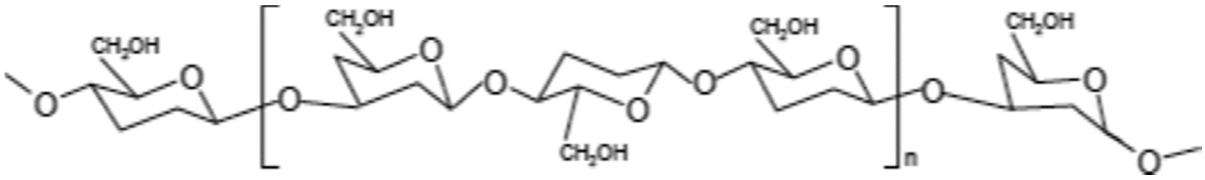

Figure 2. Structure of oat beta-glucan (Daou and Zhang 2012)

Protein in oats

Oat groats commercially grown in United States have protein content in the range of 12.4 to 24.4 % (Fulcher and Miller 1993; Pomeranz et al. 1973; Robert et al. 1983), and changes in this content do not influence the proportion of amino acids in oat (Humphreys et al. 1994b). In the oat seed, the embryo contains the highest amount of protein (30%) and the endosperm has about 10%. Hulls have relatively low amounts of protein at approximately 2% (Youngs 1972). Difference in total protein content in oat cultivars are mainly brought about by changes in lysine, methionine, and tryptophan (Frey 1952). Globulins (protein being soluble in salt and insoluble in water) account for the major portion of total protein (40-50% to 70-80%) in oats (Anja 1994; Lásztity 1998). Oat is the only cereal where the primary storage protein is classified as globulins (Peterson 1978). Organic solvent-soluble prolamines of oats, unlike those of other cereals, account for a lower percentage of the total protein for oats (Frey 1951; Peterson and Smith 1976). Oat protein is considered nearly equivalent to the protein quality of soybean and soy protein, which are in turn, considered equivalent to milk, meat, and egg protein (Ahmad et al. 2014). So, protein in oats are considered to have high nutritive value (Hischke et al. 1968) along with functional properties (Ma et al. 2001; Yung Ma 1983).

Oil content in oats

Lipid content in oats is considerably high when compared to other cereals with high level of essential linoleic acid (Mattila et al. 2005). Fat content in oats is about 3-12% on a dry weight basis (Brown and Craddock 1972). The fatty acid composition in oats is important from a nutritional viewpoint. It consists of unsaturated fatty acids such as oleic acid, linoleic acid, and linolenic acid and saturated fatty acids such as myristic acid, palmitic acid, and stearic acid. Oleic acid, linoleic acid, and palmitic acid, however, are found in high levels (Hammond 1983; Youngs 1986) whereas myristic, palmitoleic, eicosenoic, arachidic and erucic acids are found in very low amount (Saastamoinen et al. 1989). Linoleic and linolenic acids are essential fatty acids and are important for human health (Krishnan et al. 2000). Various studies have shown that both genetic and environmental conditions affect the amount of fat content in oats (Humphreys et al. 1994a; Saastamoinen et al. 1989; Welch 1975). It has been also reported that there is a negative correlation between protein and oil content among different varieties of oats (Brown et al. 1966; Forsberg et al. 1974) whereas some reported no correlation between protein and oil concentration (Schipper and Prey 1992; Silva et al. 2008).

Varieties and growth condition

Mostly, oats are grown in cool and moist climates and they are sensitive to hot and dry weather. Oat is an annual plant and can be grown in autumn and spring. Most spring sown cultivars contribute to the major production of oats. However, autumn cultivation is also practiced in higher altitude areas such as the Himalayan Hindu Kush range and those places where the climate is hot and dry (Ahmad et al. 2014).

Near-Infrared Reflectance Spectroscopy (NIRS)

Historical perspective

NIR technology was first discovered by Frederick William Herschel in 1800. NIRS was later revived in the 1950s and was put into use in the 1970s, about 150 years later. Coblentz in 1900 was the pioneer who was able to obtain the spectra of pure substances and verified the use of Near Infrared in the detection of organic compounds (Pasquini 2003). The revival of NIRS and its use, was first documented by Karl Norris (Norris 1996) and he is also known as a pioneer of NIRS development. NIRS technology was first used in the cereal industry and later became the choice for various applications because of the development in instrumentation, chemistry, and multivariate data analysis system (Manley 2014). Determination of moisture, protein and oil was the first successful work on NIR during 1970's (Law and Tkachuk 1977). Over past 30 years, this technology has proved to be one of the most efficient ways of continuous monitoring and control of product quality in food manufacturing industries (Huang et al. 2008). With time, NIRS instruments are also continually changing with the availability of new and more advanced software, features and flexibilities (McClure 2003).

Uses of NIRS

NIRS enables the measurement of samples with very little or no sample preparation and can be used on solid objects. Advantages such as being chemical free, rapid, low cost and effort makes NIRS more applicable in high throughput analyses. In principle, NIRS can only be used to measure the properties of constituents which are made of organic components and whose molecular bands absorb near infrared rays in the electromagnetic spectrum. NIRS can be used to measure the quality of both food and non-food materials.

It can be used to measure properties of non-food products like wood and wood products (Meder et al. 2010), soil (for example: soil composition and soil texture) (Stenberg et al. 2010), medical (Ferrari et al. 2012) and pharmaceutical products (Jamrógiewicz 2012). It is also equally applicable to measure food and food products such as meat and meat products (Prieto et al. 2009), milk and milk products (Cattaneoa and Holroydb 2013), fruits and vegetables (Nicolai et al. 2007), beverages (Cozzolino et al. 2006) and cereals and cereal products (Woodcock et al. 2008). NIRS has been used for the direct measurement of various constituents in grain and grain products such as corn (Campbell et al. 1999; Wehling et al. 1996), rice (Kawamura et al. 1999) and wheat (Delwiche and Hruschka 2000; Ozdemir 2006).

Use of NIRS for oat quality

Considerable literature shows the utility and robustness of measurements generated using NIRS for the testing of grain nutritional characteristics. Few works have been done on the calibration of NIRS for the measurement of constituents in oats (Krishnan et al. 1994b; Krishnan et al. 2000; Silva et al. 2008) and its use for the routine analysis of oat constituents. (Williams et al. 1991) studied the analysis of oat bran products through NIRS. This technology has been successfully used for the determination of protein and oil content in oats (Hymowitz et al. 1974; Krishnan et al. 1994a; Silva et al. 2008), evaluation of groat percentage in oats (Redaelli and Berardo 2002), and the determination of fiber components in oats (Redaelli and Berardo 2007). NIRS was used for the determination of soluble fiber β-glucan in barley (Allison et al. 1978; Henry 1985). Limited work, however, has been done to measure β-glucan concentration in oat. Others have studied the use of NIRS (Bellato et al. 2011) and FT-NIR (Wang et al. 2014) for the

measurement of nutritional contents of oats including β-glucan. NIRS measurement of β-glucan is more challenging and is less accurate than the measurement of protein in oats (Cervantes-Martinez et al. 2002).

Calibration Development

NIRS spectra are resulted when there is absorption of energy by organic molecules through molecular bond vibration and consist of overtones and combination bands. Due to having large number of vibrations in molecular bonds, it is very difficult to extract useful information from the spectra (Manley 2014). So, appropriate regression techniques are applied to establish relationship between spectra data points and data from chemical analysis through which appropriate calibration model is developed.

Multivariate data analysis

NIRS spectral data are multivariate in nature since they contain large number of data points i.e. one data point in each wavelength for each sample. Besides that, differences among samples are only through small spectral differences. For this reason, multivariate data analysis is necessary to relate spectral data to compound concentration. Multivariate data analysis consists of two steps i.e. spectral preprocessing and calibration model development (Manley 2014). Different calibration methodologies such as principal component regression (PCR) (Thomas and Haaland 1990), partial least square (PLS) (Chen et al. 2005; Geladi and Kowalski 1986), artificial neural network (ANN) (Borggard and Thodberg 1992) and support vector regression (SVR) (Belousov et al. 2002) are widely used in quantitative calibration analysis.

Spectral pre-processing

Because of the differences in small wavelengths in NIR electromagnetic spectrum and differences in size of particles of samples, undesirable scatter effects are created that lead to non-linearity in spectra. So, spectral pre-processing is important to reduce or remove undesirable background information and to increase the signal (Rinnan et al. 2009). The common pre-processing methods are normalization, derivatives, multiplicative scatter correction (MSC) (also called normalization) and standard normal variate (SNV) (Agelet and Hurburgh Jr 2010; Rinnan et al. 2009). The first step in the spectral preprocessing is called averaging. It is used to decrease the number of wavelengths and increase smoothing in spectra. Most spectrophotometers provide better spectral resolution which ranges from 1 to 2nm. A study was done by artificially inflating the spectra from 2nm to 0.2nm and the accuracy of model was lowered. However, accuracy was improved by removing high spectral resolution through wavelength compression (Nicolaï et al. 2007). The second step is called centering, and this is obtained by subtracting averages from each of the variables. Centering is done to interpret all the results around the mean (Nicolai et al. 2007) and usually done with principal component analysis (PCA). Smoothing is done to remove noise from the spectra. The next step in spectral preprocessing is called normalization. There are various techniques in chemometrics to normalize the spectra, but multiple scatter correction is the most popular one (Næs et al. 2002). It is used to remove baseline shift (additive effect) and multiplicative effects (tilt), which are created due to difference in light path length because of uncontrollable physical variations such as unequal distribution of particles, size of particles, sample packing (density) variability and refractive index. These unwanted variations become

unrelated to chemical response and might affect subsequent analysis. The normalization process tries to remove the scattering effect by linearizing each spectrum with the ideal spectrum of the sample. Standard normal variate correction is also a normalization technique in which each spectrum is normalized to zero mean and unit variance. The final step in data preprocessing is Derivation. Derivative is the measurement of slope of spectrum at each point of the spectrum and this is suitable method to remove baseline shift (Barnes et al. 1989). Derivatives are also applied to remove overlapping peaks (Manley 2014).

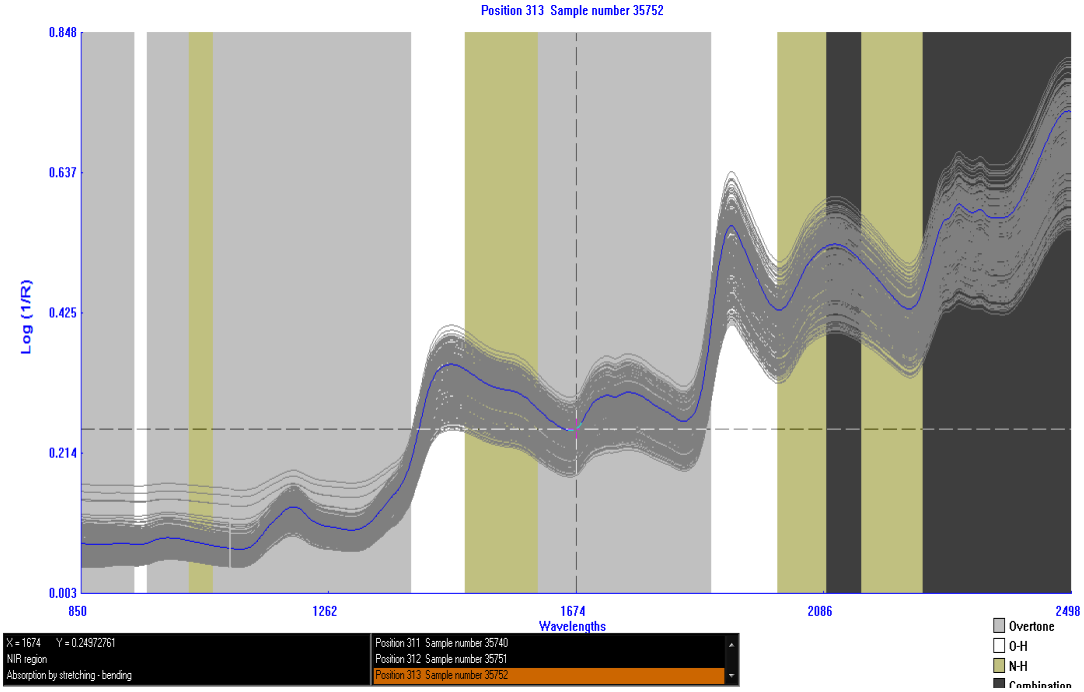

Figure 3. Spectra images on NIRS

Development of calibration model and validation

Coefficient of determination of calibration (RSQ) and validation (RSQ_{val}) dataset, standard error of calibration (SEC), standard error of cross validation (SECV), standard error of prediction (SEP), variance ratio (1-VR), bias and residual predictive deviation (RPD) are the statistical terms which determine the accuracy of NIRS calibration models and validate that the model is robust enough to measure unknown samples (Konieczka and Namiesnik 2016). RSQ, SEC, SECV and 1-VR values are obtained through calculation based on samples used for calibration while RSQ_{val}, SEP and bias are obtained through calculation based on the validation datasets i.e. samples which were not used in the calibration model development. Bias is the statistical term which shows the difference in results that is predicted by NIRS and obtained through reference analysis (Shenk et al. 2001).

The term 'validation' describes an independent set of samples which are used to measure the accuracy of calibration model developed (Mark and Workman Jr 2010). Appropriate external validation dataset is very important to determine the robustness and successful application of multivariate calibration models. Most of the NIRS calibration models that have been cited are based on the validation dataset which have been pooled out from the same sample batches and it is important to prove that the model developed is robust enough for different batches (Nicolai et al. 2007). A study tested the accuracy of calibration model developed for dry matter by using one season's samples and validated by different season samples (Lovász et al. 1994). Lack of robustness in the calibration model to measure soluble solid content (SSC) in pineapple and melon was found in another study (Guthrie et al. 1998). Thus, to obtain robustness in the calibration model,

samples from different seasons and different locations should be included and robustness is the only issue of concern in NIRS calibrations development (Nicolai et al. 2007).

Importance of Repeatability file in NIRS calibration

The Repeatability file is made by scanning few samples (usually 3-4) for multiple times under different conditions. It helps to minimize the unwanted information in the calibration model development of NIRS by correcting error sources. These files contain the same fixed samples and designated with same name. The variation in any single spectra relates to changes in condition applied during scanning such as temperature effects, particle size effects, and compaction of sample. During calibration equation development, the repeatability file is used to negate the effects of those spectral wavelengths which are significantly affected by change in conditions during scanning (Tillmann and Paul 1998). Use of the Repeatability file has been successfully used and it has improved the calibration model (Shenk and Westerhaus 1991).

Disadvantages of NIRS

One of the major disadvantages of NIRS is that it depends on obtaining successful calibration procedures. In addition, this technique has low sensitivity to minor constituents (Büning-Pfaue 2003). Sensitivity limit is 0.1% for major quality constituents (Iwamoto and Kawano 1992). Because of the difference in optical instrumentation, transfer of calibration equation from one instrument to another instrument is limited (Givens et al. 1997). There are no accurate calibration models which account for the interaction between NIRS light and constituents and hence the calibration is purely empirical in most of the cases. Though a lot advancement has been made in the

calibration procedure of NIRS, specified and widespread accepted methodology has not occurred yet (Blanco and Villarroya 2002).

CHAPTER 3. MATERIALS AND METHODOLOGY

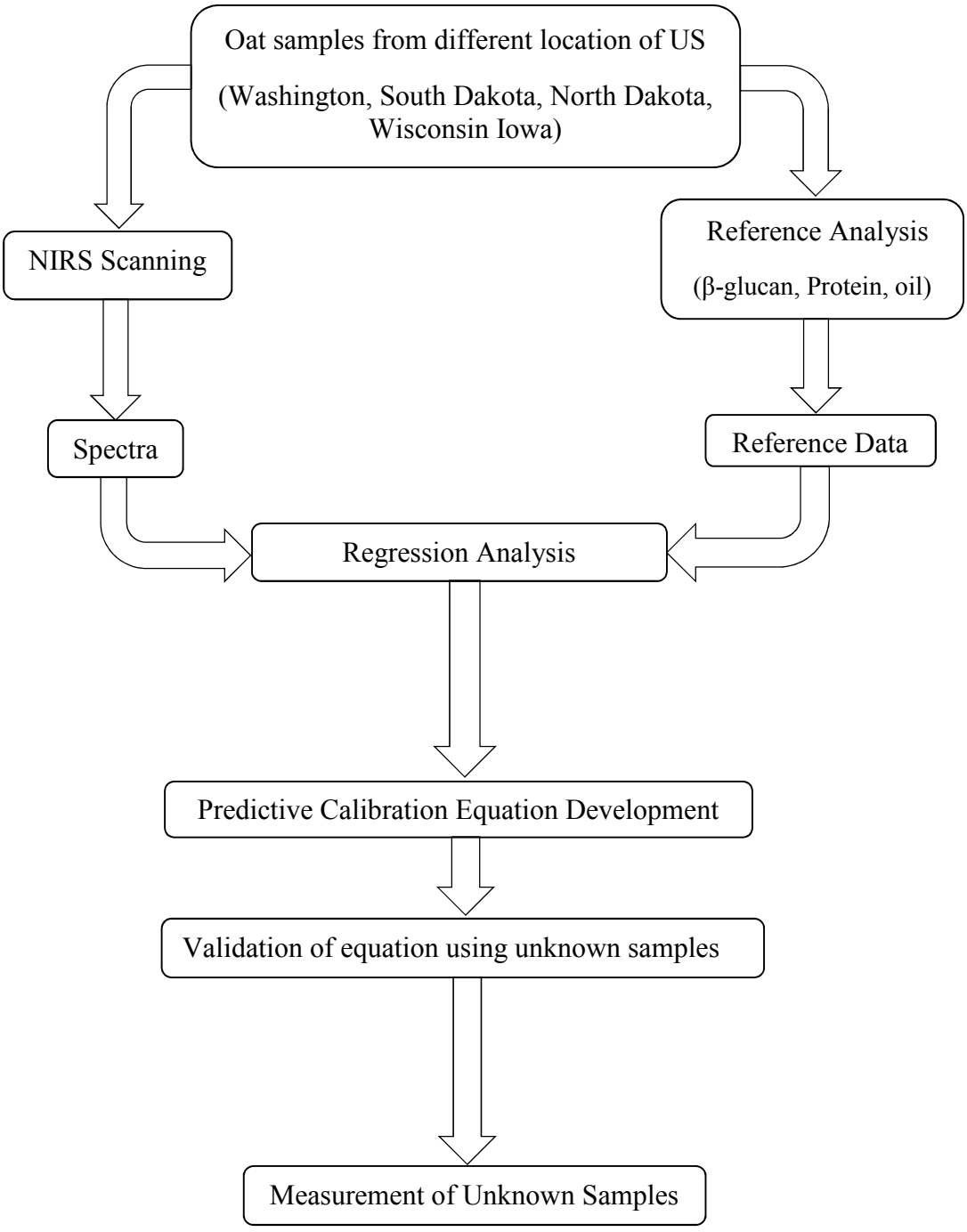

Figure 4. Flow diagram summarizing experimental methodology for development of predictive NIRS calibrations and their validation

Sample preparation

A total of 535 oat samples from South Dakota (2015 and 2016 crop year) and Washington (2014 and 2015 crop year) were used for protein and oil determination and 417 oat samples from different locations of South Dakota, North Dakota, Iowa, and Wisconsin (2014-2016 crop year) were used for beta-glucan determination, representing a large genetic range. Seventy-five grams (75 g) of subsamples were taken and dehulled by using a laboratory dehuller LH5095 (Codema LLC). After dehulling, hulls and undehulled kernels were removed. About half of the dehulled samples were dried-milled to a particle size of <500μm using a Retsch Brinkmann centrifugal grinding mill (Retsch GmbH, Hann, Germany) before reflectance measurement and chemical analysis for β-glucan, protein content and oil content in oat samples. The remaining half of the sample was scanned on the NIRS for the whole grain calibration development.

Analytical Procedure

Approved methods of analytical procedures for the reference analysis of β-glucan, protein and oil content in oat were used and the data were used for NIRS calibration. All the methods were approved by American Association of Cereal Chemists International (AACCI method 32-23.01; AACCI method 46-30.01) and American Oil Chemists Society (AOCS Am 5-04).

Moisture Analysis

Moisture content of all oat samples was determined so that the β-glucan, protein, and oil content values could be estimated on a dry weight basis. Moisture content was

determined by a standard air oven method approved by AACCI (AACCI method 44-15.02).

Chemical analysis of β-glucan

β-glucan content in different varieties of oat samples were determined by using AACCI method 32-23.01 (AACCI, 1999) which consists in employing a mixed β-glucan linkage kit (Megazyme international Ireland Ltd., Wicklow, Ireland). Data were expressed on a dry weight basis through moisture correction. The reference analysis of β-glucan, protein and fat was done in duplicate. For β-glucan analysis, samples from each location were used in replicate. All the laboratory values of β-glucan, protein and fat were expressed on a dry weight basis. Standard barley flour and oat flour with known level of β-glucan i.e. 4.1% and 8% or 4.1% and 7.5% (on a dry weight basis) were included in every set of analyses.

Method of β-glucan analysis

In determination of β-glucan, oat samples were first hydrated by adding sodium phosphate buffer solution (pH 6.5) and then treated with the enzyme Lichenase which depolymerize the β-glucan polysaccharide into β-gluco-oligosaccharides. An aliquot of the resulting filtrate was then treated with the enzyme β-glucosidase which degraded the oligosaccharide molecules into D-glucose monomers. The concentration of resulting glucose was then measured using GOPOD (Glucose oxidase peroxidase) reagent in spectrophotometer. The principle of β-glucan analysis is illustrated in Figure 5.

About 80-120mg of ground oat sample was weighed accurately into a glass centrifuge tube of about 17ml capacity. Sample tubes were tapped well to ensure that all the sample

material fell to the bottom of the tube. Exactly 200µl (0.2ml) of aqueous ethanol (50% v/v) was added to wet the sample. Addition of ethanol helped in dispersion of sample. Small magnetic stirrer was added to the tube to prevent clumping of samples. An aliquot (4ml) of Sodium phosphate buffer (20mM, pH 6.5) was added and samples were stirred on a vortex mixer. After mixing, sample tubes were placed immediately in a boiling water bath and incubated for 1 minute. Then the tubes were vigorously stirred and again incubated in the boiling water bath for a further 2 minutes and then stirred again. Then, the tubes were taken out and incubated at 50ºC and allowed to equilibrate for 5 minutes. Lichenase enzyme of about 0.2ml was added to each sample tubes and the content of the tube were stirred. The tubes were then sealed with parafilm and incubated for 1 hour at 50ºC with regular vigorous stirring on a vortex mixer for about 3 to 4 times to ensure that the enzyme lichenase digested all the contents of the tube. After the incubation is completed, 5ml of sodium acetate buffer (200mM, P^H 4.0) was added and contents of the tube were vigorously mixed using a vortex mixer. The sample tubes were allowed to equilibrate at room temperature for about 5 minutes and centrifuged for 10 minutes at 1000 g or filtered through grade 41 filter paper. After the supernatant or filtrate was separated, 0.1ml of sample was carefully dispensed to the bottom of three test tubes of 12 ml capacity. An aliquot (0.1 ml) of β-glucosidase enzyme in 50mM sodium acetate buffer was added to two of these tubes which are reaction tubes. The enzyme β-glucosidase digests the content of the tubes. To the third tube, 0.1ml of 50mM sodium acetate buffer (without enzyme) was added. All the tubes were incubated at 50ºC for 10 minutes. After that, 3ml of GOPOD (Glucose Oxidase Peroxidase) reagent was added to each tube and the tubes were incubated at 50ºC for further 20 minutes. Then, the tubes were removed

from incubation bath and absorbance reading was taken at 510nm against a reagent blank on a spectrophotometer within 1 hour. One and half milliliter (1.5ml) cuvettes were used for taking absorbance readings. The values were imported to Mega Calc. software provided by Megazyme Inc. where the amount of β-glucan was calculated on an "as is" basis. Control samples of barley flour and oat flour provided in the kit was used on every run to ensure that the same protocol was applied to all the samples. With each set of enzymatic analyses, reagent blank and D-glucose standards of 50µg and/or 100µg were used in duplicate to calculate the F-factor. Reagent blanks contained 0.1ml of distilled water, 0.1ml of sodium acetate buffer (50mM) and 3ml of GOPOD reagent. The glucose standard solution had 0.1ml of sodium acetate buffer (50mM), 0.1ml of D-glucose standard (50µg/0.1ml or 100µg/0.1ml) and 3ml of GOPOD reagent (McCleary and Codd 1991).

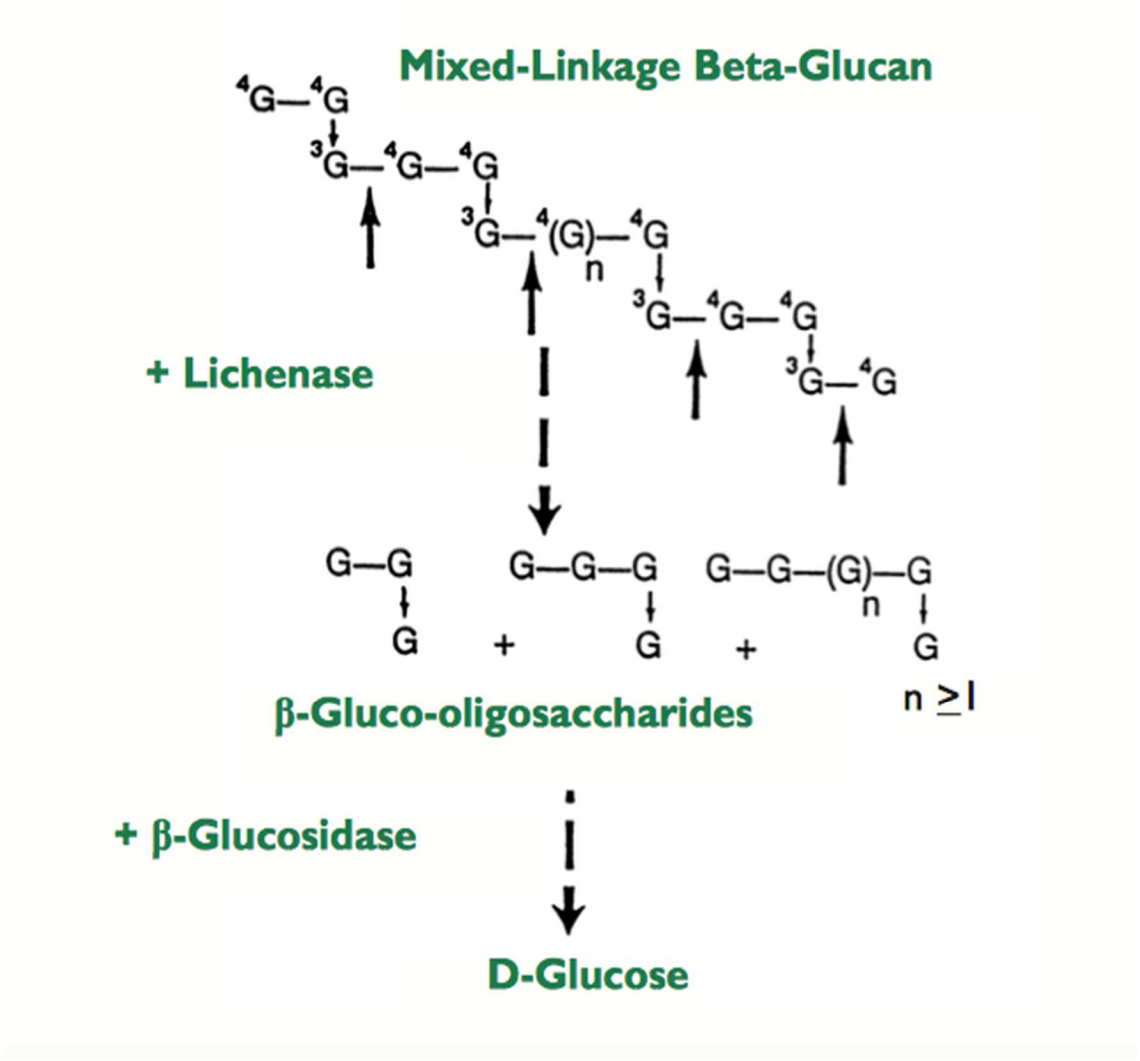

Figure 5. Principle of the enzymatic analysis of β-glucan (Source: www.megazyme.com)

Protein Analysis

Protein analysis was carried out using the Enhanced Dumas combustion method 46-30.01 (AACCI, 2000) using a CE Elantech Flash EA 1112 (ThermoFinnigan Italia S.p.A., Rodano (MI) Italy). In this method, samples of known mass, about 75mg, were incinerated in a high temperature oven of around 900˚C chamber temperature in the presence of oxygen. Due to the high temperature, N_2, CO_2 and H_2O were released from the samples. These gases passed through special columns where H_2O and CO_2 were absorbed by magnesium perchlorate and soda lime in the column. The N_2 gas was then measured by passing the remaining gas through the column called a thermal conductivity detector (TCD). The signal from TCD was converted to N_2 content. A conversion factor of 5.83 was used to calculate percentage protein from percentage nitrogen content in various oat samples. All the protein values were expressed on a dry weight basis through moisture correction to enhance the NIRS calibration equation development.

Oil Analysis

Oil content analysis was carried out using standard AOCS procedure Am 5-04 using the instrument called ANKOM^{XT15} Crude Fat extractor (ANKOM Technology, Macedon, New York, USA). This instrument extracted crude oil using conventional solvents, mainly petroleum ether. The extracted compounds were triacyclglycerols along with small amount of lipids. The analysis was done by measuring the loss of mass after extraction of fat or oil from the sample contained in the filter bags. The isolation of sample was completed by surrounding the sample in a sealed filter bag with a filtering capacity of 2 to 3 microns. The filter bags were made of polymeric material which could

withstand high temperature and solvents used in the instrument. Samples needed to be pre-dried before extraction by heating at 103ºC for 3 hours. Moisture content was calculated after drying the samples. About 15 samples could be run at one time and it was relatively easy to use this system compared to other conventional Soxhlet-based fat extraction instruments. Filter bags with samples were placed in the extraction vessel and the extraction time and temperature were selected. Then, the instrument automatically filled the extraction vessel with solvent, extracted the fat from samples and recycled the solvent.

This procedure began with the numbering of all the empty filter bags using a solvent resistant marker. Weights of all filter bags were taken prior to filling them with samples. Then, an empty filter bag was placed in the bag holder. Weights of filter bag in the holder were tared together. Approximately 1.5 to 2gm of samples were added to each filter bags. Then initial weight of samples with filter bags were recorded (W1). Then, percentage of moisture was calculated:

$$\text{Percentage Moisture (\%)} = \frac{\text{(Filter bag weight + sample weight) – weight after drying}}{\text{Sample weight}}$$

Filter bags were sealed using a heat sealer within 4mm of its open end. The seal could be observed as a solid melted stripe towards the top edge of the filter bag. If the seal was not tight, bag was then resealed. After this, all the samples were dried at 102ºC ± 2ºC for 3 hours to remove moisture prior to extraction. Then, all the samples were removed from oven and placed in desiccant pouch. Samples were allowed to cool to room temperature for 10 to15 minutes. All the filter bags were reweighed (W2), samples were now ready

for extraction. After the extraction time was completed, the Teflon insert from extraction vessel was removed and bag holder was taken out from Teflon insert. All the filter bags were removed from bag holder and placed in an oven for about 15-30 minutes at 102ºC ± 2ºC. The filter bags were then placed in a desiccant pouch and allowed to cool for about 10-15 minutes. Weights of each filter bag was taken immediately after removal from desiccant pouch (W3). Finally, the bottom of the vessel was cleaned using applying petroleum ether in a paper towel and stiff wire brush ensuring that no oil residue left in the bottom of the vessel.

Crude oil contained within a sample can be calculated by applying following formula:

% Crude oil = ((W2-W3)/W1) *100

Where, W1 = Original weight of sample

W2 = Weight of pre-extraction dried sample and filter bag

W3 = Weight of dried sample and filter bag after extraction

Spectroscopic analysis

The Near Infrared Reflectance spectra of whole and ground oat samples were obtained by scanning the samples on NIRS DS2500 analyzer (Foss North America).

Calibration Development

Spectral measurements were related with the reference data from chemical analysis by using multiple variables-based regression methods to develop a calibration model. Data were centered using modified PLS and spectral outliers were identified and removed.

Spectra were processed with multiplicative scatter correction to partially correct the baseline differences by implementing standard normal variate (SNV) and detrend. Mathematical treatment of 1,4,4,1 was applied for spectra with 2nm wavelength resolution and 1,16,16,1 was applied for spectra with 0.5nm wavelength resolution. Cross validation was used to identify outliers, to choose the optimum number of Partial Least Squares (PLS) or principal component analysis (PCA) factors in the calibration model and to estimate the performance of calibration model while predicting unknown samples (Bellato et al. 2011; Kays et al. 2005). Best calibration models were chosen based on the lowest possible standard error of calibration (SEC), lowest standard error of cross validation (SECV), greatest value of one minus variance ratio (1-VR) and greatest coefficient of determination (RSQ). Separate set of samples whose values were known through chemical analysis were separated from the pool of the total sample sets and they were called the Validation data set. About 25% of the total samples were used as a validation set.

Software

To collect the spectra by scanning of samples, ISIscan Nova version 7.0.0.187 was used and the spectra was auto-synchronized with the program software called Mosaic Solo. Spectra were exported from Mosaic solo. WinISI Project Manager version 4.8.00 was used for calibration development.

Statistical Analysis

The correlation study was used in the validation of NIRS prediction based on calibration equation. Paired comparison t-test was applied to determine the difference between

means at 95% confidence interval ($P < 0.05$). Differences among varieties and growing locations in terms of β-glucan, protein and fat content were studied through analysis of variance components by using Analysis of Variance (ANOVA) and Duncan Multiple Range Test. Analysis was done using R software version 1.0.136.

CHAPTER 4. RESULTS AND DISCUSSION

Determination of β-glucan, protein and oil content in oats were performed by using standard reference analytical procedures. Determination were made both on the calibration sample set and validation sample set. Validation determined the accuracy and robustness of the model. Means and standard deviation (SD) were reported for both the calibration as well as the validation sample sets to determine if the means were statistically different. The coefficient of determination (RSQ), one minus variance ratio (1-VR), standard error of calibration (SEC) and standard error of cross validation (SECV) were employed to determine the accuracy of the model. The robustness of the model was determined by computing the standard error of prediction (SEP) and coefficient of determination (RSQ_{val}). These parameters were important criteria to consider the robustness of model. Best equations for beta-glucan, protein and oil content were achieved with the spectra exported with 2nm wavelength resolution. The best models were obtained where spectra were pre-processed using standard normal variate (SNV) and with math treatment of 1,4,4,1 and detrend was used for scatter correction. Use of repeatability file to remove the effect on spectra due to temperature and compaction of samples improved the calibration equation for all three components.

Moisture Analysis

The information on moisture content of oat samples were determined on a dry weight basis by adjusting for sample moisture content. The mean value of moisture content of the samples used for ground oat β-glucan calibration was 9.17% with a standard deviation (SD) of 0.69 (N = 432) and the samples used for whole oat β-glucan calibration had a

mean moisture content of 9.05% with a standard deviation (SD) of 0.6 (N = 341). The same number of samples were used for whole groat as well as ground groat protein and oil content calibrations where the mean moisture content and standard deviation (SD) were 8.88% and 0.65(N = 535), respectively.

Table 1. Beta-glucan, protein and oil content of oat samples analyzed using reference procedures[a]

Constituents	N	Range %	Mean %	S.D.
Beta-glucan[b]	417	3.42-7.61	4.86	0.66
Protein[c]	535	10.99-22.94	16.4	2.43
Oil[d]	535	3.08-8.26	5.56	0.89

[a]S.D.: Standard Deviation, N: Number of samples

[b]Beta-glucan determined by using AACCI standard procedure 32-23.01

[c]Protein determined by AACCI standard procedure 46-30.01

[d]Oil content determined by AOCS standard procedure Am 5-04

Analysis of Beta-glucan

Reference Analysis

A wide range of concentration of β-glucan (g per 100g of oat groat) on a dry weight basis was observed through reference analysis of β-glucan (table 1). The β-glucan content ranged from 3.42 to 7.61% with a mean value of 4.86% and standard deviation of 0.66. For accuracy and precise result, barley control flour (4.1% on dry weight basis) and oat control flour (8% and 7.5% on dry weight basis) were used. Table 2 and Table 3 shows the accuracy and precision of the result by comparison of mean value, standard deviation (SD) and coefficient of variation (CV). Mean values of barley control flour and oat control flour shows closeness with their standard values along with low SD and CV.

Table 2. Summary of beta-glucan determination of control samples

	Barley Control	**Oat Control**
Mean	4.09 %	7.97 %
Standard Deviation (SD)	0.06	0.14
Coefficient of Variation (CV)	1.57 %	1.76 %

Reported value for Standard barley control flour: 4.1%, Reported value for Standard oat control flour: 8%

Table 3. Summary of beta-glucan determination of control samples

	Barley Control	**Oat Control**
Mean	4.10 %	7.50 %
Standard Deviation (SD)	0.09	0.15
Coefficient of Variation (CV)	2.15 %	2.10 %

Standard barley control flour: 4.1%, Standard oat control flour: 7%

NIRS Analysis

Of a total 417 ground oat samples, 313 samples were used for calibration model development and 104 samples were used for validation. For whole oat calibration, 251 samples were used for calibration model development and 85 samples were used for validation out of total 336 samples. During calibration equation development, software selected 308 samples out of 313 samples in ground oats and 247 samples out of 251 samples in whole oats. The accuracy of the calibration models developed for ground oats and whole oats were assessed. Calibration and validation statistical details are provided in the Table 4. Accuracy of calibration model was determined based on various statistical terms including RSQ, 1-VR, SEC, SECV for equation development and SEP, bias, and RSQ_{val} for validation. High RSQ and low standard errors were indicative of good calibrations. Excellent predictive equations were obtained for the determination of β-glucan (Table 4). In comparing ground oat groat and whole oat groat calibrations, the ground oat groat calibration data set, yielded higher coefficients of determination (RSQ=0.93) value, 1-variance ratio (1-VR=0.88) value and lower standard error of calibration (SEC=0.16), and standard error of cross validation (SECV=0.22). The validation data set for the ground oat groat sample set, yielded a low standard error of prediction (SEP) value of 0.24, a low bias of 0.001 and a high coefficient of determination (RSQ_{val}) of 0.87. The whole oat groats calibration yielded an RSQ value of 0.92, 1-VR value of 0.88, SEC value of 0.19 and SECV value of 0.24. The validation data set for whole oat groat samples yielded an SEP value of 0.22, bias value of 0.023 and RSQ_{val} value of 0.87. All the NIRS predictions provided accurate results compared with the reference methods for both ground oat groat as well as whole oat calibration and

validation data sets. No significant difference was found through paired comparison t-tests of means between chemically analyzed and NIRS predicted values of β-glucan in both calibration and validation data sets in both ground and whole oat samples as shown in table 5. All NIRS predicted β-glucan data were expressed on a dry weight basis (g per 100g of oat groat). Additional validation of the effectiveness of NIRS predictions was provided by correlation plots relating reference analytical values with NIRS predicted values on the same samples. Figures 6-9 shows the graphic evidence correlating the two methods.

Table 4. Near infrared-reflectance spectroscopy (NIRS) calibration and validation statistics on β-glucan content in ground and unground oat groats[a]

	Calibration Set						Validation Set			
Constituent	**N**	**Mean**	**RSQ**	**SEC**	**SECV**	**1-VR**	**N**	**RSQ_{val}**	**Bias**	**SEP**
Ground oat	308	4.84	0.94	0.16	0.22	0.88	104	0.87	0.001	0.24
Whole oat	247	4.87	0.93	0.18	0.23	0.89	85	0.89	-0.025	0.21

[a]RSQ: coefficient of determination; SEC: standard error of calibration; SECV: standard error of cross validation; 1-VR: one minus variance ratio; SEP: standard error of prediction; RSQ_{val}: Coefficient of correlation.

Table 5. Comparison of AACCI procedure and near infrared reflectance spectroscopy (NIRS) prediction for beta-glucan content in oats of calibration and validation samples of Ground and Whole oat groat samples[a]

		AACCI Procedure		NIRS Prediction	
Constituents	**N**	**Mean%**	**SD**	**Mean%**	**SD**
Calibration Set					
Ground oat	313	4.84 a	0.66	4.84 a	0.64
Whole oat	251	4.90 a	0.69	4.90 a	0.74
Validation Set					
Ground oat	104	4.90 a	0.67	4.90a	0.66
Whole oat	85	4.82 a	0.63	4.84 a	0.57

[a]Means with the same letter within rows are not significantly different from each other ($P \leq 0.05$).

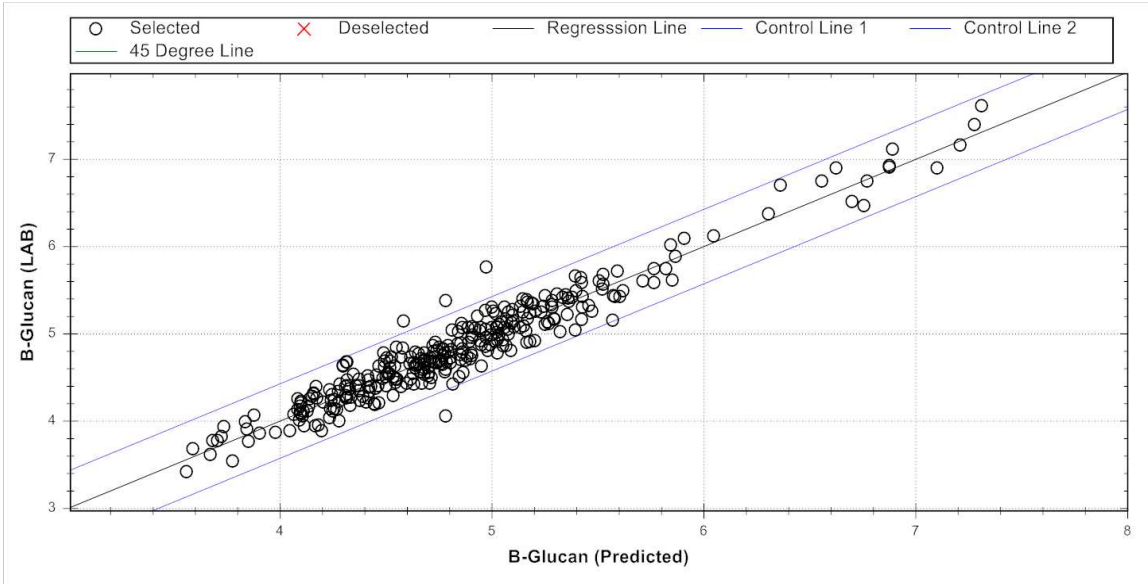

Figure 6. Correlation between reference data and NIRS methods for estimation of percent beta-glucan in ground oats in calibration dataset

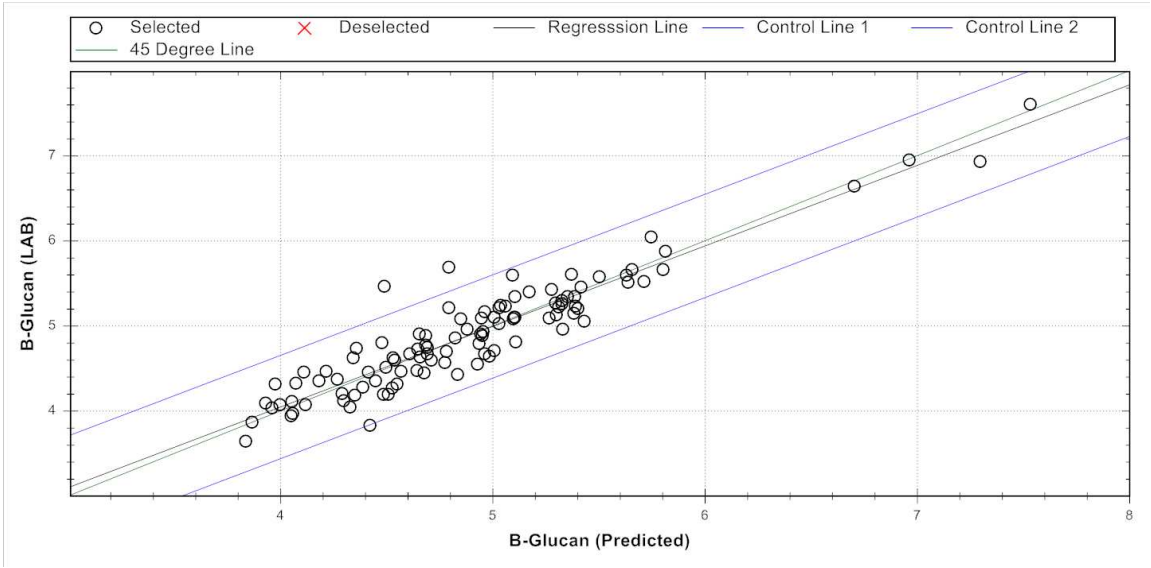

Figure 7. Correlation between reference data and NIRS methods for estimation of percent beta-glucan in ground oat groats in validation dataset

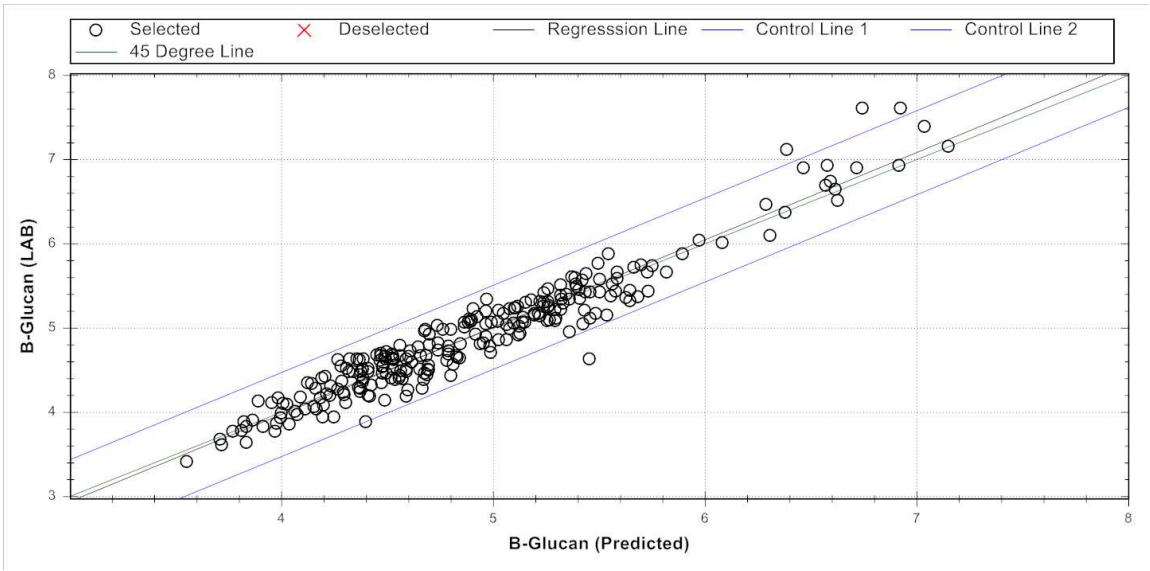

Figure 8. Correlation between reference data and NIRS methods for estimation of percent beta-glucan in whole oats groats in calibration dataset

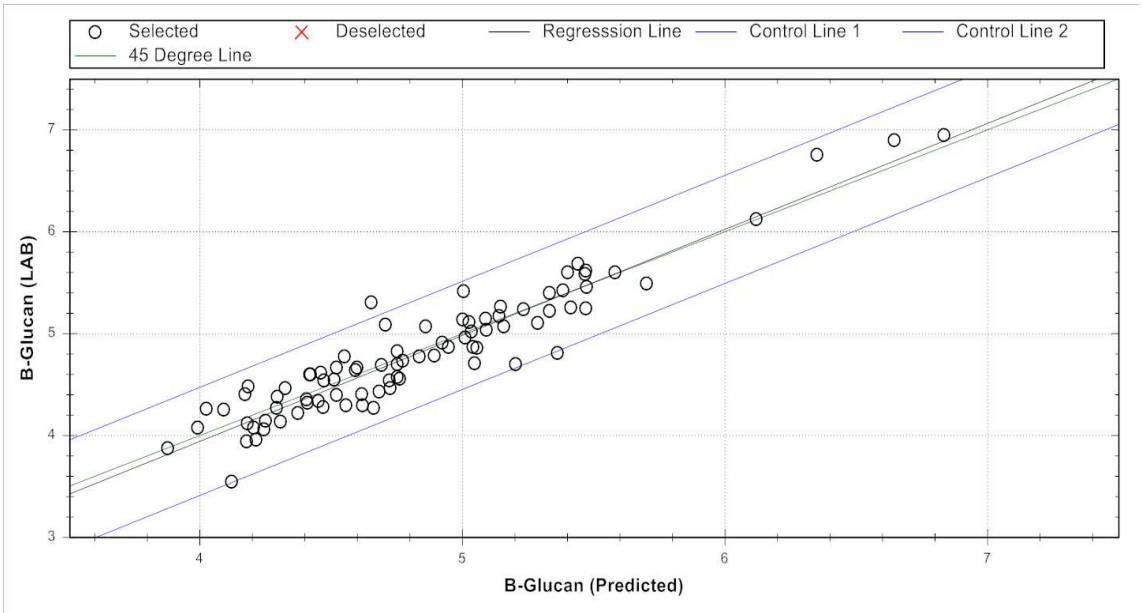

Figure 9. Correlation between reference data and NIRS methods for estimation of percent beta-glucan in whole oat groats in validation dataset

Analysis of Protein

Reference analysis

Protein content as measured by official reference methods (AACCI 46-30.01) yielded a range of 10.99 to 22.94% on a dry weight basis. The mean protein content was 16.40% with a standard deviation (SD) of 2.43.

NIRS analysis

NIRS calibration models were developed for both whole oat groats as well as ground oat groats and the accuracy of the model was determined based on the values of statistical parameters including RSQ, 1-VR, SEC and SECV as provided in Table 6 below. Paired comparison t-test of mean values of protein content in oats from reference analysis and NIRS analysis showed no difference (Table 7). An independent data set called the validation sample set was also used to check the accuracy and robustness of the model. For validation, the statistical terms included SEP, RSQ_{val} and bias. Values of 0.94, 0.93, 0.61 and 0.64 for RSQ, 1-VR, SEC, and SECV, respectively, were obtained in the assessment of accuracy of the calibration equation of ground oat groats. Similarly, values of 0.91, -0.052 and 0.72 of $RSQ_{val,}$ bias and SEP were obtained for validation dataset of ground oat groats. For whole oat groats, RSQ value of 0.92, 1-VR of 0.89, SEC of 0.70 and SECV of 0.80 were obtained for the calibration equation. An RSQ_{val} of 0.89, bias of -0.045 and SEP of 0.81 were obtained to validate the calibration equation by use of the validation data set.

Table 6. Near infrared-reflectance spectroscopy calibration and validation statistics on protein content in oats[a]

	Calibration Set					Validation Set			
Constituent	**N**	**Mean**	**RSQ**	**SEC**	**SECV**	**1-VR**	**N**	**RSQ_{val}**	**Bias**	**SEP**
Ground oat	394	16.35	0.94	0.61	0.64	0.93	134	0.91	-0.052	0.72
Whole oat	394	16.59	0.92	0.70	0.80	0.89	133	0.89	-0.045	0.81

[a]RSQ: coefficient of determination; SEC: standard error of calibration; SECV: standard error of cross validation; 1-VR: one minus variance ratio; SEP: standard error of prediction; RSQ_{val}: Coefficient of correlation.

An equal number of samples were used for NIRS protein calibration for each of whole oat groats and ground oat groat samples. For the ground oat calibration, 401 samples were used for calibration model development and 134 samples were used for validation out of 535 samples. A total of 394 samples out of 401 available samples were selected in developing the calibration equation while the remaining 7 samples were considered as outliers by the software. Similarly, 402 samples were used for the calibration model development and 133 samples were used for the validation set out of a total of 535 samples. Out of 402 samples, 394 were used in calibration process and the rest were considered as outliers by the software. Paired comparison t-test showed that the mean protein content predicted or estimated through NIRS was statistically similar to the protein values obtained from reference analysis in both the calibration and validation sample sets. This was true of both the whole groat as wells as ground groats sample sets. Correlation plots (Fig. 10 to 13) provide graphic evidence of excellent correlations established between the two methods.

Table 7. Comparison of AACCI procedure and near infrared reflectance spectroscopy (NIRS) prediction for protein content in calibration and validation samples of Ground and whole oat groats[a]

		AACCI Procedure		NIRS Prediction	
Constituents	**N**	**Mean%**	**SD**	**Mean%**	**SD**
Calibration Set					
Ground oat	401	16.40 a	2.42	16.36 a	2.32
Whole oat	402	16.60 a	2.41	16.59 a	2.29
Validation Set					
Ground oat	134	16.40 a	2.44	16.46 a	2.23
Whole oat	133	15.89 a	2.40	15.92 a	2.20

[a] Means with the same letter within rows are not significantly different from each other ($P \leq 0.05$).

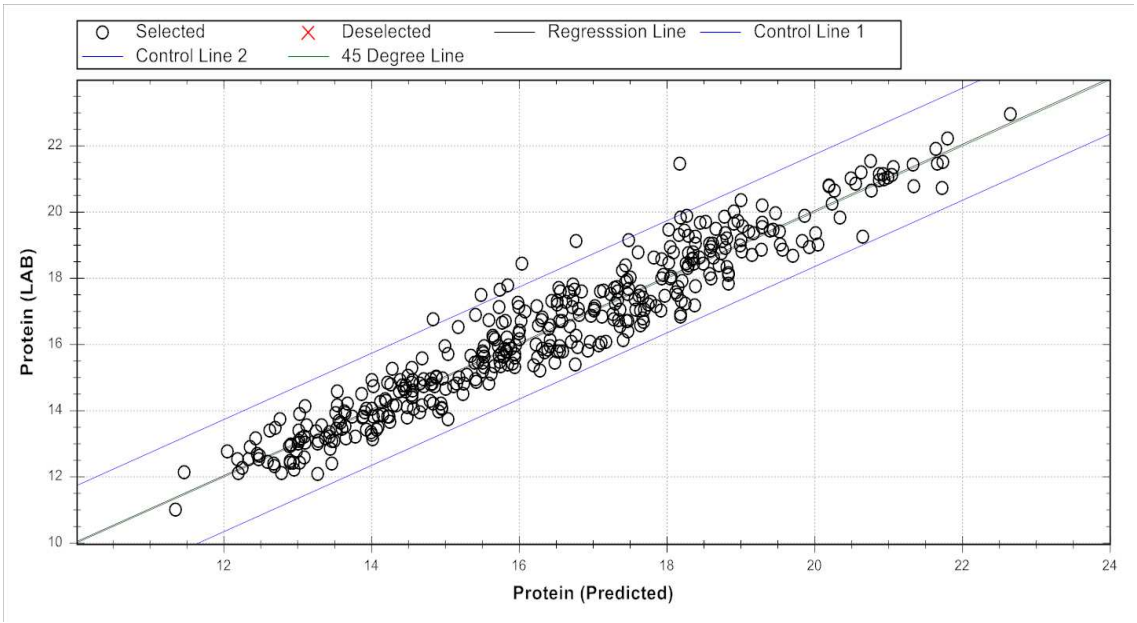

Figure 10. Correlation between reference data and NIRS methods for estimation of percent protein in ground oats in the calibration dataset

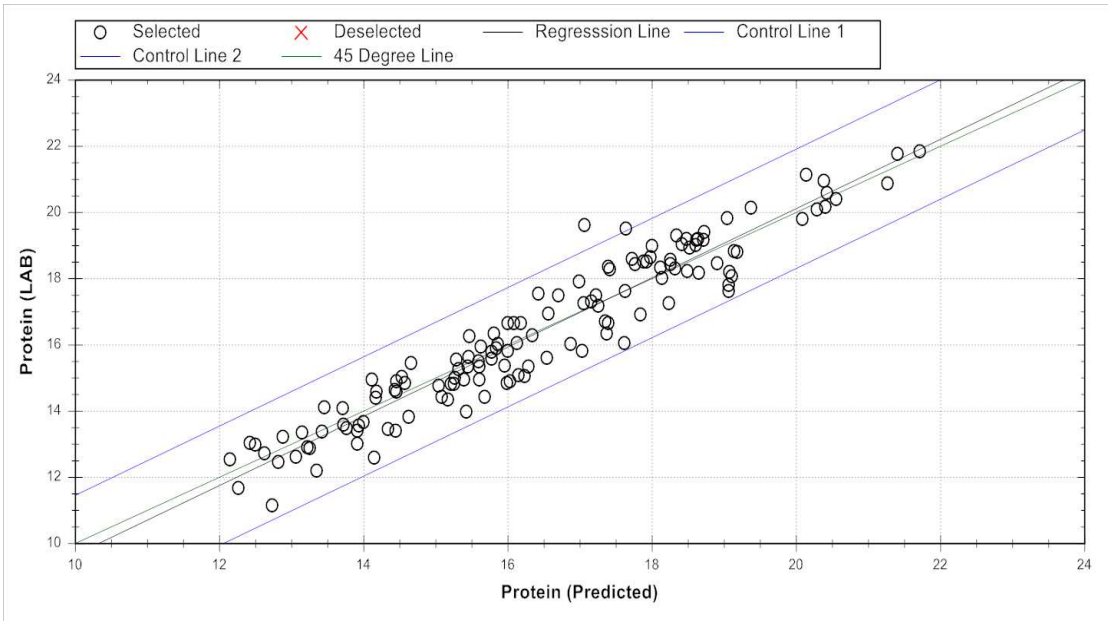

Figure 11. Correlation between reference data and NIRS methods for estimation of percent protein in ground oats in the validation dataset

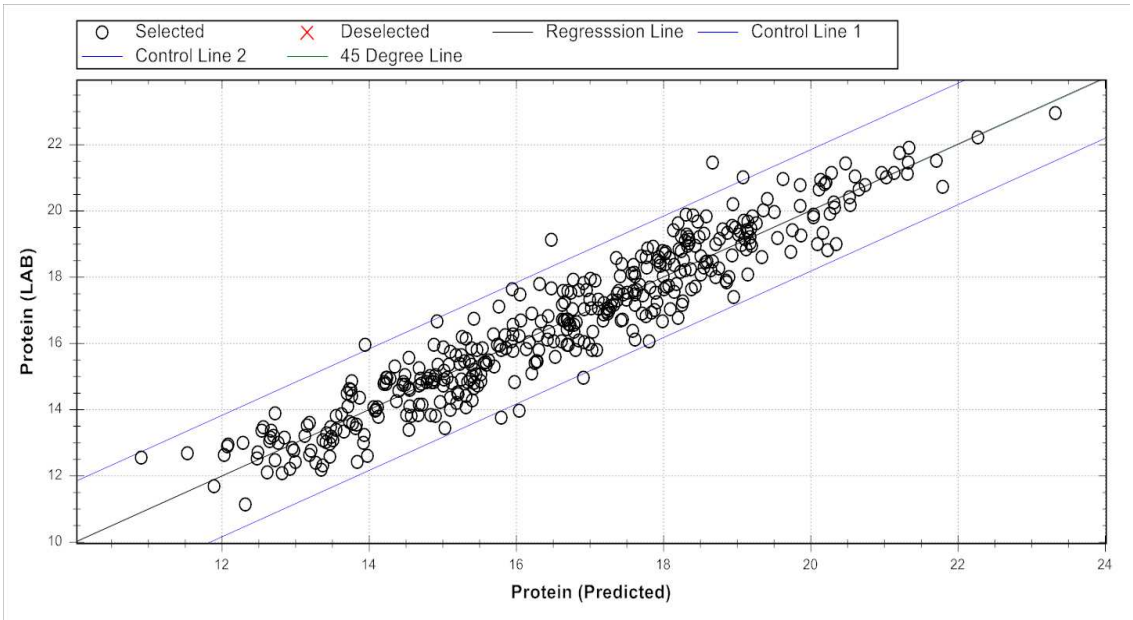

Figure 12. Correlation between reference data and NIRS methods for estimation of percent protein in whole oats groats in the calibration dataset

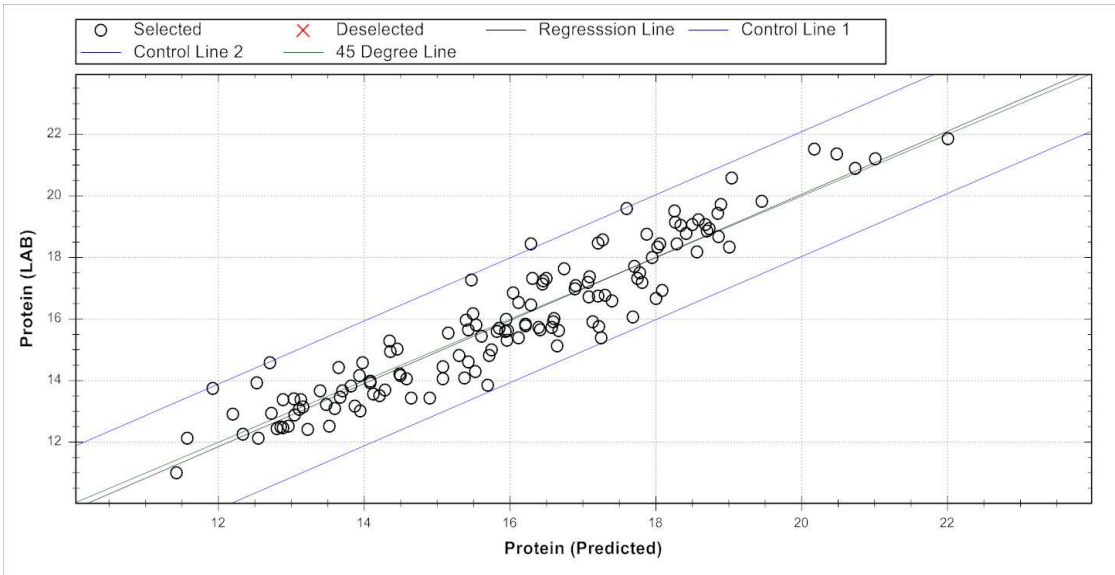

Figure 13. Correlation between reference data and NIRS methods for estimation of percent protein in whole oat groat in the validation dataset

Analysis of oil content

Reference analysis

Oil content was expressed as percentage (g per 100g of sample) of the samples on a dry weight basis. Oil content in all samples ranged from 3.08 to 8.26%. The mean oil content was 5.56% with a standard deviation (SD) of 0.89. Oil content analysis was done by using standard AOCS procedure (AOCS Am 5-04).

NIRS analysis

Accuracy of calibration equation and its validation were assessed using the statistical terms including RSQ, 1-VR, SEC, SECV, SEP, bias and RSQ_{val}. Equal numbers of samples (about 535) were used for both whole oat groat and ground oat groat NIRS oil calibrations. The details of the values of various statistical terms which define the accuracy of calibration models are provided in Table 8. From the table, we can observe that the NIRS oil calibration equations for ground oat groats yielded an RSQ value of 0.93, 1-VR value of 0.92, SEC of 0.23 and SECV of 0.26. For validation samples of ground oat oil calibration, RSQ_{val} was 0.91, bias was 0.018 and SEP was 0.25. Similarly, for the whole oat groats oil calibration, RSQ was 0.90, 1-VR was 0.88, SEC was 0.27 and SECV was 0.3. Validation data sets in whole oat oil calibration, yielded an RSQ_{val} of 0.88, a bias of -0.016, and an SEP of 0.31.

Table 8. Near infrared-reflectance spectroscopy calibration and validation statistics on oil content in oats[a]

	Calibration Set					Validation Set			
Constituent	**N**	**Mean**	**RSQ**	**SEC**	**SECV**	**1-VR**	**N**	**RSQ_{val}**	**Bias**	**SEP**
Ground oat	385	5.52	0.93	0.23	0.26	0.92	133	0.91	0.018	0.25
Whole oat	394	5.52	0.90	0.27	0.30	0.88	133	0.88	-0.016	0.31

[a]RSQ: coefficient of determination; SEC: standard error of calibration; SECV: standard error of cross validation; 1-VR: one minus variance ratio; SEP: standard error of prediction; RSQ_{val}: Coefficient of correlation.

Table 8 shows that an equal number of samples were used in the calibration development for NIRS oil content estimation for both the ground oat groat and whole oat groat samples. Paired comparison t-test showed no significant difference between reference mean value and NIRS predicted mean value as shown in table 9. Out of 535 samples used for ground oat calibration, 402 samples were selected in the calibration equation development and 133 were used as the validation sample set. Out of the 402 samples, few spectral outliers were removed and only 385 samples were used in actual calibration equation development. Similarly, 402 out of 535 total number of samples were selected for calibration equation development and only 394 samples were used as calibration dataset. The rest were removed as some were spectral outliers. One hundred and thirty-three (133) samples were used for validation of calibration equation for whole oat groats NIRS oil calibration. Statistical similarity was observed between the means of NIRS predicted oil content and the values obtained from reference analysis through paired comparison t-test. No statistical differences were observed between NIRS predicted and actual values in both the calibration and validation datasets. This was true of both the whole groat and ground groat samples. This similarity can also be observed through the correlation plots (Fig.14-17) between actual and predicted values in both the calibration and validation dataset (for both whole oat groat and ground oat groat samples).

Table 9. Comparison of AOCS procedure and near infrared reflectance spectroscopy (NIRS) prediction for oil content in calibration and validation samples of Ground and whole oat groats[a]

		AOCS Procedure		NIRS Prediction	
Constituents	**N**	**Mean%**	**SD**	**Mean%**	**SD**
Calibration Set					
Ground oat	402	5.53 a	0.91	5.53 a	0.88
Whole oat	402	5.53 a	0.89	5.53 a	0.84
Validation Set					
Ground oat	133	5.64 a	0.84	5.63 a	0.81
Whole oat	133	5.62 a	0.90	5.60 a	0.83

[a] Means with the same letter within rows are not significantly different from each other ($P \leq 0.05$).

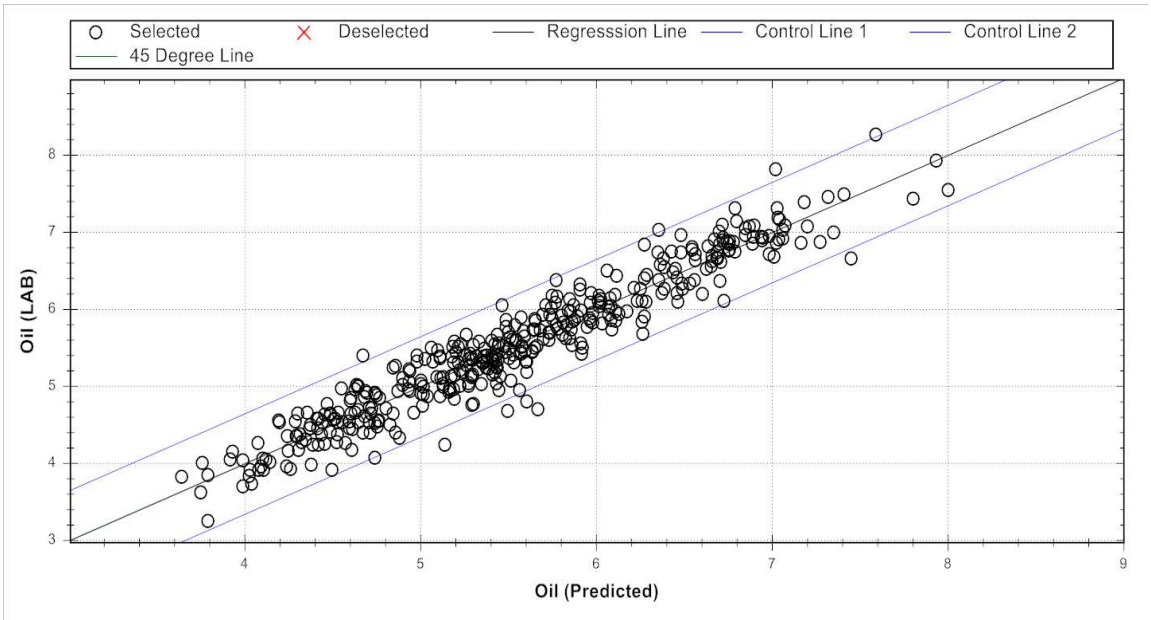

Figure 14. Correlation between reference data and NIRS methods for estimation of percent oil in ground oat groats in calibration dataset

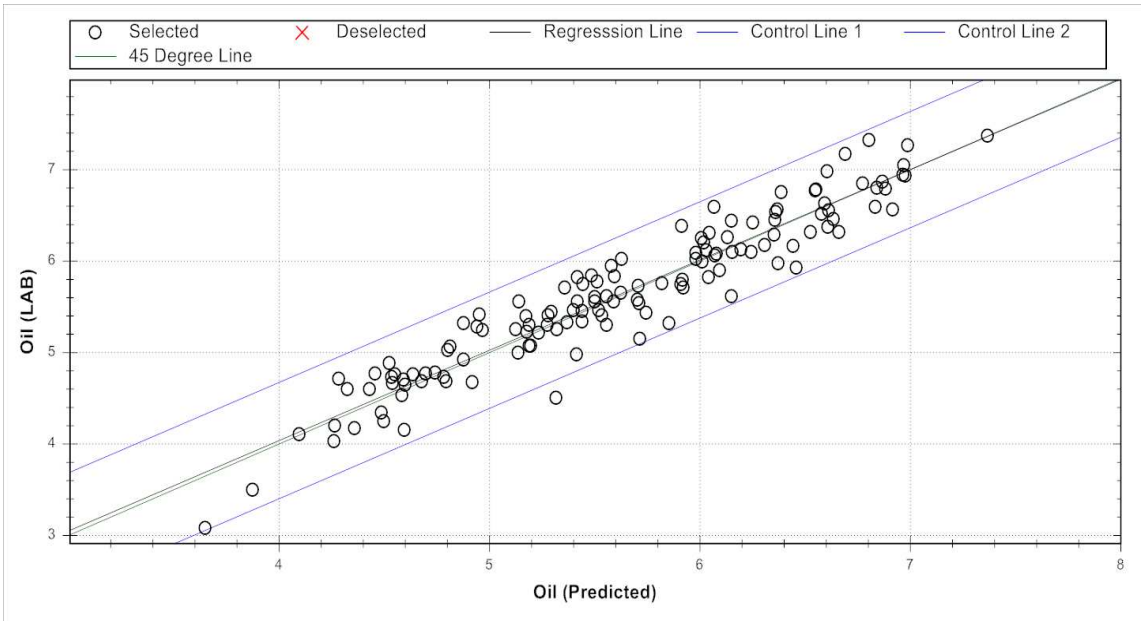

Figure 15. Correlation between reference data and NIRS methods for estimation of percent oil in ground oat groats in validation dataset

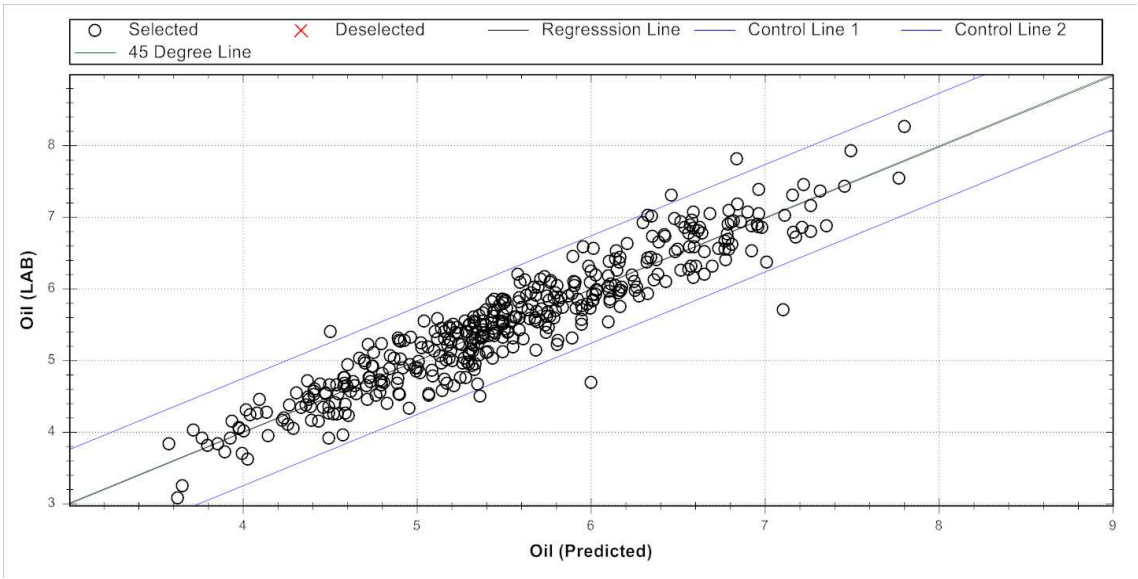

Figure 16. Correlation between reference data and NIRS methods for estimation of percent oil in whole oat groats in the calibration dataset

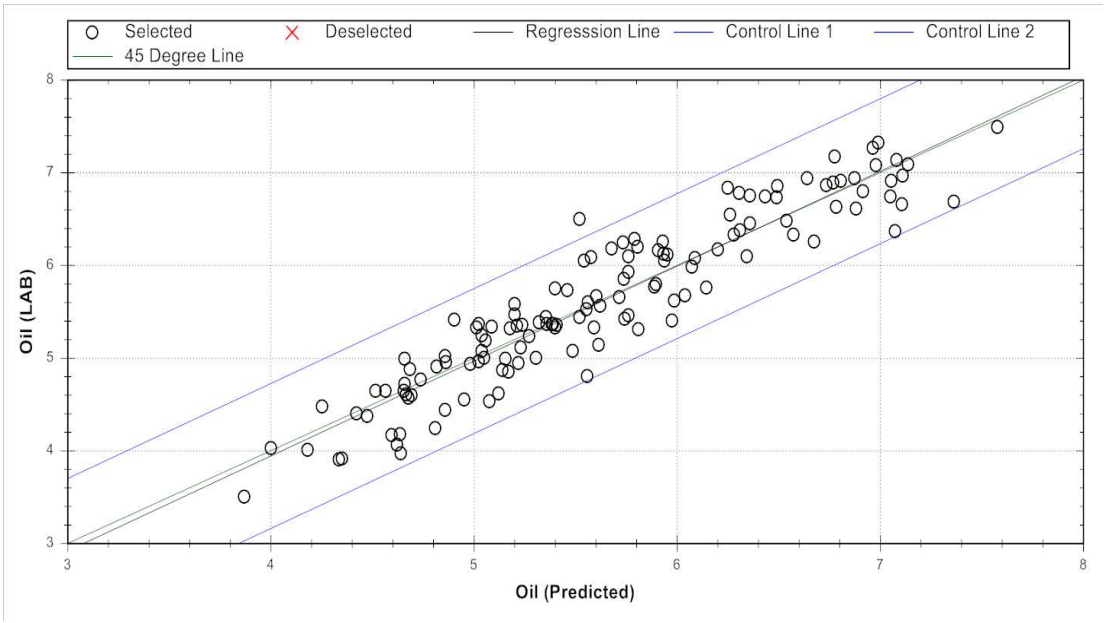

Figure 17. Correlation between reference data and NIRS methods for estimation of percent oil in whole oat groats in validation dataset

Variability study of beta-glucan, protein, and oil content of South Dakota oat cultivars

Sixteen cultivars grown in four growing locations were analyzed over two growing seasons. Table 10 provides a summary of the analysis of variance (ANOVA) of data obtained by chemical reference analysis and NIRS. The table shows statistically significant effects of cultivar (genetics), growing location and growing year on the variability of protein, oil and beta glucan content of oats grown in SD during the 2015 and 2016 crop year. Interactions among some of these factors were also significant. Analysis of variance on beta-glucan showed the significant effect of genotype, location and their interaction which supports the findings from previous studies as well (Lim et. al 1992; Peterson 1991). Table 10 also shows the comparison of variance analysis in protein, oil, and beta-glucan content among reference analysis and NIRS ground and whole oat calibration. Comparison of results shows similarity among reference analysis and NIRS prediction. Ground oat calibration prediction more resembles with reference analysis. From this result, we can say that ground oat calibration model is better than whole oat calibration.

Tables 11, 12 and 13 provide the means of the three constituents (protein, oil, and beta glucan content) for the cultivars used in the study. The Duncan Multiple Range Test was performed to compare the significant difference in values of beta-glucan, protein, and oil content for all the varieties grown in South Dakota in the crop year 2015 and 2016. The tables show the ranking of different varieties as revealed by chemical, NIRS whole groat and NIRS ground groats estimation. Variety GMI423 had highest beta-glucan content while variety Natty had the lowest beta-glucan content. Variety SD110466 had highest protein content and variety Souris the lowest protein content. Variety Rockford has the

highest oil content and variety Natty the lowest oil content. The ranking of all the varieties were similar whether the data were ordered according to chemical analysis or NIRS estimation for all three compounds. There were slight differences in ranking in few varieties, but the parameters were statistically same. The rankings visibly demonstrate the effectiveness of the NIRS ground oat groat and NIRS whole oat groat predictive calibrations in discriminating between named cultivars based on nutrient and fiber content.

Table 10. Analyses of Variance of beta-glucan, protein and oil content of Oats grown in South Dakota in 2016 and 2017

Source	df	Official Method[a]			NIRS method (Ground Oat)[b]			NIRS method (Whole Oat)[c]		
		Mean Square	F Ratio	Significance level	Mean Square	F Ratio	Significance level	Mean Square	F Ratio	Significance level
Beta-glucan										
Cultivar	15	0.188745	104.458	***	5.6659	160.095	***	4.7967	199.306	***
Location	3	0.054769	30.311	***	1.2325	34.826	***	0.913	37.9369	***
Year	1	0.039736	21.9911	***	0.7165	20.2459	***	0.3503	14.5535	***
Cultivar x Location	45	0.003021	1.6718	*	0.0632	1.7868	*	0.0656	2.7246	***
Location x Year	3	0.020693	11.4521	***	0.4456	12.59	***	0.4076	16.9376	***
Cultivar x Year	15	0.003602	1.9937	*	0.0554	1.5656		0.0575	2.3904	**
Cultivar x Location x Year	43	0.001341	0.742		0.0372	1.0506		0.0365	1.5151	
Residuals	62	0.11203	0.001		2.194	0.0354		1.492	0.0241	
Groat Protein										
Cultivar	15	0.000117	10.699	***	6.22	8.0097	***	5.53	17.4145	***
Location	3	0.00027	24.7656	***	19.49	2.51E+01	***	25.13	79.0605	***
Year	1	0.006655	611.067	***	506.52	6.53E+02	***	451.43	1420.47	***
Cultivar x Location	45	1.91E-05	1.7496	*	0.87	1.1177		0.72	2.2603	**
Location x Year	3	0.000725	66.6074	***	28.01	3.61E+01	***	36.53	114.933	***

Cultivar x Year	15	1.56E-05	1.4308		0.63	0.8102		0.58	1.8297	
Cultivar x Location x Year	43	1.26E-05	1.1577		0.56	0.72		0.64	2.0073	**
Residuals	62	0.000675	1.09E-5		50.45	0.78		19.7	0.32	
Groat Oil										
Cultivar	15	9.3077	155.1	***	8.738	335.7736	***	7.2278	99.1164	***
Location	3	8.9094	148.461	***	6.6824	256.7857	***	7.8262	107.323	***
Year	1	0.2936	4.8916	*	0.3722	14.3011	***	0.6265	8.5914	**
Cultivar x Location	45	0.1368	2.2789	**	0.1216	4.6743	***	0.135	1.8515	*
Location x Year	3	6.8996	114.971	***	6	230.561	***	4.4507	61.0341	***
Cultivar x Year	15	0.2544	4.2385	***	0.0819	3.1464	***	0.0721	0.9888	
Cultivar x Location x Year	43	0.111	1.8497	*	0.0721	2.7721	***	0.0907	1.2441	
Residuals	62	3.721	0.06		1.613	0.026		4.521	0.0729	

Significant. codes: '***' 0.001 '**' 0.01 '*' 0.05

[a] AACC Approved Methods 32-23.01 for B-glucan analysis, 46-30.01 for protein analysis and AOCS Am 5-04 for oil analysis

[b] Near Infrared Reflectance Spectroscopy method for Ground oat

[c] Near Infrared Reflectance spectroscopy method for Whole oat

Table 11. Ranking of oat cultivars based on beta-glucan content in 2015 and 2016 South Dakota Samples[a]

Reference Analysis			NIRS[b] Analysis (Ground Oat Groat Calibration)			NIRS[b] Analysis (Whole Oat Groat Calibration)		
GMI423	6.93	a	GMI423	6.86	a	GMI423	6.60	a
Newburg	5.35	b	Newburg	5.37	b	Newburg	5.35	b
Jury	5.20	bc	Horsepower	5.24	bc	Jury	5.30	b
Horsepower	5.14	c	Jury	5.14	cd	Horsepower	5.14	c
Rockford	5.13	c	Rockford	4.98	de	Rockford	5.06	c
Goliath	4.92	d	Goliath	4.93	e	Souris	4.87	d
Souris	4.89	de	Souris	4.88	e	Hayden	4.87	d
Hayden	4.75	ef	Hayden	4.86	e	Goliath	4.77	de
Deon	4.65	fg	Deon	4.62	f	Deon	4.68	e
Shelby427	4.53	gh	Shelby427	4.49	fg	Streaker	4.46	f
Streaker	4.38	hi	Streaker	4.36	gh	Shelby427	4.42	f
Stallion	4.32	ij	Stallion	4.31	hi	Stallion	4.35	fg
Jerry	4.18	j	Jerry	4.24	hi	Jerry	4.25	gh
SD110466	4.17	jk	SD110466	4.16	ij	SD110466	4.17	h
Colt	4.00	kl	Colt	4.02	jk	Colt	4.13	h
Natty	3.90	l	Natty	3.99	k	Natty	3.97	i

[a] Means with the same letter within each column are not statistically different from each other ($P<0.05$).

[b] NIRS: Near infrared-reflectance spectroscopy

Table 12. Ranking of oat cultivars based on protein content in 2015 and 2016 South Dakota Samples[a]

Reference Analysis			NIRS[b] Analysis (Ground Oat Groat Calibration)			NIRS[b] Analysis (Whole Oat Groat Calibration)		
SD110466	17.24	a	SD110466	17.47	a	SD110466	17.53	a
Stallion	16.88	ab	Stallion	17.23	ab	Jerry	16.90	b
Jerry	16.79	ab	Jerry	17.20	ab	Streaker	16.83	b
Streaker	16.79	ab	Streaker	16.59	bc	GMI423	16.78	b
GMI423	16.49	b	GMI423	16.58	bc	Stallion	16.71	b
Goliath	15.91	c	Shelby427	16.20	cd	Goliath	16.06	c
Deon	15.87	c	Deon	16.08	cde	Deon	16.04	c
Shelby427	15.83	c	Goliath	15.82	cdef	Jury	15.90	cd
Horsepower	15.68	cd	Horsepower	15.80	cdef	Shelby427	15.86	cd
Natty	15.42	cd	Jury	15.79	cdef	Rockford	15.82	cde
Rockford	15.41	cd	Rockford	15.67	def	Natty	15.61	cdef
Jury	15.40	cde	Newburg	15.48	def	Hayden	15.43	def
Colt	15.37	cde	Souris	15.48	def	Colt	15.42	def
Hayden	15.15	de	Natty	15.42	def	Horsepower	15.42	def
Newburg	15.15	de	Hayden	15.33	ef	Souris	15.34	ef
Souris	14.77	e	Colt	15.24	f	Newburg	15.31	f

[a] Means with the same letter within each column are not statistically different from each other ($P<0.05$).

[b] NIRS: Near infrared-reflectance spectroscopy

Table 13. Ranking of oat cultivars based on oil content in 2015 and 2016 South Dakota Samples[a]

Reference Analysis			NIRS[b] Analysis (Ground Oat Calibration)			NIRS[b] Analysis (Whole Oat Calibration)		
Rockford	6.91	a	Rockford	6.89	a	Rockford	6.77	a
Stallion	6.49	b	Stallion	6.54	b	Stallion	6.45	b
GMI423	6.46	b	Streaker	6.29	c	GMI423	6.37	bc
Streaker	6.36	bc	GMI423	6.27	c	Hayden	6.29	bcd
Hayden	6.23	cd	Hayden	6.26	cd	Streaker	6.12	cde
Jury	6.20	cd	Jury	6.22	cd	Jury	6.06	def
Newburg	6.10	d	Newburg	6.11	de	Newburg	6.03	efg
Shelby427	6.07	d	Horsepower	6.00	e	Shelby427	5.85	fg
Horsepower	5.78	e	Shelby427	5.98	e	Horsepower	5.82	g
Deon	5.40	f	Deon	5.55	f	Deon	5.42	h
Goliath	5.26	fg	Goliath	5.29	g	Goliath	5.31	hi
Souris	5.07	g	Souris	5.07	h	Souris	5.08	ij
Jerry	4.66	h	Jerry	4.74	i	Jerry	4.83	jk
Colt	4.54	h	Colt	4.40	j	Colt	4.82	k
SD110466	4.25	i	SD110466	4.35	j	SD110466	4.39	l
Natty	3.99	j	Natty	4.10	k	Natty	4.10	m

[a] Means with the same letter within each column are not statistically different from each other ($P<0.05$).

[b] NIRS: Near infrared-reflectance spectroscopy

CHAPTER 5. SUMMARY AND CONCLUSIONS

NIRS is a powerful screening tool for the rapid and accurate determination of many samples within a short time. This study involved the development of predictive NIRS calibrations for the estimation of protein, oil, and beta glucan content in ground and whole oat groats. Separate calibrations were developed for ground and un-gound oat groats and the accuracy of the calibrations were verified by using validation sample sets that were independent of calibration samples. Reference analyses were done by using officially validated approved methods for protein content, oil content, and beta glucan content. NIRS data were compared with reference data obtained by official chemical analyses. The three methods, namely, NIRS whole groat calibration, NIRS ground groat calibration and reference methods were judged to be not significantly different from each other by analysis of variance (ANOVA) statistical analysis. Evaluation of 16 cultivars grown in four locations over two growing years (N=16x4x2) showed that all the factors i.e. genotype, location, year and their interaction had statistically significant influence on the variability of protein, oil, and beta glucan content in SD oats.

Side by side comparisons of the rankings of the 16 cultivars showed that the order of cultivars was essentially the same when ranked by the NIRS whole oat calibration, ground oat groat calibration or reference chemical/enzymatic analysis. Paired T-test revealed that NIRS methods were not significantly different from the reference methods in evaluation of the nutrients evaluated in this study. A wide range of occurrence of the constituents allowed for robust predictive calibration equations. The NIRS calibrations were deemed to be accurate and precise as assessed by calibration statistics, as well as

using samples independent of the calibration sample set for the purpose of method validation.

A robust base calibration has been set up for the determination of beta-glucan, protein, and oil content in oats in this study. But because new varieties are being developed through breeding and because the growing environment changes from year to year, it is likely that outlier samples will be identified in the succeeding years. Those samples will need to be included in the calibration model to expand the equation and improve the robustness of the calibration model.

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